

EVALUATING MICROEMULSIONS FOR PURIFICATION OF
BETA-GALACTOSIDASE FROM *Kluyveromyces lactis*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

BEKİR GÖKÇEN MAZİ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
FOOD ENGINEERING

NOVEMBER 2010

Approval of the thesis:

**EVALUATING MICROEMULSIONS FOR PURIFICATION OF
BETA-GALACTOSIDASE FROM *Kluyveromyces lactis***

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ABSTRACT

EVALUATING MICROEMULSIONS FOR PURIFICATION OF BETA-GALACTOSIDASE FROM *Kluyveromyces lactis*

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November 2010, 118 pages

In this study, we evaluated the potential of water-in-oil microemulsions for the separation of β -galactosidase (lactase) from other proteins. The ability of β -galactosidase to break down the milk carbohydrate lactose gives the enzyme considerable commercial importance. The extent of solubilization of a commercial *Kluyveromyces lactis* preparation of β -galactosidase into microemulsion droplets formed from 200 mM bis (2-ethylhexyl) sodium sulfosuccinate (AOT) in isooctane was measured as a function of buffer type, pH, ionic strength, and protein concentration. Our results showed that, due to the large molecular weight of β -galactosidase (MW~ 220-240 kDa, dimeric form), the enzyme was taken up by the microemulsion droplets mainly under very low salt conditions. Based on these results, we designed a one-step separation procedure, in which a small volume of aqueous buffer containing the protein mixture is added to an organic surfactant solution. Microemulsion droplets form in the oil and capture protein impurities of smaller molecular weights, while excluding the high molecular weight target protein. This causes the β -galactosidase to be expelled into a newly formed aqueous phase. The feasibility of this one-step process as a bioseparation tool was demonstrated on a feed consisting of an equal mixture of β -galactosidase and the test protein β -lactoglobulin. Recovery and separation of the two proteins was analyzed as function

of buffer type, pH, ionic strength, and protein concentration. Results showed that separation was most complete at 100 mM KCl salt concentration, where the droplets were big enough to carry β -lactoglobulin but too small for lactase. At 100 mM salt concentration, we recovered 92% of the total lactase activity in a virtually pure form.

The same separation scheme was then tested on crude extract obtained from a cell culture broth of the yeast *Kluyveromyces lactis*. Cells of the yeast *K. lactis* were disrupted by minibeadbeater, forming a crude extract that was used as the feed in our separation process. A 5.4-fold purification factor of the extract was achieved, with 96% activity recovery. The results showed our one-step separation process to be an interesting method for the production of β -galactosidase as a technical enzyme: it has the potential to achieve a continuous, large-scale partial purification of the enzyme, potentially reducing the number of steps required in downstream process.

Keywords: AOT, Beta-galactosidase, *Kluyveromyces lactis*, Microemulsion, Protein Purification, Reversed Micelles

ÖZ

Kluyveromyces lactis BETA-GALAKTOZİDAZININ SAFLAŞTIRILMASI İÇİN MİKROEMÜLSİYONLARIN DEĞERLENDİRİLMESİ

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Tez Yöneticisi: Prof. Dr. Haluk Hamamcı

Kasım 2010, 118 sayfa

Bu çalışmada, β -galaktosidaz'ın (laktazın) diğer proteinlerden ayrılması için yağ içerisinde su mikroemülsiyonlarının kullanılma potansiyelini değerlendirdik. β -Galaktosidaz'ın süt karbonhidratı laktozu parçalayabilme özelliği bu enzime dikkate değer ticari bir önem vermektedir. *Kluyveromyces lactis*'den hazırlanmış ticari bir β -galaktosidaz preparatının, izooktan içinde 200 mM bis (2-ethylhexyl) sodium sulfosuccinate (AOT) den oluşturulmuş mikroemülsiyon su damlacıkları içinde çözünme derecesi tampon çeşidi, pH, iyon gücü ve protein konsantrasyonunun fonksiyonu olarak ölçülmüştür. Elde ettiğimiz sonuçlar, β -galaktosidaz'ın büyük molekül ağırlığından dolayı (MA ~ 220-240 kDa, dimeric formu) bu enzimin mikroemülsiyon su damlacıkları tarafından büyük ölçüde çok düşük tuz konsantrasyonları altında absorplandığını göstermiştir. Bu sonuçlara dayanarak, tek-aşamalı bir ayırma yöntemi geliştirdik, bu yöntemde protein karışımı içeren düşük hacimli su bazlı tampon, organik bir yüzey aktif madde çözeltisine eklenmiştir. Mikroemülsiyon su damlacıkları yağ içerisinde oluşur ve büyük molekül ağırlığına sahip istenen proteinleri dışarıda bırakırken küçük molekül ağırlıklı protein safsızlıklarını yakalar. Bu β -galaktosidaz'ın yeni oluşan su fazı içine atılmasına neden olur. Bu yöntemin bir biyo-ayırma aracı olarak uygulanabilirliği β -galaktosidaz ve test proteini β -laktoglobulini eşit miktarda içeren besleme karışımı

üzerinde gösterilmiştir. Bu iki proteinin birbirinden ayrıştırılması ve geri kazanımı tampon çeşidi, pH, iyon gücü ve protein konsantrasyonunun fonksiyonu olarak incelenmiştir. Sonuçlar, ayırmanın 100 mM KCl tuz konsantrasyonunda büyük ölçüde tamamlandığını göstermiştir, bu noktada su damlacıkları β -laktoglobulini taşıyacak kadar büyük fakat laktaz için çok küçüktür. 100 mM tuz konsantrasyonunda neredeyse saf bir şekilde toplam laktaz aktivitesinin % 92'si geri kazanıldı.

Aynı ayırma planı daha sonra *Kluyveromyces lactis* mayası hücrelerinden elde edilen ham özüt üzerinde de denenmiştir. Ayırma yöntemimizde besleme olarak kullanılan ham özüt, *K. lactis* maya hücrelerinin boncuklu hücre kırıcı ile parçalanmasıyla elde edilmiştir. % 96 aktivite geri kazanımı ile birlikte özütün 5.4 katı saflaştırma faktörüne ulaşılmıştır. Bu sonuçlar tek-aşamalı ayırma yöntemimizin β -galaktosidaz'ın teknik enzim olarak üretimi için dikkate değer bir yöntem olduğunu göstermiştir: bu yöntem aşağı-akış işlemlerinde gereksinim duyulan işlem basamaklarını azaltma potansiyeli nedeniyle enzimin, sürekli bir sistemde, büyük ölçekli kısmi saflaştırılmasının gerçekleştirilmesi potansiyeline sahiptir.

Anahtar Kelimeler: AOT, Beta-galaktosidaz, *Kluyveromyces lactis*, Mikroemülsiyon, Protein Saflaştırması, Ters Misel

To my family

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Prof. Dr. Haluk Hamamcı for his guidance, criticism, valuable discussions and encouraging advices throughout my study. I wish to thank for his academic support that made this thesis possible.

I would like to extent my sincere appreciation to thesis committee member Prof Dr. Zümürüt Begüm Ögel, Prof. Dr. Hüseyin Avni Öktem, Prof. Dr. Şebnem Harsa and Prof. Dr. Pınar Çalık for their very valuable suggestions and comments.

I appreciate the opportunity to be a part of a wonderful lab group headed by Prof. Dr. Stephanie R. Dungan, who I admire greatly. Her scientific ethics, dedication to teaching, personalities and priorities in life will influence and guide me for many years to come. I want to specially thank to Prof. Dr. Stephanie R. Dungan and Prof. Dr. David M. Ogrydziak for their understanding, support and guidance and for providing me facilities during my research at University of California, Davis. I would like to express my warmest thanks to Prof. Dr Ahmet Palazoğlu for his kindness and good advice during my study in Davis. My special thanks go to H. Mecit Öztop and Dr. Şeyda Açar who have made my life easier with their friendship, encouraging attitude and suggestions during my stay in Davis.

My ever friends Kemal Şen and B. Levent Alpsal. Your presence always made me feel warm and happy, thanks your friendship...

I would sincerely thank to my friends Dr. Erkan Karacabey and Dr. Halil İbrahim Çetin for their endless friendship, encouragement and help in all parts of my life, making my stay in METU happy and memorable.

I would like to thank deeply to my dear friends and colleagues, Dr. A. Oğuz Büyükkileci, Dr. Neslihan Altay Dede, Dr. Nadide Seyhun, Dr. Özge Şakıyan

Demirkol, Cem Baltacıoğlu, Hande Baltacıoğlu and Mete Çevik for their helps and motivation, and the times we have spent together.

Love and thanks also go to all my laboratory friends for their support and help throughout this study.

I would like to mention valuable friendship and help of my friends, Dr. Yoo Jung Kim (Jun), Dr. Vannarith Leang (Van), Dr. Ariyaprakai, Suwimon (Vicky), Dr. Nathan Lloyd, Wyatt Musnicki and Dr.Yuyi Shen. Life would not be easy without their contributions when being away from home.

It is impossible to thank all the people who have contributed to my work or have just been good friends but I remember you all with great pleasure.

Finally, I would like to dedicate this work to my dear parents and my loved wife Dr. Işıl Barutçu Mazi for their love, patience and belief in me and for making those hard times much easier. Thanks to my cute daughter Karya Mina who fancied my life. Words are incapable to express my gratitude and my love to my family, Gülhan Mazi, Samet Mazi, Meliha Arıkan, Süreyya Nuçen Menemencioğlu and Emin Tuçen Mazi.

This study was supported by the grant BAP-08-11-DPT2002K120510 from METU, by the Scientific and Technical Research Council of Turkey (TUBITAK) BİDEB-2214 (Research in Foreign Countries Fellowship Program), by the Robert Mondavi Institute - Center of Advance Materials, Methods and Processing, University of California, Davis (RMI-CAMMP) and by the UC Davis University Outreach and International Programs (UO&IP) Office.

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CHAPTER 1

INTRODUCTION

β -Galactosidases are a family of enzymes that hydrolyze the linkage in β -galactosides. Lactase is the important member of this family that hydrolyzes the milk carbohydrate lactose, a disaccharide with poor water solubility and low sweetness. Lactose is also poorly digested by many people worldwide. The ability of the β -galactosidase to break down lactase, converting it to the sweeter and more soluble glucose and galactose, gives the enzyme considerable commercial importance. β -Galactosidase can be used to convert whey into a sweet syrup useful as a food additive, to mitigate problems of lactase crystallization in food products, to increase ripening rates in cheese production, and reduce levels of lactase waste. Perhaps most importantly, it can significantly enhance the availability of milk and dairy foods to consumers suffering from some degree of lactose intolerance. These applications all add to the value of milk and milk products.

Because of the high value of β -galactosidase, there has been considerable research into methods of its production, especially using microbiological sources. Enzyme produced from *E.coli* has played a prominent role in scientific studies, but is not an acceptable source for use in foods. Commercial production of lactase is primarily from the yeasts *Kluyveromyces sp.* or *Aspergillus sp. fungi*. The former produces an enzyme which operates optimally at neutral pH, and is therefore appropriate for use in milk hydrolysis (Panesar et al., 2006). *Kluyveromyces sp.* produces lactase intracellularly, and thus recovery of the enzyme involves cell breakage, removal of cell debris and nucleic acid, and purification of β -galactosidase from other proteins in the extract. These downstream processing steps can add significant cost to the production of this enzyme. For many enzymatic production processes, downstream production steps can contribute as much as 60-90% of the processing cost (Banik et

al., 2003). For lactase in particular, it is generally recognized that the cost of extraction of the protein from the cell broth is high, and that this hampers its industrial utilization (Rodriguez et al., 2006). Improving processing cost efficiencies is particularly important for food enzymes such as β -galactosidase, where price point is much lower than for the pharmaceutical proteins for which many separation approaches have been developed.

We propose in this study to explore the potential of water-in-oil microemulsions for extracting and purifying β -galactosidase from a cell extract produced from *Kluyveromyces lactis*. Water-in-oil microemulsions (also called reversed micellar solutions) are nanometer water droplets, stabilized by a monolayer of surfactant, are dispersed in organic solvents. The water containing microemulsion droplets can selectively extract protein molecules - in some cases removing nearly 100% of the protein from water - by tuning the pH and salt concentration of the system. These microemulsions are largely immiscible with water, and have been extensively explored for their potential in effecting a liquid-liquid separation of a target protein from other components in the cell broth (Göklen and Hatton, 1985; Luisi, 1985; Luisi and Magid, 1986; Luisi and Laane, 1986; Dekker et al., 1986; Giovenco et al., 1987; Luisi et al. 1988; Wolbert et al., 1989; Krei and Hustedt, 1992; Pires et al., 1996; Krishna et al., 2002). Such an extraction approach has the potential for continuous purification of the enzyme, and may be able to reduce the number of steps required in downstream processing (Giovenco et al., 1987).

1.1 Surfactant, Micelles, Reversed Micelles and Microemulsions

If an organic solvent is placed in contact with an aqueous solution then solutes will partition between the two phases: components that are hydrophobic will partition to the organic phase and those that are hydrophilic will partition to the aqueous phase. A level of complexity is added when surfactants, which are amphiphilic molecules, are added to the system, since surfactant molecules exhibit affinity for each phase.

The term surfactant is a contraction of “surface-active-agent”. Surfactant, which is an amphiphilic molecule, is made up of two different chemical groups (i) the hydrophilic (water-loving) head and (ii) the hydrophobic (water-fearing) tail (Figure 1.1). Surfactants are classified into three groups depending on their nature and hydrophilic head: anionic, cationic, and nonionic surfactants. The hydrophobic tail of a surfactant may consist of a single chain (i.e. alkyl chain in soaps) or up to four chains (i.e. quaternary ammonium salts).

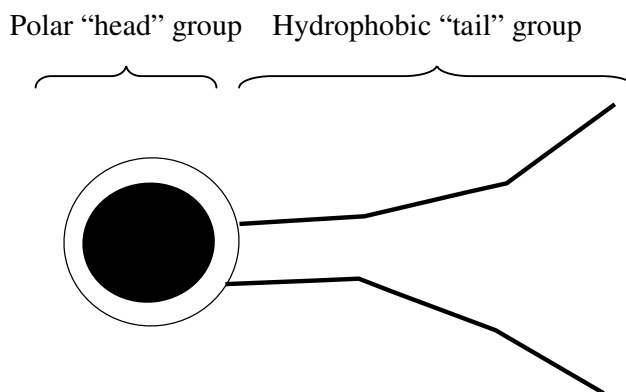


Figure 1.1 Cartoon of surfactant with two hydrocarbon chains.

In oil/water mixtures, surfactants spontaneously aggregate with their head groups pointing toward water and tail groups pointing toward oil. Depending on the surfactant geometric structure and oil/water solution conditions, surfactants form a variety of self-assembled structures such as monolayer, bilayer, lamellar (liquid crystalline phase), vesicles (liposome), micelles, and reversed micelles (Figure 1.2).

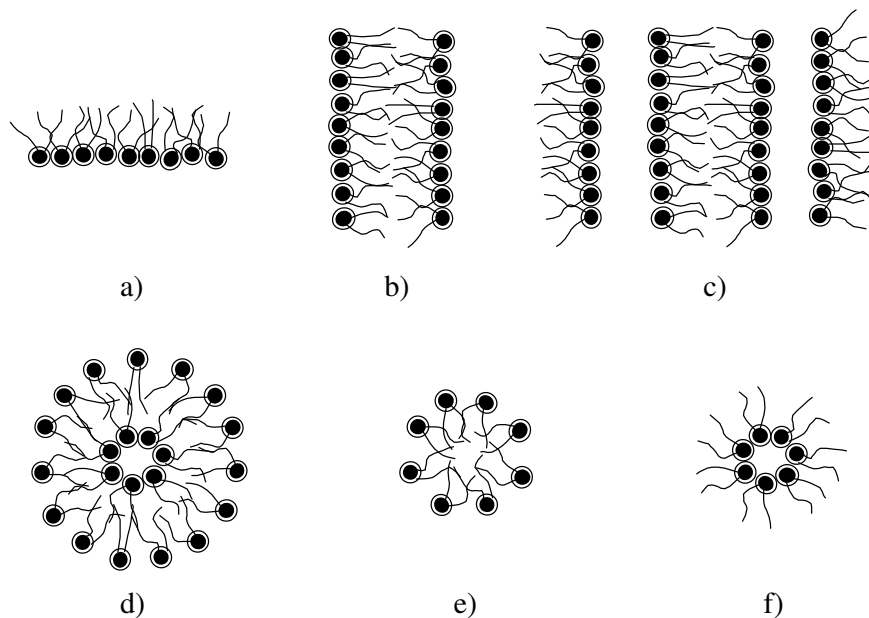


Figure 1.2 Various forms of surfactant aggregations in solution

(a) monolayer, (b) bilayer, (c) lamellar, (d) vesicles (liposome), (e) micelle, (f) reversed micelle.

The molecular geometry of the surfactant can be described by the packing parameter, v/a_0l_c , where l_c is the hydrocarbon chain length, a_0 is the optimal headgroup area, and v is the volume of the hydrocarbon chains (Figure 1.3). When the packing parameter is greater than 1, the amphiphile tends to form reversed micelles (Israelachvili, 1992). Factors that alter these parameters will affect the structures the surfactant form.

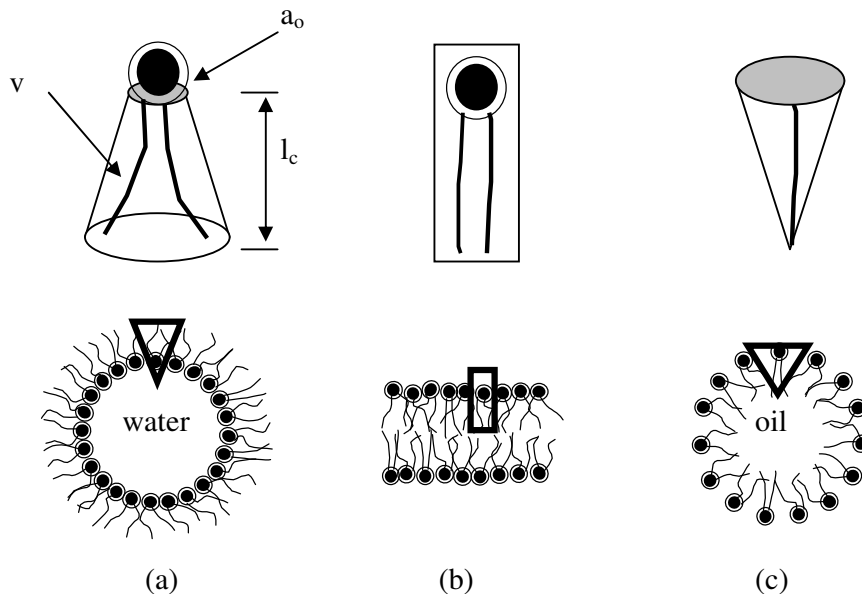


Figure 1.3 Molecular geometry of the surfactant

a) water-in-oil microemulsion, b) lamellar, c) oil-in-water microemulsion.

Most of the synthetic ionic surfactants have only one lipophilic chain. Single-chain surfactant, such as sodium dodecyl sulphate (SDS) or cetyltrimethyl-ammonium bromide (CTAB), have a much higher affinity towards the water than towards the oil (i.e. they are much more hydrophilic than lipophilic) and, therefore, need a lipophilic co-surfactant in order to increase their oil solubility and aid reversed micellar formation (Shinoda and Lindman, 1987). Successfully used cosurfactants are short-chain alcohols (e.g. butanol, pentanol, hexanol, or octanol) or short-chain amines. However, ionic surfactants having more than one hydrocarbon chain are mostly able to form reversed micelles without the aid of cosurfactants. Their larger surfactant tail cross sectional area compared to the polar head group area makes them more likely to form an interfacial film with reverse curvature, i.e., reversed micelles. Moreover, the two hydrocarbon chains give the ionic surfactant molecule more balanced hydrophilic-lipophilic properties compared to single-chain ionic surfactants. An example of an ionic surfactant that forms reversed micelles in organic solvents without the addition of cosurfactants is the anionic, double chained, branched surfactant bis (2-ethylhexyl) sodium sulfosuccinate (AOT).

In the aqueous phase, micelles form primarily due to hydrophobic interactions that drive the surfactant tail groups together. In an oil phase, reversed micelles form due to the dipole-dipole interactions and hydrogen bond that form between surfactant head groups (El Seoud, 1994). If micelles or reversed micelles solubilize an additional, significant quantity of water or oil in their core they are referred to as water-in-oil (w/o) or oil-in-water (o/w) microemulsions (Figure 1.4), respectively (El Seoud, 1994).

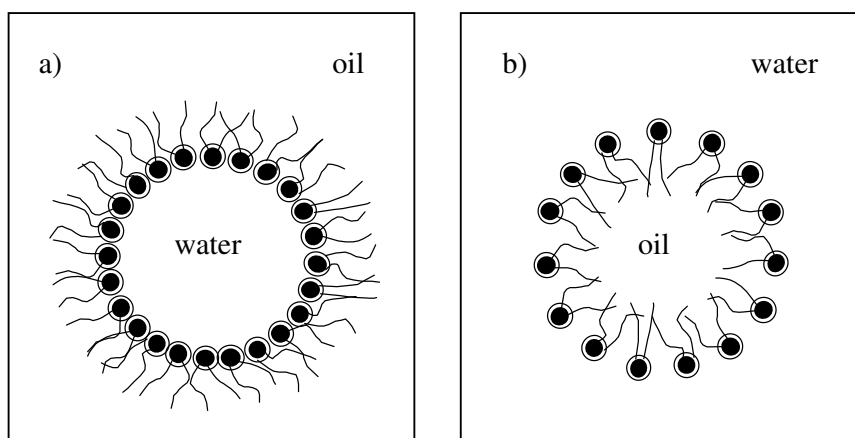


Figure 1.4 Schematic diagram of a spherical microemulsion droplet
(a) water-in-oil (w/o), (b) oil-in-water (o/w) microemulsion droplet.

While having some intermediate properties, microemulsions differ in several important ways from micelles and macroemulsions (Table 1.1). Microemulsion droplets have average sizes that are larger than micelles and smaller than emulsions, typically 10-100 nm (Evans and Wennerström, 1999). Like emulsion, the dispersed liquid is immiscible with the continuous phase. However, microemulsions are thermodynamically stable and form spontaneously, similar to micelles (Wennerström et al., 1997).

Table 1.1 Technical differentiation between emulsions, microemulsions and micelles.

Emulsions	Microemulsions	Micelles
Thermodynamically unstable	Thermodynamically stable	Thermodynamically stable
Cloudy colloidal systems	Optically transparent (Isotropic)	Optically transparent (Isotropic)
Micelle diameter ≥ 1000 nm	Micelle diameter 10-100 nm	Micelle diameter 2-5nm
The dispersed liquid is immiscible with the continuous phase	The dispersed liquid is immiscible with the continuous phase	No dispersed liquid

The free energy of a surfactant film depends on how much it is curved. The spontaneous curvature (c_0) is defined as the curvature an unconstrained surfactant film would adopt (Evans and Wennerström, 1999). When conditions favor a negative spontaneous curvature the surfactant monolayer bend inward to form small w/o microemulsion droplets (Figure 1.5). As the curvature becomes less negative, microemulsion droplets increase in size until flatter lamellar or bicontinuous phases form, which possess zero curvature. If the spontaneous curvature becomes positive the surfactant monolayer bends to form o/w microemulsions of variable size and composition (Kelley et al., 1994).

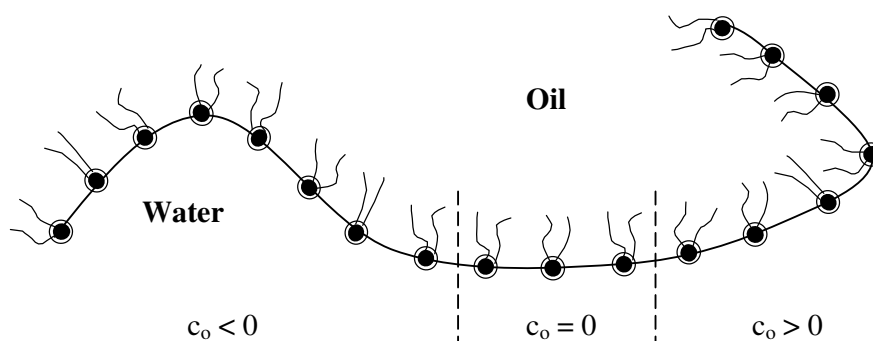


Figure 1.5 Spontaneous curvature.

Perpendicular cut through an oil-water interface showing the curved surfactant film.

Types of organic solvent significantly influence the water solubilization capacity of the reversed micelle for a certain surfactant. Hou and Shah (1987) reported that maximum water solubilization ($w_o = 60$) can be obtained with organic solvent *n*-heptan when AOT used as a surfactant. Water solubilization capacity of the reversed micelle significantly decreases with increasing the carbon number of organic solvent. Only about five water molecules per AOT molecule ($w_o = 5$) can be solubilized with hexadecane. Lang et al. (1988) showed that size of the reversed micelle decreased with increasing the molecular volume of the organic solvent. In this research, authors explained that larger oil molecules were more hindered in their ability to penetrate into AOT tail groups than smaller oil molecules. Consequently, the intermicellar attractive interaction between the surfactant tails (i.e., attraction between micelles) increased and this caused the formation of smaller w/o microemulsion droplets.

1.2 Reversed Micellar Extraction, Separation and Purification of Proteins / Enzymes

For many years liquid-liquid extraction technology which is a conventional unit operation in the chemical engineering has been successfully used in a variety of industries (i.e. chemical, petrochemical, or hydrometallurgical) for separation of compounds. In recent years, however, increased attention has been given to the potential use of liquid-liquid extraction in biotechnology for the separation, concentration and purification of proteins and other biomolecules (Kadam, 1986; Abbott and Hatton, 1988).

