IMMOBILIZATION OF LACTASE TO PERLOZA CELLULOSE RESINS

This thesis was presented in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at Massey University

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AMENDMENTS

p. ii, line 15 should read '**the** best of these results'
p. iv, paragraph 3 – 'thank Dave Elgar for his **valuable** advice' and 'paragraph 5 – '**heaps** of help?'
p. xi 'NaOH sodium hydrox**ide**'
p. 2, line 1, imid**a**zole, line 3 should be **sulphydryl**
p. 4, Table 1.1, Streptococcus thermoph**i**lus
p. 15, line 6, '**of** the commercial matrices available'
p. 26, line 15 'ECH (0.75**ml**)'
p. 28, line 28, 'the beads **were**'
p. 30, line 24 '**rpm**' not rmp, also p. 32, line 24
p. 31, line 14, '1 mM $MgSO_4$'
p. 32, line 2 ' **were** warmed to'
p. 33, line 10, '**2.11**' , not 1.11
p. 33, line 17 should be acetonitrile and p. 34, line 2
p. 37, line 6 '**Matsumoto**'
p. 65 line 23, 'in other w**o**rds'
p. 72, line 8, change 'Binding Buffer **2 two** tested' to 'Binding Buffer 2 tested'
p. 86, line 5, '**shifted**' not shift.
p. 87, line 11, 'highest activity of immobilised lactase **being** achieved at low ECH activation level**s**'

ABSTRACT

A bead cellulose matrix, Perloza, was chemically modified by two attachment chemistries to prepare inexpensive resins for immobilization of lactase. A commercial product, the base-activated matrix Eupergit C was studied for comparison.

Three types of Perloza (Perloza 100 MT, Perloza 200 MT, Perloza 500 TM) were activated by epichlorohydrin (ECH) to achieve different activation levels. The best result for lactase immobilization was gained at low activation level (activated at 2% NaOH) for two attachment chemistries. The first attachment chemistry studied was that lactase immobilized directly to ECH activated Perloza. The second chemistry again used ECH activation and followed by attachment of the 6-amino caproic acid (ACA) spacer arm and then the lactase.

In the first chemistry, Perloza 100-ECH-Lactase obtained the highest activity 11.4 NLU/g (wet resin) over Perloza 200-ECH-Lactase and Perloza 500-ECH-Lactase (40 hours immobilization). In the second chemistry, Perloza 200-ECH-ACA-Lactase retained the highest activity 30.9 NLU/L (wet resin) over Perloza 100-ECH-ACA-Lactase and Perloza 500-ECH-ACA-Lactase. Overall the best results were obtained for the ECH-ACA resins. This best of these results showed about 3 times better immobilization than without ACA spacer arm.

The activity of immobilized lactase on Eupergit C obtained was 124~131.3 NLU/g (wet resin) for 24 hours immobilization. Although this result is about four times greater than Perloza, Perloza is a much cheaper matrix.

In the storage stability studies, both Perloza and Eupergit C immobilized lactase showed a sharp drop in activity initially within 1 day, then activity loss leveled out. Perloza 200-ECH-ACA-Lactase retained 82% of its original activity after 9 days storage. However, Eupergit-Lactase only retained 39% of its original activity after the same storage period.

This result indicated that Perloza 200-ECH-ACA-Lactase may possess much better storage stability than that of Eupergit-Lactase.

Studies on the inter-relationships between pH, temperature and Perloza immobilized lactase using the substrate (ONPG) indicated that maximum hydrolysis was attained at pH 6.5-7.2 and over a temperature range of 30-42°C. No shift in the pH and temperature optima in comparison to free enzyme was observed as a result of the process of immobilization of lactase on Perloza for both attachment chemistries.

The pH-activity curve of Eupergit-Lactase shifted towards more acidic pH values in the pH optimum in comparison to free lactase. The temperature optimum of Eupergit-Lactase shifted towards higher temperature compared to free lactase.

This study showed that Perloza has potential for the large scale use as a matrix of lactase immobilization.

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ACA	6-aminocaproic acid
ADH	alcohol dehydrogenase
BCA	bicinchoninic acid
CDI	1,1'-carbonyldiimidazole
CMC	1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide
ECH	epichlorohydrin
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
FPLC	fast protein liquid chromatography
GRAS	generally recognized as safe
HCl	hydrochloric acid
HL	hydrolyzed lactose
K-phos	potassium phosphate buffer
NaOH	sodium hydroxyl
NaCl	sodium chloride
NHS	N-hydroxysuccinimide
ONP	o-nitrophenyl
ONPG	o-nitrophenyl-β-D-galactopyranoside
UF	ultrafiltration

CHAPTER ONE

INTRODUCTION

1.1 The lactose problem

Lactose is a disaccharide found in mammalian milk. Hydrolysis of lactose to its simple constituent monose sugars: glucose and galactose is catalyzed by the enzyme lactase. The problem of lactose in milk is well described in the literature (Gekas and Lopez-leiva, 1985). Because of intestinal lactase insufficiency, some individuals show lactose intolerance and they have difficulty in consuming milk and dairy products (Richmond et al., 1981; Shukla, 1975). In the case of lactose intolerance, unhydrolyzed lactose is not absorbed and moves through the large intestine where bacterial actions and osmotic effects often produce abdominal discomfort, bloating, and flatulence. The physiological imbalance and the accompanying symptoms of fermentative diarrhoea, cramps, bloated feeling, belching, and even the watery explosive diarrhoea are the same whether an individual lacks sufficient lactase activity or has ingested an amount of lactose that exceeds the hydrolytic capacity of the available lactase in the intestine.

Technologically, lactose is easily crystallized and this sets limits on certain processes in dairy industry. Cheese manufactured from hydrolyzed milk ripens more quickly than that made from normal milk. The problem of lactose in whey is mainly related to environmental pollution created when large quantities of whey are discharged.

1.2 The enzyme lactase

Lactase is the common name for the enzyme β-D-galactosidase or more formerly β-D-galatoside galactohydrolase. The enzyme typically catalyzes the hydrolysis of β-D-galactosides and α-L-arabinosides. Hydrolysis of lactose is depicted in Figure 1.1 (Shukla, 1975). In catalysis, lactose was hydrolyzed to an isomolecular mixture of glucose and galactose. The active site of the lactase molecule is characterized by one

HS and one imidizole group (Wallenfels, 1972). The hydrolysis reaction corresponds to a S_N2-like displacement mechanism, i.e. an electron pair donating reagent (nucleophile) attacks C-1 for the removal of the galactosyl group. The sulfahydryl group at the active site acts as a general acid to protonate the galactosidic oxygen atom, while the imidizole group acts as a nucleophile in that it attacks the nucleophilic center at C-1 of the glycone. A covalent intermediate involving a carbon-nitrogen bond has been proposed (Figure 1.1a).

In practice depending on the conditions, the isomolecular pattern is sometimes not followed because galactose can polymerize or join to form oligosaccarides. The enzyme is capable of catalyzing the synthesis of certain oligosaccharides through the galactosyl transfer reaction (Figure 1.1b). The β-D-galactosyl transfer occurs preferentially at the primary alcohol of D-glucose with the formation of various di- and oligosaccharides. In the removal of the galactosyl group, the sulfahydryl anion (S^-) acts as a general base to abstract a proton from water, which assists in the attack of OH^- at C-1 position. There is no inversion of the anomeric carbon at either step in the reaction, and the product retains the β configuration around the anomeric carbon.

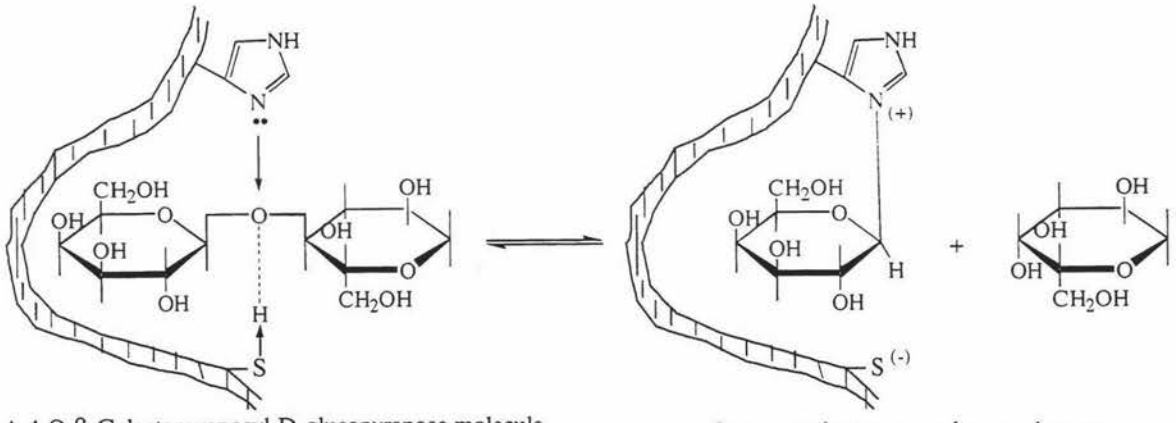


Figure 1.1a Proposed mechanism of lactose hydrolysis by lactase.

(Shukla, 1975)

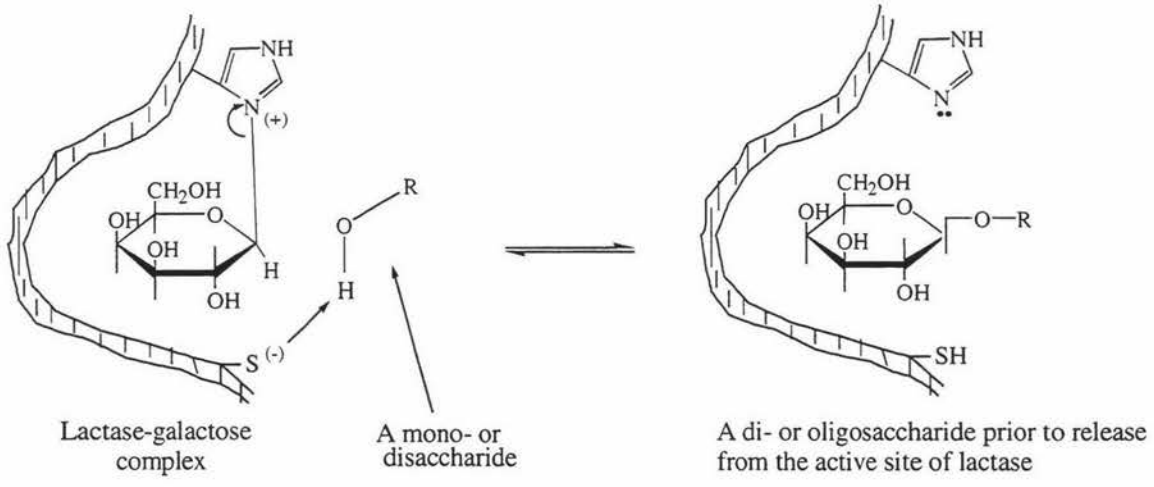


Figure 1.1b Proposed mechanism of galactosyl transfer reaction by lactase.

(Shukla, 1975)

Sources

Lactases occur widely in nature and are produced by plants, animal organs and micro-organisms such as bacteria, yeasts (intracellular enzyme), fungi of moulds (extracellular enzyme). Table 1.1 summarizes possible sources of lactase.

Possible Sources of Lactase	
Plants:	**Yeast:**
Peach	*Kluyveromyces (saccharomyces) lactis*
Apricot	*Kluyveromyces (saccharomyces) fragilis*
Almond	*Candida pseudotropicalis*
Kefir grains	*Brettanomyces anomalus*
Tips of wild roses	*Wingea roberstsii*
Alfalfa seed	
Coffee berries	**Fungi:**
	Neurospora crassa
Animal organs:	*Aspergillus foetidus*
Intestine	*Aspergillus niger*
Brain and skin tissue	*Aspergillus flavus*
	Aspergillus oryzae
Bacteria:	*Aspergillus phoenicis*
Escherichia coli	*Mucor pucillus*
Bacillus megaterium	*Mucor miehei*
Thermus aquaticus	*Scopuloriopsis*
Streptococcus lactic	*Alternaria palmi*
Streptococcus thermophlus	*Curvularia inaegulis*
Lactobacillus bulgaricus	*Fusarium moniliforme*
Lactobacillus helveticus	*Alternaria alternara*
Bicillus sp.	
bacillus Circulans	

Table 1.1 Possible sources of lactase (Gekas and Lopez-leiva, 1985).

Some of these sources are used for commercial enzyme preparations (Table 1.2). However, not all lactase sources are acceptable or generally recognized as safe (GRAS) when the enzyme is going to be used in food systems (Coughlin and Charles, 1980).

Lactase preparations from *A. niger, A. oryzae* and from *Saccharomyces sp (lactis* or *fragilis*) are considered safe because those sources have already a history of safe use

and have been subjected to numerous tests (Parizia and Foster, 1983). Maxilact, which is currently applied in dairy industry and considered safe, was employed in this study.

Commercial Preparations of Lactase
Aspergillus niger Wallerstein Co., Morton Grove, III. Baxter Laboratories, Chicage, II, USA Dairyland Food Labs, Waukesha, Wi. USA
Kluyveromyces (saccharomyces) lactis Gist-Brocades, Holland ('Maxilact') Nutritional Biochemicals Co. Ltd. Cleveland. USA. Sigma chemicals Co., St. Louis, USA
Escherchia coli C. F. Boehringer GmbH, Mannheim, Germany.
Yeast preparations British Drug House Ltd., London, England.

Table 1.2 Commercial preparations of lactase (Gekas and Lopez-leiva, 1985).

E. coli lactase, although the species most investigated, is not used in food processing because of its cost and the fact that is gives toxicity problems with crude extracts of coliforms (Greenberg and Mahoney, 1981). Safety considerations also must allow for the eventual appearance of non-desirable by-products during production. Additional factors, other than those mentioned, such as the suitability of the carrier (from food-use point of view) or the other chemicals used (binding, activation, cross-linking agents) have to be taken into consideration for the immobilized enzymes.

Properties of lactase

The properties of the enzyme differ markedly according to their source. The largest molecular size is possessed by *E. coli* lactase, (520 up to 850 kD are reported) while for *S. fragilis* and *A. oryzae* enzymes the molecular weights are 201 and 90 kD respectively (Richmond et al., 1981). *Kluyveromyces lactis* (Maxilact), which was used in this study, has a molecular weight of 200 kD and is composed of two identical subunits (Cavaille and Combes, 1995).

Micro-organisms offer high yields for production and microbial lactase are of technological interest, the major enzymes being those from *kluyveromyces lactis*, *K. fragilis* and *Aspergillus niger*. Temperature and pH optima differ also according to the source and even according to the particular commercial preparation. Immobilization of the enzymes, method of immobilization, and type of carrier can also influence those optima (White and Kennedy, 1980). In general, fungal lactase have pH optima in the acid range (2.5-4.5) and yeast and bacterial lactase in the almost neutral region (6-7 and 6.5-7.5 respectively, Coughlin et al., 1980). This pH optimum property makes each lactase suitable for a specific application. Thus, fungal lactases are used for acid whey hydrolysis while yeast and bacterial lactase are suited for milk (pH 6.6) and sweet whey (pH 6.1) hydrolysis. Yeast enzymes are less stable than the fungal enzymes and can only be used of moderate temperatures. However, they are also less inhibited by the reaction products.

Product inhibition (namely inhibition by galactose) is another property, which also depends on the source of lactase. The enzyme from *A. niger* is more strongly inhibited by galactose than that from *A. oryzae* (Sprossler and Plainer, 1983). An enzyme preparation from a *Bacillus sp.* showed less inhibition than that from *K. fragilis* (MacBean et al., 1979).

Some thermophilic organisms are very interesting in respect of enzyme thermostabilities. Thermostable enzymes, able to retain their activity at temperatures of 60°C or above for prolonged periods, have two advantages: they give higher conversion rates (or shorter residence time for a given conversion rate) and are less prone to microbial contamination (Wasserman, 1984).

1.3 Maxilact

Maxilact is a purified liquid lactase preparation, isolated from a special strain of the dairy yeast *Kluyveromyces (Saccharomyces) lactis.* The enzyme is a glycoprotein with 45% glycosylation (w/w). This yeast, first described by Beigerinck in 1889 (Maxilact

product data sheet), is a well known dairy organism which is used in the production of certain types of yogurt (Rizvanov, 1960). The yeast is also being used, in the form of an inactivated powder, as a health food and protein supplement for children.

Maxilact separates or hydrolyzes the milk sugar, lactose, into the two monosaccharides, glucose and galactose. The reaction conditions, i.e. temperature, acidity, processing time, lactose and enzyme concentration, determine the speed of the reaction. It is because Maxilact is derived from a dairy yeast, the optimal conditions are close to the natural pH and temperature of milk. The pH optimum is 6.3-7.0. The activity of Maxilact decreases rapidly below pH 5.9 (Guy and Bingham, 1978). The range of 35-40°C is the temperature optimum. However, Maxilact is still active at lower temperatures, even down to 4°C. This property is especially important because growth of spoilage bacteria at these low temperatures is very moderate. Milk (or whey) can therefor be treated during the usual overnight storage period.

Maxilact is available in three different purity grades:

- Maxilact LX 5000, a highly purified lactase for hydrolysis of milk for retail sale.
- Maxilact L2000, for whey-treatment and for the production of fermented dairy products, including yogurt and quark.
- Maxilact LC, a crude lactase preparation for agricultural applications (animal feed).

Maxilact can be employed in milk or sweet whey without any modification. Maxilact used as free lactase in hydrolysis of milk will be discussed in Section 1.4. In this study, Maxilact L2000 was employed.

Heavy metals, such as zinc ($5x10^{-4}$ M) and copper ($5x10^{-4}$ M) have a strong inactivating effect on the enzyme. In another words, heavy metals are strong inhibitors of Maxilact (Dahlqvist et al., 1977). Ionogenic or free calcium in higher concentrations (10^{-4} M) and sodium inhibit the enzyme as well. By contrast, lactase activity and/or stability is enhanced by magnesium (10^{-4} M), manganese (10^{-4} M) and potassium (10^{-1} M, Guy and Bingham, 1978). Phosphate concentrations up to 10^{-2} M influence stability positively because phosphate binds calcium ion, however, too much is inhibitory (Pivarnik and Rand, 1992). In practice, both activating and inactivating minerals are

present and the actual influence of metallic ions will have to be determined for each substrate.

Glycerol at 10-20% improves stability of the enzyme. Maxilact is 564.5 g/l glycerol. Dilution of enzyme causes lost of its stability (Dahlqvist et al., 1977). Enzyme diluted in sterile milk is stable at room temperature.

Maxilact was selected for this study because it is commercially available at relatively high purity and it is widely used in the dairy industry.

1.4 Lactase application to food science

The use of lactase as a solution to the problems of lactose intolerance, whey utilization, and lactose crystallization and as a means for producing a sweetener for the dairy and food industry has been well recognized. Shukla (1975) reviewed some of the new product developments for lactase at the time, and his compilation of reported product development is listed in Table 1.3. Numerous references were cited for the new product work.

Use of Lactase
1. Low lactose milk processing.
2. Low lactose dairy products.
3. Low lactose yogurt.
4. Sweetened yogurt.
5. Low lactose concentrate for ice cream.
6. Lactose processing of acid and sweet whey.
7. Food syrups and sweetener.
8. Lactase treatment during cheese making.

Table 1.3 Use of lactase: new product development (Richmond et al., 1980).

Maxilact was applied as free enzyme to treat milk for modifying yogurt (Engel, 1973). Engel's data indicated that 50% lactose hydrolysis rate should provide an acceptable consumer product. Gyuricsek and Thompson (1976) used hydrolyzed lactose milks to

manufacture yogurt, buttermilk, and cottage cheese. Times to reach desired pH for yogurt and cottage cheese were reduced significantly from conventional methods.

Woychik and Holsinger (1977) reviewed in detail a number of applications for hydrolyzed lactose (HL) milk, including HL milk as a fluid product and a skim milk powder. They discussed the use of HL milk in cultured dairy products. Much experimental data in support of these HL product was presented. The authors also indicated that the use of lactase (either free or immobilized) could result in quality improvements for many products and provide processing economies for others. The use of commercial soluble lactase for addition to aseptically packaged fluid milk has been summarized (Richmond et al., 1981).

Young et al. (1980) used neutralized lactose hydrolyzed fluid cottage cheese whey to supply 53% of the total solids in ice cream type frozen dairy desserts. Maxilact was used to hydrolyze lactose in the whey. These fluid whey based desserts were highly acceptable. Lactose hydrolysis of 75% should be used to retard sandiness in the products.

MacBean (1979) reviewed literature on lactose crystallization and hydrolysis. He discussed the use of syrups produced from whey or ultrafiltrate and listed many products that could take advantage of this process including flavored yogurt, imitation maple syrup, juices, canned fruit, wine, and beer. He also discussed different enzymatic and acid processes available to hydrolyze lactose.

Significant cost savings could be made by using immobilized enzyme. In some cases hydrolyzed milk has been used in order to accelerate the ripening cheddar cheese (Ridha et al., 1983). The whey product was already hydrolyzed with the possibility of being utilized in confectionery products. Hydrolyzed (80%) milk was used in the manufacture of blue cheese.

1.5 Methods for hydrolysis of lactose

There are two main methods for lactose hydrolysis (Gekas and Lopez-leiva, 1985). One is the catalytic (or acid) method, the other is the enzymatic method which uses lactase. The first method is characterized by very severe pH and temperature conditions, i.e. pH 1-2, temperature 100-150°C. The second method, depending on the properties of the enzyme used, is carried out under considerably milder conditions, i.e. pH 3.5-8, temperature 5-60°C.

A high degree of hydrolysis can be achieved in a short period of time by acid hydrolysis. A typical example is 80% hydrolysis in three minutes at pH 1.2 and temperature 150°C (Robbertson et al., 1984). The advantages of the method include simplicity and no need of expensive enzymes.

However, the disadvantages of this method are:

- Because the severe conditions used in acid hydrolysis cause denaturation of protein, this method cannot be applied to hydrolysis of milk and protein-containing lactose solution, e.g. cheese whey. Its use is limited to UF-permeate whey.
- Demineralization to 90-95% is required to reduce deactivation of the acid due to the presence of salts.
- A decolourization step is needed to reduce the brown colour.
- Formation of undesirable by-products.
- Cost of specific materials for plant construction to resist chemical corrosion.

