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ENCAPSULATION OF UREASE IN ALGINATE BEADS AND PROTECTION FROM ALPHA-CHYMOTRYPSIN WITH CHITOSAN MEMBRANE

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August 1997

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements of the degree of
Master in Engineering

ABSTRACT

Oral administration of encapsulated proteins for the treatment of metabolic enzyme disorders has received recent attention. In this study alginate was used as the immobilization matrix because of its ease of use and mild formulation conditions which are more compatible with proteins than other encapsulation methods. Alginate gels tend to have a wide pore size distribution and the larger pores allow undesired substrates to diffuse into the bead. In the treatment of enzyme disorders, proteins encapsulated in alginate gels may be deactivated by proteolytic enzymes such as chymotrypsin, which are present in gastric fluids. To reduce the permeability to chymotrypsin (MW 21,600), the alginate beads were coated with polycationic membranes. Chitosan, poly-L-lysine, and poly(methylene co-guanidine) membranes were evaluated for their capacity to exclude α-chymotrypsin and other proteases from alginate beads. The exclusion of chymotrypsin was quantified by measuring the deactivation of the encapsulated urease.

When alginate beads were exposed to 0.5% chymotrypsin for 10 min, 98% activity of the encapsulated urease was lost due to deactivation. Polymer coating of the alginate beads resulted in higher chymotrypsin exclusion. Chitosan provided approximately 50% activity retention after chymotrypsin exposure. A combination of low and high molecular weight chitosans offered 30% activity retention, while urease within poly-L-lysine membranes only had 12% residual activity. Poly(methylene co-guanidine) membranes did not provide any protection from chymotrypsin.

Lyophilization causes the beads to shrink and results in a more compact structure and a reduced pore size. After rehydration the beads do not swell to their original

diameter, effectively reducing the pore size. Lyophilization and rehydration resulted in beads with a higher chymotrypsin exclusion than polymer coated beads. After exposure to chymotrypsin, the activity retention of the encapsulated urease for uncoated beads was 71%, and 89% for chitosan coated beads. Thus lyophilization and rehydration of coated and uncoated beads resulted in the highest level of urease protection from extracapsular chymotrypsin.

Gastric fluids contain many proteases that can affect the activity of encapsulated proteins. The effects of two other proteases, trypsin and proteinase K in addition to chymotrypsin, were evaluated. Chitosan coated alginate beads were exposed to trypsin and the results were similar to those obtained from chymotrypsin, probably due to their similar molecular weights. The exposure of chitosan coated beads to the slightly larger molecular weight proteinase K resulted in a higher level of retained activity.

RÉSUMÉ

L'administration par voie orale de protéines encapsulées pour le traitement des enzymopathies métaboliques retient depuis peu l'attention des chercheurs. Dans la présente étude, c'est l'alginate qui a servi de matrice d'immobilisation en raison de sa facilité d'utilisation et de sa plus grande innocuité pour les protéines que les autres méthodes d'immobilisation. Les gels d'alginate présentent généralement une répartition plus lâche de la taille de leurs pores dont la plus grande taille permet en outre la diffusion de substrats indésirables dans les billes. Les protéines encapsulées dans des gels d'alginate utilisées dans le traitement des enzymopathies sont parfois désactivées par des enzymes protéolytiques comme la chymotrypsine, qui sont présentes dans les sucs gastriques. On a recouvert les billes d'alginate de membranes polycationiques pour réduire la perméabilité de la chymotrypsine (MM 21 600). On a ainsi pu déterminer la capacité de membranes de chitosane, de poly-L-lysine et de poly(méthylène co-guanidine) à prévenir la pénétration de l'α-chymotrypsine et d'autres protéases dans les billes d'alginate. On a quantifié l'imperméabilité à la chymotrypsine en mesurant le taux de désactivation de l'uréase immobilisée.

Dans des billes d'alginate exposées à une solution de chymotrypsine à 0,5 % pendant 10 mn, l'uréase immobilisée a été désactivée à 98 %. En recouvrant les billes d'alginate d'une couche de polymère, on a obtenu un plus fort taux d'exclusion de la chymotrypsine. Les billes recouvertes de chitosane ont conservé environ 50 % de leur activité après avoir été exposées à la chymotrypsine. L'association de chitosanes de faible et forte masse moléculaire a permis de maintenir un taux d'activité de 30 %, tandis que

l'uréase protégée par des membranes de poly-L-lysine n'a conservé que 12 % de son activité. Les membranes de poly(méthylène co-guanidine) n'ont offert aucune protection contre l'action de la chymotrypsine.

La lyophilisation entraîne la contraction des billes qui présentent de ce fait une structure plus compacte et des pores de taille réduite. Lorsqu'elles sont réhydratées, les billes ne retrouvent pas leur diamètre initial, ce qui a pour effet de réduire la taille des pores. La lyophilisation et la réhydratation ont permis d'obtenir des billes plus imperméables à la chymotrypsine que les billes recouvertes de polymère. Après exposition à la chymotrypsine, l'uréase encapsulée conserve un taux d'activité de 71 % dans les billes non recouvertes et de 89 % dans les billes recouvertes de chitosane. Ce sont donc les billes recouvertes et non recouvertes soumises à une lyophilisation et à une réhydratation qui protègent le plus efficacement l'uréase contre l'action de la chymotrypsine extracapsulaire.

Les sucs gastriques contiennent de nombreuses protéases qui peuvent affecter l'activité des protéines encapsulées. On a donc évalué l'effet de deux autres protéases, la trypsine et la protéinase K. Des billes d'alginate recouvertes de chitosane ont été exposées à la trypsine avec des effets semblables à ceux résultant de l'exposition à la chymotrypsine, ce qui est probablement attribuable au fait que ces deux enzymes ont des masses moléculaires semblables. Les billes recouvertes de chitosane exposées à la protéinase K, dont la masse moléculaire est légèrement plus élevée, ont conservé un taux d'activité plus élevé.

ACKNOWLEDGMENTS

I would like to give my deepest appreciation to my supervisor, Prof. Ronald J. Neufeld for all his guidance and patience throughout the work of this Master's. Special thanks to all members of Lab 24: Douglas Quong, Antonio Ribeiro, and Nicholas Blahuta for their ideas, assistance and enjoyment through long research hours. Special thanks to Edgar and Betty Ramirez for their kind hospitality.

To my family: my mother, father, aunt, uncle and especially my brother Alex, for their encouragement and support in the good and not-so-good times. Thank you, I owe it all to you! Finally I wish to express my deepest gratitude to my wife Colleen for all her patience and moral support over the past couple of years.

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1.0 INTRODUCTION

1.1 Immobilization

Immobilization is a method of physically confining enzymes. There are many methods used to immobilize enzymes, depending mostly on the application for the immobilized system. In carrier binding, the enzyme attaches to water insoluble supports by adsorption, ionic binding and covalent binding. Another way is to cross-link the enzyme to the support using multifunctional reagents. Enzymes may also be encapsulated within microcapsules or microspheres. Microcapsules consist of enzymes dissolved or suspended in a liquid core contained by a semi-permeable membrane. Microspheres consist of enzymes entrapped within the lattice structure of a semi-permeable gel matrix (Alexakis 1995). The following work focuses exclusively on the microencapsulation of urease.

The impact of microencapsulation on our daily lives has been large. Applications include the encapsulation of inks used in carbonless copy paper, vitamins, drugs, dyes, and food ingredients, to the encapsulation of water droplets used in microwavable cake mixes to keep them moist.

Chang (1964) pioneered the semi-permeable membrane bound microcapsule that allowed a selective interaction between the fluids inside and outside the microcapsule. Cells and other biologically active materials were being microencapsulated to reduce the possibility of immuno-reactions because the membranes or the polymer matrix are permeable to lower weight solutes but impermeable to larger molecular weight molecules such as antigens and other proteins.

1.2 Microencapsulation methods

The products of microencapsulation may be classified as microcapsules or microspheres. Microcapsules are formed via various interfacial polymerization techniques (Alexakis 1992, Monshipouri 1991), which consist of a reaction between reactants in two phases. An aqueous solution consisting of the material to be encapsulated and a water soluble reactant is emulsified in an organic fluid. A water-insoluble reactant is added to initiate membrane polymerization at the aqueous droplet interface. The advantage of interfacial polymerization is that membrane properties may be selected by the use of appropriate combinations of monomers, reactants, and cross-linkers. The disadvantages are that the reactants and solvents might damage fragile biological core materials during the encapsulation process. Interfacial precipitation as an alternative (Chang 1957), does not involve a chemical reaction. Microcapsules are formed by dispersing an aqueous solution in an organic solvent and the membrane is formed via polymer precipitation at the droplet interface.

Microspheres are produced with an anionic polysaccharide that is extruded dropwise into a solution containing multivalent cations (in this case Ca^{2+}). The droplets form gel spheres, a three dimensional matrix of ionically cross-linked polysaccharide. One advantage of this method is that it is a mild and gentle procedure, compatible with delicate biomaterials. Some examples of droplet forming techniques include adding a concentric air stream to a nozzle (Klein et al. 1983) and applying an electrical potential between the liquid surface and the nozzle (Okhamafe et al. 1996). Another way of making

microspheres is using the emulsification / internal gelation method (Poncelet et al. 1992), where beads are formed by internal gelation of an emulsified alginate solution.

1.3 Alginate as encapsulation matrix

As noted in the previous section, there are many techniques being used for immobilization. Alginate as an immobilization medium has been extensively used (Vorlop and Klein 1983). The popularity of alginate is mainly due to its ease of use, mild gelation conditions, biocompatibility, and acceptability as food additive and use in oral drug delivery systems (Hari et al. 1996, Smidsrod and Skjak-Braek 1990).

The main source of alginate is from the giant kelp *Marcrocystis pyrifera*, but it is also produced from other seaweeds including *Laminaria hyperborea* and *Ascophyllum nodosum*. Alginate is a linear copolymer made up of D-mannuronic acid (M) and L-guluronic acid (G) units. These alginate "building blocks" are not randomly distributed along the polymer chain, but rather they exhibit three types of sequences; continuous blocks of mannuronic acid units, continuous blocks of guluronic acid units, and blocks where the two types of units alternate regularly, all bound by (1-4) linkages. G-G and G-M units are linked with α(1-4) bonds and M-M and M-G units are linked with β(1-4) bonds (Klein et al. 1983, Smidsrod and Skjak-Braek 1990)

The gelation of alginate by calcium ions is due mainly to the ionic cross-linking between guluronic acid units located on adjacent alginate chains. The calcium ions will preferentially interact with G units that are part of a G block before interacting with M units located in the M blocks. As the guluronic acid blocks associate, they leave cavities

where the calcium ions can bind. Therefore the number of guluronic acid units in a polyguluronic block is the main structural feature of the alginate gel of interest for gelation. Alginate that has a high guluronic block content forms a stronger, more rigid, and possibly more brittle matrix than alginate with fewer guluronic acid blocks. Poncelet (1992) found that low guluronic acid content in the alginate favored a smaller bead diameter and a wider size distribution for emulsification systems. This may be due to easier "cracking and splitting" of the more rigid beads by the impeller.