Reversed micellar extraction is one of the attractive liquid-liquid extraction methods. Reversed (reverse or inverted) micelle is a nanometer size droplet of an aqueous solution stabilized in organic solvent by the surfactant present at the interface (Eicke, 1980). The aqueous phase can also contain hydrophilic compounds, such as ions, peptides, proteins, or enzymes. In the reversed micelle system, these biomaterials are solubilized into the polar core of surfactant shell that protects the biomolecules from the denaturation by organic solvent. Reversed micelles formed in ternary surfactant (<10%) – water (1-10%) – oil (80-90%) mixture have generally spherical shape and these solutions are also called water-in-oil (w/o) microemulsions (Dekker and Leser, 1994). The possibility of using water-in-oil microemulsions to extract protein molecules from an aqueous phase was demonstrated by Göklen and Hatton (1985; 1987) and by others (Dekker et al., 1986; Leser et al., 1986). In this approach, an aqueous protein-containing phase is contacted with a water-in-oil microemulsion. Given the right conditions, the protein then preferentially solubilizes within the microemulsion phase, at which time the two phases can be separated. Recovery of the protein may be accomplished by contacting the protein-containing microemulsion with a fresh aqueous solution under conditions favoring the transfer of protein out of the aqueous phase. This process has some nice features in that the microemulsion phase can be a gentle solvent for extracting the protein without altering its enzymatic or functional properties, and yet the process can be readily scaled up using conventional liquid-liquid extraction technology (Dungan, 1997).

Another remarkable finding was that the solubilization of different proteins into micellar solutions is a selective process (Luisi et al. 1979). This and following studies in which the solubilization behavior of pure, single proteins was investigated led to the conclusion that the micellar aggregates can be used as an instrument with the ability to extract proteins selectively from aqueous mixtures (Dekker et al. 1989; Hatton, 1989; Leser and Luisi, 1990). Selectivity is thereby governed by properties of both the micellar aggregates and the proteins, and can be tuned by optimizing protein-micellar aggregate interactions. Different reversed micellar systems (both forward and backward extraction) have been used to extract and purify various proteins/enzymes (Table 1.2).

Table 1.2 Extraction and purification of proteins / enzymes using reversed micelles.

Biomolecule	Source	Reversed micellar system	Reference
α -Chymotrypsin	Bovine pancreas	AOT/isooctane	Barbaric and Luisi., 1981
α -Amylase	<i>Bacillus licheniformis</i>	CTAB/isobutanol/hexanol/isooctane	Lazarova and Tonova, 1999
Lysozyme	Chicken egg white	AOT/isooctane	Naoe et al., 1995; 1996
Trypsin	Porcine pancreas	Tetra-oxyethylene-monodecylether /n-hexane	Adachi et al., 1998
Glucoamylase	<i>Aspergillus awamori</i>	TOMAC/Revopal HV5/n-octanol /isooctane	Forney and Glatz, 1994; 1995
Cytochrome C	Horse heart	AOT/isooctane	Ichikawa et al., 1992
Lipase	<i>Chromobacterium viscosum</i>	AOT/isooctane	Aires-Barros and Cabral, 1991
	<i>Penicillium citrinum</i>	AOT/isooctane	Krieger et al., 1997
	<i>Rhizopus delemar</i>	AOT/isooctane	Nagayama et al., 1999
Recombinate Cytocrome b ₅	<i>E.coli</i>	CTAB/cyclohexane/decanol	Pires and Cabral, 1993
Recombinate Cytocrome C ₅₅₃	<i>E.coli</i> periplasm	AOT/isooctane	Jarudilokkul et al., 1999
Alkaline protease	<i>Bacillus sp.</i>	AOT/isooctane	Rahaman et al., 1998
Ribonuclease A,	Bovine pancreas	AOT/isooctane	Andrews et al., 1994
A-Lactalbumine,	Whey		
Thaumatococin,			
Soybean trypsin inhibitor.			
Ribonuclease A,	Bovine pancreas,	AOT/isooctane	Imai et al., 1997
Cytochrome C,	Horse heart,		
Lysozyme.	Chicken egg		

Table 1.2 (continued)

Biomolecule	Source	Reversed micellar system	Reference
Lysozyme, α -Chymotrypsin, Pepsin.	Chicken egg	AOT/isooctane	Chang et al., 1994
Pepsin, Cymosin.	Porcine, Bovine	AOT/isooctane	Carlson and Nagarajan, 1992
β -Glucosidase, β -Xylosidase, β -Xylanase.	<i>Trichoderma reesei</i>	AOT/isooctane	Zamarro et al., 1996
β -galactosidase, BSA, Catalase, Hemoglobine.	<i>E.coli</i> Bovine	AOT/isooctane	Shiomori et al., 1995
α -Lactalbumine, β -Lactoglobulin.	Whey	AOT/isooctane	Lee and Dungan, 1998; Kawakami and Dungan, 1996
α -Chymotrypsin, Cytochrome C.	Bovine pancreas, Horse heart	AOT/isooctane	Dungan et al., 1991
Isocitrate dehydrogenase, β -Hydroxybutyrate dehydrogenase, Glucose-6-phosphate dehydrogenase.	<i>Azotobacter vinelandii</i>	CTAB/hexanol/octane	Giovenco et al., 1987
Immunoglobulin	Bovine	AOT/isooctane	Gerhardt and Dungan, 2002

1.3 Factors Affecting Protein Solubilization into Microemulsion Droplets

Solubilization of proteins into microemulsion droplets is controlled by electrostatic, steric and hydrophobic interactions between proteins and micelles (Wolbert et al., 1989; Dungan et al., 1991; Yamada et al., 1994; Shiomori et al., 1995; Pires et al., 1996; Kawakami and Dungan, 1996). These interactions will depend on the specific characteristics of the proteins and all parameters which influence the condition in the aqueous and organic phase. The condition in the aqueous phase, such as type of salt, ionic strength and pH largely determines the distributions of proteins between an aqueous phase and a conjugated reversed micellar phase. The partition of proteins between phases is also influenced by the parameters which are related to the organic phase, for example type of solvent, type and concentration of surfactant and presence of cosurfactant. The solubilization of biomolecules can also be affected by the changes in the system temperature. The phase transfer relies on the specific characteristics of the proteins, i.e. size and shape, isoelectric point, charge distribution and hydrophobicity (Pires et al., 1996; Carvalho and Cabral, 2000). Obviously all these parameters influence not only the physicochemical properties of the microemulsion: size, shape, charge of the interfacial layer composed of the surfactant head groups and their counterions (for ionic surfactants) which can dissociate into the micellar water pool, but also the properties of the protein molecules (overall charge) in the system (Dekker and Leser, 1994). Selective separation of the desired protein from mixtures can be achieved by manipulating these parameters.

1.3.1 Aqueous Phase pH

Net charge of the proteins is determined by the aqueous phase pH. Electrostatic interactions dominate the solubilization of the protein into microemulsion droplets when the ionic surfactants are used (Sadana, 1998). Favorable electrostatic interactions between the charged protein molecules and the inner micellar wall (surfactant head group) are obtained at pH values below the isoelectric point (pI) of the protein in the case of anionic surfactants (Göklen and Hatton, 1987), while the opposite is true for

cationic surfactants (Dekker et al., 1986). Dependence of protein solubility on pI was also indicated by many other studies (Aires-Barros and Cabral, 1991; Jolivald et al., 1993; Regalado et al., 1994; Huang and Lee, 1994; Chang and Chen, 1995). Each protein shows a characteristic pH range where solubilization starts to increase dramatically. For small proteins whose size is smaller than the size of the microemulsion droplets, solubilization occurs as soon as the net charge is opposite to that of the reversed micellar interface ($[\text{pH}-\text{pI}] < 2$). For large molecular weight proteins, however, the $[\text{pH}-\text{pI}]$ value required for optimum solubilization is much higher. For example, the solubilization of lysozyme ($\text{pI} = 11.0$) begins at about pH 12, whereas cytochrome c ($\text{pI} = 10.6$) and ribonuclease ($\text{pI} = 7.8$) require a lower pH, namely pH 10.5 and 8 respectively. Comparisons of these values with the protein pI suggest a direct correlation. The molecular weights of these proteins are relatively low (all in the range 12.5 – 14.5 kDa). For larger proteins such as α -amylase (MW 48 kDa) (Dekker and Leser, 1994) and alkaline protease (MW 33 kDa) (Rahaman et al., 1988) optimum solubilization was achieved when the $[\text{pH}-\text{pI}]$ is around 5. The reason of this can be explained as that in order to incorporate the large protein molecule, size of reversed micelles has to increase with increasing protein size. Higher energy which is obtained by increasing the number of charged groups on the protein surface is required to increase size of the reversed micelles (Hilhorst et al., 1995; Dekker and Leser, 1994). The number of charged groups on the protein molecule can be increased by arranging the aqueous solution pH (i.e. by increasing the difference between pH of the aqueous solution and isoelectric point of the protein $[\text{pH}-\text{pI}]$) (Wolbert et al., 1989). It may be noted that, the interpretation of the phase-transfer pattern is additionally difficult at extreme pH values for the reason that protein denaturation and changes in the ionization state of the surfactant.

1.3.2 Ionic Strength

The influence of ionic strength (salt concentration) on the solubilization of proteins in microemulsion droplets is explained by a number of ways. The ionic strength of the aqueous phase determines the degree of shielding of the electrostatic potential imposed by a charged surface (Leodidis and Hatton, 1990). In general, it was observed that increasing ionic strength of the aqueous phase will reduce the protein intake capacity of the microemulsion droplets (Aires-Barros and Cabral 1991; Marcozzi et al., 1991; Caroso et al., 1999). This phenomenon causes at least two important effects in the reversed micellar extraction: first, increasing the ionic strength reduces the electrostatic interaction between the charged protein molecules and charged interface in the reversed micelles by decreasing the Debye length, and second, increasing the ionic strength reduces the electrostatic repulsion between the charged surfactant head groups, resulting in a decrease in size of the reversed micelles at higher ionic strength (Dekker and Leser, 1994). This can lead to a decrease in solubilization capacity through a size exclusion effect. Effect of ionic strength on the phase transfer of lysozyme, cytochrome c and ribonuclease A in an AOT/isooctane reversed micellar system was showed by Göklen and Hatton (1987). According to this study, there is not any solubilization at high ionic strength (1.0 M KCl) whereas all of the test proteins completely solubilized at low ionic strength (0.1 M KCl). For these three proteins, increasing the concentration of KCl in the aqueous solution causes to decrease in the extent of protein transfer from aqueous to reversed micellar phase. However, ionic strength required to initiate this decrease was found to be different for each of them. Lysozyme (pI=11) was extracted at values below 0.8 M KCl, ribonuclease A (pI=7.8) and cytochrome c (pI=10.6) could only be transferred at lower KCl concentrations, namely at 0.6 M and 0.3 M respectively.

Additional effect of the ionic strength is to salt out the protein from the micellar phase because of the increased tendency of the ionic species to migrate to the micellar water pools and to displace the protein. Finally, specific and nonspecific salt interaction with the protein or surfactant can modify the solubilization behavior, and these effects will be more pronounced the higher the salt strength (Hatton, 1989).

It may be noted that, reversed micelles and phase separation do not occur while the ionic strength of the aqueous phase is below a certain limit, as a result a cloudy stable microemulsion is formed. In addition, the aqueous phase should also provide the minimum value of the ionic strength for the transfer of proteins between phases. For example, Göklen and Hatton (1985) showed that the quantitative transfer of cytochrome *c* into the AOT/ isooctane reversed micellar system was obtained when the minimum concentration of KCl is around 0.1 M.

1.3.3 Type of Electrolyte

Salt type has been reported to have a strong effect on solubilization characteristics of different proteins between aqueous and organic phases (Leser et al., 1986; Marcozzi et al., 1991; Nishiki et al., 1993; Kelley et al., 1994). Several hypotheses have been proposed to explain the specific ion effect.

Andrews et al. (1994) suggested that smaller ions produce less screening of protein-micelle electrostatic interactions and, therefore, allow more protein transfer. Their analysis, however, was based on bare ion size rather than hydrated ion size, whereas the latter may be a more relevant description of the ions in the micelle water pool. They also hypothesized that hydrophobic interactions may be stronger in the presence of sodium ions as compared to potassium ion. Moreover, the authors indicated that this effect is consistent with the lyotropic series of cations.

Nishiki et al. (1994) also observed significant protein solubilization over a much broader range of ionic strengths in the presence of sodium ion than in the presence of potassium or barium ion. They proposed that hydration of the salt molecules weakens their screening ability. Thus ions such as sodium, which are hydrated to a greater extent (Figure A.1), screen electrostatic interactions less efficiently, allowing more protein transfer.

Leodidis and Hatton (1989) observed that ions of smaller hydrated sizes than sodium ions can displace the larger sodium counterion from the vicinity of the surfactant head group in the micellar water pool, because the smaller ions can form a more close association with the head group. Consequently, these smaller ions partition more effectively into the micellar droplet, leading to better screening of the surfactant-surfactant repulsive electrostatic interactions within the droplet. Such screening results in a smaller micelle size. They also published a model which predicts the equilibrium solubilization of monovalent and divalent cations in a biphasic AOT reversed micellar-aqueous phase system. The model distinguishes between different cations by their charge, hydrated size, and electrostatic free energy of hydration.

Kelley et al. (1994) observed that the differentiation in α -chymotrypsin solubilization behavior was virtually lost when the data were plotted as a function of w_o which is approximately proportional to the micelle size for the different salt types. Kawakami and Dungan (1996), however, observed that the solubility behavior of α -lactalbumin and β -lactoglobulin is qualitatively different when different cations are present, even when the data are compared as a function of w_o . The effect of counterion type on protein solubilization appears to be more than its effect on the water transfer or micellar water pool size. They hypothesized that this distinction may be the result of specific protein-salt interactions, which could alter the conformation of the protein and hence the way the protein interacts with surfactant. Alternatively, the specific ions may influence the nature of hydrophobic interactions more directly.

1.3.4 Surfactant Type and Concentration

Structure and size or aggregation number of the reversed micelles are almost independent of the surfactant concentration when reversed micellar phase is in equilibrium with an aqueous phase (Battistel and Luisi, 1989). However, increase in the surfactant concentration of organic phase causes increase in the number of microemulsion droplets. Moreover, protein solubilization capacity of the reversed micellar phase increase with increasing microemulsion droplet number (Fletcher and

Parrott, 1988; Woll and Hatton, 1989; Krei et al., 1995). In other words, increasing the surfactant concentration favors the solubilization of protein into microemulsion droplets by enhancing capacity of the reversed micellar phase (Hatton, 1989; Hentsch et al., 1992; Carneiro-da-Cunha et al., 1994). This was clearly observed by Hatton (1989) who noted that the pH solubilization peak broadened as the surfactant concentration was increased.

On the other hand, further increase in the surfactant concentrations decrease solubilization of biomolecules (Pessoa and Vitolo, 1998; Cardoso et al., 1999) and also make difficult the backward transfer of proteins into a second aqueous phase (Hentsch et al., 1992; Carneiro-da-Cunha et al., 1994). Micellar interactions may happen at high surfactant concentration and it causes percolation and interfacial deformation with an alteration in the micellar clustering and micellar shape. Therefore, monodisperse spherical micelles might not be present predominantly in the solution. Interfacial area available to host the biomolecules is decreased by the micellar clustering which decreases the solubilization capacity of the reversed micelles (Krishna et al., 2002)

At low surfactant concentration, however, protein increasingly moves from the organic microemulsion phase to the precipitate phase, solid phase at the interface between aqueous and organic phase, with no increase in aqueous concentration. This was obviously observed by Gerhardt and Dungan (2004), who noted that the immunoglobulin G (MW 155 kDa, pI 7.7) moved from the organic microemulsion phase to the precipitate phase as the surfactant concentration (AOT) was decreased below 0.2 M at low salt. According to the Shiomori et al. (1995) work, very large proteins (hemoglobin, β -galactosidase, and BSA) were easily and completely solubilized into microemulsion droplets without any precipitate by the injection method at $w_o = 20$ when the AOT concentration equals to 0.2 M.

1.3.5 Size of Reversed Micelles

Micellar size of the established aggregates in a surfactant-water-oil mixture is depend on following parameters: (a) surfactant type; (b) oil type; (c) water content, which is often represented by the molar ratio of the water and surfactant ($w_o = [\text{H}_2\text{O}] / [\text{surfactant}]$) (Luisi et al., 1988) is the amount of water solubilized in reversed micelles, and (d) ionic strength, which is important in systems with ionic amphiphiles. If the surfactant and the oil type are kept constant it has been shown (Zulauf and Eicke, 1979) that the aggregate size is mainly dependent on the w_o value but not on surfactant or the water concentrations.

The reversed micellar size is expressed in terms of the water pool radius R_{wp} , defined as the mean radius of the water core of the aggregates, including the hydrophilic head group, but excluding the length of the hydrophobic tails (Figure 1.6), and can be quantitatively related to the micellar water content:

$$R_{wp} \text{ [nm]} = (3 v_w / \Sigma) w_o \quad (1.1)$$

where v_w is the volume of a single water molecule (0.03 nm^3) and Σ is the area occupied by a single surfactant molecule in the interface (area per head group).

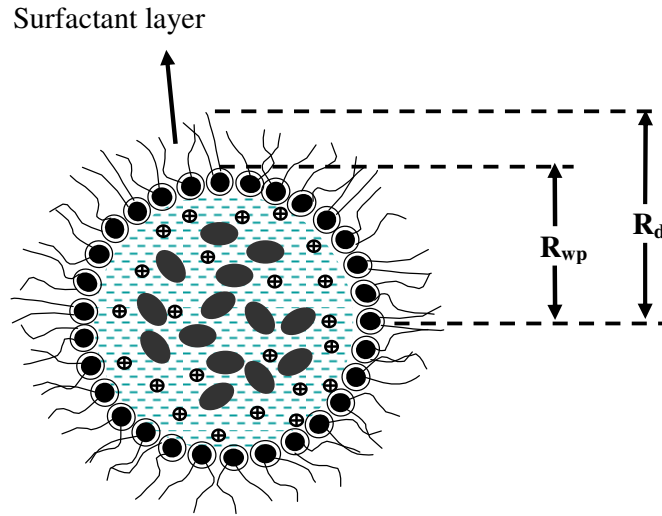


Figure 1.6 Schematic representation of water pool radius (R_{wp}) and overall droplet radius (R_d).

Taking AOT for example, $\Sigma_{AOT} \cong 0.55 \text{ nm}^2$ and assuming Σ to be independent of w_o then $(3v_w / \Sigma)$ is 0.16-0.17. For AOT this relation has been confirmed experimentally in the past by using different physical methods (Figure B.1). Therefore, for example, at $w_o = 20$, R_{wp} is 4 nm; adding to it the length of the AOT tails (1 nm) the total diameter of an aggregate is 10 nm. The average equilibrium micellar size is not significantly influenced by the absolute values of either the $[\text{H}_2\text{O}]$ or the $[\text{Surfactant}]$. However, when increasing the AOT content at constant water content, the droplet diameter decreases whereas, when increasing the water at constant AOT the droplets are getting larger as shown in Figure 1.7 (Dekker and Leser, 1994).

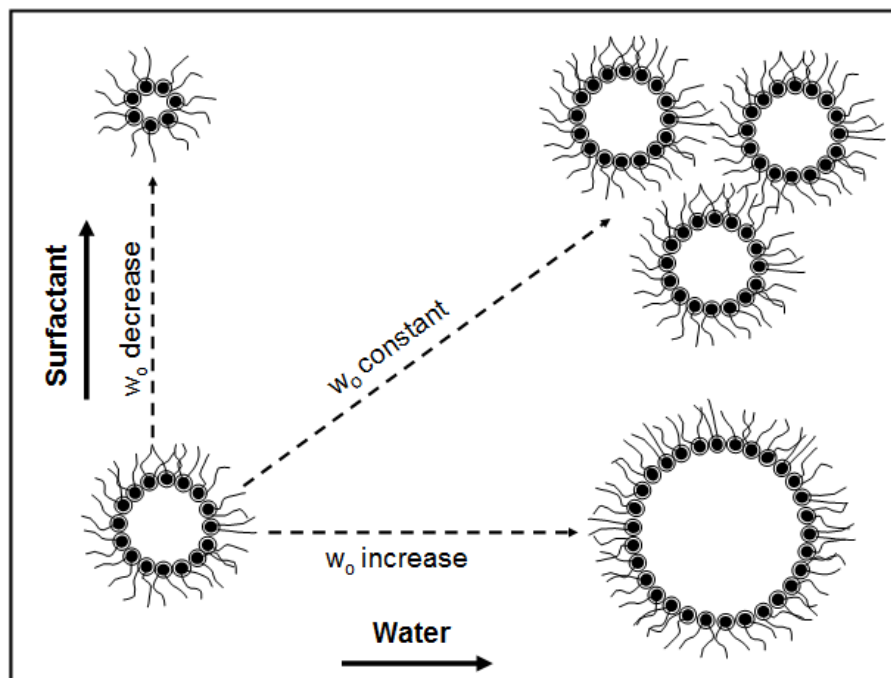


Figure 1.7 Schematic illustration of the relation between surfactant and water concentration in the system.

An effect of other parameters, such as temperature, on the AOT reversed micellar size is much less significant. Furthermore, the size distribution of the formed reversed micelle is generally quite narrow. A polydispersity index of only 12% was estimated with light scattering for AOT reversed micelles (Ricka et al., 1991).

The monodisperse small sized reversed micelles can host only proteins of certain dimensions (Luisi et al., 1979). Consequently, micellar size possibly will be used to exclude or include certain proteins. On the other hand, it should be noted that alteration in the certain operating conditions may cause regroup of several micelles to form larger micelles. It was also hypothesized that a new larger micelle of a required size can be formed around a protein to ease solubilization. Wolf and Luisi (1979) stated that configuration of reversed micelles with large enough size for the protein solubilization is induced by given protein.

1.3.6 Specific Characteristics of Proteins

Protein transfer from an aqueous into a reversed micellar phase depends not only on the composition of both phases but also on the properties of the protein under investigation (Göklen and Hatton, 1985; Kadam, 1986). Effect of protein size, isoelectric point and charge distribution was investigated by Wolbert et al. (1989). In this study, authors indicated that proteins with molecular weights larger than approximately 120 kDa should be unable to transfer significantly into w/o microemulsion solutions. On the other hand, Gerhardt and Dungan (2002; 2004) found that even very large proteins can be taken up from an aqueous phase into the microemulsion, but that there they are often metastable, as their presence seems to promote droplet clustering and eventual precipitate formation. It is also known that it is possible to form single-phase microemulsions by “injecting” aqueous solution containing very large molecules or bodies directly into the organic surfactant solution, and thus entrapping the large bodies inside the w/o droplets that form (Pfammeter et al., 1989; Pietrini and Luisi, 2002). The latter approach was shown by Shiomori et al. (1995) as a way to incorporate β -galactosidase from *E.coli* and other very large proteins in a water-in-oil microemulsion.

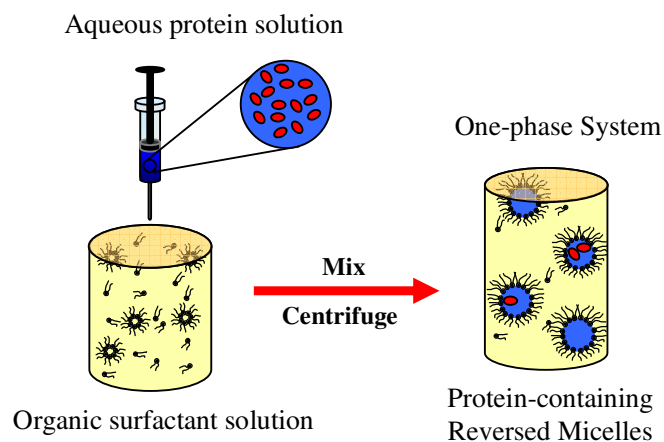
Wolbert et al. (1989) also indicated that, low molecular weight proteins are solubilized in reversed micellar phase around to their isoelectric points, while for high molecular weight proteins the difference between pI and the pH of solubilization increases. The explanation for this observation is that, as protein solubilization into organic phase requires adjustment of the reversed micelles (Levashov et al., 1982; Zampieri et al., 1986; Sheu et al., 1986; Chatenay et al., 1987) energy for this process is gained from interactions between surfactant and oppositely charged side of the protein surface. High molecular proteins require large reversed micelles for their uptake therefore more energy is needed for larger increase in size of the reversed micelles. The energy expenses for this rearrangement compensate from the larger increase in the difference between pH and the pI of the protein.

Overall charge of the protein surface strongly depends on the aqueous phase pH and it determines the yield of extraction. The question “Is there any effect of charge distribution of the protein surface on the extraction yield” was addressed for the first time on a paper by Wolbert et al. (1989). In this study, relation between the degree of charge asymmetry of the protein (from the research of Barlow and Thornton, 1986) and the yield of extraction was obtained. Wolbert and coworkers used the cationic surfactant tri-octyl-methyl-ammonium chloride (TOMAC) and found that proteins with higher degree of charge asymmetry are more easily extracted into reversed micellar phase. However, this is not a general observation, because using anionic surfactant AOT containing organic phase the same correlation was not valid. For example, 100% solubilization was reported for lysozyme (overall charge symmetrically distributed on its surfaces) in AOT containing reversed micellar system, while there was not any solubilization in TOMAC containing system for the same protein (Göklen and Hatton, 1985). This study shows that for AOT containing reversed micellar system the symmetry of charge distribution on protein surface can not be used to predict its solubilization pattern.

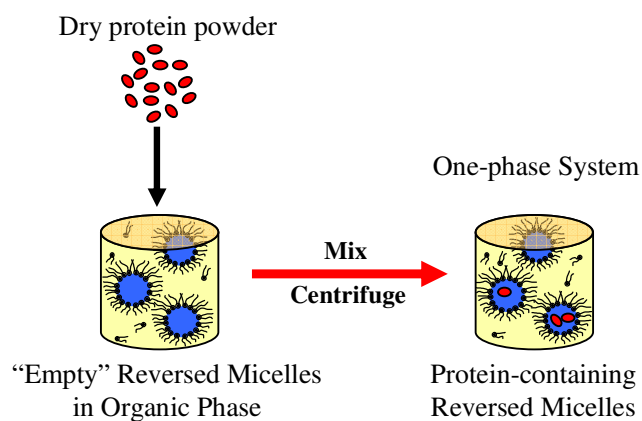
1.4 Methods of Protein Solubilization

There are three principal experimental methods to solubilize proteins into the water core of reversed micellar aggregates (Luisi, 1985): (i) the injection method, in which a few microliters of an aqueous stock solution of protein are added to a surfactant-oil mixture, and the mixture is shaken until total solubilization has occurred; (ii) the solid-liquid extraction method, where, the dry lyophilized protein powder is stirred with the w/o microemulsion already containing a given amount of water; and (iii) the phase transfer method, in which bulk aqueous protein solution is equilibrated with an organic reversed micellar solution, and the proteins are transferred from the bulk water phase into the micellar dispersed water phase (Dekker and Leser, 1994). Schematic illustration of the three reversed micellar protein solubilization methods is shown in Figure 1.8.

