COVALENT CONJUGATION OF LACTASE TO CARBOXYLIC ACID-ACTIVATED HYDROPHOBIC CARRIERS: INTERFACIAL INFLUENCES AND STRUCTURAL STABILIZATION

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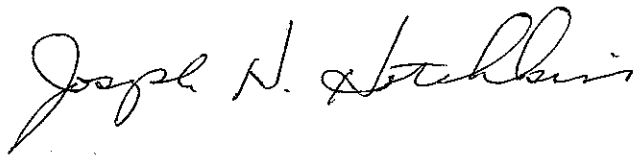

COVALENT CONJUGATION OF LACTASE TO CARBOXYLIC ACID-ACTIVATED HYDROPHOBIC CARRIERS: INTERFACIAL INFLUENCES AND STRUCTURAL STABILIZATION

Joey Nathaniel Talbert, Ph.D.

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Estimates suggest that 70% of the world's population is lactose intolerant, with certain genetic populations and aged individuals being most susceptible. To address this problem, lactase immobilized reactor systems and packaging films have been explored as economical means to produce lactose-reduced products. Hydrophobic polymers are attractive carriers for enzyme-immobilized applications in the food industry because of mechanical rigidity, cost, ability to mold into different size and shape arrangements, use as food packaging films, low swelling in aqueous solution, and regulatory approval for food contact. The surface of these supports can be modified to produce functional chemical moieties, such as carboxylic acid groups, that are cable of reacting with biological molecules in a covalent fashion to prevent leakage of the enzyme. The enzyme, lactase (β-galactosidase), however, can lose activity when conjugated to these industry-viable carriers—limiting application.

The objectives of this research were to covalently attach lactase to hydrophobic supports in a manner that retains enzymatic activity, determine the mechanism for loss of enzymatic activity upon attachment to a functionalized model system, develop methods for retaining enzyme activity when attached to the carrier, and apply the system to the development of lactase-immobilized packaging films.

Lactase (*A. oryzae*) was conjugated to polystyrene-co-acrylic acid microspheres via carbodiimide chemistry. Immobilization resulted in a decrease in enzymatic activity compared to the intrinsic specific activity. Increasing the density of

surface carboxylic acid groups on the carrier yielded a further reduction in specific activity. Blocking of the carboxylic acid-bearing amino acids of lactase with glucosamine produced no significant change in free enzyme activity, but yielded an increase in specific activity compared to the unblocked enzyme when conjugated to the microspheres. The modification of enzyme carboxylic acid groups, also, aided in the retention of specific activity when immobilized to oxidized to low density poly(ethylene) films. Changing the surface properties of polystyrene-co-acrylic acid microspheres by tethering chitosan (β-1-4-poly-D-glucosamine), resulted in increased protein loading to the support with no significant change in specific activity compared to the free enzyme under optimum conditions. The results indicate that retention of lactase (*A. oryzae*) activity upon conjugation to carboxylic acid functionalized hydrophobic carriers is dependent on the interfacial influences associated with the density of carboxylic acid groups on the surface of the support. Altering interactions of the carrier-protein interface by modifying enzyme carboxylic acid groups or changing the surface characteristics of the carrier promotes structural stabilization and retention of lactase specific activity. These methods can be applied to the development of a lactase-reducing food packaging film or an economically feasible polymer-based immobilized enzyme reactor system.

For Papaw

BIOGRAPHICAL SKETCH

Joey Talbert was born April 28, 1979 and was raised in Lebanon, Virginia. He graduated, *Summa Cum Laude* from Virginia Polytechnic Institute and State University (Virginia Tech) in May 2001 with a B.S. in Food Science and Technology. In August 2001, he began pursuing a Masters of Science in the Department of Food Science at Cornell University under the guidance of Dr. Joseph Hotchkiss—receiving an M.S. from the department in August 2004 prior to continuation of his Ph.D. studies.

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LIST OF ABBREVIATIONS

EDC: *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide

NHS: *N*-Hydroxysulfosuccinimide

PEI: Poly(ethyleneimine)

PS-co-AA: Poly(styrene)-co-acrylic acid

LDPE: Low density poly(ethylene)

PEG: Poly(ethylene glycol)

ONPG: 2-Nitrophenyl β-D-galactopyranoside

CLEC: Crosslinked enzyme crystal

CLEA: Crosslinked enzyme aggregate

MANAE: Monoamino-N-aminoethyl

BCA: Bicinchoninic acid

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

IEF: Isoelectric focusing

CHAPTER 1

LITERATURE REVIEW

Lactose Intolerance

The nutritional problems associated with lactose, a disaccharide consisting of glucose and galactose, which in whole bovine milk constitutes 4.8 w/v% of the product, are well documented (Kretchme.N 1972; Gekas and Lopezleiva 1985; Houts 1988). After weaning, humans may lose their ability to digest lactose due to a decrease in cellular production of the intestinal enzyme, lactase/β-galactosidase (β-D-galactoside galactohydrase, EC 3.2.1.23), which catalyzes the hydrolysis of the β(1-4) glycosidic bonds of lactose to obtain glucose and galactose monosaccharides at the brush border of the small intestine, allowing for uptake (Kretchme.N 1972; Nijpels 1981; Godfrey 1996). The enzyme deficiency results in a variety of adverse health effects including gastrointestinal problems due to the inability of sugar to be hydrolyzed, and; consequently, absorbed (Houts 1988). The sugar then goes to the large intestine where it is fermented by microorganisms, producing acids and gas--resulting in flatulence, diarrhea, and cramping (Kretchme.N 1972; Houts 1988).

Estimates suggest that nearly 70% of the world's population is lactose intolerant, and that lactase deficiency corresponds to genetic populations originating from areas where milk and dairy are/were not staples in traditional diets, particularly affecting those of Asian and African decent (Kretchme.N 1972; Savaino 1988; Jackson and Savaiano 2001). Likewise, those originating from areas with a history of milk consumption have adapted accordingly (Savaino 1988). Ethnically, the breakdown of lactose intolerance in the United States is: Caucasian-15%, Hispanic-53%, African-American-80%, and Asian-90%(Sahi 1994; McBean and Miller 1998).

There is, also, evidence that lactose intolerance increases with aging and loss of cellular production of the enzyme (Elbon, Johnson et al. 1998; Goulding, Taylor et al. 1999). In the United States, intolerance varies, with about one-third of the U.S. population having problems with lactose consumption (Houts 1988). Lactose intolerance can result in the avoidance of dairy products, which account for over half of the daily calcium intake in the US (Phillips and Briggs 1975; McBean and Miller 1998). Insufficient calcium intake, in return, results in corresponding health issues, including osteoporosis. Adverse nutritional and physical effects, as well as economic potential, have prompted research and opportunity in the utilization of lactose-reducing methods for the improvement and modification of milk and other dairy products.

Lactose, in food products can be reduced both prior to and after consumption of dairy. Methods of reduction include chemical hydrolysis, ultrafiltration, and utilization of external sources of the enzyme, β-galactosidase (lactase). In fluid milk products, the goal of lactose reduction is to hydrolyze the sugar between 70-100%. Less than 100% reduction is targeted because lactose has low sweetness relative to its monomers--glucose and galactose; which have a sweetness approximately 80% that of sucrose (Pivarnik 1995). The enhanced sweetness associated with hydrolysis is often unappealing to consumers, who have shown to significantly detect differences in sweetness at 80% lactose reduction (BeMiller 1996). Chemical methods, which rely on acid hydrolysis of the sugar prior to consumption, are not useful in food products because of whole-food changes in sensory and nutritional loss that occur with the treatment (Gekas and Lopezleiva 1985). Ultrafiltration, as a processing step, has yet to find a market, because of energy and capital inputs, and the difficulty in separating lactose from similar sized molecules (ie vitamins). In the food industry, applications employing lactase to reduce lactose have been most successful (Pivarnik 1995).

Lactases are produced by microbial and in mammalian organisms, and are commercially available from yeast, mold, and bacterial sources. The common sources of commercially viable lactases are *Kluyveromyces lactis, Candida pseudotropicalis, Aspergillus niger*, and *Aspergillus oryae* (Pivarnik 1995; Mahoney 1997). Mahoney makes the distinction that all lactases are β-galactosidases, while not all β-galactosidases are lactases because some plant cells and mammalian organs possess β-galactosidases that have little or no enzymatic activity on lactose (Mahoney 1997). For food applications the diversity is reduced due to regulatory restrictions, being limited, as a direct food additive, to *Kluyveromyces lactis* and *Candida pseudotropicalis,* as well as *Aspergillus oryzae* and *niger*, which have GRAS approval (Baret 1987; Anon 2003). Lactases have been used to produce reduced lactose milk, improve the properties of ice cream in regards to crystallization, whipping, and viscosity properties, and for yogurt to improve gelation, body, texture, and taste (Nijpels 1981).

Lactase activity is affected by pH, temperatures, purity, and interaction with chemicals, which effects the application of the enzyme. Optimal pH and temperature varies with the source, modification, and method of preparation (Pivarnik 1995). Fungal lactases have an optimum pH range from 2.5-5.0, and yeast lactases range from 6.0-7.0 (Park, Desanti et al. 1979; Hussein, Elsayed et al. 1989). Yeast lactases are suited for fluid milk applications (pH 6.6-6.8), while fungal lactase are utilized in fermented diary products and for whey processing. The optimum temperature for fungal lactase is between 50-55^{0}C, while yeast lactases range from 30-40^{0}C, and are rapidly denatured over 40^{0}C (Pivarnik 1995). Stability of lactase is affected by not only pH and temperature, but also interactions with other components, including divalent cations, lactose, and proteins found in milk. Mahoney and Wilder found that the half life of lactase from *K. marxianus* in milk was 20 times greater than in milk salts and 50 times greater than in phosphate buffer, presumably due to enhancement of

hydrophobic interactions (Mahoney and Wilder 1988). Lactase purity is an important, though often overlooked, property of enzyme applications (Pivarnik 1995). Commercial sources of lactase have proteases present from incomplete purification of whole cell extracts that may degrade the product when in direct contact for a period of time (Dahlqvist, Asp et al. 1977; Mahoney and Wilder 1988). For milk applications, the presence of proteases can result in the production of undesirable texture and flavor changes (Mahoney 1997). This factor is of special consideration for ultra high temperature (UHT) processed milk, which is processed to be stored for an extended time, and often have problems associated with proteases from sources other than lactase, and must be considered prior to application.

Applications that employ lactase to reduce lactose are useful for both fluid milk and whey processing. Lactose from whey, a byproduct of cheese manufacturing, can be hydrolyzed by the enzyme to produce sugars for use as prebiotics and sweeteners. Processing of whey with lactase provides not only nutritional advantages but also waste management improvements. Sweet whey, derived from the manufacturing of cheeses with rennet and acid whey, from manufacturing using acid coagulation, has, in the past been treated as a waste product (Yang and Silva 1995). Whey components, particularly proteins, have found niche markets as nutritional supplements, edible films, and dried whey powder for emulsification and protein solubility in food applications (Yang and Silva 1995; Min, Harris et al. 2005). Whey powder, whey protein concentrate, and whey isolate, as ingredients for food applications, contain lactose. Reduction of lactose in these products provides not only advantages for lactose intolerant consumers, but also enhancements in food texture and consumer acceptability when using the ingredients because of the higher solubility of glucose and galactose compared to lactose and the decrease in lactose crystallization. The protein from whey can be removed by filtration making it even

more suited for lactase activity (Saboya and Maubois 2000). The permeate, which contains primarily lactose, vitamins and minerals, can be used for prebiotics, as syrup for utilized as a sweetener with increased solubility, or for animal feed (Yang and Silva 1995). In a similar manner, permeate from skim milk is produced when microorganisms (and because of size similarities, proteins) are removed from the milk by ultra and microfiltration. The resulting permeate, which has a substantially reduced microbial load, is similar to that of sweet whey permeate. After processing, the fractionated portions can be added back to form a product with a lower bacterial count (Saboya and Maubois 2000; Nelson and Barbano 2005). Processing in this form may allow for the use of lactase, with minimal interferences from microorganisms, fat, and protein, to be used in the production of a lactose-reduced fluid milk product. Other applications of lactase to reduce lactose for consumption include: tablets that are taken prior to ingesting dairy, processing of fluid milk with lactase by the consumer, addition of the enzyme prior to or after pasteurization, and use of immobilized enzyme reactor (Nijpels 1981; Mahoney 1997)

Dosage forms, in which the enzyme is entrapped in a capsule and taken orally prior to the consumption of milk, can be used directly by the consumer (Holsinger and Kligerman 1991). Doses must be taken in an appropriate time period to allow the enzyme to reach the intestine prior to consumption of dairy. Lactases added to milk are developed from yeast; however, those taken orally are from fungal sources to better accommodate the acidic conditions of the stomach (Holsinger and Kligerman 1991). This method is, particularly, useful to consumers who eat-out and may ingest lactose. Lactose reduction by the consumer can, also, be achieved by the addition of a lactase tablets that is treated overnight at refrigeration temperatures.(Holsinger and Kligerman 1991).

The addition of lactase in milk prior to purchase by the consumer has been developed to provide convenience and decreased cost for the consumer while meeting a market demand and increasing profits for the producer (Pivarnik 1995). Lactase has been added prior or after pasteurization because of the susceptibility of yeast lactases to denaturation at processing temperatures used for batch, high temperature short time (HTST) and ultra high temperature (UHT) pasteurization, which occur at temperatures of 135^0C and higher (Nijpels 1981). The addition of lactase prior to pasteurization has the advantage of producing a reduced lactose milk that can be processed, after reduction is completed, in a continuous manner that is identical to non-reduced milk (Guy and Bingham 1978). The disadvantages of this concept include an increase in holding time of the raw milk and vulnerability of the enzyme to proteases associated with the microbial load of the raw milk as well as ensuring completion of lactose reduction within the maximum 72 hour holding period in the plant.

Lactase addition after pasteurization is advantageous due to an increase in efficacy of the enzyme from a reduction in the microbial load and more time available for reaction. The disadvantage of this application is that a holding time at the enzyme's optimum temperature (30-35^0C for *K. lactis*) or lower is required after pasteurization to achieve the desired level of lactose reduction, after which the product goes through a second heat treatment to inactive biological contaminants that accompany commercial treatments of lactase and to stop lactose reduction if the desired level has been achieved (Mahoney and Wilder 1988). An alternative approach to the addition of lactase after pasteurization has been to inject a very small amount of yeast lactase through a sterile, 0.22μm filter into UHT sterilized, packaged milk (Dahlqvist, Asp et al. 1977). The UHT product can be stored at room temperature, achieving complete hydrolysis in 7 to 10 days (Pivarnik 1995).

Lactase-Based Bioactive Systems

Immobilized Lactase for Industrial Reactors

Immobilized enzyme reactors have been utilized by the food industry to reduce the cost of enzymes during processing by binding the enzyme to an insoluble support. The advantages of immobilization include reuse, stability enhancement, and separation from the product. The enzyme may be conjugated to surface of the support by covalent bonding, ionic attachment, or hydrophobic interactions. The enzyme may, also, be incorporated into a polymer backbone during polymerization or physically entrapped in the bulk of a material. Lactase and whole cells contain lactase have been immobilized to a variety of supports by adsorption, entrapment, and covalent conjugation (Table 1 and Table 2).

Though immobilized enzyme systems have many advantages, they are limited in practice by enzyme leakage, fouling, cost, enzyme activity, carrier stability, and microbial growth (Pivarnik 1995). Fouling can occur in both filtration membranes and in porous beads by constituents such as proteins found in milk, which limits the accessibility of substrate to the enzyme (Shuler and Kargi, 2002). For fluid milk, which has neutral pH, microbial growth is encouraged on immobilized supports (Gekas and Lopezleiva 1985; Baret 1987). Sanitation of enzyme carriers is necessary to provide consumer safety, but is difficult and may be lead to loss of enzymatic activity (Baret 1987; Panesar, Panesar et al. 2006). For immobilized lactase, cleaning methods have been developed for immobilized *A. oryzae* lactase using substituted diethylenetriamines (Baret 1987).

Table 1. Immobilization of lactase (as adapted from Gekas and Lopezleiva 1985)

SUPPORT	TYPE OF LACTASE	METHOD OF IMMOBILIZATION	NOTES	REFERENCE
Phenol-formaldehyde resin	A. niger	Adsorption/glutaraldehyde	70% hydrolysis of lactose	(Boer 1982)
Porous alumina	A. niger	Adsorption/glutaraldehyde	70-80% hydrolysis	(Crippen and Jeon 1984)
Phenol-formaldehyde resin	A. niger	Adsorption/glutaraldehyde	25mg/g; 220U/g	(Harju 1979)
Egg shell	Lactobacillus bulgaricus	Adsorption/glutaraldehyde	25% maximum activity	(Makkar, Sharma et al. 1983)
Phenol-formaldehyde resin	B. circulans	Adsorption/glutaraldehyde	225U/g (wet)	(Nakanishi, Matsuno et al. 1983)
Phenol-formaldehyde resin	A. niger	Adsorption/glutaraldehyde	250mg/g; 4000U/g	N/A
Egg white powder	E. coli	Adsorption/glutaraldehyde	50% hydrolysis in 8 hrs	(Kaul, Dsouza et al. 1984)
Feather protein	A. niger	Adsorption/glutaraldehyde	100mg/g; 300U/g protein	(Stanley, Watters et al. 1976)
DEAE cellulose	Scopulariopsis	Adsorption/glutaraldehyde	1-2U/g resin	(Park and Pastore 1981)
Phenol-formaldehyde resin	A. niger	Adsorption/glutaraldehyde	70-75% hydrolysis	(Peter, Prenosil et al. 1981)
Polyacrylamide	*K. lactis*	Hydrophobic bond	70U/g	50,51,129
Brushite	E. coli	Adsorption	N/A	(Hjerten, Kunquan et al. 1981)
Tritylagarose	E. coli	Hydrophobic bond	75-90% relative activity	(Cashion, Javed et al. 1982)
Nylon-acrylonitrile	E. coli	Covalent-CMC or carbodiimide	236.5U/mg; Poor stability	(Beddows, Guthrie et al. 1981)
Amino-carbonilated cellulose	K. fragilis/E. coli	Covalent-diazo/glutaraldehyde	3U/g; 93U/g	(Beddows, Mirauer et al. 1980)
Cellulose	E. coli	Covalent-benzoquinone/oxirane	109mg/g, 4130U/g; 40mg/g, 1780U/g	(Hong, Kwon et al. 1982)
Oxirane acrylic	E. coli	Covalent	40mg/g, 1300U/g of carrier	(Hannibalfriedrich, Chun et al. 1980)
Sepharose	*A. oryzae*	Covalent	63.9U/mg	(Friend and Shahani 1982)
Oxirane polyacrylamide	*K. lactis*/K. fragilis	Covalent-oxirane	poor results	(Griffiths and Muir 1980)
Mn-Zn ferrite particles	A. niger	Covalent-silanization/glutaraldhyde	1U/g; 16U/g	(Halling and Dunnill 1979)
Plexiglass-like material	*A. oryzae*	Covalent	80% hydrolysis	(Sprossler and Plainer 1983)
Ion exchange resin	*A. oryzae* (purified)	Covalent-glutaraldehyde	1000U/g; 80% conversion	(Hirohara, Yamamoto et al. 1982)
Cellulose triacetate	*K. lactis*	Entrapment	30mg/g; 22U/g	(Dinelli 1972)
PVOH	*K. lactis*	Entrapment	100% hydrolysis in 200min	(Jancsik, Beleznai et al. 1982)
Polyacrylamide	Lactobacillus bulgaricus	Entrapment	Maximum 31% activity	(Makkar and Sharma 1983)
Collagen	A. niger	Entrapment	1680U/g	(Thomas, Kalra et al. 1980)
Hollow fibre membrane	K. fragilis	Entrapment	40% hydrolysis	(Broome, Roginski et al. 1983)
Hollow fibre membrane	*K. lactis*	Entrapment	10mg/930cm square	(Kohlwey and Cheryan 1981)

Table 2. Covalent immobilization of lactase

SUPPORT	NATURE OF SUPPORT	TYPE OF LACTASE	RETAINED IMMOBILIZED ACTVITY	AMOUNT IMMOBILIZED	NOTES	REFERENCE
Eupergit C	Copolymer of methacrylamide, N,N'-methylen-bis(acrylamide) and a monomer carrying oxirane groups; Macroporous, ~150um	*A. oryzae*	30% activity			(Hernaiz and Crout 2000)
Eupergit C-250L	Copolymer of methacrylamide, N,N'-methylen-bis(acrylamide) and a monomer carrying oxirane groups; Macroporous, ~250um, higher oxirane content	*B. circulans*	90% activity	33mg/g	increase ionic concentration, increased loading and activity (upto 1M); neutral and basic coupling pH needed; 10-24hr coupling time; immobilized on Euperit C with higher loading and lower activity	(Hernaiz and Crout 2000)
Eupergit C-glutaraldehye; Eupergit C-epoxy boronate	Copolymer of methacrylamide, N,N'-methylen-bis(acrylamide) and a monomer carrying oxirane groups; Macroporous, ~150um	*K. lactis*	70-75%	0.2mg/g	linked by carbohydrate area decreased product inhibition; >20hrs immobilization time	(Mateo, 2004)
Sepabeads	Macroporous; polymethacrylate with oxirane	*A. oryzae*	no activity			(Torres, Mateo et al. 2003)
Sepabeads-Amino-Epoxy	Macroporous; polymethacrylate with oxirane	*A. oryzae*	3500U/g	Unclear (28-40mg/g)	>20hrs reaction time for complete immobilization	(Torres, Mateo et al. 2003)
Graphite	Graphite modified using anhydrous methanol to introduce carboxyl groups	*K. lactis*	8800 X decrease in activity	0.63-1.30mg/cm square		(Zhou and Chen 2001)
Cotton	Tosylated	*A. oryzae*	55%	50mg/g		(Albayrak, 2002)

