UNIVERSITY OF WALES INSTITUTE, CARDIFF

THE DEVELOPMENT OF NEW AND IMPROVED METHODS FOR THE DOWNSTREAM PURIFICATION OF PROTEINS AND ENZYMES FROM CRUDE MIXTURES

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SCHOOL OF APPLIED SCIENCES

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ABSTRACT

New economically viable methods for the industrial scale production and processing of proteins are constantly being sought. This project concentrates on two techniques which may be utilised in the purification of non-recombinant proteins.

First, aqueous two-phase systems were investigated as a potential tool for the separation of individual proteose peptones from a total proteose peptone fraction of bovine milk. The optimum pH for the separation of β-CN-5P from the total proteose peptone components was pH 7. Aqueous two-phase systems containing PEG with a mean molecular weight of less than 8000 were not found to separate the total proteose peptone components. The addition of sodium chloride into the aqueous two-phase systems encouraged a more effective extraction of β-CN-5P, although this effect was found to plateau at 5% NaCl.

Second, a model enzyme was immobilised onto nylon film and the optimisation of binding conditions was studied using spectrophotometric procedures. Results suggested that slight variations in the concentration of the incubating solutions had a profound effect on the efficacy of the procedure. Additionally, a considerable quantity of observed binding appeared to be due to non-specific interactions between the ligand and the matrix. This binding was investigated and was found to be a result of both ionic and hydrophobic interactions.

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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STATEMENT ONE

This thesis is the result of my own investigations, except where otherwise cited. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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LIST OF ABBREVIATIONS

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	apartic acid
Asx	aspartic acid or asparagine
BAPNA	Nα-Benzoyl-DL-Arginine ρ-nitroanilide
Bis	*N,N'*-methylene bisacrylamide
CM	carboxymethyl
Cys	cystine
DEAE	diethylaminoethyl
Gln	glutamine
Glu	glutamic acid
Gly	glycine
Glx	glutamine or glutamic acid
His	histidine
HPLC	high performance liquid chromatography
HIC	hydrophobic interaction chromatography
IEF	isoelectric focusing
Ile	isoleucine
kd	kilodalton
Leu	leucine
Lys	lysine
Met	methionine
MW	molecular weight
M_r	relative molecular mass
NMWC	nominal molecular weight cut-off
ON	overnight
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PEI	polyethyleneimine

Phe	phenylalanine
pI	isoelectric point
pNPG	ρ-nitrophenyl-β-D-glucopyranoside
Pro	proline
RPC	reversed phase chromatography
r.p.m.	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SBTI	soybean trypsin inhibitor
Ser	serine
TCA	trichloroacetic acid
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
Thr	threonine
Tris	2-amino-2-hydroxymethyl-propane-1, 3-diol tris(hydroxymethyl)methylamine)
Trp	tryptophan
Tyr	tyrosine
Val	valine
Xaa	any amino acid
α-La	α-lactalbumin
β-Lg	β-lactoglobulin
λ	wavelength

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

The continuing development of efficient and reliable techniques to isolate and recover proteins and enzymes from heterogeneous mixtures is of vital importance to industries as varied as food-processing, brewing, pharmaceutical and medical. These industries rely heavily on the purification schemes devised by biotechnologists to provide them with high quantities of high quality products in the most economical way possible. [Watanabe *et al.*, 1994; Stanbury and Whitaker, 1993; Boyer and Hsu, 1993]. Biotechnologists are therefore always searching for the elusive methodology that will provide them with the exacting purity of target protein required in the smallest possible number of steps within the purification scheme.

The processes that the biotechnologists choose for a given separation are largely governed by the amount of material needed and the final purity of target protein required. Unfortunately, the economic cost of purifying every desired molecule to an exacting degree of purity would often render it an impractical procedure [Berthold and Walter, 1994; Boyer and Hsu, 1993]. The pharmaceutical industry, for instance, seeking to harvest human factor VIII molecules for the treatment of haemophiliacs will require their protein to be of the highest purity possible hence minimising undesirable side effects or allergic reactions in the individuals concerned. The pharmaceutical industry represents a special case, whereby all the procedures within a process must

conform to legal requirements and Good Manufacturing Practice. Their definition of purity requires all the molecules of a protein to be correctly folded and for the protein to be bioactive in addition to being viewed as a single homologous band on a PAGE gel [Scopes, 1996, see Section 1.7 electrophoresis]. Conversely, proteins involved in the food industry, for example, will not only be required in a considerably lower degree of purity, but in much larger quantities [Scopes, 1996]. Additionally, the pharmaceutical industry is more able than the food industry to process enzymes and proteins to such a high level of purity due to the high monetary value of product that they produce [Niven, 1995]. The ideal method for the biotechnologist is one which provides a large quantity of high quality product whilst keeping time and operational costs to a minimum, yielding 100% recovery of the target protein with no contaminating material in a single step. In reality, a process with a number of steps is required, and the target protein typically reaches a purity level of 95-99% [Watanabe *et al.*, 1994; Andersson, 1992].

Traditionally, the main sources of proteins and enzymes have been animal or plant tissue, or from micro-organisms [Bonnerjea *et al.*, 1986; Stanbury and Whitaker, 1993; Smith, 1996]. Following the discovery of restriction enzymes in the 1970s [Roberts, 1976], it became possible to combine pieces of DNA from totally different organisms, to make recombinant DNA. The technique involves the insertion of a gene into a virus, a plasmid, or another vector system, and the incorporation of this vector into the DNA of a host organism [Conzelmann and Meyers, 1996]. The inserted gene will thus be expressed in the host organism, from which the gene products can be isolated. The processes of genetically engineering specific genes into host micro-organisms, or

amplifying the production of host proteins are well documented and have become commonplace [Alikhanian, 1976; Miller, 1979; Gingold, 1985; Jones and Karp, 1986; Clark, 1992]. One such example is the introduction of the human insulin gene into genetically altered micro-organisms which then produce human-identical insulin. This is of importance to diabetes sufferers who are intolerant to the porcine insulin commonly used to treat their condition [The, 1989; Ladisch and Kohlmann, 1992; Groskreutz *et al.,* 1994; Clark *et al.*, 1997].

A survey of 100 papers relating to protein purification published up to and including 1984 showed that 46% of papers reported isolation from microbial sources, and only 10% reported isolation from genetically altered hosts [Bonnerjea *et al.,* 1985]. Animal tissue was found to represent the largest provider of protein, with 49 purification schemes obtaining starting material from this source. The only stipulation of the authors for the inclusion of a scheme into their study was that the papers should provide adequate information on purification and yield; hence laboratory methods were analysed alongside large-scale purification procedures. It is highly likely that in the fourteen years following this study, the emphasis towards purification from genetically engineered hosts will have increased, as the methodology has developed, and become more available on a wide scale. Indeed Scopes reports 'an explosive increase in activity in DNA technology' in the 1980s in his recent review [Scopes, 1996].

One problem encountered from the production of human proteins by micro-organisms is the observed lack of post translational modification of proteins and enzymes. An example of this would be the glycosylation of proteins which routinely occurs *in vivo*

and to date has required mammalian cells as hosts [Berthold and Walter, 1994; McCarthy *et al.*, 1998; Varki, 1998]. If a therapeutic protein designed to be injected into an individual does not exhibit the conventional modifications prior to dosage, the immune system of the individual will be activated, and the protein may be destroyed. Additionally, without the successful post-translational modification of the protein, it may not be biologically active or its action may be impaired [Wright and Morrison, 1997; Haynes, 1998; Kulakosky *et al.*, 1998]. Indeed, post translational modifications such as glycosylation have been shown often to be vital to the function of a protein, enabling functions as diverse as adhesion, cellular trafficking, wound healing, inflammation, cancer metastasis and molecular mimicry to occur *in vivo* [Gingold, 1985; Haynes, 1998].

The two main classes of glycosylation are N-linked, (where the oligosaccharide generally links to the consensus sequence Asn-Xaa-Ser/Thr via a terminal N-acetylglucosamine (GlcNAc) residue), and O-linked, (which takes on many forms, the most abundant of which is a link via N-acetyl-galactosamine (GalNAc) to a serine or a threonine residue). Recently further examples of glycosylation have been discovered, and classes such as phosphoglycosylation may prove to be widespread in nature [Haynes, 1998]. Indeed, modification of membrane-bound proteins by a glycosylphosphatidylinositol (GPI) moiety consisting of mainly mannose constituents is thought to have considerable implications in cell signalling and membrane trafficking [Wright and Morrison, 1997; Kulakosky *et al.*, 1998]. For these reasons, transgenic mammals which will modify the expressed proteins in a similar way to humans *in vivo* have begun to be utilised as hosts in addition to micro-organisms. These mammals

have expressed the genetically introduced, post-translationally modified protein in their milk, from which it may be harvested [Wall *et al.*, 1997(a) and (b)]. One such example is the production of human α-antitrypsinogen by transgenic sheep [Harris *et al.*, 1994]. Once the gene has been introduced, the transgenic animal will produce the encoded protein as self for the duration of its lactation. The technology to mass produce transgenic cattle is difficult, and financial restrictions imposed by the long time scale involved (7-8 yr.) have limited it to only a few groups world wide [Clark, 1992]. The techniques are similar to the recombinant technology used with micro-organisms, but have had to be revised to overcome problems not previously encountered when expressing a hybrid protein in micro-organisms, such as ensuring that the hybrid protein will be successfully produced in the mammary glands of the host [Wall *et al.*, 1997(b)].

A transgenic animal is described as one which carries recombinant DNA introduced by intentional human intervention. Originally, the genes were incorporated into the host DNA at the pre-implantation embryonic stage of development, but recent developments have allowed expansion to include postnatal gene transfer, otherwise known as somatic cell engineering (SCE) and the rapidly emerging field of nucleic acid vaccines (NAV) [Bondioli and Wall, 1998; Wall *et al.*, 1997(a) and (b)]. Nucleic acid vaccinations are more appropriately called DNA-mediated immunisation, and refer to injection (commonly intramuscular) of a plasmid DNA (or RNA) which is capable of inducing expression of an antigen in the host cells [Whalen, 1998]. Although in the early stages of development, this potential ability to introduce a therapeutic structural gene directly into the DNA of an individual who will benefit from the gene product has

major implications for the future of the pharmaceutical industry. In addition to the DNA encoding the therapeutic gene, a regulatory gene sequence, (usually incorporating the immediate early promoter sequence of the cytomegalovirus and the bovine growth hormone polyadenylation sequence) must be included in the treatment to regulate the expression of the gene product. By injecting the product directly, the need for protein separation technology in these cases could become almost obsolete, offsetting the high production costs of the gene. However, such methodology is not yet possible, and the current introduction of new genetic modification technology and new hosts is providing a new challenge to separations specialists. [Bondioli and Wall, 1998; Wall *et al.*, 1997(a) and Wall *et al.*, 1997 (b)].

Current trends in the biotechnological industry have focused on optimising the methodology required to extract the desired recombinant protein, either from the fermentation broth of the host micro-organism or from the milk of the host mammal, as the development of efficient large-scale purification schemes is lagging behind progress in the expression and fermentation technology of recombinant proteins [Hochuli, 1988]. The three main requirements for the design of this process are to achieve the greatest possible yield using the minimum cost whilst reaching the desired level of purity, hence much work is directed towards developing a successful combination of an initial clarifying step with the more specific techniques required to provide the homogeneity expected by industry [Watanabe *et al.*, 1994; Hochuli, 1988; Andersson, 1992].

Any proteins expressed in the fermentation broth of host organisms or the milk of

transgenic animals will generally only be present in low concentrations, with components such as intact micro-organisms, cell fragments, medium products and host proteins making up much of the contaminating material. There are many factors to be taken into account when purifying a protein from such complex liquids, including the minimum degree of purity required, the cellular location of the target protein, and the susceptibility of the protein to heat or mechanical denaturation and hydrolysis by indigenous host proteinases. Many of these considerations are thoroughly reviewed and discussed in a number of books and reviews [Whittington, 1989; Scopes, 1993 and 1996; Stanbury and Whitaker, 1993].

1.2 Clarification Steps

The initial step of a protein purification procedure usually involves the removal of contaminating solids or larger particles. The actual methodology used naturally varies with the type of material the product is present in. For animal or plant tissue, homogenisation is frequently the initial step, often followed by precipitation of cell structures such as the cell wall, or organelles. For proteins expressed in mammalian milk, removal of milk fat is a priority, and skimming is often the first step. In the case of microbial protein production, the initial step depends on the cellular location of the product; extracellular proteins are separated from large solid particles and microbial cells by centrifugation or filtration, intracellular proteins require an initial homogenisation or cell lysis step [Stanbury and Whitaker, 1993].

These initial stages are clarification steps, used to prepare the material for the

subsequent more sophisticated protein purification and final product "polishing" stages. Relatively crude steps such as filtration, ultrafiltration or precipitation tend to be able to deal with large quantities of impure material more effectively than some of the more specific techniques such as chromatographic procedures. For a high resolution chromatographic fractionation of a complex protein mixture, the components must first be brought into a clear solution which may often be achieved by an efficient extraction and centrifugation procedure [Andersson, 1992].

Both filtration and centrifugation are effective clarification steps, and as such are often the initial stages in the recovery of extracellular proteins. However, when a protein is expressed intracellularly in micro-organisms, the microbial cells must first be disrupted in order to release the desired protein. Mammalian cells in culture are easily lysed (e.g. By osmotic shock, sonication etc.) but bacterial, fungal and plant cells differ widely in cellular properties and some have extremely strong cell walls. Although the more fragile microbial membranes (e.g. some Gram negative bacteria such as Azobacter species) may be broken down by osmotic shock and cell wall degrading enzymes, the more sturdy varieties require less gentle procedures [Harris, 1989; Chaplin and Bucke, 1990]. A number of physical and chemical methods have been developed to successfully cleave these walls, without denaturing the protein and enzymes contained within them.

Occasionally pre-treatment of cells is required to enable sufficient cell disruption e.g. the removal of fat from animal tissue or concentration of cells grown in a fermenter. Once a cell has been disrupted, digestive proteases may be released and must be

prevented from degrading the target protein. To achieve this, a mixture of protease inhibitors is often added to the culture broth [Whittington, 1989].

The most successful and widely used of these disruption methods on an industrial scale is a physical process, namely liquid shear, which exploits the use of high pressure homogenisers to shear the individual cell walls releasing the intracellular proteins. Other physical methods that may be used to disrupt the cell walls of the micro-organisms include solid shear which combines liquid shear techniques with a temperature in the region of -25°C which causes the formation of ice crystals within the cellular matrix, hence aiding the disruption process [Stanbury and Whitaker, 1993]. The action of freezing then thawing the cells causes lysis of the cells through cold shock.

The French press (or freeze press) is an example of a vigorous cell disintegration technique. In this process, frozen cell suspensions are forced through a very small orifice at a very high pressure, (typically 150-230 MPa [Chaplin and Bucke, 1990]), which causes disruption of the cells. Other examples of vigorous techniques include bead mills where cell suspensions are subjected to a rapid vibration with glass beads, (typically 0.2-1.0 mm diameter [Chaplin and Bucke, 1990]; and ultrasonication where cells within a suspension are ruptured by high pressure sound waves [Scopes, 1993].

The sonication process relies on the rapid sinusoidal movement of a probe within a liquid. This causes the production of a large number of very small bubbles which collapse during the compression phase of the sound wave. The shock wave produced

by this collapse is sufficient to cause lysis of the cell walls by the conversion of sonic energy into mechanical energy. A disadvantage associated with this procedure is the simultaneous production of free radicals, particularly the hydrogen peroxide and the oxygen radicals which have been shown to damage the target enzyme [Chaplin and Bucke, 1990].

The main disadvantage associated with chemical processes such as the use of detergents e.g. Tween or Triton X-100 is that proteins are often damaged concomitantly with the cell walls. Any added chemicals may therefore have to be removed from the broth to protect the intracellular proteins before any further purification can take place. Alkali treatment of microbial cell walls has proved a suitable and economical, (cheap chemicals such as aqueous ammonia or sodium hydroxide are used to adjust the pH), disruption procedure in the isolation of enzymes that are tolerant of relatively high pH values [Salusbury, 1989]. However, as many enzymes will be denatured by a pH of 12 or over, this process remains of limited value to the biotechnologist [Stanbury and Whitaker, 1993]. Non-mechanical cell disruption methodology such as heat treatment is useful when the target protein is not biologically active, or is a heat stable enzyme, and several techniques exist that cause breakage of the cells including spray drying, drum drying and direct steam injection [Salusbury, 1989]. This can also be useful for removing heat-denatured protein impurities rendered insoluble by the heating process.

Any preparation of an intracellular enzyme will contain nucleic acids at this stage. These must be removed to facilitate further purification as they increase the viscosity

of the solution and, hence, reduce the manageability of the process. The most cost effective approach for their removal is by the addition of an exogenous nuclease, e.g. bovine pancreatic nuclease, although alternatively they may be precipitated out using polyethylenimine [Chaplin and Bucke, 1990].

Flocculation often precedes filtration and centrifugation and is described as a process by which particulate material formed during the disruption techniques is encouraged to come into contact and form aggregates [Whittington, 1989]. These aggregates are formed through manipulation of the surface charges of the particles, e.g. by the addition of salt to reverse charge repulsion between the particles; the use of surfactants to alter the hydrophobic/hydrophilic nature of the particles, or the use of perikinetic flocculation, or Brownian motion, which will increase the frequency of collisions between the particles. It is also advantageous to agitate the solution to increase the frequency of collisions. As long as the target protein or enzyme is not denatured by such conditions, flocculation provides a useful step in a purification scheme as it has been shown to improve centrifugation and filtration speed and efficacy [Priest, 1984; Whittington, 1989].

The purification scheme of industrial extracellular enzymes is often as simple as concentrating the fermentation broth, and adding additives to stabilise the target enzyme's activity. The main objective in such procedures is to either remove any residual protease or to selectively denature or inhibit it.

1.3 Filtration

Filtration is a general process by which suspended particles may be separated from a liquid using a porous membrane to selectively retain the particles. Many types of filtration are available to the separation scientist, and the method chosen for the effective removal of contaminating material will be dependent upon a number of factors relating to the specific requirements of the process. These include:-

The size and shape of the particles

The viscosity of the filtrate

The scale of the process

The need to enhance the flow rate by vacuum or pressure

The need for batch/continuous flow

One of the most notable disadvantages to any filtration operation is the build-up of particulate material such as fragments of microbial cell walls at the membrane surface. As time passes, this membrane fouling becomes more prominent, and a filter cake is formed causing the flow rate to lessen as the resistance to the flow of liquid increases. It is imperative to remove this filter cake at regular intervals to allow filtration to proceed freely.

Under batch flow conditions, the process must be paused and the apparatus may have to be dismantled in order to remove the cake, a costly process both in terms of manpower and time lost, when the contaminants are worthless and present in large

quantities. However, batch flow filtration provides the opportunity to clarify fermentation broths with low solids content, and is also useful in the collection of high value solids.

Conversely, during continuous flow filtration a knife blade constantly removes the build up of filter cake eliminating the need for the process to be paused, but at the cost of more complex and expensive equipment. Generally a rotating, hollow drum covered with a filter is submerged in the broth to be filtered. The filtrate is drawn through the filter using a vacuum, and is directed into one of the internal compartments of the drum. The growth of the resulting filter cake is carefully monitored and may be removed in a number of ways, using string or scrapers, typically knife blades. Ways in which filtration may be adapted to the individual needs of a particular purification process include the adjustment of the membrane pore size, or the introduction of pressure or a vacuum into the system to aid the removal of particulate material. Obviously the more material collected by the filter, the larger the filter cake will be, and the greater the potential need for a pressure or vacuum to be introduced. Alternatively a back-flushing or washing cycle can be included, but again at the cost of greater complexity and with an increase in processing time. Although filtration is a viable clarification step , the level of resolution that it affords renders it ineffective for the separation of a mixture of similar substances [Priest, 1984; Stanbury and Whitaker, 1993]. Indeed, the most effective use of filtration is either early in processing when large volumes are involved and crude processing required, or at the end of processing to concentrate a product through the removal of bulk liquid.

Ultrafiltration, the process where water and other small molecules are driven through a semi-permeable membrane by a force such as high pressure is a procedure that is often used in industry to concentrate protein solutions [Harris, 1989]. Through a reduction in pore size, this procedure provides greater purification than microfiltration, but less than gel filtration (permeation) (Section 1.4.1) as it is a much less discriminating technique. Only two fractions will result from ultrafiltration, that which will not pass through the membrane, and that which will. If the membrane pores are such that only small protein molecules will pass through the membrane, then a slight purification will result. The nominal molecular weight cut-off (NMWC- the maximum molecular weight molecule that will pass through the membrane) is not, however, the only condition to affect whether a protein will pass though the pores. If a protein is large with little tertiary or quaternary structure then it will pass though smaller pore sizes than a smaller molecular weight, but globular protein. Proteins close in molecular weight to the NMWC are likely to be evenly distributed between the two solutions as the NMWC is unlikely to be uniform across the membrane. It is also important to bear in mind that the pH, the ionic strength and the concentration of polyelectrolytes in the solution will all have some effect on the actual size of the protein, usually increasing it. Fouling of the membrane by particulate material will also lower the amount of ultrafiltrate formed by lowering the NMWC of the membrane [Harris, 1989]. To reduce the occurrence of fouling, the solution is commonly agitated or stirred close to the membrane. The membranes used for ultrafiltration procedures are generally constructed from cellulose acetate, but a range of synthetic polymer membranes are available e.g. polyamides, polysulphone, polyvinylchloride (PVC), and acrylonitrile allowing filtration over a larger pH range with a variety of aqueous and organic

solvents over a larger temperature range than the traditional ceramic filters [Harris, 1989].

1.3.1 Dialysis

Although dialysis is most commonly used to desalt a sample, it may also be used to remove small contaminating molecules and ions. It shows similarities to the methodology used during ultrafiltration, as it relies on small molecules being separated across a semi-permeable membrane which is subject to a Nominal Molecular Weight Cut-off point. The protein solution that is required to be concentrated is put into a semi-permeable dialysis bag, then added to a solution e.g. polyethylene glycol, (PEG) at 20% (w/v) or a powder, e.g. Sephadex, that will draw water through the membrane until an osmotic equilibrium is reached. It is, therefore, of considerable importance to replace the solution or solid regularly. The advantages of using a solution in place of a solid is that the protein will be protected from denaturation through losing all its water, as could happen using a solid. Conversely, PEG has been shown to contain impurities, several of which may damage the protein [Harris, 1989]. An alternative procedure involves applying a vacuum to a dialysis bag at atmospheric pressure in order to draw out water molecules, which amounts essentially to ultrafiltration.

1.4 Centrifugation

Centrifugation provides a useful alternative means of separating micro-organisms from a broth. It is a more expensive process than filtration and is generally used when the equivalent filtration would be too slow, or a higher standard of hygiene is required. Centrifugation is also preferable to filtration when the resulting filtrate is required to be free of the contamination which could potentially occur from the filter aids e.g. the scrapers, knife blades or string commonly used to free the membrane from the build up of filter cake.

In harvesting cells from fermentation broths, centrifugation generally relies on a continuous mode of action. The main factors affecting the rate of cell sedimentation are density differences between the cell and the culture medium, cell diameter, and viscosity of the broth. In an ideal situation, there would be a large density difference between the cell and the medium, the diameter of the cell would be large, and the viscosity of the broth low. In day to day situations, the converse occurs. Stokes law indicates that when cells aggregate they will sediment more rapidly due to their increased diameter, a point which is commonly exploited in large-scale schemes, typically by the brewing industry which chills the wort at the end of fermentation to encourage cell aggregation [Stanbury and Whitaker, 1993].

Centrifuges used on an industrial scale are operated on a continuous basis since non-continuous centrifuges are of limited capacity. A range of centrifuges exist, each

designed to fulfil the needs of a specific process (Table 1.1) [Stanbury and Whitaker, 1993; Whittington, 1989; Scopes, 1993].

Type of Centrifuge	Common Applications
Basket (Perforated-Bowl Basket)	Separating mould mycelia or crystalline compounds at low centrifugal speeds
Multichamber	Separating fine slurries. Regular dismantling is required as part of the cleaning process.
Solid-Bowl Scroll (Decanter)	Separating coarse materials at low centrifugal speeds to counter balancing problems.
Disc Bowl	Allows direct removal of the solid phase, although as solids still contain an appreciably high quantity of water, cleaning the many discs inside the centrifuge remains problematic. May be used to separate liquids e. g. Milk from cream
Tubular Bowl	Alternative to Multichamber centrifuges. Up to 10% solids may be processed at up to 60,000g. Heterogeneous liquids may also be separated. Problems arising from a gradual loss of activity are commonplace, as the machinery becomes clogged with particulate material.

Table 1.1 A range of large-scale centrifuges, and their applications

1.5 Precipitation

The general theory behind precipitation relies on the premise that the solubility of most proteins is lowered at high salt concentrations, thus causing a 'salting out' effect which is useful in concentrating dilute protein solutions. Salting out steps are also useful in purification processes as they provide a convenient opportunity to pause a scheme overnight, helping to protect the target protein or enzyme against degradation, denaturation, or contamination via bacteria [Harris, 1989]. Although this 'salting out' effect is not completely understood the differing relative solubilities of proteins in a salt concentration are exploited, and a crude fractionation occurs. One theory for the salting out mechanism is the involvement of hydrophobic patches. As the ionic concentration increases, the water around the patches interacts with the salt, exposing the patches. These patches begin to interact with patches on neighbouring molecules causing aggregation between proteins with a similar number and size of patches. Proteins with a greater number, or larger hydrophobic patches are thus the first to be precipitated [Harris, 1989; Scopes, 1993]. Most proteins exhibit minimum solubility at or around their isoelectric point.

At pH values higher than pI, the net negative charges on the surface of the globular proteins serve to repel other negatively charged molecules; conversely at pH values lower than pI the net positive charges on adjacent molecules will cause repulsion. At the pI of a protein when its surface exhibits a net neutral charge, repulsion is at a minimum between molecules and aggregation followed by precipitation is most likely.

As the pI values of proteins differ considerably, isoelectric precipitation (precipitation at the isoelectric point of a protein) can be a valuable purification tool [Scopes, 1993].

In the literature, 57% of the purification schemes studied included a precipitation step, over 75% using ammonium sulphate to precipitate out the protein [Bonnerjea *et al.*, 1986]. Precipitation often followed an initial homogenising stage, as it is capable of processing large quantities of material whilst remaining unaffected by contaminating non-protein materials. However, a poor purification factor occurs as many other proteins and organelles often get caught up in the aggregations and precipitate alongside the target molecule, hence the technique is not suitable for the specific purification of a target protein from a crude, heterogeneous mixture [Scopes, 1993]. The precipitate is commonly removed via centrifugation, (Section 1.2), or sometimes filtration (Section 1.1). Precipitation generally provides a high average yield, (81% observed by Bonnerjea *et al.*, [1986]), which compensates for the relatively low purification factor (average of three fold observed by Bonnerjea *et al.*, [1986]).

Strong acids e.g. perchloric or trichloracetic acid (TCA) may be used occasionally to purify proteins, although they are not suitable for purifying enzymes as they will affect the specific activity of the enzyme and may denature it. Most proteins are precipitated at TCA concentrations of 10%, and peptides will be precipitated concomitantly if the concentration is increased to approximately 20%. The precipitate may be washed in buffer and filtered to remove the excess TCA [Harris, 1989].

A common example of purification through precipitation involves the adjustment of

skim milk to pH 4.6. This precipitates the caseins in the milk, and separates them from the whey proteins which remain dissolved [Andrews and Alichanidis, 1983; Isgrove *et al.*, 1998]. The caseins are then removed either by filtration or centrifugation.

Proteins may also be precipitated using water-miscible solvents such as ethanol or acetone. The net effect of adding such a solvent to an aqueous protein solution is the decrease in solubility of the protein, primarily through aggregation caused by electrostatic and dipolar forces, and is more apparent around the pI of a protein. Unlike the interactions involved in the salting out mechanism, hydrophobic interactions have little or no effect as the hydrophobic patches on the surfaces of proteins attract the organic solvent, and if anything, enhance solubility [Scopes, 1993]. It is important that the ambient temperature does not exceed 10°C, as above this, the solvent will start to penetrate the globular domains of the proteins, and interact with the inner hydrophobic residues causing rapid denaturation of the proteins [Scopes, 1993].

Certain high molecular weight organic molecules are also successful at precipitating proteins out of solution without causing denaturation. Polyethylene glycol (PEG) (see Section 1.8, aqueous two-phase systems for further details) at molecular weights above 4000, has been shown to be suitable for the precipitation of proteins such as plasma proteins. Although PEG is quite difficult to remove from the solution, follow on techniques such as salting out, ion-exchange chromatography (Section 1.6.2) gel filtration chromatography (Section 1.6.1) and affinity chromatography (Section 1.6.3) are relatively unaffected by low PEG concentrations [Scopes, 1993].

Affinity precipitation takes advantage of the specific interactions between a protein and its ligand in much the same way as other affinity methods (affinity chromatography, Section 1.6.3, affinity aqueous two-phase systems, Section 1.8.5). In general it is not possible to precipitate out an enzyme merely by the addition of its ligand, although some exceptions to this observation occur e.g. when the ligand is a particularly large molecule such as a nucleic acid.

Once one has obtained a relatively crude extract solution free of particulate material it is then normal practice to proceed to higher resolution purification stages, such as those represented by chromatographic procedures.

1.6 Chromatography

Chromatographic procedures are useful tools to purify and isolate low concentrations of biomolecules from a relatively pure starting solution [Hochuli, 1988]. Liquid chromatography relies on a heterogeneous protein solution being passed though a stationary solid phase of gel beads tightly packed into a gel column [Andersson, 1992]. Separation of molecules is achieved by the different ways in which they interact with the stationary phase [Jonsson *et al.*, 1989]. All molecules are eluted by disruption of these interactions e.g. in ion-exchange chromatography by increasing the salt concentration of the liquid phase. The column matrix used depends on the application required, with size-based or Gel Filtration chromatography (Section 1.6.1), Ion-Exchange Chromatography (Section 1.6.2), Hydrophobic Interaction Chromatography (Section 1.6.5) and Affinity Chromatography (Section 1.6.3) comprising the four main

groups, while techniques such as Immobilised Metal Ion Chromatography (Section 1.6.4) are often valuable in specific applications. The advantages that chromatographic procedures have is that a very pure product can be obtained, particularly in the case of affinity or high performance methods. Unfortunately, chromatographic methods are unable to deal with relatively impure starting materials, as clogging of the column matrix occurs creating a build up of pressure, and ultimately causing damage to the column matrix. Similarly they are unable to cope with a large quantity of starting material.

1.6.1 Gel Filtration Chromatography

In gel filtration or size exclusion chromatography, the heterogeneous protein sample is applied to a column consisting of semi-porous gel beads made of an insoluble, but highly hydrated polymer such as cross-linked agarose, dextran, polyacrylamide *etc.* (commercially available as Sephadex, Sepharose, Superose, Ultrogel, Fractogel, Biogel, *etc.* [Andersson, 1992]). The pore size of beads is typically in the region of 100μm, meaning that whilst small molecules may penetrate the beads, larger molecules cannot, and remain in the spaces between the beads. This results in the larger molecules being eluted from the column first, as a smaller volume is accessible to them whilst the smallest molecules, which are distributed throughout the aqueous solution between and within the beads, emerge last [Stanbury and Whitaker, 1993].

1.6.2 Ion-Exchange Chromatography

Ion-exchange chromatography allows the separation of proteins according to their net charge, and may be defined as the reversible transfer of ions between the column matrix and the liquid phase. Columns are commercially available as either positively or negatively charged matrices, and as many proteins are negatively charged at physiological pH values, positively charged matrices are commonplace [Roe, 1989]. For a column with a negative matrix, positively charged proteins will selectively bind as the negatively charged species pass straight through. The matrix bound proteins may be eluted from the column by a change in pH, or more usually by the introduction of a salt concentration gradient, typically NaCl, into the system. The Na^+ ions compete with the positively charged groups of the bound protein for binding to the column, hence the less densely charged species will be eluted before the proteins with a higher charge density. For a separation to be successful, the mobile phase must be buffered to minimise pH fluctuations around the matrix which would cause diffusion between the mobile phase and the matrix at best, and at worst would denature the protein. The four main types of matrix commonly used in ion-exchange extractions are the positively charged Diethylaminoethyl (DEAE) groups and quaternary amino (QA), and the negatively charged Carboxymethyl (CM) or Sulphopropyl (SP) groups.

Ion-exchange chromatography may also be used to concentrate material. For example, the antibiotic streptomycin is easily and efficiently recovered from a filtered culture medium using a cationic matrix such as Amberlite IRC 50. The streptomycin is absorbed onto the column by displacement of the sodium ions that are loosely bound to the carboxylic group on the matrix. Using a slow flow through the column, and by adding only a small volume of culture medium, the antibiotic may be concentrated one hundred fold, and purified [Stanbury and Whitaker, 1993].

Ion-exchange chromatography is often used in association with gel filtration methods allowing a purification scheme that combines both size and ionic differences between the proteins in a heterogeneous mixture.

1.6.3 Affinity Chromatography

Affinity chromatography allows separation of biomolecules on the basis of their specific binding activities to the functional groups of substances such as enzyme-inhibitors, hormone-receptors, antigen-antibodies, lectin-carbohydrates *etc.* [Andersson, 1992; Stanbury and Whitaker, 1993]. The specificity provided means that affinity separations are considered as potentially one of the most powerful separation procedures, particularly on a preparative scale. The column matrix is modified by the covalent attachment of ligands for the target protein. Typical ligands used in affinity separations are the natural substrate or inhibitor of a target enzyme, or if an antigen is to be purified, the corresponding antibody may be immobilised. Following the flushing of the column with feedstock, the conditions are altered to allow elution of the target

molecule through disruption of the ligand/target molecule interactions.

Reactive dyes, originally developed for use in the textile industry, are also able to bind a range of proteins through a series of specific and/or non-specific interactions e.g. Cibacron Blue F-3GA which will selectively and specifically bind nucleotide-dependent enzymes such as oxidoreductases and dehydrogenases have also been proven to be important tools in the separation of biomaterials [Boyer and Hsu, 1993]. Indeed Scawen and Atkinson [1987] showed that Cibacron Blue was used as the affinity ligand in over 50% of a list of 228 examples of dye-ligand chromatography published between 1980 and 1987.

Antibodies represent the ideal choice for the separation of biomaterials, when higher specificity and sensitivity are required, indeed antibodies are sometimes the only choice of ligand for an affinity separation [Birbaum and Mosbach, 1991]. For example, the purification of a recombinant interleukin IL_2 isolated from Chinese Hamster Ovary (CHO) cells through specific interactions with its receptor which is attached to a matrix material is comprehensively described in a paper by Weber and Bailon, [1990]. However, the production costs of antibodies is extremely high, and their shelf-life relatively short due to their sensitivity, therefore antibodies are generally only used in the purification of expensive products.

The general disadvantages associated with any chromatographic procedure such as cost, the need for scale-up, and the prevention of matrix fouling also apply to affinity chromatography. In addition, the cost of the ligand is a major factor determining the

feasibility of this process on a large scale. Another potential problem is the observed leakage of matrix bound ligand into the eluent. This can be prevented to a certain extent by careful manipulation of the pH, ionic strength and temperature of the feedstock [Andersson, 1992; Stanbury and Whitaker, 1993; Boyer and Hsu, 1993]. The risk of ligand leakage is generally considered to be little more than minimal in the case of reactive dyes. However as the toxicity of the reactive dyes used in dye-ligand techniques remains relatively untested *in vivo*, the risks are considered to be sufficient for concern in the purification of therapeutic material as "FDA ruling mandates that no trace of ligand may be present in material intended for human use" [Boyer and Hsu, 1993]. When an inhibitor is used as a ligand in a separation process any ligand leakage into the purified product will have an obvious detrimental effect on the activity of the enzyme as it will ensure that the enzyme is constantly switched off.

Recent literature also discusses the potential for the application of recombinant hybrid proteins. In this technique, the genetic coding sequence for the protein of interest is combined with both coding sequences for a peptide containing high affinity for an affinity resin and for a specific cleavage site. The combined genetic sequence is then genetically engineered into the host micro-organism. The overall aim is, therefore, to purify the fusion proteins using the affinity tail, only cleaving the affinity tail when a homogeneous solution of the protein has been isolated. An example of this methodology which has been subjected to large-scale investigations is presented below. Smith *et al.*, [1984], describe the purification of the protein urogastrone with ion-exchange chromatography, following the genetic insertion of a DNA sequence encoding a polyarginine tail into *E.coli* urogostrone.

Another example involves the addition of poly histidine "tails" to the target protein, which allows the separation of target protein using Immobilised Metal Ion Chromatography techniques (See Section 1. 6. 4). An example of such technology is the attachment of a thiol-containing tail to galactokinase, and purifying it with covalent chromatography on an Activated Thiol Sepharose 4B (Pharmacia -LKB) [Mosbach, 1988]. Finally, the fusion of protein A to Human Insulin-Like Growth Factor 1 (IGF-1). Protein A has a very strong affinity for Immunoglobulin G (IgG) allowing purification of the Human Insulin-Like Growth Factor 1 using IgG as a ligand for protein A [Hochuli, 1988; Stanbury and Whitaker, 1993].

1.6.4 Immobilised Metal Ion Affinity Chromatography

Immobilised metal ion affinity chromatography (IMAC - formerly metal chelate chromatography) is an adaptation of affinity chromatography that relies on a matrix bound metal ion binding to an individual protein. The proteins have not been observed to be damaged during this technique [Porath and Olin, 1983; Andersson, 1992; Porath, 1992]. The metal ion is generally attached to the insoluble matrix support via a chelating agent and a spacer molecule [Hochuli, 1988; Porath, 1992,]. Many types of metal ions have been demonstrated to be suitable candidates for IMAC by virtue of their strong and rapid binding to the matrix bound chelators, e.g. Cu (II), Zn (II), Ni (II), Cd (II), Co (II) and Fe (III) with copper and zinc representing the metals that will complex most efficiently with histidine and cysteine residues at neutral pH [Andersson, 1992; Roe, 1989]. The basic concepts of an IMAC separation depend on

the localisation of the immobilised metal ions on the matrix surface; for a separation to be effective the metal ions must be in exposed positions hence allowing stable complexes of proteins and metal ions to form [Porath and Olin, 1983]. Desorption of the protein following the separation may take place via disruption of either the chelator-metal bond, or the metal-protein bond. Effective desorption mechanisms include the use of a competing ligand which will disrupt the metal-protein bond, or reducing the pH of the system hence raising the proton concentration, which will replace the metal ion on the matrix by disruption of the chelator-metal bond. Any protein released from the matrix by the latter method will require a tolerance of acidic pH values and will also require further purification from the metal ions. Adsorption can take place at high sodium chloride concentrations. Indeed, these conditions appear to increase both the adsorption capacity and the degree of selectivity of the system [Porath and Olin, 1983]. IMAC separations are a useful tool, particularly as scale-up is often easy and reproducible but as metal ions are seldom specific for an individual protein the level of purification they provide is low [Porath, 1992]. IMAC techniques have also been shown to be useful for concentrating very dilute solutions of a single protein [Porath, 1992].

1.6.5 Hydrophobic Interaction Chromatography

This chromatographic technique takes advantage of the hydrophobic residues exposed on the surface of many proteins, such as valine, leucine and isoleucine. In the presence of a salt such as ammonium sulphate or sodium chloride, which will serve to disorganise the water molecules around the hydrophobic patches; attractions will occur between the hydrophobic patches on the proteins and the matrix hydrophobic groups by the same mechanisms mentioned previously in the section on precipitation (Section 1. 5). As with precipitation techniques, the maximum binding *via* the hydrophobic regions occurs at the isoelectric point of the protein. The most common matrices on the market are based on an agarose support with attached phenyl or octyl groups, and may be purchased directly from the manufacturer under the trade names of phenyl-sepharose, phenyl-superose, alkyl-superose, TSK-Phenyl [Roe, 1989, Scopes, 1996]. A suitable matrix should be chosen based on the hydrophobic properties of the target protein. If the target protein has very strong hydrophobic regions, a less hydrophobic matrix should be chosen such as octyl sepharose to prevent denaturing the protein during elution. Elution often involves raising the pH of the buffer, causing the matrix bound protein to gain a net negative charge and to be released from the matrix. Other elution methods include reducing the ionic strength or displacing the target protein with a molecule which is highly hydrophobic itself e.g. propanol, butanol. Alternatively, a non-ionic detergent such as Triton X-100 or Tween 20 may be added to the solution to displace the target protein without causing denaturation.

The main disadvantage of chromatographic procedures is the scale at which they can be performed. The movement of liquid through the column relies on pressure, whether generated by gravity or via a pump. Any excessive pressure will cause damage to the solid phase, and can cause tunnelling of proteins resulting in an ineffective separation. Tunnelling describes the overlapping of protein bands during a separation, when movement through the centre of the matrix is more rapid than at the edges, and it is not possible to collect distinct fractions. Production costs of chromatography are, therefore, extremely high for large-scale purifications.