The enzymatic hydrolysis of lactose can be achieved either by free enzymes, or by immobilized enzymes, or even by immobilized whole cells containing the enzyme. Free enzyme hydrolysis systems often are carried out in a batch fermentation process. Enzyme can only been used once.

Immobilized enzymes are defined as enzymes physically or chemically confined or localized in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously (Gerhartz, 1990). This means most

methods permitting the re-use of the enzyme are immobilization methods. Immobilization allows easy separation of enzyme from products, gives increased storage stability and in some cases alters favourably enzyme properties such as thermostablity and stability to denaturing agents and pH extremes (Rosevear et al, 1987).

1.6 Immobilization methods

Lactase has been successfully immobilized by chemical or physical binding to insoluble supporting matrices (carriers). Some of the immobilization methods have been applied to large scale industrial use. These immobilization methods include: adsorption (and crosslinking), entrapment, ultrafiltration membrane retainment, covalent binding. Enzymes may also be entrapped where they are free in solution but restricted to a limited space (Gerhartz, 1990). The methods may be used individually or in combination with each other. Some of these methods are outlined below.

1.6.1 Adsorption (and crosslinking)

Adsorption is the simple and inexpensive immobilization method since no chemical reagents are required. It is usually mild, involving weak binding forces, such as hydrogen bonds, ionic bonding or Van der Waals forces. The lactase is less likely to be denatured than by chemical methods. The weak bonding allows easy regeneration of the support. However, the weak binding can lead to desorption of enzyme from changes in pH, high substrate concentration, high ionic strength and temperature. A way to improve the stability of the adsorbed enzymes, is to cross link with glutaraldehyde or other bifunctional agents after the enzyme is adsorbed.

Both organic and inorganic supports have been used for adsorption of lactase. Natural polymers such as polysaccharides and fibrous proteins are abundant, fairly cheap, are usually GRAS materials and often have a high capacity for enzyme adsorption. Their main disadvantage is their low mechanical strength and susceptibility to microbial attack, especially when processing milk or whey. This then may require using them at

extreme temperatures and/or sterilising the substrate. In contrast, synthetic polymers such as the phenolic resins are very sturdy and also possess a high binding capacity for enzymes, which makes them a more attractive support.

Porous glass beads and metals such as alumina and stainless steel have excellent physical properties and could be ideal supports for lactase. The rigidity and high density of metals is important in fluidized bed reactors where strong forces are present. However the major disadvantage of these supports is their high cost, especially in the case of glass beads.

1.6.2 Entrapment

In this method the enzyme is trapped within the internal structure of materials (usually polymers) in the form of gels, microcapsules, fibres, films, or membranes which allow substrate in and product out. The common procedure is to prepare a solution containing the enzyme and the polymeric material (or monomer) and subsequently use a technique (e.g. drying, polymerization, wet spinning, etc) to coagulate the enzyme-containing polymeric material into the required form.

The catalytic properties are altered very little as compared to other procedures such as adsorption, ionic, and covalent bonding. The thin membrane between the enzyme and the bulk solution allows passage of lactose and the hydrolysis products, while separating the enzyme from macromolecules present in the solution. This means that milk proteins will not be deposited on the enzyme itself but will be restricted to the exterior surface, where they can be removed by cleaning procedures (Greenberg and Mahoney, 1981). Whole microbial cells can be immobilized thereby eliminating costly extraction and isolation procedures.

In some cases additional cross linking of the polymeric matrix is required in order to assure entrapment.

The entrapment method is applied to substrates of low molecular weight lactose so that free diffusion between them and the products is possible. Some leakage of the enzyme can occur through diffusion, even with small pore sizes of the matrix.

1.6.3 Ultrafiltration membrane retainment

Ultrafiltration membranes have been used as a means of retaining the enzyme, therefore separating it from the low molecular weight reaction products. The enzyme is in the soluble form and combines the advantages of free enzymes with the possibility of reuse of the enzyme. This method is considered as physical immobilization. The advantage of this method is that enzyme retains the original character, such as optimal pH, temperature and structure, which promise maximal activity of the enzyme. The disadvantages are easy leakage of enzyme and some adsorption of enzyme on the membrane surface.

Cross linking of the contained enzyme through glutaraldehyde or even covalent coupling can also be used.

1.6.4 Covalent binding

The covalent attachment method involves a reaction between a nucleophilic group (i.e. amino or carboxyl group) which is present in the enzyme and electrophilic group which is formed on a carrier through its proper activation. The activating agent is also called binding agent or coupling agent. Since neither amino or carboxyl groups are involved in the active site of lactase, there is no loss of catalytic activity, theoretically. The matrices used in covalent binding usually possess good physical properties, which promise greater binding strength than that can be obtained by adsorption. Thus, this method provides very stable attachment for the enzyme without danger of leakage. Some activity, however, is usually lost either by modification or blocking of the enzyme active site (Mosbach, 1976).

It is possible that some heterogeneity in attachment of the enzyme results due to the different numbers of bonds between enzyme and support, and different sites of attachment on the enzyme (Pye and Chance, 1976). This can give an attached enzyme with a variety of activity and stability. High density of active groups may cause too strong binding to enzyme which can then induce conformational changes in the immobilized enzyme and alter active sites, resulting in loss of activity. Especially in the case of the enzyme lactase which consists of multisubunits, strong binding between activated matrix and enzyme may disrupt the bonds between subunits, resulting in disruption of active sites of the enzyme. Relatively low substitution of active groups can minimize these problems by reducing the number of bonds individual enzymes can react with, giving freedom to the enzyme. Blocking the active site prior to coupling may be another alternative to stop bonding involving the active sites.

Activity can also be reduced by impairment of free movement of substrate to the enzyme's active site. Enzyme immobilization requires the substrate to be transported from the bulk solution phase to the active site of the enzyme attached to a solid support and then product released back into the bulk solution. This is so called mass transfer control. Stability to unfavourable conditions such as temperature (Grusek et al. 1990; Hayashi and Ikada, 1990) and pH extremes may be altered favourably due to the strong bonding stabilizing the enzyme structure.

It is often difficult to regenerate supports used in this method after reaction with the chemicals used in immobilization, so supports are generally not renewable. Optimal conditions for immobilization can be difficult to find. The chemistries used in immobilization can be expensive but this can be offset by the ability to reuse the enzyme. These disadvantages to immobilization by covalent binding, such as lower initial activity and non-renewable supports, should be outweighed by the main advantages such as higher stability, high accessibility to macromolecular substrates, enzyme reusability and easy separation of enzyme and products.

The enzyme is attached to the support by either the side chain of one of several amino acids or the N-terminus or C-terminus. Side chains commonly used include lysine,

cysteine, aspartic acid, glutamic acid and tyrosine (Table 1.4). ECH activated Perloza itself or with ACA spacer arm were assumed to react with amino group of lactase such as the side chain residue of lysine or N-terminal.

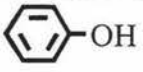
Residue	Structure
ε-amino of lysine or N-terminal	$-NH_2$
thiol of cysteine	-SH
carboxyl of glutamic acid or aspartic acid	-COOH
phenolic of tyrosine	

Table 1.4 Amino acids involved in covalent binding

1.6.5 Solid supports in covalent binding

Various solid supports have been trialed for binding. The list includes porous glass, ceramics, stainless steel, alumina, ferrites, sand, charcoal, synthetic polymers and carbohydrates such as cellulose. If the commercial matrices available, the most common and widely applicable are the polysaccharides, agarose and cellulose, and their derivatives. Both contain hydroxyl groups available for activation. Matrices containing amide and ester groups are also commercially available. This group includes polyacrylamide, gelatine and nylon. Commercially available matrices such as polystyrene porous glass Celite have been used in enzyme immobilization. Brick dust and chitin have also been trialed as solid supports. It appears almost anything may be used a solid support for immobilization of enzymes but several factors must be taken into consideration when selecting a solid support.

Factors in selecting solid supports (Cabral and Kennedy, 1991)

- Large surface area
- Permeability

- Hydrophilic character
- Insolubility
- Chemical, mechanical and thermal stability
- High rigidity
- Suitable shape and particle size
- Resistance to microbial attack
- Regenerability

1.7 Perloza beaded cellulose

Perloza is a spherical, beaded, regenerated cellulose produced by a "Thermal-Sol-Gel-Transition" process (Stamberg, 1988). Cellulose xanthate solution is dispersed in an immisicible organic solvent, shaped into a sphere by droplet formation, and solidified by an increase in temperature. Decomposition of xanthate groups occurs during solidification and is completed by alkaline hydrolysis. The resulting cellulose beads are hydrophilic, spherical particles with minimal levels of ionic groups.

The structure of the Perloza is stabilized only by hydrogen bonds and not by covalent cross-links. Porosity is excellent (molecular weight cut-off values up to 500 kDa for dextran). Perloza is available in three molecular weight exclusions 100, 200, 500 kDa, named Perloza MT 100, Perloza MT 200, Perloza MT 500, with three particle diameter ranges fine (80-100 μm), medium (100-250 μm), coarse (250-500μm) for each exclusion. Perloza MT 100, Perloza MT 200, Perloza MT 500 with medium particle dimension (called Perloza I, Perloza II, Perloza V in this thesis) were used throughout this study. A number of properties of Perloza make it suitable for enzyme immobilization.

Properties of Perloza

- Good porosity to allow unimpaired movement of large molecules.
- An ample supply of chemical groups (hydroxyl groups) which may be activated.

- Hydrophilic, therefore little, non-specific, hydrophobic interaction with proteins and the carbohydrate backbone.
- Uniform spherical shape of individual particles allowing an even distribution of enzyme.
- Mechanically rigid and chemically stable.
- Relatively cheap (underivatized prices between US $20 and $60/litre) and commercially available.

1.8 Activation methods for covalent binding

The presence of free amino, carboxylic, phenolic and thiol groups on the surface of the enzyme allows them to be attached with a wide variety of activation chemistries. A small selection of the chemistries available for activation is shown in Table 1.5. Glutaraldehyde, epichlorohydrin, carbonyldiimidazole and periodate are among of the more useful reagents due to their low toxicity and stable bonds.

Coupling reaction	Description
Diazotization	Diazo linkage between enzyme mainly His and Tyr residues and aryldiazonium groups on carrier.
Amide bond formation	Amide (peptide) bond formed between nucleophilic groups on protein (amino, hydroxy and thiol) and carrier.
Alkylation and arylation	Alkylation amino, phenolic or thiol groups on enzyme with active halides, oxirane, vinylsulfonyl or vinylketo groups on the support.
Schiff's base formation	Formation of Schiff's base (aldimine) link between free amino groups on enzyme and carbonyl groups on support.
Ugi reaction	Formation of an N-substituted amide between protein and carrier.
Amidation reactions	Imidoesters on carrier react with protein amino groups to give an amidine linkage.
Thiol-disulphide interchange	Formation of disulphide linkage between thiol groups on support and enzyme.

Table 1.5 Selection of activation chemistries.

1.9 Immobilized lactase systems

A variety of techniques and support carriers have been used. However, some specific methods were used mostly, such as adsorption and crosslinking with glutaraldehyde. Glutaraldehyde is a bifunctional crosslinking agent which is accepted in the food industry. Phenolformaldehyde resin is usually the carrier for this method. It is used in scaled-up systems in the form of fluidized bed reactor (Gekas and Lopez-leiva, 1985).

Porous silica in the form of beads is a common carrier combined with the covalent coupling method. It is activated so that silane groups are formed. Coupling to the enzyme is frequently obtained again through glutaraldehyde. Sometimes other inorganic carriers like alumina and ferrites are used for the similar technique.

Common organic supports for covalent coupling are derivatives of cellulose carrying free hydroxyl or amino groups and polyacrylic compounds activated usually by oxirane. Gels from various polymers like polyacrylamide and agar are the materials used for the entrapment technique. Some polymer fibres, which have been successfully developed and applied on the commercial and industrial scale.

Many early reviews (Finocchiaro et al, 1980, Greenberg and Mahoney, 1981, Shukla, 1975) summarized the research on immobilized lactase since 1968, when the first immobilization of this enzyme was reported.

Gekas and Lopez-leiva (1985) reviewed methods of lactose hydrolysis, including acid hydrolysis or by enzymatic catalysis with the enzyme either free in solution or immobilized by one of the several enzyme immobilization methods. In an extensive table, they summarized more than 40 immobilized lactase systems. Enzyme sources, immobilization techniques, reactor types, and general comments regarding the particular systems were provided in the table.

Greenberg and Mahoney (1981) reviewed lactose hydrolysis together with relevant properties of commercially available lactase enzymes. Procedures and supports which could be used for immobilization were discussed in detail.

Richmond et al. (1980) reported potential commercial applications of lactose hydrolyzed products and various immobilized lactase systems.

More recently numerous new developments of lactase immobilization were emerged in the literature. Only a short list of them are summarized here. Ovsejevi et al. (1998) immobilized lactase from *Kluyveromyces lactis* on to thiolsulfinate/thiolsulfonate supports. They stated that more than 80% of the activity was retained. They blocked the remaining reactive groups with glutathione resulting in an increase of the thermal stability of the derivatives almost two-fold. These derivatives achieved a high degree of

conversion (85-90%) of lactose (50 g/l) in saline solution, whey, whey permeates, and skimmed milk, either batchwise of in packed beds.

Ortega-Lopez et al. (1993) immobilized lactase from *Kluyveromyces lactis* on nylon-6 microbeads to hydrolyze lactose in skim milk (28.6% total solids), using a novel spin-basket reactor. Over 75% of lactose was hydrolyzed at 34°C within a short-space time (<7 min) without experiencing any plugging such as typically seen in packed columns.

Rogalski et al. (1994) described the properties of four different commercial preparations of lactase originating from bacteria and fungi. They compared the immobilized enzymes with their native forms and pH, temperature optimization, stability curves were presented not only for the immobilized enzymes but also for those which were additional cross-linked with glutaraldehyde or bis-oxirane. They claimed that Lactozym 3000 in an immobilized form was the most useful for lactose removal from milk.

Heng and Glatz (1994) explored the use of charged peptides fused to lactase for immobilization onto ion-exchange membranes.

Siso and Suarez (1994a) have developed and characterized a new low-cost enzymic preparation for milk whey saccharification. The preparation consisted of lactase-rich whole cells of the yeast *Kluyveromyces lactis*, previously cultured on milk whey and immobilized by covalent linkage to corn grits, which is a cheap material with good mechanical properties for use in bioreactors. They increased the measured values of intracellular lactase activity up to 240-fold by permeabilization of immobilized cells with ethanol. More than 90% milk whey lactose hydrolysis was achieved in a packed-bed bioreactor at 37°C. Later, Siso et al. (1994b) covalently linked lactase from *Saccharomyces fragilis* with glutaraldehyde to chemical modified corn grits. The immobilized enzyme was used to hydrolyze milk-whey lactose in a recycling packed-bed bioreactor. They claimed that this system for lactose hydrolysis was not limited by diffusion.

1.10 Activation methods studied

In this study Perloza was activated by epicholohydrin (ECH). Perloza has been activated successfully by ECH and then attached to some proteins in our laboratory (Burton, 1996).

At alkaline pH, ECH readily reacts with hydroxyl group of Perloza to yield derivatives containing a 3 atom long hydrophilic spacer molecule with a reactive oxirane on the end (Figure 1.2). The bond between the ECH and the matrix becomes a stable ether bond, while the other end provides ligand coupling potential. This terminal epoxy then can react with ligands containing hydroxyl, amine, or thiol groups. An amine containing ligand, which can be either an enzyme or spacer arm, was used in the study (Figure 1.2). The epoxy activation method provides an extremely stable linkage between the ligand and the matrix. The enzyme may be attached directly to the resin or though a spacer arm such as 6-amino caproic acid in the study. The spacer arm must then be activated for enzyme immobilization. ECH activation gives a linkage with no charge, which minimizes non-specific ionic binding and produces a relatively stable linkage that reduces ligand leakage.

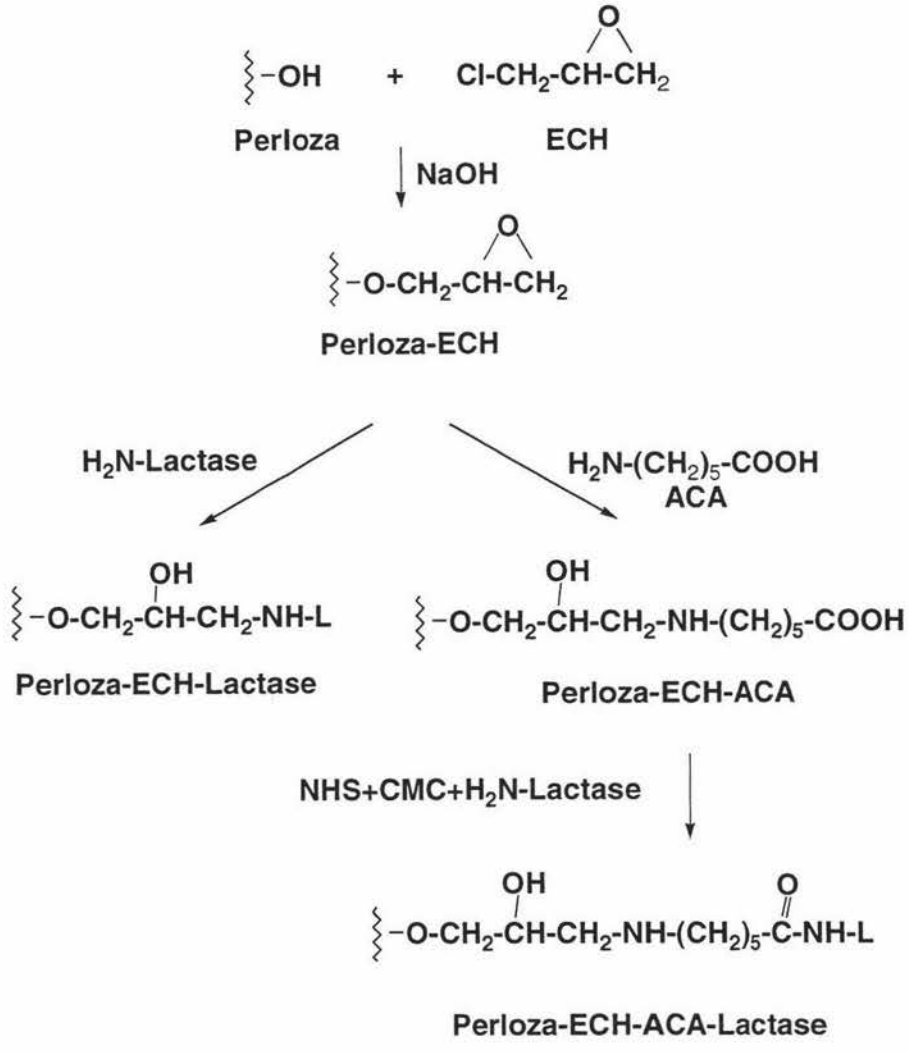


Figure 1.2 Perloza activation and lactase attachment methods used.

1.11 Analysis used in the study

The activation of Perloza by ECH was followed by titration of oxirane groups to determine the activation level. Protein substitution was measured by the bicinchoninic acid (BCA) assay. The activity of lactase was compared with free enzyme by reaction with a chromogenic substrate o-nitrophenyl galactopyranoside (ONPG). Activity of immobilized enzyme was measured by an "end point" method.

1.12 Aim of this thesis

Since cellulose is such an abundant natural material, it is an obvious choice for preparing an inexpensive gel matrix for industrial use. However some derivatized celluloses suffer from the disadvantage that they require organic solvents or carefully controlled processes for preparation. ECH activation of Perloza does not require the use of organic solvents and the preparation is a simple, one-step chemical treatment. This resulting Perloza contains the reactive group oxirane, which is frequently used in enzyme immobilization.

The aim of this thesis is to develop cheap, efficient chemical methods for Perloza activation and lactase attachment; measurement of physical and chemical properties of these resins and comparison with commercial resins. Investigation of the potential for the use in large scale of these immobilized lactases is also an interesting issue of this thesis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents and equipment

Perloza MT 100, 200, 500 of medium grade bead cellulose (Perloza I, Perloza II, Perloza V) were from Tessek Ltd. of Prague, Czechoslovakia. Sepharose 6B was from Pharmacia, Uppsala, Sweden. Eupergit C was kindly supplied by New Zealand Dairy Research Institute. Maxilact L2000 was a gift from Gist-Brocades, Mooreband NSW, Australia. 6-aminocaproic acid (ACA) was from Fluka AG, Buchs SG, Switzerland. Bicinchoninic acid (BCA), 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC), N-hydroxysuccinimide (NHS), o-Nitrophenyl β-D-Galactopyranoside (ONPG), bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, MO, USA. Epichlorohydrin (ECH) was from Dow Chemical Company, Midland, MI, USA. pH indicator paper was from Whatman Ltd., Maidstone, Kent, England. Sodium metabisulphite (lab. grade), sodium azide, were from Ajax Chemicals Ltd., Aburn, NSW, Australia. Propylene oxide was from J. T. Baker, Phillipsburg, NJ, USA. Convol sodium hydroxide (NaOH) and hydrochloric acid (HCl, Volumetric concentrate) sodium hydroxide (AR), sulphuric acid (AR) were from BDH, Dorset, England. Sodium sulphite was from May and Baker, Manchester, England. Potassium dihydrogen phosphate, di-potassium hydrogen phosphate, copper (II) sulphate pentahydrate were from Riede-de-Haën, Selze, Germany. Magnesium sulphate ($MgSO_4$, AR grade), EDTA were from BDH, Poole, England. All other reagents were analytical grade.

De-ionised water (Milli-Q grade) was used to wash all of the resins and for the preparation of all buffers and aqueous reagents.

Activity assays and BCA assays were incubated in a Julabo SW-20C water bath. Disposable 2 ml columns were from Lab Supply Pierce, Auckland, New Zealand.

Spectrophotometer was from Hewlett Packard; a model 8452A Diode Array Spectrophotometer, HP89531, with MS-DOS-UV/VIS operating software. Titrator was from Radiometer Copenhegen; model VIT90 Video titrator. FPLC was from Pharmacia, Uppsala, Sweden, a model LCC-500.