Alginate gels have a wide pore size distribution due to the open lattice structure of the matrix, where structure and molecular weight define the pore size. Larger pores can cause problems by allowing the entrapped enzymes to leak out or undesired substrates to diffuse into the bead (Tanaka et al. 1984)

Tanaka (1984) reported that the effect on the beads of increasing the concentration of $CaCl_2$ during gelation was to form a denser structure and to lower the pore size. Low molecular weight encapsulants ($< 2*10^4$) ranging from glucose to α-lactalbumin, diffuse freely in and out of Ca-alginate beads. The diffusion of these materials was unaffected by increasing the concentration of the $CaCl_2$ used in the gel preparation or the Ca-alginate concentration in the beads. Higher molecular weight encapsulants ($> 6.5 *10^4$) such as albumin and γ-globulin, did not diffuse into the alginate beads, but they all diffused out of the beads with rates similar to that of unhindered diffusion in water. The lower MW reagents did not interfere with the cross-linking of the alginate polymers, resulting in a tighter, less porous structure, while the higher MW materials affected the alginate cross-

linking. The resulting beads were more porous, explaining why large encapsulants readily diffuse out of alginate beads, but are unable to diffuse into beads.

1.4 Chitosan

Chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] is a derivative of chitin [(1→4)-2-acetamido-2-deoxy-β-D-glucan] prepared by the N-deacetylation of the latter. Chitin is a natural polymer that forms the principal constituents of the shells of crabs, lobsters, Antarctic krill and fungal walls (Thacharodi 1996, Huguet 1994) and it is available at low cost and in large quantities from the wastes of seafood processing (Krajewska 1990). Chitosan is a water soluble cationic polysaccharide and it will form a membrane on polyanionic calcium-alginate beads by displacing the calcium and forming chitosan-alginate crosslinks (Alexakis 1995).

1.5 Urease

Urease is a hydrolytic enzyme that transforms urea into ammonia and carbon dioxide. Urease was the first enzyme to be purified in a crystalline form (Sumner 1926). The jack bean urease used in this work is a hexamer consisting of identical subunits with a molecular mass of 90,800 (Takishima et al 1988), for a total molecular weight of 544,800.

The hydrolysis of urea catalyzed by urease is as follows (Lenki 1987):

$$NH_2CO\,NH_2 + H_2O \xrightarrow{\text{UREASE}} 2\,NH_3 + CO_2$$

$$NH_3 + H_2O \Longleftrightarrow NH_4^+ + OH^-$$

$$CO_2 + H_2O \Longleftrightarrow H_2CO_3$$

$$H2CO_3 \Longleftrightarrow H^+ + HCO_3^-$$

$$HCO_3^- \Longleftrightarrow H+ + CO_3^{2-}$$

$$NH_3 + CO_2 \Longleftrightarrow NH_2COO^- + H^+ \Longleftrightarrow NH_2COOH$$

The ratio between NH_3 to be protonated and CO_2 to be hydrolyzed determines the net acidity or alkalinity of the system. One mole of carbonic acid (H_2CO_3) can release two protons. At pH 7 the ammonia production rate exceeds the rate of production of carbonic acid, thus additional acid must be added to maintain constant pH in the solution.

1.6 Urease encapsulation

Urease has had a long history in encapsulation. It was first encapsulated for use in hemoperfusion applications (Chang and MacIntosh 1964) as a major constituent of artificial kidneys. Recently urease has been successfully encapsulated on chitosan membranes to increase the stability of the enzyme at higher temperatures and low pH (Krajewska et al. 1990). Monshipouri and Neufeld (1992) coencapsulated urease with hemoglobin in polyamide membranes and obtained 84% activity yield, twice as much as previously reported. Urease has also been encapsulated in xantham-alginate spheres (Elcin 1995), in polyacrylamide capsules (Shah et al. 1994) as well as calcium alginate gels (Shah et al. 1995). There have been studies on the diffusion of low molecular weight proteins in and out of alginate beads (Tanaka et al. 1984, Klein et al. 1983) and many studies on the controlled release of alginate encapsulated proteins (Polk et al 1994, Chui and Wan 1997, Okhamafe et al. 1996). The present work focuses on the effectiveness that different membranes have on the exclusion of proteases from Ca-alginate beads. The deactivation of encapsulated urease will serve as a measure of protease exclusion.

1.7 Proteases

The three proteases used in this study were chymotrypsin, trypsin and proteinase K. Chymotrypsin is a globular protein of molecular weight 21,600 (Righetti and Caravaggio 1976). The molecular weight of trypsin was reported as 23,000 (Hofmann 1964) and the molecular weight of proteinase K as 28,930 (Jany et al. 1986).

Trypsin and chymotrypsin are pancreatic proteases that are secreted into the digestive tract where they hydrolyze the peptide bonds of proteins as part of the digestive mechanism. The preferred protein cleavage sites of chymotrypsin include leucine, isoleucine, valine, and histidine (Mathews and van Holde 1990). Urease derived from jack bean (*Canavalia ensiformis*) has all these amino acids, within the region of the active site (Jose et al. 1994), thus chymotrypsin readily deactivates urease.

The encapsulation of enzymes for delivery to the lower intestine, as a treatment for disorders where metabolic enzymes are absent has been the subject of recent research. Alginate is commonly used to encapsulate the enzymes because alginate's mild gelling conditions do not adversely affect the protein's characteristics. The disadvantage presented by alginate beads is that it has large pores through which the encapsulated proteins can diffuse out (Klein et al. 1983), or the small enzymatic proteases can diffuse in, deactivating the enzyme.

Urease is a sufficiently large protein that it does not diffuse out of the alginate beads, but small proteases, such as chymotrypsin, easily diffuse into the alginate beads deactivating the encapsulated urease. In this work, urease has been encapsulated within alginate beads as a model protein and the permeability of the alginate beads will be lowered by the use of polymer membranes.

2.0 OBJECTIVES

Encapsulation of proteins in calcium-alginate beads for delivery to the lower intestine has been the subject of recent research. One of the problems encountered is that proteolytic enzymes present in gastric juices such as α-chymotrypsin, have the potential to diffuse into the bead and deactivate the protein, rendering it useless. Alginate beads tend to have a large pore size, making it difficult to exclude lower MW proteins such as α-chymotrypsin. The permeability of the alginate beads may be reduced by coating with a polymer membrane.

This study will concentrate on lowering the permeability of alginate beads by the use of polymer membranes. Urease will be used as the model protein to be encapsulated, and bead permeability will be assesed using proteolytic enzymes. The specific objectives of the study are:

1. to encapsulate urease within chitosan, poly-L-lysine, or poly(methylene co-guanidine) coated alginate beads.
2. to measure the activity of the encapsulated urease.
3. to evaluate the effect of α-chymotrypsin, trypsin, and proteinase K on encapsulated urease.
4. to measure the permeability of membrane coated alginate beads to chymotrypsin and evaluate the effectiveness of the membrane in protecting urease from α-chymotrypsin deactivation. The membranes of interest are chitosan, poly-L-lysine, and poly(methylene co-guanidine).

5. **to study the effect lyophilization has on coated and uncoated beads in regards to the protection of encapsulated urease from α-chymotrypsin deactivation.**

3.0 MATERIALS AND METHODS

3.1 Urease characterization

3.1.1. Preparation of Urease Solution

Urease stock solution was prepared daily, by dissolving 100 mg jack bean urease (Sigma, St. Louis) in 5 ml distilled, deionized water, and held on ice to stabilize the protein. The urease solution was quantified by absorbance at 280 nm (Molecular Devices, Spectramax microplate spectrophotometer system) as illustrated in Figure 3.1.

3.2 Production of alginate-urease beads

A 3% (w/v) stock alginate solution was prepared by dissolving 1.5 g sodium alginate (Saltialgine SG300, Systems Bio-Industries; formerly Sanofi; Waukesha, WI) in 50 ml distilled water on an orbital shaker. The solution was allowed to deaerate for at least 2 h.

Alginate solution (2 ml) was mixed with 0.5 ml of the urease solution for 1 min using a vortex mixer. The mixture was deaerated by centrifuging (International centrifuge, Fisher Scientific) at 3000 rpm for 2 min. The final alginate concentration was 2.4%. The mixture was extruded dropwise from a syringe connected to an air pressure droplet generator (EFD 1000XL, Providence RI) at an external air pressure of 80 psia and syringe pressure of 18 psia. The mixture was extruded through a needle (0.008 x 0.5 inches) into 25 ml 0.05M $CaCl_2$ (Anachemia, Montreal). The distance from the needle tip to the liquid surface was 16 cm, sufficient to allow formation of spherical beads. The

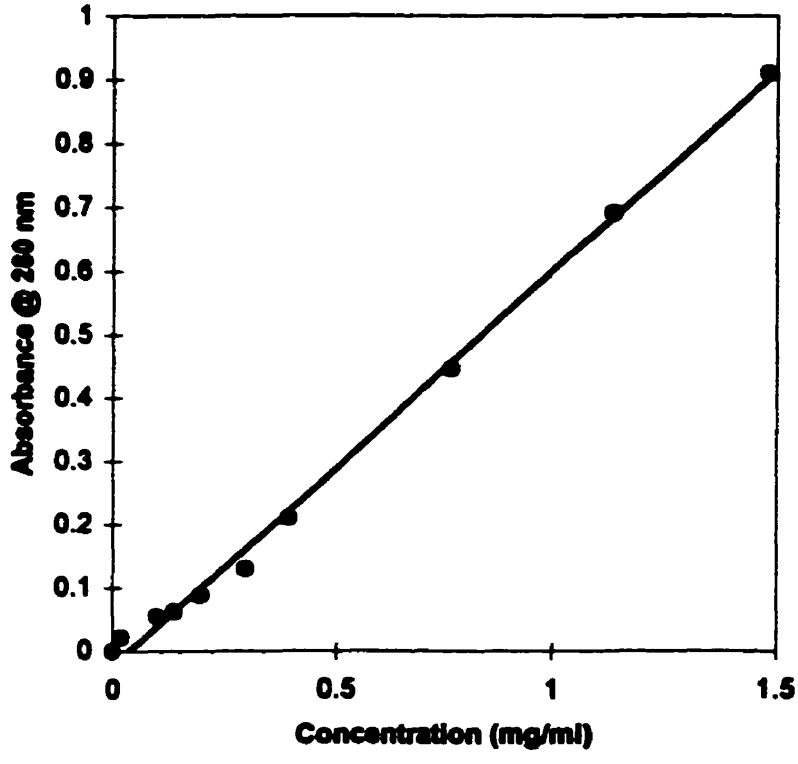

Figure 3.1

Urease Calibration Curve
Calibration equation: y=0.625x-0.024

formed beads were gently mixed during gelation using a magnetic stirrer (Fisher Scientific, Montreal), and allowed to harden for a minimum of 1h.