1.7 Electrophoresis

The process of electrophoresis is dependent on the observation that a charged molecule will migrate through a permeable solid phase within an electric field [Andrews, 1986; Dunn 1989; Laars *et al.*, 1989]. The migration of a protein or an enzyme will depend on its charge density at a given pH and temperature, hence molecules with different charge densities will be separated into distinct bands. Electrophoresis is primarily an analytical tool, used in conjunction with a separation process to monitor progress. Horizontal slab electrophoresis has been used as a preparative tool, but the observed resolution is rarely better than with ion-exchange chromatography. Similarly vertical column electrophoresis has been used in preparative processes, using a sucrose density gradient to help stabilise the buffer. Following separation, each protein band is collected by slowly allowing the liquid to run out. Common problems are the distortion of the protein bands, excessive heat

generation, and more practical difficulties such as setting up the column.

The most common gel ingredient for electrophoretic separations is polyacrylamide, although successful separations may be obtained using agarose, starch and cellulose acetate gels [Dunn, 1989]. When a PAGE gel is used as an analytical tool, as little as 5-25 μg protein can be detected using Coomassie blue staining, with silver staining techniques shown to be up to 100 times more sensitive.

1.7.1 Native gel electrophoresis

In native gel electrophoresis the proteins are retained at a pH where they remain stable and in their native form. The method exploits differences in charge and size of the proteins. To aid migration through the gel, an alkaline pH (8-9) is chosen ensuring negatively charged proteins and migration to the anode at the foot of the gel. To facilitate separation the total concentration of the acrylamide and the amount of the cross linking agent N, N'-methylene bisacrylamide (Bis) can be altered. By increasing the acrylamide concentration, the pore size of the gel is reduced, slowing the migration of the larger molecules. The separation ranges of acrylamide gels of various acrylamide concentrations are shown in Table 1.2.

Acrylamide concentration (%)	Optimum molecular weight range
3-5	Above 100 000
5-12	20 000-150 000
10-15	10 000-80 000
15+	Below 15 000

Table 1.2. The separation ranges of acrylamide gels of various concentrations [Andrews, 1986]

Polymerisation occurs following the addition of ammonium persulphate (APS), which oxidises TEMED, (N, N, N', N'-tetramethylethylenediamine), an organic base, to give free radicals which then interact with the double bonds in both acrylamide and Bis resulting in the "vinyl polymerisation" mechanism.

1.7.2 SDS-PAGE

Sodium dodeceyl sulphate (SDS) -PAGE analytical separations have many advantages including very high resolution, inexpensive costs and ease of use. The separation of proteins is obtained by virtue of a single parameter, their molecular radius (i.e. molecular weight). Because of the solubilising power of the detergent (SDS) it is particularly useful for the analysis of insoluble proteins such as membrane proteins. The overall polyacrylamide concentration will determine the separation range of an SDS-PAGE procedure. Typically 5% T (total acrylamide + bis) gels are used to

separate proteins in the size range of 20-350 Kilodaltons (kd) and 10% T gels are used for proteins in the range of 15-200 kd.

The majority of proteins and polypeptide chains will bind 1.4g of SDS for each gram of protein, effectively covering the protein and masking its charge density. The detergent disrupts quaternary structure and effectively adds three negative charges to each peptide bond ensuring that, if charge were the main separating factor, the polypeptides in the heterogeneous mixture would all migrate together. The samples are also boiled in 2-mercaptoethanol to eliminate any disulphide binding and, therefore, to disrupt tertiary structure. Obviously such a procedure causes irreversible damage to the protein, thus destroying any biological activity. The process does however have its advantages, and in addition to facilitating the study of insoluble proteins, is particularly useful for determining the subunit structure. A common problem associated with SDS-PAGE is the observation that small polypeptide chains (<10 kd) tend to form molecules with SDS that are too similar in charge and molecular radius to be separated, hence they all migrate as part of the same band. Additionally, the relationship of charge per unit length (i.e. charge density) being constant tends to break down at short polypeptide chain lengths. In an attempt to eliminate these findings, and to prevent clogging of the gel with protein aggregates, the samples are often treated with 8M urea. The urea lowers the size of the detergent micelle and, thus, the size of the peptide detergent complexes. Polypeptides in the range of 1-45 kd can then be separated.

1.7.3 Isoelectric Focusing

Isoelectric focusing allows the separation of proteins according to their net charge [Andrews, 1986]. By setting up a pH gradient within a non-sieving gel, and introducing an electric current, proteins migrate to the pH level equivalent to their isoelectric point (pI), at which their net charge is zero [Scopes, 1993]. The pH gradient is established using ampholytes which consist of low molecular weight molecules with varying pIs covering the chosen pH range. This range may be broad to separate proteins with largely different pI values, or it may be narrow to allow the separation of proteins with very similar pI values. When a current is applied to the ampholyte solution, the molecules and the proteins migrate together, the smaller size of the ampholytes facilitating their movement and allowing the rapid formation of a pH gradient. Isoelectric focusing affords a high resolution, as if a protein should happen to migrate too far, it becomes charged again, and migrates back to its pI. Isoelectric focusing has found useful applications as both a preparative and an analytical technique.

1.7.4 Capillary electrophoresis

The use of capillary electrophoresis in analytical laboratories is increasing as it provides a useful alternative to other high resolution, rapid techniques. This procedure allows the analysis of minute quantities of sample (ηg) through the utilisation of glass capillaries, thin liquid films or very narrow tubes. As the protein bands pass through a section of the capillary, they are automatically monitored and recorded. One of the main advantages of this process is that the results are quantitative. The apparatus is designed to allow fractionation by native electrophoresis, SDS electrophoresis, or isoelectric focusing. Ludi *et al.*, quoted by Andersson [1992] describe the separation of a group of closely related peptides, indicating the resolving power of this method. The method has also been shown to greatly facilitate peptide separating techniques by the exploitation of very subtle differences in charge densities between the peptides [Cobb and Novotny, 1989].

1.8 Aqueous two-phase systems

Aqueous two-phase systems represent a novel separation technique exploiting the mutual exclusion of two water soluble polymers, or a polymer and a salt. When these polymers are mixed together at concentrations exceeding a certain critical point, a two-phase system will form spontaneously [Albertsson, 1971 and 1986; Kula, 1990]. The more hydrophobic polymer forms the upper phase, and the more hydrophilic, or the salt, forms the lower phase. Aqueous two-phase systems are being investigated in an attempt to provide large scale, small step purification procedures e.g. Rito-

Palomares *et al.*, [1998] who describe a two-stage process for the isolation of proteins from cheese whey (for reviews of aqueous two-phase systems please refer to Albertsson, [1986], Andrews and Asenjo, [1986]; Walter and Johansson, [1986]; Albertsson *et al.*, [1990] and Kula [1990]).

At best, these systems have been shown to be sufficiently sensitive to distinguish between proteins differing by only 12 amino acids in length, as demonstrated by Andersson and Hahn-Hägerdal, [1990], working with native β-galactosidase and a fusion protein consisting of β-galactosidase and an additional 12 amino acids.

The phenomenon of phase separation is well documented, and is thought to have been first observed in 1896 by the Dutch microbiologist Beijerinck, when he mixed a solution of agar with gelatine [Grossman and Gainer, 1988; Kula, 1990]. Albertsson discovered the importance of aqueous two-phase systems in 1956, when he realised that in addition to phase separation, partitioning of biomaterial could take place. Aqueous two-phase extraction relies on the differentiated partitioning which occurs when a protein mixture is added to a phase-separated aqueous solution of incompatible polymers [Forciniti *et al.*, 1991(a)]. Generally, macromolecules, nucleic acids and many proteins tend to partition preferentially into the lower phase. However, by altering conditions such as polymer molecular weight and concentration, or salt type and concentration, the target protein may be encouraged to selectively partition into the upper phase.

As both polymer phases consist mostly of water, (typically exceeding 75%,

[Andersson and Hahn-Hägerdal, 1990] or 60-95% [Asenjo *et al.*, 1990]), a gentle medium is formed allowing partitioning of cells, [Walter and Johansson, 1986; Wang *et al.*, 1988; Park and Wang, 1991] enzymes and proteins, [Hustedt *et al.*, 1985; Johansson and Reczey, 1998], with little disruption of structure or activity. Additionally, the interfacial tension between the phases is very small, typically 0.1-100 μN/m, [Albertsson *et al.*, 1990].

The most common two phase systems consist of the polymers polyethylene glycol (PEG) and dextran, or PEG and a salt phase, with potassium phosphate and magnesium sulphate being the most frequently used salts. Various groups have investigated the substitution of other molecules for the lower phase, e.g. hydroxypropyl starch [Tjerneld *et al.*, 1986; Ortin *et al.*, 1992; Almeida *et al.*, 1998], citrate [Vernau and Kula, 1990; Marcos *et al.*, 1998], hydrophobically modified dextran [Lu *et al.*, 1995]; or by substitution of PEG by another molecule e.g. $C_{12}E_5$ (pentaethylene glycol mono-n-dodecyl ether) [Sivars *et al.*, 1996]. Some recent research has been directed towards utilising aqueous two-phase systems composed of ethylene oxide and propylene oxide [Johansson *et al.*, 1997].

PEG, a synthetic linear polymer has molecular weights in the region of 200-40,000, and, as described in Andersson and Hahn-Hägerdal, [1990], has the general formula;

$$HO{-}CH_2{-}(O{-}CH_2{-}CH_2)_n{-}O{-}CH_2{-}CH_2{-}OH$$

The solubilisation of PEG in water occurs due to hydrogen bonding between the water molecules and many or indeed all of the ether sites along its chain, Franco [1992].

Dextran is a natural storage polymer produced by the lactic acid bacterium, *Leuconostoc mesenteroides,* and is an α-1,3 linkage branched α-1,6 linked glucose polymer [Andersson and Hahn-Hagerdal, [1990]). Dextran produced by other bacterial and yeast species may be branched by α-1,2 or α-1,4 linkages, [Stryer, 1988]. Both polymers are relatively cheap, non toxic and biodegradable.

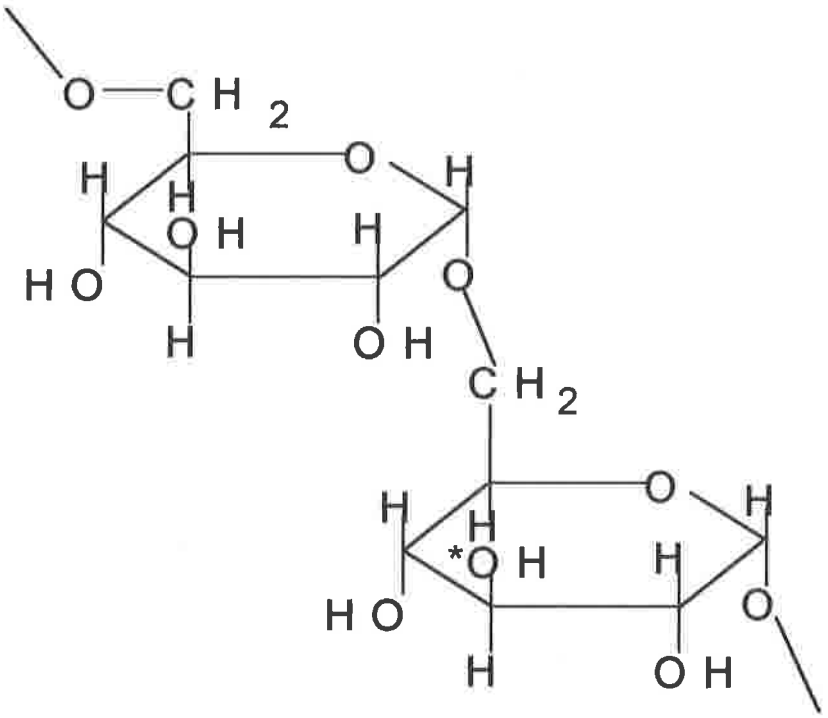


Figure 1.1. An α-1,6 branch on α-1,3 linked dextran produced by the lactic acid bacterium, *Leuconostoc mesenteroides*. Links occur at the oxygen atom labelled *.

The advantages that aqueous two phase systems have over other separation methods are extremely diverse. Once the ideal system has been established for a particular protein, the system can be easily scaled up, producing a high yield of product. A relatively fast approach to equilibrium is reached, with little damage caused to the partitioned material [Grossman and Gainer, 1988]. On a laboratory scale, the aqueous two-phase systems can also be centrifuged to further decrease the settling time caused by the low interfacial tension. Additionally, no clarification steps are required as no fouling is encountered.

Disadvantages attributed to aqueous two-phase systems relate to the observed difficulties in achieving ideal partitioning conditions of the target molecules which have resulted in rather low purification factors. Many models for partitioning exist but, they have not yet been accurately translated from theory into practice. Furthermore, the ability of a model to successfully predict the design of an aqueous two-phase systems to enable one-sided partitioning of a protein does not yet exist [Andersson and Hahn-Hägerdal, 1990].

The concentration of the polymers at which phase separation will occur can be represented by a phase diagram [Albertsson, 1986]. A phase diagram displays the dependence of phase separation on the concentrations of the aqueous two-phase components under specified conditions.

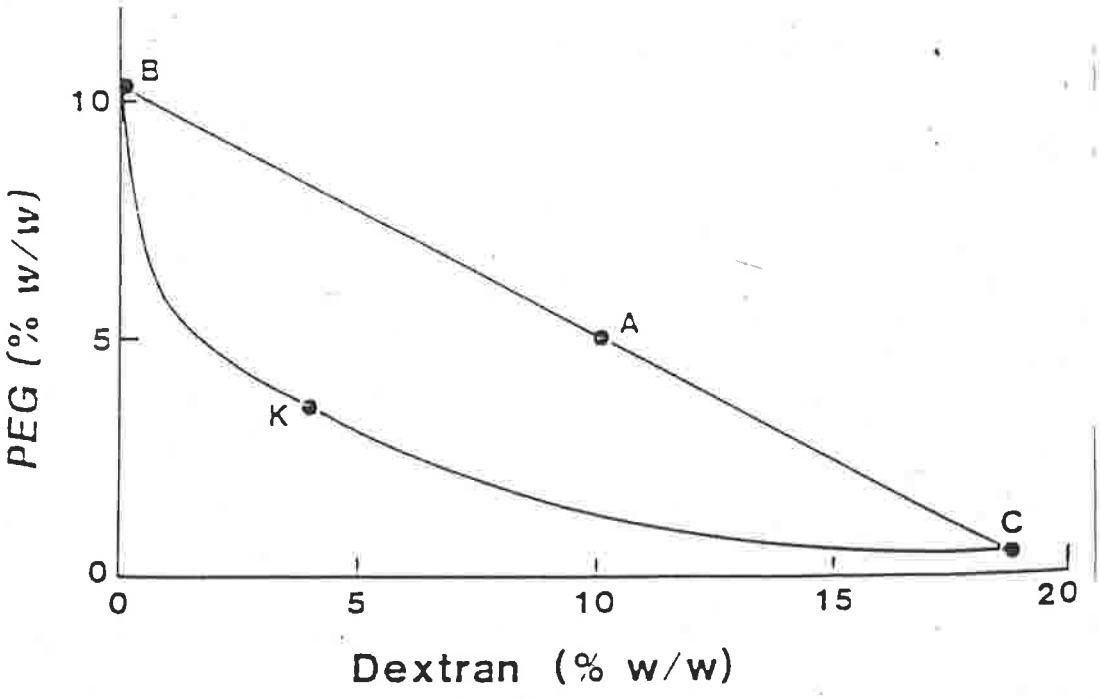


Figure 1.2 A typical phase diagram

The curve BKC in Figure 1.2 is a binodial curve. It separates the area to the left where the two components are found in a homogenous solution, from that to the right where two phases form. It passes through all the composition points of all the top phases, the critical point, K, and all the composition points of all the bottom phases. The line BAC is called a tie line. It passes through points B and C which represent the composition of the top and bottom phases of the system, and point A representing the total composition of the system. Any total composition of a system represented by a point on the same tie line will give rise to phase systems with identical phase

compositions, but different volumes of the two phases [Albertsson, 1986]. The phase volume ratio is represented by the ratio of BA:BC.

The partitioning behaviour of any material in a phase system is determined by the properties of the polymers present, their compositions, and the physio-chemical composition, as the material will interact directly with the polymers. The partition coefficient, (K) of a substance in a system is defined as the ratio between the concentrations of solute in the upper and the lower phases, as shown below.

$$K = \frac{C_T}{C_B}$$

Equation 1.1

where C_T is the concentration at equilibrium in the top phase and C_B is the concentration in the bottom phase.

It has been observed that most molecules and particles tend to partition into the dextran phase under most conditions. It is therefore usual to selectively extract the desired material into the upper phase. This is achieved by manipulation of the following factors to try to increase K of the desired substance without simultaneously increasing K of the contaminating components. The logarithm of the partition coefficient can be expressed as the sum of the logarithms of the following individual factors.

$$\ln K = \ln K_{el} + \ln K_{hphob} + \ln K_{size} + \ln K_{biosp} + \ln K_{conf} + \ln K^{\circ}$$

Equation 1.2

Additionally, a target protein with a relatively low partition coefficient may still give good yield in the upper phase, if the phase volumes are manipulated such that the upper phase is larger than the lower phase [Albertsson *et al.*, 1990].

According to the above equation, partitioning is determined by the accumulative effects of the following terms.

(i) Electrochemical effects (el). The influence of the difference in electric potential between the phases. Salts are often used to exploit any electrochemical differences between partitioning molecules, by varying the type and concentration of salt, [Merchuk *et al.*, 1998], and the pH of the system [Johansson, 1974; Bamberger *et al.*, 1984; Niven *et al.*, 1990 (b); Pfenning *et al.*, 1998]. Additionally modification of the PEG to attach a positively charged ligand e.g. trimethylamino-PEG will attract negatively charged proteins, and exclude positively charged proteins.

(ii) Hydrophobic effects (hphob). As the predominant polymer in the upper phase, PEG, is more hydrophobic than dextran, proteins with hydrophobic binding sites are encouraged to partition into the upper phase. These effects are most prominent at the isoelectric point of the protein of interest [Franco, 1992; Terstappen *et al.*, 1992].

(iii) Size effects (size). Both the size of the polymers used, and the size of the partitioned material have effects on partitioning, as will be discussed in Section 1.8.3.

(iv) Affinity effects (biosp). Affinity ligands can be attached to polymer molecules to enhance the purification of a target protein, [Flanagan and Barondes, 1975; Johansson, 1989; Nguyen and Luong, 1990; Boyer and Hsu, 1993] as described in Section 1.8.5. Examples of affinity partitioning include the partitioning of horseradish peroxidase by Bovine Serum Albumin (BSA) attached to modified PEG [Elling and Kula, 1991], and the partitioning of thaumatin using PEG bound glutathione [Asenjo *et al.*, 1990].

(v) Conformation effects (conf). The conformation of the partitioning protein is thought to have an effect on partitioning, but details of the mechanisms involved in the interactions between target molecules and phase components are scarce. Changes in protein conformation during partitioning have been suggested to be influential on the quality and yield of product [Lebreton *et al.*, 1998].

(vi) Other effects (°) are comprised of further parameters that are considered to have an effect on partitioning, e.g. temperature [Hustedt *et al.*, 1985; Albertsson, 1986], or the sequence in which a phase system is formed [Johansson, 1997; Lebreton *et al.*, 1998].

1.8.1 The effect of salts on partitioning in PEG/dextran aqueous two-phase systems

The addition of a salt to an aqueous two-phase system can affect partitioning in various ways as there are significant differences in the way salts are found to partition between the phases [Albertsson *et al.*, 1990]. The constituent ions of a salt often partition in a differential manner, introducing a net charge across the system. The ways in which salts affect partitioning are discussed below.

First, the type of salt used will influence partitioning as different salts have antagonistic effects. For example, potassium iodide introduces a net negative charge into the upper phase, encouraging partitioning of positive molecules into the upper phase; whereas lithium phosphate introduces a negative charge into the dextran phase, causing the opposite to occur [Walter and Johansson, 1986]. Cations have been found to decrease the partition coefficient of negatively charged proteins in the order $Li^+ < NH_4^+ < Na^+ < Cs^+ < K^+$, and monovalent anions in the order $F^- < Cl^- < Br^- < I^-$,with the divalent ions phosphate, sulphate and citrate all increasing the partition coefficient relative to the monovalent ions [Albertsson *et al.*, 1990]. Positively charged proteins are obviously affected in the opposite way.

Salts such as phosphate, sulphate and citrate have been shown to lower the binodial curve of an aqueous two-phase system [Bamberger *et al.*, 1983]. In other words, they reduce the polymer concentration required to form a two-phase system. A

PEG/dextran system containing one of these salts will therefore have a comparatively lower concentration of contaminating polymer in each phase when compared with an aqueous two-phase systems containing no salt. Partitioning is hence influenced according to the polymer concentration.

Second, the concentration of salt used will influence partitioning. Johansson, [1989] described how the effect of the addition of a salt will influence all protein partitioning, but particularly those proteins with a large net charge. The addition of a salt therefore, can be used to selectively extract a protein with a large overall surface charge from a mixture of proteins. As the net charge of proteins varies according to pH, salt concentration and pH are often manipulated to facilitate the purification of a target molecule.

1.8.2 The effect of hydrophobicity on partitioning in PEG/dextran aqueous two-phase systems

The hydrophobicity of biomaterial may be considered to have an important influence on partitioning in aqueous two-phase systems, as the systems consist of a hydrophobic phase and a hydrophilic phase [Hagarova and Breier, 1995]. Hence proteins with hydrophobic sites are more prone to partition into the upper phase. Additionally, the introduction of a low concentration into the PEG of bound hydrophobic groups such as palmitate will increase the partition coefficient of proteins with hydrophobic binding sites [Albertsson *et al.*, 1990].

1.8.3 The effect of size on partitioning in PEG/dextran aqueous two-phase systems

It is a generally accepted finding that when the molecular weight of one polymer is reduced, whilst the total concentration of polymers is maintained on a weight per weight basis, a protein will have an increased affinity for the system containing the lower molecular weight polymer [Albertsson 1971 & 1986; Albertsson *et al.*, 1987; Johansson 1989; Albertsson *et al.*, 1990; Forciniti *et al.*, 1991 (a) and (b)].

The main effect of increasing the polymer molecular weight of a system is that the binodial is reduced, causing a simultaneous increase in tie line length [Bamberger *et al.*, 1985; Albertsson, 1986; Johansson *et al.*, 1998]. This effect of PEG or dextran molecular weight on the tie line length appears to level off at very high polymer concentrations (average PEG molecular weights of 15,000-20,000) [Forciniti et al., 1991 (b)]. Similarly, the effects of polymer molecular weight cannot be completely isolated from hydrophobic effects, as by altering the molecular weight of PEG whilst maintaining equivalent concentrations, the hydrophobicity of the phase will also be altered. Albertsson *et al.*, [1990] showed that the effect of the polymer molecular weight is also dependent on the molecular weight of the partitioned protein, with larger proteins tending to be influenced to a greater extent than smaller proteins. The effects of polymer molecular weight on partitioning are discussed further in Chapter 3, Section 3.2.2.1.

1.8.4 The effect of polymer concentration on partitioning in aqueous two-phase systems

An increase in polymer concentration shifts the aqueous two-phase systems away from the binodial curve and results in more extreme partitioning of biomaterials through the increased exclusion effects of the polymers [Walter and Johansson, 1986; Albertsson *et al.*, 1990; Marszal *et al.*, 1995]. Hartounian and Sandler, [1991] reported that the addition of a PEG of any molecular weight causes a decrease in the concentration of the original molecular weight PEG in the dextran phase. Hence, by increasing the concentration of just one polymer, the system will exhibit more extreme polymer partitioning.

Niven *et al.*, [1990] indicated that for some proteins, partitioning could be (inversely) correlated to polymer concentration with more pronounced partitioning being observed at higher polymer concentrations. Unfortunately, the viscosity of the phases increases concomitantly with polymer concentration, and the systems become progressively more difficult to handle [Albertsson, 1986]. However, this increased viscosity can be counterbalanced by the introduction of a higher molecular weight polymer which lowers the concentration required to create an aqueous two-phase system.

1.8.5 Affinity partitioning in PEG/dextran aqueous two-phase systems

The introduction of an affinity ligand into one of the phases of a two-phase system has afforded dramatic increases in the efficiency of partitioning. PEG has been shown to be relatively easy to derivatise and, in practice, only a fraction of the PEG is required to act as a ligand carrier [Johansson, 1989].

Affinity extractions can take advantage of general protein binding ligands such as Triazine dyes, or may be biospecific for a particular protein. For example, Elling and Kula [1991] produced monoclonal antibodies for porcine lactate dehydrogenase isoenzyme 5 and purified the enzyme in aqueous two-phase systems by attaching the antibodies to PEG-oxirane molecules. The target enzyme was thus specifically partitioned into the upper phase. Other performed separations include the attachment of fatty acids to PEG to selectively extract α-lactalbumin, [Shanbhag *et al.*, 1991], and the derivatisation of PEG to PEG-epoxy-oxirane to purify BSA and protein A [Head *et al.*, 1989]. Methods for the derivatisation of PEG are constantly under investigation [Zalipsky *et al.*, 1992].

The affinity ligands will not affect the partitioning of other material in a system, hence for an effective separation, contaminants are usually required to have a low partition coefficient. The effect of the affinity ligand on the partition coefficient of the target protein is additive to its observed partition coefficient in a non affinity system, as demonstrated by equation 1.2. These effects have been shown to increase with increasing ligand concentration until a saturation effect is observed [Johansson,

1989].

1.8.6 Magnetic partitioning in PEG/dextran aqueous two-phase systems

In an attempt to increase the speed of phase separation, the addition of iron oxide particles and the influence of a magnetic field has been studied [Wikstrom *et al.*, 1987; Flygare *et al.*, 1990]. In these examples, magnetically susceptible particles (e.g. ferrofluids) are added to the phase system, and partitioning is accelerated by the application of a magnetic field to the lower phase. The authors suggest that the increase in phase separation is due to the ability of dextran to attract iron oxide particles through hydrogen bonds. The dextran is hence forced to separate faster by the movement of the iron particles towards the magnets. This application is thought to have particular significance in high polymer concentration phases where phase separation is extremely slow due to the viscosity of the system.

1.8.7 Heat induced partitioning in aqueous two-phase systems

The thermodynamic properties of PEG/dextran systems have been studied for a number of years, and various models have been provided to describe them [Kang and Sandler, 1988; Haynes *et al.*, 1989]. More recently, research has turned towards investigating novel systems which exploit temperature induced phase separation. The ability of PEG to separate from solution following sufficient heating is well known, and the temperature at which this occurs is known as the cloud point [Albertsson, 1986]. Systems including random copolymers of ethylene oxide and propylene oxide have

been shown to reduce the cloud point of a system [Alred *et al.*, 1994].

Temperature induced partitioning is proposed as a second step in an aqueous two-phase systems. Following the selective extraction of the target molecule into the upper phase, it is suggested that the polymer may be easily removed from the target by raising the temperature of the system above its cloud point. This causes the PEG (and/or the copolymers) to form a lower phase with the target remaining in the upper phase [Johansson *et al.*, 1997; Cunha *et al.*, 1998]. The traditional methods of removing the target from the PEG such as chromatography and dialysis, are therefore avoided.

1.8.8 Partitioning in PEG/salt aqueous two-phase systems

Partitioning and phase separation in PEG/salt systems are less well understood, as the majority of work has been performed using PEG/dextran systems [Albertsson, 1986; Johansson, 1989].The main salts used in aqueous two-phase systems are phosphate, sulphate and, more recently, citrate.

PEG/salt systems are considered to be more suitable for large scale procedures than PEG/dextran systems due to their low viscosity, large density differences between phases, and low cost [Grossman and Gainer, 1988]. However, much research has been directed towards recycling the salt phases to reduce environmental pollution [Kula, 1990].

One of the primary mechanisms of partitioning in a PEG/salt system is salting out of proteins from the lower phase, and the partition coefficient of proteins has been found to increase with increasing salt concentrations [Andrews and Asenjo, 1989]. However, as the solubility of a protein is dependent on its individual characteristics, proteins within a mixture will behave differently [Kula 1990; Franco 1992].

1.9 Freeze drying

It is often advisable to store proteins and /or enzymes as dry powders following their purification, as most proteolytic processes have been shown to require the presence of water. Also, the material is obviously much more convenient to handle and package when dehydrated, as is demonstrated by the availability of these products as freeze-dried powders from many of the leading manufacturers (e.g. Sigma, BDH, Fisons). It is erroneous to imagine that all the water will be eliminated from the powders, but as any salts present will also be concentrated they will ensure the effective inactivation of the contaminating enzymes. It is of importance to choose the buffer carefully as certain salts e.g. phosphates may cause a large reduction in pH on cooling and could potentially damage the target protein.

For freeze drying to be practical, the protein or enzyme solution should first be frozen from the liquid state. On a laboratory scale this is achieved by adding the protein solution to a pear-shaped flask, and by slowly rotating the flask over a mixture of dry ice and methanol. A thin layer of the protein solution is thus accomplished. On an industrial scale, the solution may be placed in a shallow tray, (the speed of freeze

drying depends on the thickness of the sample and its surface area, hence it is advisable to maximise the surface area to volume ratio), and placed in a blast freezer.

The sample is then placed in a vacuum freeze drier in the frozen state e.g. the Armfield Vacuum Freeze Drier FT33. The temperature of the freeze drier condenser reduces to -20°C and the vacuum serves to remove the water particles by a sublimation process.

1.10 Biospecific separations

Any affinity-based separation exploits the high affinity of many proteins for specific chemical groups. In most cases, the ligand is bound to a supporting molecule, such as a chromatography column matrix (Affinity chromatography, Section 1.4.3) or a phase-forming polymer (Affinity aqueous two-phase systems, Section 1.8.5). In order to achieve an effective separation, the technique must take place at conditions favourable to the formation of a ligand-target complex. To elute the target from the ligand, conditions may be altered to favour the dissociation of this complex. For example, in the purification of trypsin using soybean trypsin inhibitor (SBTI) the trypsin-SBTI complex is formed at pH 8, and the trypsin is eluted by lowering the pH to 2.3 [Niven and Scurlock, 1993]. Alternatively an elution buffer containing a high concentration of the ligand may be used. as for example in the purification of concanavalin A using column bound glucose [Stryer, 1988]. A schematic representation of a typical affinity chromatography procedure is outlined in Figure 1.3. Most affinity techniques will rely on similar binding mechanisms.

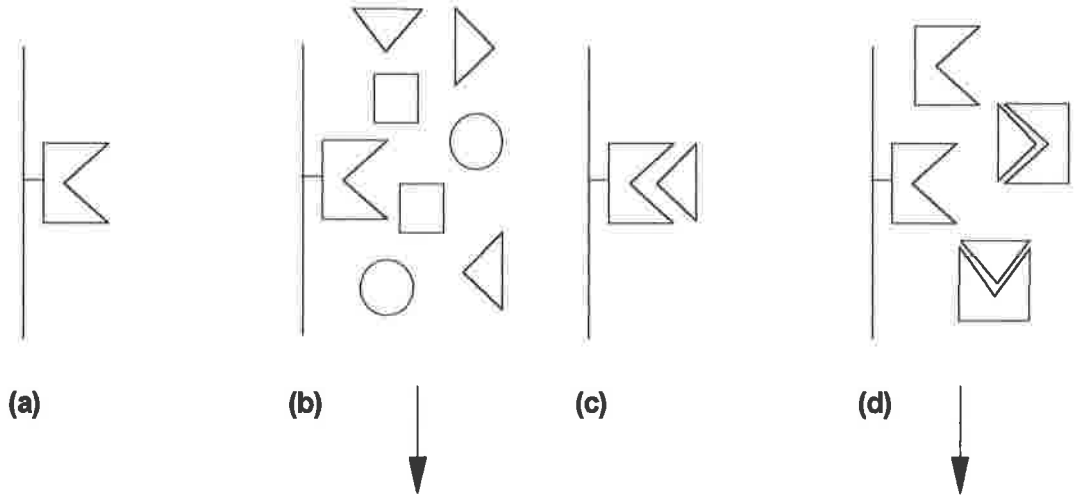


Figure 1.3. A schematic diagram showing a typical affinity chromatography separation. (a) The ligand (represented by the indented rectangle) is attached to the matrix. (b) A feedstock containing the target material (represented by a triangle) is flushed through the column. (c) The target material is specifically attached to the ligand. (d) The target is eluted from the ligand by an elution buffer containing a high concentration of the ligand.

The choice of ligand, support and the attachment of the ligand to the support are of paramount importance in an affinity separation. Much research therefore has involved optimising the supports and ligands used, and the coupling technology involved [Woodward, 1985; Scouten, 1987; Angal and Dean, 1989; Chaplin and Bucke, 1990; Scopes, 1994].

1.10.1 The choice of ligand

The main parameter defining ligand choice is its specificity for the target material. Affinity separations include either biospecific interactions between the ligand and

target enzyme e.g. enzymes-effectors/substrates, hormones-receptors, and antigen-antibodies, or more general interactions between ligands and a group of targets e.g. Triazine dyes-dehydrogenases, lectins-glycoproteins and fatty acids-fatty acid binding proteins.

The ligand should display a high level of stability at all the reaction conditions to extend the shelf life of the affinity adsorbent. It is also vital that the ligand is able to bind to the support without significant denaturation of its active site(s), and in an orientation that allows target binding.

The cost of producing a ligand and its potential shelf life also have an influence on the suitibility of a material, particularly in an industrial process where the cost of a ligand must be balanced against the cost of alternative, less specific separation techniques.

1.10.2 Choice of support matrix and spacer molecules

Any given support matrix must be strong and inert to minimise non-specific interactions. It should be stable at all reaction conditions, but should be relatively easy to derivatise. Examples of typical support molecules are polysaccharides, [Scouten, 1987] e.g. agarose, [Stults *et al.*, 1983], chitosan, [Freeman and Dror, 1994; Petach and Driscoll, 1994], PEG, [Head *et al.*, 1989; Johansson, 1989; Zalipsky *et al.*, 1992], glass, [Scouten *et al.*, 1977], and nylon [Morris *et al.*, 1975; Andrews and Mbafor, 1991; Iborra *et al.*, 1992; Aguado *et al.*, 1993; Niven and Scurlock, 1993; Niven *et al.*, 1994].

Spacers are used to spatially remove the ligand from the matrix to minimise steric hindrances. In order to reduce non-specific interactions the spacer should not itself bind with proteins.

1.10.3 The attachment of the ligand to the support matrix

It is not possible to give a detailed account of all ligand coupling techniques in this work, so for a more complete review of current methods, the reader is referred to the numerous texts available, including Woodward, [1985], Scouten, [1987], Angal and Dean, [1989] and Chaplin and Bucke, [1990].

The most widely studied mechanism for covalent enzyme coupling involves the activation of the support, generally agarose, with cyanogen bromide. This process is both rapid and relatively easy to perform on a laboratory scale. Unfortunately the by-products formed during the coupling are highly toxic, and worker safety has limited the industrial use of this mechanism [Scouten, 1987].

Another common coupling technique which takes advantage of hydroxyl groups on the support e.g. agarose and cellulose, uses sulphonyl chlorides. The procedures involved are very similar to coupling with cyanogen bromide, and typically take place overnight at room temperature [Nilsson and Mosbach 1984].

Activation of nylon, whether in powder, bead, or film form may take place in a

variety of ways. Morris *et al.*, [1975] describe the attachment of hexokinase to nylon tubes by activating the nylon with triethyloxonium tetrafluoroborate or dimethyl sulphate. Once the matrix was substituted, the enzyme was bound to the nylon using glutaraldehyde linkages. Aguado *et al.* [1993], describe a similar technique for the coupling of β-glucosidase to nylon powder. This type of mechanism is, however, thought to produce very toxic intermediates [Scouten *et al.*, 1987].

A more convenient and safer way to activate nylon film prior to the glutaraldehyde binding step, is to incubate the nylon in hydrochloric acid which produces both amino and carboxyl groups [Andrews and Mbafor, 1991; Niven and Scurlock 1993; Niven *et al.*, 1994]. The acid hydrolysis must be controlled to prevent a reduction in mechanical strength of the support. This coupling mechanism is discussed further in Chapters 5 and 6 of this work.

1.10.4 The immobilised enzyme

As mentioned previously, many enzymes have been immobilised onto a matrix for use in affinity separations. In this work, the enzyme β-glucosidase was chosen as a model ligand.

Glycosidases are a group of enzymes which catalyse the hydrolysis of glycosidic bonds in sugars, glycoproteins or glycolipids. They are present in the gastro-intestinal tract of animals, to prepare food for adsorption, and inside all cells to facilitate the degradation of complex carbohydrates. Glycosidases are of importance in the

biotechnological industries, particularly the food industry which uses them in the large scale production of invert sugar from sucrose and/or starch [Flowers and Sharon, 1979].

In terms of specificity, glycosidases are able to distinguish between the α and β moieties of sugars. Furthermore, the glycosyl moiety is also specifically recognised such that glucosidases act only on glucosides, galactosidases on galactosides and mannosidases on mannosides [Flowers and Sharon, 1979].

However, β-glucosidase has been shown to hydrolyse a range of disaccharides and substrates, provided they were linked in a β-glycosidic form [Mega and Matsushima, 1979]. Additionally, sweet almond β-glucosidase was described as having a 'relaxed specificity' by Dale *et al.*, [1985] in terms of its ability to react with artificial substrates.

Glucosidases have been purified from many sources e.g. from *Fusarium oxysporum*, [Christakopoulos *et al.*, 1994], *Trichoderma reesei*, [Chirico and Brown, 1987], almond emulsin [Grover *et al.*, 1977], *Botrytis cinerea* [Gueguen *et al.*, 1995], *Penicillium purpurogenum* [Hidalgo *et al.*, 1992] and *Aspergillus niger* [Witte and Wartenberg, 1989], with the first isolations dating back to the mid-nineteenth century [Flowers and Sharon, 1979]. The properties and characteristics of this family of proteins are, hence, well known.

1.11 The use of milk as a source of proteins and enzymes

As mentioned previously in Section 1.1, the biotechnology industry has already started to use mammals as hosts for recombinant proteins e.g. Harris *et al.*, [1995 (a) and (b)], describe the isolation of α_1-antitrypsin from transgenic sheep milk. Even before the introduction of genetic modification, many naturally occurring biomaterials were isolated from milk. Typical examples include hormones e.g. insulin and progesterone, growth factors e.g. epidermal growth factor, and immunoglobulins [Guimont *et al.*, 1997]. Today milk is a source for both native and recombinant material.

In order to design an effective isolation technique for a target protein, it is important to have a knowledge of the protein composition of milk. As bovine milk has been studied in most detail to date, this report will focus specifically on its composition.

Bovine milk contains 30-35 g/l protein, of which there are two main groups; the caseins which precipitate out at pH 4.6, and the whey proteins which remain soluble [Mulvihill, 1992 & 1994; Swaisgood, 1992].

1.11.1 The caseins

The casein fraction is comprised of four main groups of proteins, α_{s1}-, α_{s2}-, β and κ. A fifth group, the γ-caseins, are formed by indigenous proteolytic cleavage of β-casein, which also results in the formation of a group of phosphopeptides, the proteose peptones [Swaisgood, 1992; Adamson and Reynolds, 1994 & 1995]. Caseins are

generally phosphorylated, although not at all the potential phosphorylation sites. They are non-globular, but are believed to posses a small degree of tertiary structure. They are, hence, relatively resistant to heat denaturation, but susceptible to rapid proteolytic cleavage. Most milk casein is present in polydisperse micelles, which are particles containing many casein molecules complexed with inorganic salts, particularly calcium phosphate [Swaisgood, 1992].

The primary function of caseins is nutritional. However, caseins, or their derived peptides have been shown to have a major role on the regulation of some types of cell growth [Guimont *et al.*, 1997].

1.11.2 The whey proteins

The whey fraction consists primarily of two main globular proteins β-lactoglobulin, and α-lactalbumin which together make up ~75% of whey proteins. Other proteins within this fraction are immunoglobulins, serum albumin, and the proteose peptones. Whey proteins have traditionally been regarded as waste produced during the isolation of caseins, hence the commercial exploitation of these proteins has not been widespread [Smithers et al., 1996]. A schematic diagram representing the protein fraction of skimmed milk is shown in Figure 1.4.

β-lactoglobulin is the most abundant of the whey proteins, with a typical composition of 1.8-5.0 mg/ml in bovine milk [Hambling *et al.*, 1992]. It interacts with both hydrophobic molecules such as lipids and fatty acids and metal ions such as copper

(II) and calcium *in vitro* so is considered to have functions as a carrier protein [Guimont *et al.*, 1997]. β-lactoglobulin is not present in human or rodent milk, thus its true biological function remains uncertain [Hambling *et al.*, 1992].

The second main protein within the whey fraction is α-lactalbumin. Unlike β-lactoglobulin, α-lactalbumin is present in all lactating species. Its primary role is to promote the binding of glucose to galactosyltransferase which is involved in lactose synthesis.