2.2 Units used in this thesis

For Perloza and Sepharose matrix/resin, "**g**" refers to suction-dried wet weight (sometimes called wet weight for short) and "**g dry**" to oven-dried resin/matrix weight. Resin substitutions of activation are shown as "**mmol/g dry**", meaning mMoles of activated groups per gram of dry resin after the resin used for titration was dried at 110°C for one hour accordingly. Enzyme substitutions are shown as "**mg/g**", meaning mg of protein per gram of wet resin after excess water is removed from the resin in a sintered glass funnel with vacuum.

For Eupergit C resin, "**g dry**" refers to supplied dry beads of Eupergit C.

The conversions of "g dry" to "g" wet weight of the various resins are listed in Table 2.1.

	Weight of suction-dried wet resin per gram dry resin (g/g dry)	Dry weight/wet weight (%)
Perloza MT100, fine or medium	6.67	15
Perloza MT200, medium	11.1	9
Perloza MT500, medium	13.4	7
Eupergit C	3.65	27
Sepharose 6B-100	17.3	6

Table 2.1 Conversions of "g dry" dry weight to "g" wet weight of the various resins.

2.3 Perloza and Sepharose activation with epichlorohydrin (ECH)

2.3.1 Activation method 1

Initial experiments started with the standard method used by Burton et al (1996). Only Perloza 100 MT (fine) was used as a trial to investigate this method. Perloza was transferred into a 50 ml or larger sintered glass funnel and suction-dried. It was washed with 2-5 bed volumes of water, disturbing the resin bed as little as possible, suction-dried, and 20 g weighed into a reaction vessel (50-100 ml screw-top jar). Base, 20 ml of 1M NaOH was added and the vessel was left in the cold room (4-6°C) for about 30 minutes to allow it cool down. ECH (2 ml) was then added to make a final NaOH concentration of 2% reaction mixture. The vessel was sealed with Gladwrap and capped with its screw-on top and mixed by end-over-end rotation (rotator) or rollers for 23 hours in the cold room. The mixture was then transferred into a sintered glass funnel, rinsed extensively with water (e.g. 10X20 ml), suction-dried and then transferred back to a glass jar.

2.3.2 Activation method 2

Activation method 2 was based on the method used by Burton (1995). The method is similarly to activation method 1 except that Perloza 100 MT (fine) was mixed with ECH (0.75) and 0.66 M NaOH (10 ml) for 9 hours at room temperature. All the other conditions were the same to that of activation method 1.

2.3.3 Activation method 3

In order to find out what is the best activation level for enzyme immobilization, a series of reaction mixtures of varying activation were made up with different final NaOH concentration (1%, 2%, 3%, 4%, 5%, 6%, 7%). Both Perloza and Sepharose were investigated in the method. The water added into the Perloza or Sepharose (5g) was just sufficient to make a thick slurry. NaOH (30% w/v) was added to the slurry to make up the required concentration of NaOH. The water content of the starting resin was

taken into consideration when calculating the final volume and concentration of the NaOH used in the activation. The molar ratio of ECH to NaOH used was always 1.25:1. The consistency of the resin was such that good mixing was obtained, but not so thin that large amount of ECH was used unnecessarily. All other processes were the same as those in activation method 1. The amount of reagents used in Perloza activation are shown in Table **3.1.** The activated Perloza and Sepharose were named Perloza-ECH and Sepharose-ECH individually in this thesis.

2.4 Modification of Perloza 100MT with ECH and propylene oxide (PO)

Perloza was cross-linked with ECH and PO in the presence of NaOH. Earlier work by Dr J. S. Ayers at Massey University has proved that the chemical modification of cellulose was a successful way to gain high substitution levels of epoxide groups. Aqueous sodium hydroxide (15 ml of 30%), a mixture of ECH (0.5 ml) and propylene oxide (5 ml) were added simultaneously into freeze-dried Perloza 100MT powder (10 g) in a stainless steel bomb below 5°C. All the liquids were poured quickly and mixed thoroughly with a stainless steel spatula. The bomb was then sealed and stirring was commenced immediately to allow the liquids to wet all of the powder before it swelled. Stirring was continued 2-3 minutes before the bomb was placed in a water bath at room temperature. The temperature of the bath was raised to 45~50°C over 30 minutes and then held constant for a further 1 hour. The bomb was cooled down to room temperature under running tap water for about 30 minutes before being opened. The reaction mixture was transferred to a sintered-glass funnel and washed extensively with water until the washings were neutral to pH paper.

The modified Perloza was divided to two parts to carry out epoxide activation. The activation method 2 (section 2.3.2) was employed only with 3% final NaOH concentration. A high substitution level of activation at 1.1 (mmol/g dry) was obtained.

2.5 6-Aminocaproic acid (ACA) attachment to Perloza-ECH

The standard attachment method was used as described by Cross (1998). A 5 molar excess of 6-aminocaproic acid (ACA, 5 moles of spacer arm per mole of oxirane group on the activated matrix) was weighed into a small beaker and dissolved in minimum volume of 5 M NaOH (1 ml or less), water (~ 4 ml) added, then titrated with several drops of 50% HCl until pH 11.3. The mixture was added to wet Perloza-ECH to make a thick slurry and the mixture was shaken overnight at room temperature. The resin was then washed extensively with water and drained. A small sample (1~3 g) was taken for ACA titration.

2.6 Enzyme attachment

All the activated matrixes used in this study, such as Perloza-ECH, Sepharose-ECH, Eupergit C employed a similar enzyme attachment method that was described below.

2.6.1 Enzyme attachment to Perloza-ECH, Sepharose-ECH and Eupergit C

Maxilact L2000 (0.83 g) was dissolved in 1 M potassium phosphate buffer (5 ml) of pH 6.5 (where the pH is optimum pH for activity of lactase). The lactase mixture was mixed with Perloza-ECH (5 g) to make a thick slurry. The reaction vessel was sealed and mixed by end-over-end gentle rotation (rotator) or rollers for 24 to 124 hours at room temperature. At the end of reaction the beads were collected on a sintered-glass funnel (porosity 1 or 2) and the solution drained off by vacuum. The beads was washed thoroughly with approximately 200 ml of 0.1 M potassium phosphate buffer pH 6.5 until the washes had no lactase activity remaining. Activity assay used is described in section 2.9.1.

The final product was suction-dried and stored in a buffer solution containing a preservative. The initial storage buffer used was 0.1 M potassium phosphate buffer pH 6.5, containing 500 ppm ethyl p-hydroxybenzoate and 2% 2-propanol. The improved

storage buffer was 0.1 M potassium phosphate buffer, containing 1 mM magnesium sulphate ($MgSO_4$), 0.05 mM disodium EDTA and 0.01% NaN_3, pH 6.5.

Enzyme attachment to Sepharose-ECH used the same method with that for Perloza-ECH. Enzyme attachment to Eupergit C also used the similar method only small variation with the amount of reaction reagents, e.g. Maxilact L2000 (1 g) was dissolved in 1 M potassium phosphate buffer (5 ml) and mixed with Eupergit C (1 g dry) to make up the reaction mixture.

2.6.2 Enzyme attachment to Perloza-ECH-ACA

A 20 fold excess of N-hydroxysuccinimide (NHS) and 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluenesulfonate (CMC) over activatable groups (carboxylate groups) was added to wet Perloza-ECH-ACA, minimum amount of water added to make thick slurry and the mixture shaken at room temperature for 1 hour. The resin was washed thoroughly with water to remove all soluble contents at the end of reaction.

2.7 Titration methods

2.7.1 Epoxide titration

A small sample of suction-dried epoxide matrix (1-3 g) was reacted with a mixture of sodium sulphite (Na_2SO_3, 0.1 g), sodium metabisulphite ($Na_2S_2O_5$, 0.1 g) and water (6-10 ml) on a shaker for at least 4 hours at room temperature. The product was transferred to a small sintered funnel, washed thoroughly with water and 1 M hydrochloric acid (about 5X5 ml), followed by several water rinses until the effluent was neutral to pH 6-8 indicator paper. The sample was placed in a titration vessel along with 5 ml of water and about 0.5 g of sodium chloride. Using Convol 0.1 M NaOH, the sample was titrated to pH 8. The sample was then washed into a sintered glass funnel of known weight. After further washing to remove all salts, the sample was drained thoroughly and oven dried over night at 65°C. It was dried for a further 1.5 hours at

110°C, followed by cooling in a vacuum desiccator for 30 minutes with drying agent present and weighed. The substitution level was calculated as follows:

$$\text{Substitution Level (mmol/g dry)} = \frac{M * V}{W}$$

where M: Concentration of NaOH (mol/l)

V: Volume of NaOH used (ml)

W: Weight of epoxide matrix (g dry)

The value obtained for epoxide substitution level was expressed in units of millimoles/g dry (mmol/g dry, Burton, 1995).

2.7.2 6-Aminocaproic acid (ACA) titration

Resin containing carboxylate groups was washed by 4x10 bed volume of 0.1 M HCl to convert -COONa form to protonated -COOH form. The excess acid was removed by washing with at least 5x10 bed volumes of water, until the washing was neutral to pH paper. The sample was transferred to a titration vessel and mixed with a pinch of NaCl (~0.5 g) and water (~6 ml). The sample was titrated with 0.1 M NaOH to an end point of pH 11. The sample was then washed into a sintered glass filter of known weight. After further washing and oven drying, the Perloza-ECH-ACA substitution level was calculated as described in section 2.7.1.

2.8 Bicinchoninic acid (BCA) protein concentration determination

BCA (50 parts) were mixed freshly with copper (II) sulphate pentahydrate 4% solution (1 part) to make the BCA (protein determination) reagent. Bovine serum albumin (1.0 mg/ml) in 0.15 M NaCl solution was used to make a standard curve of 1-100 μg protein (0.1 ml). BCA reagent (2 ml) was added to each of the tubes, which were incubated for 30 minutes at 37°C in shaking water bath. The samples were cooled down under flowing tap water for 5 minutes and centrifuged for 3 minutes at 2500 rmp. Supernatant was removed with a Pasteur pipette for absorbance measurements.

Absorbance was measured at 562 nm against a water blank. Final absorbance was calculated by subtracting a zero value solution (0.1 ml water per 2 ml protein determination reagent incubated 37°C for 30 minutes) to produce a standard curve of absorbance versus µg protein (Stich, 1990).

Protein determination reagent (2 ml) was added to resin samples (0.05~0.1 g wet weight) and incubated as for the standard curve. Protein concentration was determined from the standard curve and expressed as mg protein per gram wet resin (mg/g).

2.9 Lactase activity assay

Lactase decomposes pseudosubstrate o-nitrophenyl-β-D-galactopyranoside (ONPG) into o-nitrophenol (ONP) and galactose at 30°C and pH 6.5. Reaction is terminated by addition of sodium carbonate. Absorbance of the resulting ONP (yellow in alkaline medium) is a measure of enzyme activity.

The buffer solution used in all activity assays was made of 0.1 M potassium phosphate, 1 mM MgS_4, 0.05 mM EDTA, pH 6.5. ONPG solution was of 5 and 6.6 mM ONPG in above buffer solution individually. Stopping solution was of 0.5 M Na_2CO_3, 0.1 M EDTA.

2.9.1 Qualitative method

This method is used for screening purposes such as qualitatively checking activity of matrix after enzyme attachment or multiple eluent fractions from chromatography. ONPG (100 µl, 5 mM) was pipetted into the wells of a 96-well micro titre plate as well as 20 µl of test sample or about 10 mg of enzyme matrix. The plate was incubated at room temperature for up to 30 minutes. Development of yellow colour indicates lactase activity.

2.9.2 Quantitative method

This method is used for measuring activity of free lactase. The buffer solution (0.9 ml) and 50 µl of 40 mM ONPG was warmed up to 30°C separately, then pipetted into a semi-micro spectrophotometer cuvette (1.5 ml) and placed in the heated cuvette holder at 30°C. The absorbance was monitored at 420 nm briefly to ensure no background increase in absorbance (blank scanning). Then the sample (50 µl) was added in the cuvette and mixed thoroughly. The increased absorbance was recorded for up to 3 minutes. The activity was expressed as follows:

Activity, L (NLU/g) = (Δabs/min) * D.F. * (1/A*1.3)*1000

Where Δabs/min: Change of absorbance per minutes
D.F.: Dilution factor
A: 4.65 abs/µmol/ml-Absorptivity of o-nitrophenyl (ONP)

Each gram of Maxilact L2000 contains 2000 Neutral Lactase Units (NLU). One NLU is the quantity of enzyme, which will form 1 µmol ONP per minute under the conditions of the test.

2.9.3 Activity measurement of immobilized enzyme by "end point" method

The enzyme-matrix sample (120~180 mg for Perloza and Sepharose, 15~30 mg for Eupergit C) was weighed into a 50 ml of beaker. The beaker was incubated in a shaking water bath at 30°C for 5 minutes before 8 ml of 6.6 mM ONPG was added. Six reaction mixtures (1 ml) were withdrawn in order to have a series of samples at 30 seconds intervals up to 3 minutes, rapidly added into 1 ml of stopping solution to halt the reaction in small vials and centrifuged for 3 minutes at 2500 rmp. Supernatant absorbances were recorded at 420 nm wavelength against a blank solution (a mixture of stopping solution and 6.6 mM ONPG 1 ml each) in a semi-micro cuvette at 30°C. Absorbances versus time were plotted to produce the change of absorbance per minutes. The activity of enzyme-matrix was calculated as follows.

Activity, L (NLU/g) = (Δabs/min) * D.F. * (8/W) * (1/A*1.3) * 1000

Where Δabs/min: Change of absorbance per minutes (abs/min, slop of absorbance vs. minute)

D.F.: Dilution factor (taken 2 here)

A: 4.65 abs/μmol/ml-Absorptivity of o-Nitrophenyl (ONP)

W: Weight of wet enzyme-matrix weighed (mg)

2.10 Temperature adjustment in temperature profile study

The ONPG substrate (pH 6.5) was divided into different samples. Each sample was then subject to hydrolysis at required temperature, using soluble as well as immobilized preparations.

1.11 pH adjustment in pH profile study

The ONPG substrate was divided into different lots and the pH of each lot was adjusted as per requirement using 50% KOH/H_3PO_4. It was then subjected to hydrolysis by enzyme preparations at 30°C.

2.12 Purification of crude β-galatosidase (Maxilact L2000) by gel filtration

The purification of Maxilact L2000 by gel filtration was carried out by the method of Ovsejevi et al (1998). Sephadex G-25 was employed for this purpose. Sephadex beads which had been kept in 10% acetonitrile were mixed with 20% actonitrile, which had been degassed and filtered though a 0.2 μm Millipore membrane in a Swinny filter, to make a thin slurry. The slurry was then poured into a 60 ml column in a steady stream, with precaution lest air bubbles become trapped in the mixture. The resin was allowed to settle under gravity flow and then the suspension was deairated by suction in the end. The inlet of the column was connected to a FPLC System. The effluent was monitored at 280 nm. The column was equilibrated with 20 mM potassium phosphate buffer

(equilibration buffer) at a flow rate of 1 ml/min overnight. The column was stored in 20% actonitrile when it was not in use.

The Maxilact L2000 (1ml) was diluted two fold with 20 mM potassium phosphate buffer before loading. Running buffer was 20 mM potassium phosphate buffer, pH 6.5. The sample was loaded onto the column at a flow rate 1 ml/minute. The lactase peak was collected and concentrated by centrifuging through a utrafiltration membrane with exclusion size of 30 kD. Then it was subjected to enzyme attachment (section 2.6.1).

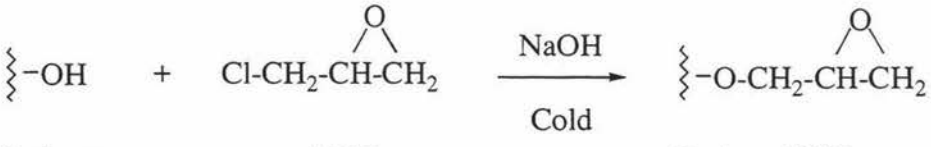
CHAPTER THREE

LACTASE IMMOBILISATION ON PERLOZA

3.1 Introduction

Bifunctional etherification reagents are particularly useful for matrix activation. ECH, used by Axen et al. (1975), and butanediol diglycidyl ether (BDE), used by Sundberg and Porath (1974), are inexpensive. Perloza was activated with ECH throughout this research (Equation 3.1). The conditions needed to substitute the maximum number of epoxide groups on a regenerated cellulose matrix have been investigated by the Ayers group at Massey University (1999). The critical factor affecting the substitution level of epoxidation relates to the ratio of sodium hydroxide and epichlorohydrin used. Sodium hydroxide is consumed by epichlorohydrin in a 1:1 mole ratio in order to react with the cellulose matrix (Equation 3.1), and also through hydrolysis of epichlorohydrin (Equation 3.2). Perloza is also regenerated cellulose, which was assumed to act in a similar fashion to the matrix used by Ayers et al.(1999).

Equation 3.1 Epoxide activation

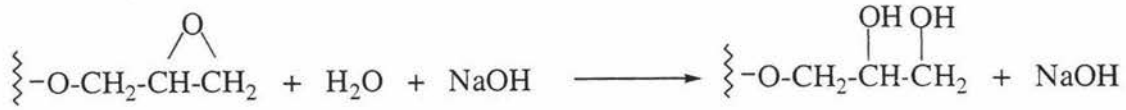


Perloza ECH Perloza-ECH

Equation 3.2 Hydrolysis of epichlorohydrin

Once the sodium hydroxide has been consumed further activation is not possible no matter how much epichlorohydrin is used. However, if excess sodium hydroxide is used hydrolysis of the active epoxide groups occurs (Equation 3.3).

Equation 3.3 Hydrolysis of activated Perloza by excess sodium hydroxide

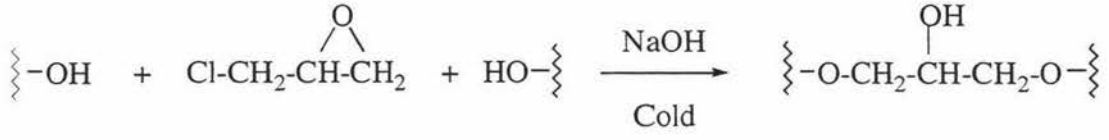


Perloza-ECH

Hydrolysis Product

In order to produce the maximum number of active epoxide groups a 25% molar excess of epichlorohydrin over sodium hydroxide was considered the necessary condition to be controlled. The final NaOH concentration of the reaction mixture of activation is also very important. Usually, the higher the NaOH concentration used the higher the epoxide substitution is obtained. However NaOH concentration above 6-8% w/v causes an extra weight of matrix without a significant increase in the number of epoxides produced (indicating that hydrolysis had occurred). Another explanation could be an increase in crosslinking had occurred (Equation 3.4).

Equation 3.4 Cross-linking of cellulose by ECH



Perloza ECH Perloza cross-linked Perloza

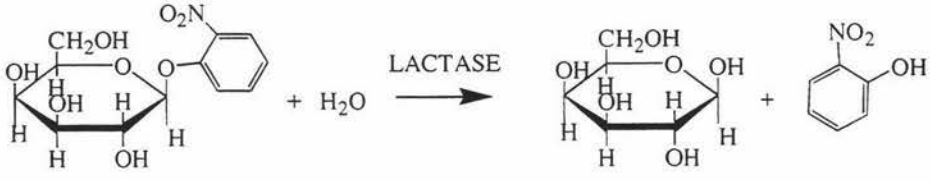
The actual volume of sodium hydroxide solution, and the number of active epoxides produced, ultimately depends on the physical properties of the Perloza used. Other factors influencing the number of epoxides produced are the temperature and time of the reaction. Maximum epoxidation is obtained by activating at low temperatures. However the time that is required for the reaction to reach completion at 4°C is in excess of 30 hours (Lilly, 1988).

It has been discovered that high levels of protein attachment can be obtained at very low activation levels (Burton et al., 1996). In order to investigate thoroughly how

different activation levels affect lactase attachment, Perloza with a range of activation levels was tested for lactase attachment in this study.

Lactase activity was determined using the ONPG assay. Lactase decomposes pseudosubstrate o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol (ONP) and galactose at 30°C and pH 6.5 (Equation 3.5). Reaction is terminated by addition of 0.5 M sodium carbonate. Absorbance of the resulting ONP is a measure of enzyme activity. ONP, which in the deprotonated form in alkaline solution shows a yellow colour, was recorded at an absorbance of 420 nm wavelength.

Equation 3.5 Hydrolysis of ONPG by lactase.



In the literature, there were quite a few studies using the same activation chemistry. Epoxy activation and ligand attachment methods have been reviewed by Klyashchitskii and Kuznetsov (1984). The stability of a coupled amine ligand was excellent under strongly alkaline conditions (pH 13), even after a period of weeks at 70°C (Sundberg and Porath, 1974). Epoxy matrices have been converted to amine resins with ammonia (Mattsumoto et al., 1980) and thiol resins with thiosuphate and reduction (Axen et al., 1975). The range of possible reactions of epoxidated matrices with proteins was reported (Zemanova et al., 1981).

Even though this successful activation method has been applied to several matrices, enzymes with a high molecular weight still can not always readily access an activated group on matrix due to steric effects (Figure 3.1). Often enzyme binding sites are buried or in a pocket just below the surface of the enzyme. An activated group on the matrix that is attached directly to support may not protrude far enough from the matrix

surface to reach the level of the binding site on an approaching enzyme molecule (Figure 3.1).