To determine the amount of the alginate-urease solution used for bead formation, the syringe was pre-weighed, then weighed with the alginate-urease mix, and weighed again after extrusion. Once formed, the beads were filtered using a 50 μm mesh, washed using distilled water, and surface dried with kimwipes and filter paper. The beads were then placed in pre-weighed, sealable sample holders to prevent further dehydration and shrinkage, then weighed.

3.3. Microsphere size distribution

Size distribution of the microspheres was determined using a laser diffraction particle analyzer (2604-LC, Malvern Instruments, England) using the normal distribution method. The mean diameter and the arithmetic standard deviation were calculated from the cumulative distribution curve.

3.4 Urease activity measurement

3.4.1 Experimental apparatus

In this study, the activity of free and encapsulated urease in urea solution, was determined with an autotitrator (Radiometer) used as a pH-stat. The activity of urease can be determined knowing the rate of acid addition to the reaction vessel, the weight of the enzyme added, and the relationship between the acid addition rate to the urea hydrolysis rate for the set pH. There are many different units to express the activity of

urease. The units used in this work are the international units (IU) defined as: 1 unit will liberate 1.0 μmole from urea per min at pH 7.0 and 25°C. Urease activity is calculated using equations 3.1. For a sample calculation see Appendix A. Monshipouri (1991) reports the ratios of ammonia production rates to acid addition rates for different pH levels. For pH 7.0 the ratio is 2.26.

$$Urease\ \ Activity\ \ (IU/mg) = \frac{d[NH_3]/dt}{mg\ \ enzyme} \tag{3.1}$$

$$d[NH_3]/d[H^+] = 2.26$$

$$d[H^+]/dt = rate\ \ of\ \ acid\ \ addition$$

Using the pH-stat to determine urease activity offers the advantage that no buffers or salts are added (with the exception of the small amounts of acid used) since certain buffers can alter urease activity (Lencki 1987).

The temperature (25°C) in the reaction vessel was controlled with a thermostated water jacket. Figure 3.2 shows the pH-stat apparatus used, and Table 3.1 lists the component parts. The glass jacketed reactor (component 13) was custom made by Montreal Glassblowing Inc. (Montreal) using the specifications in Figure 3.3.

Figure 3.2 pH-Stat Apparatus (Monshipouri 1991)

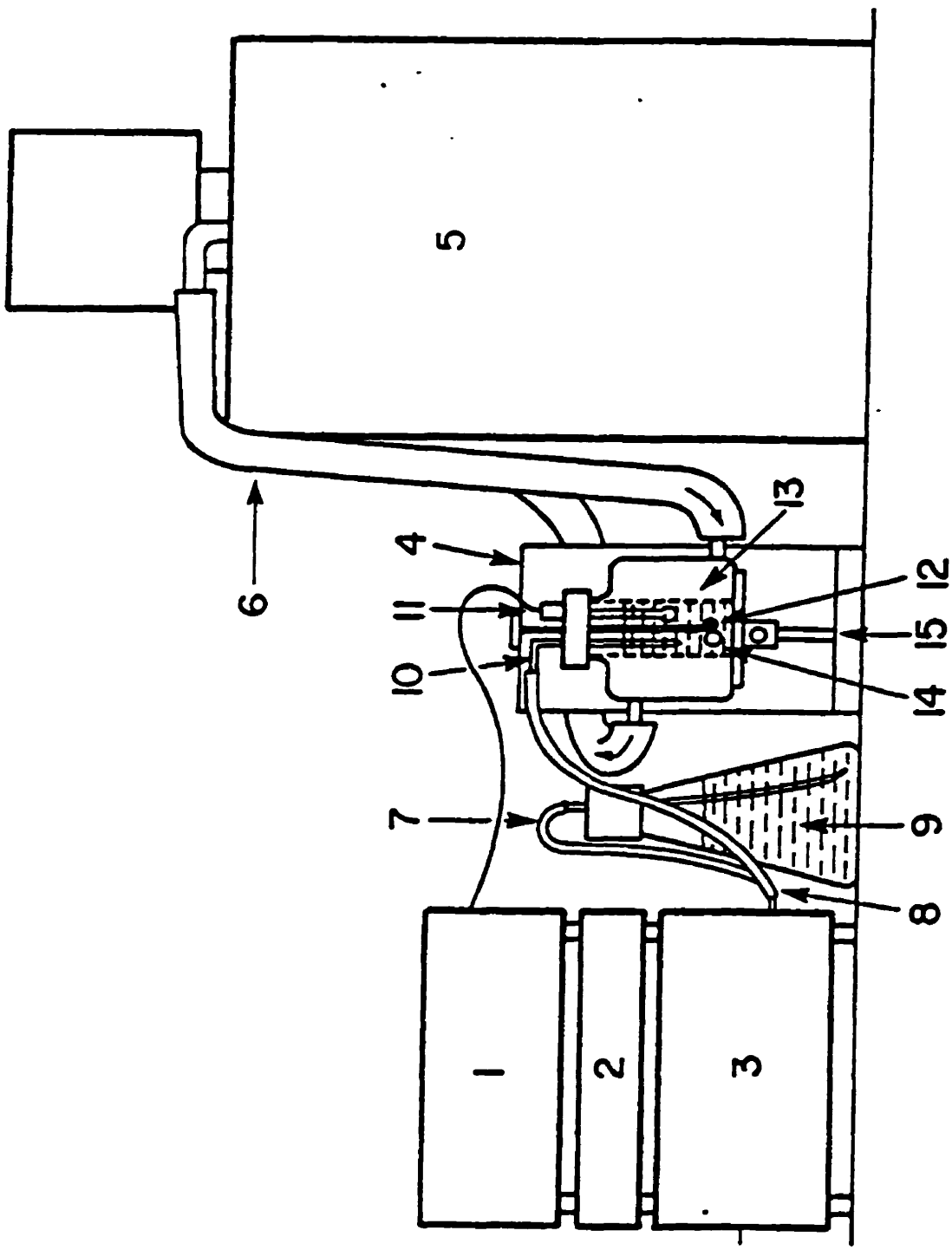

Figure 3.3 Glass jacketed reactor (Monshipouri 1991)

all values in mm

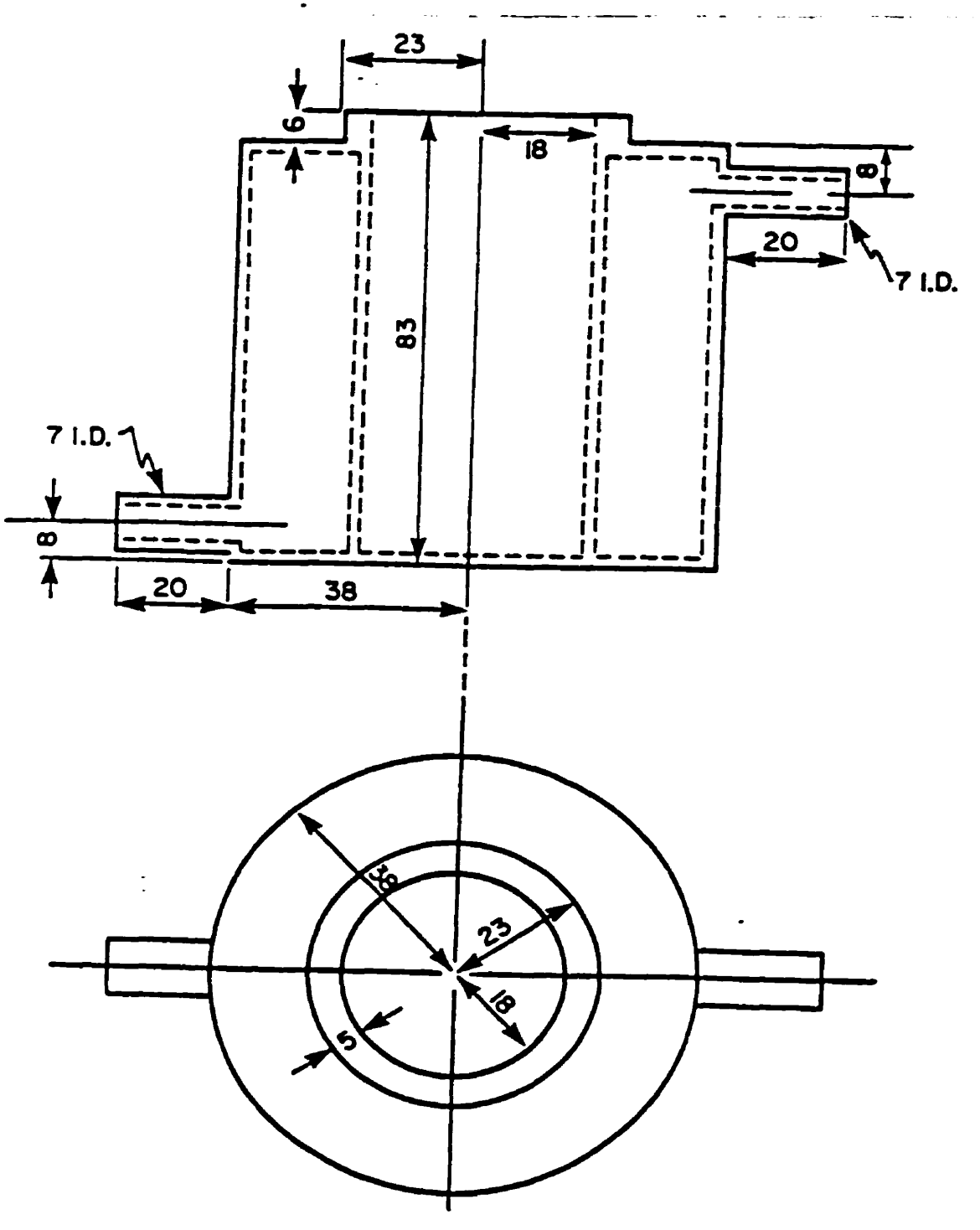

Table 3.1: pH-Stat Equipment Components (Refer to Figure 3.2)

Part Number	Part Description
1	pH Meter (Radiometer PHM 82)
2	Titrator (Radiometer TTT 80)
3	Autoburette (Radiometer ABU 80)
4	Titration Assembly (Radiometer TTA 80)
5	Polystat Refrigerated Circulator / Water Bath (Cole-Parmer)
6	Water Circulation Line - 1/2" Plastic Tubing
7	Acid Inlet Line - 3/16" Plastic Tubing
8	Acid Outlet Line - 3/16" Plastic Tubing
9	Acid Reservoir
10	Acid Addition Pipette - 3/16" OD Glass Tube (Radiometer)
11	pH Probes pH Glass Electrode (Radiometer PHG 200)
	Reference Electrode (Radiometer REF 201)
12	Plastic Stirring Rod (Radiometer)
13	Glass Water Jacket (see Figure 3.2)
14	Glass Reaction Vessel (see Figure 3.2)
15	Reactor Support (Radiometer)

3.4.2 Activity of free urease

The pH probes were calibrated using buffers at pH 4 and 7. If the efficiency was less than 98%, the probes were cleaned in a saturated solution of Pepsin (American Chemicals Ltd., Montreal) in 0.1 M HCl for 1 h. A 0.33 M Urea (American Chemicals Ltd., Montreal) solution was prepared and held at 25°C. Dithiothreitol (1 mM) was also prepared and kept on ice to prevent degradation.