Table 2 (Continued)

Cotton fibers using PEI aggregates	Glutaraldehyde cross-linked	*A. oryzae*	In slurry, high activity; After centrifugation, low activity; Immobilized activity is unclear	250mg/g	Cotton was immersed in PEI, then enzyme added which aggregated the protein, all was crosslinked	(Albayrak and Yang 2002)
Chitosan	Glutaraldehyde cross-linked	*A. oryzae*	~100%	unclear (0.1mg/g)		(Rejikumar and Devi 1995); (Rejikumar and Devi 2001)
Chitosan	Glutaraldehyde cross-linked	*K. fragilis*	11-40%	17-20mg/g	*K. fragilis* is notoriously unstable	(Carrara and Rubiolo 1994); (Carrara and Rubiolo 1996)
PVOH-formaldehyde	tosy sulfony chloride, cyanuric chloride, benzoquinone	*A. oryzae*	26-100%			(Rejikumar and Devi 1995)
Salicylic acid, resorcin, formaldehyde	tosy-sulfonyl chloride, cyanuric chloride, benzoquinone	*A. oryzae*	14-28%			
Phenol-formaldehyde resin (plexiglass)	glutaraldehyde	*A. niger*	200umol/min/g of support; 40%	5mg/g		(Olson and Stanley 1973)
Porous silica						(Di Serio, Maturo et al. 2003)
PVC/silica	PEI/glutaraldehdye	*A. oryzae*	90%	1.9mg/cm2	ribbed increases SA; rolled over like a capet around a poll	(Bakken, Hill et al. 1990); (Bakken, Hill et al. 1991); (Bakken, Hill et al. 1992)
Nylon	Glycidyl methacrylate (diazotisation) glutaraldehyde	*A. oryzae*	48-62% Vmax			(El-Masry, De Malo et al. 2001)

Table 2 (Continued)

Cellulose	Epichlorohydrin	*K. fragilis*	80%	Unclear	epoxy residues have a slow reaction, and it has been suggested by others (Mateo) that adsorption prior to covalent modification helps	(Roy and Gupta 2003)
Sepharose 4B	Agarose; 1-Cyano-4-(dimethylamino)-pyridinium tetrafluoroborate	*K. lactis*	77-112%	1-5.4mg/ml packed support	cyanating protein sulfhydryl groups; also works on amino	(Giacomini, Villarino et al. 1998)
Controlled Porous Glass	3-aminopropyltriethoxysilane activated with glutaraldehyde	*K. fragilis*	90%	Unclear	lactase cultured in lab	(Szczodrak 2000)
Siliva/alumina	aminopropyltriethoxysilane activated with glutaraldehyde	*K. fragilis*	50%			(Ladero, Santos et al. 2000)
CPC/silica	aminopropyltriethoxysilane activated with glutaraldehyde	*K. lactis*	8-34%	12.6-23mg/ml packed support		(Giacomini, Villarino et al. 1998)
Gelatin	glutaraldehyde or chromium (III) acetate	*E. Coli*	22-25%			(Sungur and Akbulut 1994)
DEAE Cellulose	glutaraldehyde	*Scopulariopsis*	6x more activity that dulolite			(Park and Pastore 1981)
Cellulose beads	Benzoquinone	*E.Coli*	23-83%	13-109mg/g	high load reduced activity but increased stability. MW of E.Coli lactase=540,000	(Hong, Kwon et al. 1982)
Nylon/acryllmide	glutaraldehyde and azide	*E.Coli*	156U/g	assumed 2mg/g based on activity	suggested poor surface grafting	(Abdelhay, Guthrie et al. 1979; Houts 1988))
Silica.Alumina	Diisocyante	*A.niger*	100 fold decrease			(Roodpeuma 1983)

Enzyme leakage and loss of activity over time has been demonstrated for a entrapment and adsorption-based systems due to continuous processing (Batsalova, Kunchev et al. 1987; Champluvier, Kamp et al. 1988; Siso and Doval 1994). Covalent modification of the enzyme to or within a support has been explored as an approach to overcome the problem of enzyme leakage (Table 2). Different supports and methods of immobilization are developed so as to maintain/enhance enzyme activity, increase enzyme loading, lower the cost of immobilization, and optimize compatibility with a reactor design. The carrier (and support chemistry) must, also, be nontoxic, approved for food use, and, for covalent immobilization, have functional groups available for bioconjugation. For lactase immobilization, compromises are made to minimize or maximize these factors, and though some systems have been developed for industry, application to dairy processing has been allusive (Baret 1987).

The nature of the support on enzyme activity is difficult to determine in part because of lack of free enzyme controls for comparison. Hydrophobic supports have been suggested to reduce the activity of the enzyme because of hydrophobic adsorption and complimentary unfoulding of the enzyme at the surface (Zhou and Chen 2001; Torres, Mateo et al. 2003). Hydrophilic natural carriers including agarose, cellulose, dextran, alginate, gelatin, and collagen have been used with high activity retention (Rejikumar and Devi 1995; Giacomini, Villarino et al. 1998; Roy and Gupta 2003). Hydrophilic carriers, though useful at the lab scale, are often not suited for industrial processing because of low mechanical rigidity (deformation), biodegradation, and swelling in aqueous solutions—leading to complications associated with pressure drops (Robinson, Dunnill et al. 1971). Inorganic carriers including silica and glass have, also, shown promise with respect to activity retetnion (Robinson, Dunnill et al. 1971; Szczodrak 2000). These supports have been used for

industrial processes, but are limited by cost and pH stability (Pivarnik 1995; Wu, Lee et al. 1998)

Active Packaging

The growing trend in consumer demand for fresh, minimally processed, natural convenient foods with fewer additives along with changes in retail and distribution practices, has presented challenges to the food-packaging industry (Vermeiren, Devlieghere et al. 1999; Vermeiren, Devlieghere et al. 2002; Suppakul, Miltz et al. 2003). In response to these trends and due the inherent limitation of traditional packaging systems to meet those demands has resulted in the development of active packaging applications. Active packaging involves interactions between a food, packaging material and the internal gaseous atmosphere (Labuza and Breene 1989). The goal of these systems is to, through the entrapment, absorption, or covalent linking of functional compounds to or within packaging materials, increase the quality and/or safety of the product after packaging. Such packaging changes the condition of the packaged food to extend shelf-life, or improve food safety or sensory attributes, while maintaining the quality of the packaged food (de Kruijf, van Beest et al. 2002). In this context, the polymer no longer has just passive properties as dictated by the chemical and physical structure, but also an active component that has been deliberately designed to serve a specific function in the food system

Enzymes that have been incorporated for active packaging include lysozyme, glucose oxidase, and nariginase. Lysozyme, an antimicrobial enzyme that is able to hydrolyze the β(1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine, aiding in the break down of the cell wall of gram positive bacteria, has been immobilized on poly(vinyl alcohol) beads, nylon 6,6 pellets, and in cellulose triacetate films (Appendini and Hotchkiss 1997). Though all polymers demonstrated activity, the cellulose triacetate films showed the greatest efficiency, retaining 60% of

their activity after 20 uses, and were showed to be inhibitory and bactericidal against *Micrococcus lysodeikticus.* Naringinase, which hydrolyzes the bitter compound naringin to naringenin and prunin, has been immobilized in cellulose acetate and cellulose triacetate polymers (Soares and Hotchkiss 1998). The films showed a decrease in K_m value, indicating an increase in the substrate affinity of the enzyme entrapped films. The films had an activity efficiency of up to 23% compared to the free enzyme at 7°C. Glucose oxidase, which converts glucose, oxygen, and water, to a glucono-delta-lactone and hydrogen peroxide, has been used in sachets and to perform as an oxygen scavenger (Brody and Budny 1995). A difficulty with this system is that glucose must be available to serve as a reactant for the enzyme to perform as an oxygen scavenger, thus limiting its application thus far.

Lactose-reducing, heat sealable, packaging films have been by developed by lactase entrapment in ethylene(vinyl acetate) and covalently bound to poly(ethylene) with reduced activity upon immobilization (Steven 2004; Talbert 2004; Goddard, Talbert et al. 2007). The enzyme has been attached to oxidized polyethylene films using a PEG intermediate and PEI-bound layer (Steven 2004; Goddard, Talbert et al. 2007). Though the PEG-intermediate did not demonstrate activity, the PEI intermediate did retain measurable activity. A PEG-modified and native lactase has been entrapped in ethylene(vinyl acetate) films with both enzymes exhibiting a significant increase in K_m and decrease in V_{max} , but with detectable enzymatic activity (Talbert 2004). PharmaCal, Ltd has reported the use of an active packaging system using incorporated lactase that could reduce lactose 30-70% in 24-36 hours (Brody and Budny 1995). However, no commercialization or patent has been developed for this product.

Loss of Enzyme Activity on Surfaces

The organization on an enzyme is that of a primary structure of covalently linked amino acids that form, along the sequence, globular, helical, and sheet folds based on hydrogen bonding (secondary structure). A tertiary structure is formed by the interactions of secondary structures--forming salt bridges, maximizing hydrophobic interactions, and satisfying requirements with the solvent. These interactions, also, form the basis of the catalytic cleft of the protein for enzymatic activity. The tertiary structure gives the enzyme a core of hydrophobic amino acids (phenylalanine, tyrosine, etc.) since exposure of these residues to a native hydrophilic environment would be unfavorable and a surface composed of hydrophilic and acid or basic amino acids because of their ability to hydrogen bond, and contains a bound water layer at the surface (Lumry and Eyring 1954; Alvaro and Russell 1991). In some cases, a quaternary structure is formed by noncovalent interactions of multiple subunits of same or different sizes.

The complexity of enzymes, though optimal for biological substrate specificity, presents challenges in the development of industrial applications. Enzymes have evolved to function under the conditions of their natural environment, and activity and stability are, subsequently, a reflection of that environment. The pH, temperature, intracellular or extracellular nature of the enzyme, protein concentration, molecular composition of the environment, salt concentration, water activity, substrate concentration, cellular function, and structure hierarchy may all influence the robustness of the enzyme and how it performs (Lumry and Eyring 1954). Removing an enzyme from its native environment for use in a designed system changes the dynamics of the molecular equilibrium between the native and unfolded state of the enzyme. This equilibrium can be thermodynamically represented in the Gibbs Free Energy Equation (Equation 1)

Equation.1:

$$\Delta G = \Delta H - T\Delta S$$

The equation can be expanded to include enthalpy (ΔH) terms for protein interactions, solvent interactions, and interactions of the two, as well as entropy (ΔS) terms for both the protein and the solvent (Creighton 1993) (Equation 2)

Equation.2:

$$\Delta G = \Delta H_{solvent/solvent} + \Delta H_{protein/protein} + \Delta H_{protein/solvent} - T\Delta S_{solvent} - T\Delta S_{protein}$$

For enzymes at a surface, the equation can be expanded further to include the insoluble surface (Equation 3)

Equation 3:

$$G = \Delta H_{solvent/solvent} + \Delta H_{protein/protein} + \Delta H_{surface/surface} + \Delta H_{protein/solvent} + \Delta H_{protein/surface} + H_{surface/solvent} - T\Delta S_{solvent} - T\Delta S_{protein} - T\Delta S_{surface}$$

The enthalpy terms account for inter- and intra-molecular interactions that can be changed to influence the enzyme state, including: bond lengths, van der Waals interactions, torsion angle, electrostatic interactions, and hydrogen bonding (Creighton 1993). The entropy terms indicate the order of the system, with the system favoring more disorder. With respect to an enzyme, dominating entropy will yield multiple, diverse confirmations of an enzyme, which, though energetically favorable, will produce an unfolded and inactive enzyme. Thermodynamic terms must; therefore, be sufficiently satisfied and work in such as way as to prevent unfolding of the catalyst.

Protein interactions at surfaces are studied at the liquid/liquid, liquid/gas, and liquid/solid interface. Proteins, because of their amphiphilic nature, are used in the formation of foams at the liquid/gas interface by denaturing of the tertiary structure to

expose hydrophobic residues to the gaseous CO_2 and liquid interface. Emulsions, similarly, incorporate proteins to stabilize solutions of oil and water constituents by reducing interfacial tensions and preventing coalescence by electrostatic repulsion (Jones and Middelberg 2003; McClements 2004). For immobilized enzymes; however, liquid/solid and solid/solid interactions are of importance, and enzyme activity can be loss or maintained due to the thermodynamic effects that occur at the surface interface between the enzyme and the carrier. Amino acids that make-up the enzyme are capable of engaging in positive and negative electrostatic attraction and repulsion, disulfide bonding, and hydrophobic interactions—all of which may lower the free energy of the system when in contact with a surface. These groups, along with the hydrogen bonding of the bound water layer and the preferential hydration of a surface, impose restrictions on the nature of a solid interface in preventing loss of the native enzyme structure when the two are in contact.

Proteins with exposed hydrophobic groups have been shown to absorb tightly to hydrophobic surfaces due to lowering of the free energy in aqueous solution that occur from decreased exposed hydrophobic surface area when the two exposed groups come into contact (Moskovitz, 2005; Czeslik, 2001; Aubin-Tam, 2005; Doshi, 2005; Lu, 1998; (Lee and Park 1994; Nishiyama, Watanabe et al. 2005; Koutsopoulos, Patzsch et al. 2007). The hydrophobic interactions cause a dehydration of the protein surface, and the subsequent adsorption presents a shift in the tertiary or secondary structure of the protein (Doshi, 2005; Moskovitz, 2005). The molecule may then spread across the surface to further minimize the free energy (Czeslik and Winter 2001; Mungikar and Forciniti 2004). Both a small or large shift may cause a loss in enzymatic activity (Kondo, Murakami et al. 1992). Experiments seeking to characterize changes in enzyme secondary structure at the surface (compared to non-denaturing surfaces) have provided limited information since the shifts, though

apparent, appear to be small compared to non-denaturing surface and random from enzyme to enzyme in regards to α-helix and β-sheet perturbations (Kondo, Murakami et al. 1992; Norde and Zoungrana 1998; Koutsopoulos, Patzsch et al. 2007). Consequently, changes in the secondary and tertiary structure are difficult to distinguish. Hydrophobic denaturation has been attributed to loss of lactase activity when the enzyme is bound to a porous polymethacrylate resin with oxirane groups (Mateo, Torres et al. 2003).

Electrostatic groups on the surface of a support may lead to attraction or repulsion of ionic amino acids on the enzyme surface. When an enzyme is in contact with the surface, minimization or maximizing those interactions can lead to distortion of the protein and a loss of enzymatic activity (Aubin-Tam and Hamad-Schifferli 2005; Haupt, Neumann et al. 2005; Lund, Akesson et al. 2005). Binding of proteins to charge supports has been shown to occur even when the net charge on the enzyme is the same as the support because of ionizable amino acid side chains (Aubin-Tam and Hamad-Schifferli 2005). The activity of lactase bound to an anionic support has been shown to be lower than activity when bound to a cationic support, suggesting a negative charge may alter the conformation of the enzyme (Hamlin, Dayton et al. 2007). Carriers with surface ionic groups can also promote a pH shift in the microenviroment of the immobilized enzyme, which may change optimum catalytic conditions (Lamb and Stuckey 2000; Wentworth, Skonberg et al. 2004). These extremes in pH can alter protonation state of amino acids causing changes in hydrogen donor/acceptor characteristics, loss or gain or electrostatic repulsions, and changes in salt bridges.

In some instances hydrophilic surfaces have been shown to reduce enzymatic activity to a greater extent than a hydrophobic surface (You, De et al. 2005). This phenomenon is attributed to competitive hydrogen bonding at the interface or breaking

of necessary salt bridges. The unique structure and chemical properties of water influences the interactions of a surface of an enzyme through hydrogen bond and dipole interactions (Raschke 2006). The hydrophilic surface changes the enzyme by competing and, ultimately, stripping the water layer from the enzyme shell, which is important from an enthalpic standpoint in maintaining tertiary structure (a hydrophobic surface may also strip the water surface by promoting dehydration--reducing the driving force to retain a hydrophobic residue inside the core).

Changing the chemical/physical nature of a carrier or distance from the surface can influence enzymatic activity, by limiting surface/protein interactions. The addition of the hydrophilic molecules to the surface of a hydrophobic material prevents proteins from adhering by reducing inter and intra-molecular hydrophobic interactions(Holmberg, Tiberg et al. 1997). Hydrophilic molecules, monomers, and polymers have been used to increase the number of functional groups on a surface or to provide a more reactive intermediate. Polyethylenimine, chitosan, polyacrylic acid, heparin, polyalylamine, alginate, and collagen have been grafted, either covalently or ionically, to facilitate protein loading or biocompatibility (Cheng and Teoh 2004); (Bahulekar, Ayyangar et al. 1991; Yang and Lin 2003); (Konig, Nitschke et al. 2002); (Vasilets, Hermel et al. 1997). The addition of the hydrophilic molecules to the surface of a hydrophobic material prevents proteins from adhering by reducing inter and intra-molecular hydrophobic interactions (Holmberg, Tiberg et al. 1997). Many of these polymers are, also, polyionic under conjugation conditions, which promotes ionic interaction with a charged protein. Initial ionic adsorption coupled with a means of covalent binding has been shown to promote protein loading on a material. (Pessela, Fernandez-Lafuente et al. 2003); (Koehler, Ulbricht et al. 2000; Jonsson and Johansson 2004). A layer-by-layer approach to immobilizing enzyme onto a support has been employed to increase the loading of the enzyme of the carrier or change

interactions of proteins with the surface (Elbert, Herbert et al. 1999; Lei, Bi et al. 2007). By this method, a surface layer is formed on an activated support, most often using an ionic polymer. An opposite charged layer is then deposited on top forming a thin layer by ionic attraction and the process is repeated until a desired thickness is achieved. Enzymes may be added between layers or as a final layer on the surface. Polymer brushes have been formed from polymeric surface by using free radical grafting. Glycidyl methacrylate, for example, has been grafted to hollow fiber polyethylene membranes for protein separation technologies by irradiation-induced free radical formation (Saito 2002).

When used in a nonaqueous environment, immobilized enzymes can denature by being contact with a hydrophobic liquid or gas surface. To minimize interactions between a solvent or gas bubble, a hydrophilic polymer can be grafted as a thin layer over the enzyme/carrier to create a stable hydrophilic nanoenvironment. Coating of an aldehyde-activated dextrose over glucose oxidase immobilized to a non-porous carrier inhibited inactivation due to gas bubbles (Betancor, Fuentes et al. 2005). Penicillian acylase was made more stable against dioxane by immobilizing the enzyme through multipoint bonding, followed by creating a hydrophilic environment on the carrier as well as on the enzyme, directly (Fernandez-Lafuente, Rosell et al. 1999). If dextran was attached to just the carrier or just enzyme, no stabilization occurred indicating the necessity for complete coverage (Fernandez-Lafuente, Rosell et al. 1999).

Use of spacer molecules such as glutaraldehyde, ethylenediamine, hexamethylene diamine, or poly(ethylene glycol) has been shown to provide an increase in the activity compared to conjugation directly to the support (Jayakumari and Pillai 1991; Ganapathy, Manolache et al. 2001; Nouaimi, Moschel et al. 2001; Torres, Mateo et al. 2003). De Maio et al., have shown that increasing the chain length aids in retaining enzymatic activity and Lopez-Gallego and others have shown that

amino groups on a surface aids in the retention of D-amino oxidase activity when conjugated to a support (Lopez-Gallego, Betancor et al. 2005); (De Maio, El-Masry et al. 2003). Likewise, studies in computational protein modeling at interfacial surfaces suggest that a hydrophilic chain on a hydrophobic surface would be necessary to preserve activity of an immobilized enzyme (Moskovitz and Srebnik 2005). Trypsin was separated from carboxylic-functionalized fleece by bovine serum albumin (BSA), aldehyde dextran, amino dextran, and PEG-diamine, and direct binding (Nouaimi, Moschel et al. 2001). Use of the spacer molecules showed an increase in activity in all cases relative to direct covalent binding. BSA showed the highest activity retention followed by amino dextran, PEG-diamine, and aldehyde dextran. Though the authors contribute enhance activity to decreased interaction of the protein with the hydrophobic fleece, charge may also be a factor since the surface of the fleece is charged, the aldehyde dextran is activated by periodiate oxidation of the dextran, and the amine spacers carry positive charges. Lactase was separated from an activated nylon support by diamines of varying chain lengths (De Maio, El-Masry et al. 2003; De Maio, El-Masry et al. 2003). Increased chain length resulted in an increased activity that was attributed to separation of the protein the densely charged surface. Distance may, also, promote mobility of the biocatalyst to decrease rigidity and allow for better dynamic motion for interaction of the protein with substrate (Ganapathy, Manolache et al. 2000).

Grafting and tethering of polymer chains onto a solid surface is used for increasing the available surface area for protein immobilization, to block activated functional groups on the surface from interaction with a substrate or fluid, and to provide a suitable surface for protein attachment or rejection. The nature of the polymer interface can be changed by grafting a polymer to the surface (Fang and Szleifer 2002; Currie, Norde et al. 2003). Grafting and tethering provides a means of

retaining the key bulk properties of a material while changing the surface for biointeractions (Faucheux, Schweiss et al. 2004; Fu, Ji et al. 2005). The choice of polymer at the surface can alter the interfacial thermodynamics and microenvironment since the enzyme will be reacting with the grafted layer, providing a more suitable platform for enzyme immobilization (Moskovitz and Srebnik 2005). Chitosan and collagen, for example, have been used to change the interface of hollow fiber membranes and nanofibers for lipase immobilization (Ye, Xu et al. 2006; Ye, Xu et al. 2006).