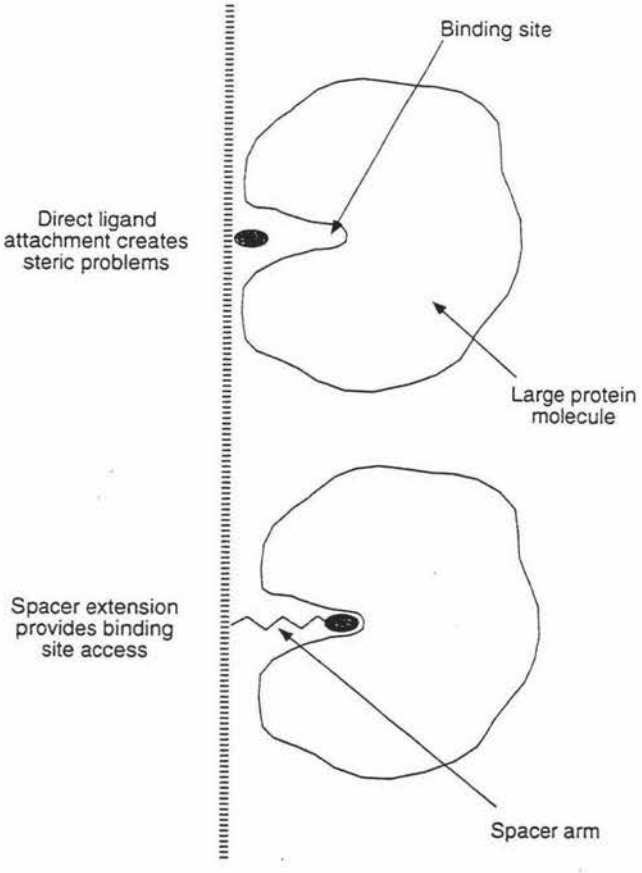


Figure 3.1 The advantage of using a spacer arm.

The principle advantage of using a spacer arm is that it provides ligand accessibility to the binding site of a target molecule. When the target molecule is a protein with a binding site somewhat beneath its outer surface, a spacer is essential to extend the ligand out far enough from the matrix to allow interaction (Hermanson et al., 1992).

The result may be a weakened interaction or no binding at all (Lowe et al., 1973). With rigid support materials, a spacer molecule may also provide greater flexibility, allowing the activated group to move into position to establish the correct binding orientation with an enzyme. The degrees of freedom that the hydrocarbon extender can provide are much greater than the movement possible within the polymeric backbone of a matrix (Cuatrecases, 1970). 6-Aminocaproic acid (ACA) was chosen as a spacer arm attached to activated Perloza in this study.

Chemical and physical properties of covalently bonded, water insoluble enzymes that have been extensively investigated in the literature are catalytic activity, pH-activity behavior, temperature-activity behavior, the Michaelis constant, substrate and inhibitor specificity, and various types of stability. Some of these properties such as activity, pH-activity behavior, temperature-activity behavior, storage stability and storage buffer selection are discussed later in this chapter.

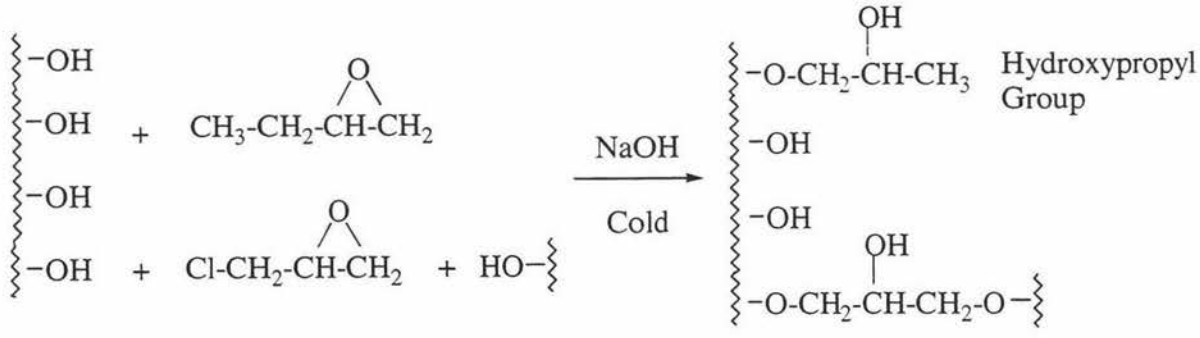
3.2 Results and discussion

3.2.1 Preliminary study

The successful activation of Perloza has been obtained in our lab using ECH chemistry (Burton et al, 1996). The commercial product Eupergit C also contains active oxirane groups. It was decided to using ECH chemistry for activation of Perloza in this study. Activated resins prepared in our laboratory, from a fine grade (80-100 μm) of Perloza have been found to have similar properties of adsorption, elution, resolution and capacity to Sepharose equivalents (Burton, 1995). Initial studies were started with Perloza 100 MT (fine). Substitution levels of ECH by activation method 1 and 2 was 0.24~0.26 and 0.22 mmol/g dry individually. Substitution levels were monitored by titration of oxirane groups. Further lactase attachment was carried out in a sealed flask and allowed to stand for 96 hours with occasionally shaking by hand. The activity of immobilized lactase on Perloza-ECH was 8.3 NLU/g of wet support.

It was demonstrated by the Ayers group that the chemical modification of cellulose can produce material suitable for derivatisation and subsequent use as chromatography media (Lilly, 1988). Cellulose was swollen with propylene oxide and cross-linked with ECH in the presence of sodium hydroxide (Equation 3.6).

Equation 3.6 Typical reactions illustrating the preparation of cross-linked hydroxypropylated Perloza.



Perloza ECH Perloza Cross-linked Perloza

Hydroxypropylated regenerated cellulose is an ideal starting matrix for the preparation of ion exchangers. The ion exchangers with this modification had a 4~5 fold increase in capacity over the one prepared without the use of propylene oxide (Egan, 1994).

In order to investigate whether high substitution levels of active groups benefit the activity of lactase immobilized, Perloza with large number of active oxirane groups was made by using a modification of Perloza before activation stage (Section 2.4). The subsequent activation was carried out by Activation Method 1 and the substitution level of ECH gained was 1.1 mmol/g dry. After the lactase attachment reaction it was shown that no or very little (about 1.39 NLU/g) activity of lactase remained on the support compared to that without chemical modification of Perloza-ECH.

3.2.2 Further activation studies

It was found that high levels of protein attachment can be obtained from very low resin activation levels (Burton et al., 1996). In order to investigate thoroughly how different activation levels affect lactase attachment, Perloza was activated with ECH in a range of NaOH concentrations to obtain varying activation levels. Perloza 200 and 500 MT were chosen to be investigated first. The amount of reagents and results of activation are shown in Table 3.1

Perloza (5 g)	Conc. NaOH (%)	30% NaOH (g)	Water (ml)	ECH (ml)	Substitution level (mmol/g)
PII	2	0.7	5.2	0.52	0.28
	3	1.05	4.95	0.78	0.41
	4	1.4	4.6	1.04	0.46
	6	2.1	3.9	1.46	0.63
	7	2.45	3.55	1.72	0.36
PV	2	0.64	4.36	0.47	0.26
	3	0.96	4.04	0.71	0.34
	4	1.27	3.73	0.94	0.44
	6	1.9	3.1	1.41	0.80
	7	2.24	2.76	1.65	0.63

Table 3.1 Epoxide activation with different NaOH concentration.

PII, PV refer to Perloza 200 MT, Perloza 500 MT (medium) individually.
Reaction conditions:

Perloza:	5 g	Temperature:	4°C
30% NaOH:	as shown	Reaction time:	16 h
Water:	as shown		
ECH:	as shown		

As the concentration of the NaOH was increased from 2 to 6%, the substitution level also increased in both PII and PV. Further increases in the NaOH concentration resulted in the substitution level falling off, indicating that the rate of hydrolysis of the epoxide had increased (Equation 3.3) and the increase of crosslinking had occurred (Equation 3.4). It was observed that NaOH concentrations above 6% caused Perloza beads to stick together, forming clumps. The crosslinking tightens Perloza beads together intramolecularly and intermolecularly, decreases the pore size of Perloza beads and even forms crosslinks between Perloza beads. The extra crosslinking formed during this stage caused a decrease in swollen volume of the final product which was also shown by Lilly (1988) and Egan (1994).

In the following lactase attachments (Table 3.2), activated Perloza with NaOH concentrations 2~4% was investigated. Lactase attachment method from Section 2.6.1 was used in the lactase immobilization. The lactase attachment levels obtained using Perloza with varying levels of activation are summarized in Table 3.2. The incubation times for attachment were 22 and 72 hours in order to study the time influence to immobilization. The results indicated that as the activation level (or concentration of NaOH) went up the activity of immobilized lactase dropped off for both Perloza 200 MT and 500 MT no matter how much lactase became attached (shown in lactase substitution level).

	Conc. NaOH (%)	Sub. level (mmol/g)	Activity1 (NLU/g)	Lactase sub.1 (mg/g)	Activity2 (NLU/g)	Lactase sub.2 (mg/g dry)
PII-ECH	2	0.28	7.40	0.24	13.4	13
	3	0.41	7.37	0.16	13.2	13
	4	0.46	5.39	0.13	10.2	10
PV-ECH	2	0.26	7.1	0.18	14.3	10
	3	0.34	6.1	0.21	13.0	9
	4	0.44	6.2	0.13	9.2	10

Table 3.2 Lactase attachment upon different activation levels.

NaOH concentration used in activation stage as shown in the table.
1M K-phos: 1M potassium phosphate buffer, pH 6.5.
Lactase sub. 1: lactase substitution level for 22 hours immobilization.
Lactase sub. 2: lactase substitution level for 72 hours immobilization.

Reaction conditions:

Activated Perloza (P-ECH):	2 g	Temperature:	room
Maxilact L2000:	0.17 g/g Perloza	Reaction time:	22 h, 72 h
1M K-phos: 2 ml			

Generally, a higher lactase substitution level, means a larger amount of lactase immobilized, and reflects higher lactase activity obtained. The lowest activation level (0.28 mmol/g dry for Perloza 200 MT, 0.26 mmol/g dry for Perloza 500 MT) gave highest activity of lactase immobilized (7.4 NLU/g for 22 hours immobilization on

Perloza 200 MT, 7.1 NLU/g for 22 hours immobilization on Perloza 500 MT). For Perloza 200 MT, higher resin substitution levels than this did not achieve further gains in lactase substitution and lactase activity. Consequently 2% NaOH for the activation stage was chosen for further investigation.

It was also discovered that the longer immobilization time (72 hours) gave higher activity of lactase immobilized than that of short immobilization time (22 hours). Some results for Perloza 500 MT could not be explained properly perhaps due to the difficulties in delivering reproducible amounts of immobilized lactase (wet weight) for weighing. Due to considerations needed for efficiency in industrial use and retention of highest lactase activity, immobilization time of 40 hours was selected as a compromise for the later studies.

3.2.3 Enzyme attachment

In Section 3.2.2, activated Perloza prepared from NaOH concentration 2% was shown to be the most effective support for regaining the highest activity of lactase immobilized. Therefore it was decided to concentrate on using 2% NaOH for the activation stage. Activation at 2% NaOH for three types of Perloza (Perloza 100 MT, Perloza 200 MT, Perloza 500 MT) were carried out for 23 hours at 4°C. The reaction time of 23 hours was chosen for obtaining the maximum completion of the reaction. The results were illustrated in Table 3.3. The activation at 3% NaOH also was carried out as a comparison.

Perloza (20 g)	Conc. NaOH (%)	30% NaOH (g)	Water (ml)	ECH (ml)	Substitution level (mmol/g dry)
PI	2	2.5	17.2	1.8	0.32
PII		2.8	21.2	2.1	0.32
PV		2.56	17.5	1.9	0.25
PI	3	7.4	32	5.4	0.36
PII			42	6.4	0.41
PV		6.6	22.8	4.9	0.32

Table 3.3 Activation of three types of Perloza at NaOH concentration 2% and 3%.

PI, PII, PV refer to Perloza 100 MT, Perloza 200 MT, Perloza 500 MT (medium) individually.
Reaction conditions:
Perloza: 20 g Temperature: 4°C
Reaction time: 23 hrs

Selection of enzyme attachment conditions

The covalent binding method involved in this study depends on nucleophilic attack of amine groups of lactase on the activated support and the inclusion of amine buffers in the reaction would obviously cause competition between this and the protein for reactive sites. Since the literature for protein immobilization onto epoxide supports claimed good results were only obtained in the presence of high concentration of lyotropic salts (high ionic strength), the immobilization was buffered with 1 M potassium phosphate solution. This is also appropriate because potassium ion is an activator of lactase.

The pH at which enzymes bind to a support is a critical parameter to be determined for any method of immobilization. It is enzymic activity which is of most concern, and this is the product of total bound protein, which generally increases at a certain pH range. The optimum pH for binding may be a complex function and usually has to be

determined empirically, taking due account of the enzyme's stability and the known protein binding character of the support. Based on these considerations, pH of the buffer was chosen at 6.5, which spans the optimal pH range of lactase between 6.3-7.2, for the preliminary study.

Most workers choose to couple enzymes at low temperatures (4°C) to minimize deactivation but it should be remembered that at temperatures nearer ambient the coupling reaction will be more rapid, reducing contact time (Rosevear et al., 1987). The coupling reaction was therefor carried out at room temperature in this study.

Maxilact L2000 (0.17 g of enzyme solution per gram of wet resin) was mixed with wet resin and minimum amount of buffer solution for a reaction period of 40 hours. Blank values were obtained for the three types of unactivated Perloza by using the same immobilization method as for activated Perloza (Table 3.4 and Figure 3.2, Perloza-lactase). These values were quite low in comparison with the values obtained with epoxide activated Perloza, indicating that the lactase substitution levels on unactivated Perloza were most probably due to non-covalent adsorption of protein that could not be readily washed away by 0.1 M potassium phosphate washing buffer.

Utilizing of spacer arm ACA

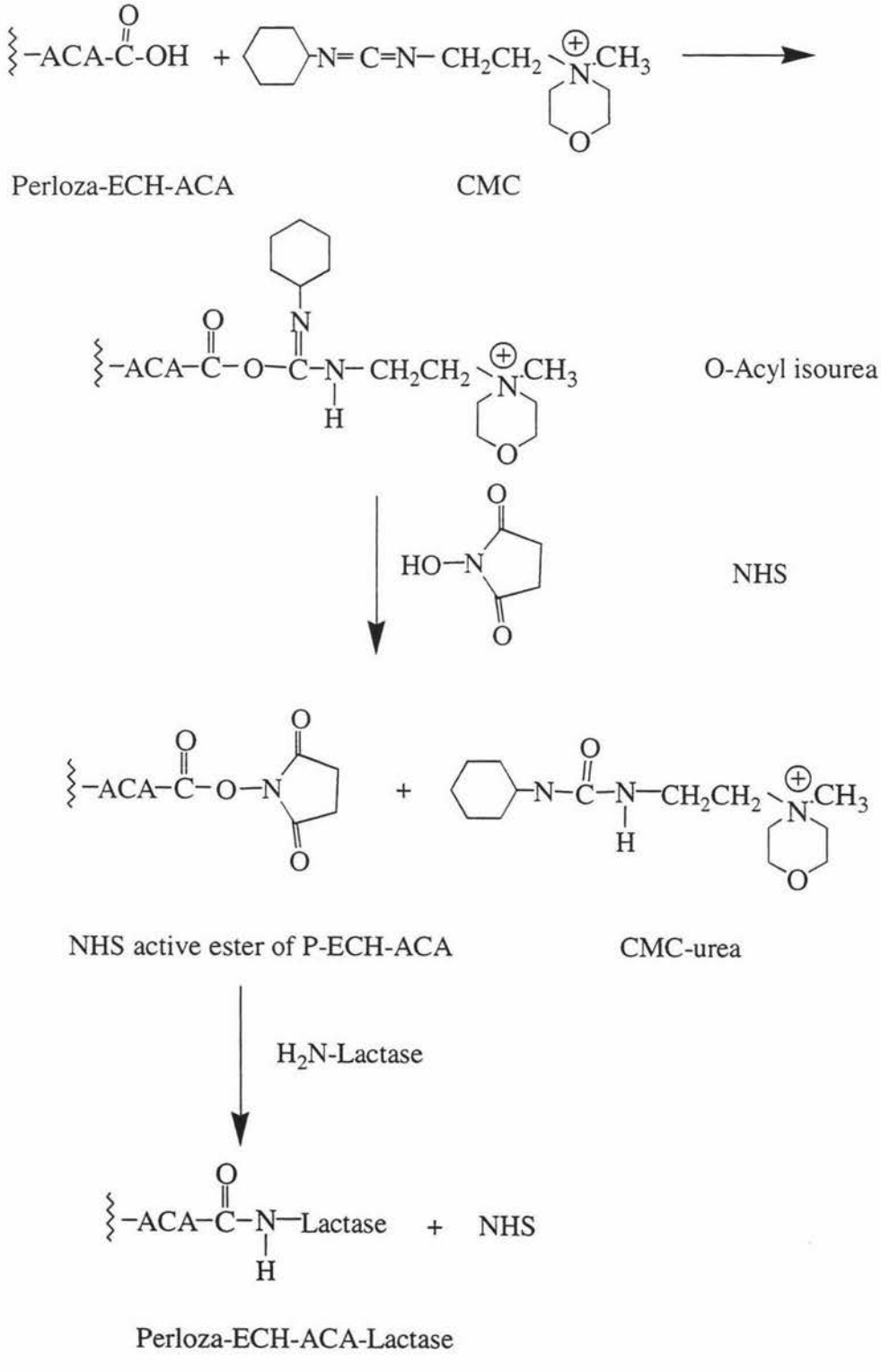
The choice of spacer molecule can affect the relative hydrophilicity of the immediate environment of an activated group. Molecules containing long hydrocarbon chains may increase the potential for nonspecific hydrophobic interactions, especially when the activated group is small and of low molecular weight (O'Carra et al., 1973). Selecting spacers that have more polar constituents, such as secondary amines, amide linkages, ether groups, or hydroxyls will help keep hydrophobic effects to a minimum.

It is also important to consider the ionic effects that a spacer molecule may impart to a gel. Spacers with terminal primary amine groups may have the potential for creating a positive charge on the support. With small affinity ligands, the residual charges can form a secondary environment that may cause considerable nonspecific interactions

with proteins. The same holds true for spacers with terminal carboxylic groups. In general, a negatively charged spacer will cause less nonspecific protein binding than a positively charged one, but blocking excess remaining groups is still a good idea. A good blocking agent for use with carboxylic residues is ethanolamine, which leaves a terminal hydroxyl group which is polar but not ionic. The blocking group is thus used after attachment of the enzymes to the activated matrix.

The best choices of spacer arm are to have appropriate coupling functionality on either end and an overall hydrophilic character. Over the years, this process of selection has narrowed the variety of spacers to a few that are used recurrently (Hermanson et al., 1992). A popular spacer used to create a terminal carboxylic group on a matrix is 6-aminocaproic acid (ACA) (Schmer, 1972). This compound provides a primary amine coupling functionality on one end and a carboxylic group on the other. Epoxide groups on the support, which are amine reactive, can first couple the spacer to the matrix. The result is a hydrophilic spacer arm containing secondary hydroxyl from the opened epoxide. A relatively stable linkage to the matrix after activation results and also produces a free carboxyl group at the end of the spacer. For second step chemistries, carbodiimides are popular reagents. 1-Cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluenesulfonate (CMC) was used in this study to activate the spacer arm (Scheme 3.1).

Scheme 3.1 Activation of Perloza-ECH-ACA and lactase attachment.



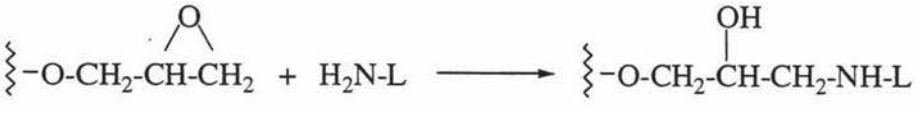
ACA may create some mild hydrophobic interactions because of its 5-carbon linear structure. In practice, however, these effects are not usually severe, especially when using hydrophilic ligands. In addition, after ligand (enzyme in this case) coupling some carboxylate groups often remain that have not been blocked by ligand. These negatively charged spacers would effectively negate the hydrophobic environment of the hydrocarbon chain. This ACA spacer arm gives a linkage with no charge, which minimises non-specific ionic binding and produces a relatively stable linkage that reduces enzyme leakage. It will favour lactase attachment and a useable activity of immobilized lactase.

It was also proven that enzymes may be attached to the activated resin through the spacer arm (ACA) by Burton et al. (1991) at Massey University. Due to the above considerations, 6-amino caproic acid (ACA) was employed as a spacer arm for building a bridge between activated Perloza and lactase in this study.

Reaction mechanisms of enzyme attachment and ACA attachment

For epoxide activated Perloza reacting with lactase it is considered that the activated group oxirane reacts with an amino group of lactase to form a reasonably stable linkage (Equation 3.7). The amine group of the lactase molecule can be either the N-terminus or a lysine side chain residue. The linkage between Perloza and lactase has no charge, which minimises non-specific ionic binding and produces a relatively stable linkage that reduces lactase leakage.

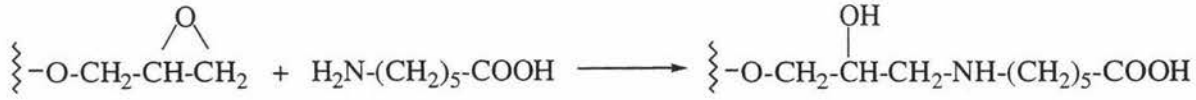
Equation 3.7 Lactase attachment to Perloza-ECH



Perloza-ECH Lactase Perloza-ECH-Lactase

Oxirane may react with amino group of ACA when ACA attaches to activated Perloza (Equation 3.8). The length of spacer is seven atoms longer than that of ECH activated Perloza.

Equation 3.8 Attachment of spacer arm ACA to Perloza-ECH



Perloza-ECH ACA Perloza-ECH-ACA

Perloza-ECH-ACA was activated by method from Section 2.6.2, using N-hydroxysuccinimide (NHS) and 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluenesulfonate (CMC) (Scheme 3.1). Titration of ACA group with the method from Section 2.7.2 proved unsuccessful (also section 3.2.5). A twenty fold excess of NHS and CMC over the amount of oxirane groups of Perloza-ECH was used in activation of Perloza-ECH-ACA for the preliminary study. It was observed that Perloza 500 MT started clumping during the process of activation of Perloza-ECH-ACA. Thus further optimization of the ratio of NHS and CMC to activated groups would be beneficial in a future study in the Perloza 500 MT case.