An assay was conducted by mixing 40 ml 0.33 M urea solution with 100 µL 1 mM dithiothreitol at 25°C. The final concentration of dithiothreitol in the reactor was 2.5 µM. Dithiothreitol is a reducing agent that inhibits enzyme agglomeration via disulphide bonds which often leads to inconsistent activity measurements (Lencki 1987). The pH was adjusted to 7.0 with 20% NaOH. Urease solution (20 mg/ml) was added and the titrator was started. The volume of acid added to maintain the pH set point of 7.0, was recorded at 1 sec intervals for 3 min. The acid addition rate is the slope of the volume of acid added vs. time graph. The slope was calculated using the least squares method with the data obtained. For sample calculations see Appendix A.

The acid used as the titrating agent was HCl. HCl was selected because it has a relatively small anion that, at low concentrations, does not significantly affect the urease activity (Lencki 1987). A balance must be struck between the amount of enzyme added to the reactor and the concentration of the titrating acid. Enzyme concentration should be high enough to provide a recordable activity. The acid concentration should also be high enough that the dilution of the reaction solution is not a factor, but not high enough that the system pH oscillates widely. The control settings on the pH stat were obtained

through trial and error and will vary as the acid or enzyme concentration is varied. The typical pH-stat settings are summarized in Table 3.2.

Table 3.2: pH-Stat Settings for Free Urease Operation

Parameters	Setting
Autoburette	
Speed	5 - 20
Volume	1 / 10
Titrator	
Proportional Band	0.05 - 0.2
Delay Sec.	pH - stat
Reactor Volume	0.040 l
Urea Concentration	0.33 M
Urease Concentration (in reactor)	6.00×10^{-4} g / L
Dithiothreitol Concentration	2.50×10^{-6} M
Acid Used	HCl
Acid Concentration	0.01 M
Run Time	3 min

3.4.3 Activity of encapsulated urease

The procedure for determining the activity of encapsulated urease is similar to the procedure for free urease. 10 beads were weighed and placed into 40 ml 0.33 M urea with 2.5 μM dithiothreitol, at 25° C and pH 7.0. The assay was run for 10 min, to compensate for the mass transfer of urea and the reaction products into and out of the beads. Data points were collected at 1 sec intervals using an automatic data acquisition program. After approximately 5 min, the rate of HCl addition became linear and the slope could be calculated by least squares fitting of a straight line through the data points. Using the activity of the free urease solution that was encapsulated, the quantity of active encapsulated urease could be determined. All assays were done within 3 h of bead preparation. See Appendix A for sample calculations.

The supernatant of the bead forming solution was tested for enzyme activity, if any. This was done to verify that no urease had diffused out of the beads during formulation.

3.5 Membrane coating of alginate - urease beads

3.5.1 Chitosan membranes

A chitosan membrane was applied to the alginate beads to protect the encapsulated urease from deactivation by the protease α-chymotrypsin. The chitosan solution was prepared by dissolving 0.8 g of chitosan (Pronova, Washington) in 90 ml distilled water. 200 μl glacial acetic acid (Baker's Analyzed, Montreal) was added to facilitate the dissolution of the chitosan. After the chitosan was fully dissolved, the solution was

filtered using Whatman #4 filter paper and brought up to 100 ml for a final concentration of 0.8% w/v). The pH was adjusted to 5.6 by dropwise addition of 20% (v/v) NaOH (Fisher Scientific, Montreal), the precipitates formed were dissolved by mixing with a magnetic stirrer. When not in use, the chitosan solutions were stored at 4°C for not more than 3 days.

Low (CL 110), medium (CL 210), and high molecular weight chitosan (CL 310) were provided by Pronova. The procedure used to make the solution and coat the alginate beads was the same for all three types of chitosan. A batch of alginate beads was added to 50 ml chitosan solution and gently mixed during membrane formation using a magnetic stirrer for 45 min at room temperature. Once coated, the beads were filtered using a 50 μm mesh, washed with distilled water, and surface dried with kimwipes and filter paper. The beads were then placed in pre-weighed, sealable sample holders to prevent further dehydration and shrinkage, then weighed.

A chitosan solution was also prepared by mixing 0.4 g low and 0.4 g high molecular weight chitosan in 90 ml distilled water and 200 μl acetic acid. After filtering the solution, distilled water was added to bring the solution volume to 100 ml, for a final concentration of 0.8% (w/v). The pH was then adjusted to 5.6 with 20% (v/v) NaOH. The alginate beads were coated using the same method described in section 3.5.1.

3.5.2 Poly-L-lysine membranes

Poly-L-lysine solution was made by dissolving 50 mg poly-L-lysine (Sigma, St. Louis) in 100 ml distilled water for a final concentration of 0.05% (w/v). The pH was adjusted to 7.0 with 20% (v/v) NaOH.

A batch of alginate beads was added to 50 ml poly-L-lysine solution and gently mixed during membrane formation using a magnetic stirrer for 10 min at room temperature. Once coated, the beads were filtered using a 50 μm mesh, washed with distilled water, and surface dried with kimwipes and filter paper. The beads were then placed in pre-weighed, sealable sample holders to prevent further dehydration and shrinkage, then weighed.

Two types of poly-L-lysine of different molecular weights were used in this work; a low molecular weight poly-L-lysine (MW=25 000) and a high molecular weight poly-L-lysine (MW=114 700). Both were prepared in the same manner.

3.5.3 Poly(methylene co-guanidine) membranes

Poly(methylene co-guanidine) (Scientific Polymer Products, Ontario NY) was available at 35% in H_2O solution. A 1% (v/v) solution was obtained by mixing 1.43 ml of the 35% poly(methylene co-guanidine) solution and 48.57 ml H_2O.

A batch of hardened alginate beads was added to 50 ml poly(methylene co-guanidine) solution and gently mixed during membrane formation using a magnetic stirrer for 20 min at room temperature. Once coated, the beads were filtered using a 50 μm mesh, washed with distilled water, and surface dried with kimwipes and filter paper. The

beads were then placed in pre-weighed, sealable sample holders to prevent further dehydration and shrinkage, then weighed.

3.5.4 Poly(methylene co-guanidine) - chitosan membranes

Another method explored to protect encapsulated urease from proteases was to double-coat the beads, first with a 0.8% (w/v) medium molecular weight chitosan, and then with a 1.0% (v/v) poly(methylene co-guanidine) solution. The procedures followed were the same as for the individual membranes.

3.6 Protease deactivation of urease

Coated and uncoated alginate beads were exposed to three proteases of varying molecular weights to determine the effectiveness of the chitosan membrane coatings. A 0.5% (w/v) α-chymotrypsin (Sigma, St. Louis), 0.67% (w/v) trypsin (Sigma, St. Louis), or 2.0% (w/v) proteinase K (Sigma, St. Louis) solution was prepared. Ten pre-weighed, surface dried beads were added to 5 ml of the enzyme solution and mixed for 10 min on a vortex mixer on shake mode, with a test tube adaptor. After 10 min, the supernatant was removed using a vacuum aspirator and the beads were immediately assayed for activity as described in section 3.4.3.

3.7 Bead lyophilization

Coated and uncoated alginate beads were weighed and placed in plastic sample holders. The samples were then lyophilized using a freeze point lyophilizer (Lyph-Lock 6 Freeze Dry System, Labconco, Kansas City) for 8 h, and rehydrated in 0.05M $CaCl_2$ solution for 3 h. The beads were filtered using a 50 μm mesh and either assayed for activity (section 3.4.3) or exposed to 0.5% chymotrypsin (Section 3.6.1).

4.0 RESULTS

4.1. Urease activity

The activity of free and encapsulated urease was measured in an unbuffered urea solution at pH 7.0, using a pH stat titrator. The urease activity was determined by measuring the rate of HCl addition needed to maintain the solution pH at the setpoint value of 7.0 as shown in Figure 4.1. Table 4.1 shows the activities of free urease within a concentration range of 0.06 to 1.0 mg/ml. As can be noted, the urease activity does not change appreciably with concentration. The average activity of free urease was 82.1 IU/mg at pH 7.0 and 25°C in a 0.3 M urea solution.

Table 4.1: Urease Activity At Different Urease Concentrations

Urease Conc. (mg/ml)	Urease Activity (IU/mg)
0.06	79.8
0.10	85.8
0.34	85.8
0.54	86.0
0.70	83.0
1.00	79.3

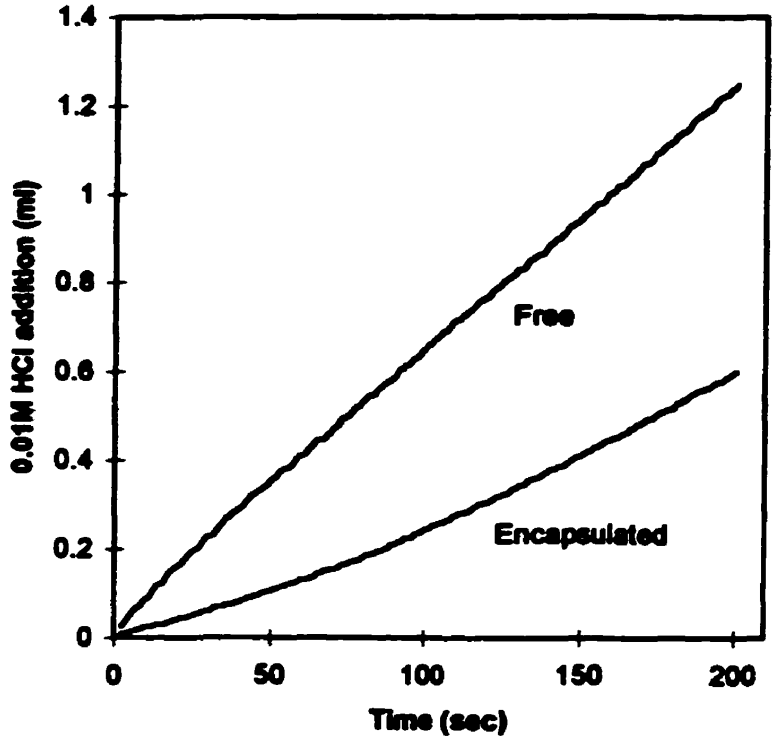

Figure 4.1

HCl addition required to maintain pH setpoint of 7.0 during urea hydrolysis with free and encapsulated urease

4.1.1. Effects of encapsulation matrix materials on urease activity

The concerns in using urease activity to determine yield of encapsulation are mass transfer effects and possibility of the matrix materials interfering with urease activity measurements. Monshipouri (1991) reported that mass transfer effects occurring at the interface were minimized by using high mixer velocities. The same conditions were used in the production of the gels and the mass transfer effects within the gel was assumed to be the same from experiment to experiment. To determine if the matrix materials interfere with urease activity, assays were conducted, while varying chitosan and poly-L-lysine concentrations and molecular weights, and co-guanidine or alginate concentrations. All experiments were conducted at pH 7.0 and 25° C in 0.33M urea with 1 mg/ml urease.