Self assembled monolayers (SAMs) are thin films of biological or chemical molecules that form spontaneously on surfaces (Bain, Troughton et al. 1989; Senaratne, Andruzzi et al. 2005). SAMs differ from graft in that the coverage formed by SAMs is an organized coverage consisting of a true monolayer, which may or may not be covalently grafted. Surface/substrates that form SAMS are limited--examples including alkanethiols on metal surfaces (particularly gold) and alkanesilanes on silicon (Senaratne, Andruzzi et al. 2005). Alkanethiols have been shown to form a self assembled monolayer on the surface of gold by oxidative addition/reductive elimination (Bain, Troughton et al. 1989; Love, Estroff et al. 2005). Alkanesilanes, likewise, self assemble on silicon oxide or silica glass (McGovern, Kallury et al. 1994; Parikh, Allara et al. 1994). With respect to enzymes on carriers, SAMs with, different pendant groups, have been used to elucidate the mechanism for loss of glucose oxidase activity when adsorbed to a surface (Guiomar, Guthrie et al. 1999)

Multipoint covalent bonding is an alternative to surface modification and has been used to enhance enzyme stability in porous carriers by, three-dimensionally, fixing the enzyme in place so as to restrict movement and denaturation associated with entropy (Martinek, Klibanov et al. 1977). Several enzymes have shown increased stability when applying this method (Balcao, Mateo et al. 2001; Ichikawa, Takano et

al. 2002; Bolivar, Wilson et al. 2006; Mateo, Palomo et al. 2007). The activity retention associated with this method, which typically incorporates, a porous agarose carrier containing glycoxal residues, (Mateo, Abian et al. 2005) may be as low as 10-15% (Kuroiwa, Shoda et al. 2005; Bolivar, Wilson et al. 2007), or a high as 100% (Guisan, Polo et al. 1997). Proper geometric alignment for the enzyme and the carrier is a limitation for this approach, and may lead to deactivation of the enzyme (Mateo, Palomo et al. 2007).

Physical Effects

The quaternary structure of an enzyme develops when subunits associate in a noncovalent manner. The association/disassociation of the subunits is dynamic, which can be problematic when conjugating to a planar surface where all the subunits may not contact the surface to be immobilized (Poltorak, Atyaksheva et al. 2004). Multipoint bonding in porous support if often effective for dimmers, but greater subunit associations may require a post-immobilization stabilization using an activated polymeric molecule (Fernandez-Lafuente, Rodriguez et al. 1999). The quaternary structure of alcohol oxidases, with 4-8 subunits, were stabilized by a two step immobilization process of 1) attaching the enzyme to a porous support and 2) conjugating aldehyde-dextran to the enzyme/support (Lopez-Gallego, Betancor et al. 2007). Though activity was reduced to 20%, quaternary structure (as determined by protein loss from the support in the presence of SDS and mercaptoethanol and analysis of the supernatant by electrophoresis) was maintained. In the same study, polyethylenimine (PEI) was used as a grafted layer, showing no loss of quaternary structure 50% activity retention when adsorbed to the support (Lopez-Gallego, Betancor et al. 2007). The enzyme; however, retained more activity on the base agarose support (80%) prior to quaternary structure stabilization approaches. Tetrameric L-asparaginase was coupled to agarose-glutaraldehyde supports followed

by conjugation with aldehyde-dextran (Balcao, Mateo et al. 2001). Then enzyme lost 60% of the intrinsic activity, but could be subjected to boiling SDS without loss of protein—demonstrating retention of the quaternary structure. Catalase from bovine liver on agarose beads showed full retention of activity; however, after washing, enzyme activity decreased and was attributed to loss of enzyme subunits in the rinse (Betancor, Hidalgo et al. 2003). Modification with aldehdye-dextran after immobilization caused a 30% reduction in enzymatic activity, but no loss of protein. Effects of washing on residual activity after immobilization; however, was not evaluated.

Crosslinking enzymes in solution to form carrier-free oligomeric microstructures are used to increase enzyme activity per unit area, provide enhanced thermostability and solvent stability, and stabilize quaternary structure (Haring and Schreier 1999; Albayrak and Yang 2002; Schoevaart, Wolbers et al. 2004). Cross-linked enzyme crystals (CLECs) are formed by cross-linking the crystallized structure of an enzyme to "freeze" the structure in place. Cross-linked enzyme aggregates, in a similar fashion, are formed by forcing enzymes to aggregate, by addition of salt or organic solvent, and cross-linking the structure. Glutaraldehyde is often used as the crosslinking agent because of its wide reactivity (Sheldon 2007). CLECs/CLEAs can be used alone as an insoluble enzymatic platform or conjugated to a carrier (Cao 2005). The limitations of CLEC/CLEAs are the small size, conditions for cross-linking, diffusion limitations, and deformation under mechanical stress (Cao, van Rantwijk et al. 2000). CLECs/CLEAs have been prepared for a number of enzymes, often resulting in greater stability under denaturing conditions (Stclair and Navia 1992; Margolin 1996; Schoevaart, Wolbers et al. 2004). Chloroperoxidase, subilisin, theromolysin, and lipase have been crosslinked as CLECs or CLEAs (Persichetti, Stclair et al. 1995; Wang, Yakovlevsky et al. 1997; Ayala, Horjales et al. 2002)

CLEAs of lipase have been immobilized in polymeric membranes to prevent leaching from the membrane without having to activate the support for covalent attachment. The process was carried out by allowing the enzyme to enter the membrane and then transferring the membrane to a solution of glutaraldehyde (Hilal, Nigmatullin et al. 2004). Catalase, which is unstable in solution because of disassociation of the quaternary structure, was modified with glutaraldehyde—producing a stable conjugate (Mishaeva, Gudkin et al. 2006). Though not an ideal CLEA, lactase has been aggregated on the surface of cotton by adding a PEI layer to the cotton, and not removing the excess PEI. When lactase is added, followed by glutaraldehyde, the lactase complexes in the PEI, and an aggregate PEI-lactase is formed (Albayrak and Yang 2002).

Site directed orientation of an enzyme on a support can be useful in preventing non-specific adsorption of the protein and alignment of the active site towards the surface, and is particularly beneficial for enzymes acting on large polymeric substrates (Butterfield, Bhattacharyya et al. 2001). Protein engineering has been used to introduce a cysteine residue on the surface of opposite the catalytic site to enhance to activity of immobilized subtilisin by rational design (Viswanath, Wang et al. 1998). A point mutation is achieved by substituting the codon calling for a cysteine residue at the desired place in the gene sequence, and introducing the gene into an expression vector to produce the new protein (Viswanath, Wang et al. 1998; Butterfield, Bhattacharyya et al. 2001). His-tagged enzymes are proteins that have genetically engineered by introducing a sequence calling for histidine residues (usually six) at the C- or N-terminus. The histidine sequence reacts specifically with divalent metal cations (Schmid, Keller et al. 1997). By covalently attaching a metal chelating agent like Ni-nitriloacetic acid (NTA), and introducing Ni^{++}, a site-specific interaction will form between the chelating agent, nickel, and the histidine sequence (Rusmini, Zhong

et al. 2007). Polyhydroxyalkanoate was immobilized by this method to silicon for the production of aliphatic polyesters (Kim, Paik et al. 2004). Another example of site-specific immobilization is that of cutinase, a serine esterase, which forms a covalent bond with phosphonate inhibitors and is specific to this enzyme (Hodneland, Lee et al. 2002). Some enzymes are glycosylated and can be conjugated to a support specifically by the carbohydrate moiety. Lipase has been immobilized in this fashion by periodate oxidation of the carbodydrate chain for conjugation to Eupergit C supports (Knezevic, Milosavic et al. 2006). Taking advantage of the pKa difference in the N-terminus has been used to site-specifically attach proteins to PEG (Roberts, Bentley et al. 2002; Lee, Jang et al. 2003). General bioconjugation schemes, targeting the same amino acid group can, likewise, result in different retention in enzyme activity (Rejikumar and Devi 1995). This phenomenon may be due to the specificity of the bioconjugation reagent with the desired amino acid groups.

Reducing the size of the carrier to the nanoscale has shown to promote increased activity of an immobilized enzyme, due to either increased Brownian motion or a reduction in protein conformational changes upon immobilization (Wang 2006). The effect of particle size was demonstrated using α-Chymotrypsin attached to polystyrene particles of 100-1000 nm as well as thin films of polystyrene (Jia, Zhu et al. 2003). k_{cat}/K_m decreased with increasing size. Though the change was slight for the nanoparticles, k_{cat}/K_m was 100 fold lower on the films, which the authors attributed to mobility effects of the catalyst (Jia, Zhu et al. 2003). Inhibition of enzyme conformational changes has, also, been attributed to the advantageous geometry of nanomaterials. Soybean peroxidase deactivation was measured after adsorption to single walled nanotubes (SWNTs) and graphite flakes (Asuri, Karajanagi et al. 2006). The deactivation kinetics of the enzyme was lower on the SWNTs and demonstrated to be independent of protein coverage, while the graphite flakes were dependent on

protein coverage. It was concluded that denaturing lateral protein-protein interactions were decreased due to the size and curvature of the SWNTs (Asuri, Karajanagi et al. 2006) (Asuri, Karajanagi et al. 2006; Kane and Stroock 2007).

The porosity of a carrier can effect apparent enzyme activity by increasing K_m of an enzyme in a three dimensional medium (Wu, Lee et al. 1998). Diffusion limitations of the substrate can be effected by the microenvironment of the carrier/solvent, inaccessibility of substrate into the pores, or blockage of available enzyme (Wu, Lee et al. 1998). Likewise, the microenvironmental pH can effect how the substrate interacts with the carrier-bound enzyme (Goldstei.L, Levin et al. 1964). Trypsin showed a 2-unit pH shift for activity when immobilized in a carboxylic acid matrix (Goldstei.L, Levin et al. 1964).

Ordering of the protein on the surface can alter the activity of the conjugated enzymes. Crowding of protein on the surface of a support with increase loading has been shown to reduce the activity of the enzyme due spatial restrictions, limited active site accessibility, or denaturing of the protein (Ganapathi, Butterfield et al. 1995; Yamada, Iizawa et al. 2006). Surface regularity has, also, been associated with maintaining enzyme activity (Matsuno, Nagasaka et al. 2007). Polymethylmethacrylate (PMMA) with an irregular surface compose of *it*-PMMA or an order surface of alternating *it*- and *st*-PMMA (which forms regular helical structures) was used to study immobilization of lactase (*E. coli*). The PMMA with the ordered surface resulted in the retention of greater lactase activity (Matsuno, Nagasaka et al. 2007). This result was attributed to enthalpic gains associated with polymer mobility, and weak/limited interactions between the enzyme and alternating surface that limited these enthalpic gains

Carrier and Enzyme Modification

Surface Modification

Functionalization of materials by copolymerization, wet chemistry, and physical-chemical methods are employed to change the characteristics of a surface or develop a surface more suitable for bioconjugation (Goddard and Hotchkiss 2007). Introduction by copolymerization insert a monomer with an inert side chain to be polymerized with a polymer containing a functional group. Polystyrene microspheres have been successfully prepared using carboxylic acid, amine, and hydroxyl monomers (Arshady 1991; Arshady 1991). Wet chemistry techniques make use of chemical groups in the polymer side chain (i.e. hydroxyls, esters, amines, aldhehydes, and carboxylic acids) or by introducing groups for an inert surface such as oxidation or nitrosylation (Sheng, Sutherland et al. 1995; Bag, Kumar et al. 1999); (Arshady 1991; Hermanson 1996). Physical techniques such as oxygen and nitrogen plasma oxidation, corona, and UV treatment have, also, been instrumental in modifying a polymer surface and are used in a number of industries including food applications (Egitto and Matienzo 1994; Chan, Ko et al. 1996; Ozdemir, Yurteri et al. 1999; Mount 2001; Aouinti, Bertrand et al. 2003). These methods have provided a means to conjugate bioactive compounds to polymeric supports, and have been described in detail by Steven and Goddard (Steven 2004; Goddard 2008)

Enzyme Modification

Biological methods of enzyme modification are focused on manipulation of the enzyme at the genetic level. Transformations in the gene and corresponding protein structure are either random (directed evolution) or intentional (rational design) (Kaur and Sharma 2006; Rubin-Pitel and Zhao 2006; Leisola and Turunen 2007). Directed evolution relies on methods such as error-prone PCR and DNA shuffling. epPCR utilizes polymerase to introduce random mutations in a gene. DNA shuffling uses a

number of homologous genes with desired characteristics. The genes are fragmented, denatured, and annealed in random fashion (Kaur and Sharma 2006). For both epPCR and DNA shuffling, the new genes are introduced in an expression vector and the corresponding protein screened for a desired property. Directed evolution has produced a number of enzymes with enhanced specificity, activity, and thermostability (Kaur and Sharma 2006; Rubin-Pitel and Zhao 2006). The method, though quick and powerful, requires large libraries of genes and multiple generations of protein producing microorganisms with no guarantee of enzyme improvement. Rational design is a technique where an intentional modification is introduced in the sequence of the gene to alter the protein. The practice aims to go from desired function to structure to sequence. Compared to directed evolution, rational design has fewer successes, attributed to the idea that knowledge of structure function of enzyme is still developing (Leisola and Turunen 2007).

Chemistry-driven modifications are employed to change the surface interactions between the enzyme and the external environment, adjust enantioselectivity and/or specificity, prevent unwanted interactions, or explore structure/function relationships. Engineering in this category include the use of surfactants, reverse micelles, extraction, and covalent modification. Surfactants may be used by directly incorporating a lyophilized protein into a solvent containing surfactant and small amounts of water (Akbar, Aschenbrenner et al. 2007). The continuous solution is then centrifuged and the supernatant removed. This method has shown 25-72% solubility of enzymes in a number of solvents of varying polarity as well as protein aggregation of up to 100 molecules (Akbar, Aschenbrenner et al. 2007).

Reverse micelle containing enzyme are produced by dissolving an aqueous solution of enzyme in an organic solvent containing a surfactant (Castro and

Knubovets 2003). The self-assembled micelles encapsulate enzyme and can be separated by centrifugation. This method allows some water to be associated with the system, which may increase the unfolding of the enzyme. Protein extraction is a unique techniques that involves ion-pairing an enzyme with a small molecule, which can be paired with a surfactant molecule and extracted into an organic layer (Paradkar and Dordick 1994; Paradkar and Dordick 1994; Novick and Dordick 2000). The method has been used with up to 95% solubilization of an enzyme, and can be applied in crosslinking an enzyme during monomer polymerization.

Polymer conjugation may be used to make the enzyme surface more hydrophilic or hydrophobic to induce solubility or thermostability (Mozhaev, Berezin et al. 1988; Davis 2003; Polizzi, Bommarius et al. 2007). This technique utilizes single polymer chains or small molecules to attach to the surface of the enzyme (DeSantis and Jones 1999). The most commonly polymeric molecules for covalent modification are carbohydrates (ie cyclodextrans, pectin, chitosan, carboxymethylcellulose, (Villalonga, Villalonga et al. 2000); (Gomez, Ramirez et al. 2000; Gomez and Villalonga 2000) and sucrose) and poly(ethylene glycol), (Longo and Combes 1999; Fernandez, Fragoso et al. 2002) . A procedure of interest for that has been utilized for the enhancement of therapeutic proteins and peptides is PEGylation, which unlike many other modifications provides simple, clean, and specific attachment chemistry for protein derivatives with low toxicity. PEGylation involves the attachment of polyethylene glycol (PEG) to a functional group of a compound so as to induce a variety of alterations to the protein including decreased immunogenicy, increased half-life due to reduced proteolysis, increased thermostability, and alterations in the solubility properties, which allows the protein to be soluble in water, toluene, 1,1,1 trichloroehane and benzene, and insoluble in ethyl ether (Mabrouk 1995; Greenwald 2001; Veronese 2001). Factors influencing these

properties are the number of PEG molecules attached, the molecular weight and structure of PEG chains attached, the location of PEG sites on the protein, and the chemistry used (Roberts, Bentley et al. 2002).

The solubility of enzymes in organic solvents is enhanced upon attachment of PEG. Kwon et al. quantified the solubility of PEG-subtilisin Carlsberg in a number of organic solvents and found PEGylation nearly always increased solubility (Kwon, Imanishi et al. 1999). Likewise, Takahashi et al., demonstrated an increase in benzene solubility of catalase with increasing degree of PEG-modification (Takahashi, Saito et al. 1988). There is indication that PEG-modified enzymes catalyze reactions in hydrophobic media and, in some cases, affinity for the substrate and velocities are increased in such solvents (Hernaiz, Sanchez-Montero et al. 1999) (Kodera, Nishimura et al. 1994), but there is little evidence to suggest whether this increase is due to stabilizing effects of PEG, solubilization that increases protein-substrate interaction, or a synergistic effect. The thermostability of effects of PEG on protein stabilization has been well documented, with a number of enzymes having demonstrated an increased thermostability after being modified with PEG (Zhang, He et al. 2001) (Hernaiz, Sanchez-Montero et al. 1999) (Longo and Combes 1999). Longo and Combes performed a detailed study on the thermostability of three enzymes after modification by glycosalation and by PEGylation (Longo and Combes 1999). Their research showed that glycosylation increased hydrophilicity of the enzymes while PEG made the enzyme more hydrophobic. Under heat denaturing conditions, the glycosylated enzymes lost more activity over time compared to the native enzymes, but PEGylated enzymes showed an increased stability over time. These results were attributed to the ability of PEG to draw water from the system to be utilized by the shell, but also increasing interaction of water with hydrophobic clusters, preventing access to the enzyme surface. Other studies; however, have shown that an increase in enzyme

thermostability upon modification with polysaccharides, though similar mechanisms for enzyme stability may be attributed to these modifications as well (Lenders and Crichton 1984; Fernandez, Fragoso et al. 2002).

Small molecules have been used to study structure function relationships of enzymes and to alter the stability of the proteins (Mozhaev, Berezin et al. 1988; DeSantis and Jones 1999). Molecules are often attached to thiol or ionic groups of the enzyme because of the reactivity; however, modifications of these groups may lead to inactivation of the enzyme (Tomschy, Brugger et al. 2002). Chymotrypsin has been glycosylated to promote stable oligomeric structures of the enzyme (Levashov, Rariy et al. 1993). The enzyme, RNase A, has also been glycosylated with glucosamine to explore thermostability of the enzyme (Baek and Vijayalakshmi 1997). Immobilized lipase has been aminated with EDC/ethylenediamine to change the selectivity of the enzyme (Palomo, Fernandez-Lorente et al. 2007). Though selectivity change, intrinsic activity was reduced by 70%. The amine and carboxylic acid groups of penicillin G acylase were converted to opposite charge by amination of the carboxylic acid groups with ethylenediamine or succinylation of the amine groups with succinic anhydride (Montes, Grazu et al. 2006). The modifications were performed to increase loading of the enzyme on ion exchange resins without dramatic changes to enzyme activity.

CHAPTER 2

RESEARCH OBJECTIVES

Given the potential applicability of packaging and reactor systems that employ the enzyme, lactase, being covalently immobilized to the surface of a hydropbobic support, the objectives of this research were to:

1. Covalently conjugate lactase to hydrophobic polymer supports in a manner that retains enzymatic activity
2. Determine the cause(s) of lactase activity loss upon conjugation to a carboxylic acid-activated, hydrophobic carrier
3. Develop methods for retaining/increasing intrinsic enzyme activity after attachment
4. Apply methods to the development of lactase-immobilized packaging films

CHAPTER 3

MATERIALS AND METHODS

Enzyme Purification and Activity

Protein Purification

A liquid preparation of commercial lactase from *Kluyveromyces lactis* (Valley Research, Inc.: Godo YNL-2), packaged in glycerol, was purified utilizing a two-step filtration method. Initially, 1.5ml of the enzyme was syringe filtered through a sterile 0.22um membrane. The product was, subsequently, added to a 2ml centrifugal ultrafiltration device with a 50,000 molecular weight cut off (MWCO) membrane (Millipore: Centricon YM-50) and centrifuged at 5000xg at 25^0C (I20 BCA centrifuge). The supernatant was reconstituted with 0.1M phosphate buffer (pH 7.0) containing 50% glycerol and 10mM $MgSO_4$ unless otherwise stated.

Lactase from *Aspergillus oryzae* (Enzyme Development Corporation; Enzeco) was purified from a dry sample by reconstituting 20mg of the commercial preparation in 1.5ml of 0.1M MES buffer (pH 5.3). The enzyme was syringe filtered through a sterile 0.22um membrane, and the supernatant added to a 2ml centrifugal ultrafiltration device with a 50,000 molecular weight cut off (MWCO) membrane (Millipore: Centricon YM-50) and centrifuged at 5000xg at 25^0C (I20 BCA centrifuge). The supernatant was reconstituted with 0.1M MES buffer (pH 5.3).

SDS PAGE

The extent of purification was determined using SDS gel electrophoresis. Samples were denatured by subjecting the enzyme to a temperature of 70^0C for 10 minutes in the presence of SDS. The samples were run on 7% acetate gels (NuPAGE; Novex) using tris-acetate running buffer and a constant voltage of 200V for 35 minutes. The

gels were stained using a Coomassie Blue solution (45% v/v water, 45% v/v methanol, 10% v/v acetic acid, and 0.25% w/v Coomassie brilliant blue R250) for 2 hours, and destained using using a solution of 50% v/v water, 40% v/v methanol, and 10% v/v acetic acid for intervals of 1 hour, then 12 hours, then 1 hour. Protein bands were compared to protein standards in the range of 40kDa to 500kDa (Invitrogen; HiMark Protein Standard).