Immunoglobulins are present in the milk of all mammals, and transfer an immune defence against disease to the young. The ratio and concentration of immunoglobulins in the milk is not only species dependent, but also depends on whether the animal is able to transfer passive immunity to its young via the placenta prior to birth (e.g. humans and rabbits) or not (cows, horses and pigs) [Larson, 1992]. Serum albumin also transfers across from the blood to the milk.

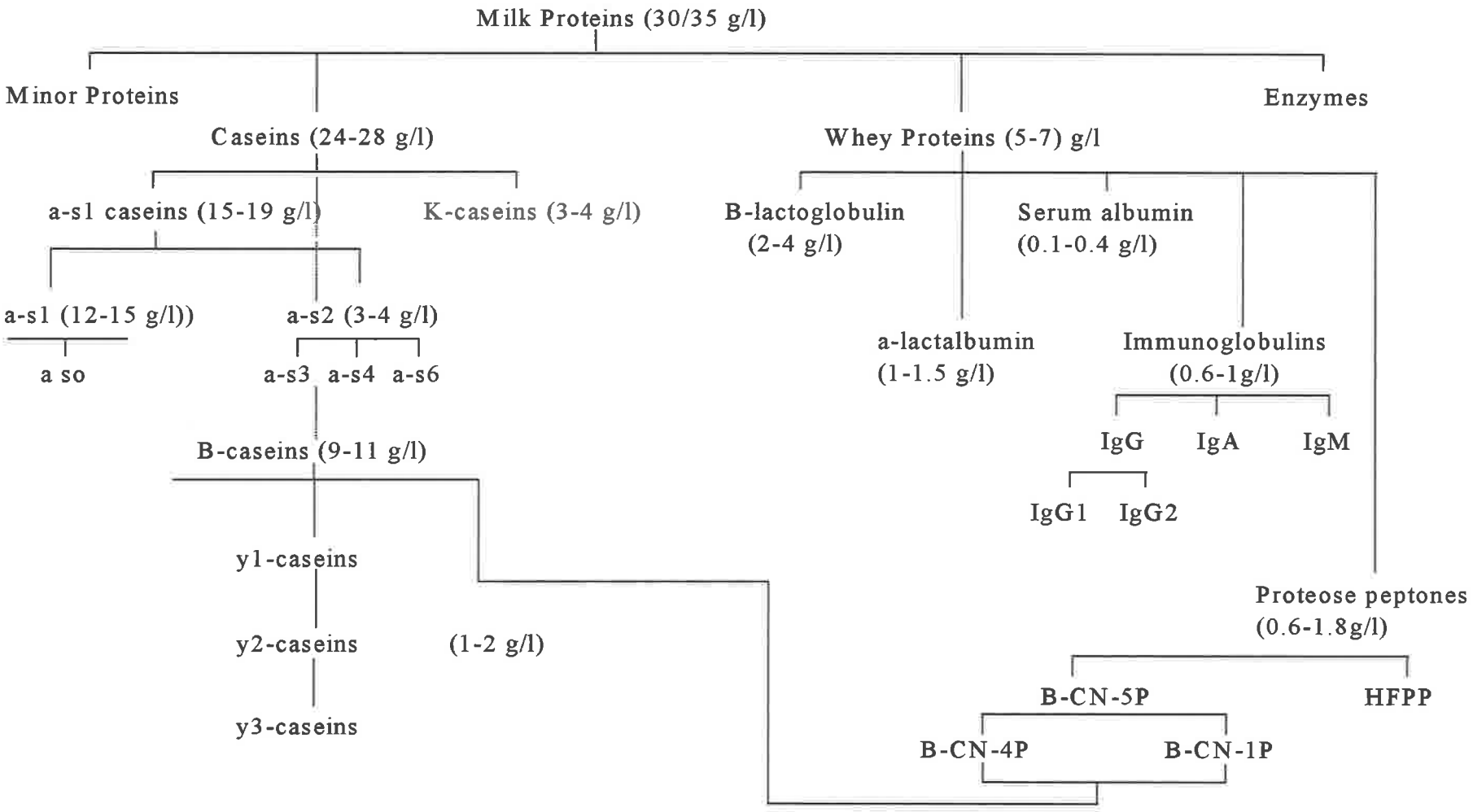


Figure 1.4 A schematic diagram of bovine milk proteins (adapted from Swaisgood, [1992])

Although the proteose peptones separate into the whey fraction, many of them are actually products of *in vivo* β-casein digestion by plasmin [Andrews and Alichanidis, 1983; Andrews, 1992; Adamson and Reynolds, 1994 and 1995; and Isgrove *et al.*, 1998].

The total proteose peptone fraction consists of over 38 components, [Andrews and Alichanidis, 1983], with investigative studies concentrating on the isolation and characterisation of four main groups, β-CN-5P, (Molecular weight 12 300) [Andrews, 1978 (a)], β-CN-4P, (Molecular weight 3468), [Andrews, 1978 (b)], β-CN-1P (Molecular weight 9000) and HFPP (hydrophobic fraction of proteose peptones) [Paquet *et al.*, 1988; Nejjar *et al.*, 1990; Sorensen and Petersen, 1993; Girardet and Linden, 1996]. β-CN-5P represents amino acids 1-105 and 1-107 of β-casein, and β-CN-4P 1-28 of the same molecule (see Chapter 3, Figure 3.1). Both these peptides are readily phosphorylated, and contain the marker sequence SerP- SerP- SerP-Glu- Glu which has been implicated in anticariogenic activity and the regulation of protein structure and enzyme activity [Adamson and Reynolds 1994 and 1995; Guimont *et al.*, 1997]. The proposed mechanism is via the interactions of the marker sequence with amorphous calcium phosphate. In the mouth, for example, these interactions are thought to buffer free calcium phosphate ions and help prevent the demineralisation of tooth enamel by acid from plaque-forming bacteria [Adamson and Reynolds, 1994 and 1995; Isgrove *et al.*, 1998]. Like β-casein, these phosphopeptides also have high nutritional value, and are often added to cell cultures to facilitate cell growth.

The HFPP fraction is a mixture of heterogeneous proteins and peptides, and is not β-casein derived. HFPP has been shown to exhibit high foaming and emulsifying properties, and much research is directed towards taking advantage of this finding [Girardet *et al.*, 1996].

1.12 Aims

The aim of this study is to find a means of facilitating protein isolations. This is achieved either through the application of existing separation methods or by the development of new methods for the isolation of proteins from a heterogeneous mixture. The advantages and limitations of the techniques will be studied in detail.

For the work on aqueous two-phase systems, casein peptides were chosen as model molecules for study as their amino acid sequences have been established. β-Glucosidase was chosen as a model enzyme in the affinity based procedure, as it is representative of a typical globular enzyme and hence acts in a comparable manner during immobilisation.

The objectives of this work are as follows.

- To isolate and purify a total proteose peptone sample by traditional and novel means.
- To investigate the partitioning of total proteose peptones in PEG/Dx aqueous two-phase systems.
- To investigate the partitioning of β-CN-5P in PEG/Dx aqueous two-phase systems.
- To improve and optimise the technique for binding a ligand onto nylon film.
- To eliminate non-specific binding of ligand during this procedure.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Phase-forming polymers

Polyethylene glycol (PEG) of mean molecular weights of 1000, 1500, 3350, 8000 and 10,000 and dextran T500 were obtained from Sigma, Poole, UK.

2.1.2 Enzymes and Substrates

Trypsin (EC 3.4.20.4), soybean trypsin inhibitor (SBTI), β-glucosidase (EC 3.2.1.21), plasmin (EC 3.4.21.7), pepsin (EC 3.4.23.1), BAPNA (Nα-Benzoyl-DL-Arginine ρ-nitroanilide), and pNPG (ρ-nitrophenyl-β-D-glucopyranoside) were purchased from Sigma, Poole, UK.

2.1.3 Proteose peptones

Raw milk was kindly provided by Farmers and Dairymen Dairies Ltd., Cardiff, UK, a proteose peptone sample prepared previously [Andrews and Alichanidis, 1983] was used as a reference material.

2.1.4 Electrophoresis

A 40% acrylamide/bis-acrylamide (19:1) solution was supplied by Sigma, Poole, UK. The gel equipment was purchased from BioRad (Mini Protean system).

2.1.5 Nylon film

Nylon film (manufactured by Portex Ltd., Hythe, Kent, UK) was purchased in the form of a roll of tubing 40mm wide (0.1 mm in thickness).

2.1.6 Ligand Binding

Glutaraldehyde was purchased as a 25 % (w/v) grade II solution, PEI (polyethyleneimine) as a 50% (w/v) solution and chitosan as a powder from Sigma, Poole, UK. Individual amino acids were also purchased from Sigma, Poole, UK.

2.1.7 Other chemicals

All other chemicals were of analytical grade and were purchased from Sigma Chemicals, Poole, UK unless otherwise stated.

2.2 METHODS

2.2.1 The preparation of Total Proteose Peptone fractions from raw bovine milk

Total proteose peptone was isolated from bovine milk according to the method of Andrews and Alichanidis [1983]. Raw milk (5 l) was warmed to 40°C, then skimmed in an Armfield FT 15 disc bowl centrifuge (Armfield Ltd., UK) to remove the cream. The milk was then heated over a bunsen burner, until the temperature reached 95°C, and held at this temperature for 30 min to denature the whey proteins. Following cooling of the milk to 40°C, the pH was adjusted to 4.6 with 1M HCl using a pH meter (8521, Hanna Instruments, UK). The mixture was stirred for 20 min to allow complete precipitation of the casein fraction and the heat denatured whey proteins. The precipitate was removed by centrifugation at 2000 *g* for 20 min (MSE Mistral 3000E,UK). The supernatant was filtered (Whatman Ltd, Maidstone, UK, No 1 paper) to ensure that all the precipitate had been removed. Trichloroacetic acid (TCA) was added to the filtrate to a final concentration of 10% (w/v) to precipitate the proteose peptone fraction. The precipitate was washed twice by suspension in acetone, followed by filtration. The resulting moist filter cake was placed in a blast freezer (Foster BOF.50 UK) at -25°C for 30 min (or until the filter cake was successfully frozen), and then placed into a freeze drier (Armfield SB 4, UK) for 48h or until a dry powder was formed.

2.2.2 The Production of β-CN-5P from raw bovine milk

Total proteose peptone was prepared as described in section 2.2.1.

2.2.2.1 Ion-Exchange Chromatography

A chromatography column containing DEAE - cellulose (DE-52) anionic exchange medium measuring 33mm x 110 mm was prepared in water according to the manufacturer's instructions (Whatman Ltd, Maidstone, UK). Buffer A consisted of 50mM Tris-HCl, pH 6.5, buffer B was buffer A supplemented with 0.5M NaCl. The column was set up for gradient elution as shown below, taking care to eliminate all air bubbles. The DEAE cellulose column was equilibrated in buffer A under gravity-flow with a small volume of salt-free buffer.

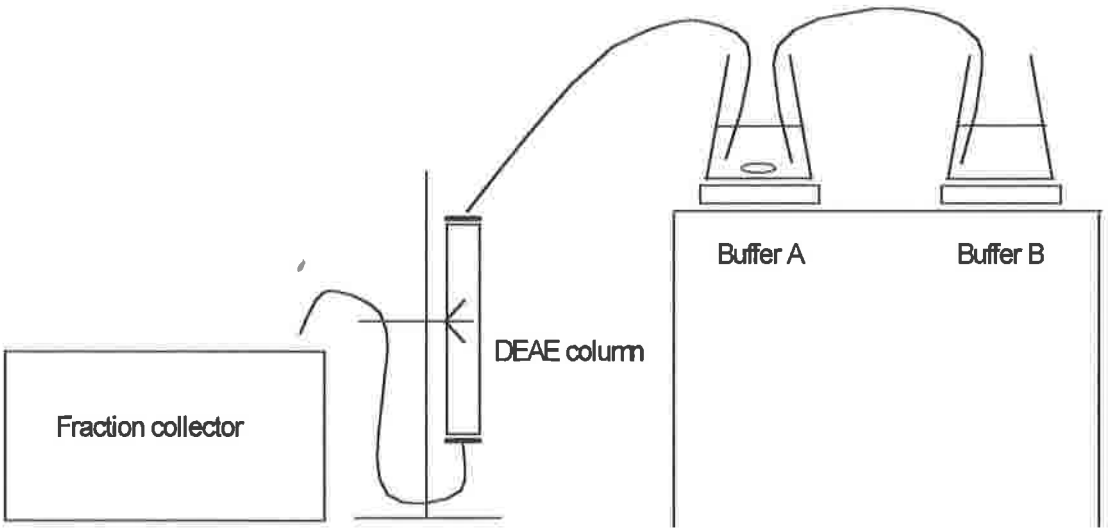


Figure 2.1 Diagram of ion-exchange chromatography

The column matrix was then washed with a further 75 ml of salt-free buffer. A sample of the total proteose peptone (0.5 g) was dissolved in 50 ml buffer, and applied to the column. When this had flowed onto the column, the column was attached to the buffer reservoirs as above.

As the two buffer chambers were at the same height, and were higher than the column, the flow rate was controlled by gravity, and the siphon bridge, creating a linear salt gradient ranging from 0-0.5M NaCl. 500 ml of buffer was passed through the column at a flow rate of 40 ml/h. Fractions of 6 ml were collected. Proteins with a net negative charge bound to the column, and were eluted according to increasing charge density, as the Cl^- concentration increased.

The eluted material was analysed using PAGE. According to the gels, eluent fractions 60-102 ml, 108-126 ml, 132-150 ml, and 156-168 ml were pooled, forming 4 groups, 1, 2, 3, 4. These were freeze dried, then subjected to gel filtration chromatography.

2.2.2.2 Gel Filtration Chromatography

A Sephadex G75 column measuring 22 x 550 mm was prepared in water according to the manufacturer's instructions (Pharmacia Fine Chemicals, UK).

The column and fraction collector were set up as shown in Figure 2.2. When adding the Sephadex, the column was placed at an angle with the plunger end up, to minimise the entrapment of air bubble bubbles. Excess water was allowed to drip through,

controlled by a gate clamp. When the Sephadex bed was at the desired level, the level was marked and water was added gently to the brim of the column. The plunger was then inserted, and forced down to a level of about 1cm below the mark, thus slightly compressing the Sephadex bed.

Figure 2.2 A photograph of the apparatus used during Gel-Filtration Chromatography.

The column was equilabrated with approximately 1 litre of 10mM phopsphate buffer before the sample was applied. The fractions obtained from the ion-exchange chromatography described above were dissolved in a volume of 10 ml with the phosphate buffer and applied to the column. The flow rate was adjusted to 40 ml h^{-1} with upwards elution using a Eyela MP-3 Micro Tube Pump (Eyela Ltd, UK), and

the fraction collector was set to 8 min, allowing 5 ml fractions to be collected (Chemlab 270 Fraction Collector, Chemlab, UK). The absorbance of the eluate was monitored at 215 nm with a Cecil Instruments CE 202 UV Spectrophotometer (Cecil Instruments, Cambridge, UK), and fractions were pooled and freeze dried according to the observed absorbance.

2.2.3 The preparation of β-CN-5P from β-casein

2.2.3.1 The Preparation of β-casein from Bovine Milk.

Raw skimmed milk (5 litres) was adjusted to pH 4.5- 4.6 with 1 M HCl and the mixture allowed to stand to let the precipitated casein settle. The bulk of the supernatant liquid was decanted off and the casein precipitate collected by centrifugation (2000 *g*, 10 min). The casein precipitate was washed three times by homogenisation with water into a final volume of 3 litres and recentrifuged. The washed precipitate was suspended in water, dissolved by addition of 1 M NaOH and the pH adjusted to 7.0 with 1 M NaOH, in a final volume of 750 ml.

Approximately 1 l of DEAE-cellulose (Whatman DE-52) was made up into a slurry in 0.05 M Tris-HCl pH 7.0, poured into an 11 cm diameter buchner funnel and washed with this buffer until the filtrate was close to pH 7.0 (6.5-7.5). The DEAE-cellulose bed was then equilibrated with 0.05 M Tris-HCl pH 7.0 containing 3.3 M urea. Solid urea (100 g) was added to 250 ml of the above casein solution (to an approximate

final concentration of 6 M urea after allowance for the volume increase on urea addition) which was then applied to the DEAE-cellulose bed. The bed was washed with 2 litres of Tris-HCl urea buffer to remove non-bound protein. Caseins were then eluted with a stepwise gradient of increasing NaCl concentration in this same buffer. Each step consisted of 2 litres of buffer, containing sequentially 0.05, 0.08, 0.12, 0.16 and 0.2 M NaCl. Eluates were collected as separate large fractions and small samples of each were anaylsed by PAGE gels in order to identify the fraction containing the purified β-casein (detected using a standard β-casein solution). Once identified, the β-casein fraction was placed in dialysis tubing, dialysed for 48 h versus water to free it from urea and most buffer salts and then stored frozen. Three such runs gave approximately 45 g of purified β-casein.

For subsequent hydrolysis experiments the solution was thawed and adjusted to pH 7.0.

2.2.3.2 Hydrolysis conditions for β-CN-5P formation

As an alternative to isolation from the proteose peptone fraction, some proteose peptone components such as β-CN-5P can be generated by the hydrolysis of β-casein, in this case using the enzyme plasmin [e.g. Andrews and Alichanidis 1983].

In order to determine the optimum plasmin concentration and incubation time to

produce the maximum amount of β-CN-5P from β-casein, 2 x 1 ml portions of casein solution were taken and tested with two concentrations of plasmin. A relatively slow reaction rate was required in order to enable a good control of the reaction process, and to minimise the amount of β-CN-5P that was further hydrolysed to β-CN-4P and other peptide fragments (β-CN-5P is an early product, but not an end product of the hydrolysis reaction).

(a) Hydrolysis with a low level of plasmin solution

- A casein sample (0.1 ml) was taken as a zero time control.
- The remaining 0.9 ml was placed in a water bath (SBI, Grant Instruments)at 37°C.
- 20 μl of a plasmin solution (containing 0.1 mg/ml in water ≡ 2 μg plasmin / 60 mg β-casein) was added
- At various time intervals, 0.1 ml portions were withdrawn and mixed with 1.0 ml of Soybean Trypsin Inhibitor (SBTI) (0.1 mg/ml in water ≡ 100 μg SBTI : 2μg plasmin) to stop the hydrolysis reaction.
- 1.0 ml of a sucrose and bromophenol blue sample buffer solution (as described in section 2.4.1) was added to each sample and 30 μl of sample was added to a lane on a 10 % PAGE gel for electrophoretic analysis.

(b) Hydrolysis with a high level of plasmin solution

The method was followed as above, but using 100 µl of the plasmin solution (hence a five fold increase of enzyme:substrate ratio).

2.2.3.3 Large scale β-CN-5P generation from β-casein

From the above small scale pilot study, 20 µl of a 0.1 mg/ml plasmin solution and an incubation time of 120 min were chosen as the optimum conditions for the maximum production of β-CN-5P from a pure β-casein solution (see Chapter 3, Figure 3.2).

Therefore this procedure was scaled up as follows.

- β-casein solution at pH 7.0 containing approximately 45 g in 750 ml was equilibrated to 37°C
- Plasmin was added to the β-Casein at a level of 20 µl of 0.1 mg/ml plasmin per ml β-casein solution (≡15 ml for 750 ml β-casein).
- The mixture was incubated at 37°C for 120 min
- The pH was adjusted to 4.5 and stirred for 20 min to precipitate residual β-casein and γ-caseins, and to stop further plasmin digestion.
- The solution was centrifuged at 2000 g (MSE Mistral 3000 E) for 15 min and the supernatant collected.
- The pelleted caseins were washed once with 250 ml water, and recentrifuged as above.

- The washing was pooled with the first supernatant, and 250 ml of a 10% TCA solution was added.
- The precipitated proteose peptone fraction was collected by filtration, washed in acetone and air-dried.
- This sample was named β-PP
- The pelleted casein fraction was redissolved in 700 ml water. 4.0 g Tris was added, and the pH adjusted to 7.0 with 1M HCl.

In order to recycle any unused β-casein and maximise the β-PP yield, the plasmin digestion was repeated with the redissolved casein, although the hydrolysis time was increased to 3h. After pH adjustment, centrifugation and TCA precipitation as above, the end product was air-dried.

Gel filtration chromatography of β-PP

The samples of β-PP prepared by the above method were redissolved in a small volume of buffer and subjected to gel filtration chromatography (as described in section 2.2.2) to purify the β-CN-5P from β-CN-4P and other small casein fragments.

2.2.4 Electrophoresis

PAGE was performed according to the following procedure.

2.2.4.1 Preparation and running of the gels

PAGE was performed in an LKB model 2001 vertical gel apparatus as follows:-

The gel stand was greased, and the mould assembled by clamping the two glass plates together, using spacers to create a 1.5 mm gap between the glass plates (16 x 18 cm). The gel mould was attached to the stand using the tightening clamps provided.

60 ml of a 10% (w/v) running (or separating) gel was prepared using the following recipe (sufficient for two gels).

- 15 ml stock 40% (w/v) acrylamide/bis-acrylamide (19:1) solution in water
- 30 ml water
- 15 ml stock running gel buffer pH 8.9, containing

    - 2.07 ml concentrated HCl (or 12 ml 2M HCl)
    - 18.3 g Tris (2-amino-2-hydroxymethyl-propane-1, 3-diol)
    - 0.2 ml TEMED (*N,N,N',N'*-tetramethylethylenediamine)
    - water to 100 ml

Freshly prepared (1 ml) ammonium persulphate solution (25 mg/ml in water) was added to the above running gel solution, which was then stirred and added to the gel mould to within 5 cm of the top of the glass plates.

A volume of water was added with a pasteur pipette to the top of the running gel solution to a level of 3-4 mm to aid polymerisation by excluding oxygen and to ensure an even surface on the top of the gel.

15 ml of a stacking gel solution was prepared as follows (sufficient for two gels).

- 2.4 ml stock 40% (w/v) acrylamide/bis-acrylamide solution
- 5.1 ml water
- 7.5 ml stock stacking gel buffer pH 6.7, containing

    - 1.03 ml concentrated HCl (or 6 ml 2M HCl)
    - 1.5 g Tris
    - 0.1 ml TEMED
    - water to 100 ml

When the running gel had polymerised (approx. 20 min) the overlying layer of water was removed. A portion (0.4 ml) of the 25 mg/ml ammonium persulphate solution was added to the stacking gel solution described above. The mixture was stirred then immediately added to the gel mould. The sample comb was added, and the mould was topped up with stacking gel solution as required.

The apparatus buffer at pH 8.3 was prepared as follows,

- 18 g Tris
- 86.4 g glycine
- water to 6 litres

Following polymerisation of the stacking gel, the sample combs were removed, and greased rubber seals were added over the top of each gel. The reservoir chamber was fixed on top of the rubber seals, and the gel stand was unclamped. The gel sandwich was added into the apparatus, already half-full of running buffer,and more running buffer was added until the electrode wires were covered by at least 0.5 cm of liquid.The cooling system was attached and switched on.

The samples were prepared by dissolving 0.5-5 mg/ml of sample in stacking gel buffer supplemented with 5% (w/v) sucrose and 0.05% (w/v) bromophenol blue. Volumes ranging from 10-120 μl were added to the gel wells using a microsyringe.

Following addition of the samples, the lid was attached, the leads connected, and the gel was run from cathode to anode at between 300-500mV until the bromophenol blue marker had nearly reached the bottom of the gel (2-4h).

2.2.4.2 Coomassie Blue staining of gels

After electrophoresis, the gels were removed from the glass plates and stained for 5-15 min with 0.25 % (w/v) Coomassie Blue G250 in 50% methanol / 10% TCA (trichloroacetic acid)). The gels were routinely destained and stored in 7% acetic acid.

2.2.4.3 Silver Staining of gels

When a more sensitive staining was required, the gels were subjected to the silver staining method of Morrissey [1981]. Following Coomassie Blue staining, the gels were soaked in water overnight, and then rinsed in fresh water for a further 30 min.

The silver staining procedure was continued as follows, using 12 ml solutions per ml of gel.

The gels were soaked in 5 μg/ml dithiothreitol for 30 min.

The dithiothreitol solution was removed, and a 0.1% (w/v) aqueous silver nitrate solution was added, again allowing 12 ml solution per ml of gel. The gels were soaked in this for 30 min, then rinsed in water.

The gels were quickly rinsed twice in a small volume of developer solution containing 0.0135% (v/v) formaldehyde in 3 % (w/v) sodium carbonate. The gels were then

soaked in the developer solution allowing 12 ml per ml of gel until the bands were clearly visible (approximately 5-15 min).

The staining was stopped by adding 5 ml of 2.3M citric acid per 100 ml developer solution, and the resulting solution was gently agitated for 10 min. As the citric acid balances the amount of carbonate in the developing solution, a neutral pH is obtained.

The gels were washed several times in water over a period of 30 min, and were stored in a 0.03% (w/v) sodium carbonate solution.

2.2.5 Preparation of Aqueous Two-Phase Systems

2.2.5.1 Preparation of Two-Phase Systems for Total Proteose Peptone Samples

Systems were generally 0.9 ml in volume. Stock solutions of polymers were prepared in water at three times the desired final concentration, and 300µl of each polymer solution was added to the systems. The proteose peptone samples were prepared at 6 mg/ml, with a volume of 150 µl added to each system. The systems were buffered by adding 150 µl of 0.6 M sodium phosphate buffer of the required pH to an end concentration of 100 mM. When NaCl was required in the aqueous two-phase system, it was added to the buffer at six times its desired end concentration. The systems were customarily prepared in the following order, dextran, PEG, sample, buffer. They were mixed by inversion and using a Jencon's Miximatic vortex, then centrifuged in a

microcentrifuge at 10 000 *g* for 15 min to aid separation. Samples (25 µl) were withdrawn from both phases of each system, and mixed with 50 µl PAGE sample buffer (as prepared in section 2.2.4.1). 10 % PAGE gels were prepared (see section 2.2.4.1) and 50µl of each sample mixture was added to a gel lane using a microsyringe. The gels were routinely stained using Coomassie Blue.

2.2.5.2 Preparation of Two-Phase Systems for Purified β-CN-5P samples

Systems of 1.0 ml were generally used. Stock solutions of polymers were prepared at 2.5 times their desired final concentration, and 0.4 ml of each polymer was added to each system. Tris/HCl buffer (0.1 M) was used with 0.1 ml added to each system, hence each system contained 10 mM buffer. Purified β-CN-5P (0.1 ml) was added to each system to a final concentration of 1 mg/ml. The systems were prepared as described and separated as above (section 2.2.5.1). Following separation, samples were withdrawn from both phases of each system and were applied to a Dionex DX 500 HPLC as described below.

2.2.5.3 Determination of the Partition Coefficient

The β-CN-5P from each aqueous two-phase system was analysed and quantified using a Superose 12 HR 10/30 gel filtration column on a Dionex DX 500 HPLC. A 0.1M Tris/HCl buffer pH 7.0 was used in each case, containing 125 mM NaCl at a flow

rate of 0.5 ml/min. The absorbance of the eluent was measured at 215 nm, as β-CN-5P does not contain any tryptophan and only 1 tyrosine residue, so therefore has very low absorbance at 280 nm. The sample was added directly to the column using a 50 µl sample loop, although it was often necessary to dilute the sample taken from the dextran phases due to their viscosity. When obtaining the standard profile for β-CN-5P, a 3 mg/ml solution was prepared and the β-CN-5P was found to be eluted after 25 min when a flow rate of 0.5 ml/min was used (Figure 8, Chapter 3). When calculating the partition coefficent of β-CN-5P in a system, both the top and bottom phases were run on the HPLC. The area under the β-CN-5P elution profile was determined and a ratio in the top/bottom phase of each system was calculated.

2.2.5.4 Construction of phase diagrams

Phase diagrams were constructed according to the cloud point method described by Albertsson [1971]. For each polymer, a stock solution (typically 20% (w/w)) was prepared in 0.1M sodium phosphate buffer pH 7. A 100 ml beaker containing a magnetic flea was weighed using a three figure top pan balance. The balance was tared to zero and 5 ml of the stock PEG solution added to the beaker, and the weight recorded. The beaker was transferred to a magnetic stirrer and the stock dextran solution added dropwise using a pasteur pipette until the solution went from clear to turbid. The weight of the solution was recorded. Deionised water was added, whilst stirring, until the solution became transparent. The weight was again recorded. The method was repeated adding first dextran then water until a substantial amount of data representing composition points close to the binodial curve was obtained. The

concentration of each polymer at each turbid phase was calculated, and a binodial curve was created by plotting the concentration of PEG against the concentration of dextran.

2.2.5.5 Construction of Tie Lines on Phase Diagrams

Tie lines were constructed for each phase diagram by mixing the polymers at concentrations that resulted in the formation of two phase systems (typically using a total volume of 40 ml). The volume of each phase was measured, and the volume ratio (top/bottom i.e. PEG/dextran) calculated. The distance on the phase diagram between the binodial and the point represented by the two-phase system corresponded to the volume ratio of the phases.

2.2.6 Ligand Binding to Nylon

In general, the ligand binding procedure outlined by Andrews and Mbafor [1991] was followed (Chapter 5, Table 5.1)

Nylon pieces measuring 3 cm by 3 cm were cut from a roll of nylon, [Portex Ltd., Hythe, Kent. UK]. They were incubated in the chosen solutions, and placed in a water bath at 40°C for the alloted time. In between incubations, the nylon pieces were thoroughly washed in water.

Immediately following the enzyme assay, the nylon pieces were again washed, and left to air dry. Their weight was recorded, and the data normalised.

2.2.6.1 Enzyme Assay Conditions

β-glucosidase bound nylon pieces were assayed with 6 ml of 5mM pNPG in 0.1M phosphate buffer at pH 5. After a 15 min assay, a small portion of the assay mixture (0.5 ml) was removed, and added to an equal volume of 2M phosphate buffer at pH 10. The absorbance was then measured at 410nm. Trypsin bound nylon pieces were assayed with 6 ml of 0.1mM BAPNA in 0.1M phosphate buffer at pH 8. After a 15 min assay, a small portion of the assay mixture (0.5 ml) was removed and added to an equal volume of 2M phosphate buffer pH 10.

2.2.6.2 Production of Casein Peptides

To produce casein peptides for use in non-specific binding site blocking experiments, a 10% (w/v) casein solution was incubated overnight with a 0.2% (w/v) pepsin solution at 37°C at pH 2. The pH was then raised to pH 8, and the peptide mixture was freeze dried. It was then used at a concentration of 2.5 mg/ml.

CHAPTER 3 THE PARTITIONING OF TOTAL PROTEOSE PEPTONES IN PEG-DEXTRAN AQUEOUS TWO-PHASE SYSTEMS.

3.1 INTRODUCTION

This chapter studies the partitioning characteristics of bovine proteose peptones in PEG-dextran aqueous two-phase systems.

The total proteose peptone fraction of milk is found in whey, and was first identified in 1918 by Osbourne and Wakeman [cited in Girardet *et al.*, 1996]. Over 38 individual components have been identified in this fraction [Andrews and Alichanidis, 1983]. The most abundant, PP3, PP5, and PP8f were renamed HFPP (Hydrophobic Fraction of Proteose Peptones), β-CN-5P and β-CN-4P, respectively by the 1984 Committee on the Nomenclature and Methodology of Milk Proteins.

Current techniques for the purification of β-CN-5P have focused on the production of a total proteose peptone fraction from raw bovine milk, and the isolation through chromatographic means of β-CN-5P from this fraction, (see Methods, Section 2.2.1). Although the level of purification afforded by these means is high, some authors agree that it is laborious, time consuming, and difficult to achieve on a large scale [Andrews and Alichanidis, 1983; Paquet *et al.*, 1985; Girardet *et al.*, 1991]. Implications of any possible involvement of β-CN-5P in anticariogenic activity in food and beverage

products add impetus to the need to find an alternative, more effective method of producing β-CN-5P in large quantities than the techniques used to date for the direct fractionation from milk. Indeed Reynolds *et al.*, [1994] suggested that new, specific techniques are required to successfully isolate and characterise anticariogenic molecules in order to facilitate studies on their structure and properties. For these reasons if β-CN-5P were to be required on a large scale, better means of generating and purifying it would be required. This is covered in Chapter 4.

β-CN-5P is one of the most abundant components within the total proteose peptone fraction [Girardet and Linden, 1996] and has striking similarities to those milk peptides thought to play a role in preventing dental caries [Adamson and Reynolds, 1995; Reynolds *et al.*, 1994; Isgrove *et al.*, 1998]. Both β-CN-5P and β-CN-4P contain multiple phosphoseryl groups, including the sequence SerP-SerP-SerP-Glu-Glu. Components containing this have been implicated in anticariogenic activity through the association of amorphous calcium phosphate with the marker sequence. It was proposed by Reynolds, [1994] that this interaction allows the phosphopeptides to act as a buffer of free calcium and phosphate ions, hence preventing the demineralisation of tooth enamel by acid generated from plaque-forming bacteria. To date, purification of such molecules has been laborious [Reynolds *et al.*, 1994], and the aim of this chapter is to investigate the potential of aqueous two-phase systems as a separation tool.

Prior to commencing studies on β-CN-5P partitioning, the Hydrophobicity Index of β-casein was determined using an online version of Kyte and Doolittle's hydrophobicity scale. This provided a clear picture of the relative hydrophobicity values of the primary sequence of β-casein, which in turn presented an image of the breakdown products of the controlled β-casein digestion described in Section 4.2.1.

The individual values for the 20 amino acids are shown in Table 3.1. A positive reading indicates a hydrophobic amino acid, and a negative value denotes a hydrophilic residue.

AA	HPI	AA	HPI	AA	HPI
Ala	1.800	His	-3.200	Thr	-0.700
Arg	-4.500	Ile	4.500	Trp	-0.900
Asn	-3.500	Leu	3.800	Tyr	-1.300
Asp	-3.500	Lys	-3.900	Val	4.200
Cys	2.500	Met	1.900		
Gln	-3.500	Phe	2.800	Asx	-3.500
Glu	-3.500	Pro	-1.600	Glx	-3.500
Gly	-0.400	Ser	-0.800	Xaa	-0.400

Table 3.1. The Hydrophobicity values for the amino acids [Kyte and Doolittle, online]

No.	7aa total	HPI	No.	7aa total	HPI	No.	7aa total	HPI	No.	7aa total	HPI	No.	7aa total	HPI	No.	7aa total	HPI	No.	7aa total	HPI
4	-10.9	-1.56	33	-11.6	-1.66	62	0.5	0.1	91	0.9	0.13	120	-12.0	-1.72	149	-10.7	-1.53	178	-5.9	-0.85
5	-2.2	-0.31	34	-19.6	-2.8	63	0.8	0.11	92	1.3	0.2	121	-9.3	-1.34	150	-9.3	-1.35	179	-5.9	-0.85
6	-0.3	-0.06	35	-19.6	-2.8	64	0.5	0.1	93	4.0	0.57	122	-3.9	-0.56	151	-9.6	-1.37	180	-6.5	-0.94
7	-4.5	-0.65	36	-19.2	-2.75	65	-1.4	-0.2	94	1.7	0.25	123	-8.6	-1.25	152	-2.2	-0.3	181	-11.8	-1.7
8	-4.5	-0.65	37	-25.5	-3.64	66	-5.0	-0.7	95	9.4	1.35	124	-2.9	-0.42	153	3.2	0.45	182	-14.1	-2.02
9	3.5	0.50	38	-23.9	-3.41	67	0.4	0.05	96	1.3	0.2	125	-1.3	-0.2	154	7.6	1.1	183	-14.1	-2.02
10	3.9	0.55	39	-22.9	-3.27	68	-0.8	-0.1	97	-4.1	-0.55	126	-4.0	-0.57	155	2.2	0.3	184	-8.3	-1.2
11	3.9	0.55	40	-22.9	-3.27	69	-2.7	-0.4	98	-1.9	-0.3	127	3.7	0.53	156	2.2	0.3	185	-10.2	-1.46
12	-4.8	-0.70	41	-22.9	-3.27	70	-10.7	-1.53	99	-4.2	-0.6	128	1.0	0.14	157	0.3	0.05	186	-4.9	-0.7
13	0.6	0.1	42	-15.6	-2.23	71	-4.6	-0.66	100	-1.6	-0.23	129	-6.3	-0.9	158	1.4	0.2	187	2.4	0.3
14	-3.5	-0.5	43	-15.6	-2.23	72	-2.7	-0.4	101	0.7	0.1	130	-0.6	-0.1	159	1.4	0.2	188	9.4	1.4
15	-4.5	-0.65	44	-15.6	-2.23	73	-3.5	-0.5	102	-7.4	-1.06	131	-7.6	-1.1	160	3.3	0.67	189	11.6	1.67
16	-13.5	-1.93	45	-17.6	-2.51	74	-3.5	-0.5	103	-6.7	-0.95	132	-1.9	-0.27	161	-0.3	-0.05	190	11.3	1.62
17	-21.2	-3.03	46	-9.6	-1.38	75	-2.6	-0.4	104	-7.1	-1.01	133	0	0	162	5.1	0.73	191	3.3	0.47
18	-21.2	-3.03	47	-9.3	-1.34	76	-2.6	-0.4	105	-12.4	-1.75	134	-5.8	-0.83	163	5.7	0.82	192	3.3	0.47
19	-17.5	-2.5	48	-7.4	-1.06	77	0.2	0.03	106	-12.4	-1.77	135	1.5	0.22	164	5.7	0.82	193	-0.1	-0.01
20	-16.8	-2.4	49	-8.4	-1.2	78	-5.9	-0.85	107	-15.8	-2.4	136	8.8	1.25	165	5.7	0.82	194	1.3	0.2
21	-13.0	-1.85	50	-3.1	-0.6	79	-0.1	-0.01	108	-11.4	-1.64	137	8.8	1.25	166	-1.8	-0.26	195	1.3	0.2
22	-13.0	-1.85	51	-3.1	-0.6	80	5.7	0.82	109	-9.1	-1.3	138	8.5	1.23	167	-1.4	-0.2	196	-2.9	-0.42
23	-4.0	-0.57	52	0.1	0.01	81	6.1	0.87	110	-9.4	-1.35	139	3.9	0.55	168	3.2	0.46	197	-2.6	-0.37
24	-4.0	-0.57	53	-7.9	-1.13	82	5.2	0.45	111	-6.8	-1	140	9.7	1.38	169	-2.2	-0.32	198	5.1	0.73
25	-4.4	-0.63	54	-5.5	-0.8	83	7.1	1.01	112	-4.9	-0.7	141	13.2	1.9	170	2.8	0.4	199	4.1	0.58
26	-7.5	-1.08	55	-0.1	-0.01	84	10.6	1.51	113	-2.6	-0.37	142	6.2	0.9	171	4.7	0.67	200	5.3	0.76
27	-7.5	-1.08	56	1.3	0.2	85	16.0	2.3	114	-4.5	-0.65	143	-1.1	-0.15	172	2.0	0.3	201	-0.5	-0.07
28	-10.3	-1.47	57	-1.8	-0.25	86	8.3	1.2	115	-8.9	-1.41	144	-6.5	-0.9	173	1.6	0.23	202	-1.5	-0.22
29	-9.7	-1.4	58	0.6	0.1	87	2.5	0.35	116	-4.5	-0.65	145	-6.2	-0.9	174	-0.8	-0.11	203	-2.7	-0.4
30	-11.4	-1.63	59	3.6	0.51	88	-5.2	-0.75	117	-2.9	-0.41	146	-8.9	-1.27	175	-0.4	-0.06	204	3.4	0.5
31	-11.4	-1.63	60	5.5	0.8	89	0.6	0.1	118	-5.1	-0.73	147	-14.7	-2.1	176	-0.4	-0.06	205	3.7	0.53
32	-12.0	-1.71	61	5.9	0.85	90	4.1	0.6	119	-4.3	-0.62	148	-12.3	-1.75	177	-5.9	-0.85	206	12.4	1.75

Table 3.2. The Hydrophobicity Index of Bovine β-casein, calculated using Kyte and Doolittle's Hydrophobicity Index [online].

The hydrophobicity index for bovine β-casein was calculated using the primary sequence shown in Figure 4.1, and Table 3.1. Windows of 7 amino acids in length were utilised, using a linear weight variation model which set the weight of an amino acid at each position to 1.00 [Table 3.2]. This table is shown in graph format in Figure 3.1.