Table 4.2 shows that there was no interference on urease activity from alginate. Initially, higher alginate concentrations resulted in markedly lower activity values. This problem was overcome by using a higher mixing speed. The higher alginate concentrations resulted in more viscous solutions, limiting activity through mass transfer effects.

Table 4.2: Urease Activity At Different Alginate Concentrations

Alginate (%)	Urease Activity (IU/mg)
0.0	79.7
0.5	84.5
1.0	76.3
1.5	79.4
2.0	78.3
2.5	69.7
3.0	79.3

Chitosan also did not interfere with urease activity as seen in table 4.3. CL 110 chitosan is a low molecular weight and CL 210 a medium molecular weight chitosan.

Table 4.3: Urease Activity At Different Chitosan Concentrations

Chitosan (%)	Chitosan Type	Urease Activity (IU/mg)
0.0	Control	82.8
0.5	CL 110 (Low MW)	81.4
0.8	CL 110 (Low MW)	68.1
0.5	CL 210 (Med MW)	78.3
0.8	CL 210 (Med MW)	82.5

Poly-L-lysine did not interfere with urease activity as seen in table 4.4. The initial urease activity (224 IU/mg) in Tables 4.4 and 4.5 is different from the initial urease activities shown in Tables 4.1, 4.2, 4.3 (≈ 80 IU/mg) because a new and fresh batch of urease was used.

Table 4.4: Urease Activity At Different Poly-L-Lysine Concentrations

Poly-L-Lysine (%)	Poly-L-Lysine Type	Urease Activity (IU/mg)
0.0	Control	217
0.05	MW=25 000	215
0.12	MW=25 000	215
0.05	MW=114 700	217
0.12	MW=114 700	218

Poly(methylene co-guanidine) appears to deactivate or inhibit urease as seen in Table 4.5. Approximately 90% of the urease activity is eliminated in the presence of co-guanidine.

Table 4.5: Urease Activity At Different Co-Guanidine Concentrations

Co-Guanidine (%)	Urease Activity (IU/mg)
0.0	224
0.5	26.8
1.0	26.3
1.5	23.5

4.1.2. Free urease deactivation by proteases

The proteases chymotrypsin, trypsin, and proteinase K inactivate urease through hydrolytic action. The activity of free urease during protease exposure is shown in Figure 4.2. All proteases rapidly decreased free urease activity. After 10 min exposure, 0.5% chymotrypsin, 2.0% proteinase K, and 0.67% trypsin reduced the urease activity to 50, 60, and 25% of the initial activity respectively.

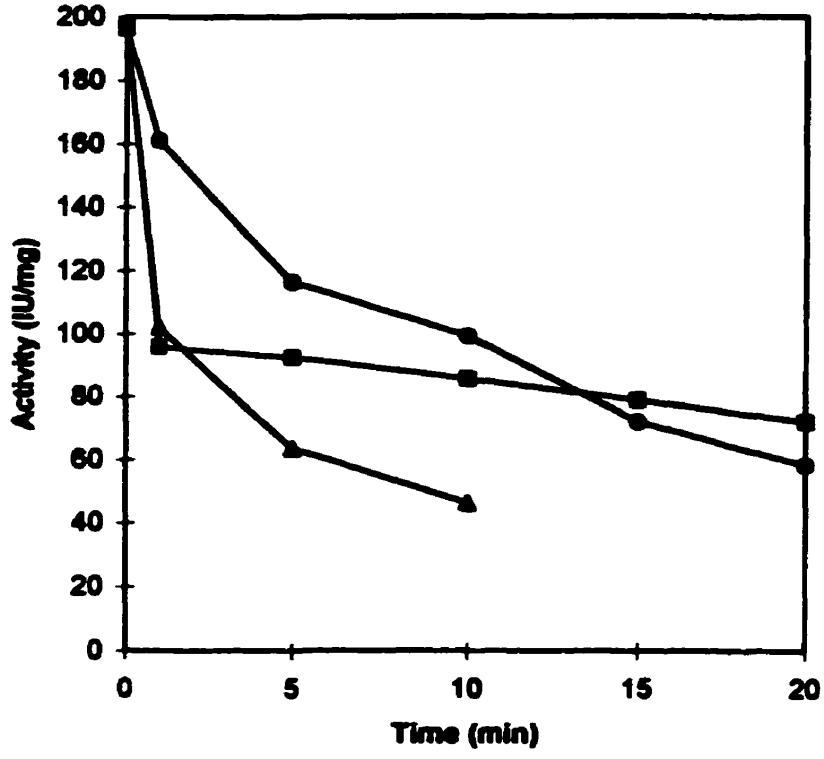

Figure 4.2

Activity loss of urease exposed to 0.5% chymotrypsin (circles), 2.0% proteinase K (squares), and 0.67% trypsin (triangles)

4.2 Membrane coated alginate beads as a protective barrier to chymotrypsin

Alginate beads containing urease were exposed to chymotrypsin for 10 min to determine if the gel was able to exclude the protease. Approximately 98% of the activity was lost, compared to beads which had not been exposed to chymotrypsin. In an effort to increase the exclusion of chymotrypsin, alginate beads were coated with poly(methylene co-guanidine), poly-L-lysine, and chitosan membranes, and the relative effectivess of these membranes in excluding chymotrypsin was evaluated. The supernatants of the solutions used to form chitosan and poly-L-lysine membrane coated beads tested negative for urease activity indicating that there was no enzyme loss or leakage during formulation. The poly(methylene co-guanidine) coating solution showed enzyme activity as described below.

4.2.1. Poly(methylene co-guanidine) membranes as a barrier to chymotrypsin

Co-guanidine membranes were applied to alginate beads to protect the encapsulated urease from chymotrypsin. Uncoated beads were used as the control and the active urease content was normalized and assigned 100% activity. When the uncoated beads were exposed to 0.5% chymotrypsin for 10 min, the residual activity was 1.8%.

Two different coating concentrations were used to evaluate the co-guanidine membrane. The activity of the encapsulated urease in the coated beads decreased with the higher co-guanidine concentration as seen in Figure 4.3. Increasing the coating concentration from 0.5 to 1.5% decreased the activity from 83 to 5.4%. When the coated

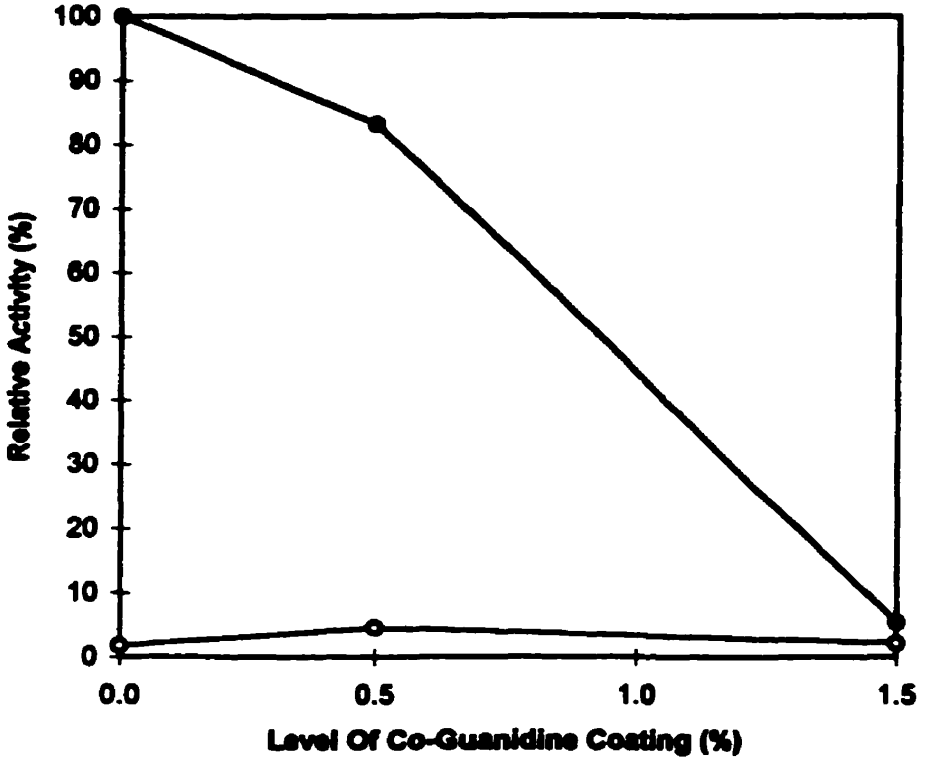

Figure 4.3

Level of urease activity within alginate beads, coated with increasing concentrations of co-guanidine. Closed symbols represent encapsulated urease activity not exposed to chymotrypsin and open symbols represent encapsulated urease activity in the presence of 0.5% chymotrypsin.

beads were exposed for 10 min to 0.5% chymotrypsin, the residual activity was 4.5 and 2.2% for the 0.5 and 1.5% coating levels respectively

The coating method was modified by applying 0.5% co-guanidine, then a second coat with 1.5% co-guanidine. The resulting activity in the coated beads was 8.5%, and when the beads were subsequently exposed to chymotrypsin, there was no residual activity. Another modification to the coating method was done by slowly increasing the co-guanidine concentration from 0.1% to 1.5%. The objective was to gradually apply the membrane coat to protect the urease from co-guanidine deactivation. The coated beads contained no active urease. Testing for urease activity in the coating solution supernatant showed that there was 1.4% active urease of the total initial urease. Earlier it was shown that co-guanidine lowered free urease activity by approximately 90% (see section 4.1.1). Using this ratio it appears that 14% of the urease leaked out of the beads during coating.