Lactase Specific Activity

Lactase specific activity was determined by a modification of Food Chemical Codex method for the determination of acid lactase units (Anon, 2003). An amount of the enzyme preparation consisting of 2-5μg was added to 2ml of a 0.012mM solution of the synthetic lactose substrate, o-nitrophenyl-β-galactopyranoside, and allowed to react under shaking for 15 minutes. At the completion of the time period, 2.5ml of 10% sodium carbonate was added to stop the reaction. The solution was diluted to 25ml with deionized water and the absorbance of 2ml of the diluted sample was read at 420nm (Jenway 6300 spectrophotometer). Specific activity was determined using Equation 4.

Equation 4:

Enzyme specific activity (U) = [(Asample – Ablank)*(25)]/[(ε)*(t)]
Asample: absorbance of test sample at 420nm
Ablank: absorbance of blank (no lactase) at 420nm
25: volume of final solution, ml
ε: extinction coefficient (4.54 ml/μmol) using a 1cm light path length
t: time of reaction, minutes
U : enzyme units (μmol/min/g of enzyme)

K. lactis Specific Activity

pH Effect

Specific activity as a function of pH was evaluated in the range of 6.0 to 8.5. Phosphate buffer was used between pH 6 and pH 7.5 (0.1M) and borate buffer was used for pH 8.0 and 8.5 (0.1M) at 30°C.

Temperature Effect

Specific activity as a function of temperature was evaluated in the range of 25°C to 55°C at pH 7.0 (0.1M phosphate buffer) using an IsoTemp 125D heating block (Fisher Scientific).

<u>*A. oryzae* Specific Activity</u>

pH Effect

Specific activity as a function of pH was evaluated in the range of 3.5 to 7.0. Citrate-dipotassium phosphate buffer was used at pH 3.5, acetate buffer (0.1M) was used from pH 4.0 to pH 6.0, and phosphate buffer (0.1M) was used for pH 6.0 to pH 7.0.

Temperature Effect

Specific activity as a function of temperature was evaluated in the range of 30°C to 70°C at pH 5.0 (0.1M acetate buffer) using an IsoTemp 125D heating block (Fisher Scientific).

<u>Stability of Lactase Preparations</u>

K. lactis Stability

Purified lactase was reconstituted with one of the following four solutions:

1) 0.1M phosphate buffer

2) 0.1M phosphate buffer containing 10mM $MgSO_4$

3) 0.1M phosphate buffer containing 50% v/v glycerol

4) 0.1M phosphate buffer containing 50% v/v glycerol and 10mM $MgSO_4$

Each preparation was stored at 4°C and 25°C, and the specific activity at 25°C, pH 7.0 (0.1M phosphate buffer) was periodically determined across a 21-day period.

A. oryzae Stability

Purified lactase was reconstituted with one of the following solutions:

1) 0.1M MES buffer pH 5.5

2) 0.1M MES buffer pH 6.8

Each preparation was stored at 4 °C and 25 °C, and the specific activity at 50 °C, pH 5.0 (0.1M acetate buffer) was periodically determined across a two week time period.

Lactase (*A. oryzae*) kinetics :

The Michaelis constant of native and immobilized β-galactosidase was determined by a method similar to that used by Cavaille (Cavaille and Combes 1995). An 8mM ortho-nitrophenyl-β-Galactopyranoside (Sigma: ONPG, N-1127, MW 301.3) solution was diluted in 0.1 M acetate buffer (pH 5.0). The 8mM ONPG solution was then serially diluted, establishing a substrate range from 0 to 8mM. Lactase (2.5-5ug) was added to individual tubes containing the ONPG (with a concentration of 0-8mM) at 50^0C. The solution was continuously shaken for four minutes with representative tubes stopped every 30 seconds by the addition of 2.5ml of 10% sodium carbonate. The absorbance was spectrophotometrically measured at 420nm. The Michaelis constant (K_m) was extrapolated from nonlinear regression using Michaelis-Menten enzyme kinetics (Equation 5) (Graphpad Prism software).

Equation 5:

$$\text{Velocity} = V_{max} * [S] / K_m + [S]$$

V_{max} = Maximum velocity (mM/min)

[S] = Substrate concentration (mM)

K_m = Substrate concentration to achieve half V_{max}

Microsphere Characterization

Microspheres (Bangs Laboratories, Inc.) were composed of polystyrene-co-acrylic acid with a size range of 1.1-1.2μm.

Microsphere Cleaning

Surfactant was removed from the commercial microsphere preparation by centrifugal spinning of an allocated volume of microspheres at 10,000 x g (Fisher microcentrifuge Model 235) in low bind polypropylene microcentrifuge tubes (Eppendorf), removing

the supernatant, replacing with water, spinning, and repeating the process with 0.1M MES buffer pH 5.5, 0.1M phosphate buffer pH 7.5, water, and finally desired conjugation or storage buffer.

Carboxylic Acid Quantification

Available carboxylic groups on the surface of the microspheres were quantified using the Toluidine Blue assay (Kang, Tan et al. 1996). Microspheres were immersed in 1ml of 5 x 10^{-4}M Toluidine Blue O in distilled water that had been adjusted to pH 10 by NaOH. After three hours of shaking at room temperature, the microspheres were centrifuged and the supernatant dye removed. The microspheres were rinsed three times with NaOH solution at pH 10 to remove non-complexed dye. Complexed dye was then desorbed by shaking microspheres three times in 1ml, 50% v/v acetic acid for 15 minutes at room temperature, followed by centrifugation and removal of the desorbed dye into a cuvette. The absorbance of the desorbed dye was read at 633 nm, and surface carboxyl groups were determined by comparison with a standard curve of dye, knowing the quantity of microspheres and the volume of the desorbing solution under the assumption of one mole of dye complexing with one mole of available carboxyl groups.

Immobilization of Lactase onto Polystyrene-co-Acrylic Acid Microspheres

Lactase was covalently immobilized onto polystyrene-co-acrylic acid microspheres through a two-step process to form an amide bond. Carboxylic acid groups on the surface of the microspheres were, initially, modified by a 10 mole excess (in relation to the number of total carboxylic acid groups) of 1-ethyl-3-(3-dimehtylaminopropyl) carbodiimide (EDC) in the presence of 100 mole excess (in relation to the number of total carboxylic acid groups) of N-hydroxysuccimide (NHS). The reaction was allowed to proceed for one hour at 25°C at pH 5.5 in 1.5ml of a 0.1M MES buffer solution to yield an activated succinimdyl ester on the surface of the microspheres.

Excess reagent was removed with the supernatant following centrifugation at 10,000 x g. The wash step was repeated with 0.1M MES buffer pH 7.0. A 1.5ml increment of lactase (*A. oryzae*) in 0.1M MES buffer, pH 7.0 was added to the activated microspheres in a 5 mole exess (in relation to the theoretical value of the amount of enzyme that could be immobilized) and allowed to react for 2 hours at 2°C under constant shaking. Enzyme that had not been immobilized was separated by centrifugation as described, previously, for excess reagent until no further protein was detected in the supernatant as determined by addition of 0.5ml of the supernatant to 3ml of Bradford reagent and compared to the control containing no enzyme.

Protein Concentration

BCA Assay

The protein concentration of free and immobilized enzyme was determined using the BCA assay (Sapan, Lundblad et al. 1999), which relies on peptide bonds associated with a protein to reduce a solution of copper in biochomic acid. Free or immobilized (0.1ml) was added to 2ml of the BCA solution and the reaction took place at 60°C for 30 minutes. After completion of the reaction period, microspheres were removed from the solution by centrifugation at 10,000xg and passing the supernatant through a 0.22μm syringe filter. The absorbance of the microsphere-free solution was read at 562nm and compared to a standard curve of 0-20μg of bovine serum albumin (BSA).

Bradford Assay

Protein concentration of free lactase was, also, determined by the dye binding method described by Bradford when BCA-interfering buffers or reagents were present. Free lactase was diluted to an estimated 0.1-1.4mg/ml in 0.1M phosphate buffer (pH 6.7). Of the diluted solutions, 0.1ml was added to a glass tube and, subsequently, 3ml of Bradford Reagent (Sigma, B 6916) that had been brought to room temperature, was added bringing the total volume to 3.1ml. The solution was then capped, vortexed,

and incubated at ambient temperature for 30 minutes. The absorbance was read in a spectrophotometer at 595nm and compared to a standard curve using bovine serum albumin (BSA). Protein concentration was extrapolated from the regression equation derived from the standard curve and the result was multiplied by the dilution factor to obtain total concentration.

Extent of Covalent Modification

Covalent immobilization was distinguished from ionic adsorption through the use of an ionic detergent. Microspheres were diluted in 1ml of a 1% w/v sodium dodecyl sulfate (SDS) in deionized water solution and heated for 10 minutes at 70^0C. Protein concentration, using the BCA method, was determined and compared to a control sample prepared in the same manner without SDS.

Enzyme Modification

Carboxyl Group Blocking

Carboxylic acid groups of lactase were blocked by the formation of an amide bond between free carboxylic acids and glucosamine and was based on the method by (Baek and Vijayalakshmi 1997). Purified lactase was added to 1ml 0.1M MES buffer, pH 5.3 to give a protein concentration of 0.5mg/ml. Glucosamine dissolved in 0.1M MES buffer, pH 5.3 was added (0.25ml) in 100 mole excess relative to the total mole amount of carboxylic acid groups present. An additional 0.25ml containing EDC dissolved in 0.1M MES buffer, pH 5.3, was, subsequently, added to give a mole ratio of EDC to lactase carboxylic acid groups of 5:1.

The artificial glycosylation reaction proceeded for 2 hours at 25 °C under constant shaking followed by centrifugal filtration at 5000xg through a 50K MWCO membrane to remove unreacted reagent and byproducts. The filtered enzyme was reconstituted with 1ml of 0.1M MES buffer, pH 5.5.

Non-denaturing isoelectric focusing was used to evaluate changes in the isoelectric point of lactase that occurred from the modification of ionically charged amino acid (ie COO^- or NH_3^+). Lactase conjugates (7μg) were added to sample buffer and loaded on pre-cast vertical isoelectric focusing gels composed of 5% polyacrylamide and 2% ampholytes with a pH gradient of 3-10. The upper chamber was filled cathode buffer (Novex) and the lower chamber filled with anode buffer (Novex) with the gel running from cathode (-) to anode (+) (ie pH 10 at the top of the gel and pH 3 at the bottom of the gel). Gels were run for 2.5 hours with the voltage step-up of 100V for 1 hour, 200V for 1 hour, and 500V for 0.5 hours. The gels were stained using a Coomassie Blue solution (45% v/v water, 45% v/v methanol, 10% v/v acetic acid, and 0.25% w/v Coomassie brilliant blue R250) for 4 hours, and destained using using a solution of 50% v/v water, 40% v/v methanol, and 10% v/v acetic acid for intervals of 1 hour, then 12 hours, then 1 hour.

Immobilization

Immobilization of lactases on chitosan-tethered and high and low carboxylic acid polystyrene-co-acrylic acid microspheres was performed as described, previously.

Stability of Immobilized Lactase (from *A. oryzae)*

The stability of immobilized lactase preparations (*A. oryzae*) were evaluated by storing the samples in 0.1M MES buffer at 4°C, pH 5.5; 4°C, pH 6.8; 25°C, pH 5.5; and 25°C, pH 6.8. Specific activity was accessed every two week over an 8 week period.

Carbodiimide Modification

The effect of carbodiimide on the free and immobilize stability of lactase was evaluated using 1-ethyl-3-(3-dimehtylaminopropyl) carbodiimide (EDC). Purified lactase was added to 1ml 0.1M MES buffer, pH 5.3 to give a protein concentration of 0.5mg/ml. An additional 0.5ml containing carbodiimide dissolved in 0.1M MES

buffer, pH 5.3, was, subsequently, added to give the following mole ratio of EDC to lactase carboxylic acid groups: 0:1, 0.05:1, 0.5:1, 5:1, and 50:1.

The reaction proceeded for 2 hours at 25°C under constant shaking followed by centrifugal filtration at 5000xg through a 50K MWCO membrane to remove unreacted reagent and byproducts. The filtered enzyme was reconstituted with 1ml of 0.1M MES buffer, pH 5.5.

Molecular Crosslinking

SDS PAGE, as described previously, was used to determine the extent of intermolecular crosslinking associated with carbodiimide modification.

The number of primary amino groups (ie lysine and N-terminus) on lactase modified by modification was determined based on methods described by Habeeb and Mokrasch (Habeeb 1966; Mokrasch 1967). Lactase conjugates were diluted in distilled water to a protein concentration of 0.2-0.6mg/ml as determined by the previously reported Bradford method. To separate tubes containing 1.95ml of 0.1M borate buffer (pH 9.6) was added 50µl of the respective diluted enzyme solutions. Picrylsufonic acid (2,4,6 trinitrobenzenesulfonic acid (TNBS)). A 5%w/v TNBS solution was made in deionized water and diluted to a 0.03M concentration by the addition of 0.3ml of the TNBS solution to 1.4 ml of distilled water to bring the total volume to 1.7ml. The 0.03M TNBS was made daily when determining the degree of modification. To the tubes containing 50µl of diluted enzymes in 0.1M borate buffer was added 20µl of 0.03M TNBS. The tubes were vortexed and incubated in the dark at 24 °C for two hours. Upon completion of the incubation period, the solutions were placed in cuvettes and the absorbance read in a spectrophotometer at 420nm and compared to a standard curve. The standard curve was created using glycine which was diluted in distilled water to a concentration of 0.266mM. The diluted glycine was added to individual glass tubes in amounts of 0, 25µl, 75µl, 125µl, 175µl, 225µl, and

275µl, and enough 0.1M borate buffer (pH 9.6) was added to bring the total volume to 2ml. The standards were treated with TNBS as described for the enzyme samples. A regression equation was derived from the standard curve of absorbance versus moles of free amino assuming a 1:1 relationship of moles of free amino group to mole of glycine, as well as a 1:1 relationship of TNBS to mole of glycine. The moles of free amino groups of lactase conjugates were extrapolated from the regression equation and expressed as a function of the quantity of protein. The degree of modification of lactase was determined by dividing the moles of free amino groups per milligram of modified-lactase by the moles of free amino groups per milligram lactase, subtracting the product from one, and multiplying the solution by 100 to obtain a percent of amino group modification. Samples were done in triplicate. Thiol concentration was determined using Ellman's reagent. A standard curve was prepared using cysteine. 250µl of a 0-1.5mM solution of cysteine in 0.1M phosphate buffer, pH 8.0 was added to glass tubes containing 50µl of Ellman's reagent and 2.5ml of 0.1M phosphate buffer, pH 8.0 giving solutions containing 0 to 3.75×10^{-7} moles of free thiol groups. The unmodified and modified lactase solutions were added to glass tubes containing 50µl of Ellman's reagent and 2.5ml of 0.1M phosphate buffer, pH 8.0 giving solutions containing 3mg of enzyme. Samples were incubated at room temperature for 15 minutes and the resulting absorbance read at 412nm. Free thiol groups were calculated using the standard curve and dilution procedure.

Carboxyl Group Modification

Isoelectric focusing, as described previously, was employed to detect modification of the enzyme.

Specific Activity of Modified-Lactase

Modified-lactase activity for the free enzyme was determined as described previously with protein concentration being determined by the Bradford method.

Immobilization

Immobilization of modified-lactases and high and low carboxylic acid polystyrene-co-acrylic acid microspheres was performed as described, previously.

Chitosan-Tethered Polystyrene-co-Acrylic Acid Microspheres

Chitosan was tethered to polystyrene-co-acrylic acid microspheres by carbodiimide coupling. The chitosan solution was obtained by the dissolution of 50mg of low molecular weight chitosan (Sigma) in 10ml of a 0.03M solution of HCl followed by stirring for 15 minutes. When the chitosan had dissolved completely, 10ml of 0.1M MES buffer, pH 5.8, was added, adjusting the final pH to 5.3 and giving a chitosan solution of 2.5mg/ml. The chitosan solution (1ml) was added to 0.5ml of 0.1M MES buffer pH 5.3 containing 0.006g of clean PS-AA, high carboxyl, microspheres and mixed. Carbodiimide (EDC) was introduced at a 10 mole excess (0.0016g) relative to the mole amount of carboxyl groups present on the microspheres. The reaction was held at 25°C for 2 hours. Free chitosan and residual EDC was removed by centrifugation using a 5x rinse of 0.1M acetic acid buffer, pH 4.0 (Lei, Bi et al. 2007)

Extent of Tethering

The extent of chitosan tethering was determined using the Toluidene Blue assay and the Acid Orange 7 assay. The Toluidiene Blue assay was used to follow the loss of carboxylic acid groups on the surface and was performed as described previously. The Acid Orange 7 assay was used to characterize the appearance of amine groups associated with chitosan (Uchida *et al*, 1993). Microspheres were immersed in 1ml of a $1x10^{-3}$M Acid Orange 7 solution in distilled water that had been adjusted to pH 3 by HCl. After 3 hours of shaking at room temperature, the microspheres were rinsed with distilled water at pH 3 to remove non-complexed dye and collected by centrifugation. Complexed dye was, then, desorbed by shaking microspheres in water, adjusted to pH 12 by NaOH, for 15 minutes at room temperature, followed by centrifugation and

removal of the desorbed dye into a cuvette. Absorbance of the desorbed dye was read at 460 nm and compared to a standard curve of dye with the assumption that one mole of dye complexes with one mole of available amine groups and knowing the volume of the desorbing solution (pH 12 NaOH).

Zeta Potential Measurements

Microspheres in 0.1M acetate buffer (pH 5.3) were diluted to low concentrations (~0.0033 μg/ml) in ultra pure water with a final pH of 5.3. A Brookhaven 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY) was fitted with the ZetaPlus option, and measurements were performed in the High Precision mode at 20°C and setting "water" as solvent. The measurement consisted of 30cycles/run, with an intercycle delay of 5seconds. Protein dilutions were adjusted in order to achieve the recommended instrument count rate of 300-350 kcps.

Scanning Electron Microscopy

Microspheres were dried on a glass microscope slide. Analysis was performed by Dr. Julie Goddard at the Cornell Nanofabrication Facility. The particles were sputter coated with Au/Pd in a Hummer V(Technics) sputtercoater. Images were produced using a scanning electron microscope (Zeiss Ultra) with SE1/InLensdetector.

Immobilization of Lactase onto Chitosan-Tethered Polystyrene Microspheres

Lactase was covalently immobilized onto chitosan-tethered polystyrene-co-acrylic acid microspheres through formation of a peptide bond. A 1.5ml increment of lactase (*A. oryzae*) in 0.1M MES buffer, pH 7.0 or 5.3 was added to chitosan-tethered microspheres in the presence of a EDC at a 0.9:1 mole ratio of EDC to enzyme carboxylic acid groups (total glutamic and aspartic acid). The reaction proceeded for 1 hour at 25°C under constant horizontal shaking. Enzyme that had not been immobilized was separated by centrifugation as described, previously, for excess reagent until no further protein was detected in the supernatant as determined by

addition of 0.5ml of the supernatant to 3ml of Bradford reagent and compared to the control containing no enzyme.

Immobilization of Lactase on Polyethylene Films

Cleaning Films

Additive-Free Low Density Polyethylene (LDPE) films (640I, Dow Chemical Company, 100um) were cut in $2x2cm^2$ pieces and cleaned by the 3-step process described by Goddard. Films were sonicated 3 times in dichloromethane for 10 minute intervals, followed by identical treatments in acetone and, then, deionized water.

Oxidation of Polyethylene

Polyethylene films were functionalized by chemical oxidation using a chromic acid solution. A weight percent ratio solution of 29:42:29 CrO_3:H_2O:H_2SO_4 was prepared and distributed to glass vials held in a heating block at 70°C. Cleaned PE films were individually submerged in 15ml of the chromic acid solution and held at 70°C for two minutes then rinsed 3 times in deionized water. The rinsed, oxidized films were submerged in 70% nitric oxide for 15 minutes at 50°C to dissolve any chromium salts present on the film, and subjected to triplicate rinse in deionized water prior to storage in deionized water until further use.

XPS Spectra

XPS (X-ray photoelectron spectroscopy) analysis was conducted at the Penn State Materials Characterization Laboratory (State College, Penn., U.S.A.) on a Kratos Analytical Axis Ultra (Kratos Anaytical, Inc, Chestnut Ridge, Ny, U.S.A.) with a monochromatic Al ka X-ray source at an X-ray power of 280 W, a 90° takeoff angle, and a spot size of 700 x 300 μm.

Contact Angle

Contact angle measurements were used to provide evidence of film modification. Films were mounted on glass microscope slides using double-sided tape for use with a Tantec contact angle meter. A single drop of reagent grade deionized water (pH 7.0) was syringe fed until it was in contact with the film. The resulting angle taken from the origin through the height of the droplet and its half-width was determined in triplicate and labeled as the contact angle of the film.

Extent of Carboxylation

Available carboxylic groups on the surface of the microspheres were quantified using the Toluidine Blue assay (Kang et al, 1996). Films ($2x2cm^2$) were immersed in 10ml of 5×10^{-4}M Toluidine Blue O in distilled water that had been adjusted to pH 10 by NaOH. After three hours of shaking at room temperature, the films were rinsed three times with NaOH solution at pH 10 to remove non-complexed dye. Complexed dye was then desorbed by shaking films three times in 5ml, 50% v/v acetic acid for 15 minutes at room temperature, and the absorbance of the desorbed dye was read at 633 nm, and surface carboxyl groups were determined by comparison with a standard curve of dye, knowing the quantity of microspheres and the volume of the desorbing solution under the assumption of one mole of dye complexing with one mole of available carboxyl groups (Uchida et al, 1993).