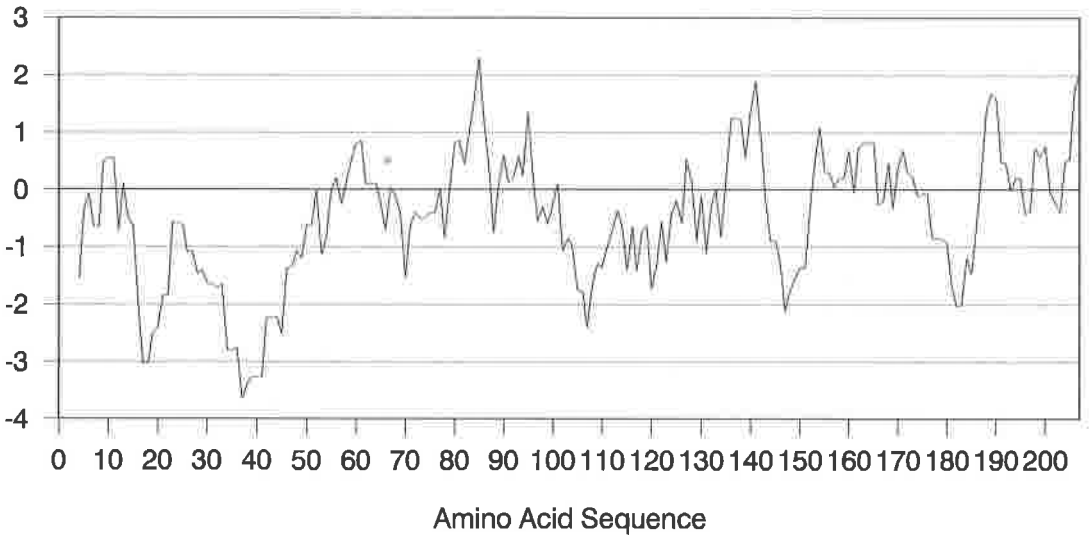


Figure 3.1 The Hydrophobicity Index of β-casein, from Kyte and Doolittle [online].

From Figure 3.1 and Table 3.2, it can be seen that β-CN-5P (residues 1-105/1-107 of β-casein) consists of predominately hydrophilic residues, particularly at its N-terminal end. Residues 80-96 are, however, hydrophobic, which indicates that the overall structure of β-CN-5P has similarities to that of an emulsifying agent, having a hydrophobic end, and a hydrophilic end. This could account for the solubility of β-

CN-5P in both the PEG and dextran phases, particularly as β-casein is known to contain little tertiary structure [Swaisgood, 1992].

The hydrophobicity of a molecule has been shown to have substantial influence on its partitioning characteristics in an aqueous two-phase system [Albertsson, 1990; Franco, 1992; Hagarova and Breier, 1995]. The relative hydrophobicity of β-CN-5P compared with β-CN-4P could account in part for the observation that β-CN-5P will partition preferentially into the upper, PEG phase whilst β-CN-4P remains in the more hydrophilic dextran phase under certain conditions. (For example, see Figure 3.4 (c), where β-CN-5P partitioned into the PEG phase at pH 7 and 1 % NaCl, whilst β-CN-4P partitioned into the dextran phase).

3.2 RESULTS AND DISCUSSION

3.2.1 The partitioning of proteose peptones in aqueous two-phase systems.

Aqueous two-phase systems were considered to have potential for the separation of β-CN-5P from either a milk total proteose peptone sample, or from a β-casein digest. In this chapter the partitioning of molecules in a milk total proteose peptone sample was investigated. The aqueous two-phase system partitioning of pure β-CN-5P produced by β-casein hydrolysis and purified using gel filtration is described and discussed in Chapter 4. The literature suggests that molecules moving in an aqueous two-phase system will partition independently of any other partitioning material [Albertsson,

1971 and 1986]. The conditions of an aqueous two-phase system can thus be manipulated to maximise purification of β-CN-5P. It was considered important to investigate the partitioning of proteose peptones to eliminate the possibility that the optimal conditions for β-CN-5P partitioning to the upper PEG phase would correspond with the conditions required for the extraction also of unwanted peptide fragments into the upper phase. Therefore in a sequence of preliminary studies, the partitioning of milk total proteose peptones in a range of aqueous two-phase systems were investigated. The aim of the study was to discover the conditions which would allow the selective extraction of β-CN-5P into the PEG phase. In this way, a comparision of the partitioning of β-CN-5P and a large number of contaminating materials was obtained. Partitioning behaviour was analysed using PAGE, and variations in systems were obtained by using PEG preparations of differing molecular weight, varying the pH of the system (adjusted using a phosphate buffer) and the presence and concentration of sodium chloride in the system.

3.2.1.1 The effect of PEG molecular weight on the partitioning of total proteose peptone components

The effects of polymer molecular weight on the partitioning of a molecule are well documented [Diamond and Hsu, 1989; Forciniti *et al.*, 1991 (a); Hartounian and Sandler, 1991; Chen, 1992]. By reducing the molecular weight of one phase-forming polymer whilst keeping the molecular weight of the second phase-forming polymer constant, partitioned material will favour the first polymer phase [Albertsson, 1971 and 1987; Johansson, 1974; Kula *et al.*, 1982; Forciniti *et al.*, 1991(a)]. Hence, the

larger the molecular weight of PEG the lower the value of K for any given partitioned material, and conversely the larger the molecular weight of dextran the higher the value of K [Andrews and Asenjo, 1989].

The effect of polymer molecular weight has been investigated in detail by a number of groups including Albertsson *et al.*, [1987 and 1990] who investigated the partitioning of a range of proteins in systems with differing molecular weight dextran. The smallest of their studied proteins, cytochrome c (mol.wt. 12,384) has a comparable molecular weight to β-CN-5P (mol.wt. 12,300), and demonstrated an independence of K from the polymer molecular weight when compared with the effects of polymer molecular weight on the larger proteins studied (Table 3.3). Although this observation suggested the possibility that β-CN-5P would be relatively unaffected by PEG molecular weight, it is important to note that partitioning is due to many factors other than protein molecular weight, and two proteins of similar molecular weight will not necessarily be influenced by polymer molecular weight in the same manner.

Proteins	molecular weight	Partition coefficient in phase systems with 6% PEG 8000 and 8% dextran of different molecular weights				
		Dextran 40	Dextran 70	Dextran 220	Dextran 500	Dextran 2000
Cytochrome c	12 384	0.18	0.14	0.15	0.17	0.21
Ovalbumin	45 000	0.58	0.69	0.74	0.78	0.86
Bovine Serum Albumin	69 000	0.18	0.23	0.31	0.34	0.41
Lactate dehydrogenase	140 000	0.06	0.05	0.09	0.16	0.10
Catalase	250 000	0.11	0.23	0.40	0.79	1.15
Phycoerythrin	290 000	1.9	2.9	-	12	42
β-Galactosidase	540 000	0.24	0.38	1.38	0.02	1.61
Phosphofructoki -nase	800 000	<0.01	0.01	0.01	0.28	0.03
Ribulose diphosphate carboxylase	800 000	0.05	0.06	0.15		0.50

Table 3.3 The effect of molecular weight of dextran on the partitioning of proteins with different molecular weights. (Buffer used was 10mM sodium phosphate (pH 6.8)) [Albertsson *et al.*, 1987].

Therefore it was not expected to find huge differences in partitioning when different PEG molecular weights were used in the work, although as the other main components of the proteose peptone fraction have typical molecular weights of 3469 (β-CN-4P), 9000 (β-CN-1P) and ~ 40 000 (the proposed molecular weight of subunits of the hydrophobic fraction of proteose peptones [Girardet and Linden, 1996]), it was conceivable that changes in PEG molecular weight would affect each molecule sufficiently differently to facilitate separation, thus providing a potential separation tool by alterations in polymer molecular weight.

Figure 3.2 shows the effect of PEG molecular weight on proteose peptone partitioning in PEG-dextran systems.

Figure 3.2. The effect of PEG molecular weight on the partitioning of proteose peptone components in PEG;Dextran T500 aqueous two-phase systems containing (a) 0% NaCl at pH 9, (b) 1% NaCl at pH 9, (c) 5% NaCl at pH 9 and (d) 10% NaCl at pH 9. All systems were 0.9 ml in volume and contained 12% PEG; 8.3% dextran T500. Lanes 1 and 2 were PEG 1000 systems, lanes 3 and 4 PEG 1500 systems, lanes 5 and 6 PEG 4000 systems, lanes 7 and 8 PEG 8000 systems and lanes 9 and 10 PEG 10 000 systems. For Figures (a), (c) and (d), odd numbered lanes depict the PEG phase of each system, and even numbered lanes represent the corresponding dextran phase. For Figure (b) even numbered lanes depict the PEG phase of each system, and odd numbered lanes represent the corresponding dextran phase

Figure 3.2 (a)

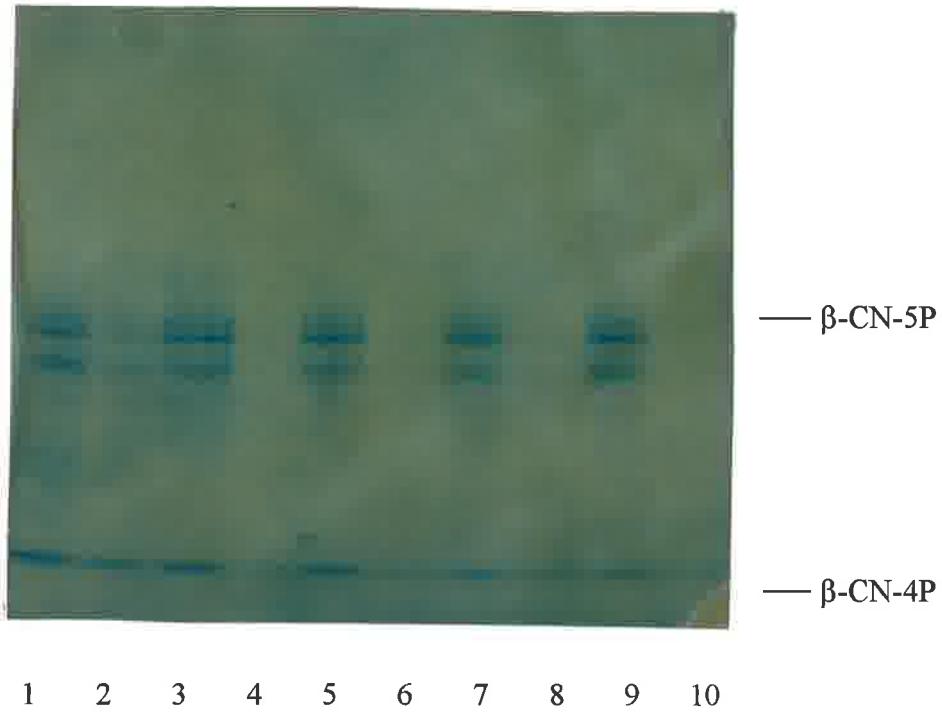


Figure 3.2 (b)

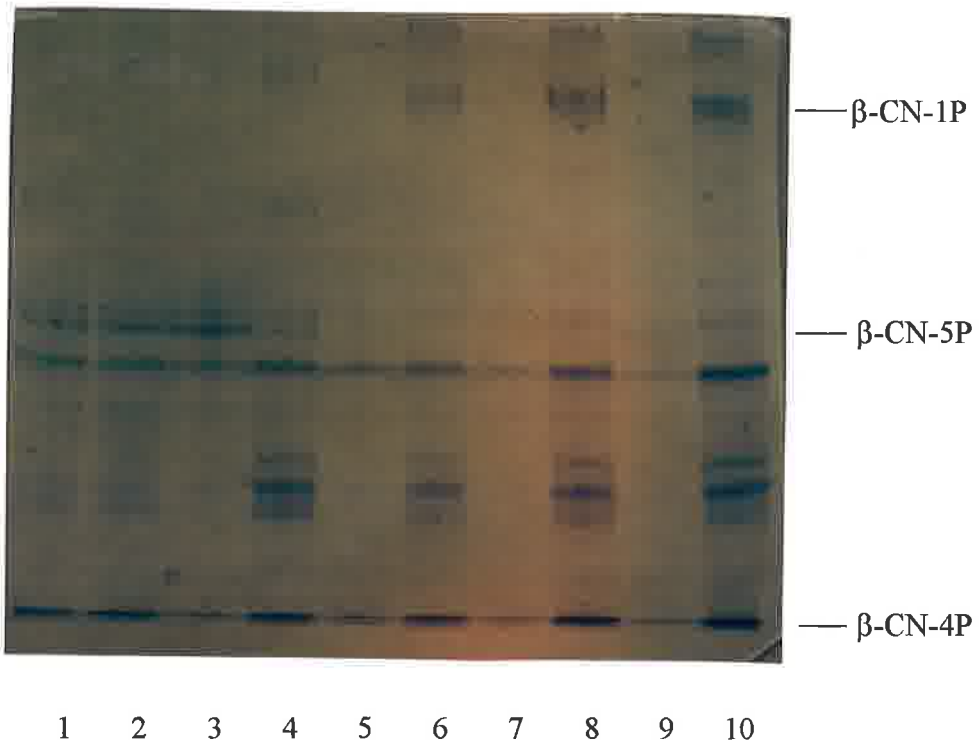


Figure 3.2 (c)

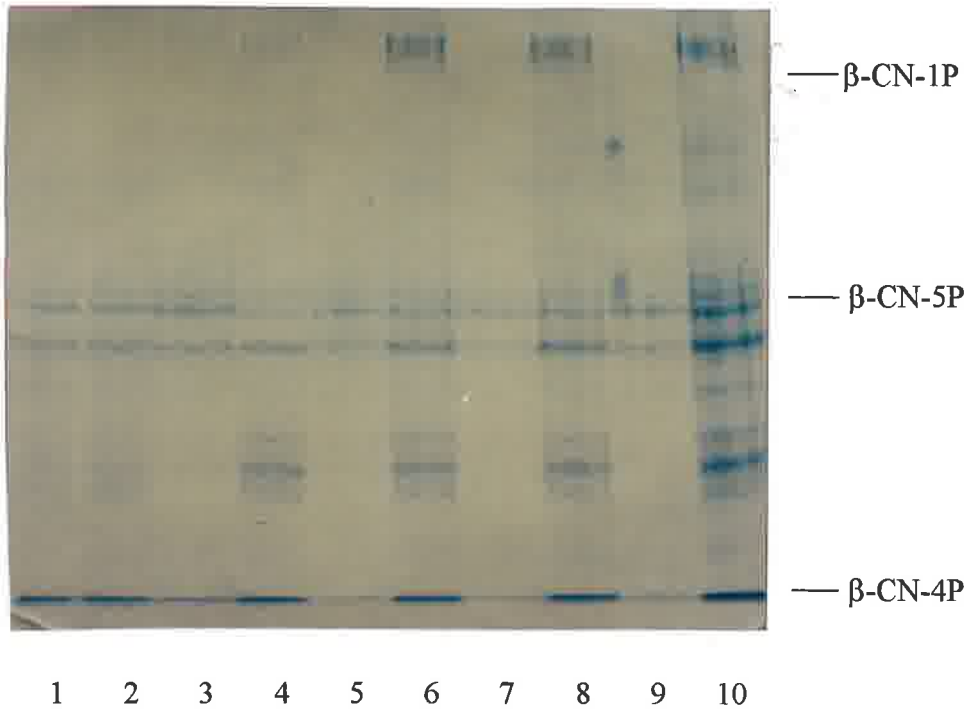


Figure 3.2 (d)

As may be seen in Figure 3.2 (a), (b), (c) and (d), the general trend of β-CN-5P partitioning indicated that a more effective separation from the rest of these components could be obtained using systems with PEG molecular weights of 4000, 8000 and 10 000 (lanes 5- 10 of each gel) at each of the studied pH ranges and salt concentrations. The act of raising the PEG molecular weight therefore helped to encourage many of the accompanying molecules to partition into the dextran phase in accordance with the literature [Albertsson, 1971 and 1987]. This also supports the observation of Diamond and Hsu [1989] that PEG with molecular weights of 4000, 8000 and 10 000 provide the greatest potential for routine aqueous two-phase systems.

They also observed that the partitioning of small dipeptides was unaffected by PEG molecular weight changes, which adds strength to the observation that as the size of the partitioned material decreases, so do the effects of polymer molecular weight too. In this case, when compared to the other components, the partitioning of β-CN-4P in particular, (as it numbers only 28 amino acids in length) would be relatively unaffected by PEG molecular weight. The data obtained in Figure 3.2 (a), (b), (c) and (d) supports this concept, as β-CN-4P remains in the dextran phase of each system at every chosen condition and at each PEG molecular weight.

An additional reason for the use of PEG with molecular weight 4000 or above relates to the ease of use of each molecular weight PEG. PEG with a mean molecular weight of 1000 or 1500 is comparatively more difficult to handle in the laboratory than higher molecular weight PEG, as it tends to be a solid mass at ambient temperature, rather than a powder, as is observed with PEG of molecular weight 4000, 8000 or 10 000.

The increase in the partition coefficient of β-CN-5P seen with the larger molecular weight PEG was consistent with the observation that an increase in polymer molecular weight results in an increase in tie line length and a decrease in the binodial curve [Forciniti *et al.*, 1991 (a); Bamberger *et al.*, 1985]. Systems with larger tie line lengths are known to give more pronounced partitioning of particulate material [Albertsson, 1986]. Similarly a decrease in the binodial curve indicates that each phase will be more concentrated in the main phase-forming polymer, thus increasing the exclusion effects of each phase and resulting in a more prominent separation of the molecules. The

effect of PEG molecular weight on binodial curves in PEG:dextran T500 systems is clearly shown in Figure 3.3 (a), (b), (c), (d) and (e).

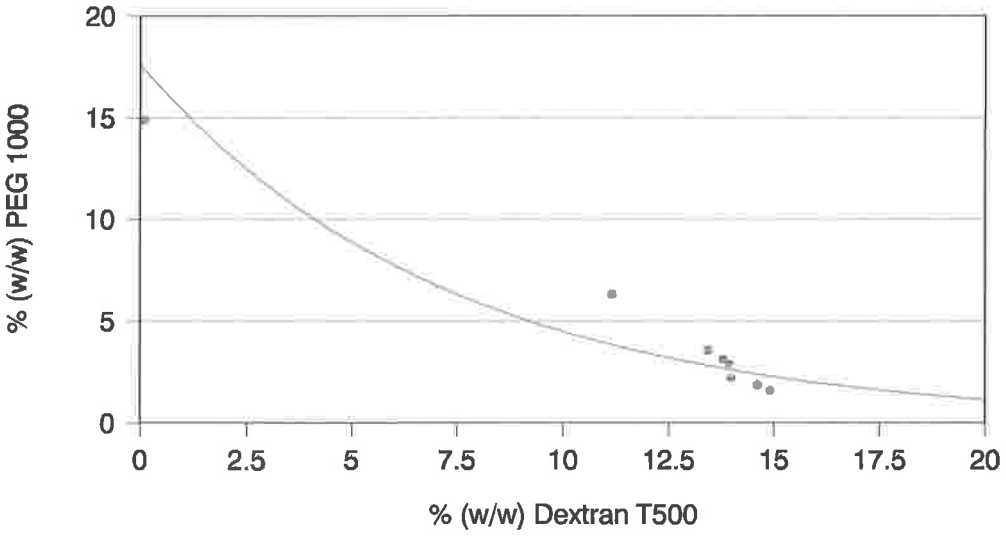


Figure 3.3 (a). A binodial curve for PEG 1000; dextran T500 at pH 7

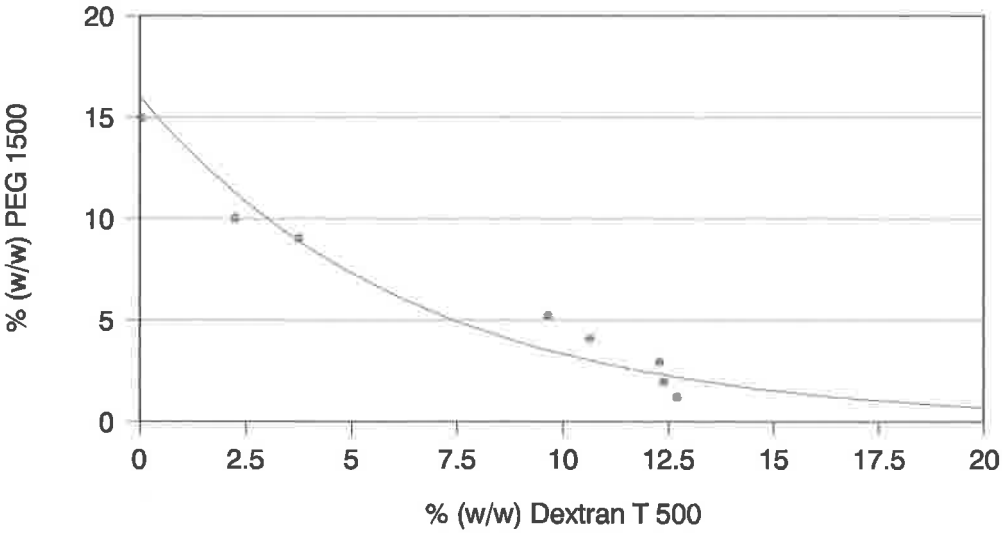


Figure 3.3 (b). A binodial curve for PEG 1500; dextran T500 at pH 7

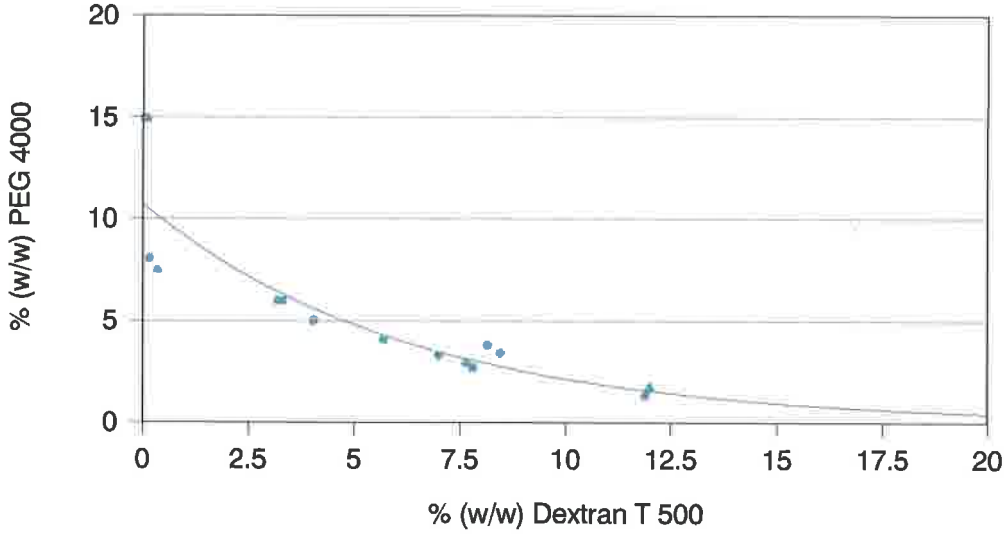


Figure 3.3 (c). A binodial curve for PEG 4000; dextran T500 at pH 7

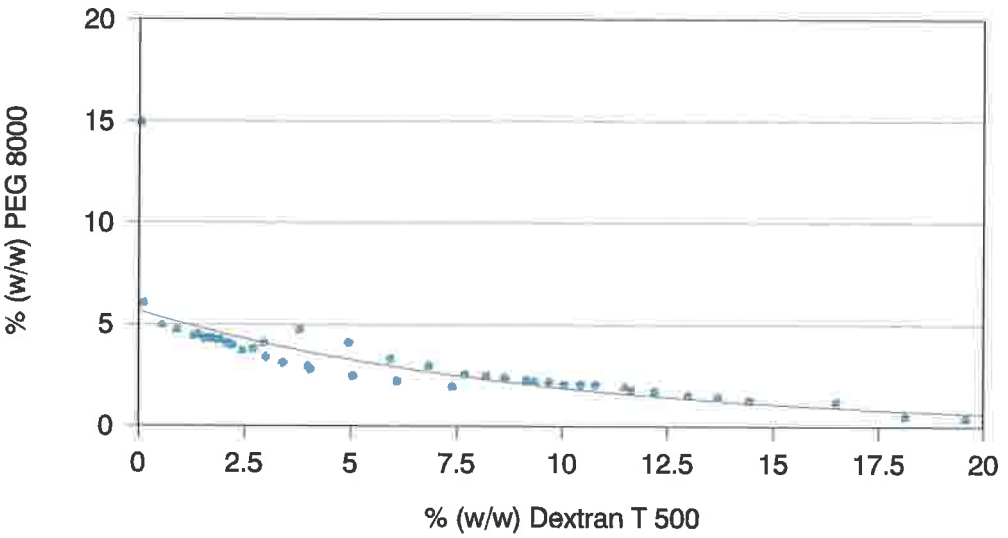


Figure 3.3 (d). A binodial curve for PEG 8000; dextran T500 at pH 7

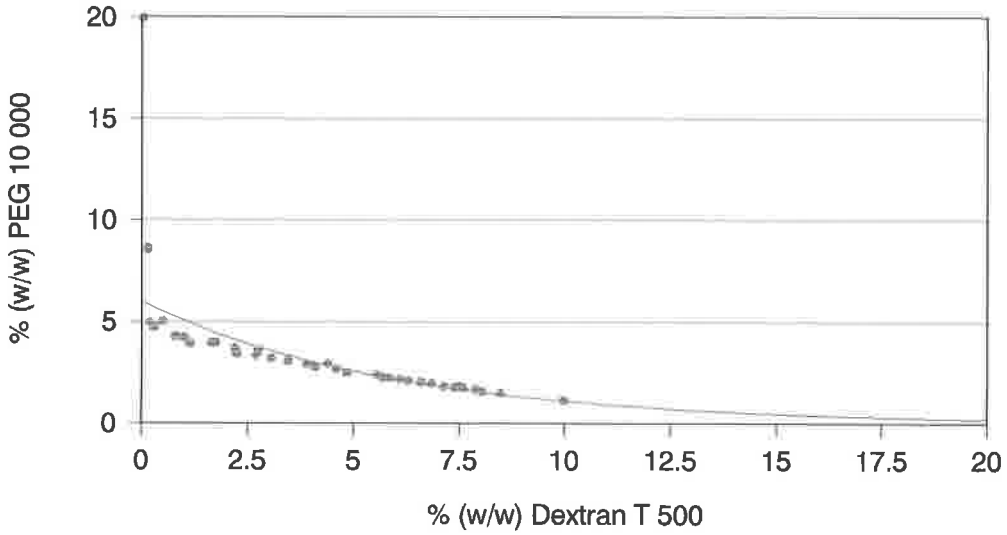


Figure 3.3 (e). A binodial curve for PEG 10 000; dextran T500 at pH 7

From the figures, it is evident that the larger molecular weight PEG polymers were required in a lower concentration to form a two-phase system than the lower molecular weight PEG polymers, a fact that is consistent with published material [Hartounian and Sandler, 1991; Albertsson *et al.*, 1987 and 1990; Forciniti *et al.*, 1991 (a) and (b); Diamond and Hsu, 1989]. Indeed it proved very difficult to construct complete binodial curves with the lower molecular weight PEGs, (Figure 3.3 (a) and (b)). This was due to the difficulty in obtaining data points at high PEG concentrations. When using the cloud point method, the starting solution consists of, for example, 20% PEG, to which dextran is added dropwise until a turbid solution representing a two-phase system is obtained. Deionised water is then added dropwise until the solution becomes clear again, representing a single heterogeneous solution. Typically, a single drop of dextran was required to produce a two-phase system, and up to 5 ml of deionised water was needed to return the solution to a single phase. Consequently a larger

volume of dextran was required to reform the two-phase system to obtain a second data point. The concentration of PEG in the solution was hence substantially diluted following the first data point, and that of dextran considerably raised. In the case of PEG 1000, this resulted in the lack of intermediate points between 15% PEG and 6.5% PEG, and 0.2% and 11.5% dextran respectively (Figure 3.3 (a)). As the molecular weight of the PEG increased, this effect became less prominent, as progressively less deionised water was required to move from the initial turbid solution into a clear solution. Less dextran was then required to produce the second turbid solution and therefore the PEG was diluted to a lesser degree, and less dextran was present *etc.*. It was hence possible to collect many data points, e.g. Figure 3.3 (e) where 25 data points were obtained over the range 0-10% dextran.

All systems were formed in the presence of dibasic phosphate salts which were used to buffer the systems to pH 7. These have been shown to lower the binodial curve, thus meaning that a lower concentration of polymers was required for a two-phase system to form than in the absence of buffer salts. This is thought to be due to the dibasic salts being excluded by the PEG, a phenomenon which has been commonly exploited in the formation of PEG-phosphate systems [Andrews and Asenjo, 1996; Grossman and Gainer, 1988; Vernau and Kula 1990].

Anomalies in the binodials may be attributed to the method used to determine the composition points of the system, which involved subjective determination of whether a solution is clear or turbid. Albertsson, [1986] states that this procedure, known as the cloud point method, is sufficient to produce data close to the binodial. Despite the

pitfalls of such a technique, it is well established, and the data obtained is generally regarded to be valid and reliable.

In real terms, both an increase in tie line length and a decrease in the binodial curve mean that each phase will be more concentrated in the main phase-forming polymer. The effect of this will be to increase the magnitude of the partition coefficient of the partitioning protein, whether the actual value of K is positive or negative [Forciniti *et al.*, 1991 (a)]. As a larger protein will contain more hydrophobic and hydrophilic domains and more charged regions than a small protein, the effects of polymer molecular weight are more pronounced in their case. The observed increase in tie line length with PEG molecular weight is less pronounced between higher molecular weight PEGs, which accounts for the similarity of results obtained between PEG of molecular weight 8000 and 10 000 [Hartounian and Sandler, 1991].

The general trend indicates that an increase in the molecular weight of PEG, whilst keeping the concentration of phase-forming polymers constant, produces a system further from the binodial. This effect is caused by the lowering of the binodial curve to a position that requires a lower concentration of phase-forming polymer to create a two-phase system. This effect can be compared to increasing the concentration of phase-forming polymers in that in each case a system further from the binodial is produced. Combined with the increase in tie line length, this serves to make the partitioning of proteins more pronounced, hence increasing the potential for an effective separation. Indeed, as an example, Chen [1992] observed a more effective

separation of α-lactalbumin and β-lactoglobulin produced from cheese whey at higher molecular weight PEGs.

In conclusion, it is evident that a better isolation of β-CN-5P from the total proteose peptone fraction in 12% (w/w) PEG; 8.3% (w/w) dextran T500 systems at pH 9 containing 0%, 5% or 10% NaCl was achieved using PEG with a molecular weight of 8000 or 10 000, as may be seen in lanes 7-10 of each gel in Figure 3.2 (a), (b), (c) and (d). Although PEG 4000 often provided a good separation of β-CN-5P it was not chosen for further investigation as some elements of the total proteose peptone fraction precipitated at the interface between the two phases in this system. This phenomenon was also observed with PEG 1500 and to a lesser extent with PEG 1000. The presence of a precipitate at the interface was undesirable as it could interfere with partitioning and subsequent phase separation. The added implication of losing a considerable amount of sample through the inability to recover it from the interface also rendered systems using lower molecular weight PEG undesirable.

3.2.1.2 The effect of pH on the partitioning of proteose peptone components in aqueous two-phase systems.

By altering the pH of the system it was hoped to achieve a partial separation of the total proteose peptone fraction such that β-CN-5P would partition preferentially into the PEG phase, with all other components partitioning into the dextran phase.

The effects of four different pH values on the partitioning of β-CN-5P in a total proteose peptone fraction were studied, using PEG with molecular weights of 1000, 1500, 4000, 8000 and 10 000 and dextran T500. Figure 3.4 (a), (b), (c) and (d) show the partitioning of a total proteose peptone fraction in 12% PEG; 8.3% dextran aqueous two-phase systems containing 1% NaCl at pH 3, 5, 7, and 9 respectively.

Figure 3. 4 The effect of pH on the partitioning of the total proteose peptone components in 12% PEG; 8.3% dextran T500 aqueous two-phase systems containing (a) 1% NaCl at pH 3, (b) 1% NaCl at pH 5, (c) 1% NaCl at pH 7 and (d) 1% NaCl at pH 9. All systems are 0.9 ml in volume. Lanes 1 and 2 represent PEG 1000 systems, lanes 3 and 4 PEG 1500 systems, lanes 5 and 6 PEG 4000 systems, lanes 7 and 8 PEG 8000 systems and lanes 9 and 10 PEG 10 000 systems. Odd numbered lanes depict the PEG phase of each system, and even numbered lanes represent the corresponding dextran phase.

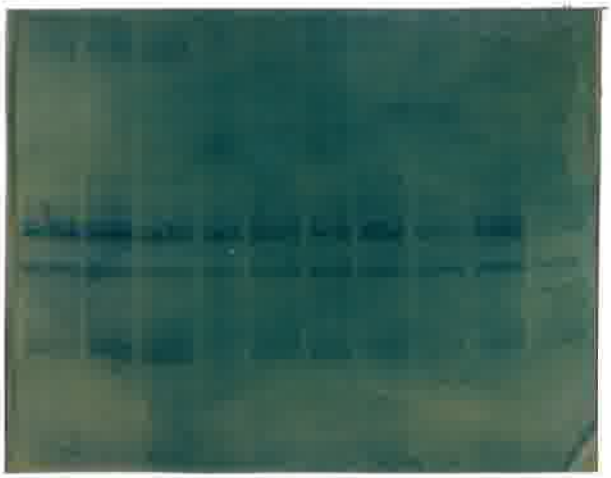


Figure 3.4 (a)

Figure 3.4 (b)

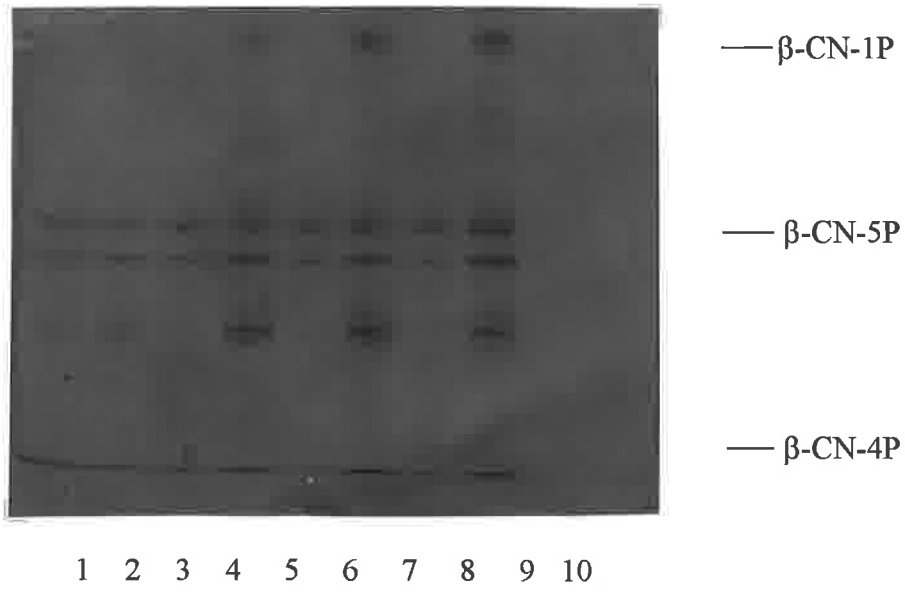


Figure 3.4 (c)

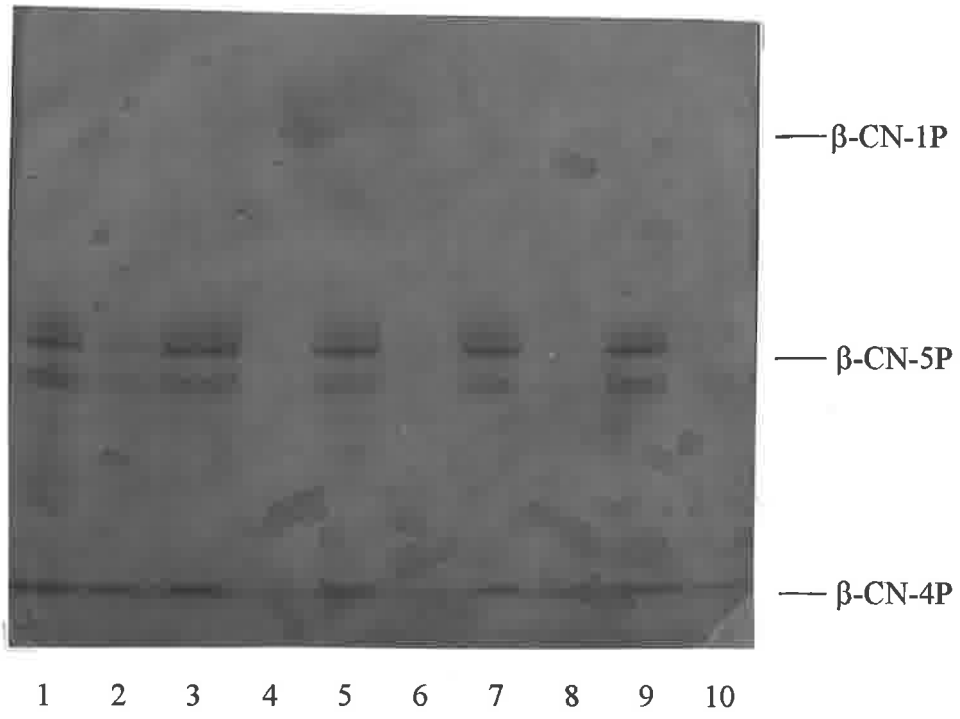


Figure 3.4 (d)

Figure 3.4 (a) shows an almost equal distribution of total proteose peptone components between the PEG and dextran phases, irrespective of the PEG molecular weight, therefore pH 3 was considered not to have potential for further study. Figure 3.4 (b), at pH 5, shows all visible material has partitioned into the upper PEG phase. However, a large amount of the fraction was lost by precipitation at the interface between phases, resulting in only a small proportion of material being recovered in the PEG phase. Similarly Figure 3.4 (d), at pH 9 shows extraction of all material into the upper, PEG phase for PEG with mean molecular weight of 1500 and above. Precipitation at the interface was also observed, but to a lesser extent than at pH 5. This resulted in the absence of some of the lower molecular weight components that were observed in Figure 3.4 (c). Systems at pH 5 and 9 were not considered suitable for further study due to the observed precipitation of material at the interface. Any precipitate at the surface interface has been shown to cause disruption in the partitioning of other phase components, often resulting in a less effective separation process, and loss of target material.

As may be seen in Figure 3.4 (c), a partial separation of the total proteose peptone components was obtained at pH 7 indicating this to be a suitable pH to conduct further work. By careful refinement of the system conditions, it was hoped to improve this partial separation by manipulating the partition coefficients of the total proteose peptones to direct β-CN-5P into the PEG phase and the contaminating molecules into the dextran phase.

3.2.1.3 The effect of the introduction of sodium chloride on the partitioning of total proteose peptone components in aqueous two-phase systems.

The effects of four different NaCl concentrations were studied at pH 3, 5, 7 and 9, using PEG with molecular weight 1000, 1500, 4000, 8000 and 10 000, and dextran T500. Figures 3.5 (a), (b), (c) and (d) show the effect of salt addition to PEG 12% (w/w); dextran 8.3% (w/w) systems at a concentration of (a) 0%, (b) 1%, (c) 5% and (d) 10% NaCl at pH 7. These results were similar to those obtained at pH 9 (Figure 3.2 (a), and (c) representing 0% and 5% NaCl respectively).

Figure 3.5 The effect of sodium chloride concentration on the partitioning of the total proteose peptone components in 12% PEG; 8.3% dextran T500 aqueous two-phase systems containing (a) 0% NaCl at pH 7, (b) 1% NaCl at pH 7, (c) 5% NaCl at pH 7, and (d) 10% NaCl at pH 7. All systems are 0.9 ml in volume and contain 12% PEG; 8.3% dextran T500. Lanes 1 and 2 represent PEG 1000 systems, lanes 3 and 4 PEG 1500 systems, lanes 5 and 6 PEG 4000 systems, lanes 7 and 8 PEG 8000 systems and lanes 9 and 10 PEG 10 000 systems. Odd numbered lanes depict the PEG phase of each system, and even numbered lanes represent the corresponding dextran phase.