(i) Injection Method



(ii) Solid-liquid Extraction Method



(iii) Phase Transfer Method

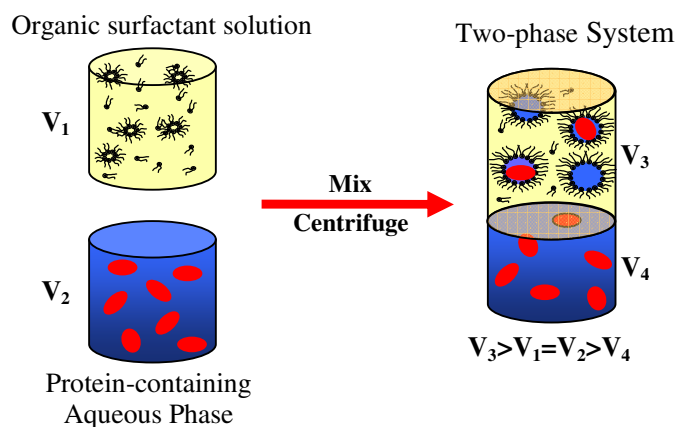


Figure 1.8 Methods of protein solubilization in reversed micelles.

Protein solubilization into reversed micelles water pool significantly depends on the protein addition method as well as on the size of reversed micelle droplets and of the size of protein. Solubilization behaviour of alcohol dehydrogenase (ADH) and α -chymotrypsin into microemulsion droplets with three protein solubilization methods were investigated in detail by Matzke et al. (1992). Protein solubilization into reversed micellar phase is less dependent on the size of reversed micelle for the injection method. On the other hand, for the solid-liquid extraction method it is strongly dependent on micellar size. Furthermore, for efficient solubilization, size of the reversed micelle must be approximately the same or larger than the protein molecule when the solid-liquid extraction method is used. For example, maximum solubilization occurred at 50-60 Å micellar diameter for small protein like α -chymotrypsin (40 x 40 x 51 Å, diameter of 44 Å, 24.8 kDa), whereas large protein like ADH (dimensions of 45 x 60 x 110 Å, diameter of 82 Å, 141 kDa) require a higher micellar size (80–90 Å) for maximum solubilization. For small micelles, energy level is too high to overcome for solubilization of the large protein into a small micelle. However, energy required for the protein solubilization into a larger micelle is lower since the micelle is not reorganized itself to incorporate the protein (Leser et al., 1987; Matzke et al., 1992).

Operationally, injection method is the simplest and fastest method (Luisi and Magid, 1986). Solubilization of protein is almost independent of micellar size in this method. Following the direct injection of a few microliters of stock aqueous solution containing very large molecules or bodies into surfactant containing organic phase, single-phase microemulsions is formed. Therefore, water in oil microemulsion droplets are forced to entrapped the large bodies inside the reversed micelles (Pfammeter et al., 1989; Pietrini and Luisi, 2002).

Phase transfer is another method to solubilize proteins in organic solvent via reversed micelles and it is basically different from the injection and solid-liquid extraction methods. In order to use such a system optimally for protein extraction this biphasic extraction system (bulk aqueous and organic phase) should be thermodynamically stable, i.e. the formation of other phases in equilibrium containing most of the

surfactant should be avoided. Luisi et al. (1979) showed first that this can be achieved at room temperature using AOT as surfactant, for example, by adding a certain concentration of salt to the extraction system. Later on, Aveyard et al. (1986) determined the exact surfactant distribution in such biphasic systems. At room temperature, they found that at low salt concentrations (i.e. $[\text{NaCl}] < 20 \text{ mM}$; heptane as oil) the AOT resides totally in the lower aqueous phase, whereas at higher ionic strength (i.e. $[\text{NaCl}] > 70 \text{ mM}$; heptane as oil) all AOT is present in the oil phase. Transfer of protein from the aqueous phase to the reversed micellar phase is obtained under certain conditions. However, achieving the same maximum solubilization value by the phase transfer method is difficult than the solid-liquid extraction and injection methods (Matzke et al., 1992; Krishna et al., 2002).

1.5 Back Extraction

Solubilization of desired protein into the reversed micellar organic phase is obtained by forward-extraction which is discussed above. The next step after forward-extraction is the backward-extraction, in which desired protein is extracted and concentrated into a fresh aqueous phase (protein recovery). The success of reversed micellar extraction processes for recovery of desired proteins will rely on the easiness with which the protein can be expelled (back-extracted) from the loaded organic phase into an aqueous phase, and on the extent to which enzymatic activity or biological function of the recovered product is maintained.

The simplest recovery method is based on the idea that the recovery efficiency is maximal at conditions under which the forward transfer is minimal. Although a number of proteins (e.g. ribonuclease A, α -chymotrypsin, α -amylase, among others) can be recovered either on the basis of steric interaction (i.e. size-exclusion), by increasing ionic strength (1-2 M) of the fresh aqueous phase, or through an electrostatic repulsion, choosing the appropriate pH (Göklen and Hatton, 1987), others, such as lysozyme, need more extreme conditions, or can hardly be recovered (albumins) using this approach (Dekker and Leser, 1994). Moreover, overall protein recovery in reversed micelle extraction is generally

less than 80% (Kelley et al. 1993). For alkaline protease, only 10–20% of recovery was achieved in the AOT/isooctane reversed micellar system. Another complication of this approach dwells in the fact that the proteins often precipitate out (denature) at the interface between the aqueous and the conjugated reversed micellar phase. This is perhaps because of a strong hydrophobic interaction between the proteins and surfactants (Aires-Barros and Cabral, 1991; Pires and Cabral, 1993).

Some alternative methods can also be performed for enhanced recovery of the protein from microemulsion droplets. Marcozzi et al. (1991) and Leser et al. (1993) proposed a recovery method which is based on the adsorption of the proteins on to an insoluble material, such as silica, from the reversed micellar solution. After decanting the organic solvent, the protein-containing silica is suspended in an aqueous solution at high ionic strength and optimized pH. By this means, the protein is desorbed from the solid silica and dissolved into the aqueous phase. Results using AOT-isooctane or short-chain lecithin-hexanol-isooctane systems as initial protein hosts showed that with this method up to 90% of the protein (α -chymotrypsin, trypsin) could be recovered without a major loss of enzymatic activity.

Carlson and Nagarajan (1992) demonstrated that the addition of 10-15% isopropyl alcohol (IPA) as a dewatering agent to the aqueous phase dramatically increases the recovery yield of bovine chymosin from AOT-isooctane reversed micelles. The solubility of AOT in the aqueous phase was enhanced by addition of IPA, facilitating the entry of the solubilized protein into the aqueous phase as an aqueous AOT-protein complex.

Another approach uses the effect of temperature on the phase behaviour of reversed micellar solutions (Dekker et al. 1991). In the system TOMAC-Rewopal HV5-octanol (0.1 vol%)-isooctane, the maximal amount of solubilized water was shown to decrease with increasing temperature. This fact was used for the enzyme recovery in the following way: by heating the reversed micellar solution containing solubilized α -amylase up to 40 °C, a certain amount of initially dispersed water was expelled. This water was shown to contain up to 80-90% of the initial enzyme activity from the

microemulsion phase. Moreover, a huge increase in enzyme concentration could be achieved since the volume of the expelled phase was extremely small.

Woll et al. (1989) reported that by adding a second water-immiscible organic solvent (e.g. 15-20% by volume of ethyl acetate) to an AOT-isooctane reversed micellar phase, the micelles are disrupted, leading to an expulsion of the protein from the organic solution to the aqueous receiving phase.

1.6 β -Galactosidase from *Kluyveromyces lactis*

β -Galactosidase or lactase (i.e. E.C. 3.2.1.23, β -D-galactoside galactohydrolase) from yeast *Kluyveromyces lactis* is very large, hydrophilic and globular protein with an isoelectric point of 5.0. It has an active dimeric form (MW~ 220-240 kDa) (Tello-Solís et al., 2005), with an active tetramer also likely (MW~460-480 kDa) (Becerra et al., 1998). Cavaille and Combes (1995) and Becerra et al. (1998) reported that the main active form of the enzyme is dimeric and it was composed of two identical subunits.

1.7 Aim of the Study

There is strong need to improve the efficiency of downstream processing of the enzyme lactase, in order to make this important enzyme cheaper and more available. By determining the level of purification that is possible with reversed micellar extraction methods, as well as the conditions that enhance that extraction, we will evaluate the potential of this separation method for more efficiently producing β -galactosidase. Improved methods for purification of this enzyme will make it more available and cost-effective on a commercial scale and expanded utilization of this protein is clearly tied to an expanded market for milk and other dairy products.

We hypothesize that water-in-oil microemulsions (reversed micellar solutions) can be used to purify the enzyme β -galactosidase. We will test this hypothesis by

exploring extraction of model protein solutions, as well as extracts produced by the yeast *Kluyveromyces lactis*. Our specific objectives for this project are:

- to compare two-step phase extraction to an injection and back extraction methodology for purifying β -galactosidases;
- to determine the purification factors achievable using these approaches, and to compare them to existing commercial technologies;
- to establish conditions that enhance purification with the minimum number of steps;
- to compare purification from model protein solution and from yeast extract.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Sodium di-ethylhexyl sulfosuccinate (Aerosol OT; AOT) of 99% purity was obtained from Sigma and used as received. Isooctane (ACS Grade) and all salts were obtained from Fisher Chemical (Pittsburgh, PA) and used without further purification. Orthonitrophenyl- β -D-galactopyranoside (ONPG) was obtained from Sigma Chemical Co. β -Lactoglobulin (β -Lg; pI = 5.2; MW of dimer 36 kDa) was obtained from Sigma Chemical Co. (St. Louis, MO) and commercial preparation of β -galactosidase (β -gal; pI=5.0; consisting primarily of dimer with MW ~ 220-240 kDa) from *Kluyveromyces lactis*, Maxilact LX5000, a gift of DSM Food Specialties (Delft, The Netherlands), was used without further purification. Other chemicals used were of analytical grade and commercially available from Sigma, Aldrich, Merck, Oxoid and Fluka. Water used to prepare all aqueous phases was distilled and passed through a Barnstead ultrapure ion exchange column.

2.1.2 Organism

Kluyveromyces lactis ATCC 8585 was purchased from Industrial Yeast Collection of Dipartimento di Biologia Vegetale di Perugia (BDVPG), Italy.

2.2 Methods

2.2.1 Growth Condition of the Yeast

Kluyveromyces lactis was cultivated in shake flasks. Culture was aerobically grown in YPL (1% yeast extract, 2% peptone, 2% lactose) at 29°C and 200 rpm (Figure C.1).

2.2.2 Dry Weight Determination

Dry weights were determined by using a standard curve. For dry weight measurements preweighted nitrocellulose filters (pore size: 0.45 µm) were used. The medium were diluted to various OD600 values ranging from 0.1 to 0.6. After filtration of 15 ml each of these media, the filters were washed with demineralized water and dried in a microwave oven for 20 min. These samples were weighed again and the difference between the reweighed filter and these samples gave the weight (dry cell weight) of the yeast samples at different OD600 values. The OD versus dry weight standard graph was prepared according to the findings (Figure D.1).

2.2.3 Preparation of Crude Extract with Glass Beads

Cell were harvested at 600 nm of 15 (about 24 mg wet wt/ml) by centrifugation at 3000 g for 10 min at 4°C and washed once with deionized water. The cells (1 g wet weight) were resuspended in ice-cold 0.01M Na-phosphate buffer (pH 7.5) containing 2 mM PMSF, 1 mM EDTA, and 1.4 mM β -mercaptoethanol to a volume of 4 ml (250 mg wet wt/ml). Glass beads (0.5 mm) were added to 2 ml microvials of the Mini-beadbeater to a mass of 1.6 g (makes the half of the volume) and were chilled on ice. Cell disruption was carried out for 3 min (six cycles of 30 s bursts with 1 min cooling intervals on ice in between). Cell-debris is removed by

centrifugation at 10000 g (in unit of standard gravitational acceleration) for 10 min and the cell-free/protein rich extract recovered in the supernatant is used for the reversed micellar extraction experiments.

2.2.4 Analytical Methods

2.2.4.1 Determination of Protein Concentration

The concentration of pure β -galactosidase or β -lactoglobulin in the aqueous and organic phase was determined by ultraviolet (UV) absorption at 280 nm on a UV-visible spectrophotometer (Shimadzu UV 160U, Kyoto, Japan). Measured absorbance was corrected by subtraction of UV absorption at 310 nm (Rahaman and Hatton, 1991; Kelley et al., 1994). Standard curves were prepared for aqueous and organic phases. The concentration of total soluble proteins after separation of β -galactosidase from the test protein and total soluble protein in the crude extract obtained from the yeast cells of *Kluyveromyces lactis* was determined by the Bradford method using bovine serum albumin as the standard protein (Bradford 1976).

2.2.4.2 Enzyme Assays

Assays of β -galactosidase activity serve not only to indicate the protein's effectiveness as an enzyme after extraction, but can also be used a specific method for the quantification of the enzymes presence. Prior to beginning of the assay, the ONPG solution (4 mg/ml ONPG in 0.1 M sodium phosphate buffer (z-buffer) at pH 7.0 containing KCl (0.075 g/L) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0246 g/L)) is prepared at room temperature for at least one hour. The appropriately diluted enzyme solution is stored at 4 °C until assay time. A 0.1 ml appropriately diluted enzyme solution is added into 0.9 ml of z-buffer with β -mercaptoethanol (2.7 ml/L) solution. This solution is

preincubated at 30°C for at least 5 minutes. Reaction is started by addition of 0.2 ml ONPG solution into 1 ml enzyme z-buffer β -mercaptoethanol solution. Final volume of reaction solution adds up to 1.2 ml after the addition of ONPG solution into the reaction solution to start reaction. The reaction is allowed to proceed for 15 minutes at 30°C. Reaction was stopped by addition of 0.5 ml 1 M sodium carbonate into reaction medium. After the addition of sodium carbonate, total volume of the reaction medium becomes 1.7 ml. The absorbance is measured at 420 nm against appropriate enzyme blank. Enzyme blank is prepared in the same way with enzyme solution except it lacked 0.1 ml enzyme solution; instead 0.1 ml of distilled water is added. Units of the enzyme activity are expressed as the amount of enzyme required to release 1 nmoles of ONP per minute under the assay conditions. The molar extinction coefficient of *o*-nitrophenol under these conditions is $4.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ($4.5 \times 10^{-3} \text{ ml nmol}^{-1} \text{ cm}^{-1}$) (Miller, 1972; Guarente, 1983).

The absorbance change (ΔA_{420}) is calculated as follows:

$$\Delta (A_{420}) = A_{420} (\text{Enzyme reaction}) - A_{420} (\text{Blank}) \quad (2.1)$$

$$\begin{aligned} \text{Enzyme Activity} &= \frac{\Delta A_{420} \times V_f \times D}{t \times \epsilon \times V_s} \times \ell \\ &= \text{units / ml} \end{aligned} \quad (2.2)$$

One units is the amount of enzyme that hydrolyze 1 nmole/min of ONPG at 30°C.

V_f = final volume, ml

V_s = sample volume, ml

D = dilution factor

ℓ = light path, cm

ϵ = molar extinction coefficient of *o*-nitrophenol, $\text{ml nmol}^{-1} \text{ cm}^{-1}$

t = time of the reaction, min

Specific activity is calculated as follows:

$$\begin{aligned} \text{Specific Enzyme Activity} &= \frac{\text{Enzyme Activity (units/ml)}}{\text{Total protein (mg/ml)}} & (2.3) \\ &= \text{units / mg} \end{aligned}$$

2.2.4.3 Protein Extraction Procedure

In our one-step separation process, 1.725 ml of crude extract (total protein concentration 0.230 mg/ml) was contacted with 12 ml organic solution (0.2M AOT in isooctane, aqueous phase to organic phase volume ratio 1:7). The amount of water is always in excess of the water solubility of a one-phase microemulsion formed at the salt concentrations used in this study. Injected aqueous phase and organic phase were contacted by magnetic agitation for 25 min, which was experimentally determined to be sufficient to reach equilibrium. Samples were centrifuged at 2000 g for 10 minutes at 25°C for phase separation. Phases were then carefully isolated and analyzed. The pH and ionic strength of the initial aqueous feed was adjusted with potassium phosphate buffer (50 mM) and addition of potassium chloride (0, 50, 100 mM). The idea is that the droplets take up most of the water, all or most of the small molecules and proteins (relative to β -galactosidase) but expel the big molecules such as β -galactosidase into the excess water pool because of its large size.

2.2.4.4 Measurement of Water Content

Water content was determined by injected a volume of 0.2 – 0.4 ml of microemulsion phase into a Mettler DL 18 Karl Fisher titrator (Mettler-Toledo, Inc., Hightstown, NJ) using a gastight syringe. The titrator automates the Karl Fisher reaction, in which water is consumed stoichiometrically by an iodine-based titration in a methanol based solvent. A two-pin platinum electrode continuously monitors the solution for the presence of iodine. When an endpoint is reached the quantity of

water injected is reported by the instrument (Scholz, 1984). A mass balance can be used to calculate the water content of the microemulsion:

$$\text{Volume of water in microemulsion (ml)} = \frac{\frac{V_w}{V_s} \times V_t}{1 - \frac{V_w}{V_s}} \quad (2.4)$$

In this equation, V_w is the volume of water measured by Karl Fisher reaction, V_s is the total volume of one injection of the microemulsion, and V_t is the volume of organic phase initially used in the equilibration experiment.

A useful (and often reported) parameter that is used to characterize the water content of w/o microemulsion is w_o , which is the ratio of the moles of water to surfactant in the organic phase:

$$w_o = \frac{[H_2O]}{[AOT]} \quad (2.5)$$

The parameter w_o can also be related to the micellar radius through geometric arguments.

2.2.5 Monitoring the Proteins

Enzyme purity was monitored by SDS-PAGE (Sodium dodecyl sulfate - polyacrylamide gel electrophoresis) (Laemmli, 1970). The apparatus used was Mini Protean II system (BIO-RAD Laboratories, Richmond, CA). SDS PAGE was performed using 5% stacking and 15% separating gel. The gels were stained with Coomassie brilliant blue R after the electrophoresis.

2.2.6 Ammonium Sulfate Precipitation and Dialysis

Crude extract obtained from *Kluyveromyces lactis* was partially purified by gradual precipitation with the addition of solid ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$ at 0-35%, 35-50%, 50-70% and 70-100% of saturation followed by centrifugation at 12000 g for 20 min. The precipitates were dissolved in the minimum amount of 0.01M sodium phosphate buffer solution (pH 7.5) containing 2 mM PMSF, 1 mM EDTA, and 1.4 mM β -mercaptoethanol and the solution was dialyzed using a dialysis bag with a 10 kDa molecular weight cut-off against 2 x 2 liters of the same buffer for 24 hours to remove salt from the sample. The dialysates were centrifuged at 10000 g for 10 minutes to remove denatured and undissolved proteins, the precipitates were discarded. The β -galactosidase activity and total protein concentration of the supernatant obtained from each fraction were measured. All purification steps were performed at 4 °C unless noted otherwise.

2.2.7 Gel Filtration Chromatography

Gel filtration chromatography was performed on ÄKTAprime plus system using a Superdex 200 preparation grade (produced by covalent bonding of dextran to highly cross-linked agarose) HiLoad 16/60 (120 ml) prepacked column (General Electric Health care, Amersham Pharmacia AB, Björkgatan, Uppsala, Sweden). Two milliliters sample was applied to the column and eluted with 10 mM potassium phosphate buffer, pH 7.0, 150 mM NaCl at 0.5 ml/min. Eluents were collected in 0.5 ml aliquots for β -galactosidase activity determination.

2.2.8 Determination of the Molecular Weight and Size of the Proteins by Gel Filtration Chromatography

Gel filtration chromatography was used for the determination of the molecular weight and size of unknown proteins in the crude extract. The high molecular weight calibration kit (GE Healthcare Bio-Sciences AB, Björkgatan, Uppsala, Sweden) (Table E.1) was used for the calibration of gel filtration columns. The molecular weight (MW) of the native unknown protein was determined from the calibration curve (plot of K_{av} vs. $\log MW$) (Figure F.1). The molecular size of the native unknown protein was also determined from a plot of the $\sqrt{-\log K_{av}}$ versus Stoke's radius (R_{St}) (Figure F.2). For accurate determination of partition coefficient (K_{av}) the calibration standards were run under the same conditions as the sample (Figure F.3).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Phase Transfer Method

In this study, anionic surfactant AOT in isooctane reversed micellar system was used for the extraction and primary purification of β -galactosidase from *Kluyveromyces lactis*. Our aim was to evaluate microemulsion (reversed micellar extraction) for the purification of β -galactosidase. For this purposes solubilization experiment was done with substantially pure commercial *Kluyveromyces lactis* preparation of β -galactosidase (Maxilact LX 5000) by the traditional reversed micellar extraction methods (i.e. phase transfer and injection method). The process parameters pH and protein concentration in the initial aqueous phase are varied to determine solubilization characteristics of β -galactosidase by phase transfer method.

The aqueous β -galactosidase solution was mixed with an equal volume of the organic phase containing the anionic surfactant AOT in isooctane. Each phase had an initial volume of 4.0 ml. The solution were mixed in a glass vial and stirred at 500 rpm for 25 min. The dispersion was then centrifuged for 10 min at 2000 g.

Desired pH and salt concentration were adjusted by using a 0.01 M sodium acetate buffer over a pH range of 5.1-5.7, by using a 0.01 M sodium phosphate buffer over a pH range of 5.8-7.9 and by adding sufficient NaCl to obtain the desired sodium concentration. Equilibration experiments were carried out at an initial aqueous protein concentration of 1 mg/ml. Organic phase consisted of 0.2 M AOT in isooctane. The pH of the aqueous phase was varied within the range 5.1-7.9. The ionic strength of the aqueous phase was held constant through the addition of NaCl to the 0.01 M buffer solution, to obtain a net sodium concentration of 0.1 M.

Figure 3.1 displayed the results of protein distribution between organic and aqueous phase and protein lost as a function of pH in the initial aqueous phase. β -Galactosidase concentration in the organic phase increased with the increase in pH at first and then decrease with pH after reaching their maximum at pH 6.5. The change in β -galactosidase solubilization in the organic phase with pH may be mainly ascribed to the electrostatic repulsion between the reversed micelles and protein. In this research, negatively charged reversed micelles were formed by using the anionic surfactant AOT. and the isoelectric point of. When the initial aqueous pH is higher than the isoelectric point of the β -galactosidase (5.0), leading to a negatively charged protein, β -galactosidase was expelled from the reversed micelles.

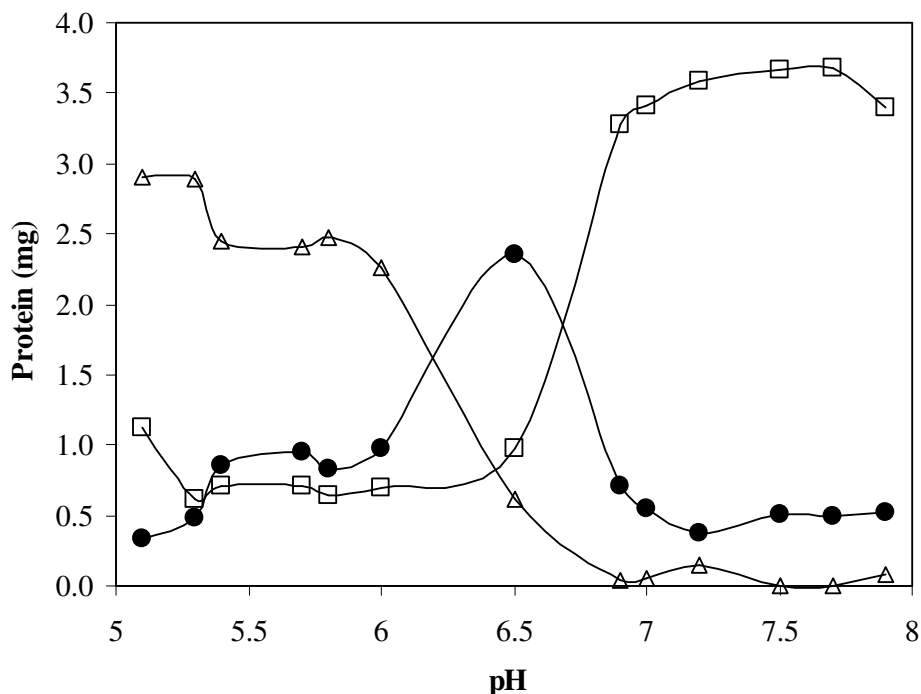


Figure 3.1 Effect of pH of initial aqueous phase during forward extraction on distribution of β -galactosidase between organic and aqueous phase.

Total protein in organic phase (●), aqueous phase (□) and protein lost (Δ).

Concentration of β -galactosidase in the aqueous phase increased with the increase in pH. However, protein lost decreased with increase in pH. The reason for higher protein loss at lower pH (pH < 7) was due to the precipitation of the enzyme.

Figure 3.2 showed that there was no effect of pH on the water content (w_o) of microemulsion phase. However, addition of protein into microemulsion system caused the small decrease in the w_o value at all pH. The results above indicated that main driving force of the β -galactosidase solubilization is electrostatic interaction at constant ionic strength.