Immobilzation of Lactase

Unmodified and glucosamine-modified lactase (*A oryzae)* were immobilized to oxidized films by a two-step process. Oxidized PE films were added to 100ml of 0.1M MES buffer, pH 5.3 under continuous stirring to keep the films submerged. 1-ethyl-3-(3-dimehtylaminopropyl) carbodiimide (EDC) was added in a 10 mole excess and N-hydroxysuccinimide (NHS) was added in a 100 mole excess relative to number of carboxyl group present on the film. The reaction was allowed to proceed for 1 hour at

room temperature. The resulting films, with an activated NHS-ester group, were rinsed in deionized water and placed in a 100ml of a 0.1M MES buffer (pH 7.0) containing 5mg of either the modified or unmodified lactase. The reaction occurred for two hours at room temperature. Films were, subsequently, rinsed three times in 0.1M MES buffer (pH 5.5) and stored in the same buffer at 4 °C until further use.

Protein Concentration of Lactase-Immobilized PE films

Protein concentration was determined using the BCA films described previously. One $2x2cm^2$ film was added to 2ml of the BCA solution and kept at 60°C for 30 minutes. The film was removed and the absorbance at 562nm was read and compared to a control film with no immobilized lactase. Protein concentration per cm^2 of film was determined by dividing the protein concentration on the $2x2cm^2$ film by eight since lactase was immobilized on both sides of the film. Covalent immobilization was distinguished from ionic adsorption through the use of SDS as described, previously.

Activity of Lactase-Immobilized PE films

Specific activity of unmodified or glucosamine-modified lactase, immobilized on oxidized films, was determined using a modification of the FCC method for free enzyme activity. One film was added to 10ml of a 0.012mM solution of the synthetic lactose substrate, o-nitrophenyl-β-galactopyranoside, and allowed to react under shaking for 15 minutes. At the completion of the time period, 2ml of the solution was pipetted into 2.5ml of 10% sodium carbonate to stop the reaction. The solution was diluted to 25ml with deionized water and the absorbance of 2ml of the diluted sample was read at 420nm (Jenway 6300 spectrophotometer). Specific activity was determined using equation 4. Temperature and pH effects were evaluated in the range described for the free enzyme. The Michaelis constant was determined by transferring a single $2x2cm^2$ film to preheated tubes (under shaking) at each time point (30second intervals) in a given substrate concentration (0-8mM). Upon completion of each time

increment, the film was transferred to the tube representing the proceeding time increment, while the preceding was, simultaneously, stopped by the addition to 10% sodium carbonate. Separate films were used for each substrate concentration and K_m was determined from Equation 5.

CHAPTER 4

RESULTS AND DISCUSSION

Characterization of Lactases from Different Sources

Lactase is derived from a number of species, though commercial applicability is limited to enzyme from fungal, yeast, and bacterial species. In the food industry, the principal lactase sources are those of *Aspergillus* (sp. *oryzae* and *niger)* and *Kluyveromyces* (sp. *lactis* and *fragilis*). Though both serve to catalyze lactose into glucose and galactose, the optimum catalytic conditions as well as stability under conjugation and storage conditions differ. To determine the suitability of the preparations for immobilization, species from the two organisms, specifically *Aspergillus oryzae* and *Kluyveromyces lactis*, were evaluated for their respective activities and stabilities across multiple conditions in free enzyme form.

Lactase was purified from commercial preparations to remove stabilizers, extraneous proteins, and cell debris. Syringe filtration (0.22um) was applied to remove yeast cells (*K. lactis*) or fungal cells (*A. oryzae*). Stabilizers (glycerol, dextrose, salts) and small non-lactase proteins (ie proteases, lipases, etc.) were, further, removed using centrifugal filtration through 50K MWCO membranes leaving the respective enzymes. The isolated protein was subjected to SDS PAGE with bands seen for *A. oryzae* (105K Da) (Figure 1) and *K. lactis* (MW 117K Da) (Figure 2). These single bands, corresponding to the molecular weight on the enzyme, indicate that the enzymes were appropriately purified, by the described methods, from the commercial preparation and constitute the total protein of the system.

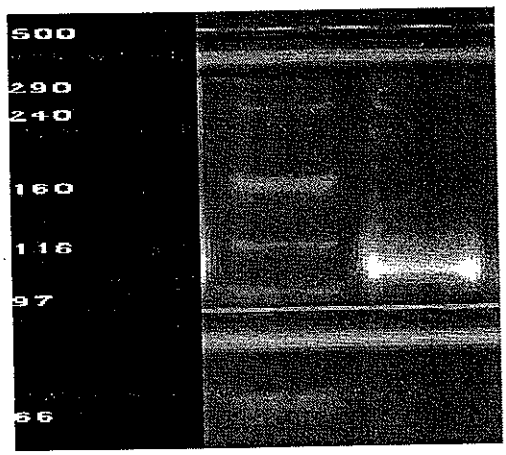

Figure 1. ***A. oryzae*** **lactase (SDS-PAGE gel)**

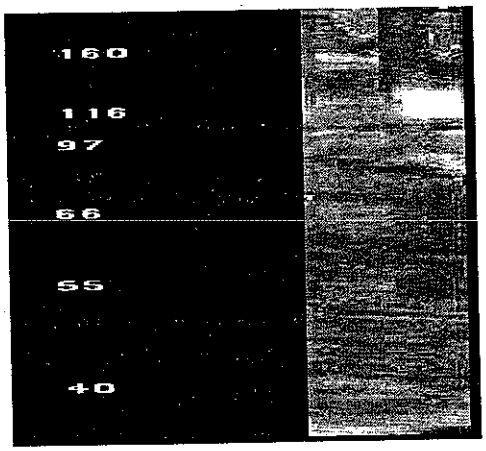

Figure 2. ***K. lactis*** **lactase (SDS-PAGE gel)**

Lactases from *K. lactis* and *A. oryzae* were characterized with respect to pH and temperature. As expected, the free *K. lactis* lactase shows an optimum activity at neutral pH (7.0) (Figure 3) and 35^{0}C (Figure 4), with a maximum activity approaching 98Kumol/min/g of lactase. Though optimum activity of *K. lactis* lactase is 35^{0}C in its native form, it has been noted that the stability of the enzyme, without stabilizers, can be effected by pH at that temperature (Cavaille and Combes 1995). Beyond 55^{0}C, the enzyme wis rapidly inactivated. *A. oryzae* lactase held optimum activity at a more acidic pH (4.0-5.0) (Figure 3) and a higher temperature (50^{0}C) (Figure 4), with

maximum activity of 133Kumol/min/g of lactase. Conditions above 50^{0}C would be beneficial for immobilized lactase preparations in deterring microbial growth rate of mesophilic organisms--favoring the aspergillus species (Madigan 2000). For lactose reduction of dairy products, lactase from *K. lactis* is favored for fluid milk processing because of the optimum activity near that of fluid milk (pH 6.8). *A. oryzae* lactase is more often used for whey processing, which takes places at acidic pH (<6.0). Though lactase from *K. lactis* would not be able to be used for whey processing because of the inactivity at the necessary acidic pH, *A. oryzae* lactase may be used for fluid milk processing because it retains activity at pH 7.0 and 50^{0}C (58Kumol/min/g of lactase), provided the enzyme is stable at that pH.

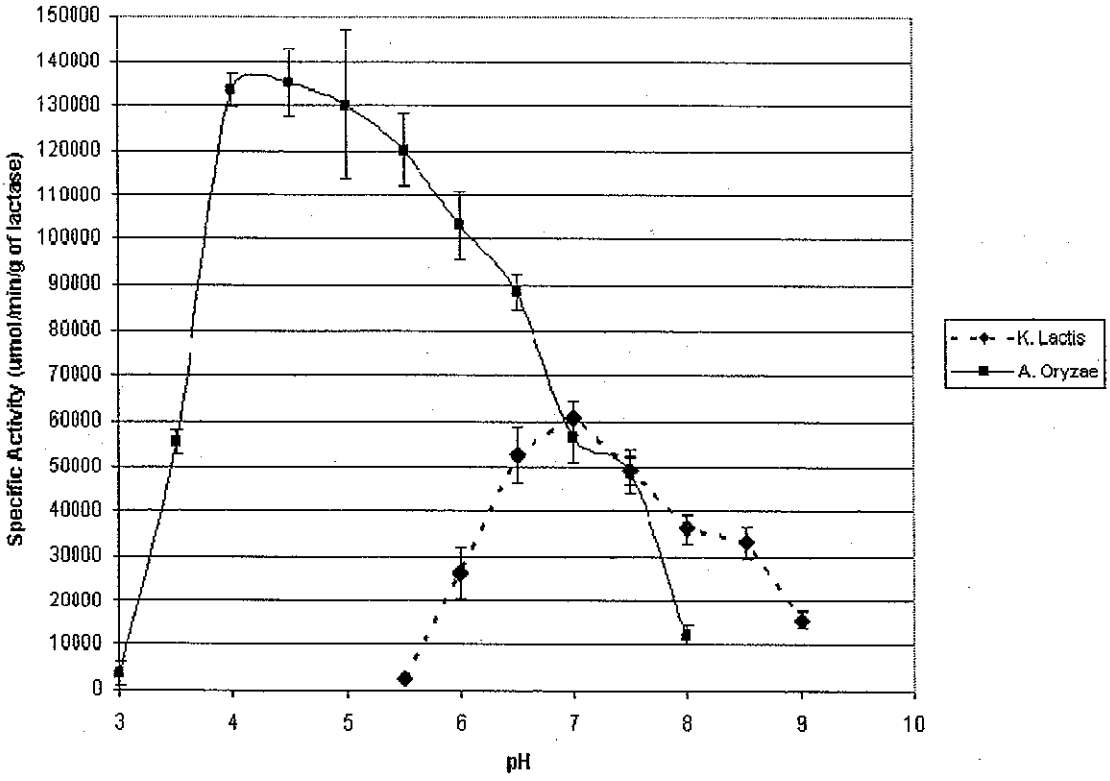

Figure 3. Effect of pH on free lactase (*A. oryzae and K. lactis*) activity. Activity at 50^{0}C for *A. oryzae* and 30^{0}C for *K. lactis* using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

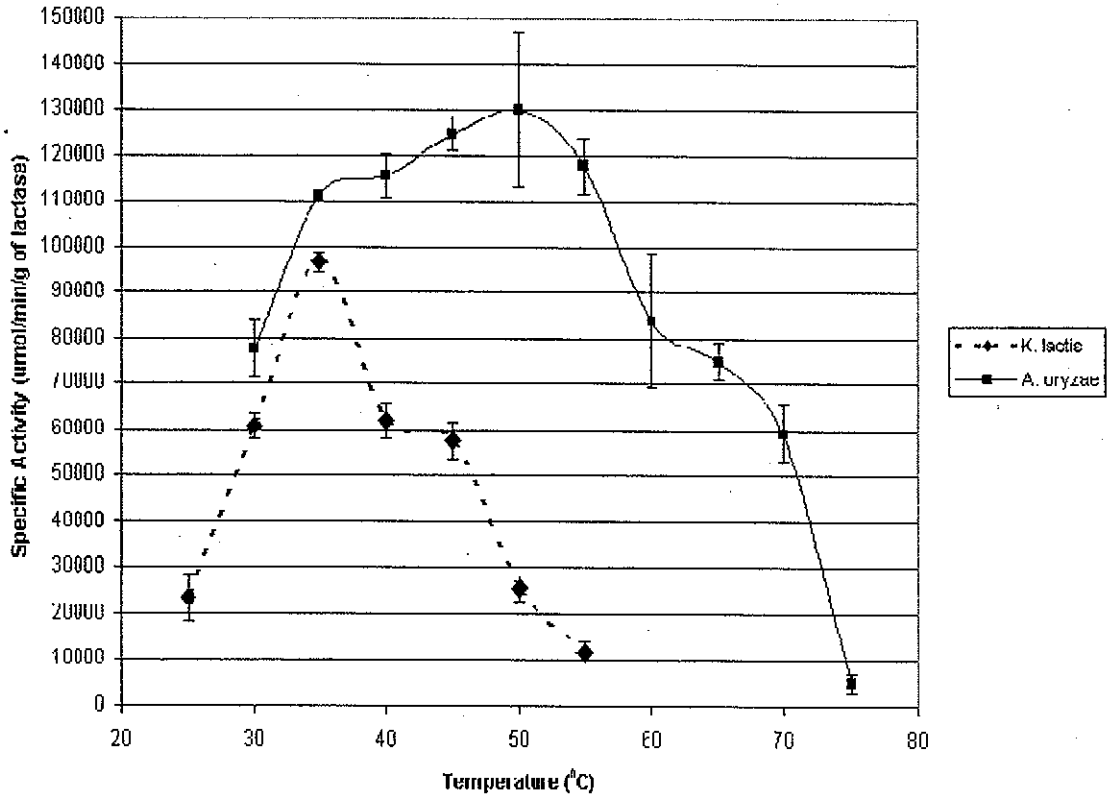

Figure 4. Effect of temperature on free lactase (*A. oryzae and K. lactis*) activity. Activity at pH 5.0 for *A. oryzae* and pH 7.0 for *K. lactis* using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

The stabilities of the two enzymes were evaluated to investigate potential applicability to lactose reduction in whey and fluid milk processing. *K. lactis* lactase stability has been shown to be influenced by magnesium and the presence of glycerol at temperatures above the optimum for catalytic activity (35^{0}C) (Cavaille and Combes 1995). Investigation was preformed into the stability of the enzyme with and without magnesium and glycerol at controlled room temperature (25^{0}C; pH 6.8) and refrigerated temperature (4^{0}C; pH 6.8). The results showed that at higher temperature (25^{0}C), the enzyme was less stable across the three week period with or without magnesium than at (4^{0}C) (Figure 5). At 4^{0}C, the control and "magnesium only"

samples showed a significant reduction in activity at the end of the three week period. Glycerol (50%), had a significant ($p<0.05$) stabilizing effect across the three week period on *K. lactis* lactase at both 4^0C and 25^0C, compared to preparation without glycerol--independent of magnesium (Figure 5). As can be seen from Figure 5, no significant loss of activity was observed across the three week period when the enzyme was in the presence of glycerol. These results indicate that glycerol, in which the commercial lactase of *K. lactis* is packaged, is used to retain the stability of the free enzyme. Glycerol has demonstrated an ability to promote the stability of a proteins, the exact mechanism for this stabilization; however, is still being investigated (Davis-Searles, Saunders et al. 2001). Glycerol can mimic the cellular environment--promoting preferential hydration/solvation of the enzyme surface, and protein association (Kim and Lee 1993). The explanation agrees with the nature of *K. lactis* lactase, which is an intracellular enzyme that is active in dimer and tetramer forms through association of identical subunits, but not as a monomer (Cavaille and Combes 1995; Becerra, Cerdan et al. 1998). Removing the enzyme from the native environment into a dilute aqueous condition will alter the quaternary and tertiary dynamics, shifting the equilibrium of the native and unfolded state. The necessity of dimer association for catalytic activity suggests that the active site of the enzyme is shared between subunits or interaction promotes a shift in catalytic cleft. In solution, the association-dissociation of subunits making up the quaternary structure is dynamic (Poltorak, Atyaksheva et al. 2004). During immobilization, the quaternary structure of the lactase, consequently, needs to be retained in the dimer position. Glycerol can, also, effect the hydrogen bonding structure of water to proteins (O'Fagain 2003). Because of the necessity of the bound water layer in supporting tertiary structure stabilization, the presence of glycerol may aid in the promotion of these bonds.

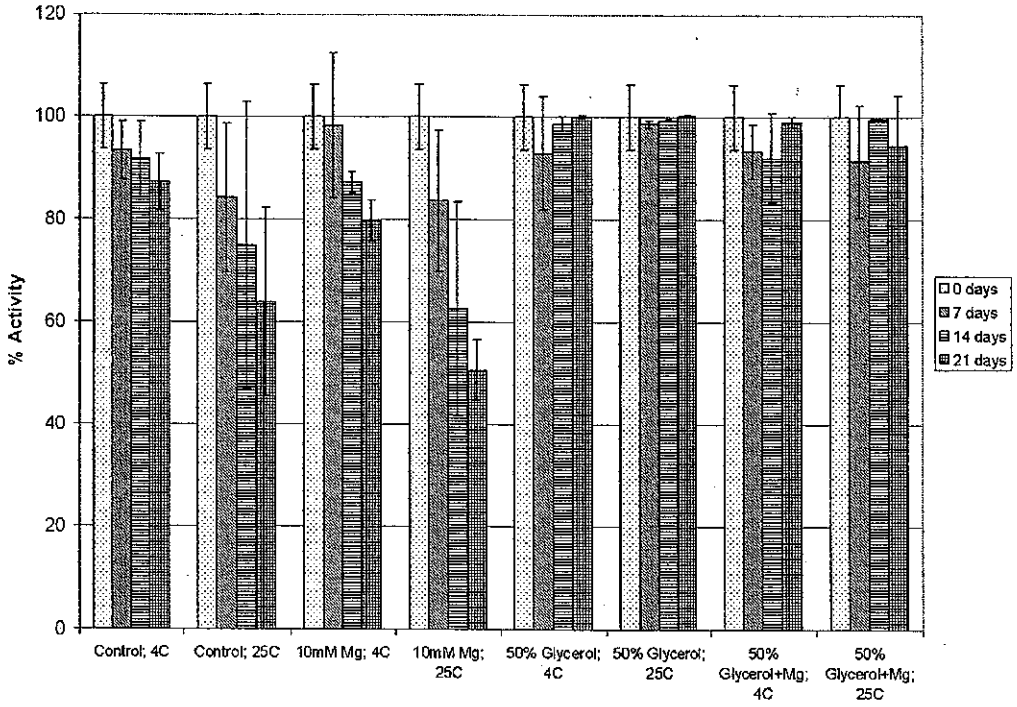

Figure 5. Stability of free lactase (*K. lactis*). Activity at 25^{0}C, pH 7.0 for *K. lactis* using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

The storage stability of *A. oryzae* lactase was evaluated under whey and fluid milk pH processing conditions (pH 5.5 and 6.8, respectively) at controlled room temperature (25^{0}C) and refrigeration conditions (4^{0}C). Temperature and pH had no significant effect ($p<0.05$) on the retained activity of the enzyme across a three week evaluation period (Figure 6). Stability results indicate that *A. oryzae* lactase can, with respect to storage pH, be used for both whey and fluid milk processing. Unlike, *K. lactis* lactase, *A. oryzae* lactase is an extracellular enzyme that is active as a monomer (ie no quaternary structure), and commercially available in a lyophilized powder form. Extracellular enzymes are typically more stable than intracellular enzymes because of localized environmental changes during intracellular folding, expression, and extracellular activity, which warrants robustness (Rodriguez, Leiro et al. 2006).

Because *A. oryzae* lactase has catalytic function as a monomer and no requirements for quaternary structure, concerns of multiple subunit stabilization during immobilization are alleviated. Lactase from *A. oryzae* was determined to be a more suitable candidate than *K. lactis* lactase for immobilization because of the stability and activity across pH conditions useful for processing and covalent conjugation, long-term stability in aqueous conditions without the need for stabilizers, high catalytic activity at temperatures above the growth optimum for mesophilic organisms, ability to retain activity after sanitation procedures (Baret 1987), and lack of quaternary structure requirements.

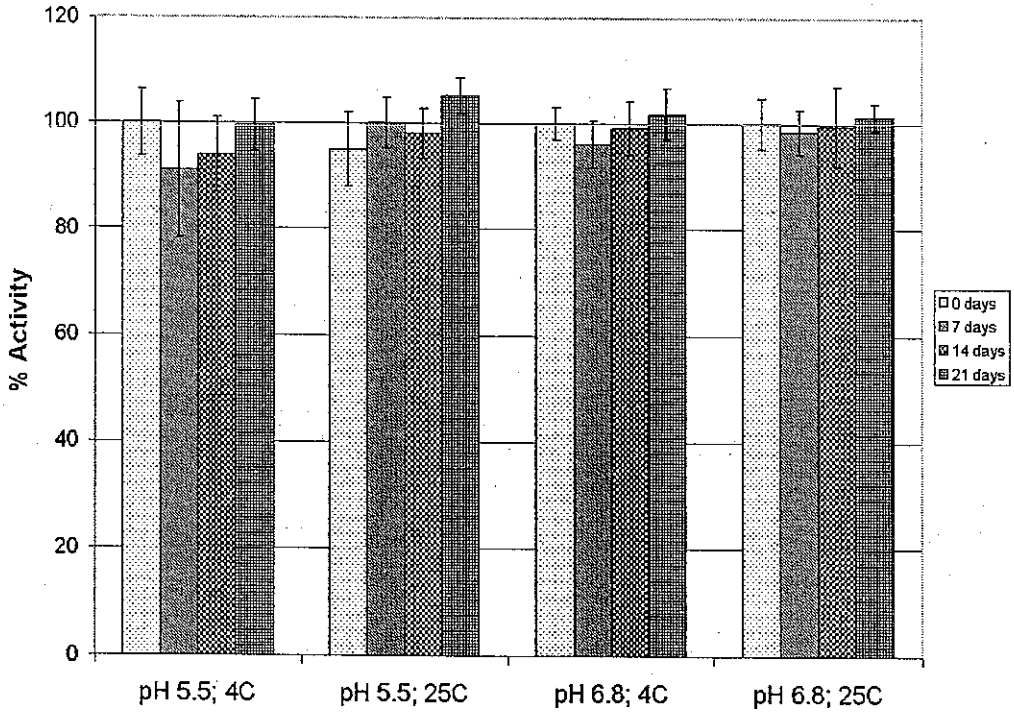

Figure 6. Stability of free lactase (*A. oryzae*). Activity at 50^{0}C, pH 5.0 for *A. oryzae* using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

Covalent Immobilization of Lactase on Polystyrene-co-Acrylic acid Microspheres

Effect of carboxylic group concentration

Previous attempts to covalently immobilize lactase to oxidized packaging films produced no measurable lactase activity (Steven, 2004). Since this loss of activity could not be explained, a model system for conjugation of the enzyme was developed to lay a foundation to eliminate and introduce variables for study. Investigations on the covalent immobilization of lactase to hydrophobic supports focused on the attachment of *A. oryzae* lactase to polystyrene-co-acrylic acid microspheres (~1μm).