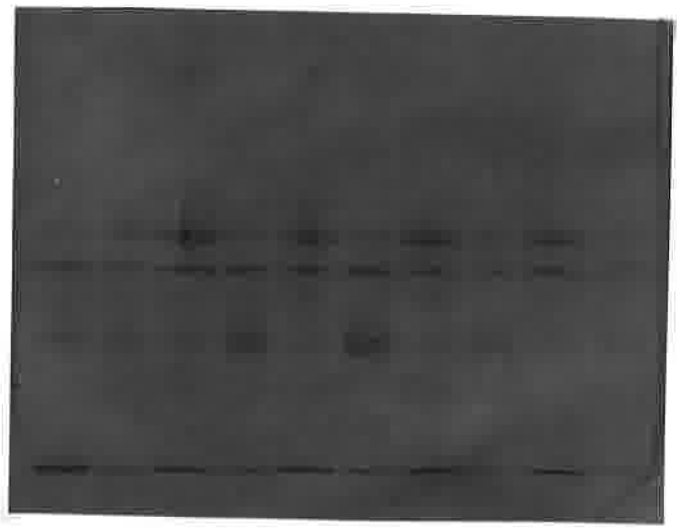


Figure 3.5 (a)

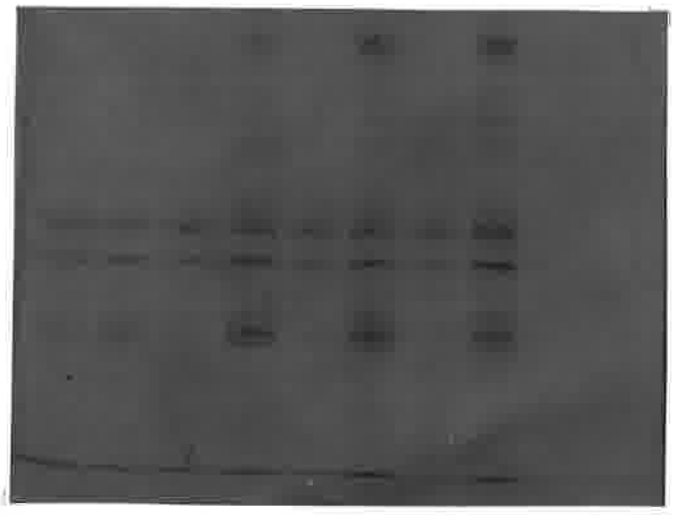


Figure 3.5 (b)

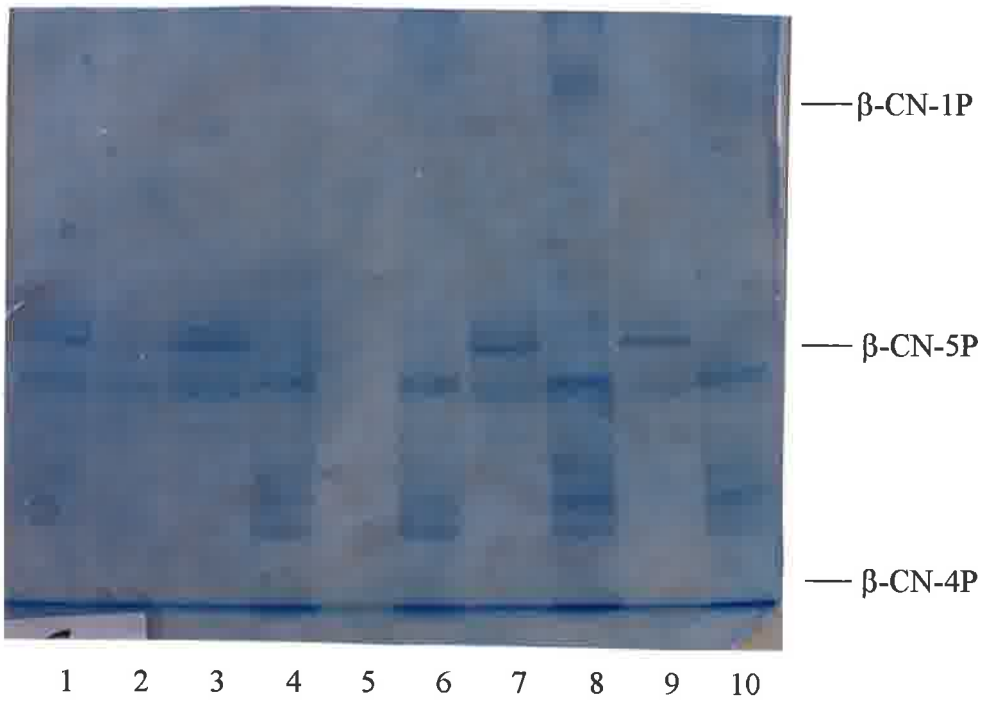


Figure 3.5 (c)

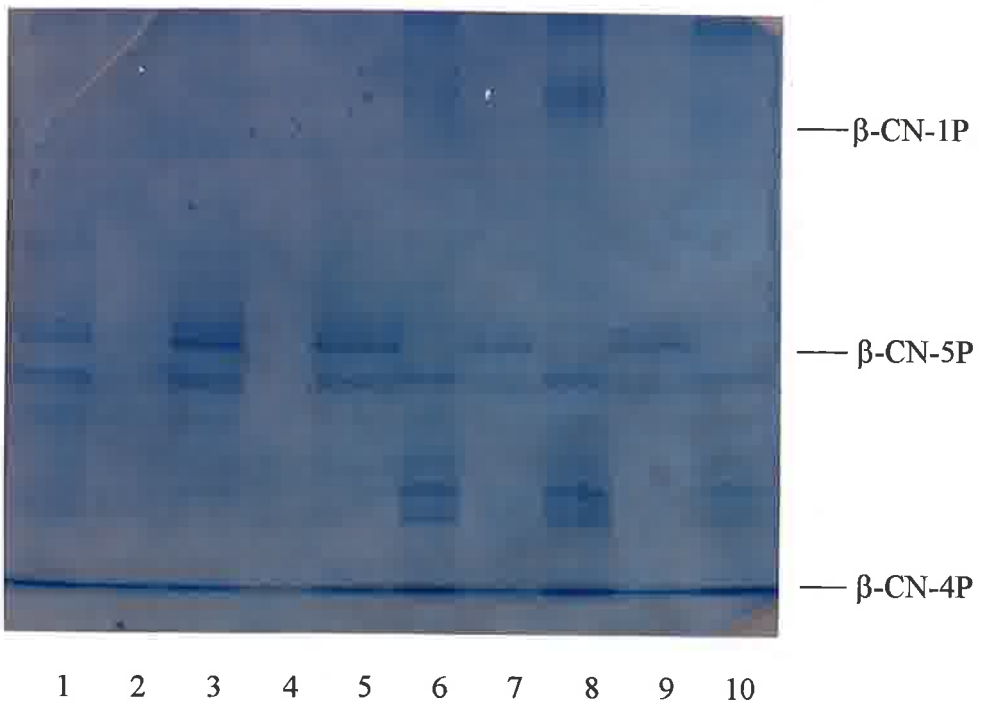


Figure 3.5 (d)

At the lower NaCl concentrations of 0% and 1% much of the lower molecular weight material precipitated at the interface between polymer phases. This material partitioned into the dextran phases in the systems containing 5% or 10% salt and was, therefore, more soluble in systems with higher ionic strength. A more effective separation of β-CN-5P was obtained using 5% salt rather than 0% or 1% (Figure 3.5 (c), (b) and (a)) although the salt concentration appeared to reach an optimum value at 5% as no further improvement was noted in the separation at 10% salt (Figure 3.5 (d)).

These changes in partition coefficients relative to the NaCl concentration in the system may be explained by studies on salt partitioning and its effect on partitioned material. Johansson [1974] observed that when a salt is added to a system the constituent ions show an unequal affinity for each of the phases and hence partition differently. This results in an electrostatic potential difference at the interface which affects the partitioning of charged macromolecules [Bamberger *et al.*, 1984; Walter and Johansson, 1986]. Although salts partition fairly evenly between the phases, small differences cause significant alterations in the partition coefficent of macromolecules.

Johansson [1989] determined the partition coefficients for a number of cations and anions, Figure (3.6).

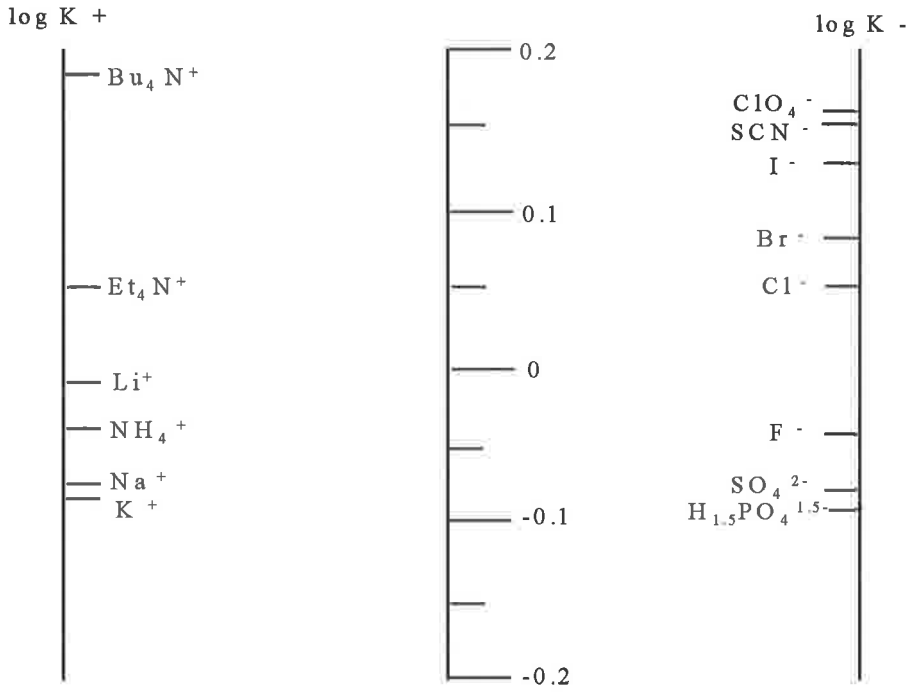


Figure 3.6 The hypothetical partition coefficients for a number of anions and cations in PEG 4000: dextran T500 systems Johansson [1989].

As may be seen in Figure 3.6, a system comprising 8% (w/w) dextran T500 and 8% (w/w) PEG 4000 contains a net negative charge at the PEG phase of the interface, and a net positive charge at the dextran phase when NaCl is added. This is due to the partitioning of the cation Na^+ predominantly into the dextran phase and the anion Cl^- into the PEG phase. The potential difference generated between the phases by these ions is relatively small when compared to a salt, the constituent ions of which have very different affinities for each phase, e.g. K^+SCN^-. However, such a salt has limited value in aqueous two-phase systems due to its denaturing properties on proteins, hence the milder NaCl is preferred.

The partitioning of a charged protein in a salt-containing system appears to follow Equation 3.1,

$$\log K = \log K_0 + \gamma Z$$

Equation 3.1

where K_0 is the partition coefficient of the protein at its isoelectric point, Z is the protein's net charge at the pH of the system, and γ is a factor that depends on the salt used and the composition of the system [Walter and Johansson, 1986; Johansson, 1989]. Any alterations in the partitioning of the total proteose peptone components observed at different pH values will consequently be due to the net charge of the molecule at each pH.

Unlike salts such as phosphate and citrate, NaCl does not lower the binodial of a system. The concentration of each polymer required to pass the critical point and turn from a single heterogeneous phase to a two-phase system is, therefore, unchanged. Correspondingly the contamination of the upper phase with the lower phase-forming polymer and the contamination of lower phase with the upper phase-forming polymer will not be different in systems containing 0%, 1%, 5% or 10% NaCl. Any differences in partitioning may then be attributed directly to the electric potential generated across the interface of the system.

Another acknowledged finding is that the presence and type of salt is more influential on partitioning than the concentration used, with K reaching limiting values at higher

salt concentrations [Johansson, 1974 and 1989]. This finding is confirmed by the data in Figure 3.4 which shows a marked similarity between results obtained at 5% NaCl and those obtained at 10% NaCl, indicating that above a minimum salt concentration, the separation effect is almost independent of salt concentration.

3.3 CONCLUSIONS

The hydrophobicity index of bovine β-casein was determined, and β-CN-5P was found to consist of predominantly hydrophilic residues, particularly clustered towards the N-terminal end, with a more hydrophobic C-terminal end.

Aqueous two-phase systems containing PEG with a molecular weight of 8000 or 10 000 were found to encourage partial separation of β-CN-5P in a total proteose peptone sample. However, systems containing PEG with a molecular weight of less than 8000 did not effectively separate the proteose peptones, and encouraged the precipitation of several components at the interface.

The aqueous two-phase systems containing PEG with a molecular weight of 8000 or 10 000 had lower binodial curves, which promoted a more pronounced separation of the proteose peptones, through the increased exclusion effects of each phase.

Aqueous two-phase systems at pH 3 gave an equal distribution of total proteose peptone components between the phases, whereas systems at pH 5 and 9 caused partitioning of all material into the upper phase. Systems at pH 7 allowed a partial extraction of β-CN-5P into the PEG phase.

The addition of sodium chloride into the aqueous two-phase systems encouraged a more efficient extraction of β-CN-5P, although this effect was found to plateau at 5% NaCl.

CHAPTER 4 THE PREPARATION OF β-CN-5P AND ITS PARTITIONING IN PEG-DEXTRAN AQUEOUS TWO-PHASE SYSTEMS.

4.1 INTRODUCTION

The preparation of β-CN-5P and its partitioning behaviour in PEG- Dextran aqueous two-phase systems is investigated in this chapter.

As explained in chapter one, β-CN-5P is composed of amino acids 1-105, or 1-107 of the β-casein molecule. Other proteose peptone components represent other fragments of β-casein and peptides derived from the other caseins, (α-$_{s1}$, α-$_{s2}$, and κ-caseins) as well as certain peptides and minor proteins that do not come from the casein family at all [Nejjar *et al.*, 1990]. The aim of the current study was to investigate the possibility of producing β-CN-5P in the laboratory by controlled hydrolysis of β-casein which can be relatively easily purified on a large scale. This approach would also avoid many of the unwanted proteose peptone fraction components which are not β-casein derived, from the sample and aid both purification of casein-derived constituents and the interpretation of proteose-peptone properties. Andrews and Alichanidis, [1983], indicated that plasmin hydrolysis of β-casein results in β-CN-5P, β-CN-4P, γ-caseins and other peptides.

A second reason for this study relates to the relative amounts of β-CN-5P in raw, bovine milk. The proteose peptones, including β-CN-5P, are only present in relatively small quantities (0.6-1.8 g proteose peptones/l) in raw bovine milk [Swaisgood, 1992], although, as demonstrated by Andrews and Alichanidis [1983], the quantities can be significantly increased by leaving the milk for several days prior to fractionation. This is due to the action of indigenous proteinases which produce more β-CN-5P and other peptides by *in situ* hydrolysis of β-casein. Their experiments on the storage of milk prior to total proteose peptone isolation showed that β-CN-5P was an intermediate product of the indigenous β-casein hydrolysis, indicating that any hydrolysis would require careful monitoring to prevent the further digestion of β-CN-5P into β-CN-4P and other peptides.

Although the technique of storing milk for up to 7 days prior to fractionation increased the amount of residual β-CN-5P, it remained difficult to predict the amount of β-CN-5P that would be recovered. This was probably due to temperature differences, (affecting the plasmin activity), and differing concentrations of plasmin (and plasminogen) in the milk. Physical factors that deteriorate during storage of untreated milk, such as its smell and consistency rendered the method unsuitable to perform in industrial quantities, and encouraged the research into an alternative method. β-Casein is one of the more common milk proteins, with concentrations typically exceeding 9 g/l [Dalgleish, 1981]. It was felt, therefore, that controlled hydrolysis of purified β-casein could provide an effective means of producing large quantities of β-CN-5P.

In order to investigate the partitioning of β-CN-5P in a range of aqueous two-phase systems, a β-CN-5P sample was prepared from β-casein as described in section 2.2.3. The method developed in this section allowed a greater quantity of β-CN-5P to be prepared in a single batch compared with the amount that could be produced from whole bovine milk, as β-CN-5P is a direct product of β-casein digestion. The purity of this sample was demonstrated by FPLC analysis (Figure 4.8).

4.2 RESULTS AND DISCUSSION

4.2.1 The production of β-CN-5P by hydrolysis of β-casein

Purified β-casein was hydrolysed by plasmin, (as described in Methods, Section 2.2.3), which selectively cleaves polypeptide molecules after the amino acid residues lysine and arginine, but especially lysine. Sites of the β-casein molecule that are particularly susceptible to cleavage by plasmin are situated after the lysine at position 28, which is followed by a second lysine residue, and after the lysines at positions 105 and 107 which are separated by a single histidine residue [Figure 4.1].

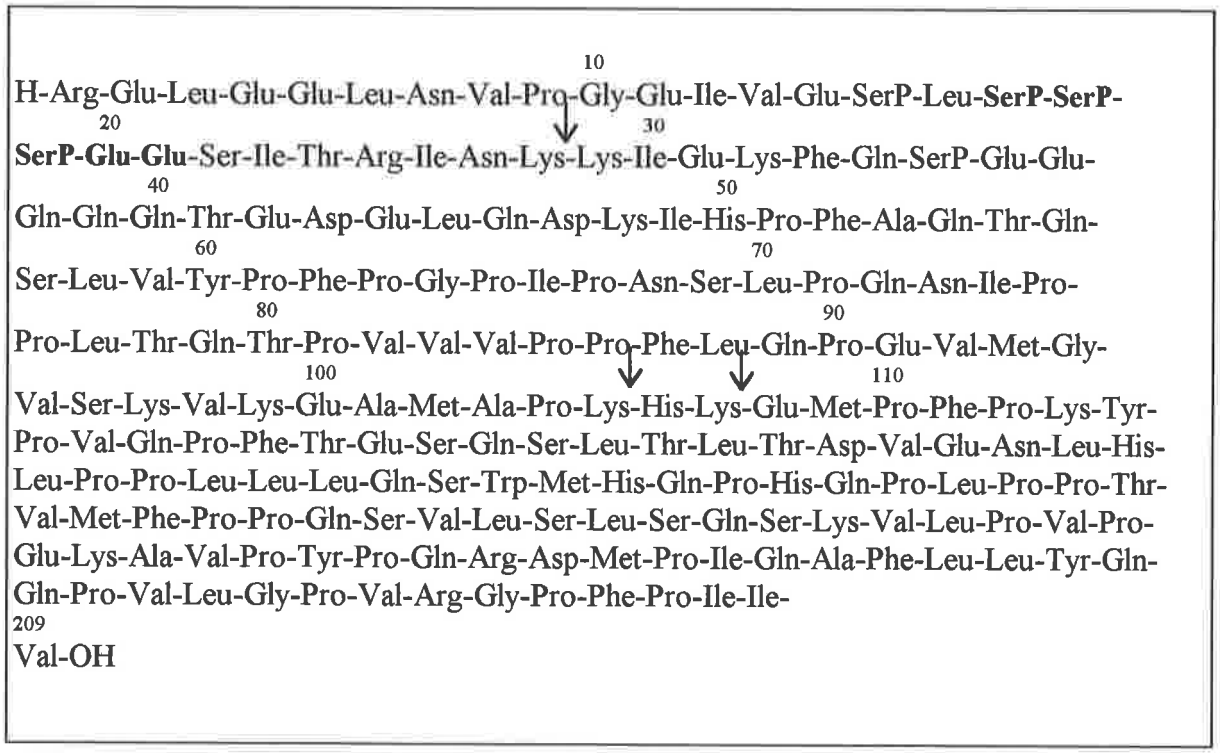


Figure 4 .1 The amino acid sequence of bovine β-casein. β-CN-5P consists of amino acids 1-105 or 1-107. β-CN-4P consists of amino acids 1-28. The arrows represent the cleavage points favoured by the enzyme plasmin, and the bold text denotes the sequence most commonly associated with anti-cariogenic properties.

As β-CN-5P consists of amino acids 1-105 or 1-107 of the β-casein molecule, a controlled hydrolysis was required to ensure the optimal amount of β-CN-5P could be produced. While plasmin readily cleaves all three bonds (Lys_{28}, Lys_{105} and Lys_{107}) the latter two are split faster than Lys_{28}, so by controlling the reaction conditions we aimed to prevent excessive cleavage at Lys_{28}, and hence produce the maximum amount of β-CN-5P. Any β-CN-5P produced through this hydrolysis would need to be separated from other materials contained within the sample, such as residual β-casein, plasmin, γ_1, γ_2, γ_3, -casein (representing amino acids 29-209, 106-209 and 108-209 of the β-casein molecule respectively), β-CN-4P (amino acids 1-28) and other small peptides, prior to studying its anticariogenic effects.

Figure 4.2 shows a PAGE gel demonstrating the progressive breakdown of β-casein over 4 h into β-CN-5P by plasmin at an enzyme:substrate ratio of 1:30 000. This gel was used to decide the optimal conditions for the production of β-CN-5P. A second study was performed using a five fold increase of enzyme (data not shown). This study showed that the same level of β-casein digestion was obtained after 20 min as was observed in Figure 4.2 after 120 min (lane 9). The lower amount of plasmin was chosen for the large scale digestion for several reasons. First, the time limit of 20 min incubation optimal with the higher level of enzyme was considered less easy to reproduce accurately than 120 min incubation. Second, the higher concentration of enzyme caused too rapid a digestion of β-casein; and hence increased the likelihood of further digestion of the recently formed β-CN-5P into β-CN-4P and other peptides (e.g. by cleavage after the lysine residues at positions 28, 29, 32, 48, 97 or 99. See Figure 4.1). Third, during industrial separations, the lowest possible amount of

enzyme that performs the separation effectively would be preferred, due to the monetary costs involved with enzyme usage.

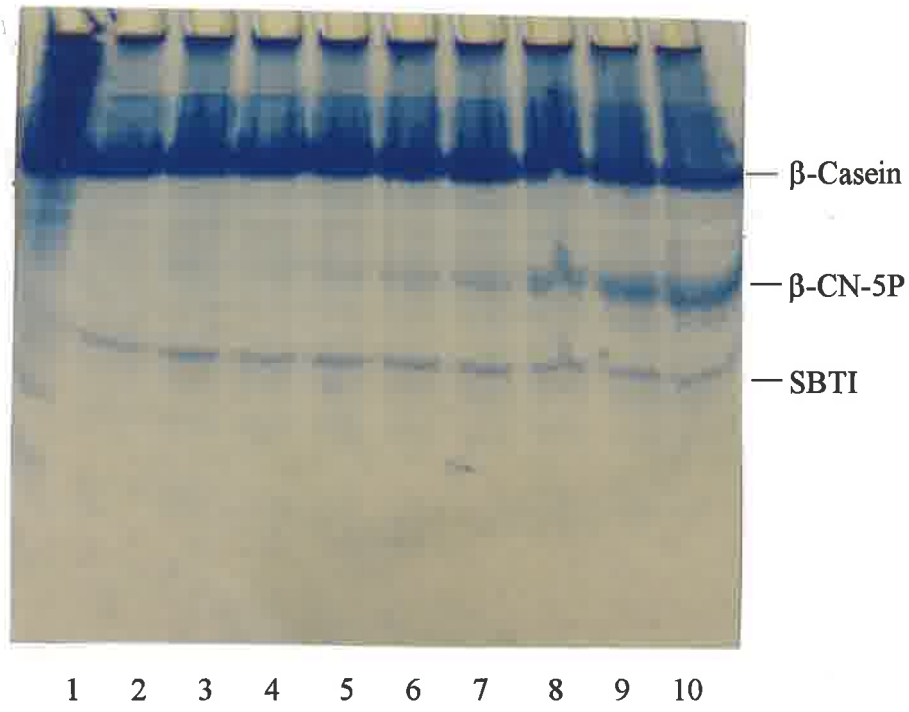


Figure 4.2 PAGE gel showing the breakdown of β-casein over time. Plasmin was added to a solution of β-casein at a ratio of 2 μg plasmin/ 60 mg β-casein. The reaction took place at 37°C and was stopped by the addition of 0.1 mg/ml SBTI (Soybean trypsin inhibitor). Lane 1 shows β-casein prior to digestion. Lane 2 shows a β-casein and SBTI control mixture. Lanes 3-8 represent the digestion of β-casein following 2, 5, 10, 20, 30, 60, 120 and 240 minutes incubation with plasmin respectively. The gel was heavily overloaded with β-casein in order to reveal peptide breakdown more clearly.

Following the results of the pilot study described above, a pure β-casein sample was digested for 120 min by plasmin as described in methods section 2.2.3.2 and the resulting sample (named β-PP) was subjected to gel filtration on a Sephadex G-75 column (Figure 4.3). The aim of the gel filtration was two fold. First as an analytical

tool, to give a more quantitative picture of the β-PP sample than was obtained from the gel profile. Second as a separation tool, to isolate the β-CN-5P from other components in the β-PP sample such as residual β-casein, β-CN-4P, and other peptide products of the hydrolysis reaction.

Gel filtration was considered in place of methods such as ion-exchange chromatography, as it previously has been shown to be more effective at separating the proteose peptones, probably due to the limited pI range demonstrated by this group of components (pH 4-5) [Andrews and Alichanidis, 1983; Paquet *et al.*, 1988].

A comparison of the profiles of a β-PP sample and a total proteose peptone sample isolated from milk may be seen in Figures 4.3 and 4.4. Perhaps the most obvious difference between the two chromatographic profiles is the presence of a substantial peak between fractions 13-20 in the total proteose peptone sample. This peak is due to the hydrophobic fraction of proteose peptones which have been shown previously to elute as a broad band from a gel filtration column close to the exclusion volume, and before other proteose peptone components [Paquet *et al.*, 1988]. The broad band indicates that the hydrophobic fraction of proteose peptones is heterogeneous and/or present in aggregate form following heat damage incurred during the initial stages of the extraction procedure (i.e. when the skimmed milk is heated at 95°C for 30 min).

The hydrophobic fraction of proteose peptones is believed to be a native milk glycoprotein, as opposed to the other proteose peptone constituents which are clearly products of enzymic hydrolysis of caseins [Girardet and Linden, 1996]. As such, this

glycoprotein is absent from the β-PP sample, facilitating the isolation process. Indeed the relative purity and amount of β-CN-5P in the β-PP sample was found to be greater than in previous work where β-CN-5P was isolated from a total proteose peptone fraction under the same conditions (Figure 4.4).

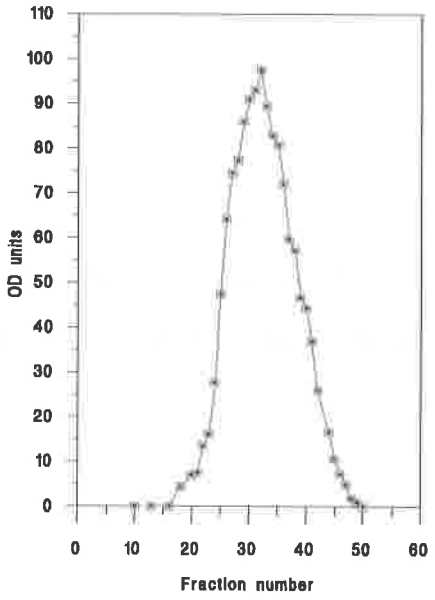


Figure 4.3 Fractionation of β-PP on a Sephadex G-75 gel filtration column containing 0.1M sodium borate buffer pH 7.5, monitoring at 215 nm.

The level of resolution afforded by the gel filtration methods in Figure 4.4 was inadequate to separate individual components of the milk total proteose peptone fraction. Substantial overlap can be seen between peptide peaks, and it appeared difficult to further separate the molecules by gel filtration, although this may be achieved to a degree using ion-exchange chromatography (data not shown).

β-CN-5P was eluted after 25 min, followed by β-CN-1P (27 min) and β-CN-4P (28 min). These peaks were in agreement with pure standards run under the same conditions (data not shown).

Figure 4.6 demonstrates that the FPLC profile provided by the β-PP sample is less contaminated with unwanted peptides than that obtained from the milk total proteose peptone sample. Furthermore, it shows a greater than two fold increase in the relative proportion of β-CN-5P when compared directly with the amount of β-CN-5P in the milk derived total proteose peptone sample. It was therefore proposed that if a reliable method for the separation of β-CN-5P from the other materials in the β-PP sample could be found, a useful means for the more efficient production and isolation of β-CN-5P from β-casein would be obtained.

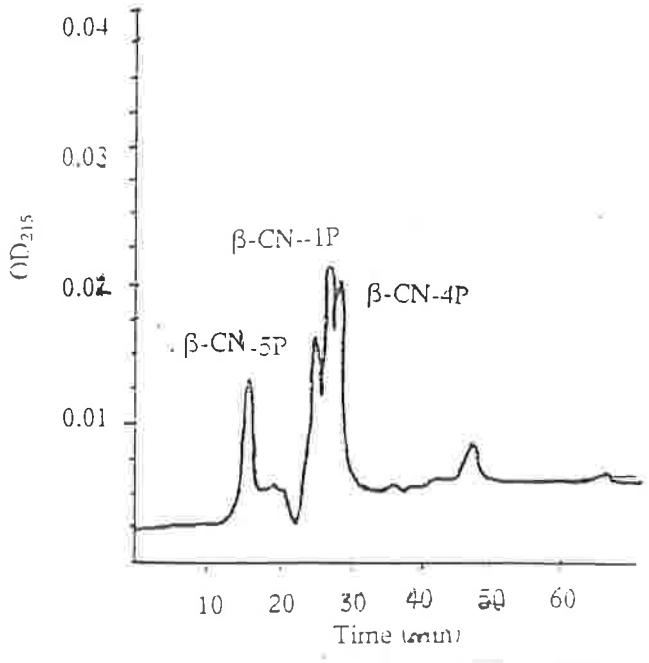


Figure 4. 5 FPLC separation of milk total proteose peptone with a Superose 12 HR 10/30 column in 0.05M Tris-HCl pH 7.0 containing 0.25M NaCl, monitoring at 215 nm.

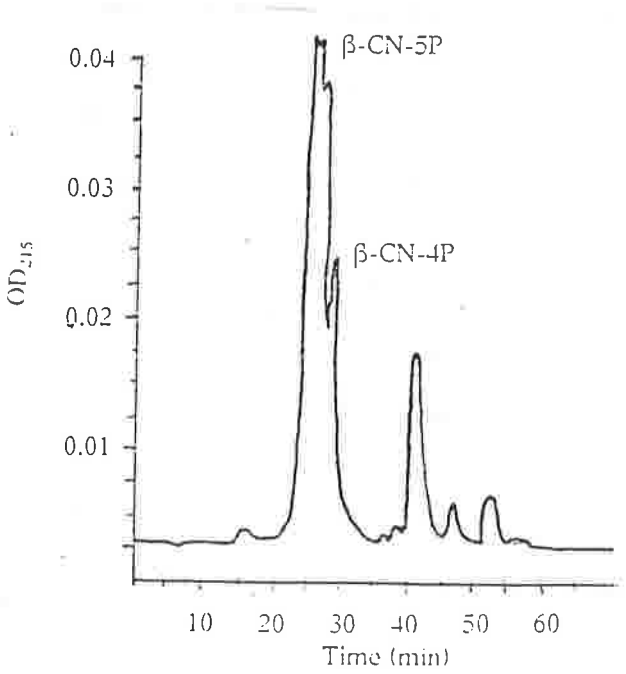


Figure 4. 6. HPLC profile of β-PP obtained using a Superose 12 HR 10/30 column in 0.05M Tris-HCl pH 7.0 containing 0.25M NaCl, monitoring at 215 nm.

In order to further purify on a larger scale the target material from β-PP, the fractions containing β-CN-5P obtained by gel filtration on the Sephadex G-75 column (Figure 4.3) were then pooled, (and a little NaCl was added to act as a salt marker), and run through a Sephadex G-25 gel filtration column (Figure 4.7). As gel filtration had taken place in the presence of a borate buffer, this step was necessary to remove the borate and other salts from the sample. A sample from each fraction was added to a silver nitrate solution to determine the elution position of the salts through precipitation of silver chloride (visual assessment). The separation of β-CN-5P from the borate ions

and NaCl is demonstrated in Figure 4.7. β-CN-5P produced in this manner was found to exhibit strong anticariogenic properties (performed by collaborators at Guy's Hospital, London, personal communication).

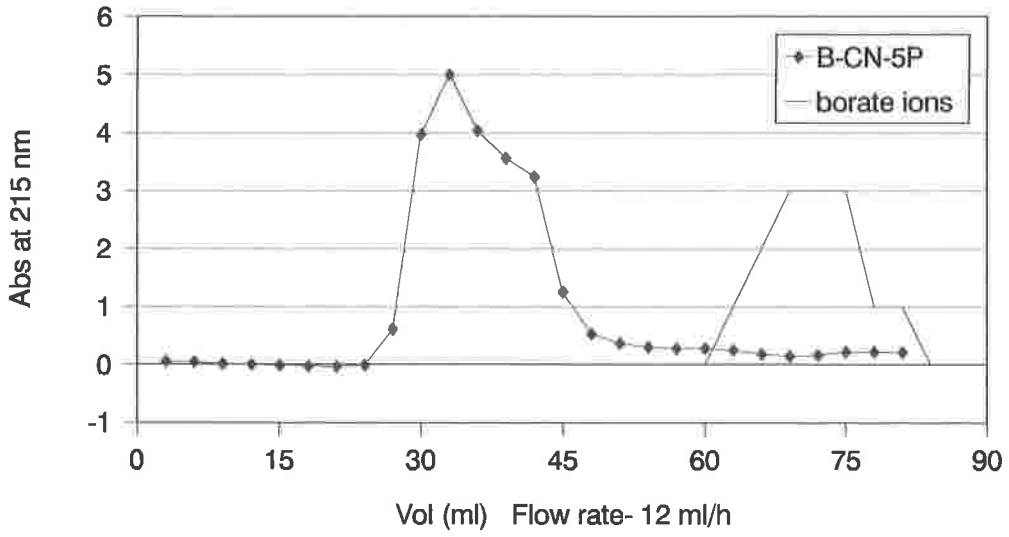


Figure 4.7 Desalting of β-CN-5P from borate ions and NaCl using a Sephadex G-25 gel filtration column

The purity of β-CN-5P produced in this manner was confirmed by FPLC (Figure 4.8).

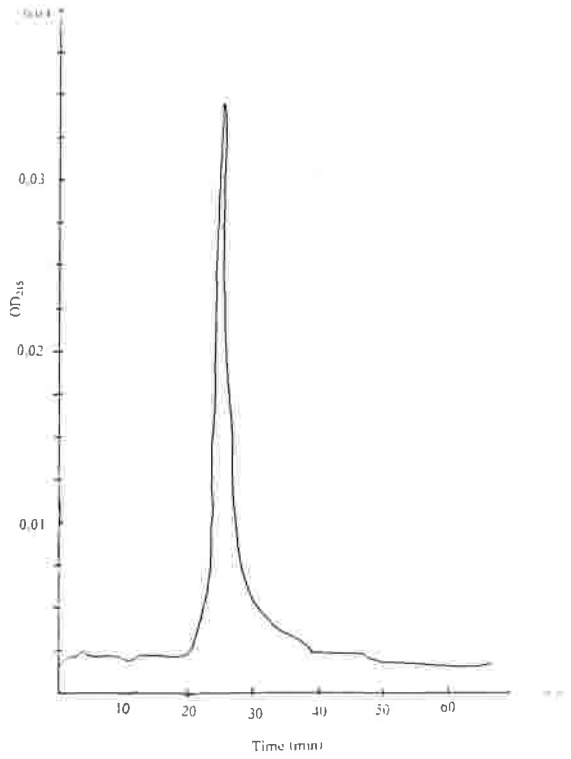


Figure 4.8. FPLC profile of purified β-CN-5P redissolved in 0.05M Tris-HCl pH 7.0 containing 0.25M NaCl, monitoring at 215 nm.

Whilst this methodology was effective on a laboratory scale, the use of chromatographic tools severely limits the technique on a large scale; therefore aqueous two-phase systems were investigated as a possible means of isolating β-CN-5P from β-casein, following digestion of the β-casein by plasmin.

The data on the partitioning of the total proteose peptone fraction isolated from bovine milk described in Chapter 3 was qualitative, i.e. was determined visually by PAGE gel analysis, and, therefore served to give a general view of partitioning. In order to provide a clearer picture of β-CN-5P partitioning in aqueous two-phase systems a

method was devised whereby quantitative data could be collected. Size exclusion (gel filtration) HPLC was chosen as a suitable analytical tool, and the absorbance of β-CN-5P was monitored at 215 nm. As β-CN-5P does not contain any tryptophan residues and only one tyrosine residue, (see Figure 4.1), it is poorly detected by absorbance at 280 nm, the normal wavelength used to detect proteins. PEG did not absorb at 215 nm, and although a peak was observed in the dextran profile, eluted at 16 min, it was easily distinguished from the β-CN-5P peak (25 min).

The partitioning of a molecule in an aqueous two-phase system may be described by its partition coefficient, K, generated by Equation 4.1;

$$K = \frac{[top]}{[bottom]}$$

Equation 4.1

Hence by measuring the concentration of β-CN-5P in the PEG phase of each aqueous two-phase system and dividing this by the concentration of β-CN-5P in the dextran phase, the partition coefficient of β-CN-5P in a number of aqueous two-phase systems was calculated.

4.2.2 THE PARTITIONING OF β-CN-5P IN AQUEOUS TWO-PHASE SYSTEMS

4.2.2.1 The effect of polymer concentration on β-CN-5P partitioning in aqueous two-phase systems.

A range of systems using PEG 8000 were chosen for investigation, as data obtained in section 3.2.2.1 suggested that this molecular weight was most useful in the partial separation of β-CN-5P from a milk total proteose peptone sample.

"Equivalent systems" representing systems with different polymer concentrations, but equal phase volumes were studied. Niven *et al.*, [1990b] devised a systematic method for investigating the effects of polymer concentration on protein partitioning. They illustrated that by preparing "equivalent systems" (defined as having different concentrations of phase-forming polymers, but equal phase volumes), systems representing equivalent points on different tie lines could be compared. Using this approach they demonstrated a direct relationship between PEG concentration and the reciprocal of the partition coefficient, where 1/K either increased or decreased linearly as the PEG concentration increased, depending on the system composition, and the protein studied. In this work, it was aimed to determine whether a linear relationship was valid for the partitioning of β-CN-5P in equivalent systems.

The systems in Table 4.3 were constructed in duplicate to a total volume of 40 ml at pH 7 to determine the phase volume ratio, and to construct tie lines on the phase

diagram (as described in Methods 2.2.5.5). Figure 4.9 confirms that these systems provided comparable phase volume ratios and tie lines that were parallel, in accordance with Albertsson, [1986]. The tie line length was observed to increase as the polymer concentration increased for each system.

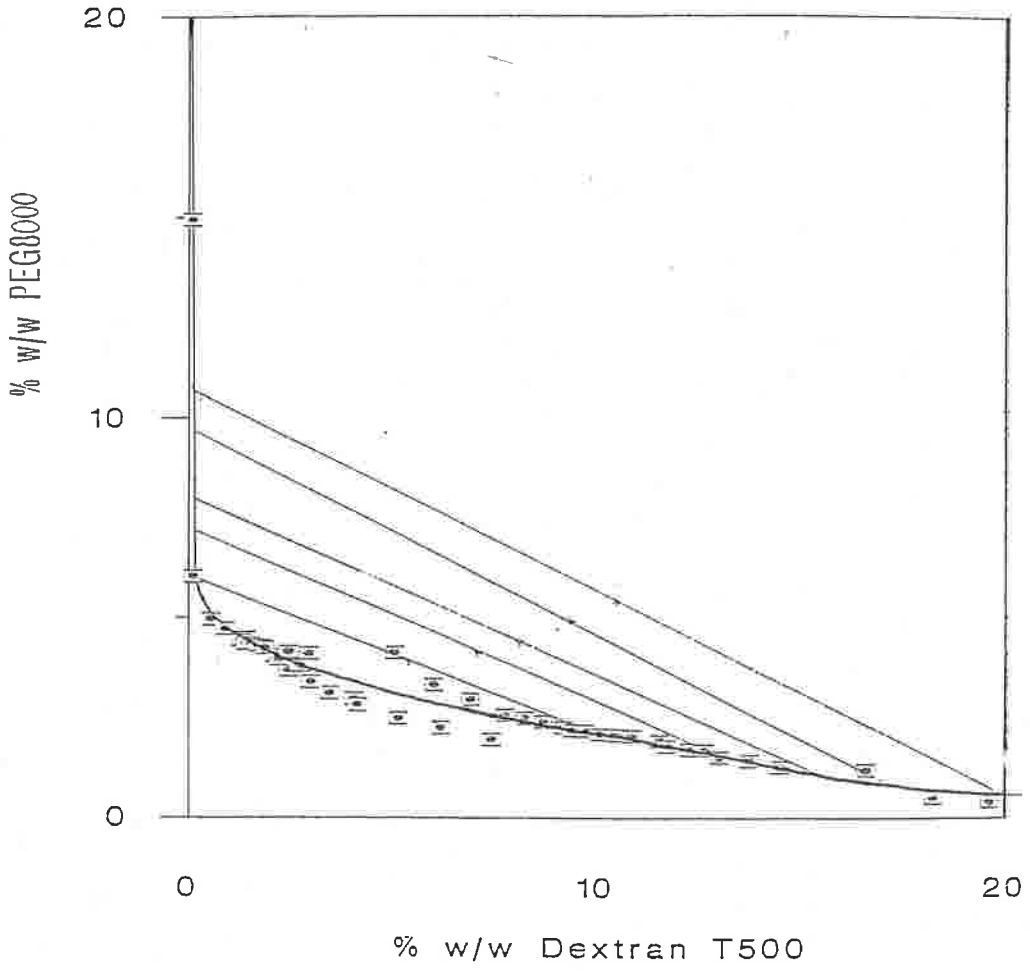


Figure 4.9 A binodial curve showing phase systems and their tie line lengths with PEG 8000; dextran T500 and pH 7

Duplicates of the systems described in Table 4.3 were made up into a total volume of 1 ml and a β-CN-5P sample was added to each to observe its partitioning behaviour.