4.2.2. Poly-l-lysine membrane as a barrier to chymotrypsin

Poly-L-lysines (PLL) of two different molecular weights were used to coat alginate beads to protect the encapsulated urease from chymotrypsin. One PLL had a molecular weight of 25,000 and the other a molecular weight of 114,700. The high molecular weight PLL was analyzed at three different coating levels and the low molecular weight PLL at two, as shown in Figure 4.4. The active urease content of uncoated beads was normalized and assigned 100% activity. When the uncoated beads were exposed for 10 min to 0.5% chymotrypsin, the residual activity was 0.9%.

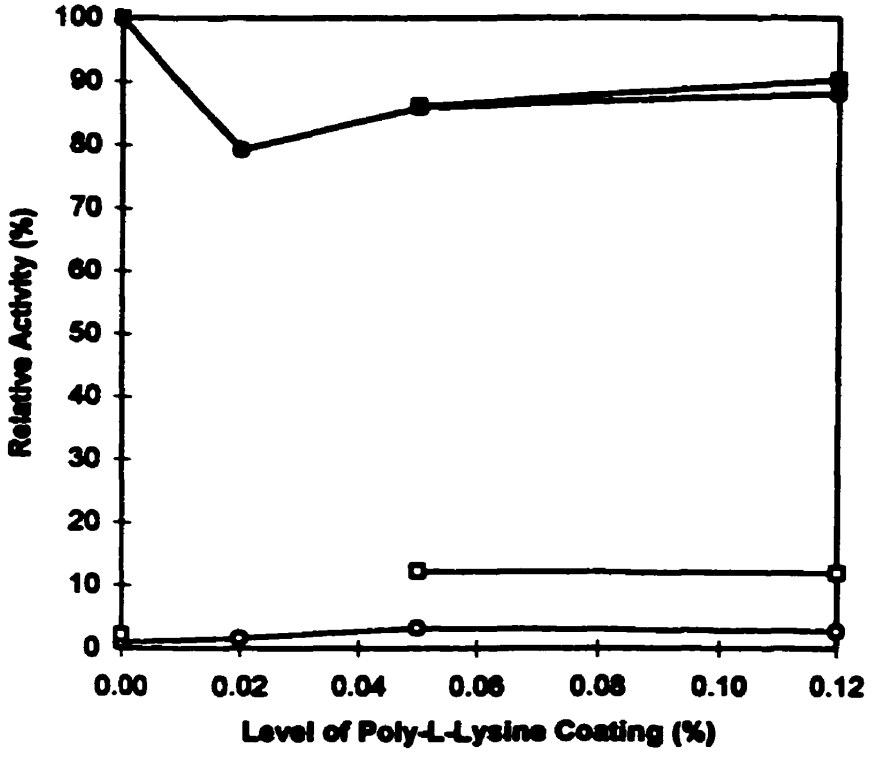

Figure 4.4

Level of urease activity within alginate beads, coated with increasing concentrations of poly-L-lysine. Closed symbols represent encapsulated urease activity and open symbols represent encapsulated urease activity in the presence of 0.5% chymotrypsin. Results compare coatings with high (circles) and low (squares) MW poly-l-lysine.

The highest level of chymotrypsin exclusion was given by the low molecular weight PLL, with an average 12% activity retention. High molecular weight PLL provided an activity retention of 2%.

Increasing the PLL concentration levels did not have a significant effect on chymotrypsin exclusion as shown in Figure 4.4. Low molecular weight PLL applied at 0.05 and 0.12% coating levels resulted in 12.5% and 11.9% activity retention. For high molecular weight PLL, the 0.02, 0.05, and 1.2% coating levels resulted in activity retentions of 1.6%, 3.2%, and 2.7% respectively.

The increase in chymotrypsin exclusion appears to be determined by the coating polymer molecular weight. PLL coatings exclude chymotrypsin more effectively than the co-guanidine coatings and PLL does not deactivate urease.

4.2.3. Chitosan membrane as a barrier to chymotrypsin

Three different chitosans of varying molecular weight were used to coat alginate beads to protect the encapsulated urease from chymotrypsin. The membranes were classified as low (CL 110), intermediate (CL 210), and high (CL 310) molecular weight. Each were analyzed at three different coating levels, as shown in Figure 4.5. The active urease content of uncoated beads was normalized and assigned 100% activity. When the uncoated beads were exposed to 0.5% chymotrypsin, the residual activity was 2.0%.

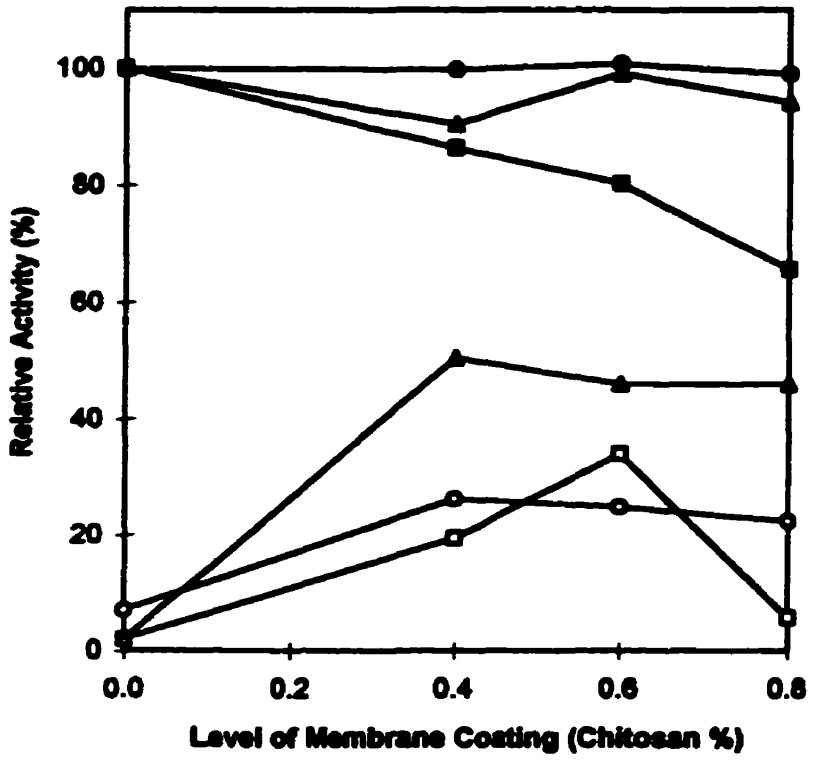

Figure 4.5

Level of urease activity within alginate beads, coated with increasing concentrations of chitosan. Closed symbols represent encapsulated urease activity and open symbols represent encapsulated urease activity in the presence of 0.5% chymotrypsin. Results compare coatings with high (circles), medium (triangles), and low (squares) MW Chitosans.

The highest chymotrypsin exclusion was provided by the intermediate molecular weight chitosan, with an average of 48% activity retention. The low and high molecular weight chitosans had lower activity retention of 20 and 25% respectively.

Increasing the chitosan concentration levels did not have a significant effect on chymotrypsin exclusion. Intermediate MW chitosan applied at 0.4% resulted in 50.5% activity retention, while 0.6 and 0.8% coating levels resulted in 46.0% retention. For high MW chitosan, the 0.4, 0.6 and 0.8% coating levels resulted in activity retentions of 26.2%, 24.8%, and 22.3% respectively. The low molecular weight chitosan offered highest protection against chymotrypsin at 0.6% coating concentration with 33.9% activity retention. For chitosan concentrations of 0.4 and 0.8%, the activity retentions were 19.4% and 5.6% respectively.

As was the case with the poly-L-lysine membranes, the difference in activity retention at different levels of membrane coating for a given molecular weight, was not very significant. It is the molecular weight of the membrane that appears to determine chymotrypsin exclusion.

4.2.4. A mixture of low and high MW chitosans as a barrier to chymotrypsin

Chitosan membranes were formed by combining equal amounts of high and low molecular weight chitosans. The membranes were used to coat alginate beads to protect the encapsulated urease from chymotrypsin. The membranes were applied at 0.8 and 1.5% chitosan coating levels. The active urease content of uncoated beads was normalized and assigned 100% activity. When the uncoated beads were exposed for 10

min to 0.5% chymotrypsin, the residual activity was 2.2% as seen in Figure 4.6. When the beads with 0.8 and 1.5% chitosan coating level were treated with chymotrypsin, the residual activities were 30.0% and 32.2% respectively.

The membrane made from the combination of the low and high MW chitosans is more impermeable to chymotrypsin than the individual chitosans, but more permeable than the intermediate MW chitosan (See section 4.2.3). As with the chitosan and PLL membranes, changing the polymer concentration did not significantly change the chymotrypsin exclusion.

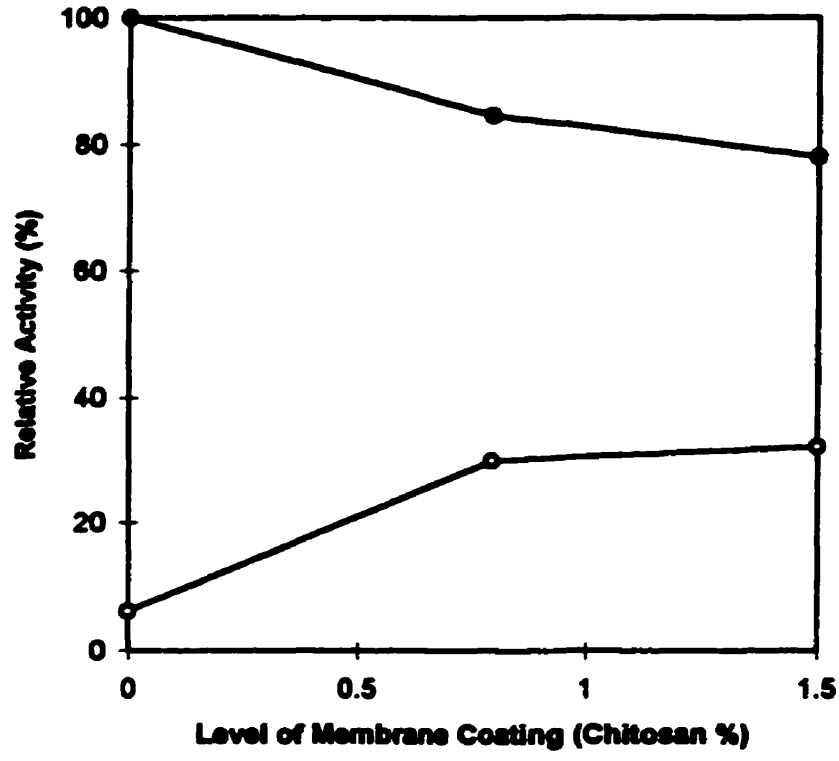

Figure 4.6

Level of urease activity within alginate beads, coated with increasing concentrations of mixed molecular weight chitosans. Closed symbols represent encapsulated urease activity and open symbols represent encapsulated urease activity in the presence of 0.5% chymotrypsin.