For covalent attachment, both the carrier and the enzyme need to have functional groups capable of reacting in a covalent fashion. Though enzymes have native functionality (carboxylic acids, amine, thiols, etc.), hydrophobic carriers are typically inert and need to be made reactive. Introduction of carboxylic groups to hydrophobic polymers is a simple means of creating surface functionality, and can be achieved using diverse methods. Methods for generating carboxylic group functionality include wet chemical treatments, plasma oxidation, corona discharge, flame ionization, and copolymerization with a carboxylic containing monomer (ie acrylic or methacrylic acid). Because of the wide potential applicability and ease of introduction of carboxylic acid functionality to other polymer systems such as food packaging films, a carboxylic acid-containing hydrophobic polymer system was deemed a desirable platform for lactase immobilization. Unlike surface oxidation, introduction of acrylic acids monomers allows the number of carboxylic groups to be tailored in a control fashion without introduction of oxidation byproducts such as hydroxyl or aldhehyde groups that may form using alternative carboxylic group generating methods.

Though particles greater than 100μm (100μm -2mm) are used in immobilized enzyme applications because of pressure drops associated with smaller particles, 1μm

microspheres were chosen because they provide high surface area for two dimensional surface, promote increased Brownian motion that enhances substrate/surface interaction, and, unlike nanomaterials, can be readily separated by centrifugation (Jia, Zhu et al. 2003). Two dimensional supports were employed to reduce variability of protein and substrate diffusion associated with porous carriers, limit swelling, and develop model applicability of two-dimensional systems to alternative polymer-enzyme systems such as packaging films (Wu, Lee et al. 1998).

Lactase was conjugated to the microspheres by a two-step carbodiimide reaction (Figure 7). The carbodiimide (EDC) reacts with the surface carboxylic groups of the microspheres, which in the presence of NHS forms an activated NHS-ester intermediate. In the second step, NHS-ester activated microspheres react with primary amine groups (lysine) on the enzyme surface, forming a "zero length" amide bond after loss of the leaving group from the nucleophilic substitution reaction. The carbodiimide can react with the enzyme and microspheres in a one-step reaction by reaction of amine groups on lactase and carboxylic acid groups on the carrier, but this method may produce intra or intermolecular modification with enzyme carboxylic groups (Yamada, Kuroki et al. 1983). An advantage of EDC/NHS chemistry is that the reagents are water soluble, which, unlike organic solvent reactions (ie carbonyldiimidazole), is beneficial to enzyme stability and prevents swelling of the synthetic polymer microspheres. For food immobilized enzyme applications, a concern is the use of bioconjugate reagents that may be detrimental to human health if ingested or in contact (Friend and Shahani 1982). By employing carbodiimide chemistry, unreacted activated groups are hydrolyzed back to the corresponding carboxylic acid. Carbodiimide hydrolyzes in water and has a half life of 2-3 seconds when forming the o-acylurea product with carboxylic groups (Hoare and Koshland 1967). In the presence of NHS; however, the activation half-life is extended to

minutes or hours (Besselink, Beugeling et al. 1993; Hermanson 1996) . The functionality of the NHS-ester, also hydrolyzes back to carboxylic acid groups, leaving no activated functional groups after immobilization that could react with components of a food system (Besselink, Beugeling et al. 1993). The EDC/NHS reaction can facilitate covalent attachment of biological free amines (ie lysine) to carboxylic acid-functionalized supports at pH 7.0—allowing for conjugation of the enzyme to the support at conditions that promote enzyme stability (Besselink, Beugeling et al. 1993; Hermanson 1996). Also, the "zero length" between the support and the enzyme allows for covalent surface-surface studies between the protein without the introduction of spacer length variability.

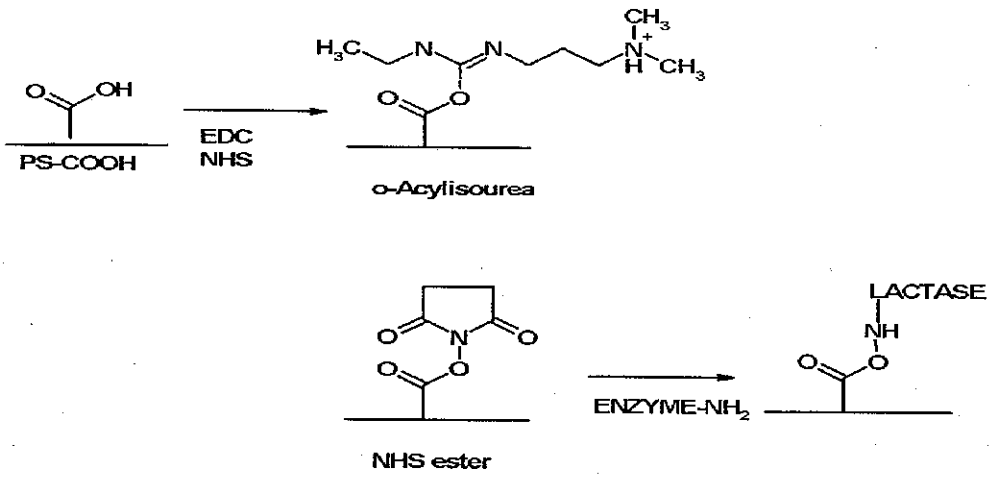

Figure 7. EDC/NHS activation of carboxylic groups followed by enzyme conjugation. R-NH_2 is indicative of available lysine residues of lactase.

Immobilization of lactase on polystyrene-co-acrylic acid microspheres (87 $Å^2$ between surface groups) yielded a protein loading on the carrier of 6.9 (±1.9)mg of lactase/g of microspheres. There was no significant change ($p<0.05$) in protein loading after subjecting the immobilized enzyme to denaturing conditions in the presence of SDS--confirming the covalent nature of the attachment. The isoelectric

point of lactase (4.6) gives the enzyme a negative net charge under conjugation conditions (pH 7.0), which favors preferential association of positively charged amino acids (Hermanson 1996). The theoretical size of a protein having the molecular weight of lactase (MW 105KDa) would be 7nm in diameter (Yang, Marchio et al. 1994). Experimental observation using dynamic light scattering indicate that *A. oryzae* lactase may be up to 12nm in diameter, but concede that observations may be skewed by enzyme purity, aggregation, and equipment limitations (Yang, Marchio et al. 1994). Based on the available surface area per gram of the microspheres and assuming lactase to be in a sphere with a diameter of 7nm with half of the enzyme surface area contributing to protein loading, a theoretical value of 10.7mg of lactase/g of microspheres can be derived for monolayer coverage (Equation 6). This result suggests that experimental surface coverage is approaching that of a monolayer.

Equation 6:

Area of a sphere = $4\pi r^2$
Enzyme diameter = 7nm
Available surface area of microspheres = $4.72 \times 10^{12}\ \mu m^2/g$
$4.72 \times 10^{12}\ \mu m^2/g\ /\ (4\pi(0.0035\mu m)^2) = 3.07 \times 10^{16}$ enzyme molecules per g of microspheres
Since the spherical enzymes are in contact with a flat surface, only the enzyme to half radius would contribute to protein loading giving
$(3.07 \times 10^{16}$ enzymes/g of MS) x (2) = 6.14×10^{16} enzymes/g of MS
Using Avargado's number and the molecular weight of lactase gives:
$(6.14 \times 10^{16}$ enzymes/g of MS) / $(6.02 \times 10^{23}$ enzyme molecules/mol)
x (105,000g of lactase/mol) = 10.7mg of lactase/g of MS

Immobilization of the enzyme resulted in a 67% mean decrease in specific activity compared to the free enzyme under optimum conditions as determined by the conversion of ONPG to ONP (Figure 8 and Figure 9). There was no change in the optimum catalytic temperature (50^0C), but the apparent optimum pH shifted from 4.5 for the free enzyme to 5.0 for the immobilized enzyme (Figure 8). The shift in pH optimum can be attributed to localized pH at the surface due to the presence of carboxylic groups. The bulk pH may, for example, be 5.0, but the hydrogen ion

concentration is greater near the negatively charged surface, yielding a more acidic pH at the surface interface (Goldstei.L, Levin et al. 1964). Factors affecting the loss of activity after immobilization on two dimensional supports can be attributed to hydrophobic interactions between the protein and the surface, loss of mobility, microenvironmental pH shifts, mass transfer limitations/substrate accessibility, surface regularity, protein-protein interactions/crowding, van der Waal forces, orientation, cofactor disassociation, bioconjugation chemistry, active site/necessary amino acid modification, Brownian motion, dehydration of the enzyme-bound water layer, distance from the surface, subunit disassociation (loss of quaternary structure, electrostatic interaction/repulsion between the enzyme and carrier surfaces, curvature of the carrier, competitive hydrogen bonding (Chauhan, Nichkawade et al. 1998; Lamb and Stuckey 2000; Gonzalez-Saiz and Pizarro 2001; Wentworth, Skonberg et al. 2004). These factors may occur alone or in combination to reduce activity of the conjugated enzyme.

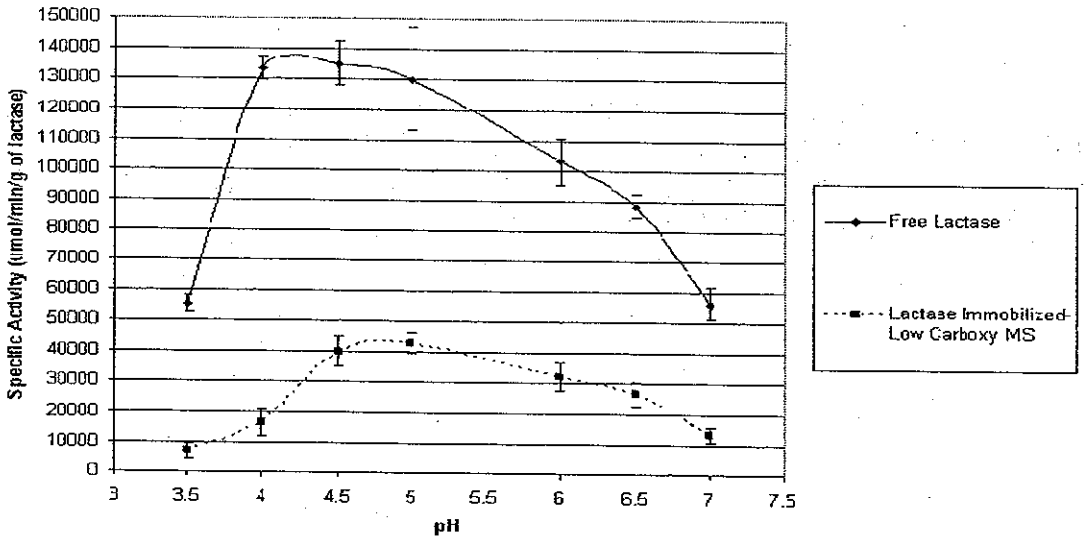

Figure 8. Effect of pH on free lactase (*A. oryzae*) and covalently immobilized lactase on polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups). Activity at 50^0C using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

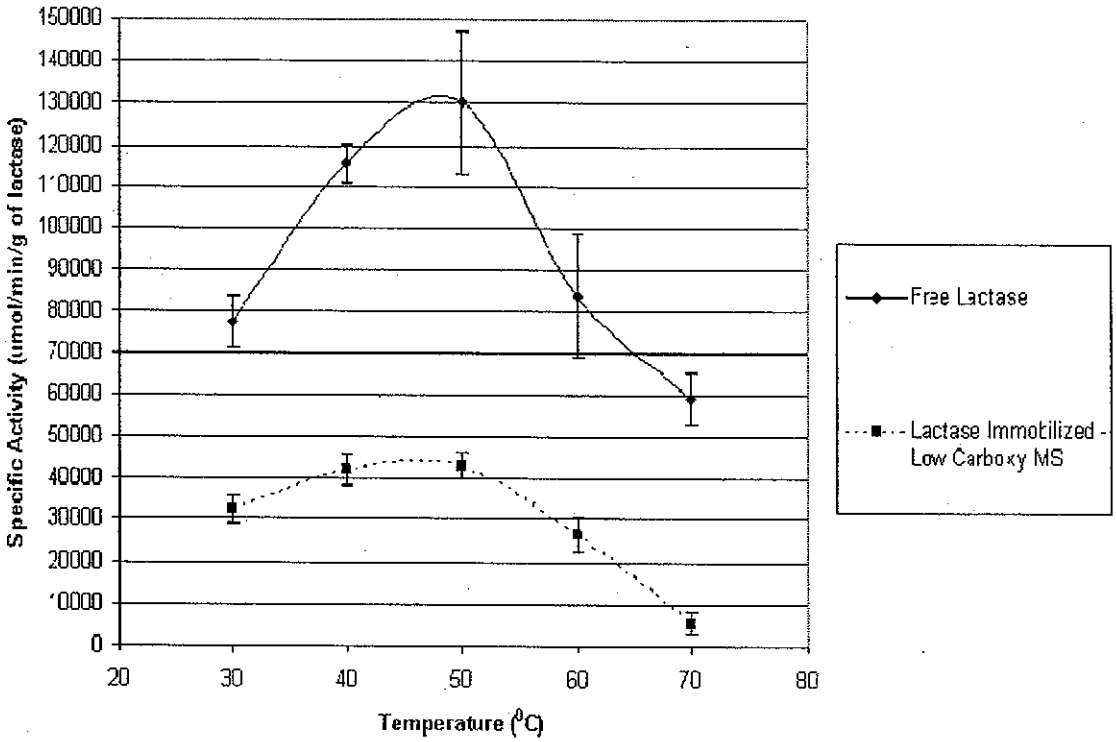

Figure 9. Effect of temperature on free lactase (*A. oryzae*) and covalently immobilized lactase on polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups). Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

During explorations to elucidate the reason(s) for loss of enzyme activity, lactase was immobilized to polystyrene-co-acrylic acid microspheres (1.2μm) having size and surface characteristics as previously described, but with a greater density of available carboxylic acid groups (6.2$Å^2$ between surface carboxylic acid groups compared to 87$Å^2$ between surface groups) (Table 3). The "high carboxylic acid" carrier increases the surface charge, hydrophilicity, and potential for multipoint binding relative to the "low carboxylic acid" support—allowing for probing of the effect of these variables on enzyme activity. Lactase was immobilized in an identical manner as before to control for conjugation effects.

Table 3: Microsphere characterization

	High Carboxyl	Low Carboxyl
Mean Diameter (μm)	1.1	1.2
Binding Capacity	0	N/A
Surface Titration Data (μeq/g)	137.3	9
Number of Microspheres/gram	1.34E+12	1.04E+12
Surface Area (μm^2/g)	5.13E+12	4.72E+12
Parking Area ($Å^2$/Surface Group)	6.2	87
Number of Microspheres/ml	1.42E+11	1.05E+11

Protein loading (4.7±1.6mg of lactase/g of support) on the "high carboxylic acid" microspheres was not significantly different ($p<0.05$) compared to loading on the "low carboxylic acid" microspheres. A significant reduction in maximum enzymatic activity ($p<0.05$); however, occurred after conjugation of lactase to the high carboxylic acid microspheres (Figure 10 and Figure 11). Relative to the activities of the free enzyme and the enzyme when conjugated to low carboxylic acid microspheres, attachment of lactase to high carboxylic acid microspheres resulted in a mean 89% reduction (relative to the free lactase) and 66% reduction (relative to the low carboxy-conjugated lactase) of specific activity under optimum conditions, respectively. There was also no significant difference ($p<0.05$) in the Michaelis constant (K_m) of the lactase on the high carboxylic acid microspheres (0.78±0.21), low carboxylic acid microspheres (0.72±0.25), or the free enzyme (0.83±0.22)—suggesting that activity is not a localized function of the substrate (as expected given the uncharged nature and high concentration of the substrate in combination with the size/two-dimensional nature of the microspheres). These results indicate that electrostatic attraction and/or repulsions between the negatively charged carrier and

the enzyme surface may promote enzyme structural instability and loss of activity on the support. Alternatively, enhanced multipoint conjugation to enzyme amine groups that results from a carrier having increased functionality, counter ion (Na^+) accumulation at the electrostatic double layer, or changes in hydrogen bonding pattern with the more hydrophilic carrier surface, provide explanations as to the loss of activity.

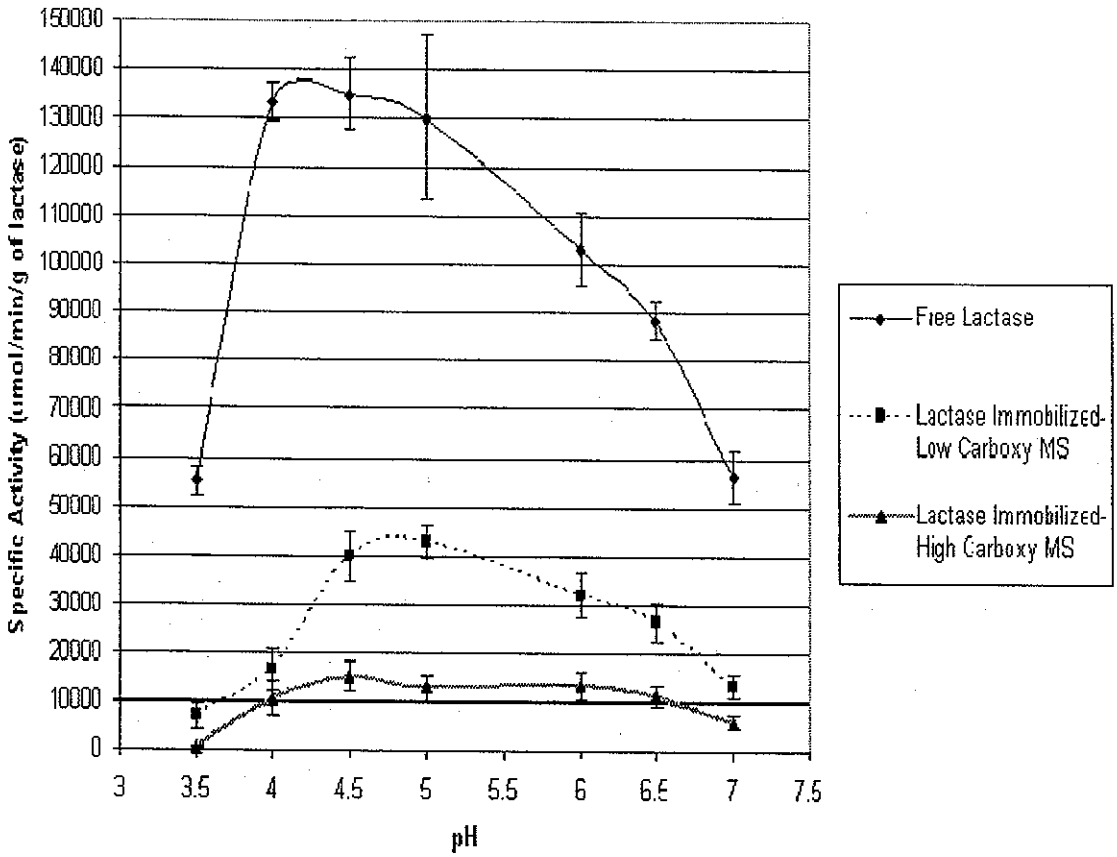

Figure 10. Effect of pH on free lactase (*A. oryzae*) and covalently immobilized lactase on low carboxylic acid polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups) and high carboxylic acid polystyrene-co-acrylic acid microspheres (1.1um; 6.2Å2 between carboxylic groups). Activity at 50^0C using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

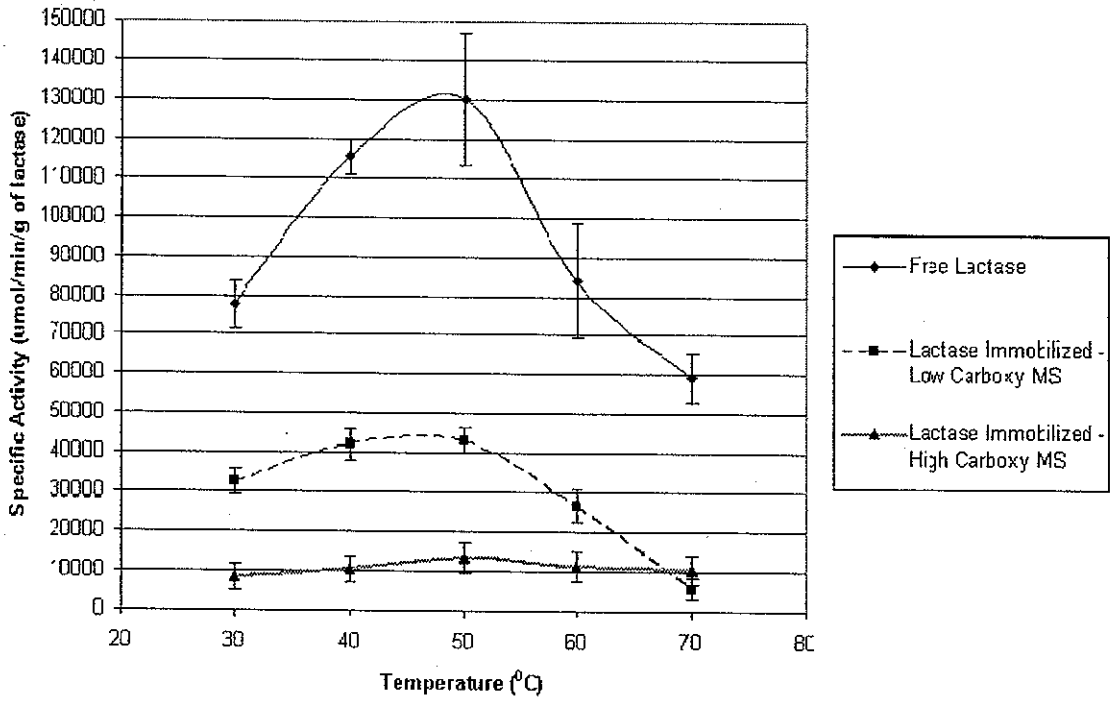

Figure 11. Effect of temperature on free lactase (*A. oryzae*) and covalently immobilized lactase on low carboxylic acid polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups) and high carboxylic acid polystyrene-co-acrylic acid microspheres (1.1um; 6.2Å2 between carboxylic groups). Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

Modification of Lactase for Immobilization on Polystyrene-co-Acrylic acid Microspheres

Controlling the extent of polymer surface functionality can be difficult when using wet chemistry, plasma oxidation, and chemical modification techniques because of the inherent nature of these processes to uniformly alter the surface (Goddard 2008). Likewise, the distribution of charged amino acids on the surface of enzymes is not always uniform; rather clusters of ionic groups have been observed (Shafferman, Ordentlich et al. 1994). With these limitations and the objectives of both 1) determining if inactivation of lactase when immobilized to carboxylic acid

functionalized polystyrene microspheres results from protein-carrier interfacial electrostatic interactions, surface hydrophilic/hydrophobic balance, counter ion accumulation, or multipoint attachment, and 2) exploring means of retaining enzymatic activity of carboxylic acid functionalized hydrophobic polymers—enzyme surface modification was investigated.