Results and discussion for enzyme attachment

The reagents used and results from lactase attachment onto both Perloza-ECH and Perloza-ECH-ACA were summarized in Table 3.4. Perloza with activation by ECH at 2% and 3% NaOH concentration were both tested. Basically, both the activity and the amount of immobilized lactase from 2% NaOH activation are higher than that from 3% NaOH activation (Figures 3.2 and 3.3). Lactase attachment to three types of Perloza for both activation levels showed similar trends. For Perloza with activated group oxirane, Perloza 100 MT-ECH obtained the highest lactase substitution level (see Figure 3.3, P-E (2%) and P-E (3%)) as well as activity (Figure 3.2, P-E (2%) and P-E (3%)), Perloza 200 MT second, Perloza 500 MT last.

The mechanism for these results is complicated. The binding force between lactase and activated Perloza is assumed to be the combination of covalent binding and some physical absorption (e.g. entrapment). Covalent binding is expected to predominate. Thus Perloza 100 may allow the lactase molecule to become embedded firmly in the matrix but the activity of lactase immobilized has not been reduced (see Figure 3.2 and Figure 3.3). The exclusion size of Perloza 200 MT may provide looser binding between resin and lactase than that of 100 MT resin, resulting in both lower activity and amount of lactase immobilized. Perloza 500 MT resin was the lowest of that all.

For Perloza with a spacer arm, Perloza 200 MT-ECH-ACA resin retained the highest activity, Perloza 100MT the second, Perloza 500 MT the last (see Figure 3.2, P-E-A-L (2%) and P-E-A-L (3%)) although Perloza 100-ECH-ACA still immobilized lactase most (the highest substitution) (see Figure 3.3, P-E-A-L (2%) and P-E-A-L (3%)). It was thought that high population of immobilized lactase on Perloza 100-ECH-ACA induced conformational changes to the individual lactase molecules attached, which disturbed the active sites of lactase and reduced total activity. In contrast, 200-ECH-ACA has a larger (but not too much bigger) pore size, which permitted more freedom to the enzyme molecules, favouring enzyme activity predominantly from covalently bound enzyme. Due to the population of immobilized lactase on Perloza 500-ECH-ACA being too low (0.5 mg/g), 500 MT resin retained the lowest activity.

Perloza type & activation	Substitution (mmol/g dry)	Maxi. (g)	K-phos (ml)	Activity (NLU/g)	Lactase level (mg/g)
PI (5 g)	-	0.83	5	0.94	-
PII (5 g)			5	0.69	-
PV (5 g)			4	0.57	-
2% NaOH activation					
PI-ECH (5 g)	0.32	0.83	5	11.41	0.47
PII-ECH (5 g)	0.32		5	7.71	0.45
PV-ECH (5 g)	0.25		4	6.93	0.31
PI-ECH-ACA (5 g)	-	0.83	5	28.53	1.00
PII-ECH-ACA (4.5 g)		0.75	4.5	30.94	0.94
PV-ECH-ACA (4 g)		0.66	4	24.70	0.75
3% NaOH activation					
PI-ECH (5 g)	0.36	0.83	5	7.33	0.3
PII-ECH (5 g)	0.41		5	5.22	0.24
PV-ECH (5 g)	0.32		4	5.18	0.2
PI-ECH-ACA (3.5 g)	-	0.5	3.5	16.97	1.42
PII-ECH-ACA (3.5 g)			3.5	23.23	0.9
PV-ECH-ACA (3.5 g)			2.5	15.43	0.5

Table 3.4 Lactase attachment upon Perloza-ECH and Perloza-ECH-ACA.

Reaction conditions:

Perloza:	5 g	Temperature:	room
Perloza-ECH:	5 g	Reaction time:	40 hrs
Perloza-ECH-ACA:	as shown		

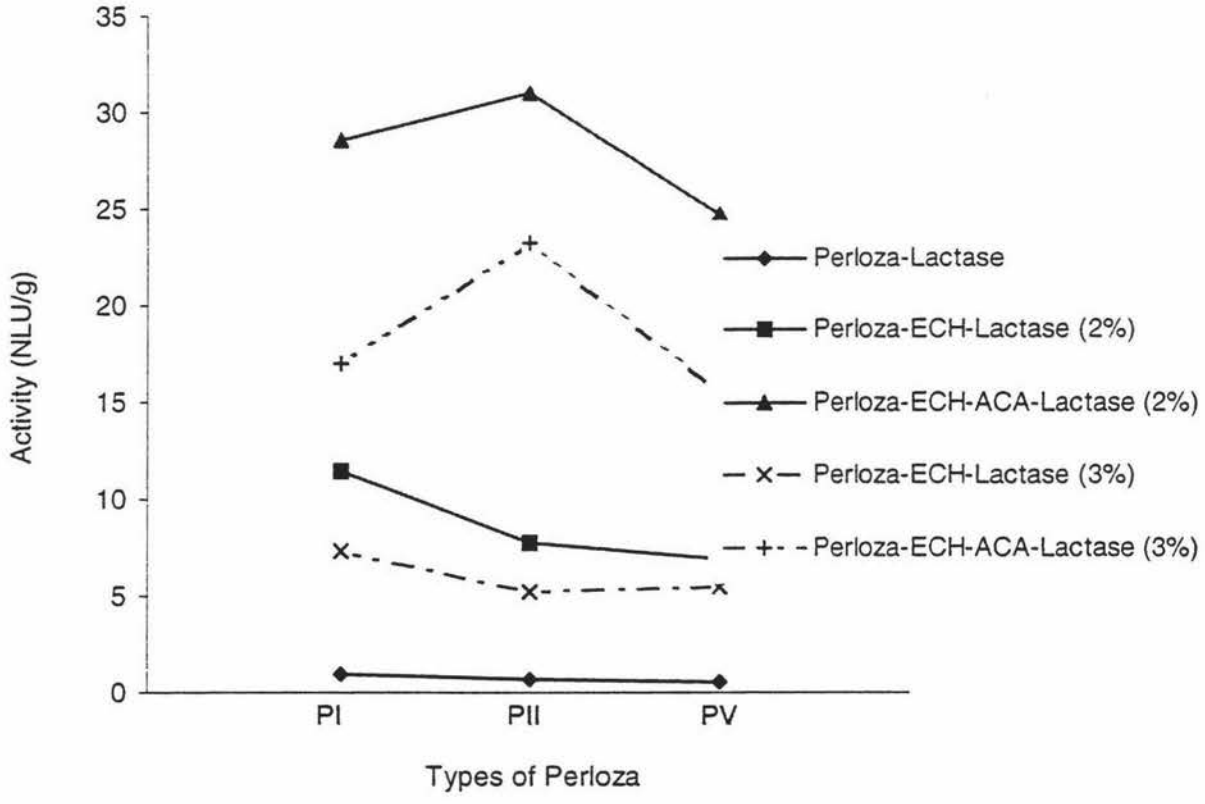


Figure 3.2 **Activity of lactase on P-ECH and P-ECH-ACA (Activition at 2% and 3% NaOH concentration).**

Immobilization conditions: Maxilact L2000 (0.17 g per gram of wet resin) reacted with resin in minimum amount of 1 M potassium phosphate buffer, pH 6.5 at room temperature for 40 hours.

Both Perloza-ECH-Lactase and Perloza-ECH-ACA-Lactase with lower activation level achieved higher activity of lactase immobilized (▲, ■) than that of higher activation level (+, ✖) for three types of Perloza. Perloza 200-ECH-ACA-Lactase obtained the highest activity over Perloza 100-ECH-ACA-Lactase and Perloza 500-ECH-ACA-Lactase. Perloza 100-ECH-Lactase retained the highest activity over Perloza 200-ECH-Lactase and Perloza 500-ECH-Lactase.

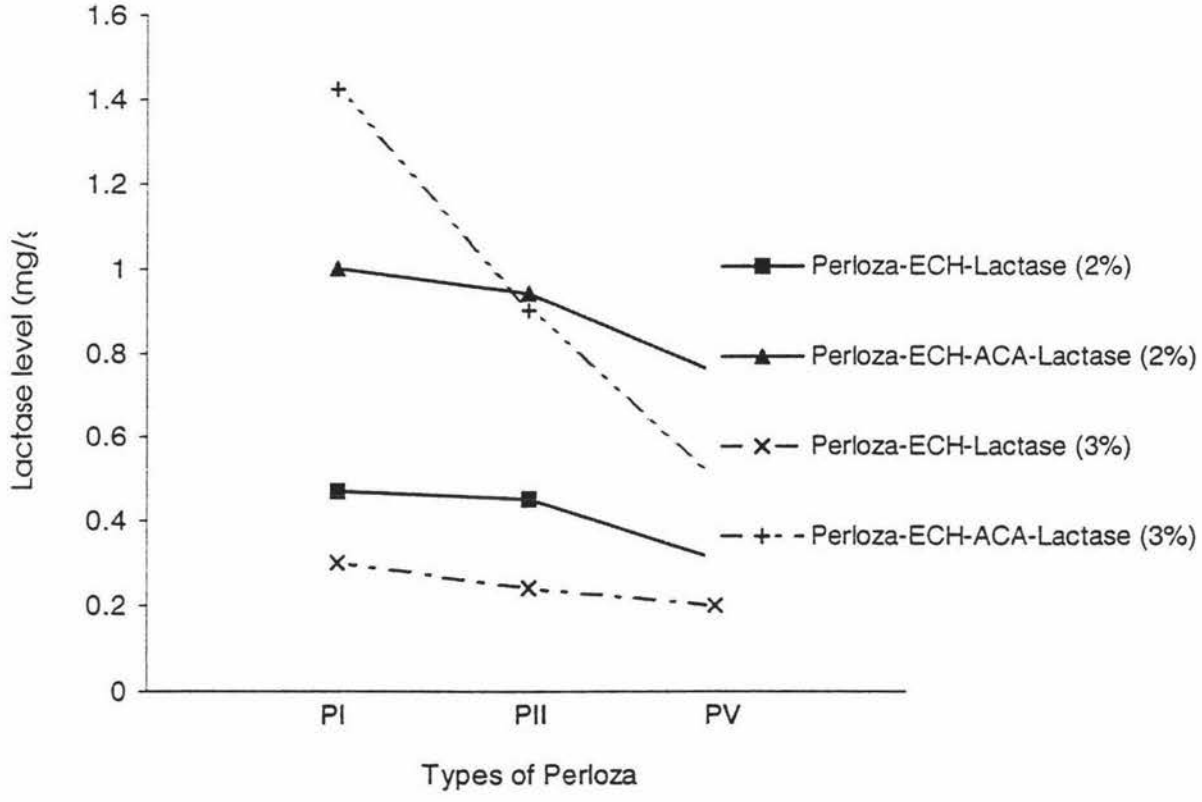


Figure 3.3 Lactase level on P-ECH and P-ECH-ACA (Activition at 2% and 3% NaOH concentration).

Immobilization conditions: as for Figure 3.2.

Both Perloza-ECH-Lactase and Perloza-ECH-ACA-Lactase with lower activation level achieved larger amount of lactase immobilized (▲, ■) than that of higher activation level (+, ✖) for three types of Perloza. Perloza 100-ECH-ACA-Lactase obtained the highest substitution level of lactase over Perloza 200-ECH-ACA-Lactase and Perloza 500-ECH-ACA-Lactase. Perloza 100-ECH-Lactase retained the highest substitution level of lactase over Perloza 200-ECH-Lactase and Perloza 500-ECH-Lactase.

3.2.4 Lactase activity assay

Initial lactase activity assay was based on the method provided by New Zealand Dairy Research Institute. The buffer used was 0.1 M potassium phosphate buffer containing 2 mM magnesium chloride, pH 6.5. o-Nitrophenyl galactopyranoside (ONPG, 2 mM) in the above buffer was used as substrate in the assay. It was found that ONPG was difficult to dissolve in such a buffer. The mixture was heated in a water bath to help dissolution but still some suspended material was seen especially after recovery from the frozen state. All the other assay procedures followed the method described in Section 2.9.3. Stopping the reaction was achieved with 0.25 M NaOH. This stopping solution was not ideal due to the slight increase in absorbance that could be seen with the spectrophotometer after termination of the reaction. Under this assay system, activity of immobilized lactase was expressed by using unit: change in absorbance per minute per gram of suction-dried (or wet) enzyme matrix.

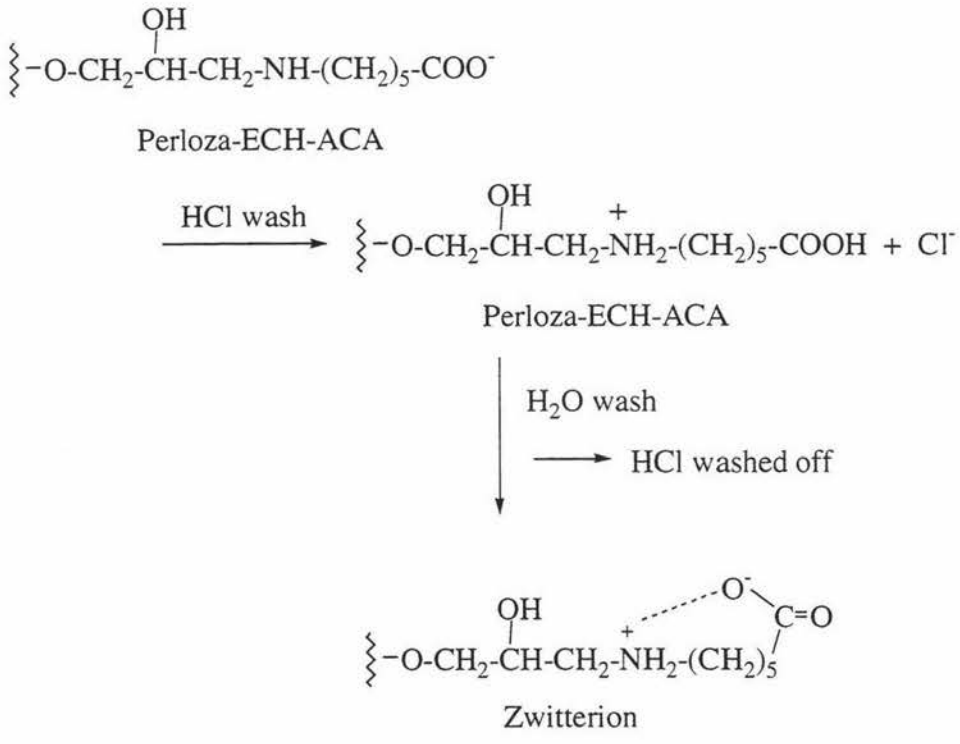
The improved assay adopted the method introduced by the Gist-Brocades company (Engelen and Randsdorp, 1999). The buffer solution used was 0.1 M potassium phosphate, 0.1 M $MgSO_4$, 0.05 mM EDTA, and pH 6.5. ONPG stock solution was 5 and 6.6 mM ONPG in the above buffer solution. The ONPG dissolved in this buffer in warm water bath and produced a clear solution. Stopping solution employed was 0.5 M Na_2CO_3, 0.1 M EDTA. After termination of the reaction, absorbance of supernatant from the sample kept constant on the spectrophotometer. Due to difficulties in delivering reproducible amounts of immobilized lactase, a few results are still questionable.

3.2.5 Titration method

Titration of epoxide groups on epoxide activated matrix was well established by the Ayers group at Massey University. Dry weight was used in order to obtain an accurate value of the substitution level. Titration of 6-aminocaproic acid (ACA) as Perloza-**CDI**-ACA has been carried out successfully by Burton (1991) and Cross (1998). This titration method was not effective for Perloza-**ECH**-ACA in this research. In the

titration process, ACA matrix was first washed with 0.1 M HCl, which was supposed to convert the -COONa form to the protonated -COOH form (Scheme 3.2). Then it was washed with water to remove excess acid. However zwitterion was presumed to form during this step, so titration of ACA groups with adding a pinch of NaCl was impossible. Further investigation of this titration method will be beneficial.

Scheme 3.2 Titration of ACA group on Perloza-ECH-ACA



3.2.6 Determination of optimum pH for immobilized lactase

The apparent activity of all enzymes shows a classic bell-shaped curve relationship with the pH of the solution in which they are dissolved (Rosevear et al., 1987). This is to be expected when one considers that enzymes are polyionic molecules with a three-dimensional structure and active site-substrate interaction, which involves charged residues. The optimum pH for the enzyme reaction closely matches the pH of the biological environment from which the enzyme was derived. The optimum pH range for lactase from Maxilact L2000 is 6.3-7.0. The pH of the environment not only affects

the efficiency of reaction but also has an effect on enzyme stability under any set of conditions. This is likely to be a bell-shaped relationship too but typically spans a wider pH range.

The pH of the medium has a similar effect on both free and immobilized enzymes but the pH stability is of even greater concern with enzymes that are to be immobilized as they have to undergo adverse conditions during coupling. Immobilization will also restrict movement of the enzyme compared to enzyme that is free in solution.

There may well be different pH optima for immobilized enzymes from different sources and different coupling methods. Weetall et al. (1974) found that immobilization of lactase from *A. niger* on zirconium coated porous glass beads resulted in a shift in the pH optimum of immobilized enzyme from that of its soluble counterpart. Mathur et al. (1983) reported no shift in the pH optimum of rennet immobilized on silanized sand. Thompkinson (1980) claimed that immobilization of lactose from *Kluyveromyces-fragilis* on an ion exchange resin showed no shift in the pH optimum of the enzyme.

The results pertaining to the pH profile determination of Perloza immobilized and soluble lactase are illustrated in Figure 3.4. The soluble enzyme preparation showed maximum hydrolysis of ONPG (expressed as relative activity above 80% of maximum) in the pH range between 6.4 to 7.2, whereas, immobilized lactase on Perloza 200-ECH (shown as Perloza 200-ECH-Lactase) showed maximum hydrolysis of substrate at pH 6.6. Then the hydrolysis dropped more rapidly than that of free enzyme as the pH increased to 7.4. Up to about pH 7.4, the activity of immobilized enzyme started to decline slower than that of free enzyme. Immobilized lactase on Perloza 200-ECH-ACA (Perloza 200-ECH-ACA-Lactase) showed maximum activity at pH 6.8. Increase in pH from 6.8 to pH 7.3 however, resulted in 20% decrease in the rate of hydrolysis by immobilized enzyme. The soluble enzyme preparation showed a greater decrease in activity at the corresponding pH. Lowering of the pH of the substrate solution from 6.8 to 6.2 resulted in a reduced rate of hydrolysis by soluble as well as insoluble enzyme

preparation. It was interesting to note that there was no shift in the pH optimum of lactase due to the process of immobilization.

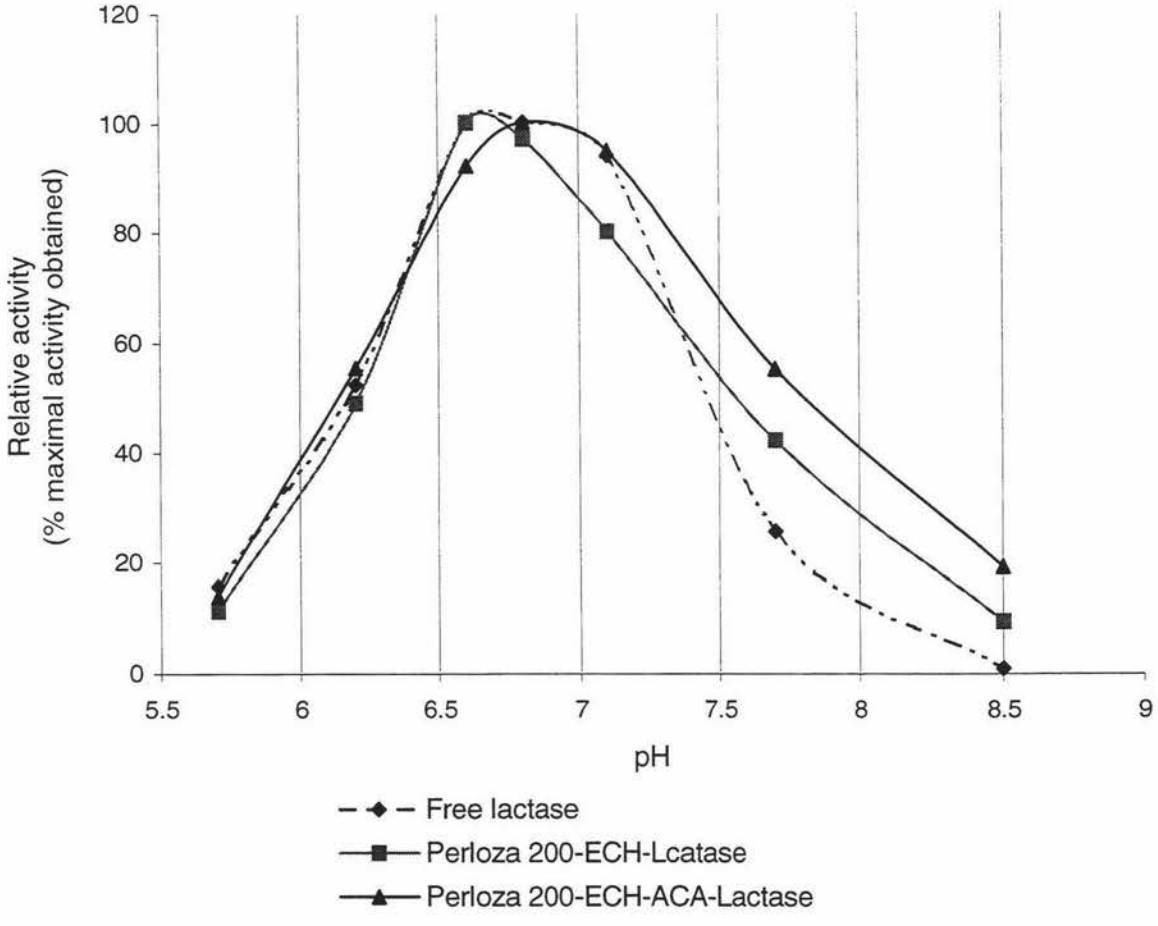


Figure 3.4 pH profile of free and immobilized lactase at 30°C.

Immobilization conditions:
Maxilact L2000 (0.17 g per gram of wet resin) reacted with activated resin (activation at 2% NaOH concentration) in minimum of 1 M potassium phosphate buffer, pH 6.5 at room temperature for 40 hours.

Maximum hydrolysis (expressed as relative activity) was attained at pH 6.5-7.2 for free lactase, as well as immobilized lactase. No shift in the pH optimum for immobilized lactase in comparison to free lactase was observed.