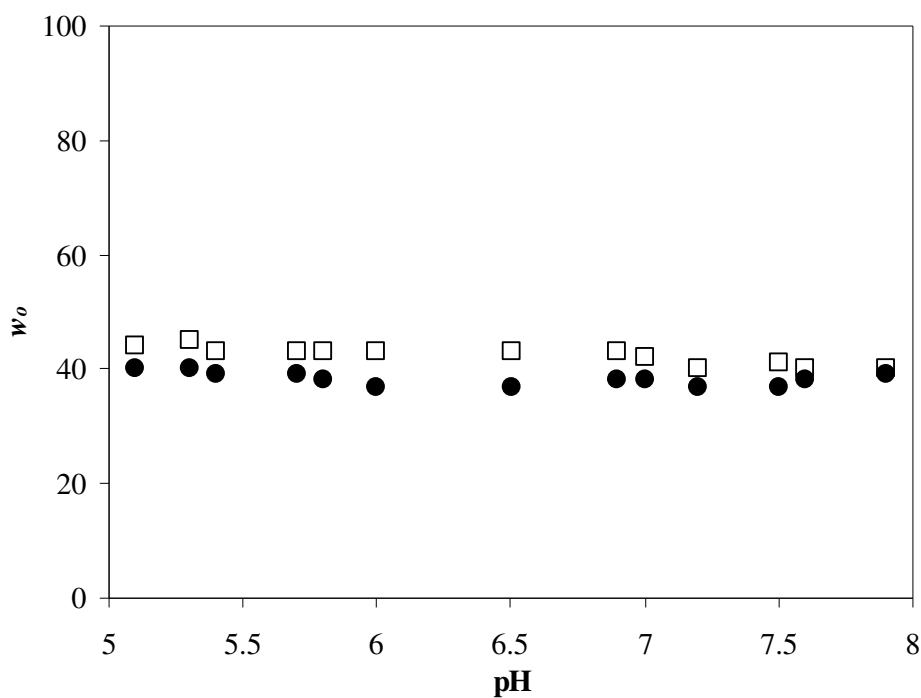


Figure 3.2 Effect of pH of initial aqueous phase during forward extraction on w_o of w/o microemulsion with (●) and without (□) protein.

3.1.1 Effect of Aqueous Phase Protein Concentration on Partitioning of β -galactosidase between Aqueous and Organic Phase

Figure 3.3 displayed the results of protein distribution between organic and aqueous phase and protein lost as a function of protein concentration in the initial aqueous phase. Concentration of β -galactosidase in the aqueous phase increased with the increase in protein concentration. However, protein lost decreased with increase in protein concentration in the initial aqueous phase. Solubilization of β -galactosidase in microemulsion phase increased with decreasing initial protein concentration.

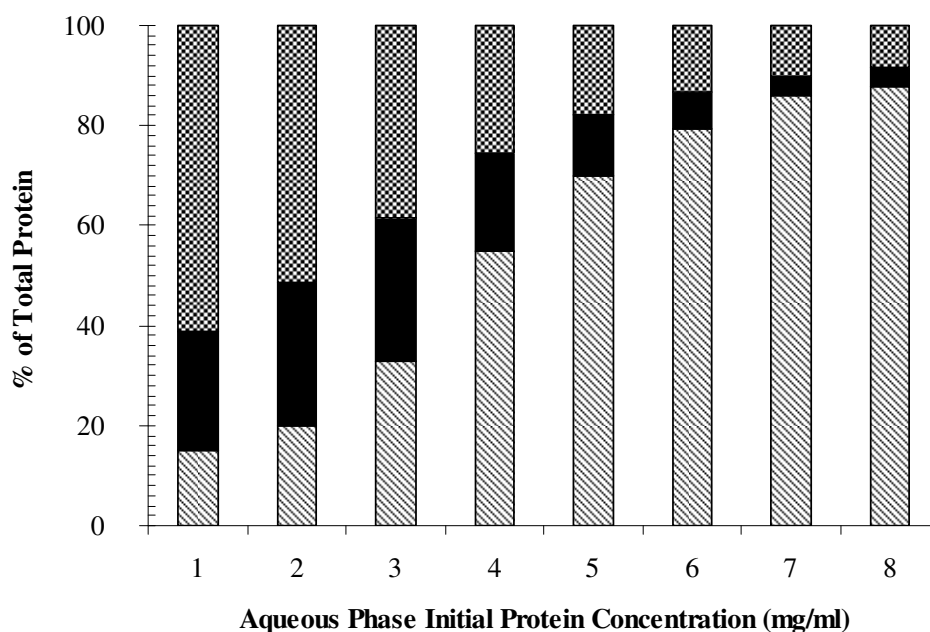


Figure 3.3 Effect of protein concentration of initial aqueous phase during forward extraction on distribution of β -galactosidase between organic (▨) and aqueous phase (▩), and protein precipitation at the interface (■).

Figure 3.4 showed the effect of initial protein concentration on w_o value of w/o microemulsion. Increasing protein concentration let to a decrease in w_o was not expected. Therefore, salt concentration in the sample was measured by using atomic emission spectroscopy. We found that Maxilact LX500 has 250 mM K ions and thus increasing sample protein concentration increased the amount of salt in the sample.

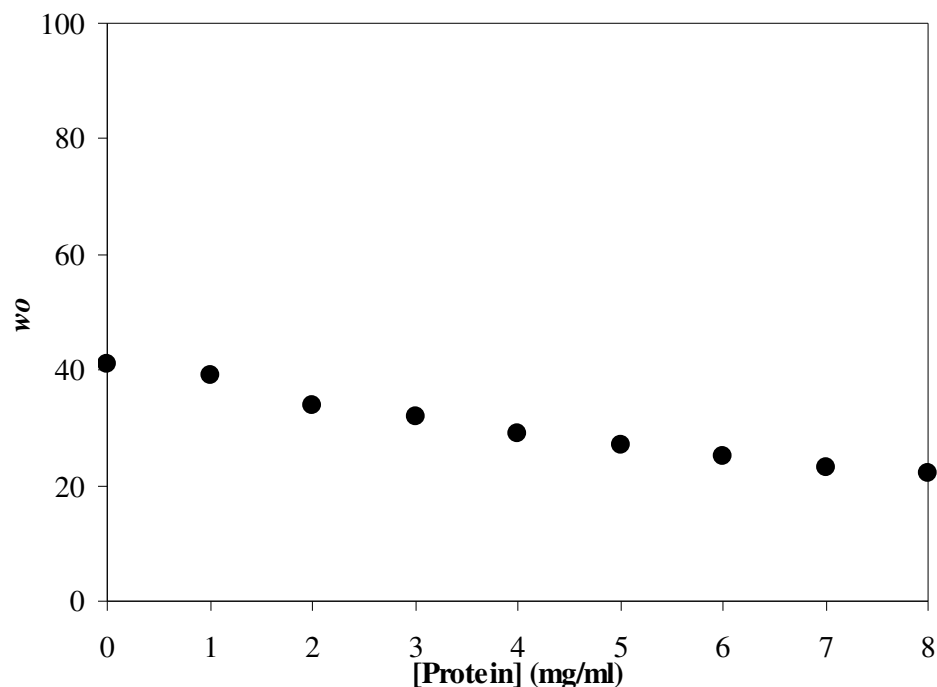


Figure 3.4 Effect of protein concentration of injected aqueous phase during forward extraction on w_o of w/o microemulsion.

Therefore, we decided to dialyze our protein sample before reversed micelle experiments. Figure 3.4 and Figure 3.5 showed that decrease in w_o was not function of protein concentration.

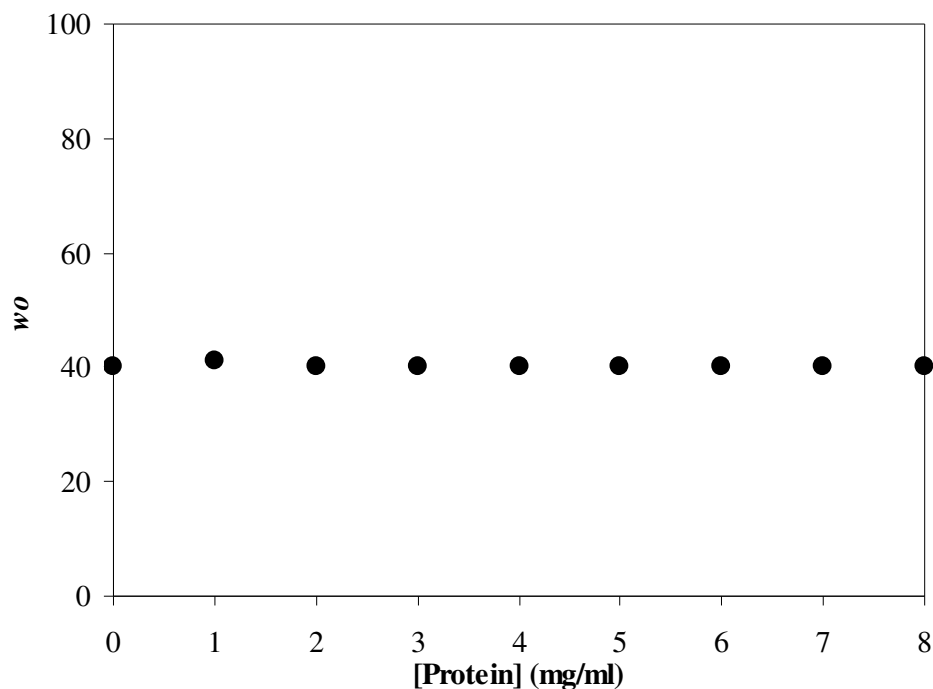


Figure 3.5 Effect of dialyzed protein concentration of injected aqueous phase during forward extraction on w_o of w/o microemulsion.

3.1.2 Effect of w_o on Extinction Coefficient of β -galactosidase

Effect of w_o on extinction coefficient of proteins was reported in the literature. For example, Matzke et al. (1992) reported that the extinction coefficient of chymotrypsin remained constant as a function of w_o . On the other hand, the extinction coefficient of alcohol dehydrogenase varied appreciably with w_o . According to the Figure 3.6 extinction coefficient of β -galactosidase remained constant as a function w_o .

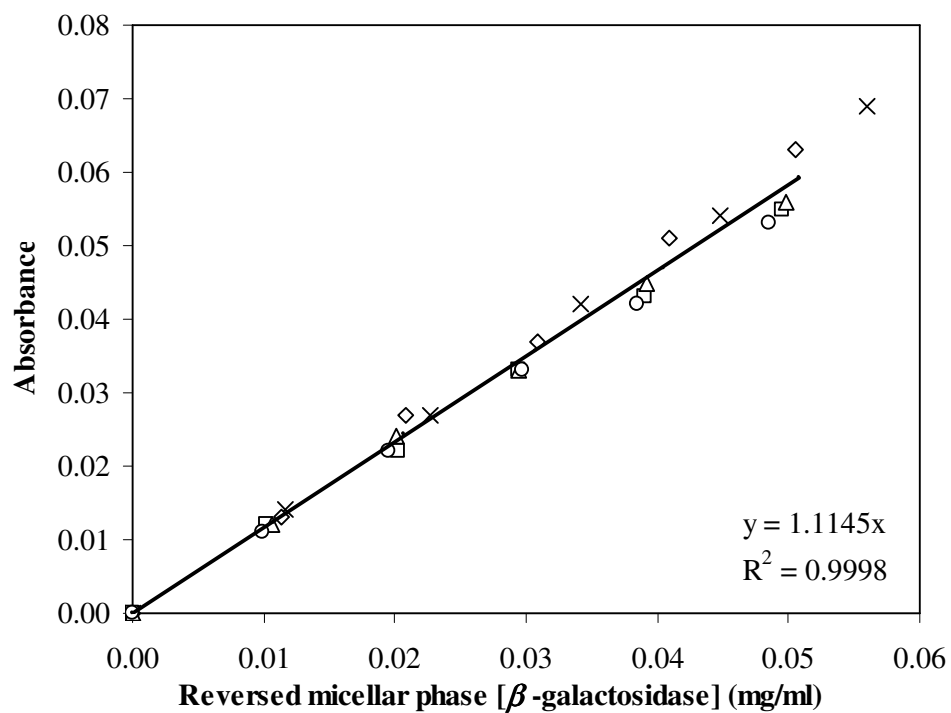


Figure 3.6 Effect of w_o on extinction coefficient of β -galactosidase.

w_o : 15 (○), 17 (△), 23 (□), 35 (◇), 40 (×).

3.2 Injection Method

Previous studies in the literature and the phase transfer experiments of this study showed that high molecular weight proteins (>120 kDa) are difficult to solubilize by phase transfer method. However, it is also known that it is possible to entrap large molecular weight proteins inside the water-in-oil microemulsion droplets by injection method. In this method, small volume of protein containing aqueous phase was directly injected into organic phase and that forms the one-phase microemulsion system. In this part of the study, solubilization and extraction of β -galactosidase was investigated by using injection method with a focus on the effect of initial aqueous phase pH, contact time before phase separation and ionic strength of the back extraction.

β -galactosidase was solubilized by the injection method at $w_o = 40$. In this method, 0.575 ml β -galactosidase aqueous solution (~3 mg/ml protein) injected into 4 ml AOT/isooctane solution and mixed solution is stirred at 500 rpm for 25 min. Back extraction of β -galactosidase from reversed micelles was carried out by contacting 4.575 ml of the reversed micellar solution, prepared by injection method, with 4.575 ml of aqueous solution (0.1 M KCl). The solution was mixed in a glass vial and stirred at 500 rpm for 25 min. The dispersion was then centrifuged for 10 min at 2000 g.

3.2.1 Effect of Aqueous Phase pH on Partitioning of β -galactosidase between Aqueous and Organic Phase

Effect of injected aqueous phase pH on forward and backward extraction of β -galactosidase was shown in Figure 3.7. Same amount of protein (~1.75 mg) was injected into the organic phase for all samples. Figure 3.7 indicates that solubilization of β -galactosidase in the organic phase decreased with an increase in pH. Amount of total protein in the extraction system (both aqueous and organic phase) increased following the back-extraction step at pH 6.5 and 7.5.

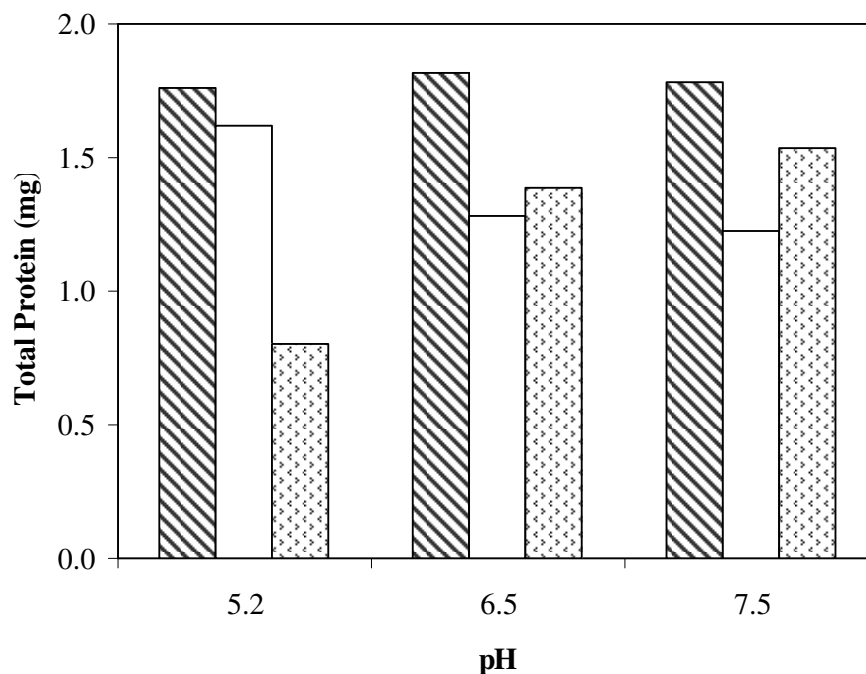


Figure 3.7 Effect of pH of injected phase on forward and backward extraction of β -galactosidase.

10 mM Na phosphate buffer was used to adjust pH. Injected protein (▨), solubilized protein in organic phase (□), total protein in the system after back-extraction (⊞).

Figure 3.8 displays the results of protein distribution between organic and aqueous phase and protein lost after back-extraction as a function of pH in the initial aqueous phase. Concentration of β -galactosidase in the aqueous phase increased with the increase in pH. However, protein lost decreased with the increase in pH. β -Galactosidase concentration in the organic phase higher at pH 6.5. The change in β -galactosidase extraction with pH mainly ascribed to the electrostatic repulsion between the reversed micelles and protein. In this research, negatively charged reversed micelles were formed by using the anionic surfactant AOT. When the initial aqueous pH was higher than the isoelectric point of the protein (isoelectric point of β -galactosidase is 5.0), leading to a negatively charged protein, the expulsion of protein from the microemulsion droplet was occurred.

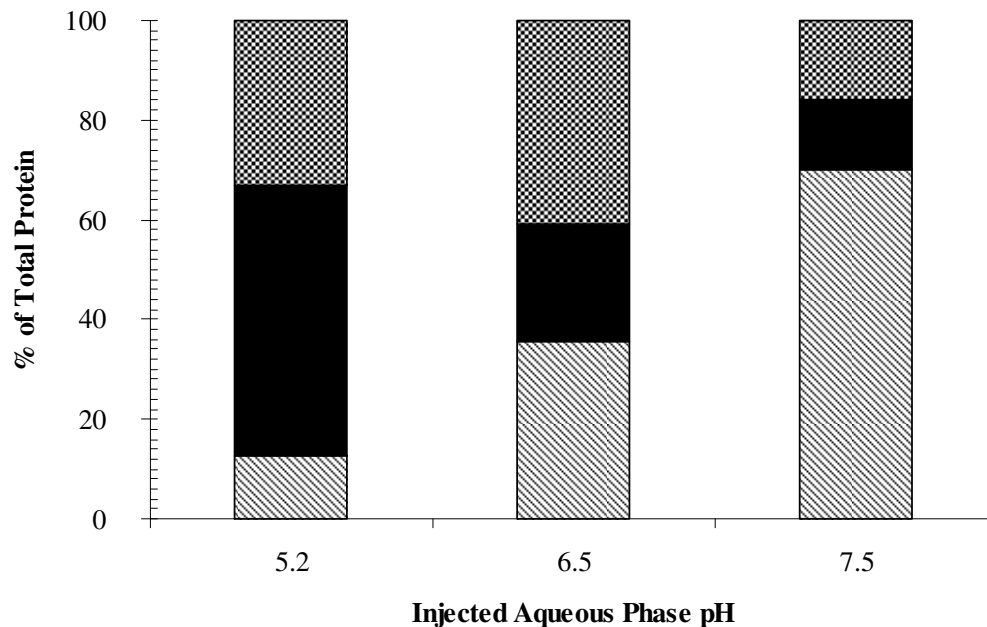


Figure 3.8 Effect of pH of injected phase during backward extraction on distribution of β -galactosidase between organic (▨) and aqueous phase (▩), and protein precipitation at the interface (■).

3.2.2 Effect of Contact Time before Phase Separation on Partitioning of β -galactosidase between Aqueous and Organic Phase

Figure 3.9 showed the effect of contact time during forward extraction on forward and backward extraction of β -galactosidase at pH 6.5. Same amount of protein in aqueous phase (3 mg/ml) was injected into the organic phase at all contact time durations. Figure 3.9 displayed that after forward extraction the total amount of protein solubilized in the organic phase was found to be lower than injected amount of protein at all contact times, but the total amount of protein in the extraction system increased following the backward-extraction.

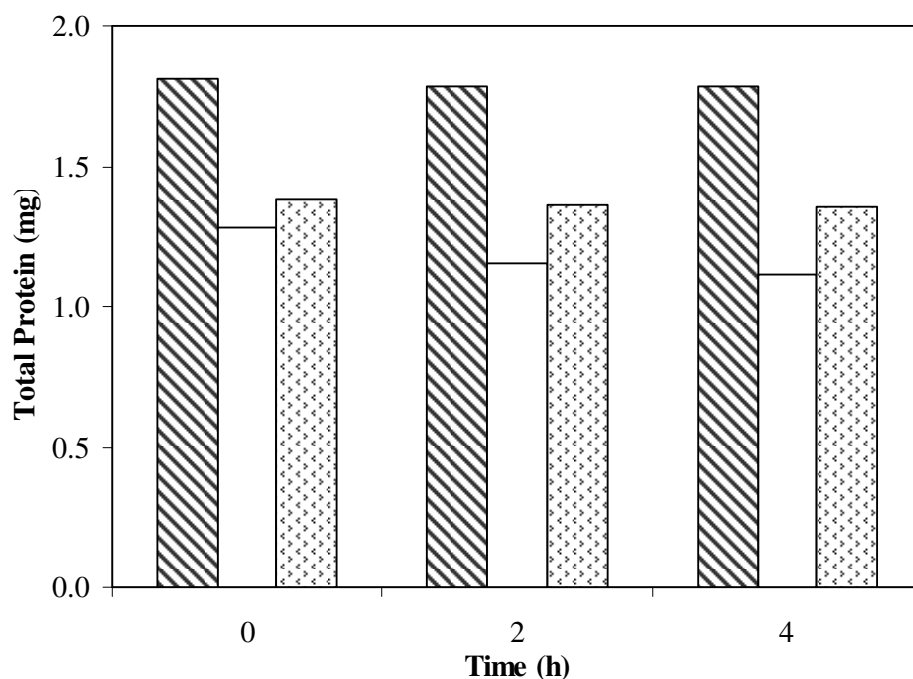


Figure 3.9 Effect of contact time during forward extraction on forward and backward extraction of β -galactosidase.

10 mM Na phosphate buffer at pH 6.5 was used to adjust pH. Injected protein (▨), solubilized protein in organic phase (□), total protein in the system after back-extraction (▩).

The differences in the measured amount of total protein before and after backward-extraction may be attributed to the presence of a little amount of invisible aqueous phase with excess active protein located at the bottom of extraction cell.

As can be seen from Figure 3.10, same amount of protein lost (~0.430 mg, 24%) was observed at all contact times after backward-extraction. The solubilization of β -galactosidase in organic phase changed very little with increase in contact time of the forward extraction between 2 h and 4 h, which demonstrate that extraction equilibrium has been achieved (Figure 3.10).

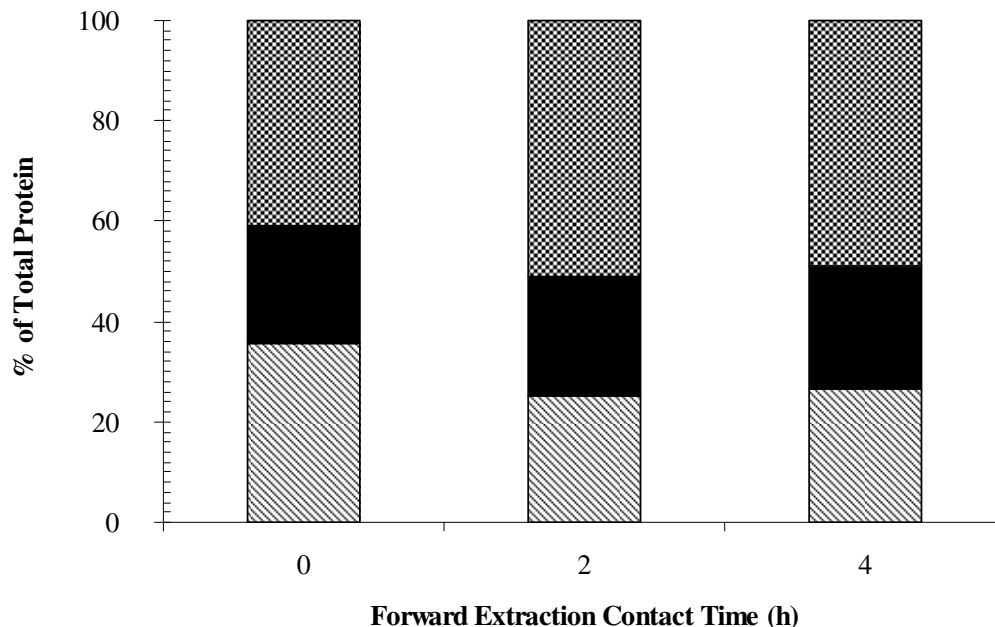


Figure 3.10 Effect of contact time (0-4 h) during forward extraction on distribution of β -galactosidase between organic (▨) and aqueous phase (▩), and protein precipitation at the interface (■) at pH 6.5.

Figure 3.11 displayed the effect of forward extraction contact time on the specific activity of β -galactosidase. Decrease in the specific activity occurred about the level of 40 % in all cases (Figure 3.11). Precipitation of protein was observed at the interface in all cases. Increasing in the contact time lowered the specific activities which were measured before and after RM extraction at pH of 6.5. Observed decrease in the specific activities was attributed to pH value instead of contact time. Measured specific activities before and after reversed micelle extraction remained at the levels of 80 kEU/mg to 50 kEU/mg, respectively, in the contact time of the forward extraction between 2 h and 4 h (Figure 3.11).

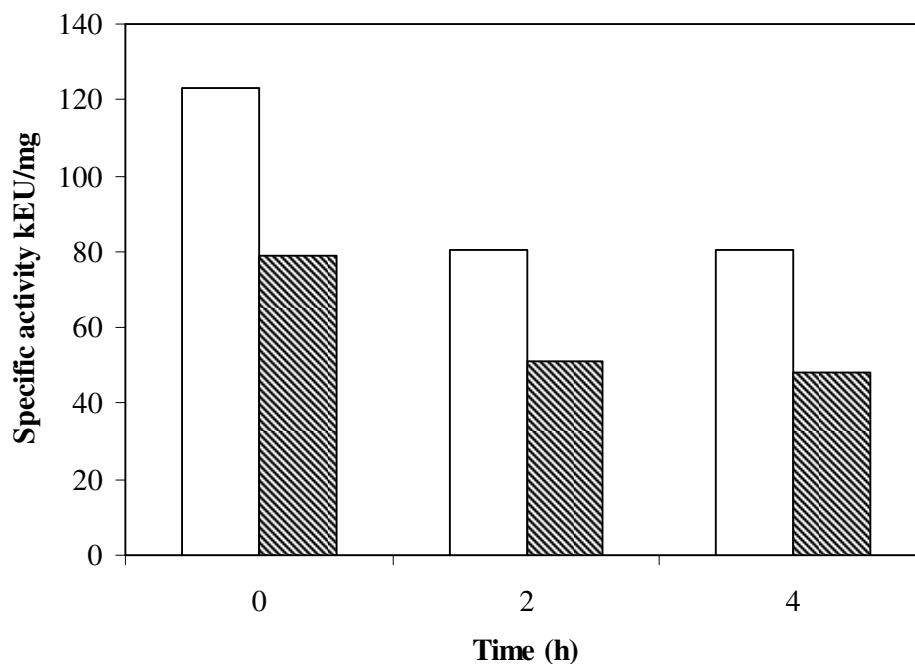


Figure 3.11 Effect of contact time during forward extraction on activity of β -galactosidase at pH 6.5.