PEG 8000 % (w/w)	Dextran T500 % (w/w)
4.0	5.3
4.2	7.0
4.5	8.0
5.0	9.2
5.5	10.3
6.0	11.1

Table 4.3 The concentrations of PEG and dextran required to form equivalent two-phase systems (Niven *et al.*, [1990])

The effect of PEG concentration on the partitioning of β-CN-5P can be seen in Figure 4.10. As the concentration of PEG increased, the partition coefficient increased ranging from 1.15 in a 4.0% PEG 8000 system to 2.19 in a 6.0% PEG 8000 system. Johansson [1989] observed that protein partition coefficients approach 1 when polymer concentrations tend towards the critical point on the binodial. In agreement with this, we found that the partition coefficient of β-CN-5P could be seen clearly to increase as the systems were further removed from the critical point.

Figure 4.10 demonstrates that β-CN-5P preferentially partitioned into the PEG phase even at the lowest PEG concentration studied (K is 1.15 in the 4.0% (w/w) PEG system). As the PEG concentration increased the amount of β-CN-5P in the PEG phase increased to a maximum in the 6.0% (w/w) PEG system (K is 2.19). This data confirmed findings by Forciniti *et al.*, [1991(a)] that the partition coefficient becomes more extreme as the tie line length and hence the polymer concentration increases. The tie line lengths of the systems used in the current study are shown in Figure 4.9,

and clearly revealed an increase in tie line length as the polymer concentration increased for each system.

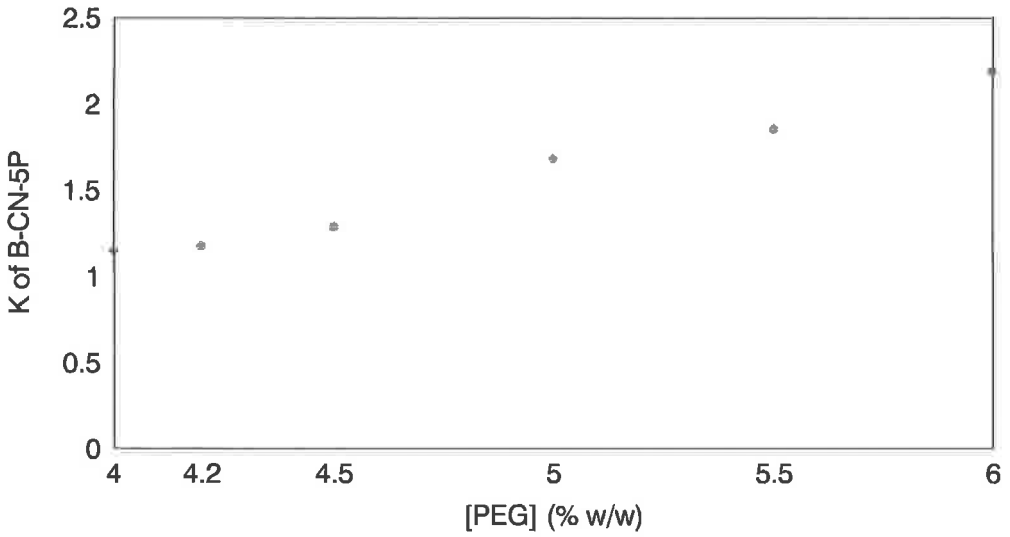


Figure 4.10 The effect of PEG concentration on the partition coefficient of β-CN-5P

Systems containing concentrations of PEG above 6% were not investigated, as the corresponding dextran phases required were progressively more viscous and hence difficult to handle.

Chen [1992] observed that an increase in polymer concentration was beneficial in terms of yield and purity for α-lactalbumin and β-lactoglobulin. The yield of β-CN-5P in the PEG phase of the systems described in Table 4.3 was investigated using Equation 4.2 adapted from Chen [1992].

$$Y_\beta = 100/ \{1+(1/R) \times (1/K_\beta)\}$$

Equation 4.2

where Y_β represents the estimated percentage yield of β-CN-5P in the upper phase; R represents the volume ratio and K_β represents the partition coefficient of β-CN-5P. The estimated percentage yields of β-CN-5P obtained using Equation 4.2 are shown in Table 4.4. The volume ratio was obtained by dividing the volume of the upper phase by the volume of the lower phase.

[PEG 8000] (% w/w)	Volume ratio	$K_{\beta\text{-CN-5P}}$	% Yield β-CN-5P
4.0	1.20	1.15	58.2
4.2	1.21	1.18	58.8
4.5	1.11	1.29	58.9
5.0	1.19	1.68	66.7
5.5	1.11	1.85	67.3
6.0	1.05	2.19	69.7

Table 4.4 The effects of PEG concentration on the estimated yield of β-CN-5P at a constant phase volume ratio.

The percentage yield of β-CN-5P was seen to increase as the polymer concentration increased ranging from 58.2% in a 4.0% (w/w) PEG 8000 system to 69.7% in a 6.0% (w/w) PEG 8000 system.

4.2.2.2 The effect of polymer concentration and the introduction of a salt, NaCl, on β-CN-5P partitioning in aqueous two-phase systems

The systems described in Table 4.3 using PEG 8000 and dextran T500 ranging from 4.0 % (w/w) to 5.5 % (w/w) PEG were made up as previously, but this time including 0% to 10% NaCl. The observed partition coefficients of β-CN-5P in each system are shown in Table 4.5.

	PEG 8000 (% w/w)				
[NaCl] (%)	**4.0**	**4.2**	**4.5**	**5.0**	**5.5**
0	1.12	1.15	1.14	1.76	1.82
0.5	1.14	1.17	1.13	1.80	1.91
1	1.17	1.26	1.22	2.00	1.99
2	1.22	1.32	1.58	2.04	2.09
5	1.29	1.38	1.60	2.15	2.17
10	1.32	1.45	1.62	2.18	2.20

Table 4.5 The effects of PEG concentration and NaCl concentration on the partition coefficient of β-CN-5P

By reading down each column, the effect of NaCl concentration may be seen at a variety of PEG 8000 concentrations. In each case, the partition coefficient increased as the NaCl concentration increased, e.g. rising from 1.14 to 1.62 in the 4.5% (w/w) PEG 8000 system. Figure 4.11 shows plots of the partition coefficient of β-CN-5P against NaCl concentration for a range of PEG 8000 concentrations.

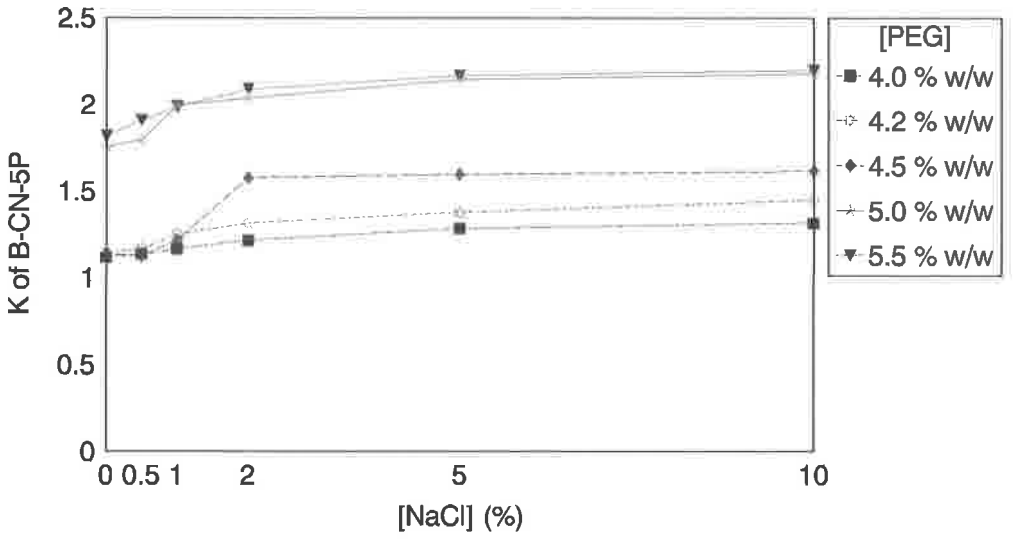


Figure 4.11 The effect of NaCl concentration on β-CN-5P partitioning at a range of PEG 8000 concentrations.

From Figure 4.11, it is evident that the effect of NaCl concentration on β-CN-5P partitioning was limited, whatever the PEG concentration of the system. For each PEG 8000 concentration studied, the partition coefficient of β-CN-5P appeared to reach a plateau at the higher salt concentrations, and little effect was observed on going from 5% to 10% NaCl (e.g. K of 1.29 at 5% NaCl to 1.32 at 10% NaCl in 4.0% PEG systems; and K of 2.17 at 5% NaCl to 2.20 at 10% NaCl in 5.5% PEG systems, Table 4.5). This finding of a plateau agreed both with published material and the experiments described in Section 3.2.1.3. As the PEG 8000 concentration increased, a higher β-CN-5P partition coefficient was produced, agreeing with the data obtained in Table 4.5. Although a large difference in the partition coefficient was obtained between 4.5% and 5.0% PEG, an increase to 5.5% PEG produced little further difference on the partition coefficient, particularly at NaCl concentrations exceeding

1%. This data agreed with findings in PEG-salt systems that indicated the effect of increasing the tie line length was less pronounced at higher polymer concentrations [Guan *et al.*, 1992].

By reading across each row of Table 4.5, the effect of PEG 8000 concentration on β-CN-5P partitioning may be examined at a variety of salt concentrations. In each case the partition coefficient increased, e.g. ranging from 1.12 to 1.82 at 0% NaCl; and from 1.32 to 2.20 at 10% NaCl. These slight increases are consistent with the observation that an increase in tie line length (and therefore polymer concentration) promotes an increase in the salt concentration of the bottom phase [Albertsson, 1985]. If this is the case, then the higher salt concentration in the dextran phase could perhaps have contributed to the increase in the partition coefficient, by effectively salting out the β-CN-5P.

Figure 4.12 gives plots of the partition coefficient of β-CN-5P against PEG 8000 concentration for a range of NaCl concentrations and shows that as PEG concentration increased, the partition coefficient of β-CN-5P increased at each NaCl concentration studied.

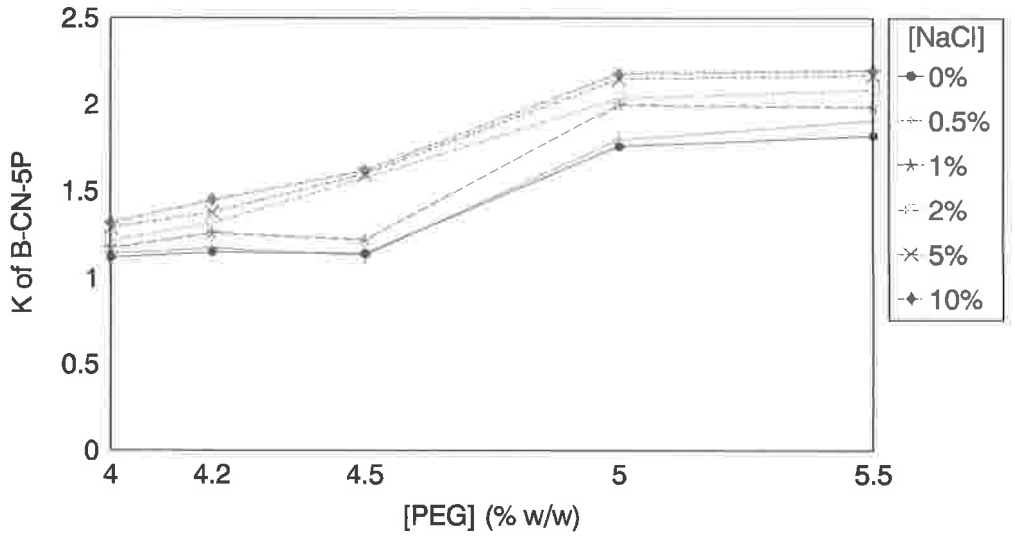


Figure 4.12 The effect of PEG concentration on β-CN-5P partitioning at a range of NaCl concentrations

The observed increases in partition coefficient were more obvious with regard to PEG concentration than those observed in Figure 4.11 when the NaCl concentration was raised. This indicated that the effect of PEG 8000 concentration on β-CN-5P partitioning was more pronounced than the effect of NaCl concentration on partitioning.

The effect of NaCl seemed slightly more pronounced at the higher PEG concentrations of 4.5% (w/w) and above, with an increase in partition coefficient in the 4.0% PEG (w/w) system from 1.12 to 1.32 at 0 and 10% NaCl respectively, and from 1.14 to 1.62 in the 4.5% (w/w) PEG system for the same salt conditions. An increase in NaCl concentrations from 0% to 1% had quite a large effect (K increased from 1.76 to 2.00 in 5% (w/w) PEG), but this appeared to reach a saturation level, as little further effect

was observed on increasing from 5% to 10% NaCl (typically K increased from 2.15 to 2.18 in 5% (w/w) PEG 8000 systems).

The estimated yield of β-CN-5P at each NaCl concentration was investigated at 5.0% (w/w) PEG 8000 using Equation 4.2, and the results are shown in Table 4.6.

[NaCl] (%)	Volume ratio	$K_{\beta\text{-CN-5P}}$	% Yield β-CN-5P
0	1.19	1.76	67.7
0.5	1.19	1.80	68.2
1	1.18	2.00	70.2
2	1.20	2.04	71.0
5	1.19	2.15	71.9
10	1.21	2.18	72.5

Table 4.6 The effects of NaCl concentration on the estimated yield of β-CN-5P at a constant phase volume ratio in 5.0% PEG 8000; 9.2% dextran T500 systems.

The increasing amount of salt in the systems was found to have no effect on the phase volume ratio. The estimated yield of β-CN-5P in the PEG phase was found to increase as the salt concentration increased, rising from 67.7% at 0% NaCl to 72.5% at 10% NaCl.

The data suggested that to produce the maximum partition coefficient and highest estimated percentage yield of β-CN-5P in the PEG phase, a minimal concentration of 5% (w/w) PEG 8000 and 5% NaCl should be used. Further increases in values

obtained at the higher PEG and salt concentrations were small and seemed to indicate a degree of saturation, and were not considered substantial enough to warrant the extra cost of phase system ingredients.

4.2.2.3 The effect of pH on β-CN-5P partitioning in aqueous two-phase systems.

Proteins and peptides exhibit net charges at a given pH depending on the composition of their various amino acid side chains and their terminal groups. At the isoelectric point of a protein its net charge will be zero; at pH values lower than its pI the molecule will be positively charged; and at values above its pI it will be negatively charged.

The terminal charges combined with the surface charges on the side groups of the individual amino acids have been shown to affect the partitioning of a polypepetide chain in an aqueous two-phase system [Walter and Sasakawa, 1971], as previously described in Equation 1, (Chapter 3)

$$\log K = \log K_0 + \gamma Z$$

Equation 3.1

where K_0 is the partition coefficient of the protein at its isoelectric point, Z is the protein's net charge at the pH of the system, and γ is a factor that depends on the salt

used and the composition of the system [Walter and Johansson, 1986; Johansson, 1989].

Therefore by altering the pH of a system it is possible to vary the partition coefficient of a substance by virtue of its surface charge.

In order to investigate the partitioning behaviour of β-CN-5P at different pH values the neutral salts NaCl and Na_2SO_4 were used. As demonstrated in Figure 4.13, many salt ions are unevenly distributed between phases. The anion Cl^- is known to preferentially partition into the upper, PEG phase, and the anion SO_4^{2-} into the lower, dextran phase [Johansson, 1974 and 1989]. By studying the partitioning of β-CN-5P in aqueous two-phase systems containing either 0.24 mole/kg NaCl or 0.12 mole/kg Na_2SO_4 for a range of pH values, it was possible, therefore, to investigate the influence of the net charge of β-CN-5P on its partitioning behaviour (Figure 4.13). Systems were made up in a volume of 1 ml at a range of pH values from 3-10.0 at 5% (w/w) PEG; 9.2% (w/w) dextran T500 in the presence of NaCl or Na_2SO_4.

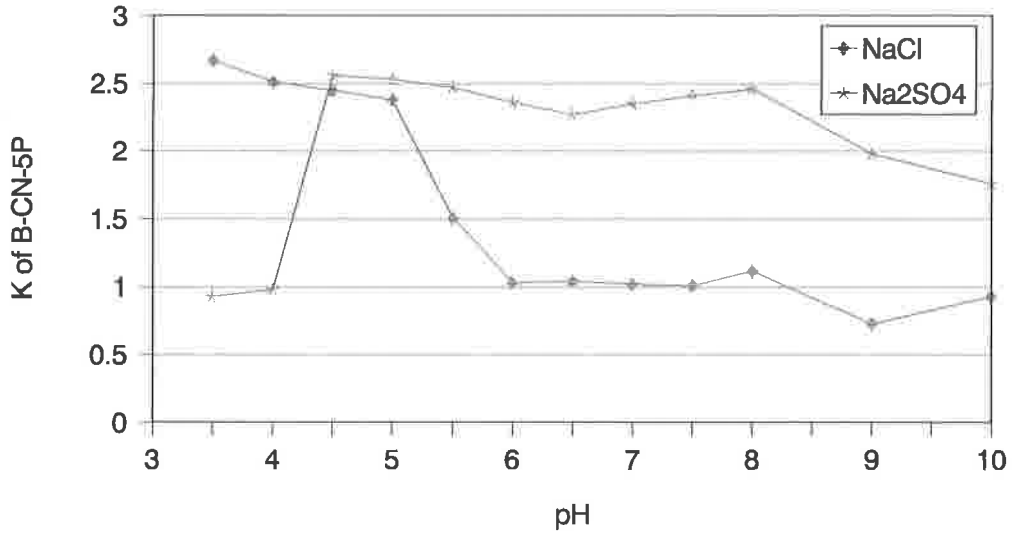


Figure 4.13 The effects of pH on the partitioning of β-CN-5P in a 5% (w/w) PEG; 9.2% (w/w) dextran system.

In the systems containing NaCl the partition coefficient was highest below pH 5.0, with a value of 2.38 more than twice that obtained at pH 7.0 (1.02). However, as demonstrated in Section 3.2.2.2, pH 5.0 also encouraged the partitioning of other proteose peptone components into the upper, PEG, phase. It was, therefore, not a suitable pH for the purification of β-CN-5P from a total proteose peptone fraction.

From pH 6.0 to pH 8.0, K was found to be fairly constant, varying from 1.03 at pH 6.0 to 1.11 at pH 8.0 with β-CN-5P therefore almost equally distributed between the phases. At pH values above 8.0, β-CN-5P partitioned predominantly into the dextran phase. A second observation related to the stability of the β-CN-5P molecule at these pH values. In all HPLC profiles obtained at pH 9.0 and 10.0, a second very small peak was visible, which may have corresponded to a β-CN-5P breakdown product.

In the systems containing Na_2SO_4, the partition coefficient was highest at pH 4.5, with a value of 2.57. A value of 2.38 was obtained at pH 7.0, which suggested that the addition of Na_2SO_4 to an aqueous two-phase systems would be more beneficial in the separation of the total proteose peptones than the observed additions of NaCl. Unfortunately, when the effects of the addition of Na_2SO_4 on the partitioning of total proteose peptone were studied, it was found to encourage the partitioning of contaminants into the PEG along with β-CN-5P (data not shown).

The β-CN-5P was found to partition preferentially into the PEG phase at all pH values above 4.5, although the partition coefficient decreased from 2.46 at pH 8.0 to 1.76 at pH 10.0. β-CN-5P was not observed to partition preferentially into the dextran phase at any pH. However at pH 3.5 (K = 0.93) and pH 4.0 (K = 0.98), the β-CN-5P was almost equally distributed between the phases, as the partition coefficient was in the region of 1.

Hence, β-CN-5P was observed to behave in an opposite manner in the presence of NaCl or Na_2SO_4. In the case of NaCl, it partitioned preferentially into the upper phase at low pH values, as the Cl^- anion rendered the PEG phase slightly more negative than the dextran phase, and β-CN-5P is positive at low pH values. Conversely, the SO_4^{2-} anion rendered the dextran phase more negative than the PEG phase, and β-CN-5P was encouraged into the lower phase at low pH values.

At higher pH values, when β-CN-5P was negative, it partitioned relatively more into the dextran phase in the presence of NaCl, and into the PEG phase in the presence of

Na_2SO_4. At the point where the two lines crossed, (the crosspoint) the pI of β-CN-5P was determined. At this pH value, in the region of pH 4.5 to 4.8, partitioning of β-CN-5P was independent of salt effect (as the net charge of the molecule was zero), and was, hence identical in the presence of each salt. The crosspoint of a protein or a peptide, therefore equates to the pI of the molecule, and the pI of β-CN-5P was determined to be in the region of 4.7. This agreed well with a value of pH 4.7 obtained by isoelectric focusing and reported previously in the literature [Girardet *et al.*, 1991]..

4.3 CONCLUSIONS

Partially digested β-casein was shown to be a suitable large scale source of β-CN-5P, and was demonstrated to contain less contaminating material than a milk total proteose peptone sample isolated directly from milk. This method is proposed as a tool to facilitate the large scale production of β-CN-5P.

By increasing the concentration of phase-forming polymers in equivalent systems, and hence the tie line length, the partition coefficient of β-CN-5P was found to increase. The estimated percentage yield of β-CN-5P in the upper phase also increased.

The addition of NaCl was found to encourage the partitioning of β-CN-5P into the PEG phase, particularly when combined with a higher PEG concentration. The estimated percentage yield of β-CN-5P in the PEG phase was also observed to increase.

The effects of pH on the partitioning of β-CN-5P were studied. In the presence of NaCl, the partition coefficient decreased as the pH increased. Conversely, in the presence of Na_2SO_4, the partition coefficient increased as the pH increased. The crosspoint was determined as being in the region of 4.0 to 4.5.

CHAPTER 5 THE OPTIMISATION OF IMMOBILISING A MODEL ENZYME TO NYLON FILM

5.1 INTRODUCTION

The binding of the enzyme β-glucosidase to pieces of nylon film is investigated in this chapter, with a view to developing a continuous separation method for the novel downstream purification of proteins.

The continuing development of efficient and reliable techniques to isolate and recover proteins and enzymes from heterogeneous mixtures is of vital importance to industries as varied as food-processing, brewing, pharmaceutical and biomedical, which often require an exacting purity of target protein, particularly if they are from bacterial and/or viral sources [Bonnerjea *et al.*, 1986; Boyer and Hsu, 1993].

The above industries have varied requirements for their products in terms of quantity , purity and value of the product. These demands range from the highly specific methodology necessary to obtain an extremely pure product on a laboratory scale, as required by the pharmaceutical industry, to simple, low resolution precipitation and filtration techniques, used to provide a crude isolation of extracellular hydrolytic enzymes for use in the food industry [Niven 1995 and 1996].

Separation technologists working in the middle ground situated between these two extremes are forced to make compromises between the intended purity of product

and the economical viability of the process. It is not possible for them to gain the desired yield and purity of a target protein from a fermentation broth in a single step. Much work is, therefore directed towards developing a successful combination of a clarifying step with the more specific techniques required to provide the homogeneity expected by industry [Hochuli, 1988; Boyer and Hsu, 1993; Watanabe *et al.*, 1994]. Affinity separations have proved to be one of the more successful approaches of the latter, high resolution techniques.

Affinity methodology for the separation of proteins traditionally relies on a ligand attached to a stationary phase coming into contact with the desired product contained within a second, mobile, phase. Ideally, the product reversibly binds to the ligand and is selectively extracted from any contaminating material present in the mobile phase [Stanbury and Whitaker, 1993]. Typically such methods take place following an initial separation of the target protein from much of the crude contaminating material that can be expected in the primary stages of a protein purification scheme [Bonnerjea *et al.*, 1986; Birnbaum and Mosbach, 1991; Andersson, 1992]. Affinity separations are generally unsuitable for the first steps of protein purification due to the expenses involved during the preparation and regeneration of matrix material, the cost of the ligand, the observed fouling of the stationary phase with particulate material, and the time and operational restrictions imposed by this fouling [Bonnerjea *et al.*, 1986].

In batch adsorption processes the amount of available ligand is a major limiting factor in the purification of target material. Moreover, practical limitations enforced by column size and throughput, and the need to introduce an already purified feedstock

have rendered these approaches difficult to achieve on a modest budget [Gill *et al.*, 1997]. A potential answer to this and other disadvantages could be to utilise a mobile support for the ligand, thus introducing a continuous separation process. A continuous process would also lower the amount of ligand required for the separation and, hence, production costs.

A continuous system has been developed to counteract the problems mentioned above in the production of less expensive products. This approach was devised by Niven and Scurlock, [1993]. The prototype apparatus known as the "Affexts" machine, and described in UK patent application No 2274843, [Niven *et al.*, 1997] is depicted in Figure 5.1.

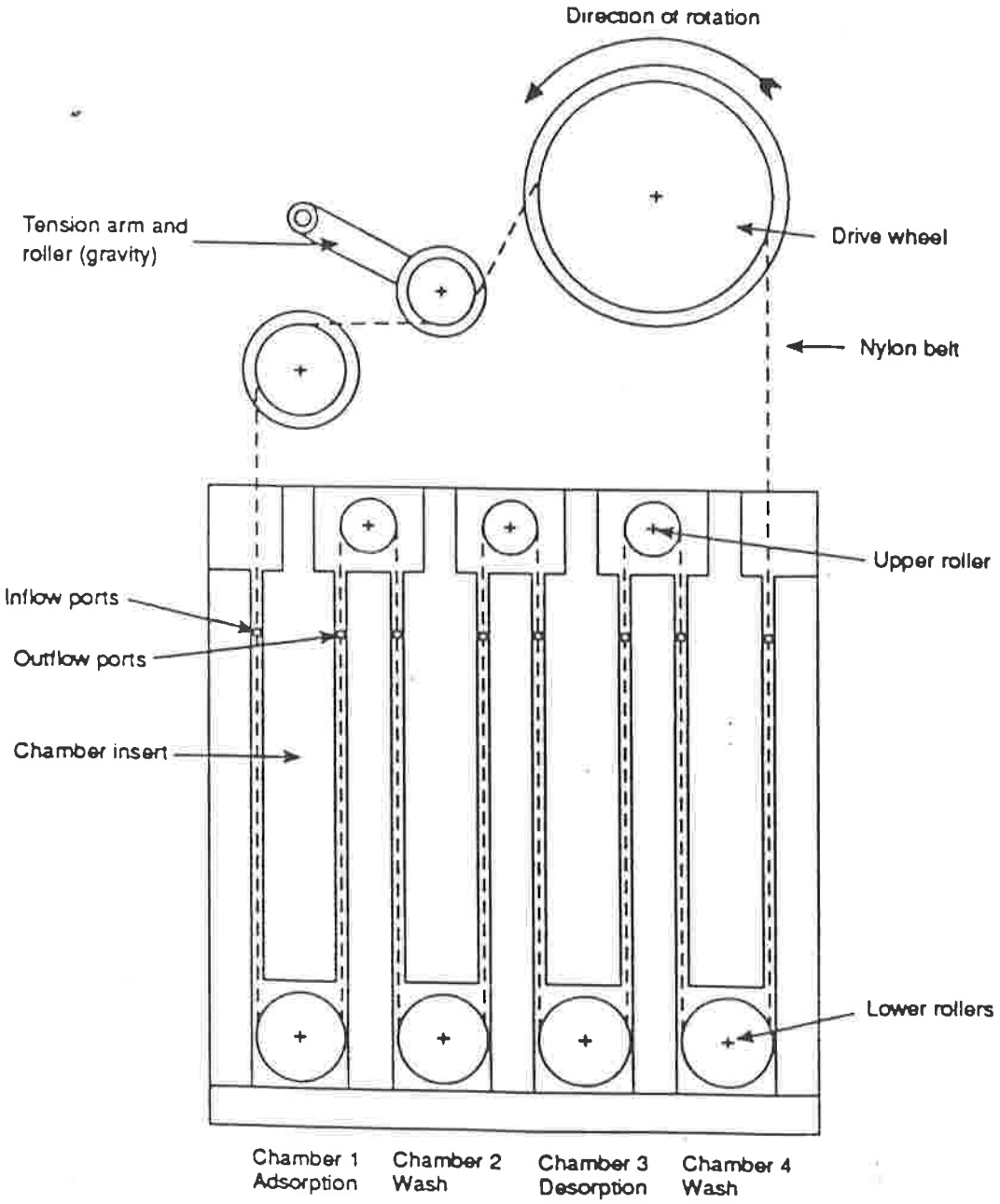


Figure 5.1 The "Affexts" machine [Niven and Scurlock, 1993]. An affinity ligand is attached to a mobile belt, the target material is collected and the ligand recycled as described in the text.

The mobile support of the above apparatus is a continuous nylon belt. A ligand is attached to the belt and is used to extract target material as follows. A target protein present in the adsorption chamber (chamber 1), specifically attaches to the nylon bound ligand and is transported via a washing chamber (chamber 2), to an elution chamber (chamber 3), where conditions are altered such that the ligand/protein complex is not favoured hence the target protein is released. The nylon bound ligand continues through the elution chamber, passes through a second washing chamber (chamber 4), and is again presented to the target protein at the adsorption chamber, thus completing a cycle. The solution in each chamber is controlled through ports which allow the recycling of eluent as required.

As the flow of the feedstock and eluent is continuous, the process can be controlled and monitored easily. No time is lost through cleaning and regeneration of the matrix, and the ligand is not prone to damage through increases in pressure. The rate-limiting step of chromatography, often attributed to the diffusion of the liquid media into the porous matrix is minimal with non-porous nylon, and a more rapid re-cycling of the ligand compensates for the low surface area of the matrix [Niven 1996]. Binding of the ligand to the matrix represents, therefore an area in which improvements would make a large difference to the procedure.

Studies have demonstrated the efficiency of this apparatus as a separation tool using soybean trypsin inhibitor as ligand and trypsin in a crude pancreatin extract as target material [Niven and Scurlock, 1993; Niven *et al.*, 1994]. The efficiency of trypsin

recovery was found to be inversely related to the input rate, indicating a compromise between throughput and recovery [Gill *et al.*, 1997].

For the potential of this procedure to be fully realised, it is of paramount importance to maximise the amount of ligand bound to the nylon film to ensure that the maximum yield of product may be obtained. Hence the binding procedure must be looked at in detail to guarantee the maximal amount of ligand can be bound.

The advantages of nylon sheet as an attractive and appropriate matrix for protein immobilisation have long been appreciated due to the strong and inert nature of nylon, the possibilities of derivatising it without reducing this strength, and its low cost and commercial availability [Carvajal *et al.*, 1977; Carvajal *et al.*, 1978; Daka and Laidler, 1980; Scouten, 1987].

This chapter describes the optimisation of current binding techniques using small pieces of nylon film and a model enzyme/substrate system. It is hoped that this protein immobilisation procedure will also be of interest to biotechnologists working with other immobilised affinity separations or with immobilised enzyme bioreactors.

Almond β-glucosidase was chosen as a convenient model enzyme for the studies as it is inexpensive and is easily assayed with the synthetic substrate p-nitrophenol-β-D-glucopyranoside (pNPG). The enzyme has been shown to run as two distinct bands on an electrophoretic gel; an intense band of 67, 000 D and a slight band of 144, 000 D, possibly due to dimer formation [Ravet *et al.*, 1993]. The glycosidases are readily

available hydrolytic enzymes whose industrial significance has been demonstrated by Agarwal and Gupta, [1996].

5.2 RESULTS AND DISCUSSION

5.2.1 Assay Optimisation

Preliminary experiments focused on optimising assay conditions for the substrate pNPG with β-glucosidase in free solution. The resulting Lineweaver-Burk plot can be seen in Figure 5.2 (a) where a concentration of 1μg/ml enzyme was used throughout. A time scale study to substantiate the suitability of a concentration of 5mM pNPG is shown in Figure 5.2 (b).

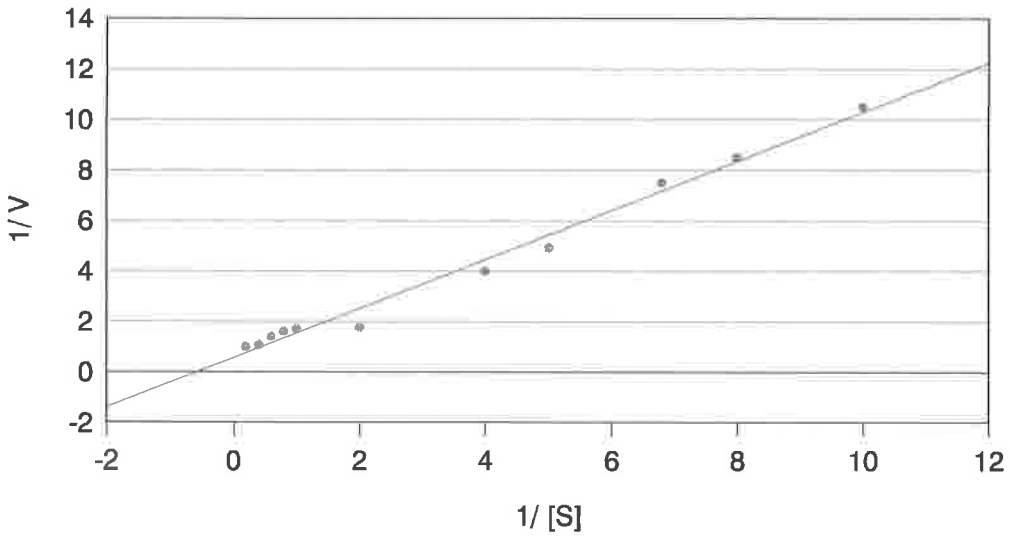


Figure 5.2 (a). A Lineweaver-Burk plot for the reaction between β-glucosidase and pNPG in free solution at pH 5. The Km was subsequently calculated as 1.85mM.

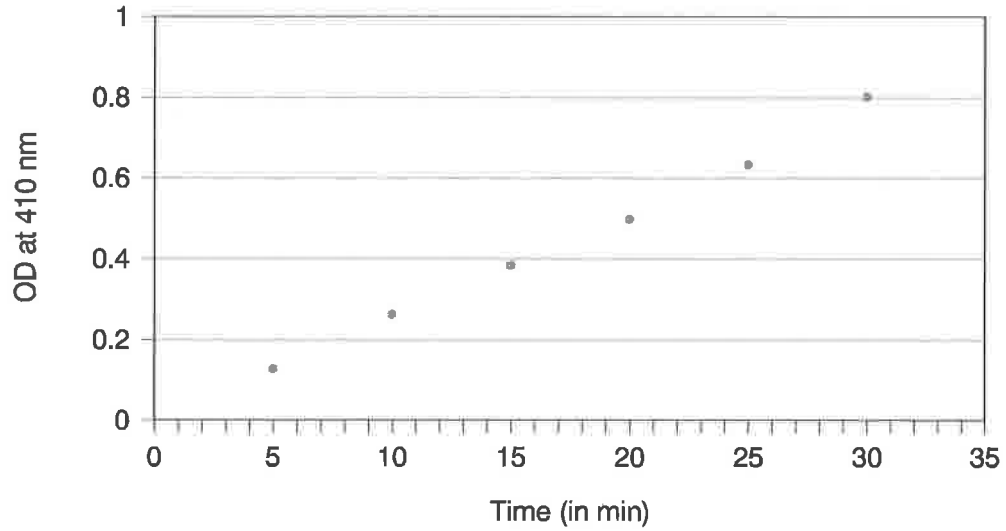


Figure 5.2 (b). A time scale study to confirm the saturating conditions of 5mM pNPG at pH 5 using 1 μg/ml β-glucosidase.

The effect of pH on the activity of free enzyme in solution was also investigated using a concentration of 1μg/ml enzyme and 5mM substrate (Figure 5.2 (c)). The pH was controlled using 50mM phosphate buffers.

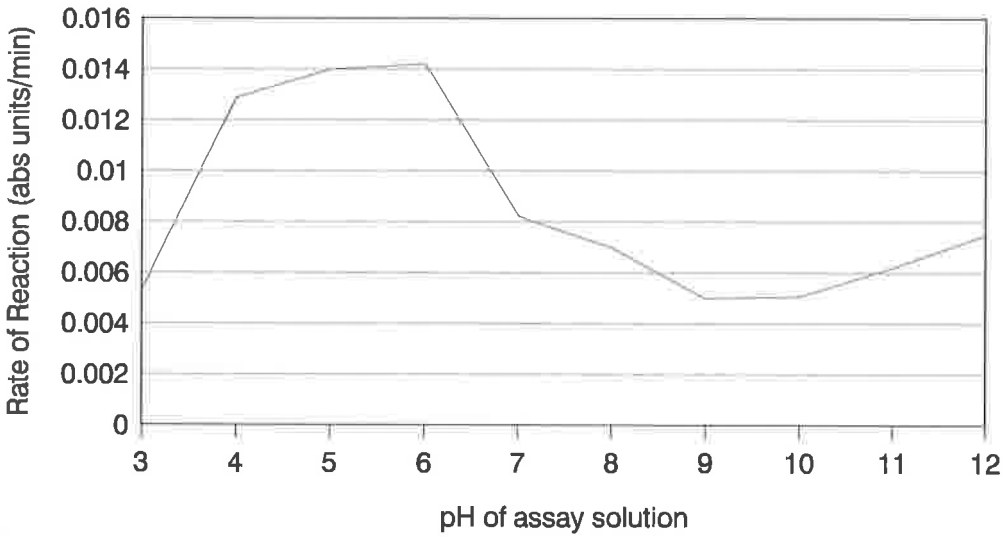


Figure 5.2 (c) The effect of pH on the activity of β-glucosidase in free solution. Activity was determined by spectrophotometrically measuring the release of p-nitrophenol from pNPG at 410nm.

The activity of almond β-glucosidase was found to be optimal at pH 5.5 which was identical to that previously reported by others [Iborra *et al.*, 1992]. β-glucosidase isolated from *S.hygroscopicus* has been shown to exhibit optimum activity at pH 4 [Spear *et al.*, 1993]. A value of pH 7.5 has been found for the enzyme isolated from the actinomycete, *Micromonospora chaleae* [Gallagher *et al.*, 1996].

Following these experiments, assay conditions were set at 15 min with 5mM pNPG at pH 5.

The procedure used to immobilise β-glucosidase onto nylon film followed a method outlined by Andrews and Mbafor [1991], whereby nylon film was activated by acid hydrolysis and the protein was coupled to the nylon in five stages using glutaraldehyde and with PEI as a spacer molecule (Table 5.1). Previous nylon coupling techniques have used reagents such as hydrazine, dimethyl sulphate, or triethyloxonium tetrafluoroborate, all of which exhibit a high level of toxicity, and were not therefore considered desirable [Iborra *et al.*, 1992; Agarwal and Gupta, 1996]. Although the toxic effects of glutaraldehyde are still considerable it is easily manipulated, and, with care risks are minimal.

Incubating Solution	Incubation Time
1. 2.9M HCl at 40°C	16 hours (overnight)
2. 2.5% glutaraldehyde pH 8	15 min
3. 5% polyethylenimine (PEI) pH 8	60 min
4. 2.5% glutaraldehyde pH 8	15 min
5. 0.25mg/ml β-glucosidase pH 8	120 min
6. (Assay) 5mM ρ-nitrophenol-β-D-glucopyranoside (pNPG). pH 5	15 min

Table 5.1. The immobilisation procedure used as a template to optimise the binding of β-glucosidase to nylon film.

Nylon pieces measuring 3 cm by 3 cm were used in each experiment, and were thoroughly washed in water between the stages outlined in Table 5.1. The assay (stage 6), was arrested by removing the nylon piece from the assay mixture. An equal volume (O.5 ml) of assay solution was added to 0.5ml 0.1M NaOH to raise the pH sufficiently to spectrophotometrically measure the release of p-nitrophenol from pNPG at 410nm. The nylon pieces were air-dried, then weighed. The absorbance readings were then normalised to allow for discrepancies in weight between the pieces of nylon.

In order to optimise the binding conditions of β-glucosidase to nylon pieces, each stage of the above procedure was studied in isolation, by altering parameters such as

incubation times and reagent concentrations whilst keeping the conditions of the other stages of the process constant.

5.2.2 Optimisation of Hydrolysis Time

To investigate the effect of altering the hydrolysis time with HCl on the amount of active enzyme that could be bound to nylon, nylon pieces measuring 3 cm by 3 cm were subjected to the methods described in Table 5.1. Four replicates were studied for each hydrolysis time, ranging from one hour to overnight (in 2.9M HCl at 40°C). As a control, two pieces of nylon were put through stages 2-5 of the procedure, following incubation initially in H_2O in place of HCl. Two additional nylon pieces were treated as blanks, following the procedure used for the test samples, but with a final incubation step in buffer in place of the enzyme. Blank readings were routinely subtracted from test readings.