3.2.7 Determination of optimum temperature for immobilized lactase

Reactions catalyzed by enzymes are subject to the general laws of thermodynamics and so the rate of reaction will increase with temperature according to the Arrhenius equation (Rosevear et al., 1987). However, reactions leading to loss of catalytic function follow the same laws.

The results of the study of the optimum operating temperature are illustrated in Figure 3.5. Increase in temperature showed a corresponding increase in the rate of hydrolysis of the substrate at pH 6.5 by free as well as immobilized enzyme preparations up to 37°C. Free enzyme and immobilized enzyme showed maximum hydrolysis (above 80% relative activity obtained) at a temperature range of 28 to 44°C. Further increase in substrate temperature, however, resulted in a sharp drop in the rate of hydrolysis by free enzyme as well as immobilized enzyme. These results suggested that no shift in the temperature optimum of the enzyme was observed due to the process of immobilization

Shukla (1975) suggested that the number of active sites in lactase per molecule is temperature dependent, i.e. a molecule with molecular weight 700 kD possesses 1 active site at 4 to 6°C, however, at 20 to 22°C it possesses 4.7 active sites. This suggests that at a low temperature the molecular conformation is such that not all active sites are available. The higher rate of hydrolysis at higher temperature for this particular enzyme may be attributed at least partially to this type of effect. However, too high temperature i.e. above 44°C, the decrease in the activity of enzyme may be caused by the inactivation of the enzyme due to protein denaturation at the higher temperature of operation.

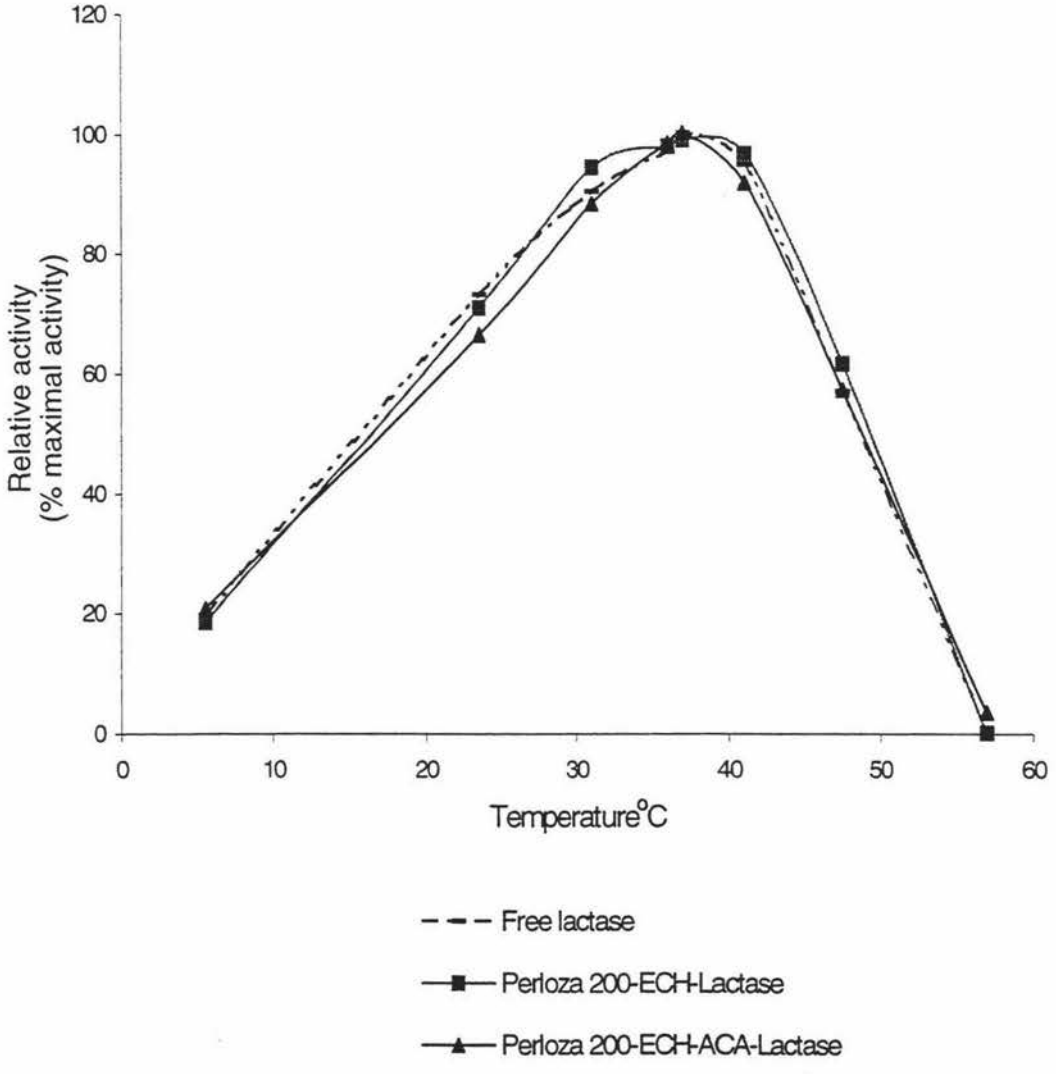


Figure 3.5 Temperature profile of free and immobilized lactase at pH 6.5.

Immobilization conditions: as for Figure 3.4.

Maximum hydrolysis was attained over a temperature range of 30-42°C. No shift in temperature optimum for immobilized lactase in comparison to free lactase was observed.

3.2.8 Storage stability of the immobilized enzymes

In principle, the stability of an immobilized enzyme can be enhanced, diminished, or remain unchanged relative to the native, water soluble enzyme, and numerous examples of each kind exist (Zaborsky, 1973).

All the immobilized enzyme samples in this study were stored at 4°C in 0.1 M potassium phosphate, 1 mM $MgSO_4$, 0.05 mM EDTA, with 0.01% NaN_3, at pH 6.5. In order to ascertain the loss of enzymatic activity with storage time, the activity of the immobilized enzyme was measured for a certain period. Lactase immobilized onto epoxide activated Perloza 100 (PI-ECH-L) for 40 hours immobilization was found to retain 70% of its initial activity after 9 days (Figure 3.6, PI). However for the lactase immobilized onto Perloza 100 with ACA spacer arm (PII-ECH-ACA-L), the activity was retained at 87% of the original after the same storage time (Figure 3.6, PIa). In the case of lactase immobilized onto Perloza 200-ECH, 73% of its initial activity was remained after 9 days (Figure 3.7, PII). Immobilized lactase on Perloza 200-ECH with spacer arm (PII-ECH-ACA-L) retained 82% of its original activity after the same storage time. These limited results indicated that lactase immobilized on spacer arm maintained better stability than lactase immobilized directly on epoxide activated Perloza for the first 9 days storage.

Figure 3.6 and Figure 3.7 also show the general shape of activity of immobilized enzyme vs time graphs. For immobilized lactase on PI-ECH-ACA and PII-ECH-ACA, there was a sharp drop to about 10% of initial activity within 1 day, after which the activity loss leveled off and kept at over 80% of initial activity until 9 days. For immobilized lactase on PI-ECH and PII-ECH, there was a sharp drop to about 20% of initial activity within 1 day, after which the activity loss leveled off and kept at over 70% of initial activity for 9 days.

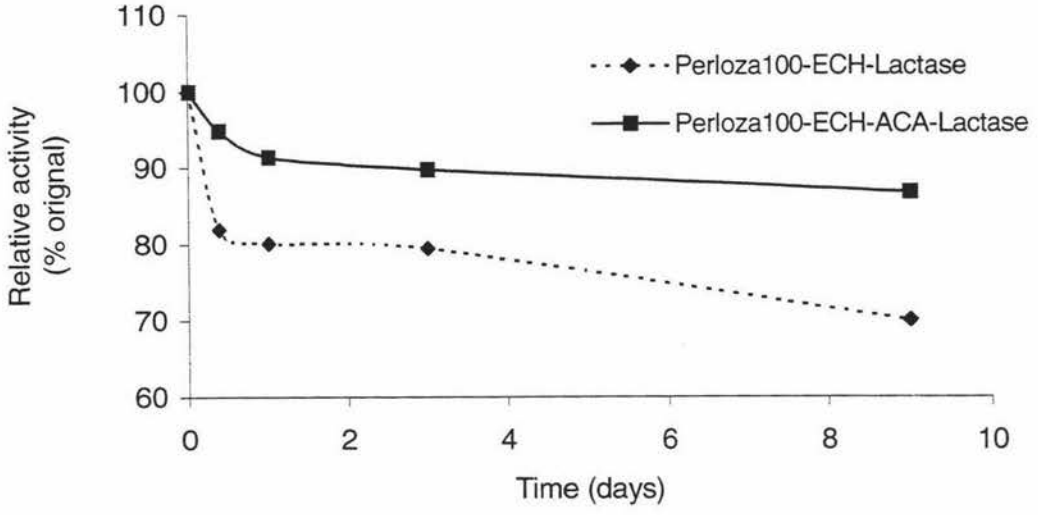


Figure 3.6 Stability study of immobilized lactase on Perloza 100.

Immobilization conditions: as for Figure 3.4.

Perloza 100-ECH-Lactase retained 70% of its original activity after 9 days storage. However, Perloza 100-ECH-ACA-Lactase retained 87% of its original activity after the same period.

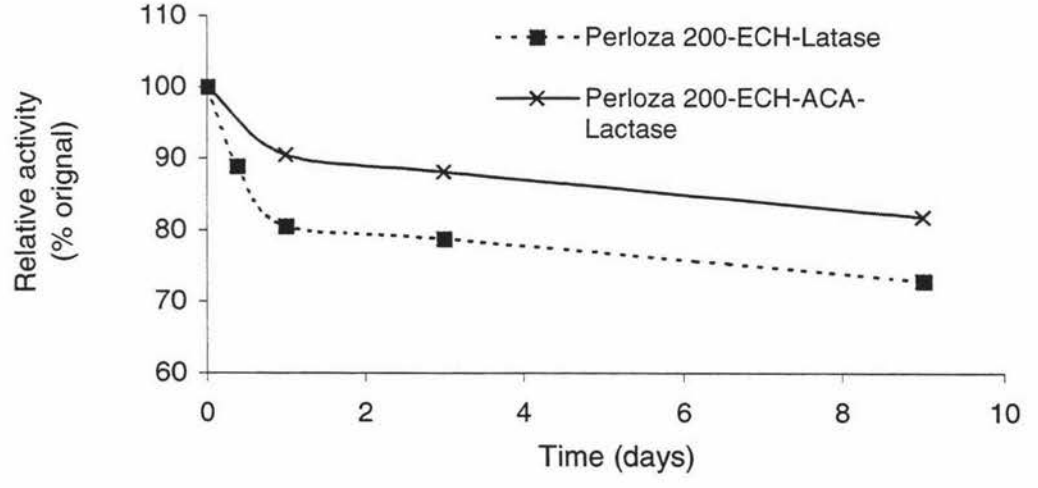


Figure 3.7 Stability study of immobilized lactase on Perloza 200.

Immobilization condition: as for Figure 3.4.

Perloza 200-ECH-Lactase retained 73% of its original activity after 9 days storage. However, Perloza 200-ECH-ACA-Lactase retained 82% of its original activity after the same period.

3.2.9 Selection of storage buffer

In general, immobilized enzymes should be stored in a similar way to the free enzyme. If the enzyme is to be used soon after preparation it is best suspended in distilled water or a buffer and stored at 4°C. Due to the fact that free lactase used in the study is stored in 45% glycerol, the storage buffer chosen for the study was 60% glycerol (PI-1 in Figure 3.8). Another storage buffer studied was 0.1 M potassium phosphate, 1 mM $MgSO_4$, 0.05 mM EDTA, 0.01% NaN_3, and pH 6.5 (PI-2 in Figure 3.8). Both potassium and magnesium ions accelerate the activity of lactase and stabilize the enzyme (Guy and Bingham, 1978). Azide added was to prevent microbial contamination of the enzyme preparation.

Figure 3.8 shows the comparison of activities of immobilized lactase on PI-ECH vs time in two buffer systems. PI-ECH-Lactase with Buffer 1 retained 28% of its original activity after 48 days storage. However, PI-ECH-Lactase with Buffer 2 remained 42% of its original activity. The result suggests that storage with Buffer 2 achieved higher retention of activity of immobilized lactase than that of storage buffer 1. Consequently storage Buffer 2 was applied for storage of immobilized lactase in the later studies.

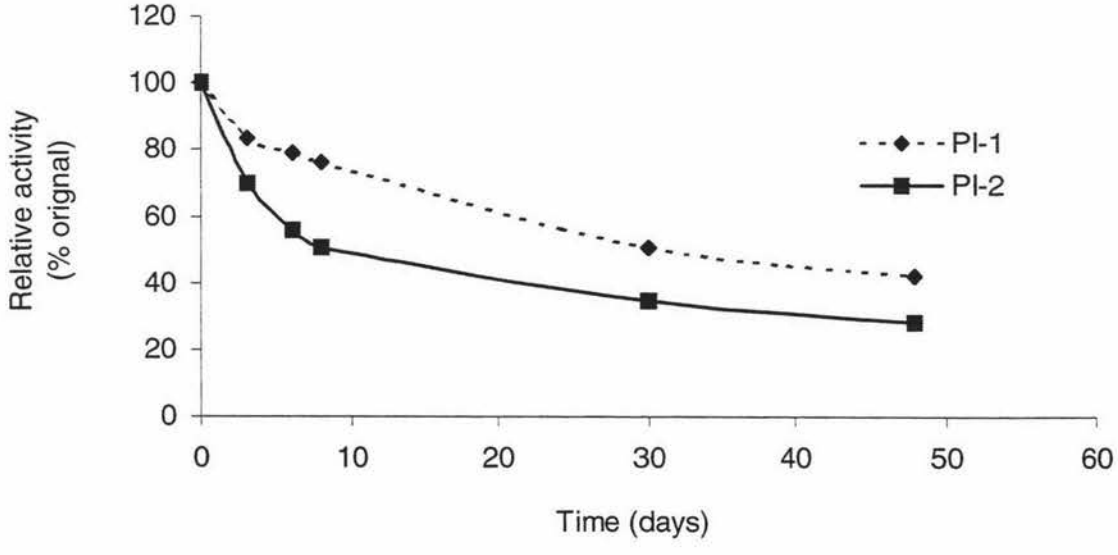


Figure 3.8 Stability study of immobilized lactase stored in different buffers.

PI-1 (◆): PI-ECH-Lactase in storage Buffer 1, which is 60% glycerol.
PI-2 (■): PI-ECH-Lactase in storage Buffer 2, which is 0.1 M potassium phosphate, 0.1M $MgSO_4$, 0.05 mM EDTA, 0.01% NaN_3, and pH 6.5.
Storage Buffer 2 is better than Buffer 1.

3.3 Conclusions

In this Chapter epoxide activation proved to be a successful activation chemistry when applied to Perloza cellulose matrix. It was discovered that Perloza-ECH with a low activation level achieved higher activity of lactase immobilized and a larger amounts of lactase immobilized for the three types of Perloza. The low activation levels (0.32 mmol/g dry resin for Perloza 100 and 200 MT, 0.25 mmol/g dry resin) were gained when activation of Perloza was performed with ECH at low NaOH concentration 2%. Even lower activation level (activation at lower than 2% NaOH) might improve the efficiency but this has not been investigated.

For enzyme attachment directly to epoxide on Perloza, Perloza 100-ECH-Lactase (2% NaOH activation) obtained the highest activity 11.41 NLU/g wet resin and substitution level 0.47 mg/g wet resin over Perloza 200-ECH-Lactase and Perloza 500-ECH-Lactase.

For enzyme attachment to ACA spacer arm, Perloza 200-ECH-ACA-Lactase retained the highest activity (30.9 NLU/g wet resin) over Perloza 100-ECH-ACA-Lactase and Perloza 500-ECH-ACA-Lactase although Perloza 100-ECH-ACA-Lactase immobilized lactase most.

Studies on the inter-relationships between pH and temperature of the substrate indicated that maximum hydrolysis (expressed as relative activity) was attained at pH 6.5-7.2 and over a temperature range of 30-42°C. No shift in the pH and temperature optima in comparison to free enzyme was observed as a result of the process of immobilization.

Storage buffer chosen was 0.1 M potassium phosphate, 1 mM $MgSO_4$, 0.05 mM EDTA, 0.01% NaN_3, and pH 6.5, which was better than 60% glycerol storage buffer, according to the remained activity of immobilized lactase after 48 days storage.

In storage stability studies, Perloza-ECH-Lactase showed a sharp drop in activity initially within 1 day, then activity loss leveled out, to 70% of its original activity retained for Perloza 100-ECH-Lactase and 73% of its original activity retained for Perloza 200-ECH-Lactase after 9 days storage, respectively. Perloza-ECH-ACA-Lactase also showed a sharp drop in activity initially within 1 day, then a gradual decrease until after 9 days to 87% and 82% of their original activity still remained for Perloza 100-ECH-ACA-Lactase and Perloza 200-ECH-ACA-Lactase, respectively. These results indicated that lactase immobilized on ACA spacer arm retained greater stability than lactase immobilized directly on epoxide activated Perloza for the first 9 days storage.

CHAPTER FOUR

LACTASE IMMOBILIZATION ON EUPERGIT C

4.1 Introduction

4.1.1 Structure and properties of Eupergit

Eupergit (pronounced 'You-per-git') is a beaded polymeric support produced by Rohm Pharma (Darmstadt, Germany). The base matrix is made by a copolymerization of methacrylamide, N,N'-methylene-bis (methacrylamide), and a component containing a reactive oxirane group (glycidyl methacrylate of allyl glycidyl ether). The structure of the polymerized product is shown in Figure 4.1. The resulting matrix is electroneutral and predominantly hydrophilic.

Eupergit C, the base-activated matrix, is available in four forms with average particle size distributions of 250 μm, 150 μm, 30 μm, and a non-porous variety at 1 μm. Analysis by electron microscopy of the porous beads has revealed the matrix morphology to contain large channels and cavities ranging in diameter from about 0.1 μm to 2.5 μm (100-2500 nm). These macropores are constructed from a network of small microbeads strung together throughout the Eupergit bead structure. However, the exclusion limit of the small pores within the microbeads is only about 200 kD, indicating a very tightly packed polymer mesh. This morphology provides excellent physical and chemical stability. The support is capable of withstanding up to 4000 psi and remaining unaffected by organic solvents, buffer salts, detergents, and chaotropic agents. Eupergit C exhibits a water uptake of approximate 3.0 ml per gram of dry beads. Changes in the pH and in the ion strength have little effect on the volume of columns packed with Eupergit C. In other wards, once fully swollen, further changes in buffer composition have only minimal effects on gel volume.

Eupergit C with particle size 150 μm was employed in this study.

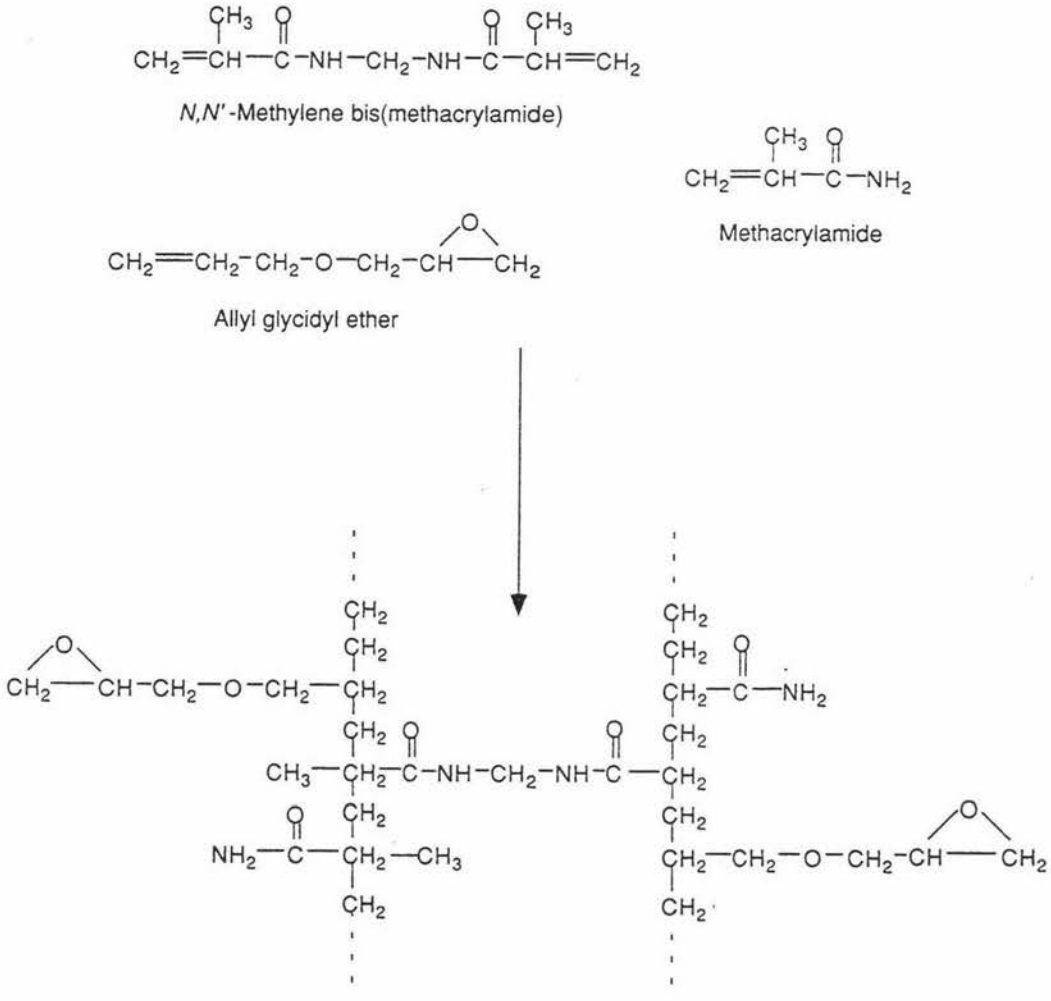


Figure 4.1 Preparation of Eupergit

Eupergit is made by the copolymerization of methacrylamide, N,N'-methylene bis (methacrylamide), and a component containing a reactive oxirane group (glycidyl methacrylate and /or allyl glycidyl ether, Hermanson, 1992).