Specific activity of β -galactosidase before ($\square$) and after ($\boxtimes$) reversed micelle extraction.

Trend of the change of total protein in the phases at pH of 7.5 was found to be similar to observed trend at pH of 6.5, but the differences in the amount of total protein before and after backward-extraction was higher than those values calculated at pH of 6.5 at all contact times (Figure 3.12). This can be associated with the different magnitudes of the electrostatic interaction (repulsion) between protein and microemulsion droplets at different pH values.

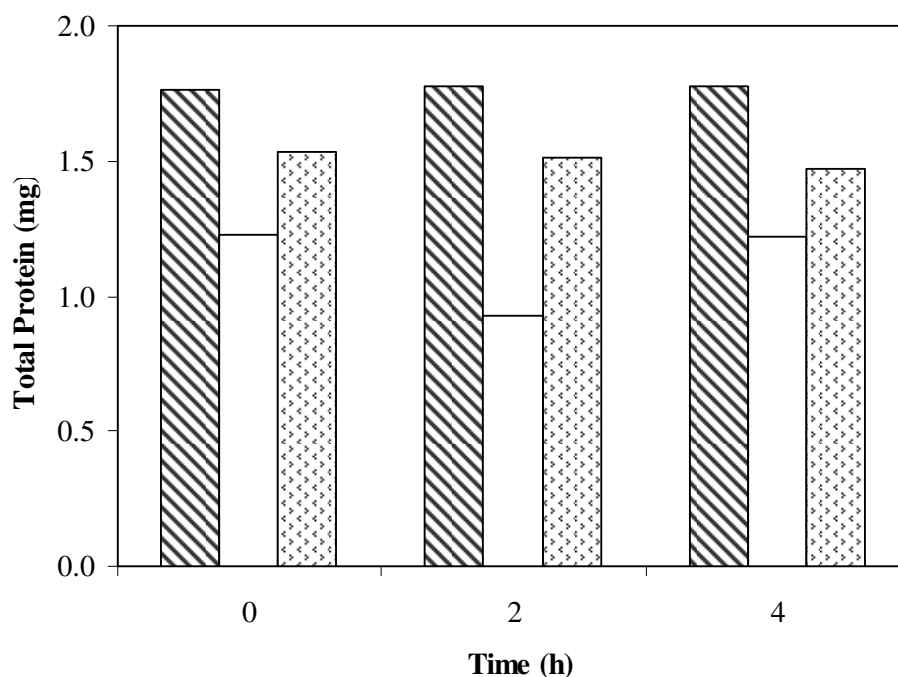


Figure 3.12 Effect of contact time during forward extraction on forward and backward extraction of β -galactosidase.

10 mM Na phosphate buffer at pH 7.5 was used to adjust pH. Injected protein (▨), solubilized protein in organic phase (□), total protein in the system after back-extraction (▩).

Protein lost after backward extraction slightly increased with increasing contact time from 0 to 4 h (Figure 3.13). The solubilization of β -galactosidase was also increased when contact time varied from 0 to 4 h. Solubilization of β -galactosidase in organic phase at pH of 7.5 was found to be lower than that value determined at pH of 6.5 since the magnitude of repulsive force between surfactant head group and oppositely charged protein increases with increase in pH value.

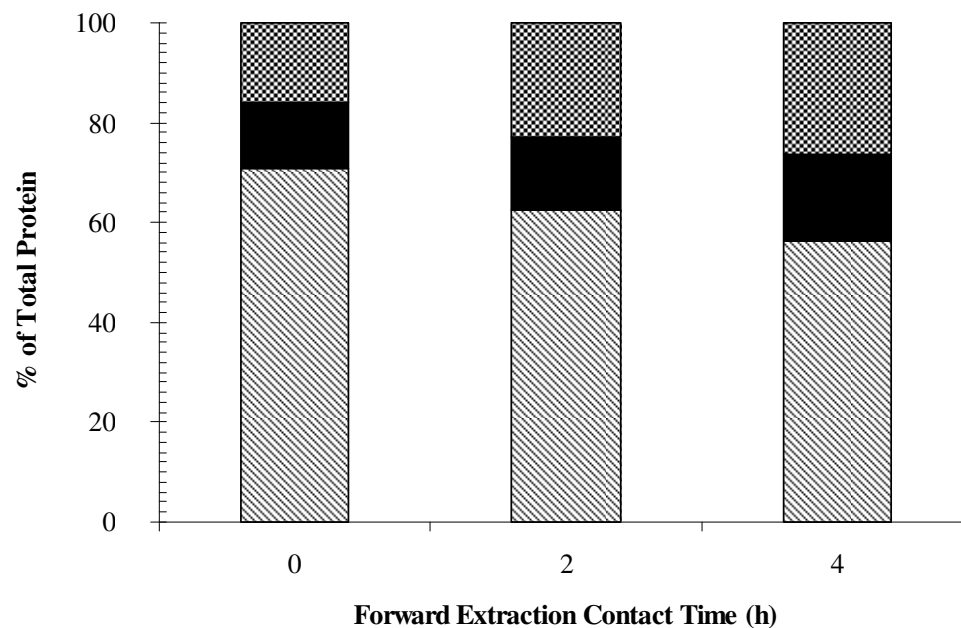


Figure 3.13 Effect of contact time (0-4) during forward extraction on distribution of β -galactosidase between organic (▨) and aqueous phase (▩), and protein precipitation at the interface (■) at pH 7.5.

In contrast to the results achieved at pH of 6.5, slight increases in the specific activities was measured after reversed micelle extraction as compared to the results obtained before reversed micelle extraction at all contact times at pH of 7.5 (Figure 3.14). Figure 3.14 indicated that the effect of contact time on the specific activities disappeared at this pH.

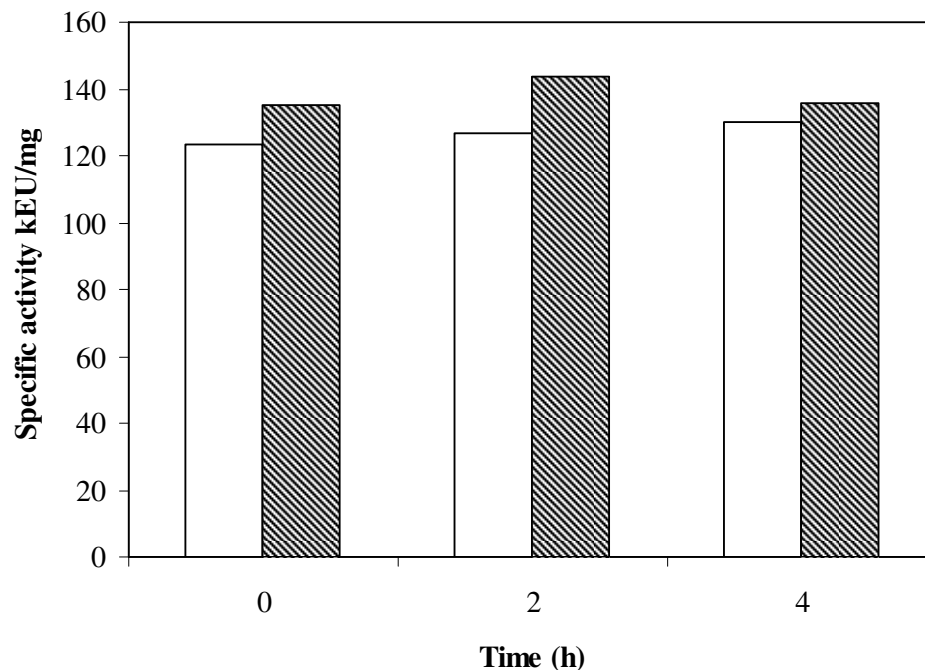


Figure 3.14 Effect of contact time during forward extraction on activity of β -galactosidase at pH 7.5.

Specific activity of lactase before ($\square$) and after ($\boxtimes$) reversed micelle extraction.

3.2.3 Effect of Ionic Strength of Back Extraction on β -galactosidase Activity Recovery

β -Galactosidase solubilized in reversed micelle by the injection method could be back extracted into an aqueous phase maintaining its enzyme activity. The effect of salt type and concentration in the aqueous phase used for back-extraction were shown in Figure 3.15, where β -galactosidase was first solubilized by the injection method at $w_o = 40$. Increasing salt concentration led to a decrease in w_o as expected. Back-extraction of β -galactosidase was influenced by NaCl concentration, and maximum recovery of the enzyme activity was obtained at 0.2 M. In the case of KCl being used in the fresh aqueous phase, back-extraction of β -galactosidase was very efficient, being nearly completed at 0.1 M, and not influenced by further increase in

KCl concentration. This suggests that selective back-extraction, i.e. effective separation of β -galactosidase from other proteins, can be achieved in the back-extraction step, since in this low-salt concentration range most proteins remain in the reversed micellar phase. It is considered that back-extraction of β -galactosidase from the microemulsion droplet into the aqueous phase is driven by electrostatic or steric repulsion, between the protein surface and the inner wall of microemulsion droplets because the protein surface is negatively charged at pH 7.0 (pI 5.0) and micelles shrink by adding salt.

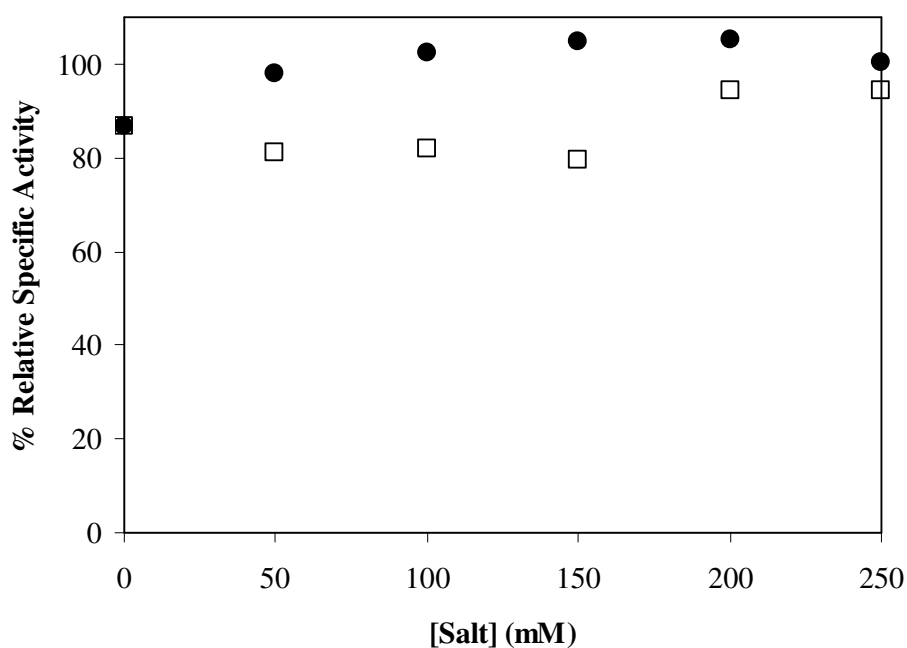


Figure 3.15 Effect of salt concentration of backward extraction on activity of β -galactosidase.

[KCl] (●), [NaCl] (□). 50 mM Na-phosphate buffers at pH 7.0.

3.3 One-step Separation Method

In this part of the study, our objective was to design a one-step method for selective separation of β -galactosidase from other proteins, using a microemulsion solution. In this strategy, a relatively small volume of aqueous protein solution would be mixed with a larger volume of organic solvent, containing surfactant. We proposed that, in such a mixture, surfactant would form water-in-oil droplets that would take up most of the water and all or most of the smaller proteins, but would expel the target β -galactosidase enzyme into an excess water pool because of its large size. β -Galactosidase was obtained as a commercial preparation, known as Maxilact LX-5000, from *Kluyveromyces lactis*. We used initial solubilization studies to determine promising conditions for this one-step separation of β -galactosidase using microemulsion droplets. Then, to test the ability of our one-step separation technique to selectively separate lactase from other proteins, we prepared binary mixtures containing lactase and β -lactoglobulin, the latter acting as a competing protein with isoelectric properties that are similar to our target enzyme. Measurements of lactase activity were used to determine the effectiveness of the separation.

3.3.1 Effects of Buffer Type and Concentration

The incorporation of β -galactosidase into microemulsion droplets was evaluated as a function of buffer type and concentration, pH, and protein concentration. Buffer solutions containing β -galactosidase were injected into the 0.2 M AOT/isooctane solution in an amount corresponding to a w_o value of 40 (1:7 aqueous to organic volume ratio). No salt were added to the aqueous solution in these studies. We found that 2 mg/ml protein dissolved in 10 mM buffer containing either potassium or sodium could be almost completely solubilized in the microemulsion. We compared the amount of protein at higher buffer concentrations to this 10 mM result (Figure 3.16).

As buffer concentration increased, the added water could no longer be completely solubilized within the microemulsion droplets, and water was expelled to a separate, excess aqueous phase. Protein was increasingly expelled to that new aqueous phase as the concentration of buffer increased. For Na-phosphate buffer concentrations exceeding 40 mM, less than 30% of the β -galactosidase was incorporated, with more than 70% released into a newly formed aqueous phase. When 40 mM K-phosphate buffers were used instead, only about 10% of the protein was solubilized. There was only a weak effect of buffer concentration above 40 mM.

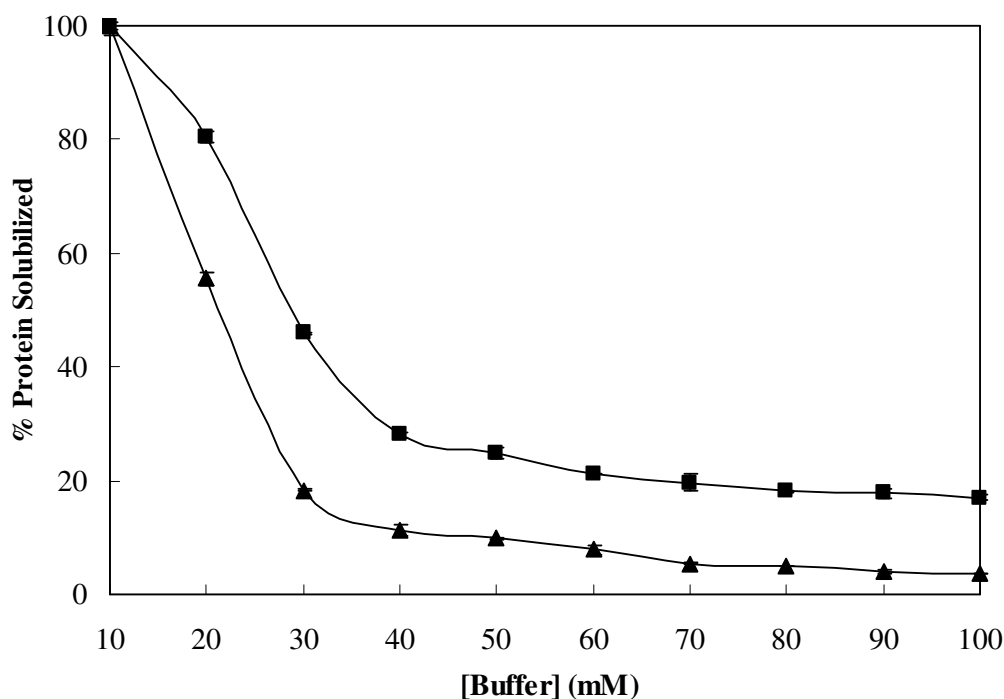


Figure 3.16 Effect of buffer concentration and type on the solubilization of β -galactosidase in the microemulsion evaluated relative to result at 10 mM buffer.

Na-Phosphate (■) and K-phosphate (▲) buffer. 200 mM AOT, injected phase protein concentration 2 mg/ml at pH 7.5.

The water content of AOT-water-in-oil microemulsion droplets is known to be a strong function of both cation type and concentration. Because of their hydrated size, volume exclusion and dielectric properties, the behavior of K^+ and Na^+ cations in this environment is quite different. Potassium ions can penetrate the surfactant layer more effectively than Na^+ ions (Leodidis and Hatton, 1989), causing smaller microemulsion droplets to form in the former case at the same cation concentration. The incorporation of the large enzyme decreases as the water content and size of the microemulsion droplet decreases, leading to the behavior exhibited in Figure 3.16.

3.3.2 Effects of pH

Effects of buffer pH on solubilization of 5 mg/ml β -galactosidase in 10 mM K-phosphate buffer were presented in Figure 3.17. pH was varied from 5.7 to 8.1. This pH range was chosen because *Kluyveromyces lactis* lactase has a native conformation at pH 7.0 and it has a neutral pH optimal range (Panesar et al., 2006). Furthermore, at pH values below 5.5 or above 8.5, protein aggregation was observed (Tello-Solis et al., 2005). These pH values are all above the isoelectric point of the enzyme, where the protein is predicted to have a net negative charge. Results in Figure 3.17 showed that higher solubilization was achieved at lower pH values, where the ratio of negative to positive charges on the protein was decreased.

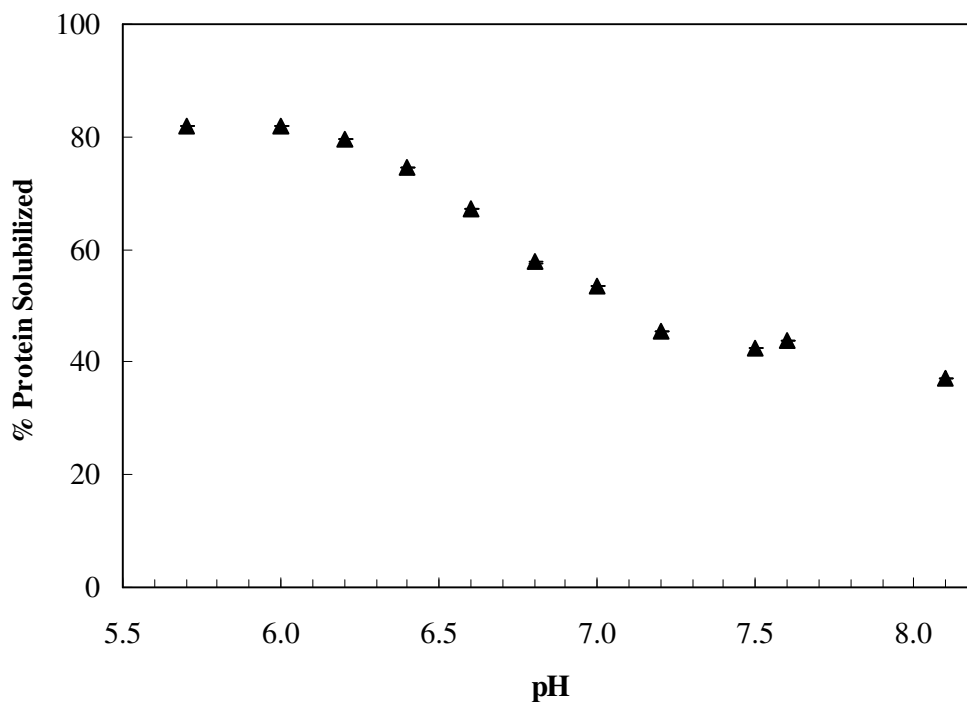


Figure 3.17 Effect of buffer pH on the solubilization of β -galactosidase in the microemulsion.

200 mM AOT, injected phase protein concentration 5 mg/ml, 10 mM K-phosphate buffer.

Charge interactions are expected to play an important role in the uptake or release of protein by microemulsion droplets when charged surfactants are used (Pires et al., 1996; Shimek et al., 2005). By forming the droplets using anionic surfactant AOT, there is repulsion between the droplets and negatively charged protein residues, which increases when the buffer pH increases and does not favors uptake of protein into the microemulsion droplets.

3.3.3 Effects of Protein Concentration

The effect of total (injected) protein concentration was also evaluated, with results shown in Figure 3.18. At higher pH and buffer concentrations, the concentration of the β -galactosidase solubilized in the microemulsion went through a maximum with increasing total protein, and the position of the maximum was shifted to higher concentrations at the lowest pH value, likely due to the more favorable environment for protein inside the droplet at this pH. The effects of pH appear significantly weaker at lower protein concentrations, at least over the narrow range of pH considered in Figure 3.18.

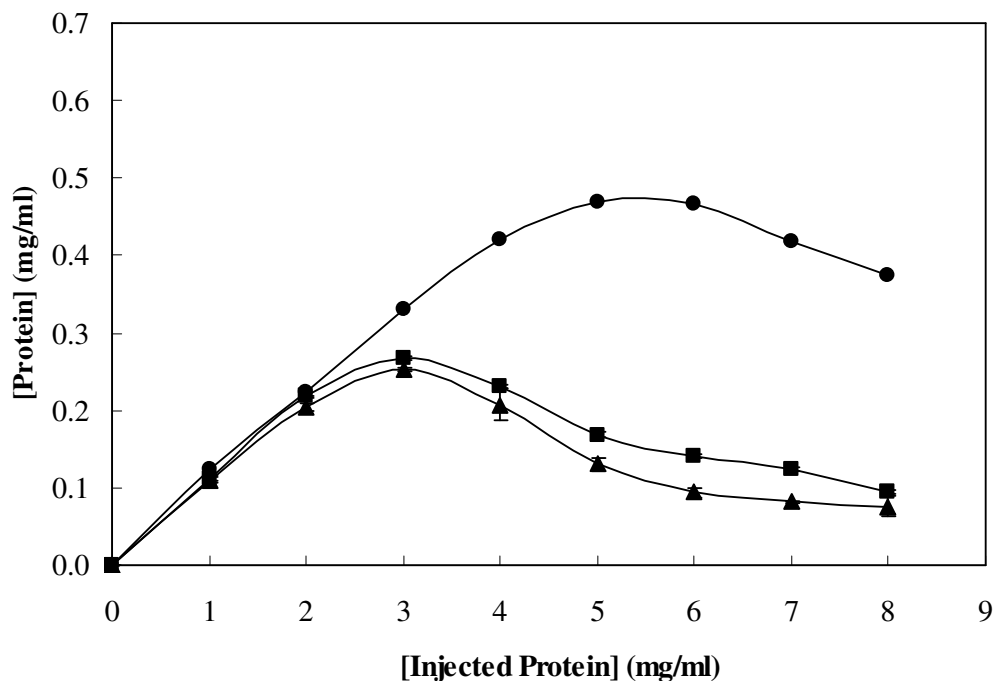


Figure 3.18 Effect of initial protein concentration and K-phosphate buffer pH on the solubilization of β -galactosidase in the microemulsion.

pH 6.6 (●), pH 7.4 (■) and pH 7.6 (▲).

3.3.4 One-Step Separation of β -galactosidase and β -lactoglobulin

The solubilization experiments above indicated that it is possible to incorporate significant quantities of β -galactosidase into w/o microemulsion droplets, but only at sufficiently low buffer concentrations. Increased buffer most likely reduces the size of the droplets, making it more difficult to accommodate this large MW protein. It is also necessary to reduce protein concentration and pH in order to achieve significant solubilization.

On the other hand, Figure 3.16 -Figure 3.18 indicate a range of conditions under which the enzyme is almost entirely excluded from the microemulsion droplets. By contacting the protein solution with organic surfactant solution at buffer concentrations greater than 40 mM, droplets that form should not retain the target enzyme. If other proteins within a mixture are solubilized under those conditions, this could result in a separation of β -galactosidase from other proteins.

This idea is the basis for a one-step separation process for β -galactosidase, which was tested by creating an aqueous feed containing an equal mixture of lactase and a test impurity protein, β -lactoglobulin (β -Lg). Total protein concentration of the injected aqueous solution was kept at 2 mg/ml, with a buffer concentration of 50 mM. Since β -galactosidase and β -lactoglobulin have very similar isoelectric points, their separation based on charge is difficult; both proteins will be more likely to be taken up by the microemulsion droplet as pH is lowered. A pH of 7.0 was chosen so that lactase activity could be maintained, whilst minimizing electrostatic repulsion between β -Lg and AOT. It is known that there is a significant β -galactosidase activity decrease at pH 6.5, with an almost complete loss of activity at pH 6.0 (Tello-Solis et al., 2005).

The difference in the molecular weights of these two proteins inspires a separation strategy that plays on their molecular dimensions. As a result, the size of the water-in-oil microemulsion droplets that form during the process was controlled by adding

salt to the injected aqueous solution. The process was designed such that the droplets should take up most of the water, all or most of the β -Lg, but expel the lactase into an excess water phase because of the enzyme's large size.

The specific activity of the feed and product aqueous solutions, before and after the one-step separation, is shown in Figure 3.19 and Figure 3.20. The specific activity is the lactase enzymatic activity divided by the mass of all protein in the system—both enzyme and non-enzyme. Figure 3.19a and Figure 3.20 a show that the specific activity was half as much in a feed containing a 50/50 mixture of lactase and β -Lg, compared to a feed with lactase only, since in the mixture the enzyme makes up only half of the mass. The specific activities of these feeds can then be compared to those in the aqueous products (Figure 3.19b and Figure 3.20b) that consist of the excess aqueous phase obtained after expulsion of water and protein from the microemulsion phase. In these product streams, the specific activities are now very similar, regardless of whether the feed contained pure lactase or a lactase/ β -Lg mixture. Such high specific activities obtained after treatment of the mixed feed indicate that the protein in the product consists of purified enzyme. β -Lg has been removed by the droplets from the mixed feed, and the remaining mass of protein that is expelled into the water corresponds closely to the active enzyme.