Figure 5.3

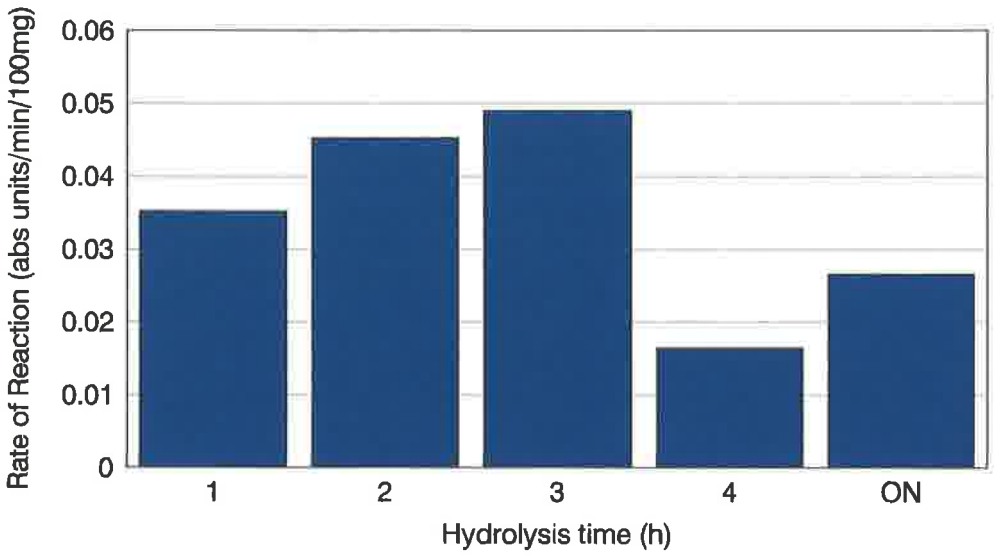
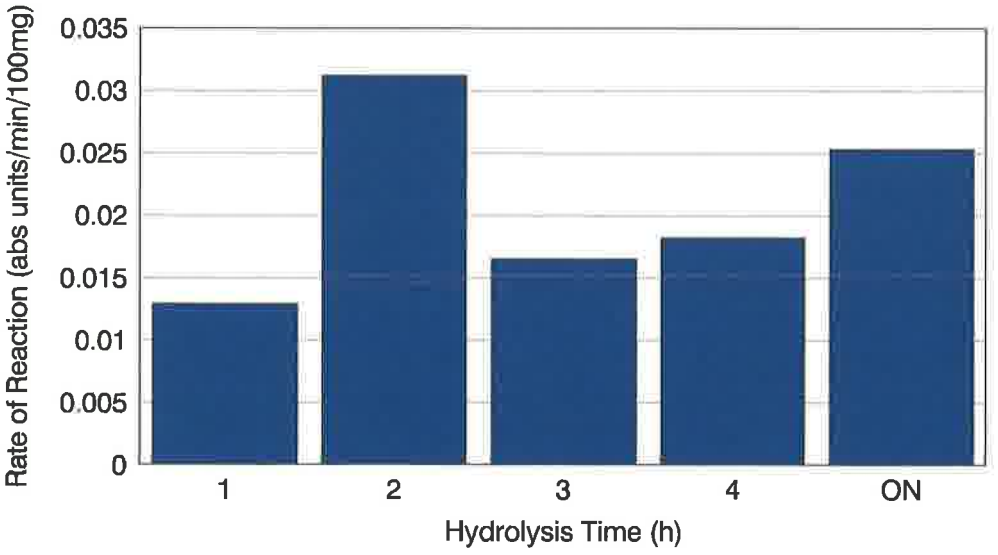


Figure 5.4



Figures 5.3 and 5.4. The effect of altering the hydrolysis time of the nylon pieces on β-glucosidase binding. Nylon pieces were submerged in 2.9M HCl at 40°C for the times stated. The activity was assayed (5.3) directly following the incubation in enzyme (step 5) or (5.4) following a prolonged incubation in deionised water (see text for details).

In the nylon pieces assayed directly following the enzyme incubation, (Figure 5.3), the rate of reaction increased from 0.0352 ΔOD/min/100mg after 1h hydrolysis to a maximum of 0.049 ΔOD/min/100mg after three hours' hydrolysis. The rate of reaction then decreased to 0.0164 ΔOD/min/100mg following 4h hydrolysis before increasing again to 0.0266 ΔOD/min/100mg after an overnight hydrolysis.

The nylon pieces assayed following a prolonged incubation in water followed a similar pattern (Figure 5.4), although the actual readings were lower. The rate of reaction increased from 0.0129 ΔOD/min/100mg after 1h to a maximum of 0.0312 ΔOD/min/100mg after 2h hydrolysis. A drop in rate of reaction to 0.0165 ΔOD/min/100mg was observed after 3h hydrolysis before the rate of reaction rose again to 0.0253 ΔOD/min/100mg after an overnight hydrolysis.

Although the data shown in Figures 5.3 and 5.4 was difficult to interpret, it was repeated in a number of experiments (12 replicates of samples overall) and it was found that the rate of reaction decreased in each of the one, two and three hour conditions after a prolonged incubation of the nylon pieces in water prior to the bound activity assay. This indicated that some of the enzyme had been removed from the nylon by the incubation of the nylon pieces in water. However, the rate of reaction remained constant, or increased slightly, with nylon pieces that had been hydrolysed in HCl for more than three hours. This would indicate that a more effective immobilisation had occurred in the pieces that were hydrolysed for longer than two

hours. One possible explanation for this trend may be that the COOH groups generated together with NH_2 groups by the partial hydrolysis of the nylon matrix, were involved in stabilising the subsequent linkages in other parts of the immobilisation scheme, perhaps by electrostatic bonding to parts of the PEI.

In view of these trends it was decided to limit the acid hydrolysis time to two hours in the present study. The possibility of stabilising the linkages formed during the shorter hydrolysis times was also investigated and will be discussed in Section 5.2.6 of this chapter. Moreover the occurrence and effects of non-specific binding will be discussed in detail in chapter 6.

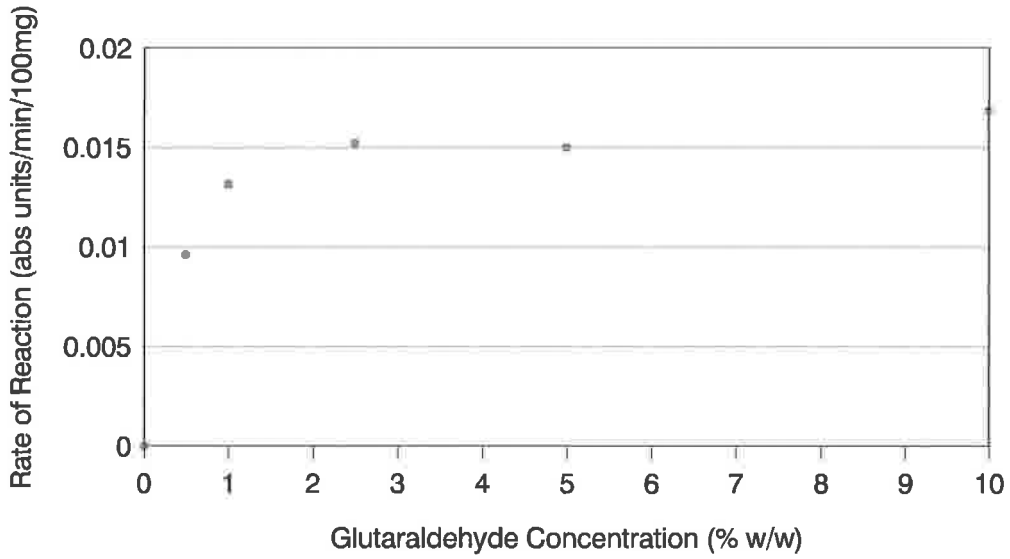
The concentration of hydrochloric acid was normally kept at 2.9M, in accordance with previous work done by Andrews and Mbafor [1991]. This concentration was chosen as it proved sufficient for the overnight hydrolysis, without causing excessive hydrolysis, and hence destruction of the nylon matrix. If the concentration exceeded 3M, the nylon pieces were observed to disintegrate, and at lower concentrations e.g. 2.5M an inferior amount of ligand was bound (data not shown).

5.2.3 Optimisation of the First Glutaraldehyde Activation

The immobilisation of enzymes to nylon film by glutaraldehyde amine linkages has previously been documented [Morris *et al.*, 1975]. The purpose of the first glutaraldehyde activation step, stage two of the overall procedure, was twofold. First

and foremost, it introduced a reactive aldehyde group onto the nylon matrix, and second, it provided a spacer arm between the nylon matrix and the enzyme, thus minimising interference of the nylon matrix with enzyme binding and activity. The optimum concentration of glutaraldehyde and length of incubation time with the nylon pieces were investigated. All other concentrations and incubation times (including stage four, the second glutaraldehyde activation step) were kept as outlined in Table 5.1. Four replicates were studied for each condition. Blank readings were routinely subtracted from test readings (Figure 5.5 (a) and (b)).

(a)



(b)

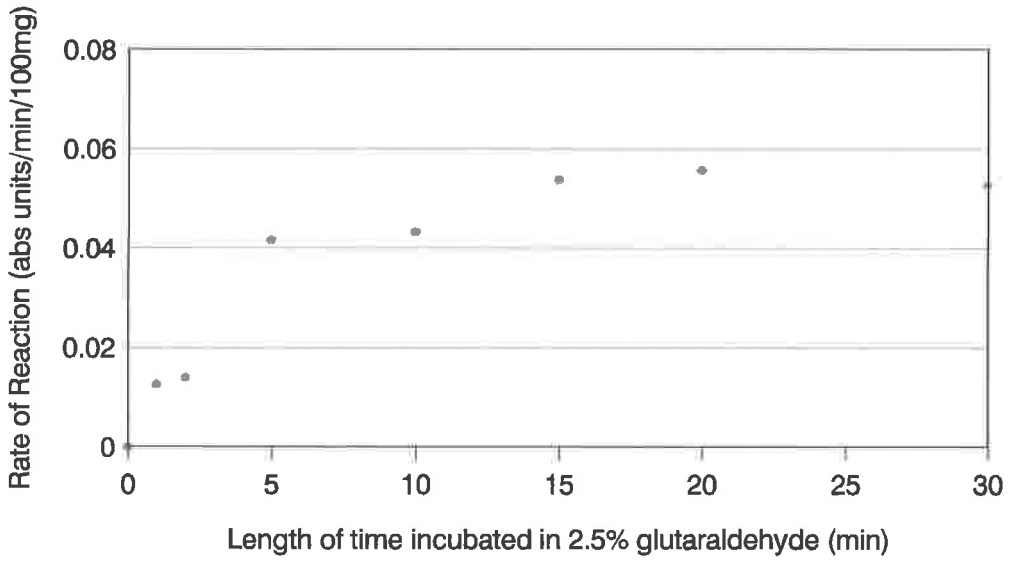


Figure 5.5. The effect of altering the concentration of glutaraldehyde and treatment time at stage 2 of the procedure (see text), on β-glucosidase binding. Nylon pieces were incubated in (a) the indicated glutaraldehyde concentrations for 15 min, or (b) in 2.5% (w/w) glutaraldehyde for the times shown.

As was expected, the amount of the β-glucosidase immobilised rose steeply then reached a plateau as the concentration of the glutaraldehyde increased. The previously chosen concentration of 2.5% w/w glutaraldehyde appeared at the start of the plateau and was hence considered to be optimal for reasons of efficiency and cost.

The incubation time of the nylon pieces in 2.5% glutaraldehyde was also investigated, with a similar plateau occurring at around 10- 15 min. It was therefore proposed to keep the incubation time of the first glutaraldehyde activation at 15 min.

5.2.4 The inclusion of a spacer molecule

Polyethylenimine was used in the third stage of the procedure in an attempt to amplify the amount of enzyme that could be bound to the nylon. This was considered possible as PEI contains many free imino groups, and should be able to act as a highly branched spacer molecule, enabling a larger amount of glutaraldehyde to become bound onto the nylon during the second glutaraldehyde activation step, (stage four of the procedure outlined in Table 5.1), and hence, increasing the amount of enzyme that could be potentially bound to the nylon. Previous work had suggested that by the inclusion of this step, an increase of 50% of bound enzyme was achieved, [Andrews and Mbafor, 1991], an observation that was supported by present data where the inclusion of PEI increased the rate of reaction from 0.029 ΔOD/min/100mg to 0.0613 ΔOD/min/100mg (Figure 5.6). Four replicates were studied for each condition. Blank readings were

subtracted from test readings. In the condition where no spacer was used, enzyme was bound immediately following the initial glutaraldehyde stage, i.e. stages 3 and 4 of Table 5.1 were omitted.

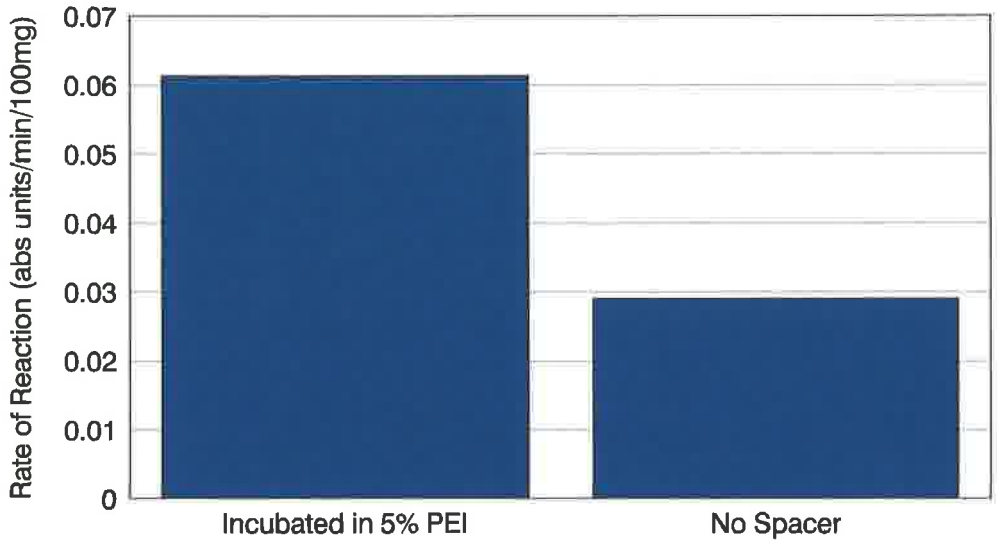


Figure 5.6. The effect of adding a spacer molecule such as PEI at stage 3 of the procedure on β-glucosidase binding.

A second branched spacer molecule, the natural polymer chitosan, was investigated as a possible alternative to PEI. Chitosan, from the deacetylated chitin of crab shells, displays similar structural properties to PEI, (i.e. provides a multiply branched spacer molecule, in this case of polyglucosamine), but required a more specific methodology to prepare as an incubating solution, as it was insoluble in phosphate buffer at pH 8. Several protocols were investigated to find a suitable procedure for the preparation of a chitosan solution, (including Muzzarelli *et al.*, [1994], and Muzzarelli *et al.*, [1995]),

and the method of Petach and Driscoll [1994] was found to be the most appropriate. Following their procedure, chitosan was dissolved in 10% acetic acid, (diluted in H_2O), and was used here at pH 3.5 in step 3 of our protocol in place of the PEI. All other stages were performed in 0.1M phosphate buffer pH 8 as previously (Figure 5.7). Four replicates were studied for each condition. Blank readings were subtracted from test readings.

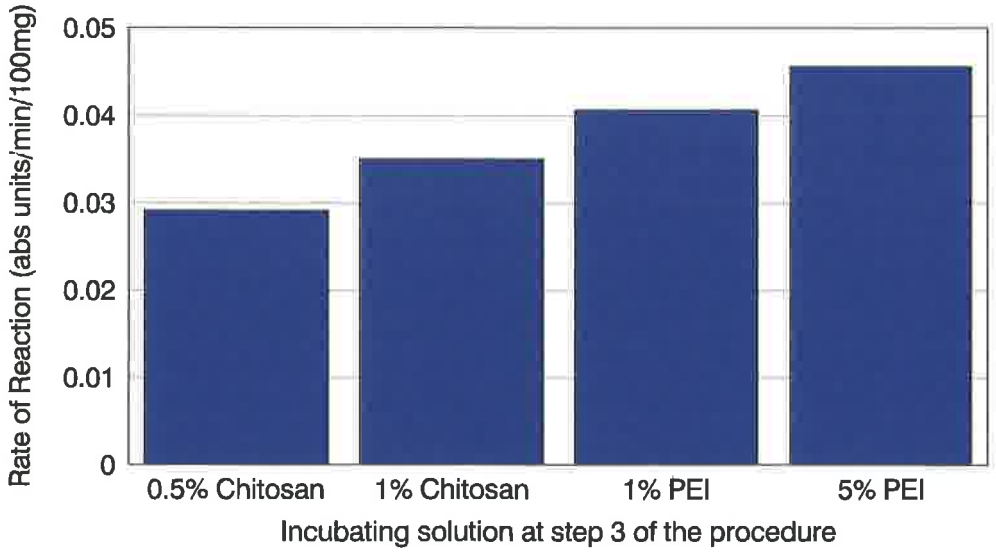


Figure 5.7. The effect of altering the concentration and type of spacer molecule on β-glucosidase binding. Nylon pieces were incubated in the solutions for 1 h at the stated concentrations (see text for details).

As can be seen in Figure 5.7, varying the concentration of the spacer molecule had little effect on the extent of nylon bound enzyme. It appeared that more enzyme bound using PEI (0.0406 ΔOD/min/100mg for 1% PEI) as a spacer molecule rather than chitosan (0.035 ΔOD/min/100mg for 1% chitosan). Further experiments to examine

the relative stability of the PEI and chitosan bonds by retesting the nylon pieces one week after they had originally been tested, (following storage in buffer at pH 8 at 4°C), showed little difference between the two spacer molecules(data not shown).The imine/aldehyde linkages formed between PEI and glutaraldehyde were hence found to compare favourably with the amine/aldehyde linkages between chitosan and glutaraldehyde in terms of stability. Thus the replacement of PEI with chitosan showed no real advantages in terms either of stability or amplification and of course chitosan is relatively much more expensive. On the basis of our results, the step was optimised by reducing the concentration of PEI from 5% to 1%. Similar results were also obtained by immobilising trypsin onto nylon by this procedure (with Nα-Benzoyl-DL-Arginine p-nitroanilide (BAPNA) used as the assay substrate) as a further model enzyme substrate system (Figure 5.8).

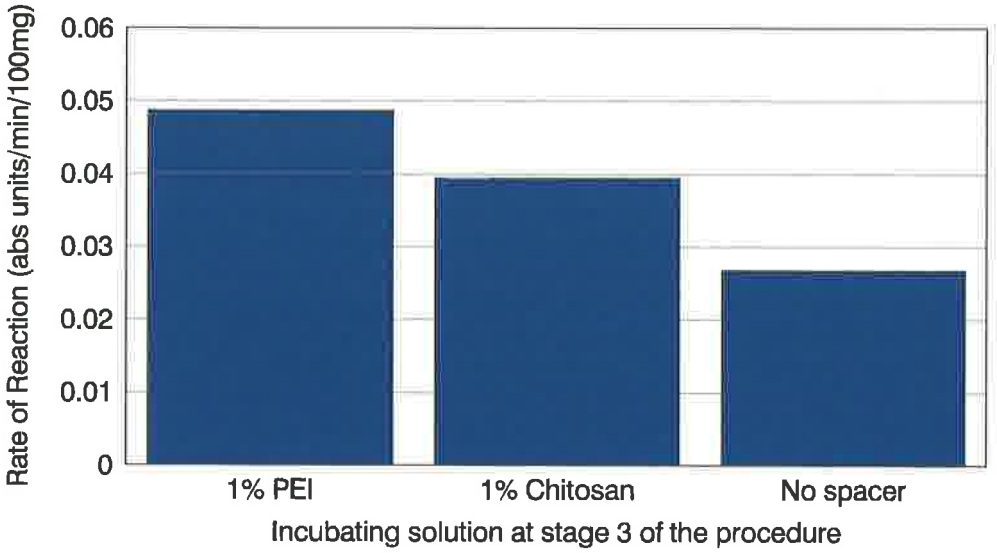


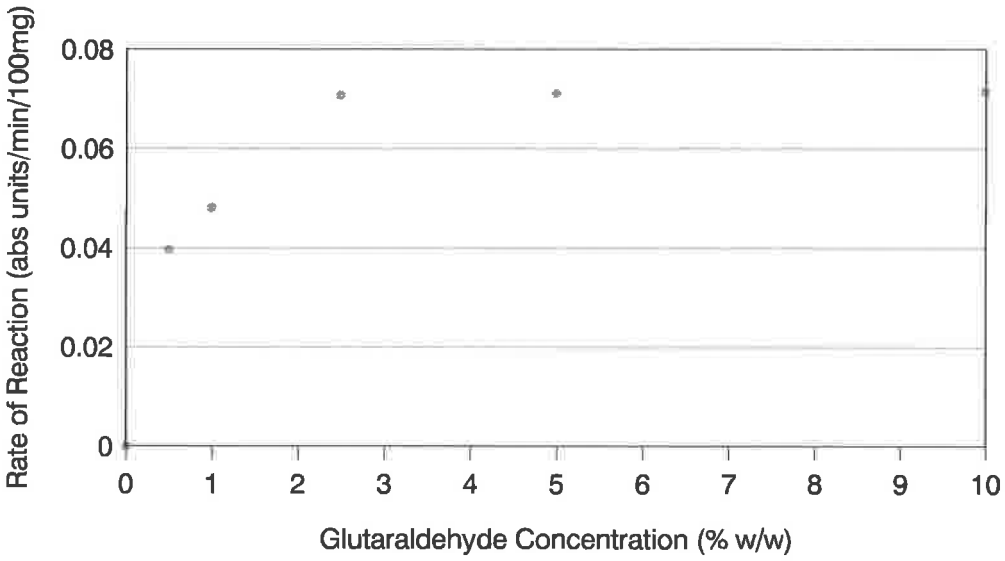
Figure 5.8. The effect of altering the concentration and type of spacer molecule on trypsin binding. Nylon pieces were incubated in the solutions for 1 h at the stated concentrations (see text for details).

Again, it was observed that a greater amount of enzyme bound onto the nylon using PEI (0.0486 ΔOD/min/100mg) rather than chitosan (0.0394 ΔOD/min/100mg) as a spacer molecule. Although the introduction of either molecule was found to amplify the amount of ligand that would bind when compared with the value obtained without a spacer (0.0267 ΔOD/min/100mg). The importance of the inclusion of an amplification step was thus confirmed.

5.2.5 Optimisation of the Second Glutaraldehyde Activation

The purpose of the fourth step in the methodology, the second glutaraldehyde activation was again to introduce both reactive groups for enzyme binding to the PEI and to introduce a further spacer molecule onto the matrix in an attempt to reduce any possible interactions between the large PEI molecule and the active site of the enzyme. This latter point was considered important as the direction of the binding of β-glucosidase could not be controlled. The spatial position of the enzyme's active site was required to be as far away as possible from the globular PEI to minimise steric hindrances during the assay. The effect of increasing the glutaraldehyde concentration from 0% w/w to 10% w/w was investigated, and a plateau similar to that obtained in Figure 5.5 (a) was observed when the nylon pieces were incubated in glutaraldehyde at a concentration of 2.5% w/w or greater (Figure 5.9 (a)). It was therefore decided that the glutaraldehyde concentration should remain at 2.5% (w/w).

(a)



(b)

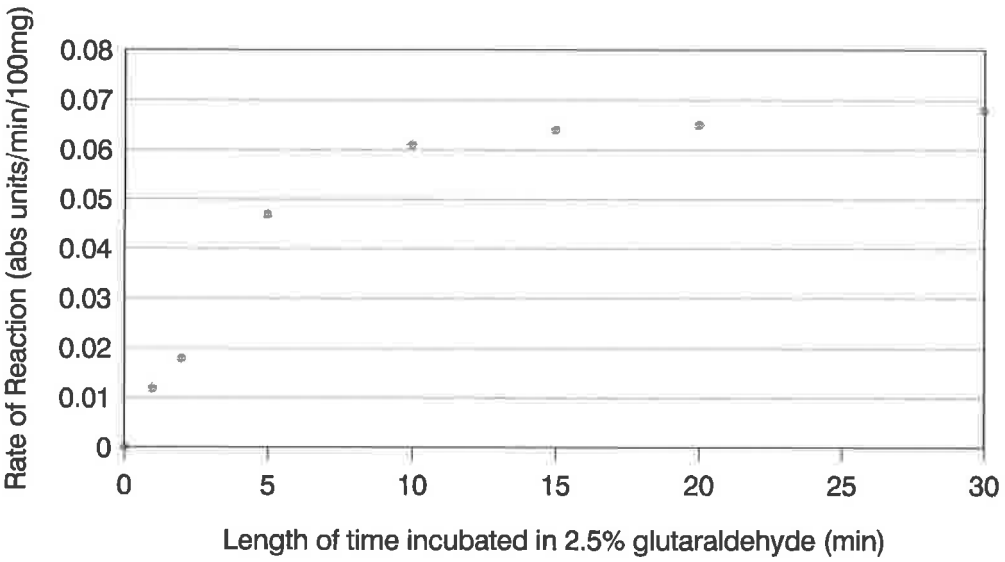


Figure 5.9. The effect of altering the concentration of glutaraldehyde and treatment time at stage 4 of the procedure (see text), on β-glucosidase binding. Nylon pieces were incubated in (a) the indicated glutaraldehyde concentrations for 15 min, or (b) in 2.5% (w/w) glutaraldehyde for the times shown.

Similarly, as in Section 5.2.2, a time scale study was performed to investigate the effect of increasing the incubation time in glutaraldehyde (Figure 5.9 (b)). All other concentrations and incubation times (including stage two, the first glutaraldehyde activation step) were kept as outlined in Table 5.1. The experiments were performed in quadruplicate with blank readings subtracted from test readings.

As previously, in Figure 5.5 (b), a plateau was observed in the region of 10-20 min. The length of incubation time in glutaraldehyde during the second incubation was set at 15 min, as no advantage was found in increasing the incubation time further.

5.2.6 Optimisation of Enzyme Binding

Optimising the β-glucosidase concentration was important in order to immobilise the maximum amount of enzyme onto the nylon film with the minimum amount of wastage due to the relative costs of using a bioreactive ligand. A range of enzyme concentrations from 0.1 mg/ml to 5 mg/ml was studied to determine the effect of increasing the concentration of the incubating solution on the amount of enzyme that would bind to the nylon bound glutaraldehyde. Nylon pieces were incubated in the chosen solution for 2 h at 37°C in 0.1 M phosphate buffer pH 8 prior to a washing step and the assay. Following the assay of the nylon pieces, the residual activity in the incubating solutions was measured to determine the amount of enzyme that had not bound to the nylon. In view of likely possible differences in the activity of free and immobilised enzyme [such differences have been reported previously, e.g. Ravet *et al.*,

1993], these readings could not be directly compared, but were used here merely as a guide as to how much enzyme remained in the incubating solution and was, hence, wasted. The level of "wasted" enzyme ranged from 0.024 ΔOD/min at 0.25 mg/ml β-glucosidase to a maximum of 0.109 ΔOD/min at 5 mg/ml β-glucosidase, indicating that progressively more enzyme was wasted as the enzyme concentration increased. Quadruplicates were performed for each condition, and blank readings were subtracted from the test readings (Figure 5.10). These readings describe the level of enzyme attached to the nylon.

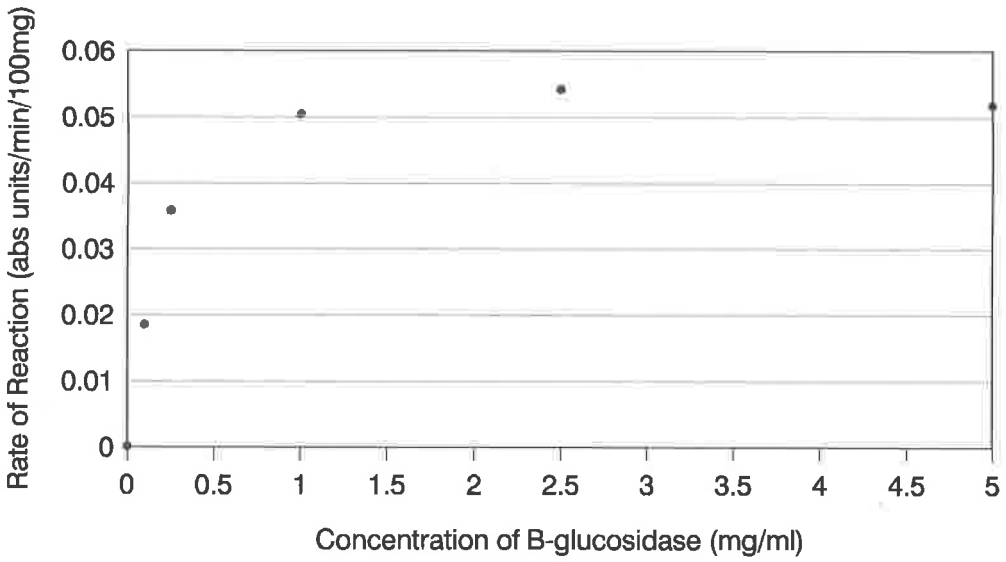


Figure 5.10. The effect of altering the concentration of enzyme in the final stage of the immobilisation scheme (see text for details) on the observed level of β-glucosidase bound.

The rate of reaction was found to plateau at concentrations above 1 mg/ml β-glucosidase, indicating saturation of the nylon pieces with enzyme at this level (Figure 5.10). However a β-glucosidase concentration of 0.25 mg/ml gave about 67% of the maximum level of enzyme binding and appears likely to represent the most cost effective approach for many enzymes or other ligands, even though at this concentration the nylon pieces were not fully saturated with the enzyme. To achieve saturation of the nylon pieces, a β-glucosidase concentration of 1 mg/ml was required, but this also results in a substantial amount of unutilised enzyme in the remaining solution.

5.2.7 The effect of using a stabilising agent such as sodium borohydride on the stability of the bound β-glucosidase.

The data provided in Figures 5.3 and 5.4 of this chapter indicated that the linkages involved during the binding of β-glucosidase to nylon might require stabilising to limit the amount of enzyme lost during a prolonged incubation of the β-glucosidase bound to nylon pieces in water. The introduction of an incubation in sodium borohydride was suggested as a means to stabilise the links, and to help prevent non-specific binding to unutilised aldehyde groupings. Following the incubation of the nylon pieces in β-glucosidase, it was postulated that some of the aldehyde groupings would remain active because steric hindrances due to the size and globular nature of β-glucosidase would prevent all of them being involved in binding the enzyme. Small, contaminating molecules however could potentially bind to these free aldehyde sites unless measures

were taken to prevent it. Through an incubation in sodium borohydride (at a pH in the basic region) these aldehyde groups would be reduced to the less reactive alcohol group. An alternative method to block these sites is described by Andrews and Mbafor [1991], who used the molecule ethanolamine to the same purpose. The advantage of using sodium borohydride is that it changes the character of the aldehyde group (by simple reduction) rather than attaching another molecule to it, thus it avoids "cluttering" on the matrix.

This experiment determined first whether the introduction of an incubation in sodium borohydride after the β-glucosidase incubation or two incubations in sodium borohydride would have any effect on the amount of enzyme bound. Where two incubations in sodium borohydride occurred, they directly followed the PEI binding stage and the β-glucosidase binding step (referred to as the initial assay). Second, all the assayed nylon pieces were incubated in water at 4°C for 48 h then reassayed to determine whether any β-glucosidase had eluted from the matrix (referred to as the second assay). As a control, nylon pieces that were incubated in all solutions bar sodium borohydride were assayed. The sodium borohydride incubations (at 2mg/ml) were performed in 0.1M phosphate buffer at pH 8 for 30 min. Each condition was performed in quadruplicate, and blank readings were routinely subtracted from all data (Figure 5.11).

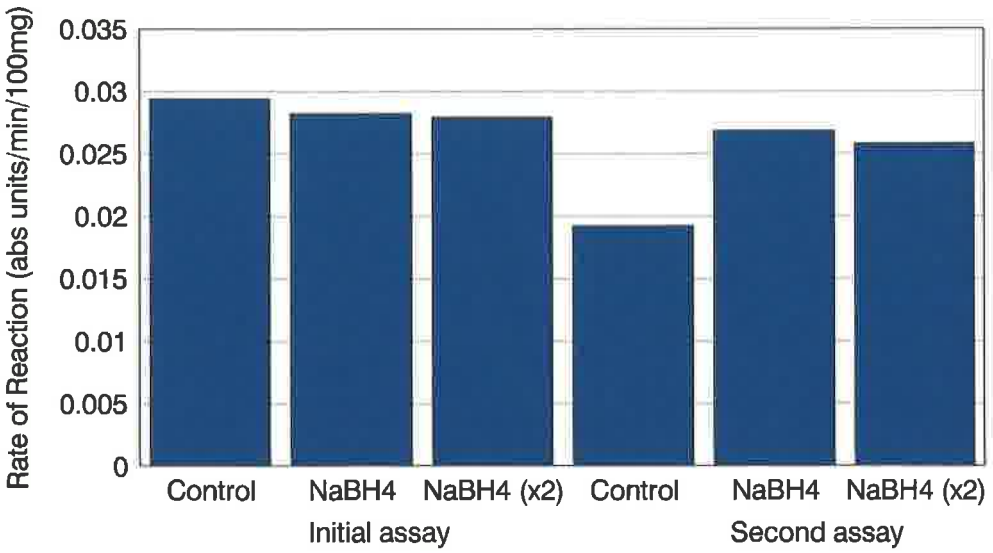


Figure 5.11. The effect of introducing a stabilising incubation in sodium borohydride on β-glucosidase binding, and the stability of this binding.

As may be seen in Figure 5.11, the addition of sodium borohydride had very little effect on the amount of enzyme that bound or on the activity of the bound enzyme. The average rate of reaction was observed to be 0.0294 ΔOD/min/100mg in the control, 0.0282 ΔOD/min/100mg in the pieces incubated once in sodium borohydride, and 0.0279 ΔOD/min/100mg in those incubated twice. Following a 48 h incubation in water, ligand was found to have eluted from all the nylon pieces. There did not appear to be a difference between the amount of ligand that eluted from the pieces that were incubated either once or twice in sodium borohydride. However, more enzyme was lost from the nylon pieces that had not been incubated in sodium borohydride at any stage. This indicated that the introduction of an incubation in sodium borohydride following the enzyme had helped to stabilise the links on the matrix to a small extent.

5.3 CONCLUSIONS

In summary a procedure for coupling a protein ligand to pieces of nylon film was optimised. Slight variations in concentrations of incubating solution, and length of incubation time were found to have a profound effect on the efficacy of the procedure, with the concentration of each of the reagents reaching a maximum when the nylon pieces were fully saturated. The natural polymer chitosan was a useful spacer molecule, and greatly amplified the amount of β-glucosidase that would bind compared with not using an amplification step. However, chitosan was not found to be as effective as PEI in amplifying the amount of enzyme that could be bound to the nylon pieces.

The introduction of a stabilising step, namely the incubation of the nylon in sodium borohydride following stage 5 of the procedure, reduced the amount of ligand that would be eluted following storage of the enzyme bound nylon pieces in water, whilst not affecting the amount of enzyme that would initially bind to the nylon. It is proposed that the inclusion of this step would be beneficial to the storage properties of ligand bound enzyme.

Compared to other procedures [Iborra *et al.*, 1992; Agarwal and Gupta, 1996] for immobilising proteins and enzymes onto nylon, this procedure is relatively simple, easily controlled, uses cheap and much lower toxicity reagents and is effective; while nylon itself is inexpensive, and readily available in powder, beaded, tube or sheet

forms. For these reasons it is believed that this method, which should be applicable to most proteins or enzymes with little or no modification, has considerable potential in a wide variety of affinity or bioreactor situations.

CHAPTER 6 AN INVESTIGATION INTO THE EXTENT OF NON-SPECIFIC LIGAND BINDING ONTO NYLON FILM

6.1 INTRODUCTION

Any affinity based separation relies on the specificity between the chosen ligand and the target substance. Any ligand that shows specificity for a group of substances could be selected for use in an affinity preparation. In affinity chromatography, for example, carbohydrate binding proteins or lectins are used to purify glycoproteins [Andersson, 1992]; protein A for immunoglobulins [Ohlson and Weislander, 1987] and Cibacron Blue F-3GA for various enzymes [Boyer and Hsu, 1993].

As Cibacron Blue F-3GA, and many other ligands are not naturally derived compounds, their interactions with proteins cannot be deemed to be 'biospecific'. Rather, Cibacron Blue F-3GA is very similar in structure to a natural substance, the coenzyme nicotinamide adenine dinucleotide, (NAD^+), and binds to enzymes by virtue of their nucleotide binding domains. Hence Cibacron Blue F-3GA is commonly used in the preparation of enzymes, (e.g. oxidoreductases, dehydrogenases and transferases), that naturally interact with nucleotide cofactors.

One aspect of affinity methods that causes concern in the biotechnology industries, particularly the therapeutic industry, is the potential for affinity ligands to leak into the medium. Indeed an Food and Drug Administration (FDA) ruling described by Scopes

[1987], insists that no trace of a ligand may be present in a protein destined for human use.

Additionally, it is imperative to chose a matrix that exhibits a low non-specific binding capacity (e.g. via a general hydrophobic or electrostatic binding in addition to the intended covalent links) to minimise the contamination of product with non-target material in the feedstock. As previously mentioned, any matrix will also have to demonstrate high stability, a high capacity for specific binding and a low cost. The stability and cost of nylon film as a matrix has been confirmed, and its specific binding capacity is fully exploited by virtue of the regeneration of the nylon belt during cycling through the Affexts machine. To finalise studies on nylon film, it is important therefore to investigate fully its non-specific binding adsorption.

The findings in chapter 5 suggest that some ligand (β-glucosidase) was attached non-specifically to the nylon belt. As the interactions involved are not as stable as those produced during the specific binding procedure, leakage of ligand, resulting in a slower rate of formation of p-nitrophenol from pNPG was observed. This was particularly demonstrated in studies which showed a decrease in rate of reaction following a sustained incubation of the nylon film with bound β-glucosidase in water (Section 5.2.1). One explanation for this could be a breakage in specific links resulting from limited stability of the imide linkages between spacer molecules. However, as a similar leakage of ligand was observed even after treatment with sodium borohydride to stabilise the imide linkages, non-specific binding of ligand to nylon film was also

considered to be a possible explanation for at least part of the observed fall in rate of reaction.

To investigate the extent of this non-specific binding, a range of experiments was performed with 'untreated' nylon film that had been hydrolysed only as in step 1 of Table 5.1 before being immersed in β-glucosidase (i.e. no covalent coupling via the glutaraldehyde and PEI steps) and with film fully treated as in Table 5.1., (chapter 5).

6.2 RESULTS AND DISCUSSION

The initial step of the normal binding procedure involved the partial hydrolysis of the nylon pieces to produce NH_2 groups for glutaraldehyde activation. It was considered possible that the COOH groups produced in conjunction with these amine groups would have the potential to be involved in non-specific ionic linkages with the ligand. Furthermore, any amine groups prevented from being involved with the glutaraldehyde activation through steric hindrance could also be involved in non-specific polar interactions. Hence in this study of non-specific binding, the 'untreated' nylon was routinely subjected to an incubation in 2.9M HCl prior to its 2 hour incubation in β-glucosidase in order to more accurately mimic any non-specific binding activity on the fully-treated nylon. Controls were performed during each experiment where nylon that had not been incubated in enzyme was assayed. These blank readings were routinely subtracted from all values.

6.2.1 Investigations into the Stability of the Specifically and Non-Specifically Bound β-Glucosidase.

Figure 6.1 gives the results of an experiment where the stability of β-glucosidase binding to treated and untreated nylon film was compared over a period of time. Half of the samples were stored in water, the rest in 1M NaCl, both at 4°C. Following a washing step in water, the nylon pieces were assayed in pNPG. The initial reading corresponds to the rate of reaction observed following incubation with β-glucosidase. The nylon pieces were then incubated in water or 1M NaCl and reassayed after 1h, 2h,

16h, 40h, 1 week and 2 weeks, with the incubation solution changed daily. All samples were in triplicate.