Chemical modification of enzymes is used to change solubility, thermostability, and/or evaluate structure/function relationships. Though often utilized to evaluate properties of the free enzyme, this fundamental concept was applied to immobilized lactase by covalently blocking surface-available carboxylic (ie aspartic and glutamic acid) of lactase with low molecular weight molecules prior to immobilization on low and high carboxylic acid polystyrene-co-acrylic acid microspheres. Carboxylic acid groups of lactase were modified by conjugation of glucosamine in the presence of EDC (Figure 12) (Baek and Vijayalakshmi 1997). The single amino residue of glucosamine has a low pKa value (6.91) that favors conjugation of the amine as a nucleophile via EDC—the reaction, of which, must take place at acidic to neutral pH because of the necessity for carboxylic acid to be in a deprotonated state. The glucosamine molecule, also, provides a lone point of attachment to the enzyme—preventing crosslinking. The neutrally charged hydroxyl groups of the carbohydrate, whose potential ester formation with the activated carboxylic acid would not be expected to compete with amide formation because of the low pKa of the terminal amine, would retain the hydrophilic nature of the enzyme after blocking the surface carboxylic acid groups so as not induce insolubilization or aggregation.

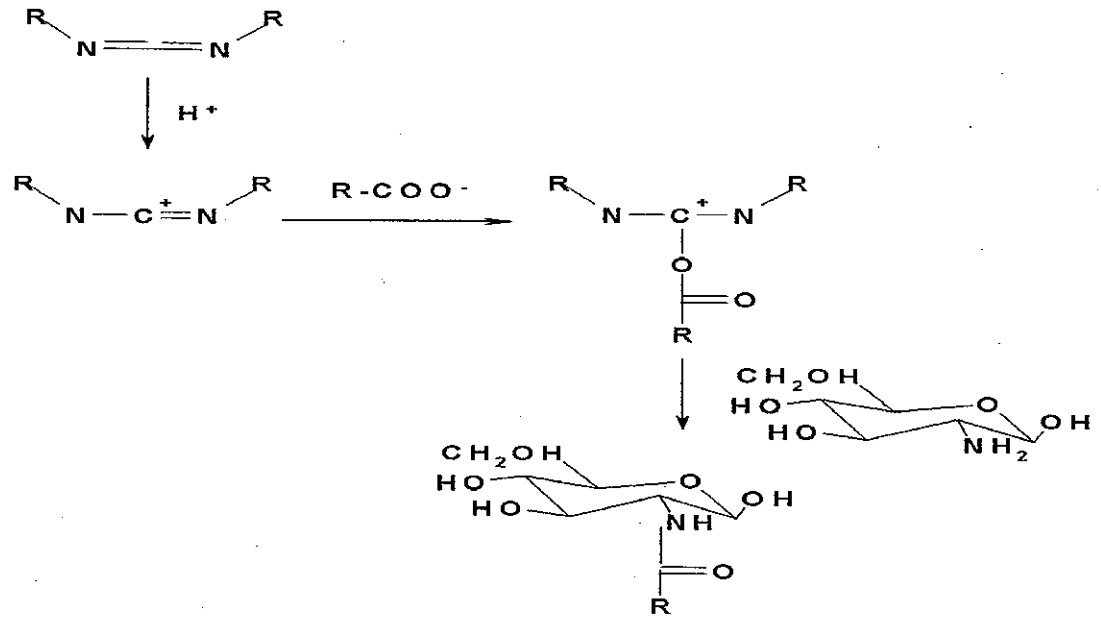

Figure 12. Glucosamine blocking of lactase carboxylic groups in the presence of EDC. R-COO$^-$ represents aspartic acid and glutamic acid residues of lactase.

The amino sequence reveals that lactase (*A. oryzae*) has 100 acid amino acid groups (aspartate and glutamate) capable of reaction with the carbodiimide (Figure 13). The majority of available amino groups would be expected to be on the surface of the enzyme to facilitate hydrogen bonding and minimize destabilization of the hydrophobic core (Creighton 1993).

MKLLSVAAVALLAAQAAGASIKHRLNGFTILEHPDPAKRDLLQDIVTWDDKSLFINGERIMLFSGE
VHPFRLPVPSLWLDIFHKIRALGFNCVSFYIDWALLEGKPGDYRAEGIFALEPFFDAAKEAGIYLIA
RPGSYINAEVSGGGFPGWLQRVNGTLRSSDEPFLKATDNYIANAAAAVAKAQITNGGPVILYQPE
NEYSGGCCGVKYTDADYMQYVMDQARKADIVVPFISNDASPSGHNAPGSGTGAVDIYGHDSYPL
GFDCANPSVWPEGKLPDNFRTLHLEQSPSAPYSLLEFQAGAFDPWGGPGFEKCYALVNHEFSRVF
YRNDLSFGVSTFNLYMTFGGTNWGNLGHPGGYTSYDYGSPITETRNVTREKYSDIKLLANFVKAS
PSYLTATPRNLTTGVYTDTSDLAVTPLIGDSPGSFFVVRHTDYSSQESTSYKLKLPTSAGNLTIPQLE
GTLSLNGRDSKIHVVDYNVSGTNIIYSTAEVFTWKKFDGNKVLVLYGGPKEHHELAIASKSNVTIIE
GSDSGIVSTRKGSSVIIGWDVSSTRRIVQVGDLRVFLLGKNSAYNYWVPELPTEGTSPGFSTSKTTA
SSIIVKAGYLLRGAHLDGADLHLTADFNATTPIEVIGAPTGAKNLFVNGEKASHTVDKNGIWSSEV
KYAAPEIKLPGLKDLDWKYLDTLPEIKSSYDDSAWVSADLPKTKNTHRPLDTPTSLYSSDYGFHTG
YLIYRGHFVANGKESEFLIRTQGGSAFGSSVWLNETYLGSWTGADYTMDGNSTYKLSQLESGNYH
VITVVIDNLGLDENWTVGEETMKNPRGILSYKLSGQDASAITWKLTGNLGGEDYQDKVRGPLNEG
GLYAERQGFHQPQPPSDSWESGSPLEGLSKPGIGFYTAQFDLDLPKRAEGPSSTS

Figure 13. Amino acid sequence for lactase (*A. oryzae*) adapted from Berka, 1998. Acidic amino acids (aspartate and glutamate) are highlighted.

Because of the solubility of the enzyme at the isoelectric point, isoelectric focusing was employed rather than titration methods to indicate modification of the enzyme via deviation of the isoelectric point (pI) of the enzyme (the pH at which the enzyme has no net electrical charge) before and after blocking. After the reaction of the enzyme with glucosamine, there is a substantial increase in the band surrounding the isoelectric point of the enzyme to a more basic pH (Figure 14). This result indicates that the carboxylic groups available to contribute to the overall net charge of the enzyme have been reduced by glycosylation—leaving, predominately, basic amino groups to donate to enzyme charge and yielding a higher pI than the control.

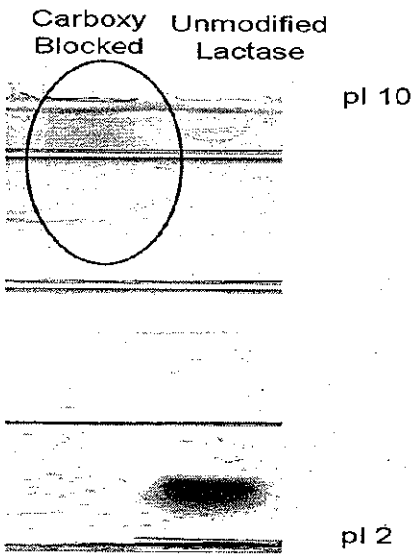

Figure 14. Isoelectric focusing gel of native and carboxylic acid-blocked lactase.

Modification of carboxylic acids groups of lactase yielded no significant change ($p<0.05$) in the activity at optimum catalytic conditions or optimum conditions of pH and temperature when compared to the unmodified free enzyme (Figure 15 and Figure 16). Active site protection via saturation with substrate or a reversible inhibitor can be used if modification lends itself to loss of activity from conjugation of amino

acids necessary for catalytic activity (Talbert 2004). *A. oryzae* lactase; however, did not require protection of the active site during blocking of charged functional groups, under the described conditions, to retain catalytic activity—suggesting carboxylic acid groups are not present in the active site, are unavailable to react with the modifying agents under the conditions of the experiment, or are kinetically restrained. This agrees with the proposed data that the active site of lactase consists of a histidine and cysteine residue (Nijpels 1981).

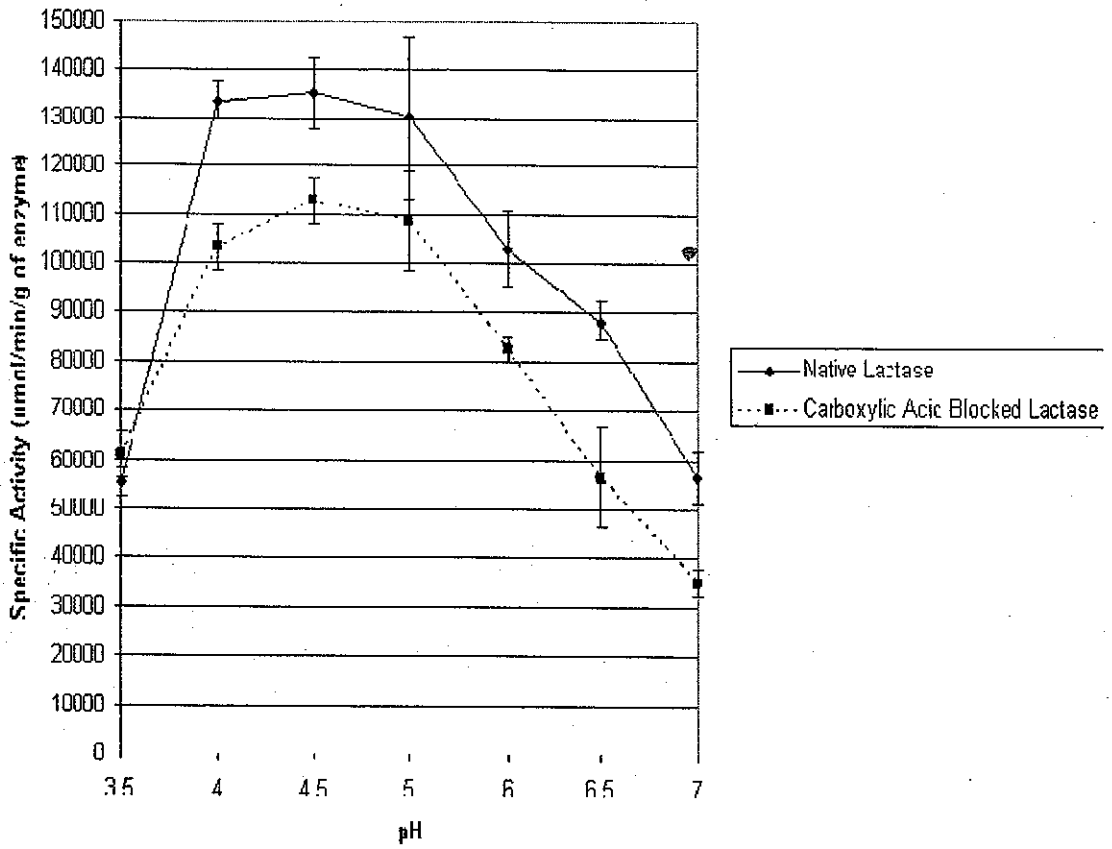

Figure 15. Effect of pH on free native and carboxylic acid-blocked lactase (*A. oryzae*). Activity at 50^{0}C using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

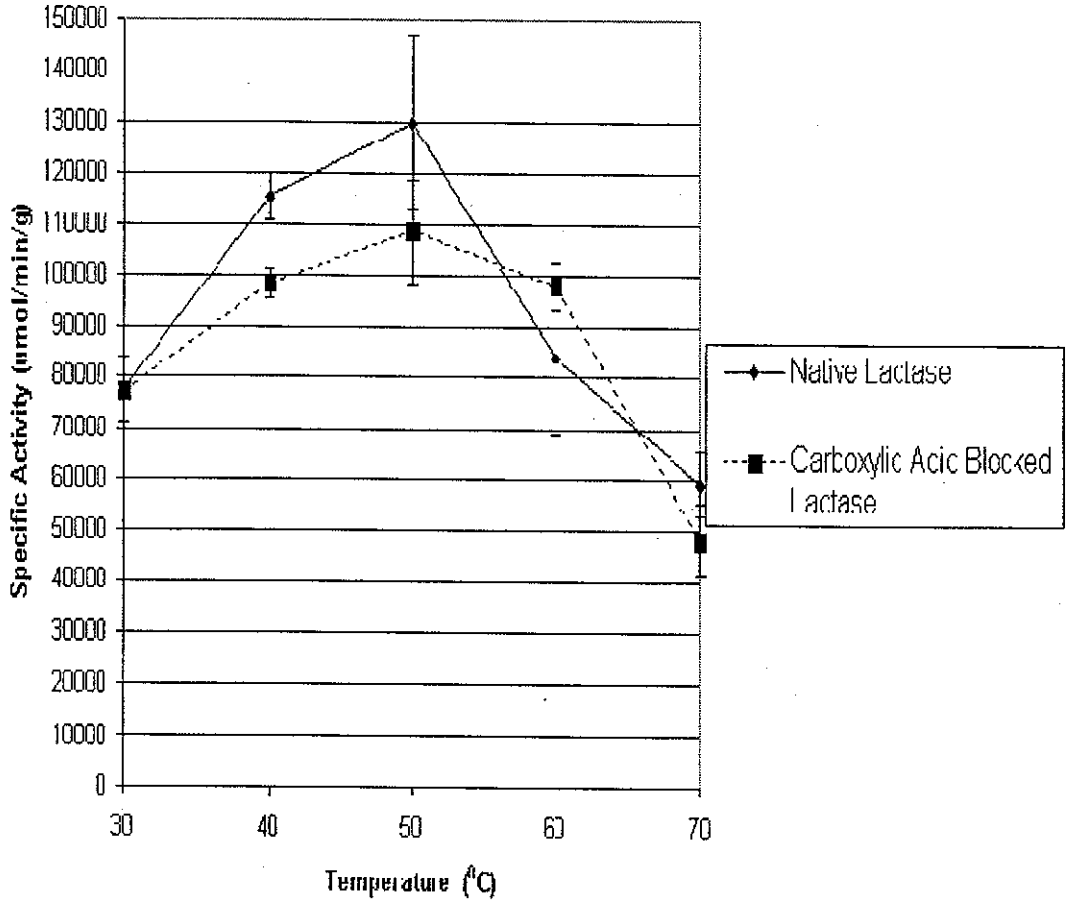

Figure 16. Effect of temperature on free native lactase (*A. oryzae*) and carboxylic acid blocked lactase. Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

Carboxylic acid-blocked lactase was conjugated to high and low carboxylic acid polystyrene-co-acrylic acid microspheres. No significant change ($p<0.05$) in the amount of enzyme conjugated to the microspheres was observed for the carboxylic acid-modified lactase (high carboxylic acid-9.8±1.9mg/g; low carboxylic acid-8.9±2.1). The specific activity of carboxylic-acid blocked lactase immobilized on high carboxylic acid functionalized polystyrene microspheres compared to the free enzyme and the unblocked immobilized enzyme can be seen in Figure 17 and Figure 18. Under optimum conditions, the immobilized, carboxylic acid-blocked lactase

exhibited a 7-fold increase in activity compared to the unblocked, immobilized lactase. With respect to the free enzyme, a 34% mean decrease in activity occurred after immobilization of the carboxylic acid blocked lactase. A shift in optimum pH, from 4.5 for the free enzyme to 5.0 for the immobilized enzyme was observed though the change was not significantly different ($p<0.05$). Activity changes with pH and temperature is similar for both the free and immobilized modified-lactase with the exception of activity at 70^0C. The change in molecular motion with high temperature may allow for unavailable electrostatic groups, such as those buried in the enzyme or involved in salt bridges to become available for interaction with the surface, leading to loss of structural stability.

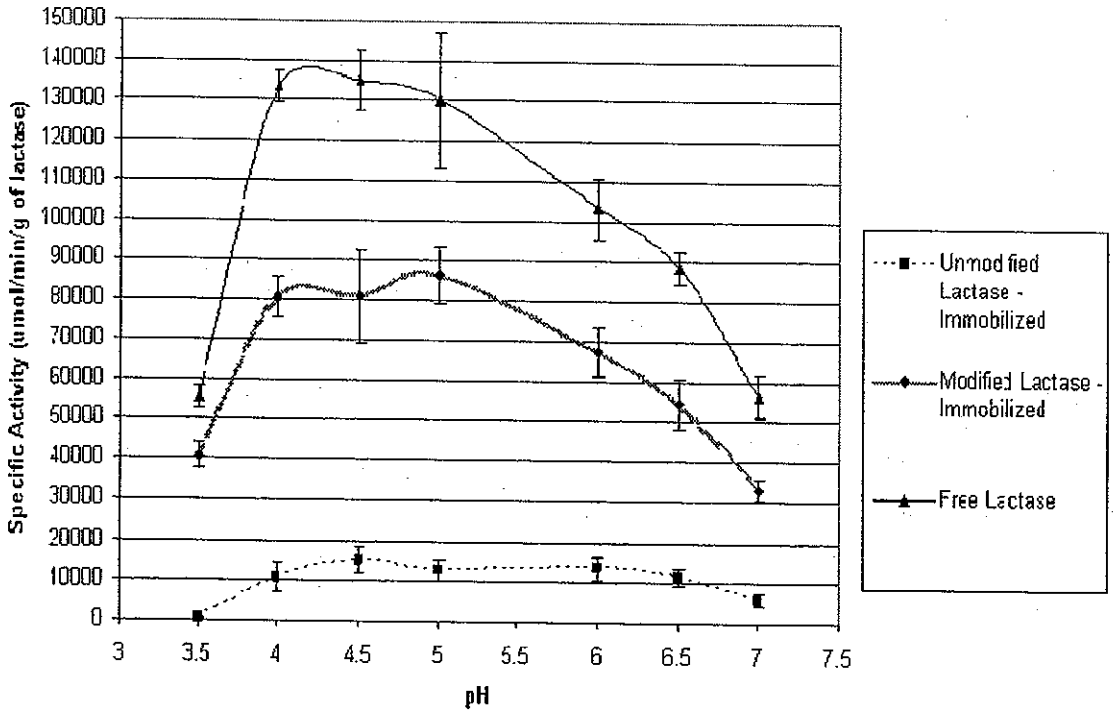

Figure 17. Effect of pH on native lactase (*A. oryzae*) and carboxylic acid-blocked lactase (modified with glucosamine), covalently immobilized on high carboxylic acid polystyrene-co-acrylic acid microspheres (1.1um; 6.2Å2 between carboxylic groups). Activity at 50^0C using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

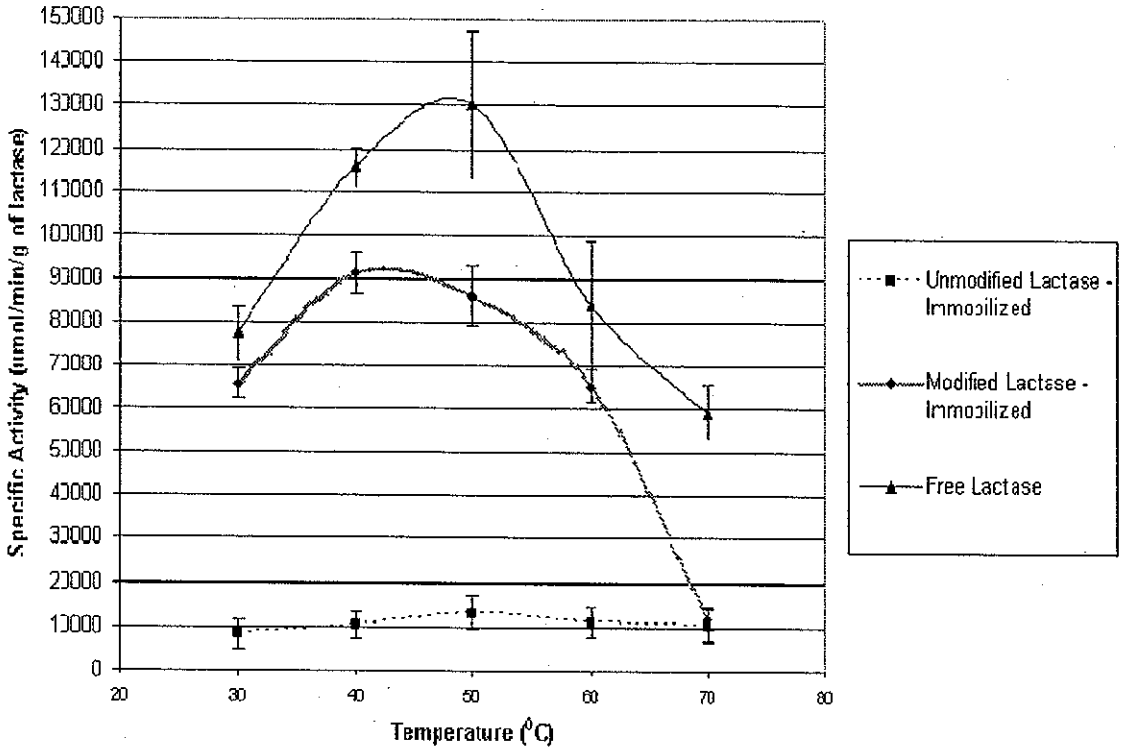

Figure 18. Effect of temperature on native lactase (*A. oryzae*) and carboxylic acid blocked lactase (modified with glucosamine), covalently immobilized on high carboxylic acid polystyrene-co-acrylic acid microspheres (1.1um; 6.2Å2 between carboxylic groups). Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

As with the high carboxylic acid microspheres, conjugation of carboxylic acid blocked lactase to low carboxylic acid functionalized microspheres exhibited an increase in activity retention. Conjugation of the blocked enzyme did not result in a significant change ($p<0.05$) in enzyme activity (116Kμmol/min/g) compared to free lactase under optimum conditions (Figure 19 and Figure 20). These results along with the activity of modified lactase on high carboxylic acid microspheres lends evidence that lactase immobilization on carboxylic acid activated polystyrene is dependent upon protein-carrier interfacial interactions.