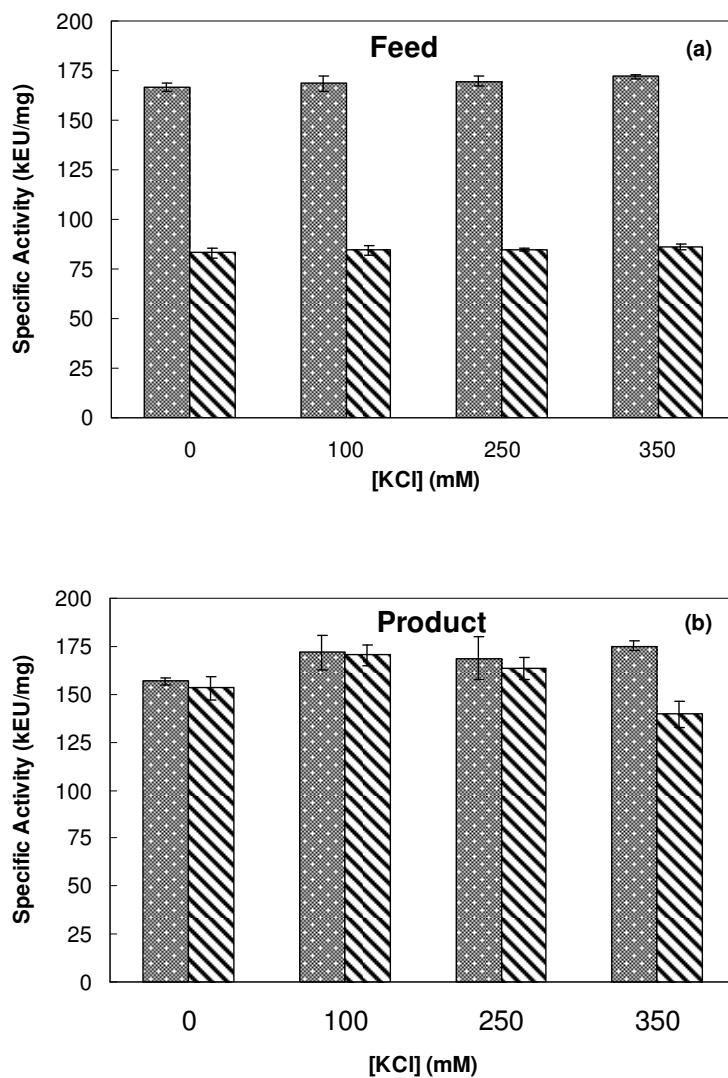


Figure 3.19 Effect of injected aqueous phase KCl concentration on selective one-step separation of β -galactosidase from pure aqueous solution of β -galactosidase (▨) or a 50:50 mixture of the β -galactosidase and β -lactoglobulin (▩).

Specific activity of β -galactosidase was measured in the feed (a) and the product (b) aqueous solution. Microemulsion contained 200 mM AOT. Aqueous phase was at pH 7.0 and contained 50 mM K-phosphate buffer.

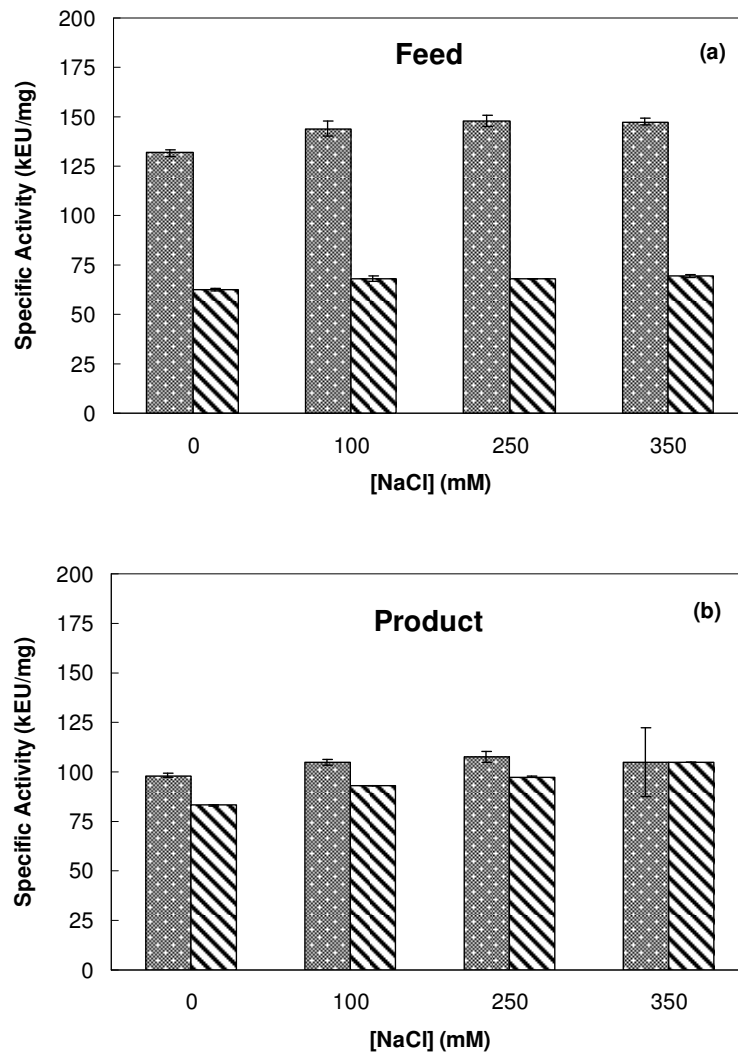


Figure 3.20 Effect of injected aqueous phase NaCl concentration on selective one-step separation of β -galactosidase from pure aqueous solution of β -galactosidase (■) or a 50:50 mixture of the β -galactosidase and β -lactoglobulin (▨).

Specific activity of β -galactosidase was measured in the feed (a) and the product (b) aqueous solution. Microemulsion contained 200 mM AOT. Aqueous phase was at pH 7.0 and contained 50 mM Na-phosphate buffer.

The results in Figure 3.19 indicated an influence of salt concentration on the specific activity of the product obtained from the lactase/ β -Lg mixture. In the presence of KCl, the specific activity of the lactase separated from the mixture matches the pure lactase control at the three lower salt concentrations, but exhibits a decrease at 350 mM KCl. The presence of salt serves to reduce electrostatic interactions within the microemulsion, thereby minimizing protein-surfactant charge interactions and also the size of the microemulsion droplet itself. The latter effect can reduce protein solubilization within the microemulsion as the droplet size becomes more restrictive. To explore this phenomenon further, the extent of solubilization of the two individual proteins, β -galactosidase and β -lactoglobulin, as single components was determined as a function of potassium chloride concentration in the feed. The molar ratio of water to surfactant (w_o), which is approximately proportional to the diameter of the droplets, was also measured in the resulting microemulsion. The percentage of the two individual proteins solubilized in the microemulsion was plotted in Figure 3.21 as a function of both KCl concentration (lower axis) and w_o (upper axis). It can be seen that the solubilization of the smaller protein, β -Lg, remains high and almost constant at lower salt concentrations, where there are correspondingly high water contents. But as w_o drops below 20, and the droplet diameter is similarly reduced, solubilization of β -Lg is reduced. This occurs when 350 mM KCl is added to the feed. The much larger lactase enzyme, on the other hand, is not substantially solubilized at any salt concentration. The results in Figure 3.21 suggest that during separation of a mixture of the two proteins using the highest KCl concentration of 350 mM (Figure 3.19b), some β -Lg is excluded from the microemulsion droplets and therefore finds its way in the final aqueous product. There it acts as an impurity that reduces the specific activity of the product.

Our results also indicate an effect of cation type on the effectiveness of the separation. As shown in Figure 3.20b, in the presence of NaCl, the specific activity of the product of the separated mixture remained below that of the pure lactase control, at the three lower salt concentrations. Kawakami and Dungan (1996) observed previously that solubilization of β -lactoglobulin in the microemulsion increased with an increase in NaCl salt concentration at low [NaCl], reaching a

maximum at 300 mM NaCl at pH 6.2. At still higher NaCl concentrations the solubilization decreased with increased salt, similar to the behavior in Figure 3.21. Comparison of these observations with the results in Figure 5b therefore suggests that there was a less complete solubilization of β -Lg that occurred at 0, 100 and 250 mM NaCl concentrations, that resulted in incomplete separation of this protein from β -gal.

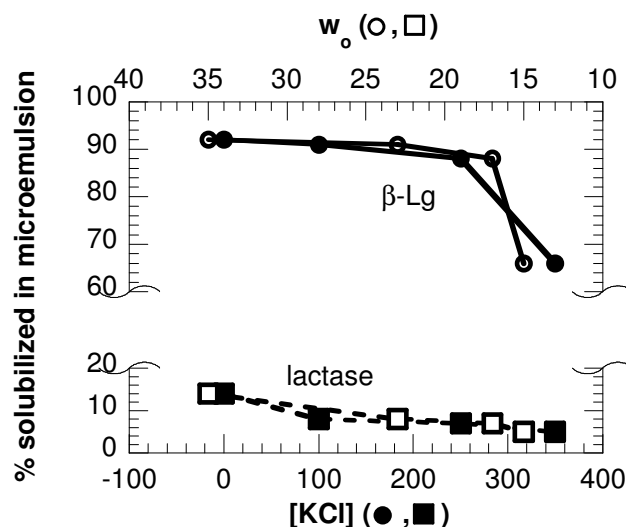


Figure 3.21. Percentage of β -lactoglobulin (O,●) and β -galactosidase (□,■) taken up by the microemulsion phase from a pure aqueous protein solution as a function of salt concentration in the feed or water content of the microemulsion. Organic phase contained 200 mM AOT. Aqueous phase contained 0.05 M K-phosphate buffer at all salt concentrations and was at pH 7.0.

It is plausible that the ion concentration and type can affect not only protein interactions with the microemulsion droplet, but also the hydrolytic action of the β -galactosidase enzyme. Some ions, such as sodium, calcium, and zinc, have been shown to have inhibitory effects on yeast lactase; other ions such as potassium, phosphate, and magnesium showed activating and/or stabilizing effects (Pivarnik and Rand, 1992). In the feed used in our study, an approximately 20% decrease in specific activity was observed with 50 mM Na-phosphate buffer compared to K-

phosphate buffer, with no added salt (Figure 3.20a). There was a slight increase in this specific enzymatic activity sodium chloride concentration increased. Thus, Na-phosphate buffer alone had some inhibitory effect on the enzyme. It appears that, for systems containing Na cations, contact with the microemulsion increased these inhibitory effects on lactase. In our control experiments containing lactase alone in the presence of sodium, the specific activity of the product was lower by ~27% compared to the feed at all salt concentrations, as shown in Figure 3.20. This inhibitory effect may come from interactions of the Na-phosphate buffer and the surfactant AOT, causing changes in protein conformation (Ugwu and Apte, 2004), or from binding of surfactant to the protein upon transfer into the aqueous product stream (Shinagawa et al., 1993, Shimek et al., 2005). Such binding of AOT to protein can be enhanced at lower NaCl concentrations (Shimek et al., 2005).

On the other hand, a microemulsion-based separation using potassium ions did not have a similar inhibitory effect on the enzyme activity. Specific activities in the product stream for the pure lactase system matched those of the feed in all cases (Figure 3.19a, b). According to the Pivarnik and Rand (1992), variations in potassium phosphate buffer concentrations between 10 and 60 mM at pH 6.5 had little effect on enzymatic activity, consistent with our results.

The separation of the commercial lactase preparation Maxilact LX 5000 (from *Kluyveromyces lactis*) from the test protein β -lactoglobulin was also monitored by SDS-PAGE (Laemmli, 1970). Figure 3.22, lane 2 represents a control feed with pure Maxilact LX 5000. The feed containing a 50/50 mixture of Maxilact and the test protein β -lactoglobulin is shown in lane 3. After the separation step, the microemulsion droplets took up all or most of the β -lactoglobulin, so that this protein is absent in lanes 4-6 of Figure 3.22. Therefore, the newly formed aqueous phase has only Maxilact LX 5000. Only at 350 mM KCl is residual β -lactoglobulin detected in the product. At this salt concentration, about 26% of the β -lactoglobulin is excluded from the microemulsion and goes into the newly formed aqueous phase (Figure 3.21), which causes the drop in specific activity of the product shown in Figure 3.19b.

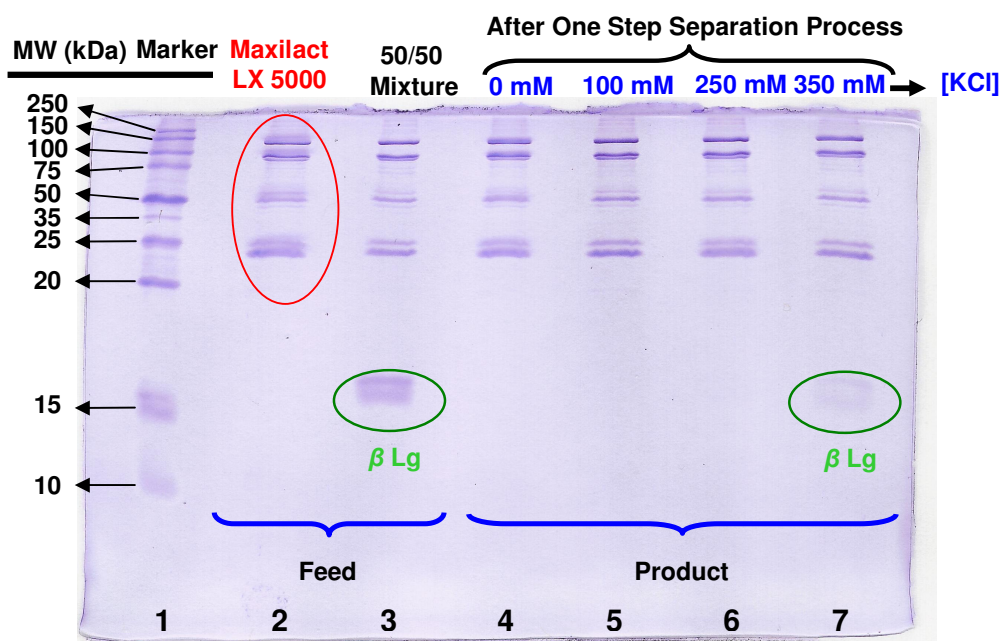


Figure 3.22 SDS PAGE analysis of the β -galactosidase from *Kluyveromyces lactis*. Fifteen percentages of denaturing gel was stained with Coomassie blue R (total protein in each line was same).

The extent of the separation of the lactase enzyme from the β -Lg impurity can be quantified by taking the ratio of the specific activity in the product relative to the feed, using the data in Figure 3.19. For the system containing potassium salts, 1.6 to 2.0-fold purification factors are achieved, with the maximum value at 100 mM KCl. The latter value matched the theoretical maximum purification possible from separation of the 1:1 lactase/ β -Lg feed mixtures we used.

In Figure 3.23 is shown the yield of the lactase enzyme, measured using activity measurements, after the one-step separation for the potassium-containing system. The yield is 92% at 100 mM, where the purity of the protein was maximized. Even higher yields of 96-97% were obtained at higher salt concentrations. Thus, the method recovers more than 90% of the enzyme in a virtually pure form. Because a small volume of aqueous feed is used in the method, an amount only slightly larger

than the water solubility in the microemulsion, the enzyme is also recovered in a more concentrated form than its value in the feed. The concentrations were enhanced by 1.5 to over 7 fold (Figure 3.23).

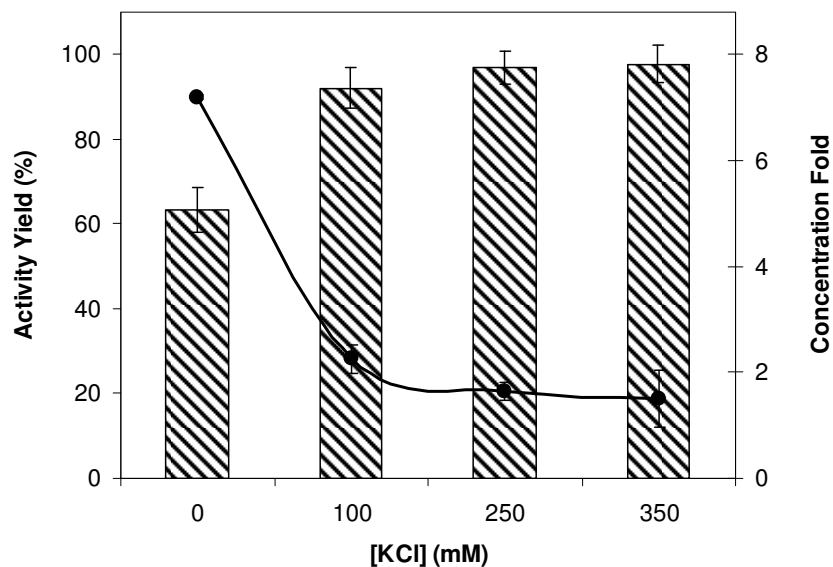


Figure 3.23 Effect of feed salt concentration on concentration (●) and yield of β -galactosidase (▨) in the aqueous product.

Organic phase contained 200 mM AOT. Aqueous phase was at pH 7.0 and contained 50 mM K-phosphate buffer.

3.4 One-step Partial Purification of β -Galactosidase from *Kluyveromyces lactis*

In this part of our study, purification of β -galactosidase from crude extract obtained from *Kluyveromyces lactis* yeast was attempted by using our one-step reversed micellar extraction method (Figure 3.24), with a focus on the effects of pH, salt and protein concentration on purification performances (activity recovery and purification fold). Results are compared with conventional separation methods and traditional forward and backward reversed micellar extraction procedures.

After cell disruption, we obtained the crude extract which is a complex mixture. The protein diversity in the crude extract is shown in Figure 3.25 (line 2 and 4). Research in the literature show that beside the β -galactosidase pyruvate decarboxylase (MW 200 kDa, pI 5.1) (Krieger et al., 2002), proteinases A (MW 42-48 kDa, pI 4.4) and B (MW 42-48 kDa, pI 6.4), carboxypeptidase Y (MW 38-42 kDa, pI 3.6) (Grieve et al., 1983) and alcohol dehydrogenase (MW 141 kDa, pI 5.4) (Gowda et al., 1988) along with other impurity proteins are present.

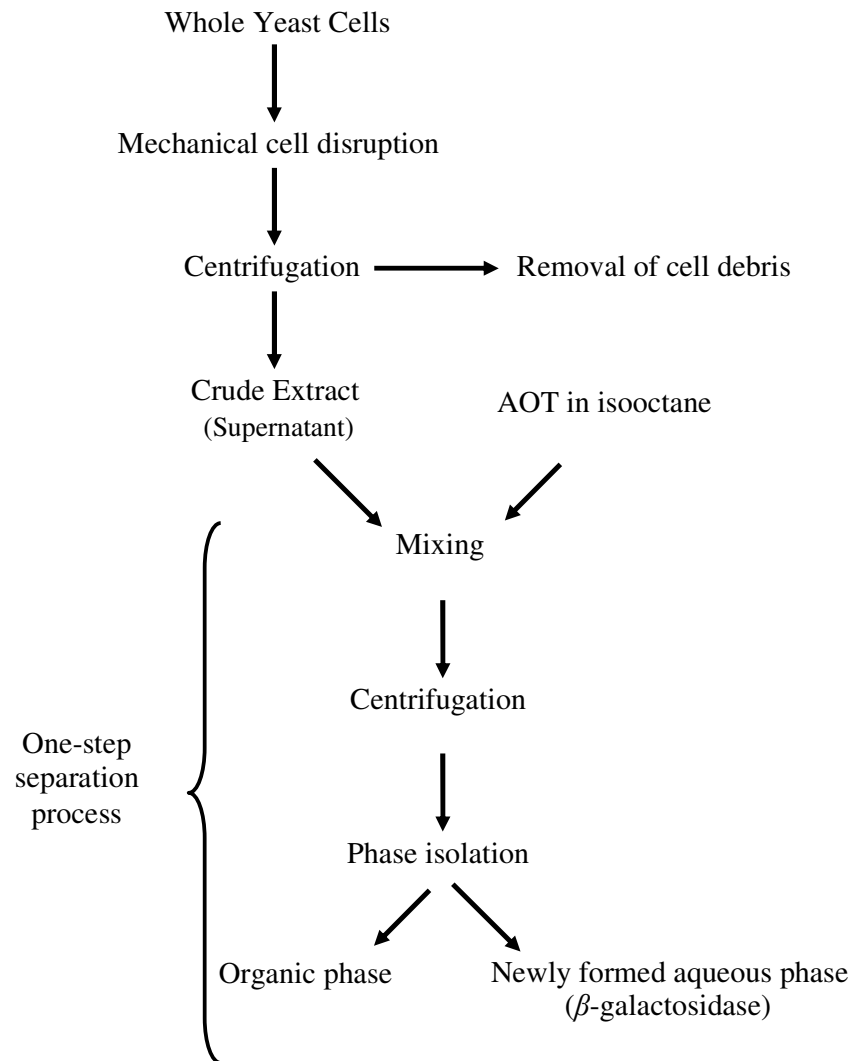


Figure 3.24 Procedure for partial purification of intracellular β -galactosidase from *Kluyveromyces lactis* yeast cells by innovative one-step reversed micelle extraction technique.

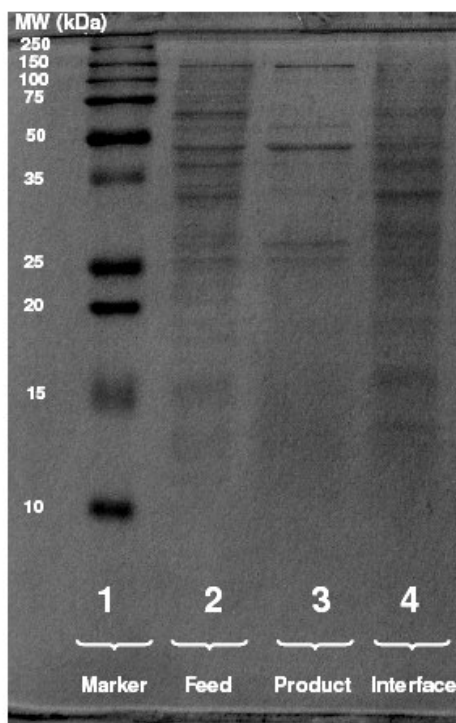


Figure 3.25 One-step reversed micelle extraction of β -galactosidase from *Kluyveromyces lactis*.

Lane 1: molecular weight marker, lane 2 is the crude extract (feed) and lane 3 is the product obtained after reversed micelle extraction. Lane 4: aggregated impurity proteins at the interface.

3.4.1 Effect of pH

Effect of injected aqueous phase pH on purification fold, protein recovery and water content of microemulsion droplets was shown in Figure 3.26. Our previous results in model system showed that lower solubilization of β -galactosidase was achieved at higher pH values. Repulsion between the negatively charged protein and anionic surfactant head group increases with increasing pH. However, we do not want to increase pH to a value higher than 7.5 since further increase in pH may decrease the

solubilization of impurity proteins which causes a decrease in purification fold. Moreover, β -galactosidase aggregation was shown to occur at pH higher than 8.0 (Tello-Solis et al., 2005). Therefore, the effect of injected aqueous phase pH on purification performances was investigated at pH 7.0 and 7.5. Results in Figure 3.26 show that purification folds increased from 2.6 to 2.9 when pH increased from 7.0 to 7.5.

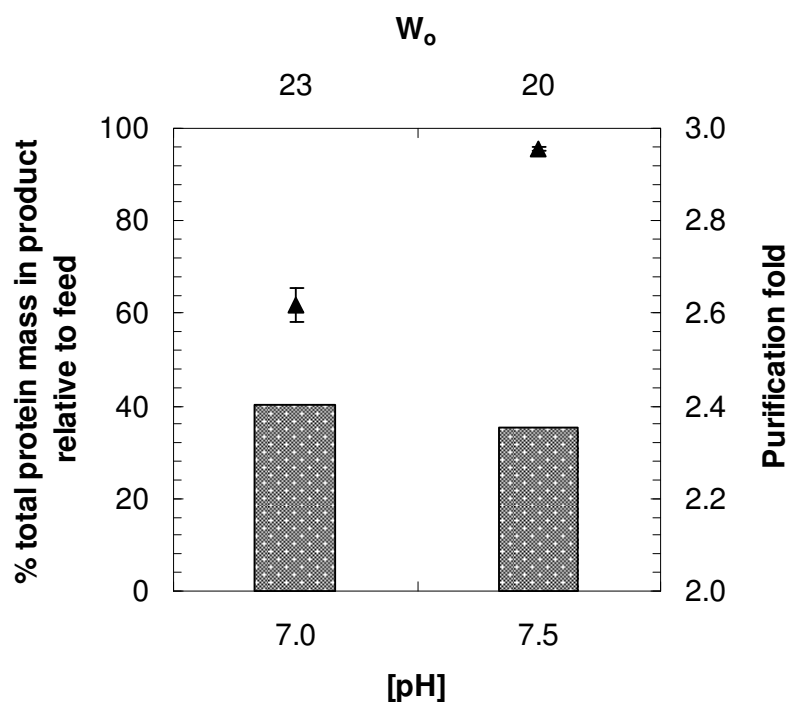


Figure 3.26 Effect of injected aqueous phase pH on purification fold ($\blacktriangle$), protein recovery ($\blacksquare$) and w_o .

200 mM AOT, injected phase protein concentration 2 mg/ml, 10 mM K-phosphate buffer and 100 mM KCl.

3.4.2 Effect of Salt Concentration

Figure 3.27 gives results for the purification fold and relative amount of protein and total enzymatic activity in aqueous solution after one step extraction. Total protein concentrations in the initial aqueous phase were ≤ 0.3 mg/ml for each sample. The total protein and % protein reflect both the target β -galactosidase and other impurity proteins in the initial crude extract. The reduction in that mass of protein after extraction reflects removal of those impurities by the method, as well as any loss of β -galactosidase enzyme.