4.2.5. Using co-guanidine on chitosan coated beads as a barrier to chymotrypsin

A double membrane was formed on alginate beads to protect the encapsulated urease from chymotrypsin. The first coating was intermediate molecular weight chitosan at a concentration of 0.4%. The chitosan coated beads were then coated with 1% co-guanidine. The purpose was to protect the urease from co-guanidine deactivation using the chitosan membrane. Following chymotrypsin exposure, there was no residual urease activity indicating that this combination of coatings did not offer any protection from chymotrypsin.

4.2.6. Lyophilizing chitosan coated beads as a barrier to chymotrypsin

Lyophilization or freeze drying, dehydrates the beads causing them to shrink, reducing the pore size. As the beads are rehydrated, they do not regain their initial diameter (Kearney et al 1990), thus pores are effectively smaller than that of fresh beads.

Coated and uncoated alginate beads containing urease were lyophilized to protect the urease from chymotrypsin. The active urease content of fresh uncoated beads was normalized and assigned 100% activity. When exposed for 10 min to 0.5% chymotrypsin, the residual activity was 5.3%. The active urease content of the lyophilized and rehydrated beads was 23.5 and 24.2% for the uncoated and chitosan coated beads respectively. Thus it appears that approximately 76% of the activity is lost during lyophilization and rehydration.

When rehydrated uncoated beads were exposed to chymotrypsin, the residual activity was 71% of the non-exposed controls. When the chitosan coated rehydrated

beads were exposed to chymotrypsin, the residual activity was 89% of the non-exposed controls. It appears that lyophilized and rehydrated beads offer a high level of protection from chymotrypsin exposure.

4.3. Chitosan membrane as a barrier to trypsin and proteinase K

Alginate beads, coated with intermediate molecular weight chitosan were used to evaluate the bead's activity retention when exposed to chymotrypsin, trypsin, and proteinase K. Chymotrypsin and trypsin were each analyzed at two different coating levels and proteinase K at one coating level, as shown in Figure 4.7. The active urease content of uncoated beads was normalized and assigned 100% activity. When the uncoated beads were exposed for 10 min to 0.5% chymotrypsin, the residual activity was 8.6%, 5.4% when exposed to 0.67% trypsin, and 69.2% when exposed to 2.0% proteinase K.

At a coating level of 0.4% chitosan, exposures to chymotrypsin, trypsin, and proteinase K resulted in activity retention of 50.6, 44.4, and 75.3% respectively. At a 0.8% chitosan coating level, exposure to chymotrypsin resulted in a retention of 47.0% of the urease activity and 43.2% following trypsin exposure.

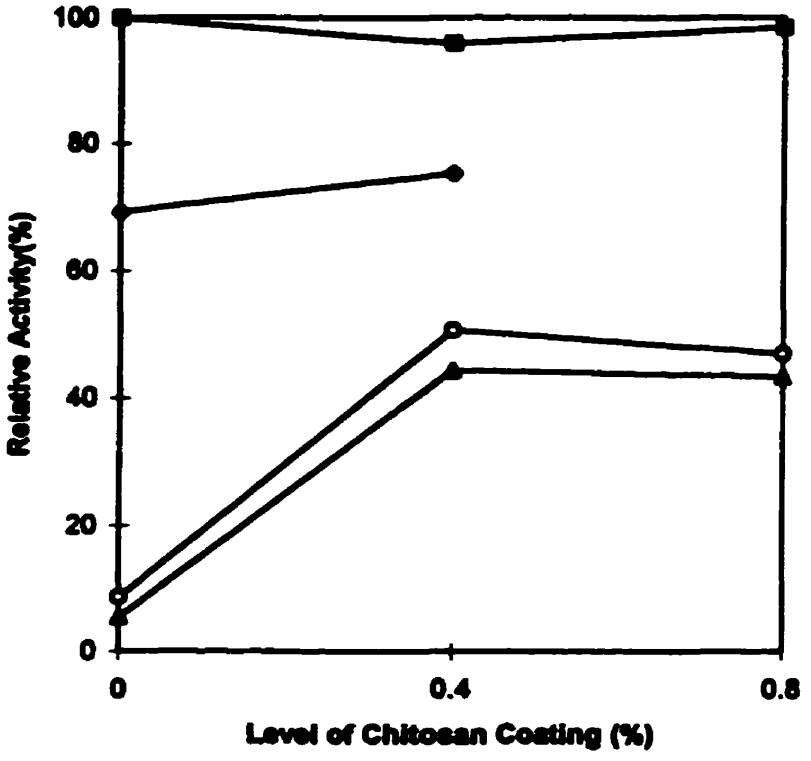

Figure 4.7

Level of encapsulated urease activity within chitosan coated alginate beads, exposed to three different proteases. Closed symbols represent encapsulated urease activity not exposed to proteases and open symbols represent encapsulated urease activity in the presence of proteases. The results compare beads treated with 0.5% chymotrypsin (circles), 0.67% trypsin (triangles), and 2.0% protease K (diamonds).

5.0 DISCUSSION

There are approximately 300 genetic disorders that result in an absence of certain metabolic enzymes or in an enzyme activity disorder. The problems associated with these disorders may have severe toxic effects. One example is phenylketonuria (PKU). This disorder is characterized by a deficiency of the enzyme phenylalanine hydroxylase (PAH), which hydroxylates the amino acid phenylalanine to tyrosine. If this condition is not treated, phenylalanine accumulates in the blood and severe mental deficiencies result (Safos 1995). One of the treatments of this disorder is to supply the patient with a diet low in phenylalanine, but these diets are usually unpalatable and difficult to monitor and implement, especially in young people. Another treatment is to administer the deficient enzyme, PAH, orally. One of the problems this presents is that the proteolytic enzymes present in gastric juices deactivate the dosed enzyme. A way to overcome this problem is to encapsulate the enzyme. Much work has been done in the field of encapsulation for intestinal delivery of drugs (Bourget and Chang 1985, Hari et al. 1996, Leusen et al. 1996, Miyazaki et al. 1994), and the use of membranes for the retention and protection of the encapsulated species.

In the present study, different membranes were evaluated for their capacity to exclude α-chymotrypsin and other proteases from alginate beads. The exclusion of chymotrypsin was quantified by measuring the deactivation of urease which was encapsulated in the alginate beads.

Alginate was selected as the immobilization matrix because it is one of the most extensively used immobilization mediums available (Vorlop and Klein 1983). Alginate has

been used to encapsulate proteases (Chui and Wan 1997), proteins (Elcin 1995, Polk et al. 1994, Shah et al. 1995, Wheatley et al. 1991), hemoglobin (Huguet et al. 1994), and microbial cells (Kearney 1990) to just name a few. The popularity of alginate is mainly due to its ease of use, its ability to form gels by ionic cross-linking with divalent cations such as calcium, and its mild formulation conditions. Other encapsulation methods may employ harsh chemicals such as organic solvents, or harsh conditions such as elevated temperatures that have the potential to destroy the activity of sensitive proteins. Alginate is also biocompatible and non-toxic, and used as a food additive and thickener. The main disadvantage of alginate beads is their low stability in the presence of phosphates, citrates or other substances that have a high affinity for Ca^{+2}. Alginate beads are also destabilized by non-gel-inducing ions such as high concentrations of sodium and magnesium that replace the calcium ions in the gel matrix (Smidsrod and Skjak-Braek 1990). Scale-up of bead production has also been problematic due to the limitations of extrusion systems. The emulsification / internal gelation technique offers the potential for large scale applications (Poncelet et al. 1992).

Alginate beads have been surface coated with polycationic polymers that form a membrane, to prevent or slow the release of encapsulated material (Huguet 1996, Hari et al. 1996). Polyelectrolyte complexes are formed when an anionic and a cationic polymer are simultaneously present in an aqueous solution. Alginate-chitosan is an example of the complex formed by two oppositely charged polymers. The electrostatic interaction of anionic carboxyl groups of alginate with the cationic amine groups of chitosan and the resulting entanglement of the polymers forms a strong and flexible membrane (Polk et al.

1994). Poly-L-lysine was also found to ionically interact with alginate and is used to prepare alginate coated microcapsules (Goosen et al. 1985).

The activity of both free and encapsulated urease was assayed using the pH-stat technique, which has several advantages over other activity assay procedures (Sumner 1951, van Slyke and Archibald 1944). The pH-stat method controls the reaction pH at the set-point value (pH 7.0) by precise addition of acid. The rate of acid addition over time and the ratio of ammonia production to the acid addition rate for the set-point pH (Monshipouri 1991) are used to determine the enzyme activity.

The traditional way of measuring urease activity uses phosphate buffer. Sumner (1951) recognized that the buffer components have an effect on the pH profiles and kinetics of urease. Dixon et al. (1980) showed that the phosphate monoanion ($H_2PO_4^-$) is a strong competitive inhibitor of urease. Urease assays performed in phosphate buffers give lower activities than the activities determined in the absence of buffer. The advantage the pH-stat method has is that it does not need buffers to maintain a constant pH. Rather it controls the reaction pH by direct addition of acid or base if the set-point pH is above the self buffering point (pH 8.8), of the urea system (Lencki 1987). In this manner the inhibitory effects of the buffers on the enzyme reaction are avoided. Another advantage is that the assay is performed quickly and reliably.

Urease encapsulated within uncoated alginate beads served as the control for this study. The activity was evaluated, normalized, and assigned 100% activity. After a 10 min exposure to 0.5% α-chymotrypsin, the residual activity in the uncoated beads was approximately 2%. Chymotrypsin is a protease of molecular weight of 21,600 (Righetti

and Caravaggio 1976), that hydrolyses urease and other proteins. Polk et al. (1994) and Tanaka (1984) found that low molecular weight proteins (MW = 65,000) freely diffused out of alginate beads. Based on Polk's and Tanaka's diffusion findings and that chymotrypsin is much smaller than the proteins used in their studies, it was expected that chymotrypsin would be able to freely diffuse into the alginate beads. The activity drop in uncoated beads after exposure to chymotrypsin confirms that it is freely diffusing into the alginate beads and deactivating the urease.

The alginate beads were coated with chitosan, poly-L-lysine, and poly(methylene co-guanidine) membranes in an effort to better exclude chymotrypsin. For the chitosan membranes, the chitosan molecular weight had the strongest effect on chymotrypsin exclusion. Low and high molecular weight chitosans individually, did not exclude chymotrypsin as well as when the beads were coated with a combination of the high and low molecular weight chitosans. Polk et al. (1994) observed two trends concerning the molecular weights of chitosan. The first was that lower molecular weight chitosans increased the permeability of the beads and the second was that a combination of low and high molecular weight chitosans provided a lower permeability than the individual chitosans. It is probable that the longer chains of the higher molecular weight chitosan form a better lattice network, resulting in a more uniform membrane, while the shorter chains of the lower molecular weight chitosan fill in the gaps left by the longer chains forming a thicker and stronger membrane, improving chymotrypsin exclusion. It was hypothesized that the combination of low and high molecular weight chitosans would have greater penetration into the bead for a thicker, stronger, and more uniform membrane, but

that was not the case. The highest level of chymotrypsin exclusion was provided by intermediate molecular weight chitosan with approximately 50% of activity retention. This may be due to a better chitosan-alginate ionic interaction by the intermediate molecular weight chitosan.