4.1.2 Immobilization methods

Because of the incorporation of oxirane groups in the manufacture of Eupergit, the matrix comes preactivated and ready to couple ligands through its epoxy groups. The direct immobilization of primary amine-, sulfhydryl-, or hydroxyl-containing ligands is possible using similar chemistry (see Chapter 3). Particular aspects of the Eupergit coupling protocol include the use of 1 M potassium phosphate, pH 7.5, in the reaction

medium. Most epoxy coupling protocols require elevated temperatures (40-45°C) and much higher pH environments (pH 11) to open the ring and drive the reaction. This is especially true when coupling amine- or hydroxyl-containing ligands. Although the recommended procedure for Eupergit coupling does include a long reaction time (16-72 hr), the reaction is carried out only at room temperature. One possible explanation for the success of this procedure, even under mild conditions, is the presence of phosphate ions. It has been suggested by the manufacturer that phosphate ion catalyzes the reaction and allows it to proceed at physiological pH (Hermanson, 1992). However, immobilization conditions for individual enzymes may be different depending on its properties. Selection of appropriate conditions for lactase immobilization on Eupergit C will be discussed in this Chapter.

The high content of active oxirane groups (> 600 μmol/g (dry) for Eupergit C) results in a high protein binding capacity. The actual amount of target protein bound will of course depend on its purity. The high oxirane content is also of benefit for chemical modifications of the matrix. After binding the protein, there are still sufficient free oxirane groups available to give the matrix a more hydrophilic, hydrophobic, anionic or cationic character by appropriate derivatization with low molecular weight components. The removal of excess of oxirane groups after protein immobilization is especially necessary in affinity chromatography. Hydrophilic mercaptans (such as thioglycerol) or hydrophilic amino compounds, (e.g. TRIS) are particularly suitable as reagents for this purpose.

4.2 Results and discussion

4.2.1 Titration of oxirane groups on Eupergit C

Titration of oxirane groups on this epoxide activated matrix used the method established by Ayer's group at Massey University. Dry weight was used in order to obtain accurate result. The titration was carried out using procedure from Section 2.7.1, which is the same with the titration method used for epoxide activated Perloza. The titration result of oxirane groups substitution level on Eupergit C applied in the study was 0.27-0.29 mmoles/g (dry resin). This result was obtained after long term storage of Eupergit C (at NZDRI). The substitution ex-factory was reported as 0.6 mmols/g. It appears that upon the storage some oxirane groups had degraded.

4.2.2 Optimum enzyme attachment conditions

4.2.2.1 Amount of Maxilact applied in immobilization

The ratio of the protein to the matrix has to be optimized for any individual enzyme and a particular matrix in order to obtain the optimum activity per volume (or weight) of the biocatalyst without an unreasonable loss of the enzyme.

To do this, various amounts of Maxilact L2000 were applied to mix with Eupergit C in 1 M potassium phosphate buffer at room temperature. The reaction period was 94.5 hours. Table 4.1 shows the relative activities (% of maximal activity obtained) of immobilized lactase (called 'wet catalyst' in Table 4.1) and binding yields for the various amount of lactase applied.

Maxilact L2000 applied* (g/g of Eupergit C dry)	Relative activity of wet catalyst** (% maximal)	Activity yield (%)
0.11	9	27
0.23	19	29
0.45	48	38
0.67	76	40
1.12	100	32

Table 4.1 Lactase immobilization with series amounts of Maxilact applied.

Plotted in Figure 4.2 and Figure 4.3.
* 1 gram of Maxilact L2000 contains 2000 lactase activity unit (NLU).
** Wet catalyst means immobilized enzyme on wet resin. 1 gram Eupergit C dry gives approximately 3.6 grams of wet catalyst.

The maximum activity was obtained for the largest amount of Maxilact (1.12g/g Eupergit C dry, Table 4.1 and Figure 4.2). Figure 4.3 shows activity yield of wet catalyst of Eupergit C vs. Maxilact applied. Maximum activity yield was achieved when about 0.67 g Maxilact/gram Eupergit C dry was applied.

Choosing an appropriate amount of free enzyme to apply for immobilization, is a consideration that aims to obtain optimum activity per weight (or volume) of the biocatalyst. On the other hand, activity yield of the activated matrix has to be taken into account to economize an immobilization system. Consequently, one gram of Maxilact L2000 applied to per gram of Eupergit C was chosen as a compromise for the later studies.

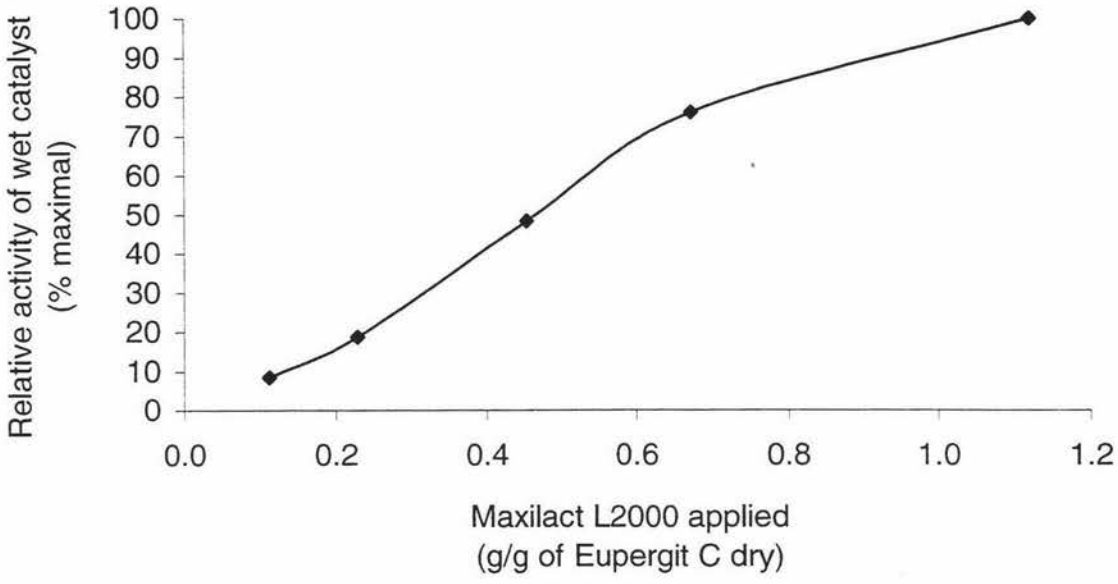


Figure 4.2 Relative activity of wet catalyst on Eupergit C vs. Maxilact applied.

Immobilization conditions:
Eupergit C (0.5g) was mixed with various amounts of Maxilact L2000 in 2.5 ml potassium phosphate buffer, pH 6.5 for 94.5 hours at room temperature.

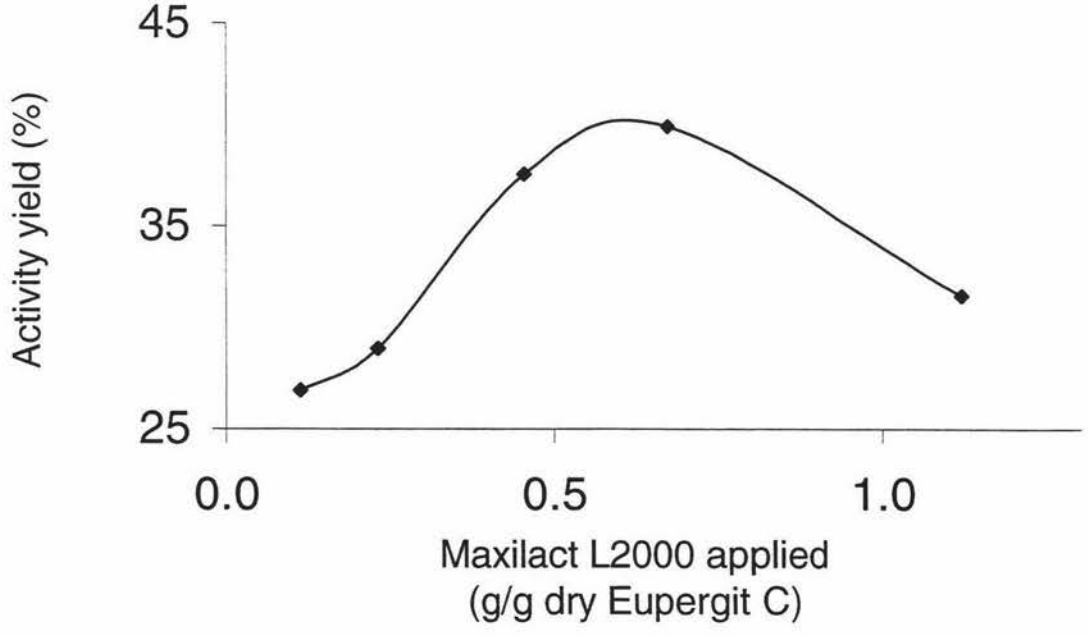


Figure 4.3 Activity yield of wet catalyst on Eupergit C vs. Maxilact applied.

Immobilization conditions: as for Figure 4.2

4.2.2.2 Optimum lactase immobilization time

When immobilizing any individual enzyme the binding yield, the activity yield and the operational stability of the immobilized enzyme depends upon the immobilization time.

Manufacturer recommends that stable enzyme preparations should be immobilized for 72-96 hours to guarantee a maximum binding yield and activity. In some special cases such a long term procedure will result in a much higher operational stability of the immobilized enzyme due to increased multipoint attachment. For rather sensitive enzymes which rapidly loose their activity, immobilization times can be reduced to 5-10 hours.

Lactase is a relative stable enzyme and can be immobilized for longer time in order to obtain some good properties. This will be discussed later in this Chapter.

Figure 4.4 shows the immobilization of lactase vs. reaction time. Approximately 90% of the maximum activity of the immobilization enzyme was reached within 24 hours. However, the activity yield of the product can be optimized by prolonging the incubation time to 48 hours or even longer. The stability of the product was found even better when prolonging the incubation time to 96 hours (Section 4.2.7).

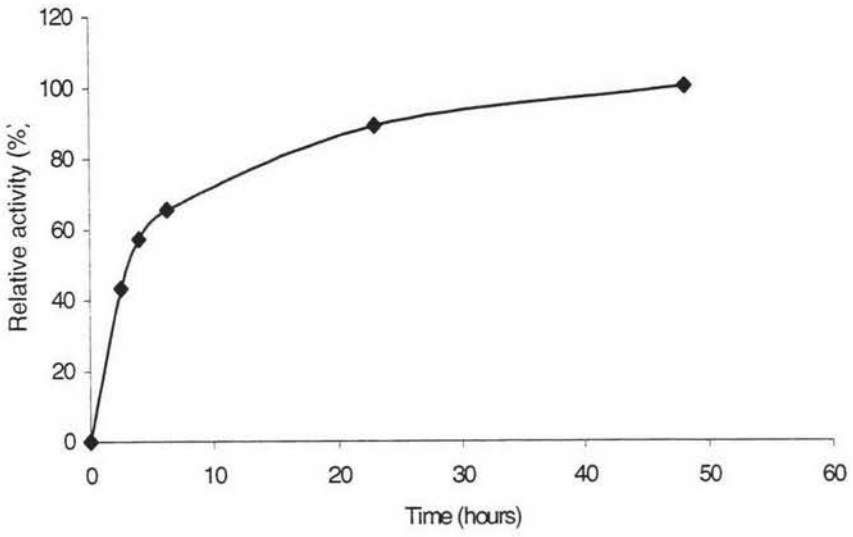


Figure 4.4 Immobilization of Lactase/time dependency.

Immobilization conditions:
Eupergit C (1 g) were mixed with Maxilact L2000 (1.15 g) in 1 M potassium phosphate buffer (5 ml), pH 6.5 at room temperature. Samples were withdrawn at different times.

4.2.2.3 Selection of pH value of the binding buffer

It was noted by the manufacturer that oxirane groups of Eupergit C can react with proteins over a wide pH range (from 1~12). An enzyme should be immobilized at the pH optimum of its stability, provided that at this pH the enzyme remains reasonably active. However, the highest activity is achieved when it is immobilized at the pH optimum of its activity.

Binding Buffer 1 used in the study was 1 M potassium phosphate, pH 6.5. This pH is in the optimal pH range of lactase activity 6.3~7.2. Binding Buffer 2 two tested was 0.2 M potassium phosphate, containing 1 M potassium citrate, pH 8.5 in order to investigate the affect of a higher pH to immobilization (Burton, 1996).

The activity yields for the two binding buffers were summarized in Table 4.2. The results showed that the activity yields little difference between the two binding buffers.

Activity with Buffer 1 was only slight higher than that of Buffer 2. In the future studies Binding Buffer 1 was employed only.

Binding buffers and pH	1 M K-phos pH 6.5	0.2 M K-phos, 1M K-citrate, pH 8.5
Activity of wet catalyst (NLU/g)	103.4	99.7

Table 4.2 Activities of immobilized lactase on Eupergit C/dependency on pH of binding.

Immobilization conditions:
Eupergit C: 0.5 g
Binding buffer: 2 ml
Temperature: ambient
Maxilact L2000: 0.5 g
Reaction time: 22 hours

4.2.3 Lactase attachment onto Eupergit C

After optimization of lactase attachment conditions, lactase immobilization on Eupergit C was carried out. Maxilact L2000 (1 g/gram Eupergit C dry beads) was dissolved in 1 M potassium phosphate buffer (5 ml) and mix with Eupergit C to make up the reaction mixture. It was necessary to mix reaction mixture gently and completely by using end-over-end rotation. Magnetic stirring often results in damage to the beads, which must be forbidden. Reaction vessels must be sealed properly in case leakage. It was a great convenience to carry out the reactions at room temperature.

Activity of immobilized lactase on Eupergit C for 24 hours immobilization obtained was between 124.1 to 131.3 NLU/g wet catalyst of Eupergit C. The variations of the results were due to the room temperature variations or other problems such as difficulties in weighing wet catalyst precisely.

The following characteristic studies of the immobilized lactase on Eupergit C were based on the above immobilization procedures.

4.2.4 Determination of optimum pH for immobilized lactase on Eupergit C

The plot of activity vs. pH of the immobilized enzyme on Eupergit C can reveal changes in both the position of the pH optimum and in the general shape of the curve in comparison with the free enzyme (Figure 4.5). Free lactase illustrated maximum hydrolysis at pH range of 6.5 to 7.2. However, the pH activity curve of the immobilized enzyme shifted toward more acid pH values, showed maximum hydrolysis at pH range of 6.2 to 6.8. The shift can be caused by the water-insoluble carrier, the chemical modification of the enzyme, or certain enzymic reactions. However, it is difficult, if possible, to ascribe precisely the cause and magnitude of the alteration in the pH profile of lactase upon its immobilization.

The shape of the pH-activity curve of the immobilized lactase was broader than that of free lactase. This is consistent with the results obtained by Goldstein et al. (1971), Line et al. (1971), Royer and Green (1971) for other enzymes. The result suggests that immobilized lactase on Eupergit C has a weak sensitivity to pH of the medium.

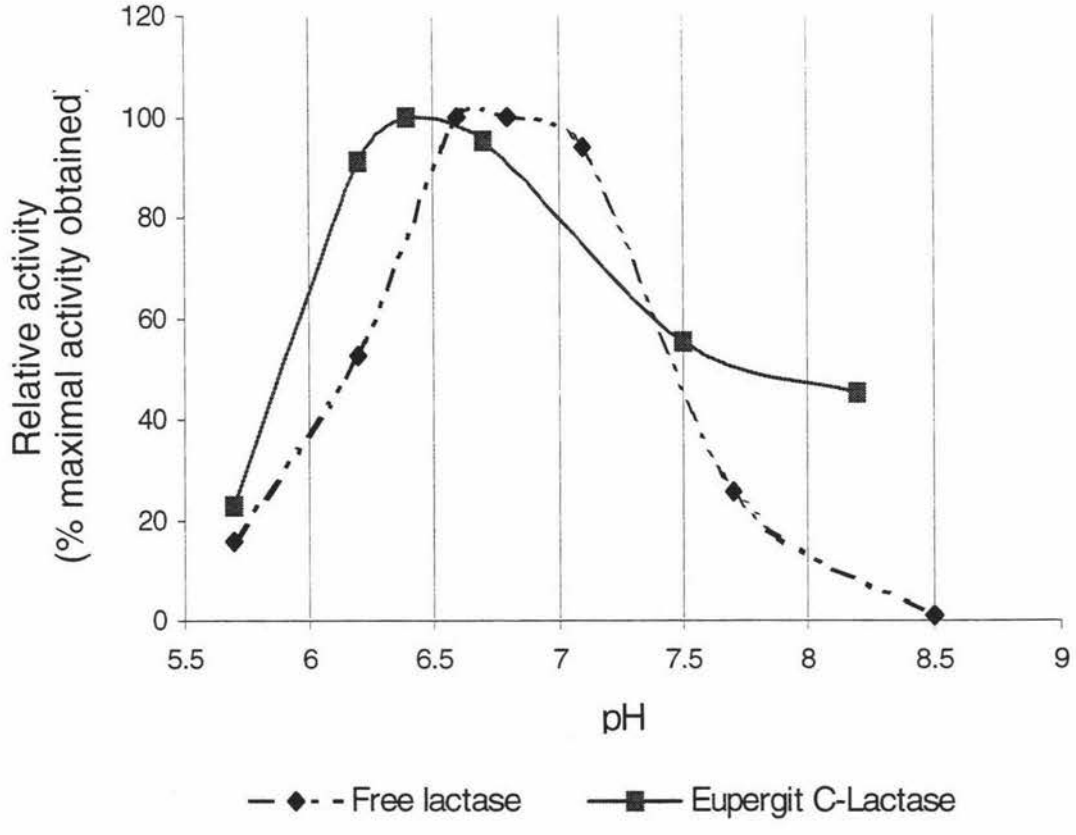


Figure 4.5 pH profile of free and Eupergit C immobilized lactase at 30°C.

Immobilization conditions:
Maxilact L2000 (1 g per gram of Eupergit C dry) reacted with Eupergit C in 5 ml of 1 M k-phos buffer, pH 6.5 at room temperature for 24 hours.

Maximum hydrolysis (expressed as relative activity) was attained at pH 6.5-7.2 for free lactase, however, 6.2~6.8 for immobilized lactase on Eupergit C. The pH activity curve of immobilized lactase shifted toward more acid pH values in the pH optimum in comparison to free lactase.

4.2.5 Determination of optimum temperature for immobilized lactase on Eupergit C.

The results of studies of optimum temperature for free and Eupergit C immobilized lactase are illustrated in Figure 4.6. Figure 4.6 indicates that the optimum temperature of the enzyme changed upon immobilization. Free lactase showed maximum activity at a temperature range of 36 to 40°C. Further increase in temperature, produced a sharp drop of hydrolysis by free enzyme. In contrast, the immobilized lactase showed a wider temperature optimum from 37 to 47°C.

The decrease in the activity of soluble enzyme may be attributed to the inactivation of the enzyme due to protein denaturation at the higher temperatures. The process of immobilization, however, seems to provide some kind of protection of the immobilized enzyme, allowing it to be used at slightly higher temperatures as compared to free enzyme.

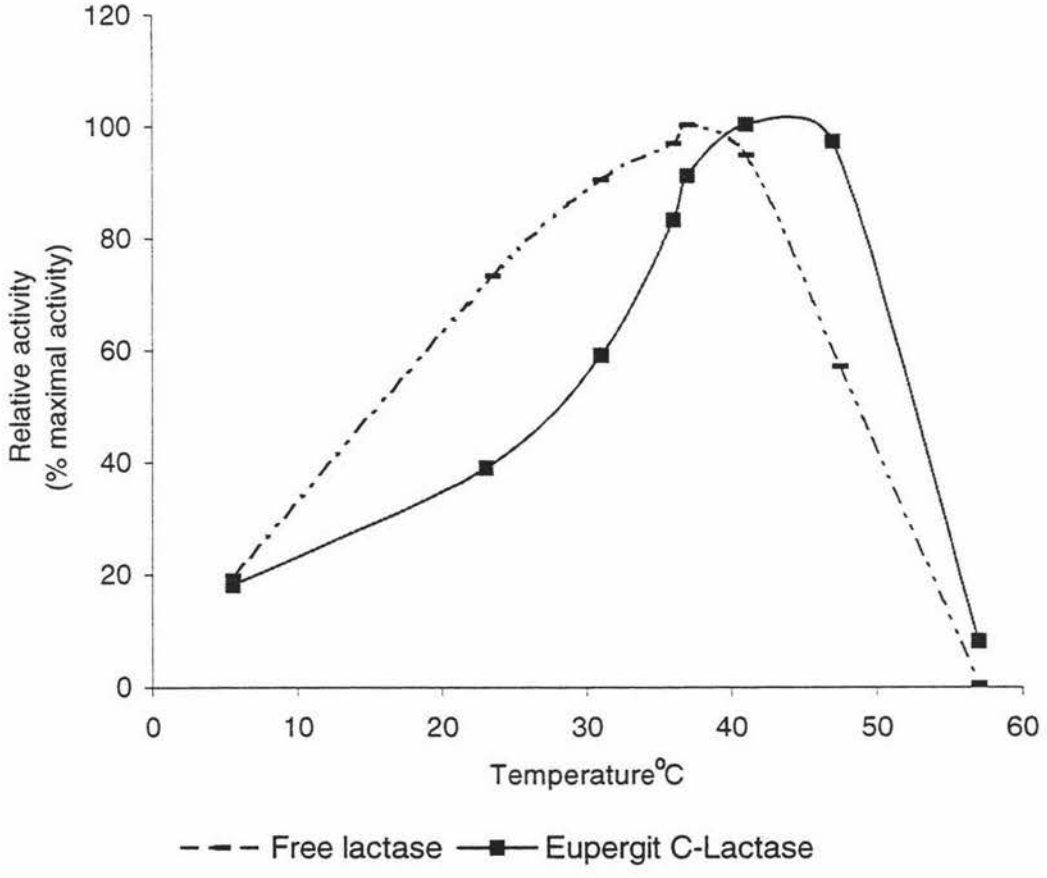


Figure 4.6 Temperature profile of free and Eupergit C immobilized lactase at pH 6.5.