Figure 6.1 (a)

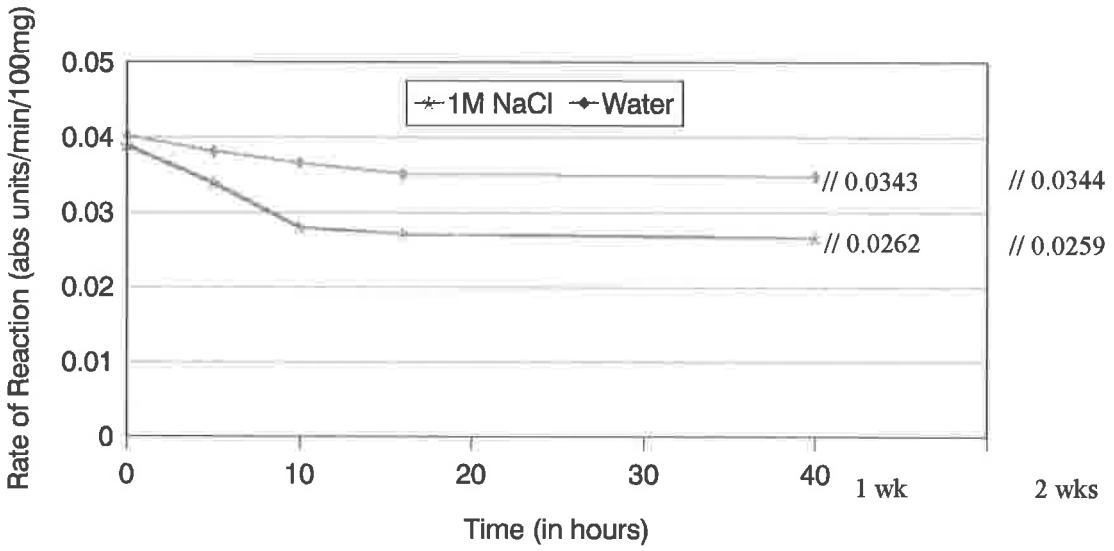


Figure 6.1 (b)

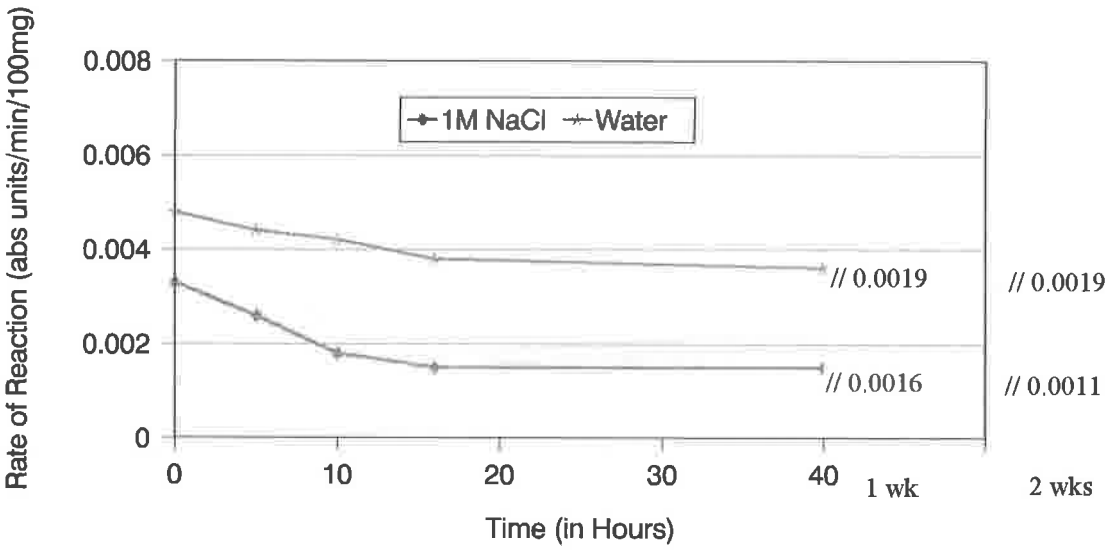


Figure 6.1 A comparison between the binding obtained on treated (a) and untreated (b) nylon film, and how this binding is affected by prolonged incubation in water or 1M NaCl.

As can be seen, although the rate of reaction decreased for the treated nylon (i.e. subjected to the binding conditions outlined in Table 5.1) nylon (Figure 6.1 (a)) in both conditions following 1 and 2 hours' incubation, this decrease was gradual, and the nylon film was still active after 2 weeks. The treated nylon stored in water gave a rate of reaction that dropped from an initial value of 0.0402 ΔOD/min/100mg to 0.0344 ΔOD/min/100mg following a 2 week incubation in water. The treated nylon film stored in 1M salt exhibited a larger overall drop in rate of reaction than that stored in water, indicating that the presence of salt facilitated the removal of β-glucosidase from the nylon, a pattern repeated in the non-specific binding example of untreated nylon. This suggested that electrostatic bonds played a major role in the non-specific binding process. However, in neither event was it possible to remove all of the associated enzyme in this way.

In the case of the untreated (i.e. hydrolysed only) nylon film (Figure 6.1 (b)), ten-fold less enzyme was bound initially to the nylon. The general pattern observed in Figure 6.1 (a) for the treated nylon where the rate of reaction decreased rapidly then reached a plateau, paralleled that for untreated samples, but in this instance readings reached a very low value. Indeed, the fall in rate of reaction observed with the treated nylon equated to a drop of approximately one quarter of the initial value, and in the untreated nylon a drop equivalent to approximately one half of the initial value was obtained. This would indicate that the non-specifically bound ligand in Fig 6.1 (b) was less stable on the nylon than the specifically bound ligand in Figure 6.1 (a), and hence, was more easily removed. The similarities observed in the profiles of each nylon type,

particularly the initial sharp fall in rate of reaction, indicated that non-specific binding could also be occurring during the glutaraldehyde activation procedure. Therefore non-specific binding could be considered to constitute a degree of all activity described thus far.

To investigate further the nature of the non-specific binding, 'untreated' nylon was incubated in detergents to minimise hydrophobic binding following an initial assay.

Figure 6.2 shows the effect of incubating 'untreated' nylon in mild non-ionic detergent (0.5% Tween 80, or 0.5% Triton X100) over a period of time. Although both detergents were effective at removing some non-specifically bound ligand, neither was able to remove as much as was obtained using salt.

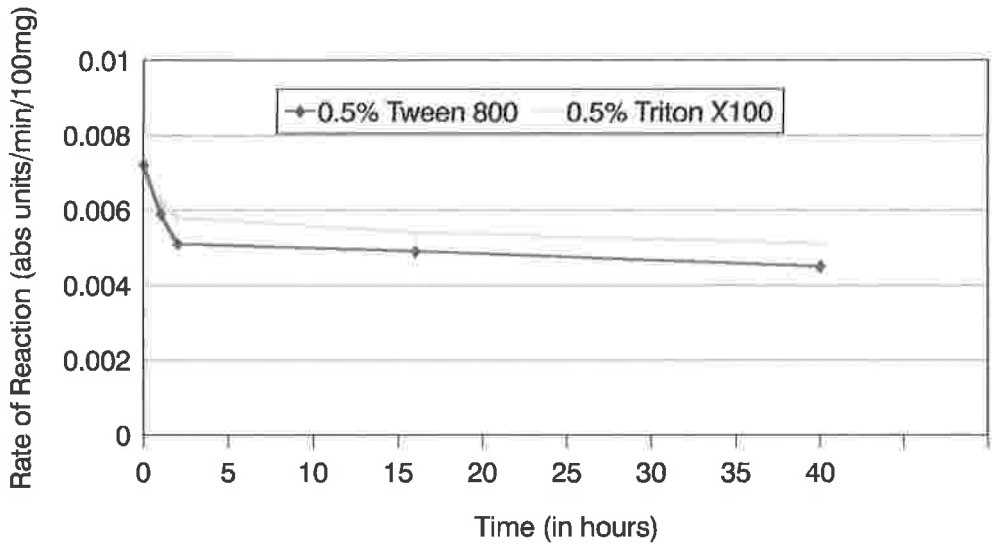


Figure 6.2 The effect of incubating 'untreated' nylon film in detergent (0.5% Tween 80, or 0.5% Triton X100) on the amount of non-specifically bound β-glucosidase.

From the above experiments it was concluded that although neither salt nor detergent was completely effective at removing the non-specifically bound proteins, both were able to remove a proportion of the ligand. This would suggest that the binding was due to a combination of ionic and hydrophobic forces. More protein was removed following the incubation with salt suggesting that in the case of β-glucosidase, at least, ionic forces are predominant.

Following the above studies, 'untreated' nylon was subjected to a gentle washing step involving both salt and non-ionic detergent to study the amount of protein that could be removed from the nylon. This step would be suitable for use with most proteins, as although it causes disruption of ionic and hydrophobic bonding, it is unlikely to denature the bound protein ligands unless more potent ionic detergents were used.

Figure 6.3 shows that although a combination of mild detergent and salt was able to remove a larger amount of non-specific binding than either in isolation over the initial 2 hour period, the method was still insufficient to completely eliminate non-specific interactions.

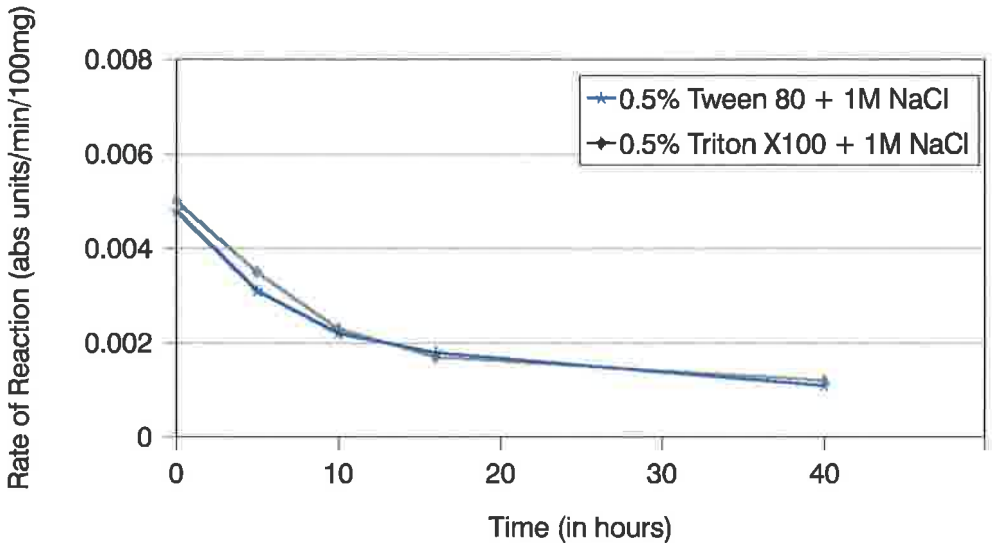


Figure 6.3 The effect of incubating 'untreated' nylon film in detergent (0.5% Tween 80, or 0.5% Triton X100) and 1M NaCl on the amount of bound β-glucosidase.

These results posed questions regarding the quality of ligand binding to nylon which would have implications regarding the use of nylon belts in a continuous process. As previously mentioned, it is of paramount importance that a target protein is free from contaminating material resulting from an affinity separation. The above results have shown that non-specific binding, followed by ligand leakage, was considerable when using nylon film as a matrix and β-glucosidase as the ligand. Some of the non-specifically bound ligand remained nylon bound following successive incubations in detergent and salt. This indicated that a proportion of the non-specifically bound β-glucosidase, at least, was strongly bound and would probably have remained nylon bound during a typical affinity purification procedure. However the remaining enzyme

that was eluted following washes in detergent and/or salt would potentially be a risk during an affinity preparation and could contaminate the target material.

At first glance, the solution to this problem appeared to be straightforward, i.e. to elute as much non-specifically bound material as possible prior to commencing the affinity separation. However, this would merely remove the ligand and expose the potential non-specific binding sites on the nylon. Any proteinaceous material could then, in theory, attach to the nylon film via these binding sites. In addition, other proteins might displace any non-specifically bound β-glucosidase, and might then also contribute to contaminants in the target material. The answer therefore would seem to be to find a way of blocking these non-specific binding sites, and a number of substances have been investigated for this purpose.

In order to confirm whether the ligand could indeed be displaced by a second protein, nylon pieces were incubated in trypsin following their initial assay. Although trypsin is known to cleave proteins, it is more effective against those with little tertiary structure. As β-glucosidase is a globular protein it was considered to be sufficiently resistant to cleavage by trypsin. The incubating solution was buffered to pH 5 to further minimise trypsin activity. Trypsin is a basic protein, therefore it represents a good model protein in this instance as it has the potential to bind ionically to the COOH groups on the hydrolysed nylon in addition to binding hydrophobically to the matrix.

Trypsin can itself be easily assayed by the synthetic substrate Nα-Benzoyl-DL-Arginine p-nitroanilide (BAPNA). The ability of trypsin to replace β-glucosidase on the nylon

film was determined in two ways; firstly by reassaying the nylon pieces with pNPG to investigate any decrease in β-glucosidase following incubation in trypsin-containing buffer, and secondly by assaying the pieces in BAPNA to see the level of non-specific trypsin binding.

Table 6.1 shows the extent of substitution of β-glucosidase by trypsin. The information shown represents an average of four nylon pieces for each set of conditions. The experiment was also performed on treated nylon, and results are expressed in terms of rate of reaction (ΔOD/min/100mg).

	1st assay (β-glucosidase)	2nd assay (β-glucosidase)	Trypsin assay	Percentage difference in β-glucosidase
Untreated nylon	0.006	0.005	0.002	↓16.7%
Treated nylon	0.034	0.022	0.008	↓32.8%

Table 6.1 An investigation into the replacement of non-specifically bound ligand by a second protein.

As can be seen from the table, trypsin was able to replace β-glucosidase on the nylon film, simply by introducing a short incubation in 2.5 mg/ml trypsin. With untreated nylon, the relative amount of non-specifically bound β-glucosidase was shown to decrease by 16.7%, as the trypsin activity rose to 0.002 ΔOD/min/100mg. The rates of

reaction show that there is considerably more β-glucosidase bound than trypsin, at a ratio of approximately 5:2.

The specifically bound β-glucosidase was also found to be 'at risk' from the incubation in trypsin. 33% of the β-glucosidase was lost following incubation in trypsin, and it would appear that the trypsin attached to the vacant sites or even displaced some of the β-glucosidase, as its activity on the nylon increased to 0.008 ΔOD/min/100mg. It is quite probable that much of the substituted β-glucosidase had been bound as a result of non-specific interactions, as observed in the preceding experiments.

6.2.2 Investigations into the Location of Non-Specifically Bound β-Glucosidase on Nylon Film

To investigate the location of the non-specific β-glucosidase binding to the nylon, the following experiments were performed. First, to determine whether casein could be bound successfully to the nylon, the rate of reaction of β-glucosidase was obtained from nylon pieces that had been subjected to all methods outlined in chapter 5, (Figure 6.4 (a)), and nylon pieces that were subjected to all methods outlined in chapter 5, but subjected to a 2 hour incubation in 2.5 mg/ml casein prior to the 2 hour incubation in 2.5 mg/ml β-glucosidase (Figure 6.4 (b)). The rate of reaction obtained from the former set of nylon was 0.0237 ΔOD/min/100mg, and from the latter, 0.0045 ΔOD/min/100mg. This discrepancy in rate of reaction indicated that casein had been

specifically attached to the nylon via the glutaraldehyde linkages, as depicted in Figure 6.4 (b), hence blocking these sites for β-glucosidase. Subsequent incubation in β-glucosidase had enabled some of the enzyme to bind either directly to the nylon matrix, to the casein, or to one of the substances involved in the linkage procedure (i.e. PEI or glutaraldehyde). This β-glucosidase could be deemed to be bound non-specifically.

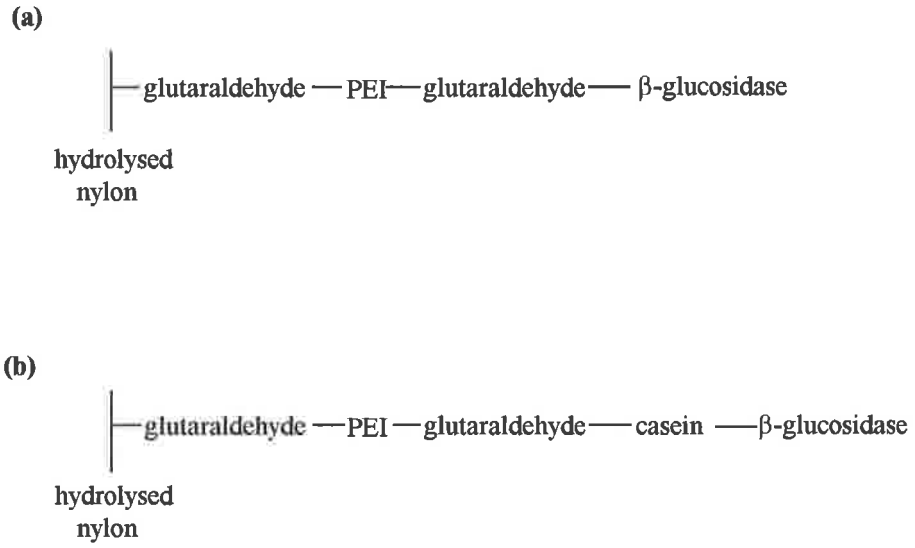


Figure 6.4. A schematic diagram describing the proposed attachment of (a) β-glucosidase and (b) casein and β-glucosidase to the treated nylon.

A second series of experiments revealed the location of the non-specifically bound β-glucosidase in more detail. It is important to remember that β-glucosidase was chosen as a model protein, and in this instance represents binding that could occur by

contaminants in an affinity separation. One possibility it was necessary to eliminate was that following the binding of a ligand to the nylon, and the target protein to this ligand, a second protein, a contaminant, could attach to the target protein. To test this theory, casein was chosen to represent the target protein, and β-glucosidase to represent any contaminating material.

Casein was bound to the treated nylon as described above in Figure 6.4 (b), and this nylon was submerged in a β-glucosidase solution to attach the β-glucosidase to any free non-specific binding sites. In order to investigate whether any β-glucosidase had directly attached to the casein the nylon was submerged in a 20 μg/ml trypsin solution at pH 8 (50mM sodium phosphate) for 10 min. Casein is a protein with very little tertiary structure and, hence, is more susceptible to degradation by trypsin than the globular β-glucosidase. It was hoped therefore that the trypsin would digest the casein, hence releasing any β-glucosidase non-covalently bound to it (Figure 6.5).

The assay measured the release of β-glucosidase from the nylon by reassaying the nylon pieces rather than by assaying the washing solution that the casein and β-glucosidase had eluted into, as the slight differences observed between the rate of reaction of free and immobilised β-glucosidase [Ravet *et al.*, 1993] could contribute to significant variation between observed rate of reactions when the values are so low, as in this case. The experiments were performed in quadruplicate.

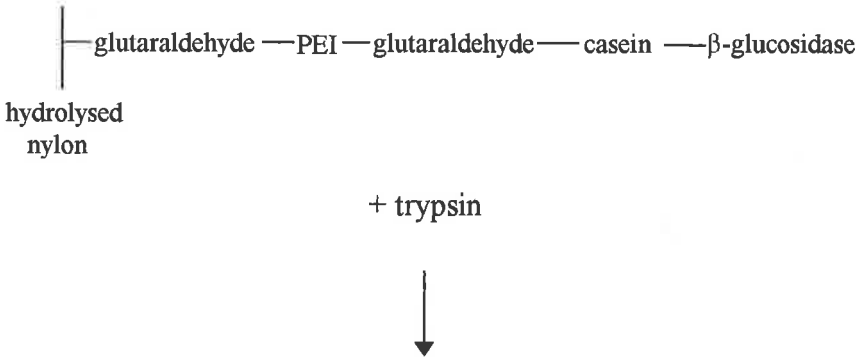


Figure 6.5 A schematic diagram describing the proposed effect of incubating treated nylon in trypsin following incubations in both casein and β-glucosidase.

After an initial incubation in β-glucosidase, the rate of reaction was 0.0042 ΔOD/min/100mg with pNPG comparable to that obtained in the previous section where the observed rate of reaction was 0.0045 ΔOD/min/100mg. Following the incubation in 20 μg/ml trypsin solution, a washing step, then a second assay in pNPG the rate of reaction reduced to 0.0033 ΔOD/min/100mg. This showed that only 21% of the β-glucosidase had been attached directly to the casein, and, hence up to 79% was still attached to the nylon matrix. The schematic provided by Figure 6.5 therefore only represented a small part of the actual β-glucosidase binding. This result indicated that in an affinity separation using nylon and the binding procedure described in chapter 5, any contaminants would be more likely to attach to the matrix (the hydrolysed nylon film) or the intermediate substances (glutaraldehyde and PEI) rather than non-specifically onto the ligand (in this instance, casein).

In order to investigate whether the non-specific binding sites on the nylon matrix itself could be blocked and, hence, made unavailable to β-glucosidase, a similar experiment using untreated, i.e. hydrolysed only nylon was designed. This study involved a comparison of two sets of nylon pieces. The first were hydrolysed then incubated in 2.5 mg/ml β-glucosidase for 2 hours at pH 8. The second, as depicted in Figure 6.6, were hydrolysed then incubated in a 2.5 mg/ml casein solution at pH 8 for 2 hours prior to the β-glucosidase incubation.

Both sets of nylon pieces were assayed. The second set was then subjected to a trypsin digest as described previously, was washed, and was reassayed with pNPG. Both conditions were performed in quadruplicate.

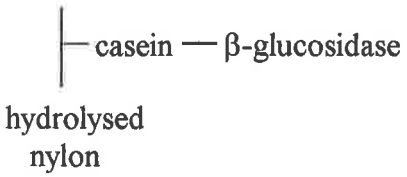


Figure 6.6 A schematic diagram describing the proposed attachment of β-glucosidase to the non-specifically bound casein

In the first set of nylon pieces where β-glucosidase was attached directly to the hydrolysed nylon the observed rate of reaction was 0.007 ΔOD/min/100mg. In the second set of nylon pieces where an incubation in casein had been introduced, the rate

of reaction was 0.002 ΔOD/min/100mg. This suggested that the casein had successfully blocked the majority of the non-specific binding sites on the hydrolysed nylon.

Following digestion of the casein by trypsin it was not possible to measure any β-glucosidase activity on the nylon. The β-glucosidase activity observed during the initial assay of the second set of nylon pieces was, hence, deemed to be a result of the direct association of β-glucosidase with the bound casein, and confirmed the matrix-blocking capabilities of casein.

The above study was repeated using the protein Bovine Serum Albumin (BSA) as a blocking agent in the place of casein, and comparable readings of the initial assay were obtained (data not shown).

As the above studies demonstrated that proteins could be used to block the non-specific binding sites a series of investigations using peptides and amino acids were performed. These were considered to be of greater practical use than whole proteins as their size would allow them access to a larger number of non-specific binding sites, whilst at the same time they would not cause a steric hindrance to the other substances involved in the binding procedure.

6.2.3 Investigations into the efficacy of peptides and amino acids as blocking agents for the non-specific binding sites on hydrolysed nylon

As an initial study into the blocking capabilities of peptides and amino acids, untreated, i.e. hydrolysed only, nylon pieces were routinely used. These pieces were incubated in the chosen peptide or amino acid prior to an incubation in β-glucosidase. The nylon pieces were then washed before being assayed to determine the level of β-glucosidase binding, and hence the ability of each substance to block the non-specific binding sites. Hydrolysed nylon pieces that had been incubated in β-glucosidase only were used to help gauge the maximal amount of non-specific β-glucosidase binding throughout these experiments. Nylon pieces that had not been incubated in enzyme were used routinely as a control, and their readings were subtracted from all shown values.

The first experiment compared the blocking capabilities of casein peptides with those of whole casein molecules. To produce casein peptides, whole casein that had been isolated from skimmed milk through precipitation at pH 4.6 followed by filtration, was digested using the proteinase enzyme pepsin to form a variety of casein peptides (as detailed in Chapter 2.2.6.2) These peptides represent fragments of α-s1, α-s2, β and κ caseins, cleaved preferentially between Phenylalanine (or Tyrosine or Leucine) and Tryptophan (or Phenylalanine or Tyrosine) residues [Chaplin and Bucke, 1990].

Hydrolysed nylon pieces were incubated for 2 hours in either 2.5 mg/ml casein, 2.5 mg/ml casein peptides or water (control) prior to a 2 hour incubation in β-glucosidase.

The pieces were then washed, and assayed in pNPG for 15 min. Each condition was performed in quadruplicate.

In the control example where the hydrolysed nylon pieces were incubated in water, the observed rate of reaction was 0.008 ΔOD/min/100mg. In the nylon pieces incubated in whole casein, the rate of reaction was 0.002 ΔOD/min/100mg and in the pieces incubated in casein peptides, 0.004 ΔOD/min/100mg.

This suggested that although the casein peptides were able to prevent some β-glucosidase binding, they were not as effective as whole casein molecules at blocking the non-specific binding sites.

Next the blocking capabilities of a range of amino acids were compared. Five amino acids were chosen, and are depicted along with their functional groups in Table 6.2. No neutral hydrophilic amino acid was chosen as the nylon matrix represents a considerable hydrophobic surface, thus it was thought that a poor degree of binding would be observed using hydrophilic amino acids.

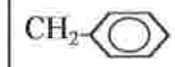
Amino Acid	Type	Functional Group
Glycine	Standard	H
Aspartic Acid	Acidic	CH_2COOH
Arginine	Basic	$CH_2CH_2CH_2NHCNH_2NH_2^+$
Leucine	Hydrophobic,aliphatic	$CH_2CHCH_3CH_3$
Phenylalanine	Hydrophobic aromatic	

Table 6.2 The amino acids used to attempt to block non-specific binding sites on the nylon matrix. Their functional R groups are shown.

Hydrolysed nylon pieces were incubated in either one of the individual amino acids (at 2.5 mg/ml), a mixture of all five amino acids (at a total concentration of 2.5 mg/ml), or water, before a 2 hour incubation in β-glucosidase. They were then washed, and assayed.

Amino Acid	Rate of Reaction (ΔOD/min/100mg)
Control	0.008
Glycine	0.008
Aspartic Acid	0.007
Leucine	0.008
Arginine	0.005
Phenylalanine	0.004
Mixture of above Amino Acids	0.002

Table 6.3. The blocking of non-specific binding using amino acids. The data provided refers to the observed rate of reaction of β-glucosidase following a blocking step by the amino acid stated.

The nylon that had been submerged in water only, gave a rate of reaction of 0.008 ΔOD/min/100mg, as did the nylon submerged in glycine. Glycine could be concluded therefore to have no effect on β-glucosidase binding. The nylon submerged in the acidic aspartic acid , or the aliphatic hydrophobic leucine did not prevent the binding of β-glucosidase in an effective manner either, with observed rates of reaction of 0.007 ΔOD/min/100mg. The basic amino acid arginine and the hydrophobic, aromatic phenylalanine were most competent of the individual amino acids at reducing non-specific binding with rates of reaction of 0.005 ΔOD/min/100mg and 0.004 ΔOD/min/100mg respectively. The efficacy of each amino acid at preventing non-specific binding followed the trend, phe > arg > asp/leu > gly.

The lowest amount of β-glucosidase that bound was observed using the mixture of amino acids, (0.002 ΔOD/min/100mg).

The prevention of β-glucosidase binding was therefore a combination of different factors. The presence of the large benzene ring on phenylalanine seemed more important than the hydrophobicity of the molecule, as the hydrophobic leucine was inferior at preventing β-glucosidase binding. The individual factors were cumulative as was demonstrated by the rate of reaction of the amino acid mixture being lower than any individual amino acid. As the blocking capabilities of casein and the amino acid mixture were comparable, it was decided to test their ability to block the non-specific binding sites on treated nylon pieces.

6.2.4 Investigation into the efficacy of casein and amino acids as blocking agents of the non-specific binding sites on treated nylon

In order to block non-specific binding sites on the nylon matrix, and hence reduce elution of the ligand (β-glucosidase) and the potential contamination of the target protein, a range of experiments was performed.

It was decided to introduce this blocking step after the binding of PEI. The sequence for the addition of β-glucosidase to nylon would then be: hydrolysis in 2.9M HCl, 1st glutaraldehyde activation, PEI, blocking step (with casein or an amino acid mixture), 2nd glutaraldehyde activation, and ligand binding.

By introducing the blocking step at the above stage of the procedure, it was hoped to minimise disruption to the specific binding procedure. If, for example, the blocking step had been introduced immediately following the initial hydrolysis, the binding of glutaraldehyde could have been impaired. Similarly, if the blocking step had followed the second glutaraldehyde activation, the ligand binding would have been impeded through interactions between the glutaraldehyde and the blocking agent (as described in the case of casein in Section 6.2.2 and Figure 6.4 (b)).

Figure 6.7 shows the effect of introducing a blocking step on β-glucosidase binding. Nylon pieces were incubated in the solutions described in Table 5.1 (control), plus subjected to a 2 hour incubation in either 2.5 mg/ml casein or a 2.5 mg/ml amino acid mixture (containing 0.5 mg/ml phenylalanine, leucine, glycine, arginine and aspartic

acid as previously) in the binding sequence indicated above. The experiment was performed in quadruplicate, and blank readings were subtracted from test readings.

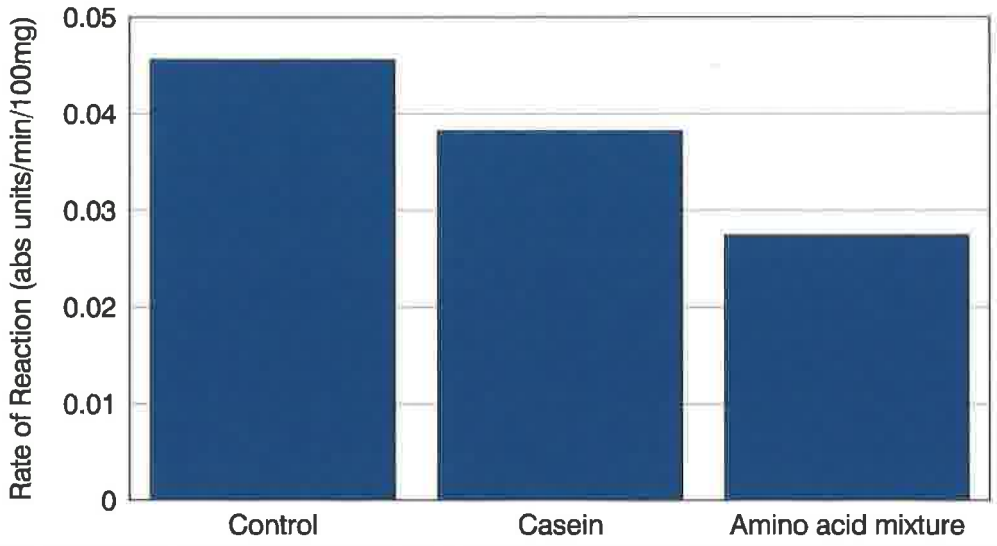


Figure 6.7. The effect of introducing a blocking step to prevent non-specific attachments on the level of β-glucosidase binding.

As can be seen, the observed rate of reaction was less in both the pieces incubated in casein (0.0382 ΔOD/min/100mg), or the amino acid mixture (0.0274 ΔOD/min/100mg) than in the control 0.045 ΔOD/min/100mg). This decrease could be deemed to be due to the blocking of non-specific binding sites. The rate of reaction was lower in the amino acid mixture than with whole casein, as presumably the smaller amino acid molecules were able to block the non-specific sites not reachable by the larger casein.

The stability of the bound ligand was then tested by subjecting each condition to an overnight incubation in 0.5 % Tween 80 and 1M NaCl at 4°C. This mix of a mild detergent and salt was formerly found to be most effective at removing non-covalently bound β-glucosidase (Figure 6.3), and therefore provided the sternest test of the stability of the β-glucosidase binding.

Figure 6.8 shows the observed rate of reaction for each condition following the initial assay and after an overnight incubation in mild detergent and salt.

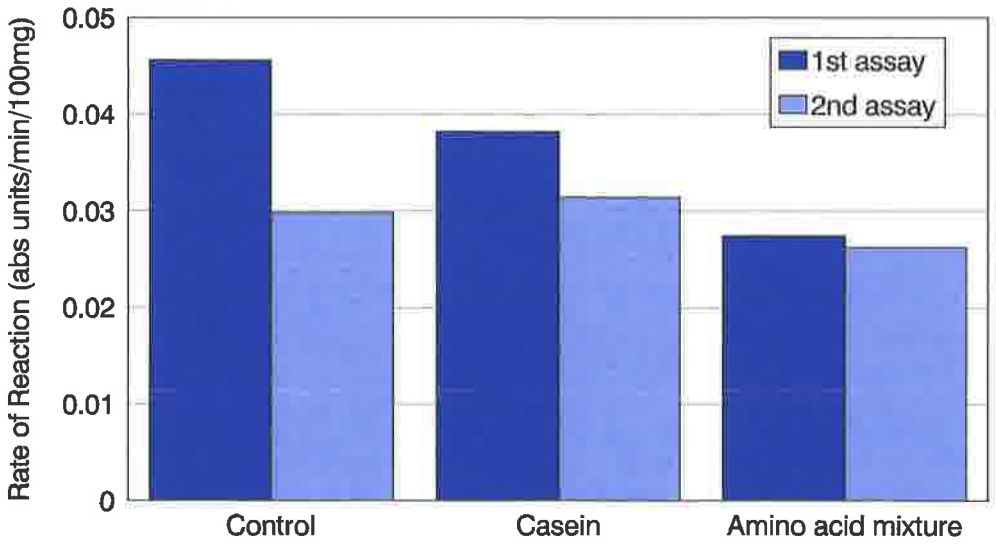


Figure 6.8. The effect of the introduction a of blocking step to prevent non-specific attachments on the stability of β-glucosidase binding.

The data provided in Figure 6.8 shows that the β-glucosidase bound onto the nylon most strongly through the addition of a blocking step using an amino acid mixture. This is indicated by a fall in rate of reaction of just 4 %, compared with a decrease of 18% when casein was used to block the non-specific binding sites, and 35 % when no blocking agent was used.

The decrease in rate of reaction observed in the initial assay through the introduction of a blocking step with the amino acid mixture (0.0274 ΔOD/min/100mg), compared with the control 0.0456 ΔOD/min/100mg, appeared to be justified by the increased stability of the remaining binding. The increased stability of ligand binding would therefore help keep contaminants to a minimum in an affinity separation. It was hence proposed to include a blocking step into the ligand binding procedure outlined in Table 5.1. Non-specific binding appeared to be the cause of the observed ligand leakage rather than the presumed instability of some of the links.

6.3 CONCLUSIONS

The stability of β-glucosidase binding to the nylon matrix during the glutaraldehyde binding procedure was investigated, and non-specific binding was suggested as a possible cause for ligand leakage. A combination of mild detergent and salt was found to be more effective at eluting non-specific bound β-glucosidase than either in isolation or water, indicating that the non-specific binding was a result of a combination of ionic and hydrophobic forces.

The instability of the non-specifically bound β-glucosidase was demonstrated by the facility of replacing the β-glucosidase with a second enzyme, trypsin.

The location of the non-specifically bound β-glucosidase was investigated further using casein as a linking agent, which was easily cleaved to elute β-glucosidase by trypsin. Through these experiments, it was discovered that non-specific binding was more likely to occur on the nylon matrix or the intermediate substances, rather than onto the ligand.

Several molecules, including casein, casein peptides, and the amino acids phenylalanine, leucine, glycine, arginine and aspartic acid were studied as potential blocking agents for the non-specific binding sites on the nylon matrix. The most effective using hydrolysed nylon pieces was found to be either an amino acid mixture or whole casein. The amino acid mixture was found to be more effective than casein when used as a blocking agent on treated nylon.

The level of β-glucosidase binding using the amino acid mixture was lower than with the control, (0.0274 ΔOD/min/100mg and 0.0456 ΔOD/min/100mg respectively). However the β-glucosidase was found to be bound in a more stable manner with the amino acid mixture than with the control, with only 4% eluted following an overnight incubation in detergent and salt, compared with 35% in the control.

The introduction of a blocking step was suggested for the procedure outlined in Table 5.1, in order to minimise ligand leakage into the eluent of an affinity separation.

CHAPTER 7 GENERAL CONCLUSIONS

Aqueous two-phase systems were studied as a potential tool in the separation of β-CN-5P from a total proteose peptone fraction. Systems using PEG with average molecular weights below 8000 were not found to be of use, as the total proteose peptones were evenly distributed between the phases, and in some cases precipitated out at the interface.

Systems with PEG of average molecular weight 8000 or 10 000 encouraged the partial separation of β-CN-5P into the upper, PEG phase. One reason was provided by the binodial curves, which were lower in the systems containing PEG with average molecular weights of 8000 and 10 000. This indicated, therefore, that a system containing equal polymer concentrations would be further from the binodial in systems with higher molecular weight PEGs, and partitioning in these systems would be more pronounced through the increased exclusion effects of the phases.

The most suitable pH for the partial separation of the total proteose peptones was determined as pH 7 as β-CN-5P partitioned preferentially into the upper phase, and the contaminants were contained within the dextran phase. At pH 3, all material partitioned equally between the phases whilst at pH 5 and 9, all material tended to partition into the lower, dextran phase. Therefore no partial separation of the total proteose peptones was obtained at these pH values.

The introduction of NaCl was found to have a pronounced effect on separation, allowing a more efficient extraction of β-CN-5P into the upper, PEG, phase, although this effect reached a plateau at 5% NaCl. The addition of salt also increased the solubility of the material that had previously precipitated at the interface between the phases.

The possibility of isolating β-CN-5P directly from a bovine β-casein digest using chromatographic techniques was shown to provide a useful alternative large scale method of β-CN-5P production, when compared with its traditional purification from bovine milk. The β-casein digest contained less contaminating material than the milk total proteose peptone fraction prepared using the older methods, e.g. IgG and PP3 were not present in the β-casein digest. The reduction of contaminating substances facilitated the isolation of β-CN-5P.

The partitioning characteristics of purified β-CN-5P were studied in PEG; dextran aqueous two-phase systems. By increasing the polymer concentration and, hence, the tie line length, β-CN-5P was encouraged to partition more strongly into the PEG phase. The addition of NaCl also encouraged partitioning into the upper phase. The estimated percentage yield of β-CN-5P was found to increase with both increasing PEG and NaCl concentrations.

The effects of pH on purified β-CN-5P partitioning were studied in the presence of two neutral salts NaCl and Na_2SO_4. In systems containing NaCl and, hence, a more

systems containing Na_2SO_4 and, hence, a more negative dextran phase, the partition coefficient was higher at pH values above 4.5 (see Figure 4.6). The crosspoint, equating to the pI of β-CN-5P was determined as pH 4.7.

In the rather different topic area of affinity-based biopurifications, the coupling of the enzyme β-glucosidase as a model ligand to nylon pieces was developed and optimised. For each individual binding stage, saturating conditions were determined and a plateau was reached.

A stabilising step was introduced to prolong the storage life of the active nylon, and to prevent elution of the enzyme. This step did not alter the amount of enzyme that bound onto the nylon.

Non-specific binding was proposed as a possible cause of the observed enzyme leakage from the nylon, and was studied in depth.

Enzyme was found to elute from the nylon following incubation in water, salt, detergent or salt and detergent solutions. More enzyme was lost during incubations in both salt and detergent, which suggested that the non-specific binding was a result of both ionic and hydrophobic forces.

The location of the non-specific binding was studied, and it was found to be more likely to be a result of interactions between the enzyme and the nylon matrix, rather than between the enzyme and the ligand.

than between the enzyme and the ligand.

Various molecules were studied as potential blocking agents for the non-specific binding sites on hydrolysed nylon pieces. The most effective blocking agent was found to be either whole casein molecules, or an amino acid mixture containing Phe, Leu, Gly, Arg and Asp. Following testing using treated nylon pieces, the amino acid mixture was found to be a more effective blocking agent than whole casein molecules.

The observed level of enzyme binding on treated nylon pieces was lower when using the amino acid mixture than with the control. However, only 4% of enzyme eluted following an incubation in detergent and salt, compared with 35% in the control showing that less reversibly bound β-glucosidase was linked onto the nylon when an amino acid mixture was used as a blocking agent for non-specific binding sites. The introduction of a blocking step was suggested as a means to combat non-specific binding of enzyme to the nylon and, therefore, to prevent loss of enzyme which would potentially contaminate the eluent.

The following table outlines the proposed method of binding enzyme or protein to nylon film. Data from chapters 5 and 6 of this work has been incorporated into the original methodology outlined in Table 5.1 to optimise each binding step and to eliminate non-specific reactions. Nylon pieces should be washed thoroughly in water in between each stage. Sodium phosphate buffers (0.1 M) are suitable for use.

Step	Incubating conditions
1	2h in 2.9 M HCl at 37°C
2	15 min in 2.5% glutaraldehyde, pH 8 at 20°C
3	60 min in 1% (v/v) PEI, pH 8 at 37°C
4	2h in either 2.5 mg/ml casein, pH 8 at 20°C, or 2.5 mg/ml amino acid mixture (containing 0.5 mg/ml of phenylalanine, leucine, glycine arginine and aspartic acid), pH 8 at 20°C
5	15 min in 2.5% glutaraldehyde, pH 8 at 20°C
6	≥ 2h in ≥ 1.0 mg/ml enzyme, pH 8 at 20°C
7	30 min in 2 mg/ml sodium borohydride, pH 8 at 20°C
8	30 min in 1M NaCl + 0.5% (v/v) Tween 80 or Triton X-100, pH 8 at 20°C

Table 7.1. A revised methodolgy for the coupling of enzyme to nylon film

The above procedure is proposed to be suitable for the coupling of enzymes to nylon film, and is currently under investigation at UWIC. It may not prove necessary to subject all enzymes to all 8 stages of the procedure, particularly when a low level of non-specific binding may be tolerated, hence steps 4, 7 and 8 may be omitted. Additionally, the enzyme binding step, step 6, may take place at other pH values if the chosen enzyme is not stable at pH 8.0.

From all the information obtained during this work, it is apparent that traditional separation technology methods can be greatly enhanced by using more novel processes. Whilst the elusive one step purification process remains out of reach at present, other techniques may be implimented to cut down time and operational costs.

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