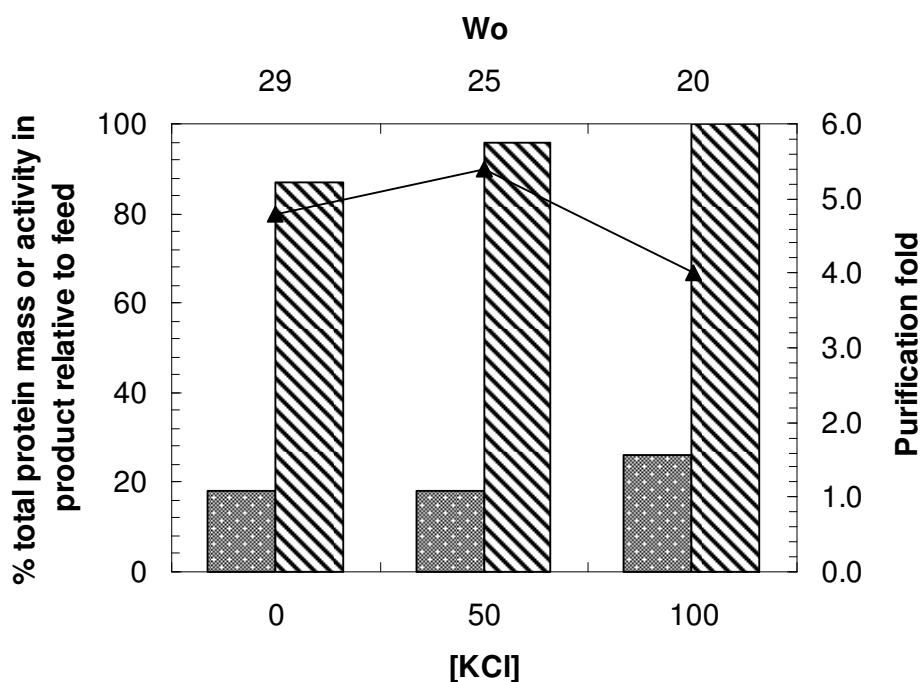


Figure 3.27 Effect of injected aqueous phase salt concentration on one-step partial purification of β -galactosidase from crude extract of *Kluyveromyces lactis*. Purification fold (▲), total protein (■), total activity (▨). 200 mM AOT, pH 7.5, 50 mM K-phosphate buffer.

The amount of β -galactosidase is specifically detected by measuring total enzyme activity. The total activity was calculated by multiplying the enzyme activity to the volume of aqueous solution. The only small drop in that total activity after extraction indicates that most of our enzyme is recovered and in an active form. Percentages of total activity recovery tell us how much β -galactosidase was recovered. Specific activity was calculated by dividing the total enzyme activity by the total amount of protein in the sample volume. It shows the purity of our enzyme. The purification fold was calculated by dividing the specific activity of product by the specific activity of feed. Purification fold and activity recovery is the two imported indicators show the purification method success.

After one-step reversed micellar extraction, up to a 5.4-fold purification factor was achieved with 96% of initial β -galactosidase activity recovered in the newly formed aqueous phase (Figure 3.27). This result was achieved with a 50 mM KCl salt concentration added to the buffer. An even higher activity recovery (and thus yield of enzyme) was obtained at higher [KCl] but with lower purity.

Salt concentration of the applied sample affected purification and reversed micellar extraction performance (Figure 3.27). Total activity in the newly formed aqueous phase increased with increase in salt concentration and reached the initial aqueous phase total activity (100% recovery) at 100 mM KCl. It means that at that salt concentration all β -galactosidase was excluded into newly formed aqueous phase. Increase in the salt concentration cause increase in the recovered protein. However, purification fold increased at first and reached highest value (5.4) when the [KCl] was 50 mM and then decreased with increase in [KCl]. It means that when we increased the [KCl] from 50 to 100 mM all β -galactosidase + some impurity protein was excluded from the microemulsion droplets. It caused the increase amount of impurity protein in the newly formed aqueous phase and purification fold decreased. Decrease in the solubilization of β -galactosidase and some impurity proteins can be explained by steric interaction. Increasing salt concentration decreased the size of the droplet (Figure 3.27).

One-step partial purification of β -galactosidase from *Kluyveromyces lactis* was also monitored by SDS-PAGE (Laemmli, 1970). Comparison of results from feed (lanes 2, 4 and 6) and product (lanes 3, 5 and 7) clearly show that most of the unwanted proteins in the crude extract were successfully removed by the microemulsion droplets during the extraction (Figure 3.28).

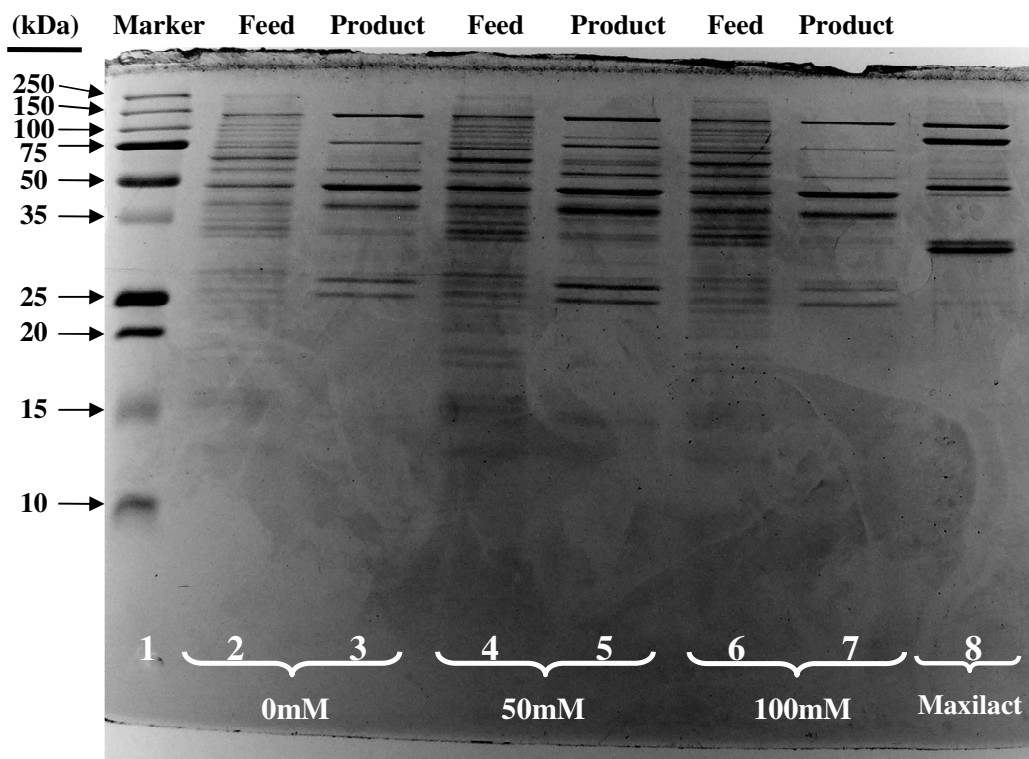


Figure 3.28 SDS PAGE analysis of the β -galactosidase from *Kluyveromyces lactis*. Fifteen percentages of denaturing gel was stained with coomassie blue R. Lane 1: molecular weight marker (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa), lane 2 and 3 are the crude extract and crude extract after reversed micelle extraction with 0 mM KCl, lane 4 and 5 are the crude extract and crude extract after reversed micelle extraction with 50 mM KCl, lane 6 and 7 are the crude extract and crude extract after reversed micelle extraction with 100 mM KCl, respectively (total protein in each lane 1.8 μ g). Lane 8: commercial pure enzyme (Maxilact LX 5000, 1.8 μ g).

3.4.3 Effect of Protein Concentration

The effect of feed protein concentration was studied in extract with 100 mM KCl and a pH of 7.5 (Figure 3.29). As the feed protein concentration increased, the extent of extraction of contaminant proteins from crude extract decreased, while the amount of protein aggregates observed at the interface between the organic and the aqueous phase after extraction increased. This showed the interaction between AOT and contaminant proteins in the crude extract, which may cause aggregation. It could thus be reduced by decreasing the feed protein concentration.

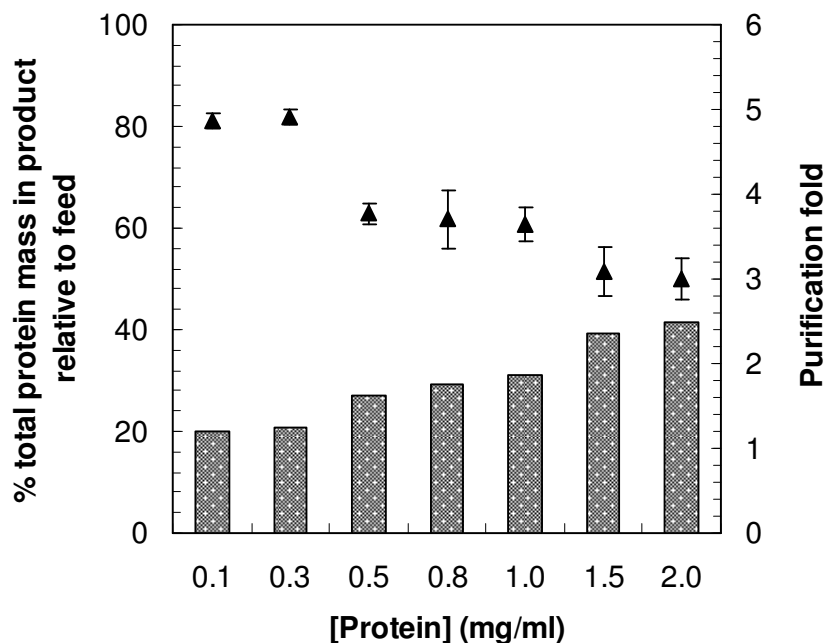


Figure 3.29 Effect of injected protein concentration on purification fold (▲) and protein recovery (▨).

200 mM AOT, w_o : 20, feed volume injected 1725 μ l, pH 7.5, 50 mM K-phosphate buffer and 100 mM KCl.

The percent activity recovered after extraction remained almost constant at 100% at all feed protein concentrations. However, the total protein concentrations in the newly forward aqueous phase increased (21% - 42%) with increasing feed protein concentration (0.1 – 2 mg/ml). Taken together, these results indicated that the percent of contaminant protein in the newly formed aqueous phase was increased with increased feed protein concentration.

3.4.4 Recovery of Other Proteins from the Microemulsion

Back-extraction of the impurities (proteins, enzymes, amino acids) which were solubilized in microemulsion droplets during the one-step separation process, would enable the reuse of reversed micellar solution. After extraction of β -galactosidase from crude extract, the microemulsion solution and newly formed aqueous phase were carefully isolated from each other. The microemulsion solution then was contacted with an equal volume of fresh aqueous solution with a high ionic strength (1M KCl) to promote partitioning of impurity proteins to the new aqueous phase. Because of this high KCl concentration, aggregation of impurity proteins at the interface was observed. Such formation of a third aggregate phase under high salt conditions has been reported previously.

Dungan et al. (1991) reported that back extraction rates can be orders of magnitude slower than those for forward transfer. We probed the influence of back extraction time in our system by measuring the absorbance of organic phase at 280 nm on a UV-visible spectrophotometer. Reading was corrected by subtractions of absorbance at 310 nm. Figure 3.30 shows the effect of contact time during back extraction on recovery of impurity proteins. Back-extraction of impurity proteins was ~ 60% after 30 h of mixing.

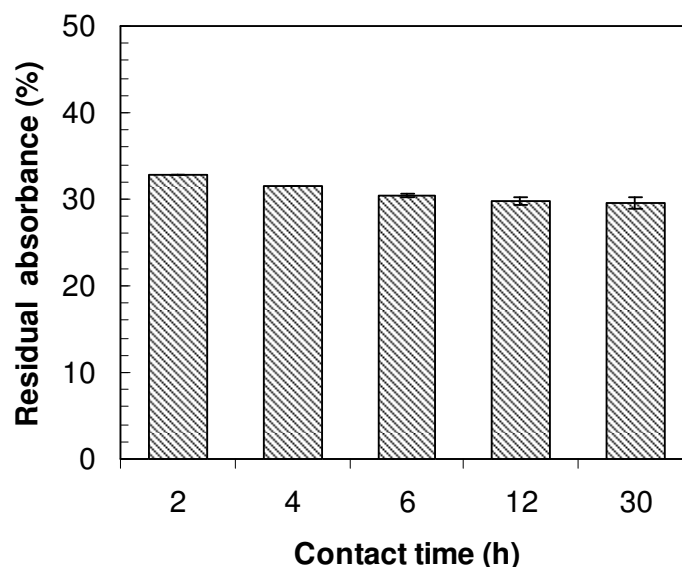


Figure 3.30 Effects of contact time during back-extraction on removal of water soluble impurities from microemulsion droplets.

Injected aqueous phase: pH 7.5, protein concentration 1.4 mg/ml, [KCl]: 100 mM.

Fresh aqueous phase for back-extraction: [KCl]: 1M.

3.4.5 One-step Separation vs. Traditional Forward and Backward Extraction Procedure

There are two common approaches (phase transfer and injection method) to extract, separate or purify protein by reversed micellar extraction. Phase transfer is a two-phase system by which target proteins selectively partition into microemulsion solution and then expelled from microemulsion into fresh aqueous phase which was contacted with isolated microemulsion phase by back-extraction (Matzke et al., 1992; Kelley et al., 1994; Kawakami and Dungan, 1996). Second approach is the injection method. In this method, a tiny amount of aqueous solution is directly injected into organic surfactant solution and thus one-phase microemulsion droplets are formed. In this approach all the proteins present are entrapped into microemulsion droplets and then target proteins are selectively extracted into fresh aqueous phase by back-extraction. The goal of these two methods is to bring target protein into microemulsion droplet and then get it out selectively. However, the very

large size of yeast β -galactosidase makes the solubilization of this protein difficult in microemulsion droplets. If incorporated in a one-phase system, it is likely to be metastable (Gerhardt and Dungan, 2002; 2004). In our two-phase system one-step reversed micellar extraction method, all except target protein partitions into microemulsion droplets. The target protein selectively expels into newly formed aqueous phase by tuning the initial aqueous phase pH, ionic strength and protein concentration and no back-transfer needed.

In this part of our study, we compared the “injection/recovery” approach proposed by Shiomori et al. (1994) and our one-step separation process. Shiomori et al. (1994) reported that 71% of the total initial lactase activity was recovered with a 4.2 fold purification factor with the injection method (2 step separation -forward and backward extractions). In our method maximum purification fold (5.4) with 96% of the initial activity recovery was achieved at pH 7.5 with 50 mM K-phosphate buffer and 100 mM KCl concentrations. The results represented in Table 3.1 showed our one-step separation process to be a successful method for the partial purification of β -galactosidase.

Table 3.1 Comparison of different reversed micellar extraction techniques employed for lactase purification.

(* from Shiomori et al., 1994).

Methods	Aqueous Phase	Total Protein (mg)	Total Activity (kEU)	Specific Activity (kEU/mg)	% Protein in Product Relative to Feed Mixture	Activity Recovery (%)	Purification (fold)
Our Study	Feed	0.398	2.6	6.6	100	100	1.0
	Product	0.071	2.5	35.4	18	96	5.4
Injection*	Feed	0.583	1.5	2.6	100	100	1.0
	Product	0.098	1.1	11	17	71	4.2

3.4.6 One-step Separation vs. Conventional Separation Methods

In this part of the study, separation of β -galactosidase from crude extract of *Kluyveromyces lactis* was investigated by using two conventional separation methods. Results obtained from ammonium sulfate precipitation and size exclusion chromatography were compared to our one-step separation results and results obtained from literature.

Different separation methods for the extraction and purification of β -galactosidase from the yeast *Kluyveromyces lactis* have been published in the literature. Summary of these purification methods for β -galactosidase purification and their performance was shown in the Table.3.2.

Table 3.2 Chromatographic methods used for the purification of β -galactosidase from *Kluyveromyces lactis*.

Purification Methods	Purification Fold	Activity Recovery (%)	Specific Activity (kEU/mg)	References
C, SP, SEC, IEX*	78.6	19	143	Biermann and Glantz, 1968
C, SP, PP, SP, DEAE-IEX, HA*	80.0	27	139	Dickson et al., 1979
SEC, IEX, UF, SEC*	11.0	0.33	23.22	Becerra et al., 1998
SEC*	1.8	50	3.87	Becerra et al., 2001
IEX*	1.6	33	3.39	Becerra et al., 2001
AC*	2.5	5.3	5.30	Becerra et al., 2001
EE*	1.7	80	17.5	Ganeva et al., 2001
OSP*	7.4	50.8	26.66	Matheus and Rivas, 2003

* UF (ultrafiltration), AC (affinity chromatography), C (centrifugation), SP (salt precipitation), DEAE-IEX (diethylaminoethyl ion exchange chromatography), EE (electroinduced extraction), HA (Hydroxyapatite chromatography), IEX (ion exchange chromatography), OSP (organic solvent precipitation; toluene precipitation followed by acetone precipitation), PP (polymer precipitation), SEC (size exclusion chromatography),

Biermann and Glantz (1968) reported that β -galactosidase from the crude extract of *K. lactis* was purified by procedures including centrifugation, precipitation with ammonium sulfate, size exclusion chromatography, and ion exchange chromatography. These steps resulted a purification of 78.6 folds, a 19% activity recovery, and a specific activity of 143 kEU/mg. Dickson et al. (1979) achieved almost same specific activity and purification fold with higher activity recovery (27%) after a 6 step separation (centrifugation, salt precipitation, polymer

precipitation, salt precipitation, ion-exchange chromatography on DEAE–Sephadex, and hydroxyapatite chromatography).

Becerra et al. (1998) used several chromatographic techniques in sequence (gel filtration, ion-exchange, ultrafiltration, and gel filtration) to purify β -galactosidase from crude yeast extract. In his study, only 0.33 % of the total initial lactase activity was recovered, with a 11 fold purification factor after a 4 step separation. Becerra et al. (2001) also achieved 1.8 folds of purification with 50% activity recovery, 1.6 folds of purification with 33% activity recovery, and 2.5 folds of purification with 5.3% activity recovery after a single size exclusion, ion exchange, and affinity chromatography step, respectively.

When β -galactosidase was purified by electroinduced extraction, the purification factor of 1.7 folds with 80% of activity recovery was reported by Ganeva et al. (2001). Matheus and Rivas (2003) declared that 7.4 folds purification was achieved with 50.8% activity recovery by two-step organic solvent precipitation.

Partial purification of β -galactosidase from *Kluyveromyces lactis* by gradual ammonium sulfate precipitation was summarized in Table.3.3. Among the concentration ranges of ammonium sulfate, sample obtained from 50-70% ammonium sulfate precipitation exhibited the highest β -galactosidase activity (Table. 3.3). These results indicate that β -galactosidase was precipitated between 50 to 70% of ammonium sulfate saturation. In this fraction, 2.5 folds increase in the specific activity was achieved, with 61% of total enzymatic activity recovery.

Table 3.3 Purification of β -galactosidase from a crude extract of *K. lactis* by ammonium sulfate precipitation.

Fraction	Sample Volume (ml)	Total Protein (mg)	Total Activity (kEU)	Specific Activity (kEU/mg)	% Protein in Product Relative to Feed Mixture	Activity Recovery (%)	Purification (fold)
Crude extract	150	298.35	2569	8.61	100	100	1.0
Ammonium Sulfate Precipitation							
0-35%	11.8	4.43	10	2.14	1.5	0.4	0.2
35-50%	17.0	67.23	142	2.12	22.5	5.5	0.2
50-70%	15.7	73.21	1568	21.42	24.5	61.0	2.5
70-100%	16.4	72.59	161	2.22	24.3	6.3	0.3

The purification of β -galactosidase enzyme from a crude extract of *K. lactis* was accomplished by means of gel filtration chromatography resulted in a purification factor of 15.5 folds with 73% of enzyme yield based on total enzymatic activity recovery. The results of the β -galactosidase purification by gel filtration chromatography were shown in Table 3.4. Gel filtration chromatogram of crude extract from the yeast *K. lactis* exhibits nine peaks (Figure 3.31). The β -galactosidase activity was assayed in the collected fractions obtained from gel filtration chromatography. Only second peak of the chromatogram showed β -galactosidase activity.

Table 3.4 Purification of β -galactosidase from a crude extract of *K. lactis* by gel filtration chromatography.

Step	Sample Volume (ml)	Total Protein (mg)	Total Activity (kEU)	Specific Activity (kEU/mg)	% Protein in Product Relative to Feed Mixture	Activity Recovery (%)	Purification (fold)
Crude extract	2	3.127	28.48	9.1	100	100	1.0
Gel filtration	6	0.147	20.70	141.1	5	73	15.5

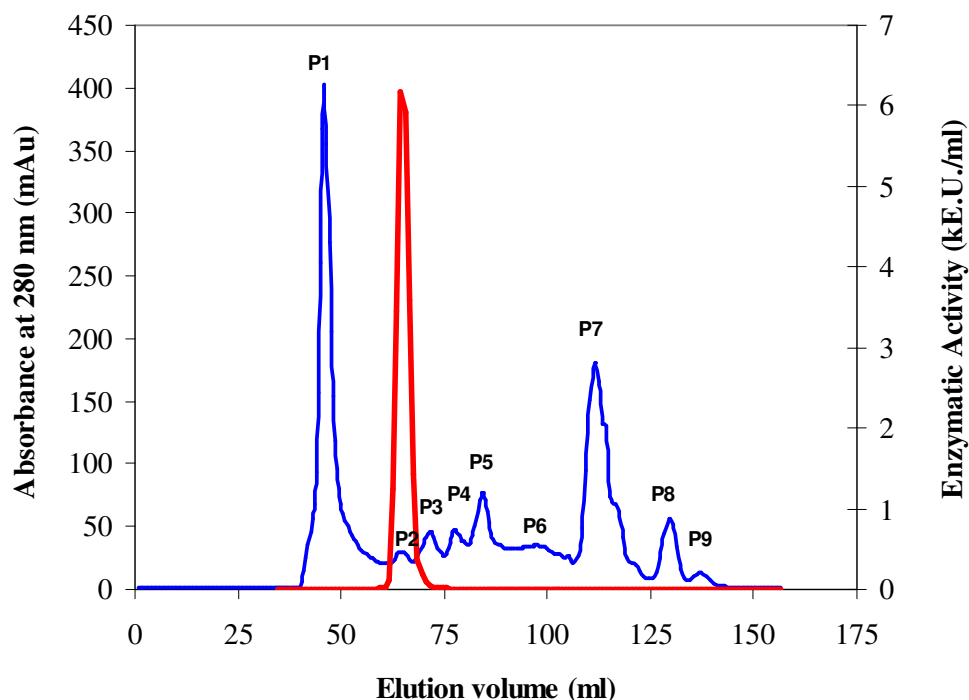


Figure 3.31 Elution profile of 3.13 mg of crude extract (—) from *K. lactis* by gel filtration chromatography and β -Galactosidase activity (—) in the collected fractions.

The purity of the β -galactosidase activity containing peak (second peak, P2) (Figure 3.32) was analyzed by a second gel filtration chromatography as in the previous procedure. For this purpose, the second peaks (Figure 3.32, A) obtained from three replicates of the first gel filtration chromatograms were combined (15 ml) and concentrated by ultrafiltration with Amicon Ultra-15 centrifugal filter devices with 10 kDa molecular-weight cut-off (Millipore Corporation, MA, USA). The Amicon filter was centrifuged at 4850 g, 4°C for 15 minutes using a Universal 320 R refrigerated fixed-rotor centrifuge (Hettich Zentrifugen, Germany). The concentrate (2 ml) was then subjected to second gel filtration chromatography. Gel filtration chromatogram of the second peak had two major peaks (Figure 3.32, B). These peaks were P2 (240 kDa) and P7 (> 42 kDa) as in the first gel filtration chromatogram (Figure 3.32, A). The P2 was the most dominant peak on the chromatogram. Therefore, this means that β -galactosidase was the major protein in the eluents.

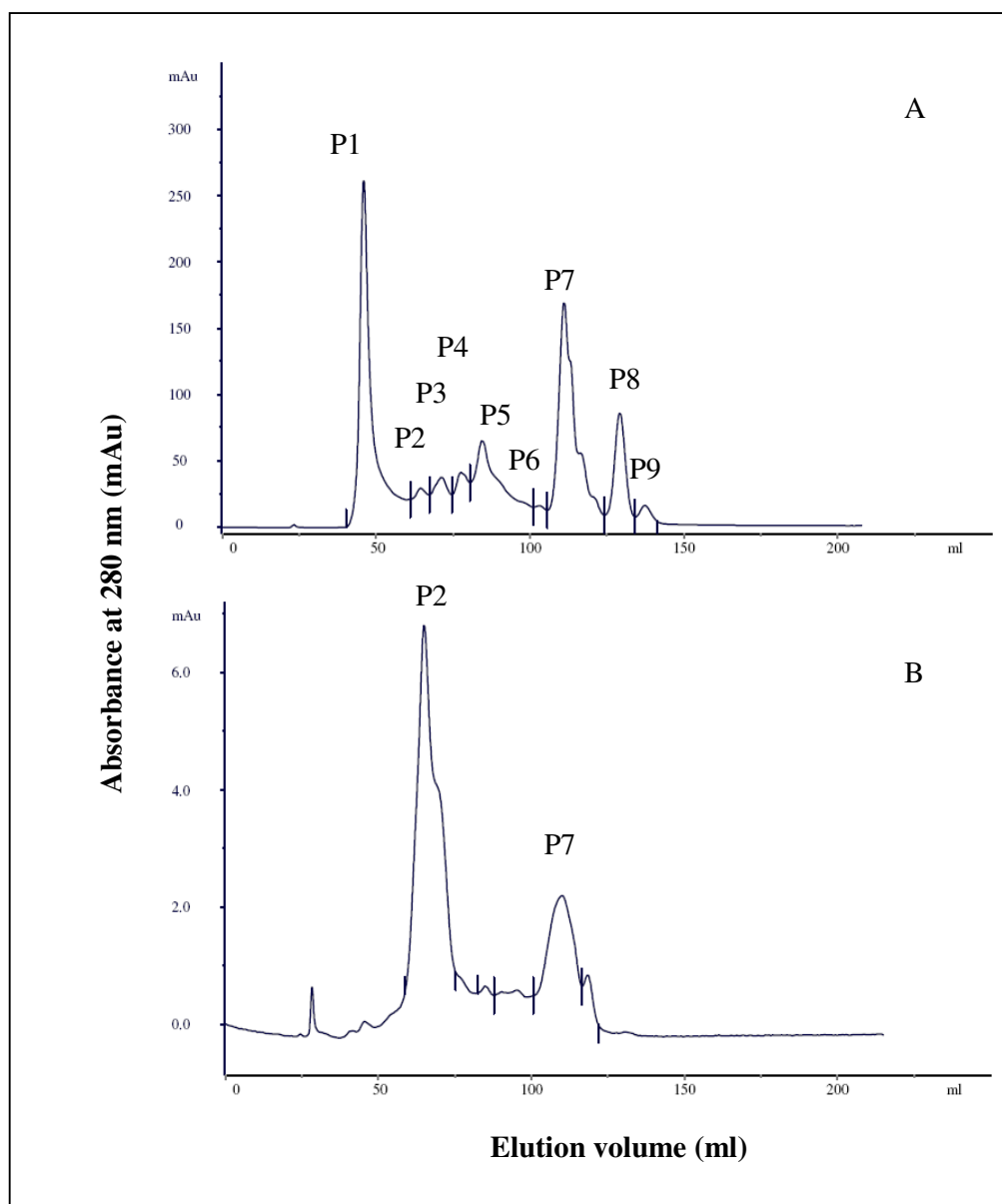


Figure 3.32 Protein profile obtained after gel filtration chromatography. (A) Chromatography of a crude extract from *Kluyveromyces lactis*. (B) Chromatography of β -galactosidase activity containing fraction (P2).