As was the case with the chitosan membranes, the poly-L-lysine molecular weight had the greatest impact on the exclusion of chymotrypsin from alginate beads. The highest exclusion of chymotrypsin, with 12% activity retention, was obtained by coating the beads with poly-L-lysine of MW = 25,000. The higher molecular weight poly-L-lysine (MW = 114,000) had a much lower activity retention after treatment with chymotrypsin. This trend follows that discussed by Goosen et al. (1985) and Zou (1997). They observed that higher molecular weight poly-L-lysine membranes increased the permeability of the beads. As is the case with chitosan, the longer chain lengths of poly-L-lysine have more difficulty penetrating the alginate gel matrix, which results in a poorer poly-L-lysine-alginate ionic contact than would occur with shorter polymer chain lengths that can better penetrate into the gel matrix. Goosen reports achieving molecular weight cut-offs in the order of 67,000 using poly-L-lysine of MW = 17,000 and speculates that with a poly-L-lysine of MW = 4,000, even lower cut-off values could be achieved. A probable explanation for the difference in permeabilities of the chitosan and poly-L-lysine membranes might be the difference in membrane thickness. Zou's findings indicated that the chitosan membranes ranged from 30 μm to 50 μm, while the poly-L-lysine membranes ranged from 3 μm to 13 μm. Thus the thicker chitosan membrane is less permeable.

Poly(methylene co-guanidine) was tested as a membrane material, but the results showed no improvement in protection from chymotrypsin as compared to the uncoated beads. One of the reasons for this low activity retention might be that at high coating levels, the co-guanidine penetrated into the bead and deactivated the urease. When free urease was exposed to co-guanidine, the enzyme lost over 90% of its activity. At lower coating levels, it seems that co-guanidine diffusion into the bead was slower than at the higher coating level as evidenced by the higher retention of activity at the lower coating level (Figure 4.3), but the resulting membrane was not able to prevent chymotrypsin from penetrating the bead and deactivating the encapsulated urease. In an attempt to slow the diffusion of co-guanidine into the alginate beads at low coating levels, the coating method was modified. One modification consisted of forming a membrane on the beads at low level and then another membrane at high coating level. Another modification was to slowly increase the co-guanidine concentration from 0.1% to 1.5%. The purpose was to gradually apply the membrane coat to protect the urease from co-guanidine deactivation. A third modification was done by a 1% co-guanidine coating on alginate beads that had been pre-coated with chitosan. None of the modifications offered protection from chymotrypsin and in case of the third modification, the co-guanidine actually increased the permeability of the chitosan membrane.

Lyophilization and rehydration of alginate beads was evaluated as a possible way to protect the encapsulated urease from chymotrypsin. The dehydration process causes the beads to shrink and results in a more compact structure with a reduced pore size. After rehydration, the beads do not swell to their initial diameter (Kearney et al. 1990),

thus pores are effectively smaller than that of un-lyophilized beads. After lyophilization and rehydration, the beads retained about 25% activity, before exposure to chymotrypsin. Thus lyophilization deactivated 75% of the encapsulated urease, possibly due to protein denaturation. Alternatively it may be that the denser alginate matrix reduces urea diffusion, hindering access to the active site of urease.

The best protection against chymotrypsin deactivation was provided by the rehydrated beads. The chitosan coated rehydrated beads retained almost 90% of the activity after exposure to chymotrypsin, compared to the rehydrated beads not exposed to chymotrypsin. The uncoated beads retained 70% of their activity after being exposed to chymotrypsin. Kearney et al. (1990) reported that alginate beads had a 250% increase in mechanical strength after lyophilization. The combination of a stronger and denser structure and smaller pore size due to the decrease in bead diameter, contribute to the improved exclusion of chymotrypsin from lyophilized and rehydrated alginate beads.

Chitosan coated alginate beads were exposed to proteases of different molecular weight to evaluate the exclusion capacity of the membrane. The three proteases, chymotrypsin, trypsin, and proteinase K, were selected because of their ability to hydrolyze urease. Chymotrypsin has a molecular weight of 21,600 (Righetti and Caravaggio 1976), trypsin 23,000 (Hofmann 1964), and proteinase K 28,930 (Jany et al. 1986). The residual activity values for the coated and uncoated beads exposed to chymotrypsin were similar to the values for the beads exposed to trypsin. This may be expected, due to the small difference in molecular weight between the two proteases. Based on the molecular weight of proteinase K (MW = 28,930) it was expected that the

residual activity values after exposure, would be similar to those of chymotrypsin and trypsin. However, the retained activity after exposure to proteinase K was almost 70% compared to unexposed beads. The low permeability of the beads to proteinase K are probably due to a hydrophobic interaction between the protease and the hydrophilic alginate, instead of a molecular weight size exclusion. Charge effects seem unlikely since the isoelectric points of the three proteases are similar.

In summary the best exclusion of chymotrypsin (MW = 21,600) from alginate beads was provided by lyophilization and rehydration of the beads, with 89% encapsulated urease activity retention for chitosan coated beads, compared with rehydrated beads not exposed to chymotrypsin, and 70% activity retention for uncoated beads.

Chitosan and poly-L-lysine membrane coating of alginate beads offered 50 and 12% activity retention respectively, after treatment with chymotrypsin. Poly(methylene co-guanidine) membranes did not provide chymotrypsin exclusion.

6.0 CONCLUSIONS

Encapsulation of proteins within alginate gels, for the treatment of metabolic enzyme disorders has recieved recent attention. A drawback of alginate as encapsulation matrix is that it has a wide pore size distribution and the encapsulated proteins may be deactivated by proteolytic enzymes present in gastric juices, that diffuse into the bead. One of these proteolytic enzymes is α-chymotrypsin (MW = 21,600). In this study different membranes were evaluated for their capacity to exclude α-chymotrypsin and other proteases from alginate beads. The exclusion of chymotrypsin was quantified by measuring the deactivation of encapsulated urease. The activity of both free and encapsulated urease was assayed using the pH-stat technique, which controls the reaction pH by direct addition of acid. The advantage of using this technique is that it does not involve the use of phosphate buffers, that have inhibitory effects on the enzyme reaction and lower the enzyme's activity.

The lowest level of urease deactivation by chymotrypsin was given by lyophilizing and rehydrating the beads. By lyophilizing alginate beads containing urease, the activity retention after exposure to chymotrypsin, was increased from 2 to 70%. By coating the beads with a chitosan membrane prior to lyophilization, the activity retention was raised to 90%. The problem associated with lyophilization is that the encapsulated urease initially looses about 75% of its activity, but this may be improved by modifying the encapsulation and lyophilization methods.

As an alternative way to exclude chymotrypsin from alginate beads, chitosan, poly-L-lysine, and poly(methylene co-guanidine) membrane coatings were applied. The highest

level of chymotrypsin exclusion was provided by intermediate molecular weight chitosan with approximately 50% activity retention. A combination of high and low molecular weight chitosans provided 30% activity retention. Beads coated with poly-L-lysine only provided 12% activity retention after exposure to chymotrypsin. Beads were also coated with co-guanidine membranes, but following chymotrypsin exposure there was no residual urease activity, indicating that co-guanidine did not provide protection from chymotrypsin.

Gastric fluids contain many proteases that can affect the activity of encapsulated proteins. The effects of two other proteases, trypsin and proteinase K in addition to chymotrypsin, were evaluated. Chitosan coated alginate beads were exposed to trypsinresulting in 44.4% urease activity retention, similar to that obtained from chymotrypsin (50%). Both proteases have roughly the same molecular weight, chymotrypsin 21,600 and trypsin 23,000. The exposure of chitosan coated beads to proteinase K (MW = 28,930) resulted in 75% activity retention. Increased exclusion of proteinase K may be due to the larger molecular weight.

7.0 REFERENCES

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8.0 APPENDIX

Appendix A

Sample calculation of free urease activity:

Urease activity measurement at pH 7.0 and 25°C was conducted as follows:

Urease dilution concentration = 400 mg/l

Amount added = 1.0×10^{-4} l

Weight of urease added = 1.0×10^{-4} l x 400 mg/l = 0.040 mg

Reactor volume = 40×10^{-3} l

Urea concentration = 0.33 M

Acid concentration = 0.01 M

Acid addition rate = 4.15×10^{-4} l/min

$$d[NH_3]/d[H^+] = 2.26$$

$$d[H^+]/dt = (4.15x10^{-4} l/\min)\ \ (0.01\ \ mol/l) = 4.15x10^{-6} mol/\min$$

Rate of ammonia production:

$$d[NH_3]/dt = \frac{d[NH_3]}{d[H^+]}\ \ \frac{d[H^+]}{dt} = (2.26)\ \ (4.15x10^{-6} mol/\min) = 9.38x10^{-6} mol/\min$$

Urease activity:

$$Activity = \frac{d[NH_3]/dt}{mg\ \ enzyme} = \frac{9.38x10^{-6} mol/\min}{0.040\ \ mg} = 2.34x10^{-4} mol/\min * mg$$

1 IU = 10^{-6} mol/min ammonia produced at pH 7.0 and 25°C.

Urease activity = 234 IU/mg

Sample calculation of encapsulated urease activity:

Urease activity measurement at pH 7.0 and 25°C was conducted as follows:

Initial urease encapsulated = 5.044 mg

Beads produced = 0.7573 gr

10 beads = 0.0603 gr

Reactor volume = 40 x 10^{-3} l

Urea concentration = 0.33 M

Acid concentration = 0.01 M

Acid addition rate = 1.498 x 10^{-3} l/min

$$d[NH_3]/d[H^+] = 2.26$$

$$d[H^+]/dt = (1.498x10^{-3} l/\min)\ \ (0.01\ \ mol/l) = 1.498x10^{-5} mol/\min$$

Rate of ammonia production:

$$d[NH_3]/dt = \frac{d[NH_3]}{d[H^+]}\ \ \frac{d[H^+]}{dt} = (2.26)\ \ (1.498x10^{-5} mol/\min) = 3.38x10^{-5} mol/\min$$

Active Urease:

$$mg\ \ active\ \ urease = \frac{d[NH_3]/dt}{free\ \ urease\ \ activity} = \frac{3.38x10^{-5} mol/\min}{2.34x10^{-4} mol/\min * mg} = 0.145mg$$

$$Encapsulation\ \ yield = \frac{(0.7573gr\ \ beads)\ \ (0.145mg\ \ urease)}{(0.0603gr\ \ beads)\ \ (5.044mg\ \ urease)} = 0.36$$