Immobilization conditions: as for Figure 4.5.

Maximum hydrolysis (expressed as relative activity) was attained at temperature 36 to 40°C for free lactase, 37 to 47°C for immobilized lactase on Eupergit C. A shift towards higher temperature in the temperature optimum for immobilized lactase in comparison to free lactase was observed.

4.2.6 Storage stability study of immobilized lactase on Eupergit C

The Eupergit C immobilized lactase samples in this study were stored under the same conditions as for Perloza immobilized lactase, i.e. at 4°C in 0.1 M potassium phosphate, 1 mM $MgSO_4$, 0.05 mM EDTA, 0.01% NaN_3 and pH 6.5. The loss of enzymatic activity with storage time for Eupergit-Lactase (24 hours immobilization) is shown in Figure 4.7. Eupergit-Lactase, which originally achieved activity 131.3 NLU/g of wet catalyst, retained 31% of its original activity after stored for 19 days.

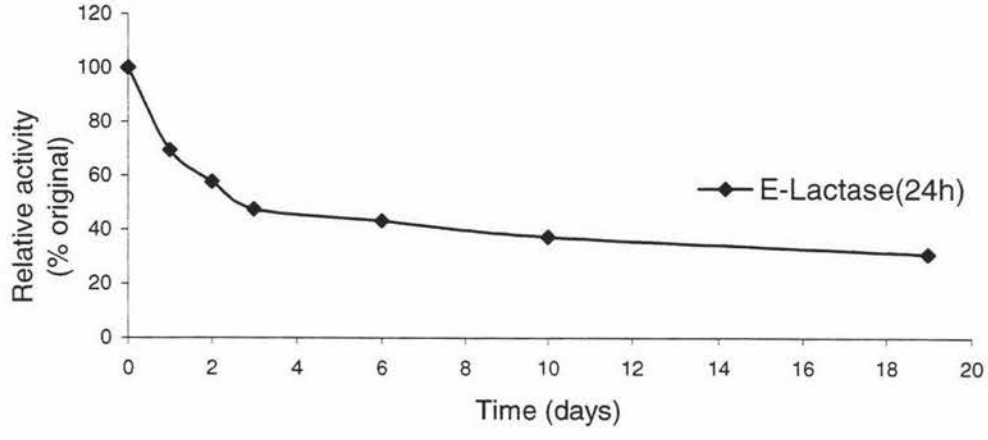


Figure 4.7 Stability study of immobilized lactase on Eupergit C (24 hours immobilization).

Immobilization conditions: as for Figure 4.5.

4.2.7 Comparison of stability of E-Lactase upon different immobilization times

Prolonging the incubation time of the immobilization reaction for too long may result in activity loss of immobilized enzyme due to denaturation of proteins or other reasons. For an increased immobilization time to 96 hours, the activity retained on the Eupergit-Lactase was lower than that for 24 hours immobilization time. Shown in Figure 4.8a, original activities for 24 and 96 hours immobilization were 131.3 and 103.4 NLU/g wet catalyst, respectively.

However, on the other hand, this long term immobilization procedure may result in a much higher stability of immobilized enzyme. Figure 4.8a and Figure 4.8b are consistent with this presumption.

The immobilized lactase with long immobilization time 96 hours only achieved lower activity 103.4 NLU/g, originally, but retained activity 61.6 NLU/g (60% of its original) after 16 days storage, which is greater than of 24 hours Eupergit-Lactase (activity 42.3 NLU/g, 32% of its original) after the same storage period (Figure 4.8a), although 24 hours immobilization achieved higher activity (131.3 NLU/g) at the beginning. This suggests that 96 hours immobilization time may be a better option for practical immobilization.

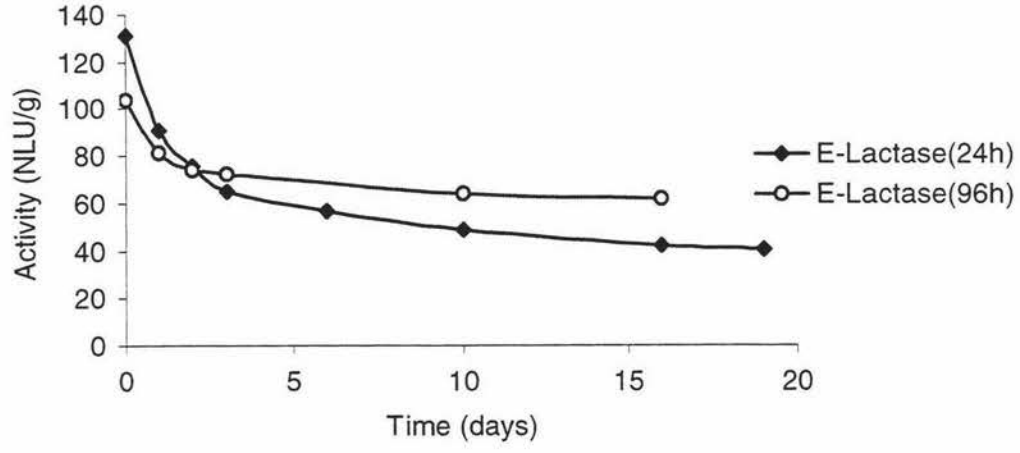


Figure 4.8a Activities of Eupergit-Lactase upon 24 and 96 hours immobilization vs. storage time.

Immobilization conditions: as for Figure 4.5.

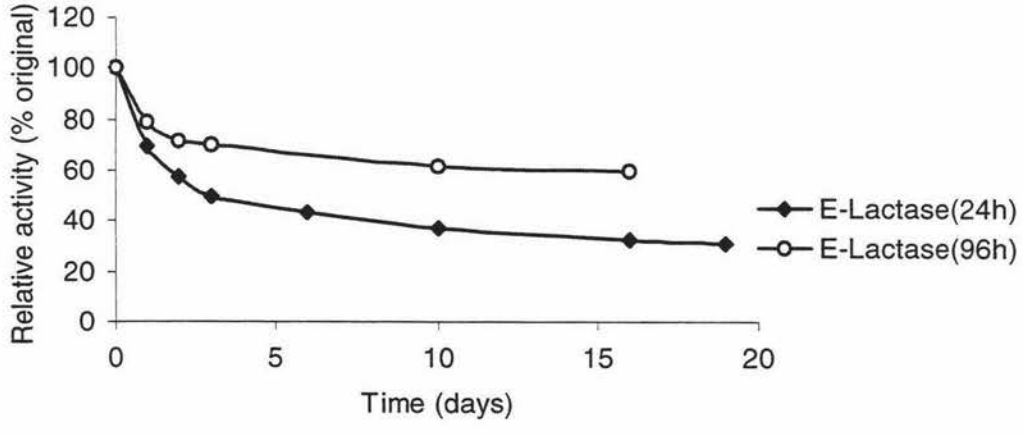


Figure 4.8b Stability study of Eupergit-Lactase upon 24 and 96 hours immobilization.

Immobilization conditions: as for Figure 4.5.

4.3 Conclusions

The conditions of immobilization of lactase on Eupergit C beaded polymeric support were carried out at room temperature. One gram of Maxilact L2000 was used per gram of Eupergit C. Immobilization time was chosen at 24 hours, however, 96 hours immobilization retained higher activity (61.6 NLU/g) than of 24 hours (42.3 NLU/g) after 16 days storage. Binding buffer used was 1 M potassium phosphate buffer pH 6.5 (5 ml per gram of Eupergit C dry).

Activity of immobilized lactase on Eupergit C under the above conditions obtained was between 124.1 to 131.3 NLU/g wet catalyst.

The optimum pH for immobilized lactase on Eupergit C was determined to be 6.2 to 6.8. The pH-activity curve of immobilized lactase shifted towards more acidic pH values in the pH optimum in comparison to free lactase.

The study on the relationship between operating temperature and activity of Eupergit-Lactase indicated that the temperature optimum for Eupergit-Lactase shift towards higher temperature in comparison to free lactase.

In storage stability studies, Eupergit-Lactase with 24 hours immobilization retained 32% of its original activity after 16 days storage (retained 42.3 NLU/g). However, Eupergit-Lactase with 96 hours immobilization remained 60% of its original activity after the same storage period (retained 61.6 NLU/g).

CHAPTER FIVE

SUMMARY AND FUTURE WORK

5.1 Summary

In the studies reported in Chapter Three, the immobilization of lactase to three types of Perloza was investigated, using two covalent binding attachment chemistries. In order to compare these results with a commercial product, lactase was immobilized onto the base-activated commercial matrix Eupergit C. This was discussed in Chapter Four.

The three types of Perloza used were Perloza 100 MT, Perloza 200 MT, Perloza 500 MT. Activation of the three types of Perloza with ECH at different concentrations of NaOH (2%~7%) were investigated. It was found that activation at low NaOH concentration (2%) gave low activation levels (0.32 mmol/g dry resin for Perloza 100 and 200, 0.25 mmol/g dry for Perloza 500). Activation at higher NaOH concentration gave higher activation levels as might be expected. Activated Perlozas with different activation levels were studied for the immobilization of lactase. It was discovered that the lower activation level (activated at 2% NaOH) achieved high activity of lactase immobilized for the two chemistries.

Two methods of covalent binding were compared for the three types of Perloza. One chemistry was that lactase immobilized directly to ECH activated Perloza to produce Perloza-ECH-Lactase. Another chemistry again used ECH activation followed by attachment of 6-amino caproic acid as a spacer arm and then the enzyme to form Perloza-ECH-ACA-Lactase.

In lactase immobilization to Perloza-ECH, Perloza 100-ECH-Lactase (2% NaOH activation) obtained the highest activity at 11.4 NLU/g (wet resin) and enzyme substitution level 0.47mg/g (wet resin) over Perloza 200-ECH-Lactase and Perloza

500-ECH-Lactase. The immobilization was carried out at room temperature for 40 hours.

In lactase immobilization to Perloza-ECH-ACA, Perloza 200-ECH-ACA-Lactase retained the highest activity (30.9 NLU/g wet resin) over Perloza 100-ECH-ACA-Lactase and Perloza 500-ECH-ACA-Lactase. This created about a 3 times better result than the case without the ACA spacer arm.

Immobilization of lactase on Eupergit C was carried out at room temperature and different immobilization times were used. The activity of immobilized lactase on Eupergit C obtained was 131.3 NLU/g (wet resin) and 103.4 NLU/g (wet resin) for 24 hours and 96 hours immobilization, respectively.

In the storage stability studies, both Perloza and Eupergit C immobilized lactase showed a sharp drop in activity initially within 1 day, then activity loss leveled out. Perloza 200-ECH-ACA-Lactase (40 hrs immobilization) showed a gradual decrease after first day down to 82% of its original activity which was then retained for 9 days storage (Figure 5.1). However, Eupergit-Lactase immobilized for 24 and 96 hours only retained 39% and 62% of its original activity after the same storage time, respectively.

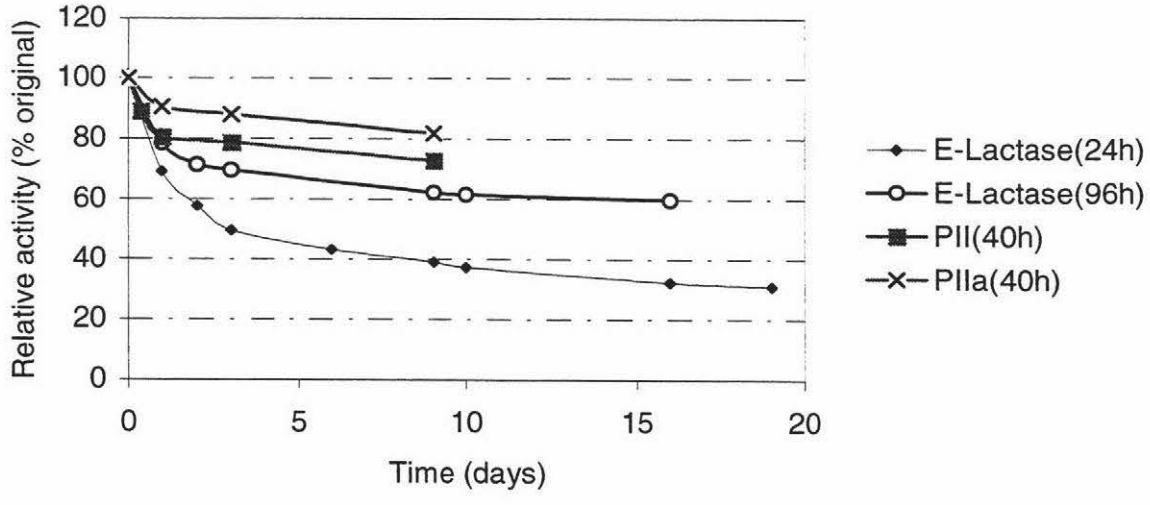


Figure 5.1 Comparison of storage stability of immobilize lactase on Perloza and Eupergit C

These results indicated that Perloza 200-ECH-ACA-lactase might possess better storage stability than that of Eupergit-Lactase. Since Perloza is a cheap resin ($20~$60 dollars/litre), this result once again showed ECH activated Perloza 200 with spacer arm may possess potential for large scale use as a matrix for lactase immobilization.

An interesting phenomenon worth mentioning is that long term immobilization of Eupergit (96 hours) gave lower initial activity (131.3 NLU/g) than short term immobilization (24 hours, 103.4 NLU/g), however, 96 hours immobilization retained higher activity (61.6 NLU/g, 60% of original) than 24 hours (42.3 NLU/g, 32% of original) after 16 days storage.

Studies on the relationships between pH, temperature and activities of the immobilized lactases on Perloza indicated that maximum activity was attained at pH 6.5-7.2 (Figure 5.2) and over a temperature range of 30-42°C (Figure 5.3). No shift in the pH and temperature optima in comparison to free enzyme was observed as a result of the process of immobilization of lactase on Perloza for both attachment chemistries.

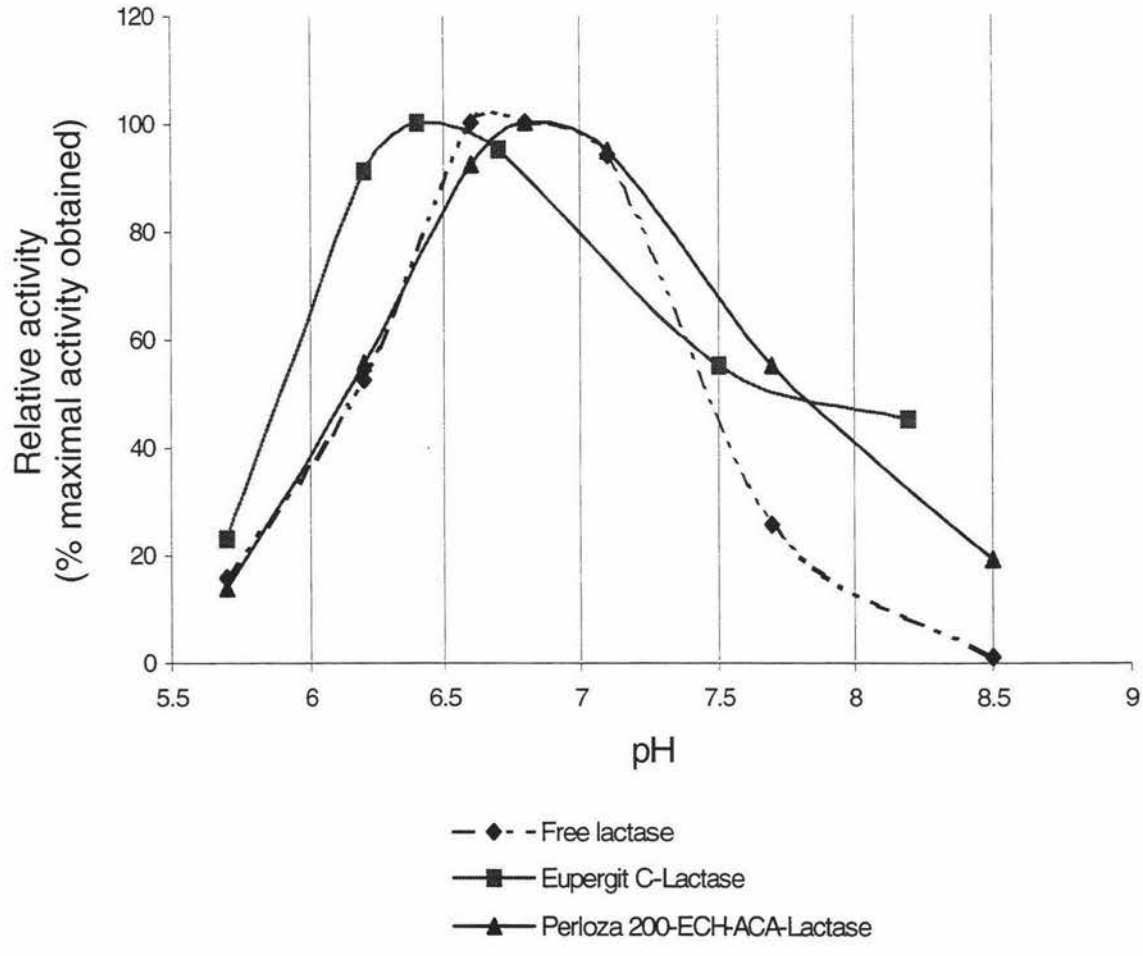


Figure 5.2 **pH profile of free, Perloza 200-ECH-ACA and Eupergit C immobilized lactase.**

The optimum pH for immobilized lactase on Eupergit C was determined to be 6.2 to 6.8 (Figure 5.2). The pH-activity curve of immobilized lactase on Eupergit C shifted towards more acidic pH values in the pH optimum in comparison to free lactase (Figure 5.2). The study on the relationship between operating temperature and activity of Eupergit-Lactase indicated that the temperature optimum of Eupergit-Lactase shift towards higher temperature in comparison to free lactase (Figure 5.3).

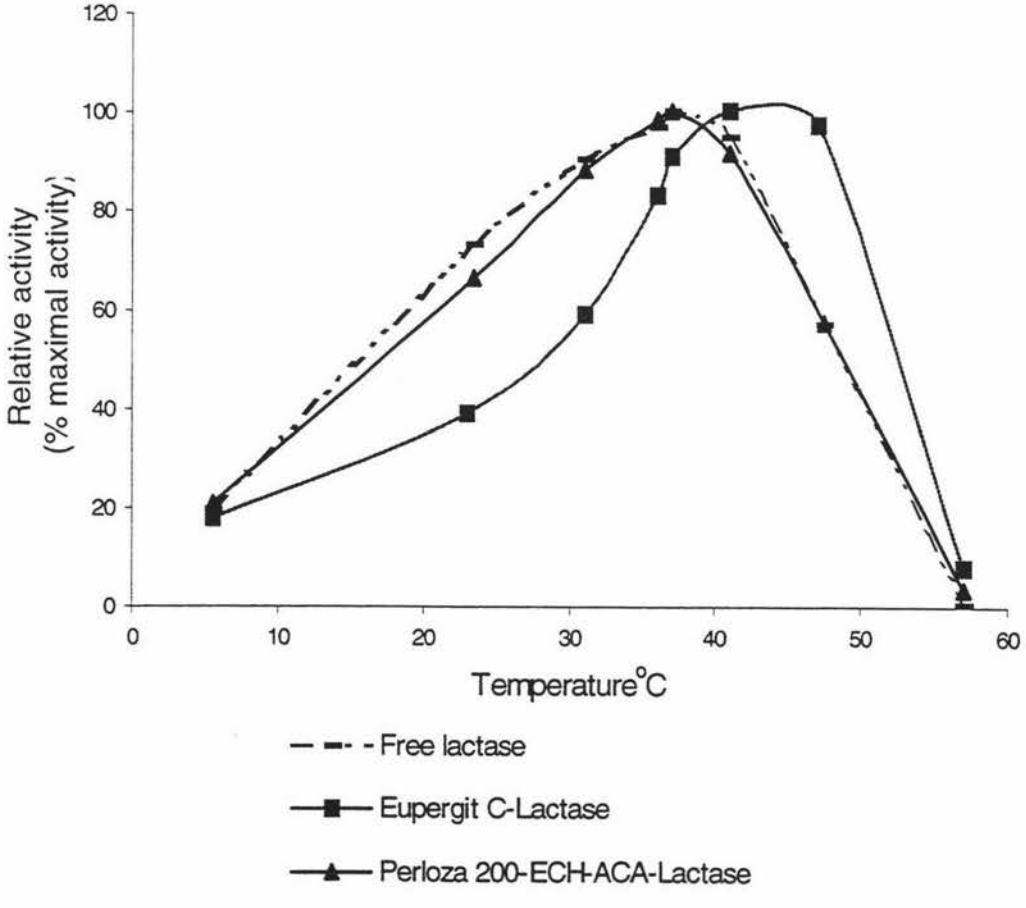


Figure 5.3 Temperature profile of free, Perloza-ECH-ACA and Eupergit C immobilized lactase.

5.2 Future work

In this research, ECH activated Perloza with the 6-aminocaproic acid (ACA) spacer arm achieved great success in immobilization of lactase. These resins were prepared using CMC catalyzed condensation. Nevertheless, the epoxide resin substituted with the ACA spacer arm using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) rather than CMC should be tried in order to compare the activities of the immobilized lactase. The epoxide substituted resin with either an amine or thiol linked spacer arm containing a carboxyl group could also be trialed. Other attachment chemistries should also be considered such as carbonyldiimidazole (CDI) can be used to couple the target enzyme either directly or after spacer arm attachment.

Due to the highest activity of immobilized lactase achieved at low ECH activation level, the enzyme optimization of the activation level could be investigated more thoroughly. In other words lower ECH activation levels (ECH activation carried out at NaOH concentration lower than 2%) could be beneficial. This may be because the lactase (Maxilact preparation) from dairy yeast consists of two subunits. The bonds between subunits are easily disrupted by strong binding due to high density of activated groups on matrix. Two previous studies immobilizing yeast ADH (alcohol dehydrogenase), which is a dimer, encountered similar problems with the activity of immobilized enzyme (Cross, 1998; Fisher, 1997).

Carrying out practical lactose hydrolysis in whey or milk by the immobilized lactase produced in this study remains to be done.

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