The approximate molecular mass of the native β -galactosidase and other impurities were also obtained by gel filtration on a HiLoad 16/60 column (Table 3.5). The gel filtration column was calibrated using the high molecular weight calibration kit (Thyroglobulin 669 kDa, Ferritin 440 kDa, Aldolase 158 kDa, Conalbumin 75 kDa and Ovalbumin 43 kDa) (Figure F.1).

Table 3.5 The approximate molecular mass of the nine peaks in the gel filtration chromatogram of crude extract.

Peak	Retention (ml)	Molecular Weight (kDa)
P1	46.01	1230
P2	64.60	240
P3	71.25	134
P4	77.52	77
P5	84.51	42
P6	96.88	<42
P7	111.12	
P8	129.22	
P9	137.38	

The β -galactosidase activity assay performed in the eluents showed that enzyme activity was limited to molecular weight region 110 – 370 kDa (Figure 3.31). At molecular weight 240 kDa, maximum β -galactosidase activity was observed, indicating that the main active form of the β -galactosidase in the crude extract is dimeric (approximate hydrodynamic diameter was calculated 10.9 nm). In the literature, a molecular weight of 240 kDa was reported for the dimeric form of the same enzyme (Becerra et al., 1998).

In our one-step separation experiment, innovative reversed micellar extraction technique was tested for the recovery of β -galactosidase. A 5.4 fold purification factor was achieved with 96% activity recovery. The results represented above show that our one-step separation process is an efficient and rapid method (process took 30 minutes) for the substantial purification of β -galactosidase. These are advantages over the other approaches.

CHAPTER 4

CONCLUSIONS

The separation of the very large protein β -galactosidase from the milk protein β -lactoglobulin was investigated using a one-step, microemulsion-based method. Conditions for the separation were determined based on solubilization experiments employing β -galactosidase alone, to determine uptake of this enzyme by the microemulsion droplets as a function of buffer concentration and type, pH, and protein concentration. These experiments showed that this large enzyme could be incorporated into the microemulsion droplets at low buffer concentrations, especially under conditions of lower pH and protein concentration. Conversely, by contacting an enzyme-containing aqueous feed with the organic surfactant solution under conditions of higher buffer concentration (> 40 mM), the microemulsion droplets that form would effectively exclude the protein and ensure its entrance into the excess aqueous product stream.

Consequently, conditions in the aqueous feed solution (ionic strength, buffer type and pH) were chosen to favor extraction of proteins smaller than β -gal into microemulsion droplets, but to exclude the large enzyme. This feed was contacted with a 200 mM AOT in isooctane solution, and an excess aqueous product phase containing mainly β -gal was formed after mixing. Binary feed mixtures containing lactase and one competing protein (β -lactoglobulin) were separated using this approach, with maximum purification of the enzyme achieved for feeds containing 100 mM KCl. Under these conditions, 92% of the lactase was recovered in a virtually pure form, and at a concentration that was 2.3-fold higher than the feed solution. Higher potassium chloride concentrations decreased the microemulsion droplet size, which thus shifted some of the β -Lg impurity into the product, but

further increased the enzyme yield, while replacement of potassium with sodium in the system caused some activity loss of the enzyme.

The promising nature of these results motivated further studies on the purification of β -galactosidase from a crude extract of *Kluyveromyces lactis*. In order to obtain a technical enzyme, yeast cells of *Kluyveromyces lactis* were disrupted by mini-beadbeater and recently developed, one-step reversed micellar extraction method was tested for the recovery of β -galactosidase.

Using this approach, a 5.4-fold purification factor for β -galactosidase was achieved with 96% activity recovery when 50 mM K-phosphate buffer (pH 7.5) was used with 50 mM KCl. Ionic strength and total protein concentration of the applied sample affected purification and reversed micellar extraction performance. Purification fold decreased from 5 to 3 when protein concentration was increased from 0.1 to 2 mg/ml in the initial aqueous phase. The results presented show our one-step separation process to be an interesting method for the production of β -galactosidase as a technical enzyme, since it can also be applied on a continuous large scale, quickly achieve a substantial purification of the enzyme and thereby reduce the number of steps required in downstream processing.

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APPENDIX A

VARIATION IN HYDRATED RADIUS OF IONS

Hydration of an ion depends on the electrostatic attraction of water molecules to that ion. Because attraction of water molecules around an ion depends on that ion's density of charge, smaller ions (and thus ions of greater ionic potential) attract more water molecules. The result is the *inverse* relationship between non-hydrated radius and hydrated radius shown below.

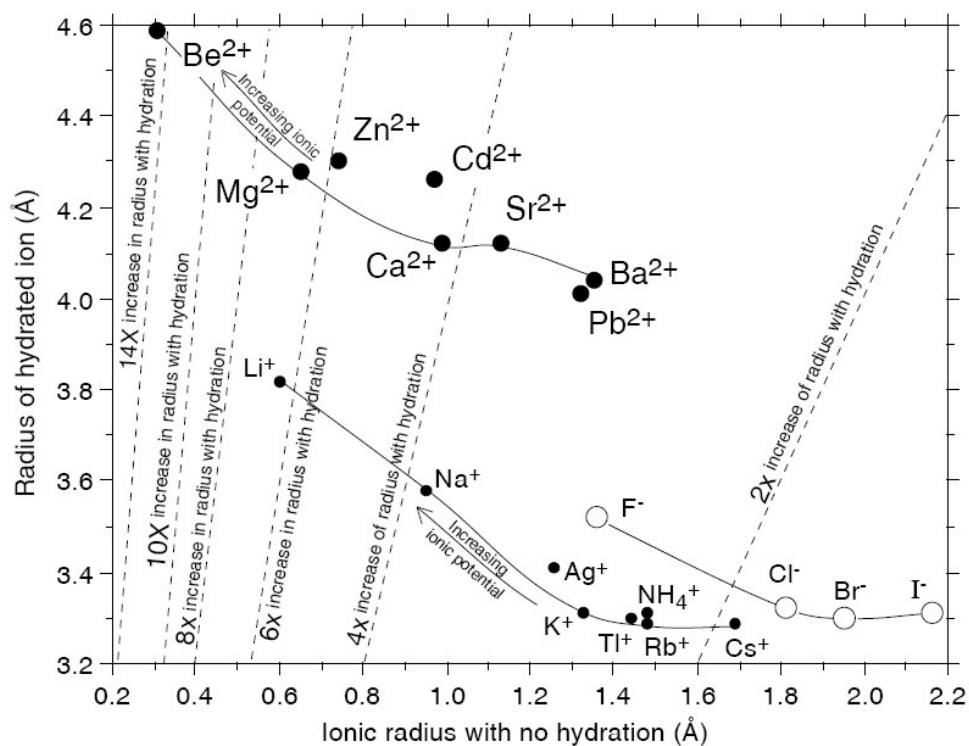


Figure A.1 Variation in hydrated radius of ions

APPENDIX B

RELATION BETWEEN WATER POOL RADIUS AND WATER CONTENT

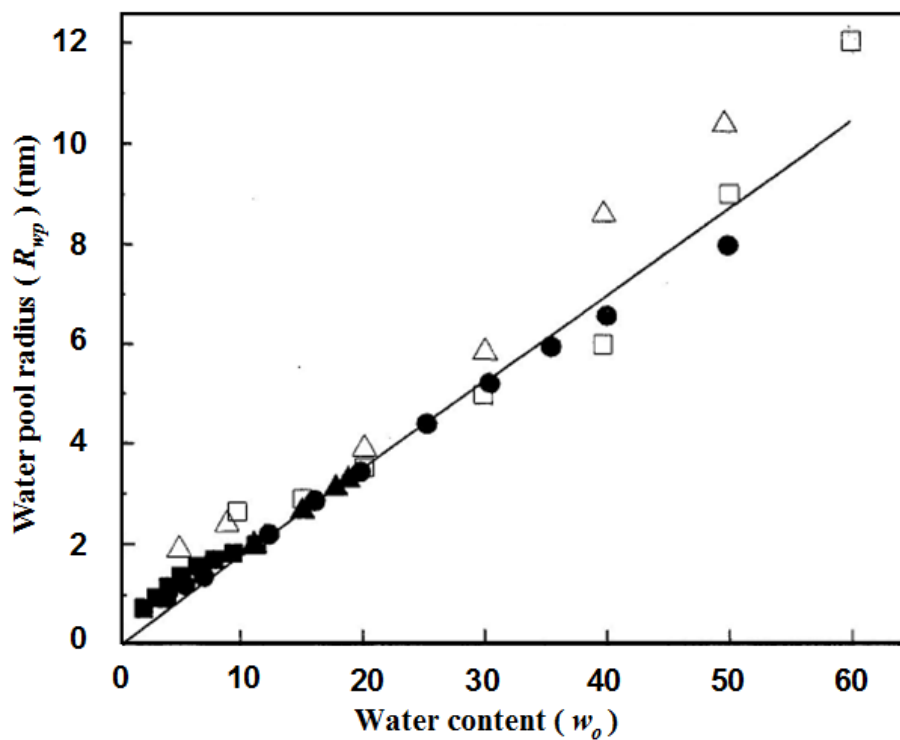


Figure B.1 Relation between measured water pool radius R_{wp} and the water/surfactant molar ratio w_o .

Small-angle neutron scattering (SANS) (□), nuclear magnetic resonance (NMR) (●), quasi-elastic light scattering QELS (△), fluorescence (■), fluorescence (▲).

APPENDIX C

GROWTH OF *Kluyveromyces lactis*

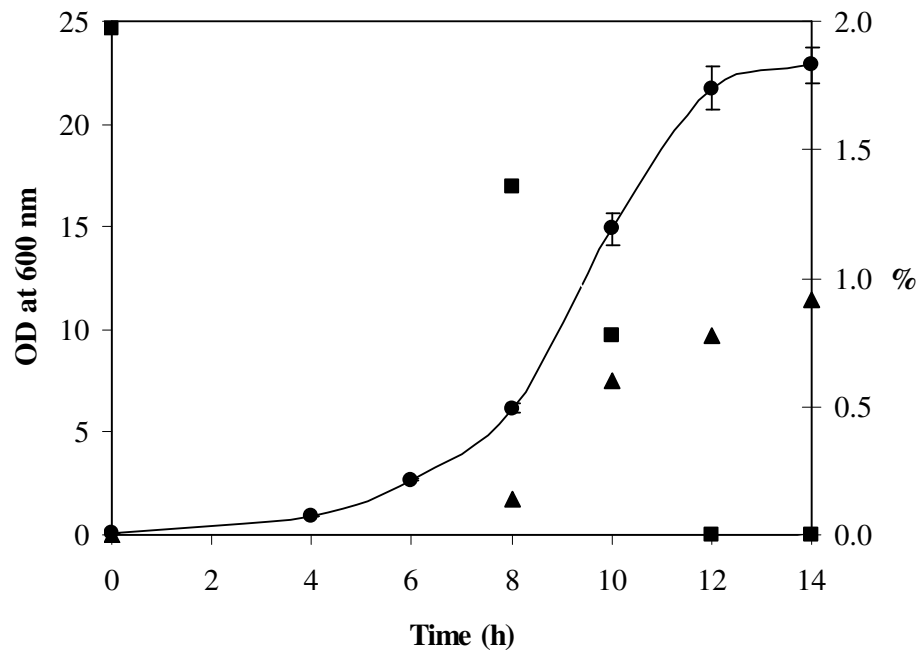


Figure C.1 Growth of *Kluyveromyces lactis* (●) and change of lactose (■) and ethanol (▲) concentration of the media during growth.

APPENDIX D

STANDARD CURVE FOR DRY CELL WEIGHT DETERMINATION

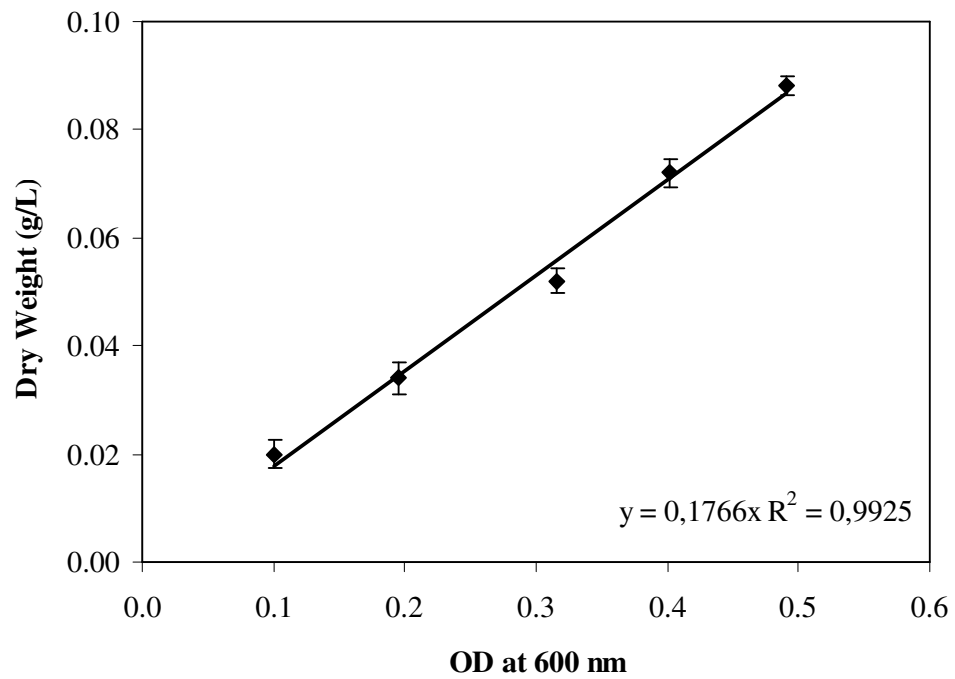


Figure D.1 Standard curve for dry cell weight of *Kluyveromyces lactis*.

APPENDIX E

THE HIGH MOLECULAR WEIGHT CALIBRATION KIT

Table E.1 Characteristics of high molecular weight (HMW) gel filtration calibration kit.

Protein	Molecular weight (kDa)	Source
Ovalbumin	43	Hen egg
Conalbumin	75	Chicken egg white
Aldolase	158	Rabbit muscle
Ferritin	440	Horse spleen
Thyroglobulin	669	Bovine thyroid
Blue dextran 2000	2000	

APPENDIX F

MOLECULAR WEIGHT AND SIZE CALIBRATION CURVE

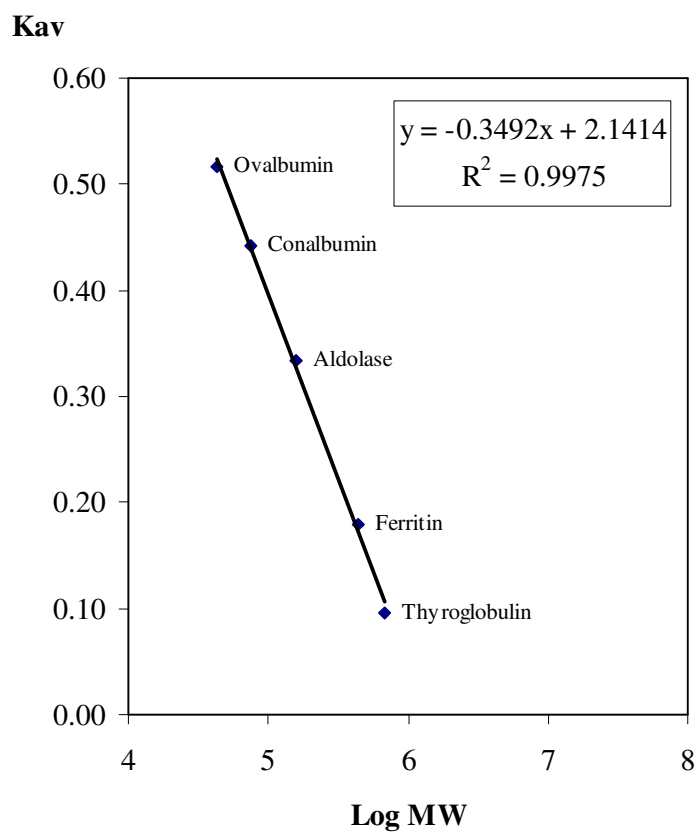


Figure F.1 Molecular weight calibration curve for the standard proteins on HiLoad 16/60 Superdex 200 pg column.

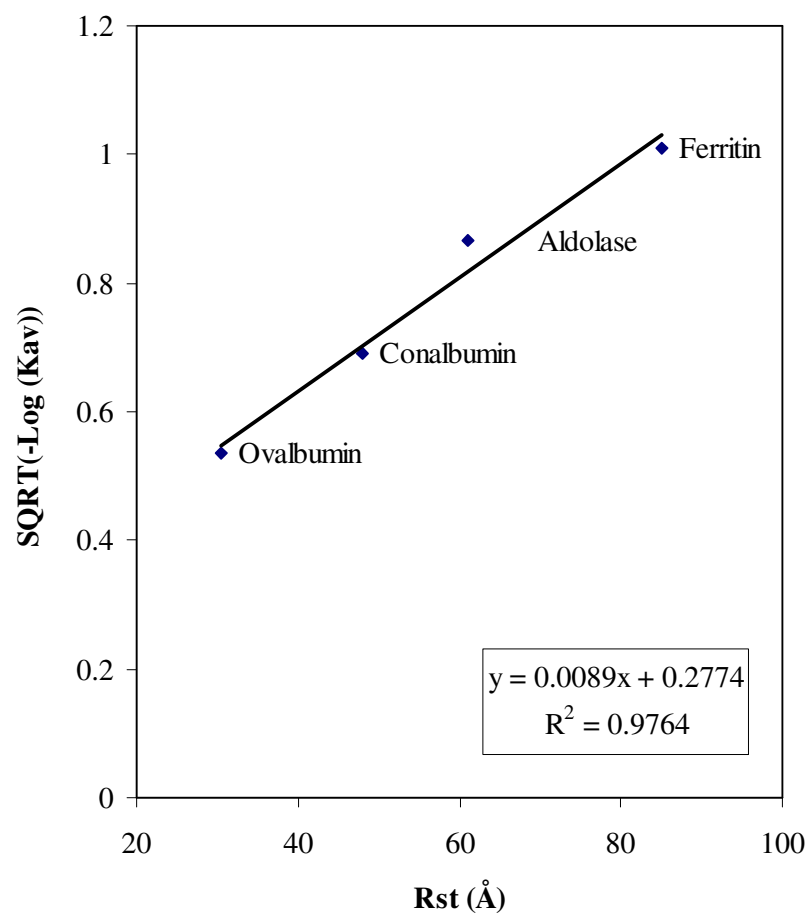


Figure F.2 Molecular size calibration curve for the standard proteins on HiLoad 16/60 Superdex 200 pg column.

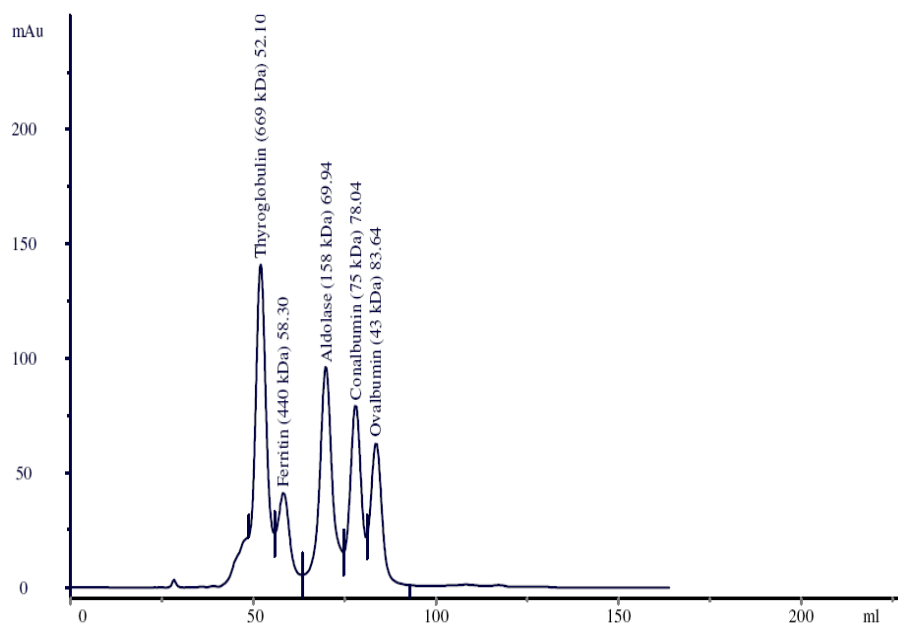


Figure F.3 Chromatographic separation of the standard proteins on HiLoad 16/60 Superdex 200 pg column.

The method used for Figure F.3.

Sample: Proteins from HMW Gel Filtration Calibration Kits

Sample vol.: 500 µl

Buffer: 10 mM K-phosphate buffer, 150 mM NaCl, pH 7.0

Flow rate: 0.5 ml/min

System: ÄKTAprime plus

Detection: 280 nm

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HONOURS AND AWARDS

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Ranked 1st in the poster competition of 6th Food Engineering Symposium, Antalya, 2009

UC Davis University Outreach and International Programs (UO&IP) Office, Research Fellowship. Apr 2008- Oct 2008

TUBITAK – BİDEB – 2214 – Research in Foreign Countries Fellowship Program. (1 year) Apr 2007- Apr 2008

Ranked 1st in the Faculty of Agriculture 2001

Ranked 1st in the Food Engineering Department 2001

PROJECT WORK

Purification of β -galactosidase from Fermentation Broths, M.E.T.U Research Fund Project, BAP-08-11-DPT.2002K120510, Researcher, 2004-2009.

Evaluating Microemulsions for Lactase Purification, Robert Mondavi Institute - Center of Advance Materials, Methods and Processing, University of California, Davis (RMI-CAMMP), Researcher, 2007-2008.

Purification of β -galactosidase by reverse micelle extraction: determination of the temperature effect and enzyme subunit composition, TÜBİTAK-TOVAG 108O823, Researcher, 01/03/2010

PUBLICATIONS

Journal Paper

1. Mazi, B.G., Hamamcı, H. & Dungan, R.S., 2010. One-Step Separation of β -Galactosidase from β -Lactoglobulin Using Water-in-Oil Microemulsions. Journal of Food Engineering (paper submitted)

Conference Paper (International)

1. Mazi, B.G., Dungan, R.S., & Hamamcı, H., 2008. Investigation of Conditions Favoring the Uptake and Release of β -Galactosidase by Microemulsion Droplets. 2008 IFT Annual Meeting & Food Expo, New Orleans, Louisiana, USA. (Oral Presentation)
2. Mazi, B.G., Dungan, R.S., & Hamamcı, H., 2008. Selective One-Step Separation of β -Galactosidase and β -Lactoglobulin by Microemulsion Droplets. 2008 International Enzyme Engineering Symposium, Kuşadası, Turkey (Oral Presentation).
3. Mazi, B.G., Dungan, R.S., & Hamamcı, H., Isolating β -Galactosidase by Microemulsion Droplets from *Kluyveromyces lactis*. 2009 IFT Annual Meeting & Food Expo, Anaheim, California, USA.
4. Mazi, B.G., Hamamcı, H., Ogrydziak, D.M. & Dungan, R.S., 2009. One Step Partial Purification of β -Galactosidase from *Kluyveromyces lactis* Using Microemulsion Droplets. 2009 CIGR Section VI International Symposium on Food Processing, Monitoring Technology in Bioprocesses and Food Quality Management, Potsdam, Germany, p. 387.

5. Mazi, B.G., Hamamcı, H. & Dungan, R.S., 2010. A New Approach to Purify High Molecular Weight Proteins by Reversed Micelles. Vth International Bioengineering Congress, Bioprocess Engineering Section, İzmir, Turkey, p. 54. (Oral Presentation)

Conference Paper (National)

1. Mazi, B.G., Hamamcı, H., Ogrydziak, D.M. & Dungan, R.S., 2008. β -Galaktosidaz'ın Ters Misel İçerisinde Çözünürlüğü. Türkiye 10. Gıda Kongresi, Erzurum, 2008, p. 933-936.
2. Mazi, B.G., Hamamcı, H., Ogrydziak, D.M. & Dungan, R.S., 2009. Farklı Kaynaklardan Elde Edilen β -Galaktosidaz'ın Nanoboyuttaki Mikroemülsiyon Su Damlacıkları ile Özütlenmesi. Gıda Mühendisleri Odası, 6. Gıda Mühendisliği Kongresi, Antalya, 2009, p. 237-238.