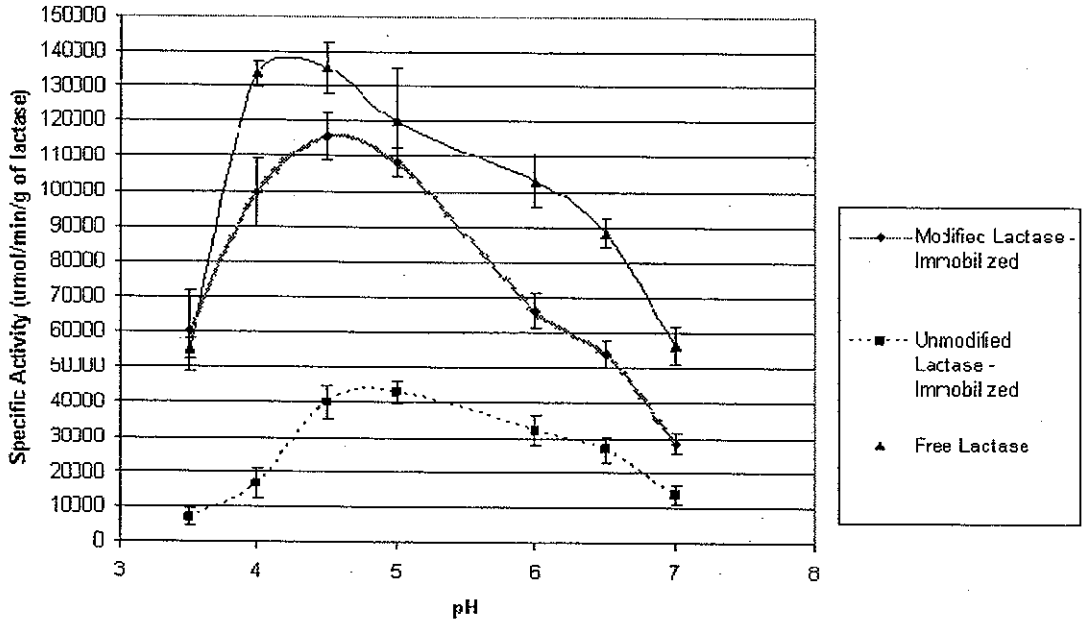

Figure 19. Effect of pH on native lactase (*A. oryzae*) and carboxylic acid-blocked lactase (modified with glucosamine), covalently immobilized lactase on low carboxylic acid polystyrene-co-acrylic acid microspheres. Activity at 50^{0}C using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

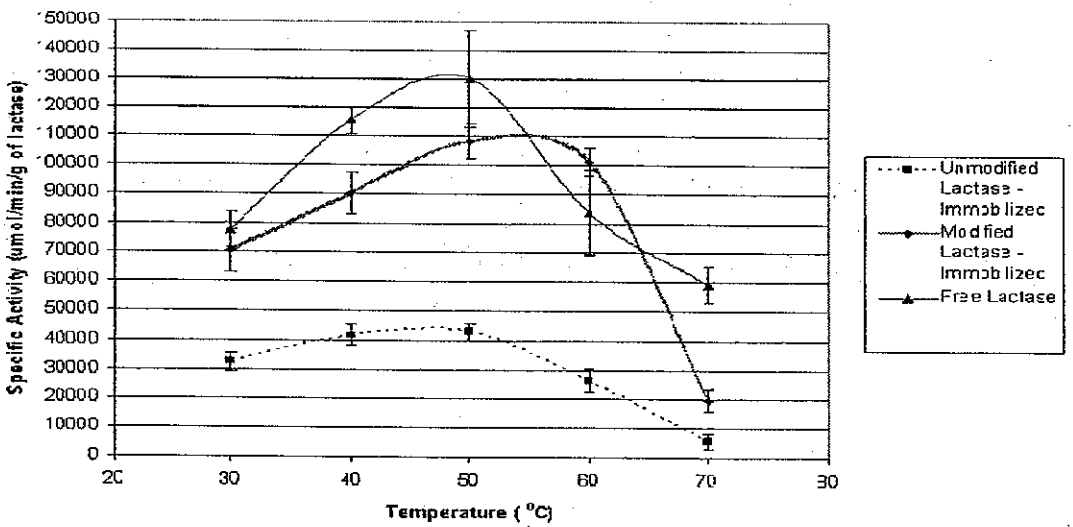

Figure 20. Effect of temperature on native lactase (*A. oryzae*) and carboxylic acid blocked lactase (modified with glucosamine), covalently immobilized on low carboxylic acid polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups). Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

As the pH becomes more basic from pH 5.0, there is a significant drop in activity. With increasing pH comes an increase in deprotonation of the carboxylic acid groups of the enzyme—the pKa of amino acids in a protein being variable. Net electrostatic repulsion as well as deprotonation and repulsion of carboxylic acid chains, which were unavailable at pH 5.3 for blocking, would be expected to increase with increasing pH, introducing detrimental negative-negative interfacial electrostatic interaction—yielding activity values that are not significantly different from that of the modified enzyme on high carboxylic acid at the same corresponding pH. The temperature profile of the immobilized modified-enzyme was shifted compared to the free enzyme (Figure 19). At 70^0C, there is a significant loss in activity ($p<0.05$) for the immobilized modified-enzyme compared to the free enzyme. As described for the high carboxylic acid, higher temperature promotes enhanced molecular motion and, subsequent, structural confirmation sampling and interaction with, not only the aqueous medium, but also the carrier, which seems to promote loss of activity.

These results provide further evidence that inactivation of the enzyme after conjugation to carboxylic acid functionalized polystyrene microspheres is due to negative-negative interfacial charge repulsion between the carrier and enzyme and not multipoint binding, since the same quantity of amine groups on the enzyme are free to react with the carrier before and after blocking of the carboxylic acids on the enzyme. The repulsion of the enzyme from the surface may be localized from charge amino groups or result from net charge above the isoelectric point of the enzyme. Literature results regarding conjugation of lactase on negatively charged surface appears conflicting. The activity of bacterial (*E. coli*) lactase was shown to be substantially inhibited on a polyanionic structure compared to an anionic surface (Hamlin, Dayton et al. 2007). Contrary, the activity of lactase adsorbed to sulfate dextran-modified MANAE-agarose was reported to be 100%; however, the activity appears to result

from activity measurements taken after desorption of the enzyme from the carrier (Fuentes, Pessela et al. 2004). If activity is inhibited on the surface but not after desorption (ie the protein is distorted on the surface but recovers in solution), ionic supports may be used for separation of the protein without concerns of denaturation. Blocking the carboxylic acid groups of lactase, by changing the pI of the enzyme and retaining enzyme activity, may have application for, not only covalently bound enzyme, but also recycling of enzyme on ionic supports (Montes, Grazu et al. 2006).

Stability of Lactase Conjugates

The activity of the carboxylic-acid blocked lactase and unmodified lactase (control) on carboxylic acid microspheres was monitored across a 4-week period to evaluate long-term stability of the conjugates at potential processing storage conditions (Figure 21). Across the evaluation period, no significant changes ($p<0.05$) were observed at the respective pH and temperature storage conditions, indicating that that conjugates are stable and changes in activity as a function of conjugation occur prior to activity measurement.

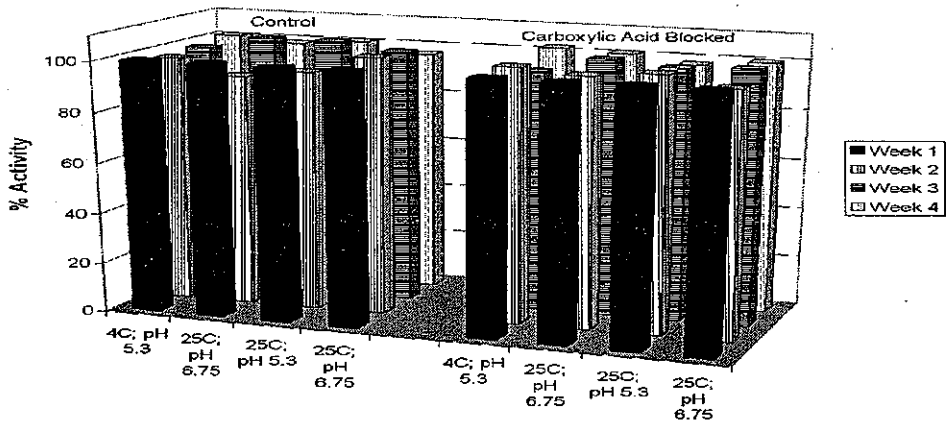

Figure 21. Stability of immobilized lactase (*A. oryzae*) on low carboxylic acid polystyrene-co-acrylic acid microspheres. Activity at 50^0C, pH 5.0 using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

Carbodiimide Modification of Lactase for Conjugation to Polystyrene-co-Acrylic acid Microspheres

Carbodiimide addition to lactase in the absence of glucosamine was investigated to explore the possibility of non-specific inter and intramolecular crosslinking reactions that may occur when using the reagent in solution. Molecular crosslinking has been shown to occur when using EDC with proteins because of the ability of the carbodiimide to react with protein carboxylic acid groups with available amine groups present on the protein (Yamada, Kuroki et al. 1983; Grabarek and Gergely 1990). Activity was assessed for lactase after incubation with EDC at 0, 0.5, 5, and 50 molar excess with respect to the moles of carboxylic acid groups associated with the enzyme (Park, Desanti et al. 1979; Berka 1998; Samoshina and Samoshin 2005). As can be seen from (Figure 22 and Figure 23), there is a lowering in the activity of lactase after incubation with 50 molar excess EDC—suggesting modification of the enzyme. Decrease in activity after the addition of high concentrations of carbodiimide to lactase has been observed, previously, though details for the loss of activity were not described (Fernandezlafuente, Rosell et al. 1993).

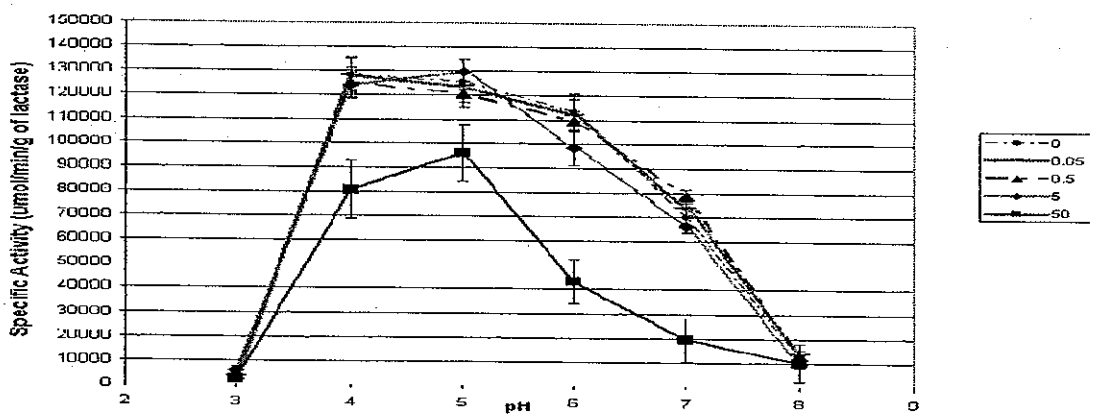

Figure 22. Effect of pH on carbodiimide-modified lactase (*A. oryzae*) activity. Activity at 50^{0}C (0.1M acetate buffer) using ONPG as enzyme substrate. Legend indicates molar excess of carbodiimide relative to available carboxylic acid of lactase. Values represent mean values ± standard deviation (N=3).

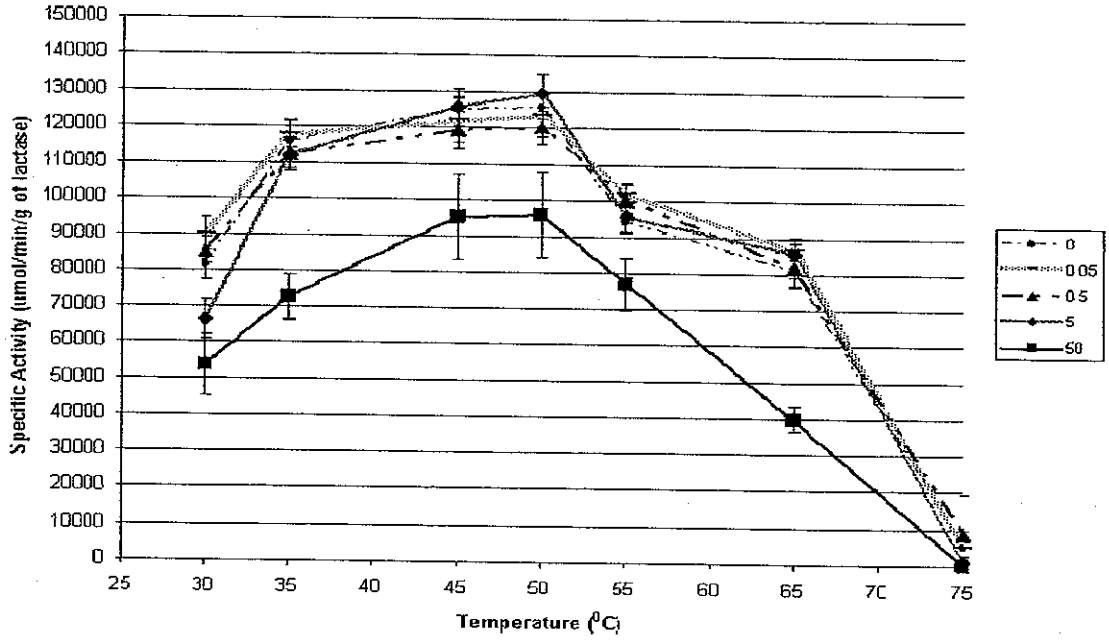

Figure 23. Effect of temperature on carbodiimide-modified lactase (*A. oryzae*) activity. Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Legend indicates molar excess of carbodiimide relative to available carboxylic acid of lactase. Values represent mean values ± standard deviation (N=3).

SDS-PAGE of the enzyme preps after incubation with EDC reveals all preps to be monomers of the same molecular weight—indicating that EDC does not promote intermolecular crosslinking of lactase (Figure 24). Likewise, evidence of intramolecular crosslinking was pursued by determining the availability of amine and thiol groups. If intramolecular crosslinking occurs during incubation, free amines and thiols would be expected to react and fewer of those groups would be available for detection. TNBS and Ellman's reagent, used for detecting free amines and thiols, respectively, showed no significant difference ($p<0.05$) between the quantity of available amine and thiol groups associated with the enzyme incubated without EDC and those incubated with EDC, suggesting that intramolecular crosslinking does not

occur, is at a level below assay detection limits, or reacts with other amino acids such as tyrosine (Hermanson 1996).

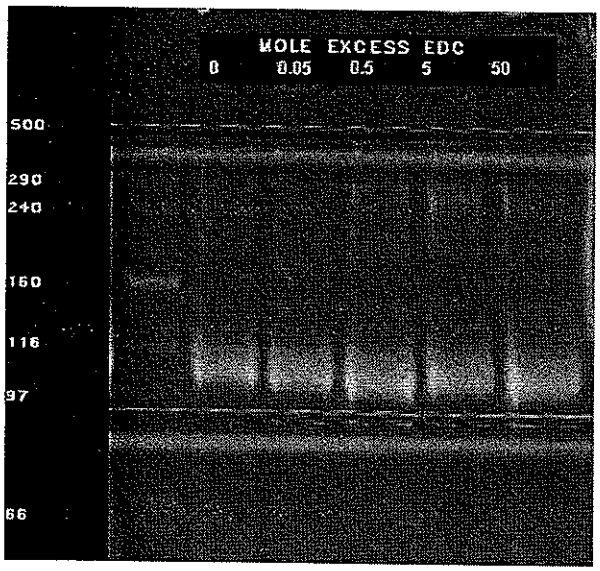

Figure 24. SDS-PAGE gel of lactase modified with molar excess of carbodiimide (EDC).

Lactase preps modified with the indicated molar excess of EDC were immobilized on high and low carboxylic acid polystyrene-co-acrylic acid microspheres. Loading on the microspheres was not significantly ($p<0.05$) different across incubation levels. When immobilized to high carboxylic acid polystyrene-co-acrylic acid microspheres, there was no significant ($p<0.05$) difference in lactase activity associated with EDC incubation until 5 molar excess (relative to the moles of carboxylic acid groups of lactase) of EDC was used. For 5 molar excess, a significant increase ($p<0.05$) in activity was observed with a 50% mean activity retention compared to the same free enzyme prep under optimum conditions (Figure 25 and Figure 26). Enzyme prep incubated at 50 molar excess had a greater increase in activity—yielding an 83% mean activity retention compared to the same free enzyme prep under optimum conditions.

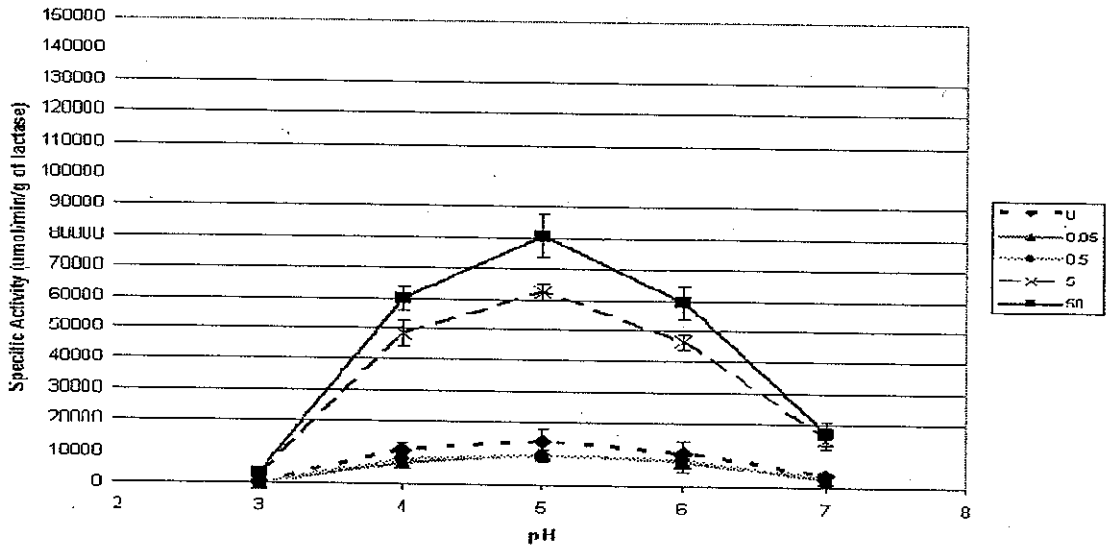

Figure 25. Effect of pH on carbodiimide (EDC) modified lactase (*A. oryzae*), covalently immobilized on high carboxylic acid polystyrene-co-acrylic acid microspheres (1.1um; 6.2Å2 between carboxylic groups). Activity at 50^{0}C using ONPG as enzyme substrate. Legend represents molar excess of EDC to enzyme carboxylic acid groups. Values represent mean values ± standard deviation (N=3).

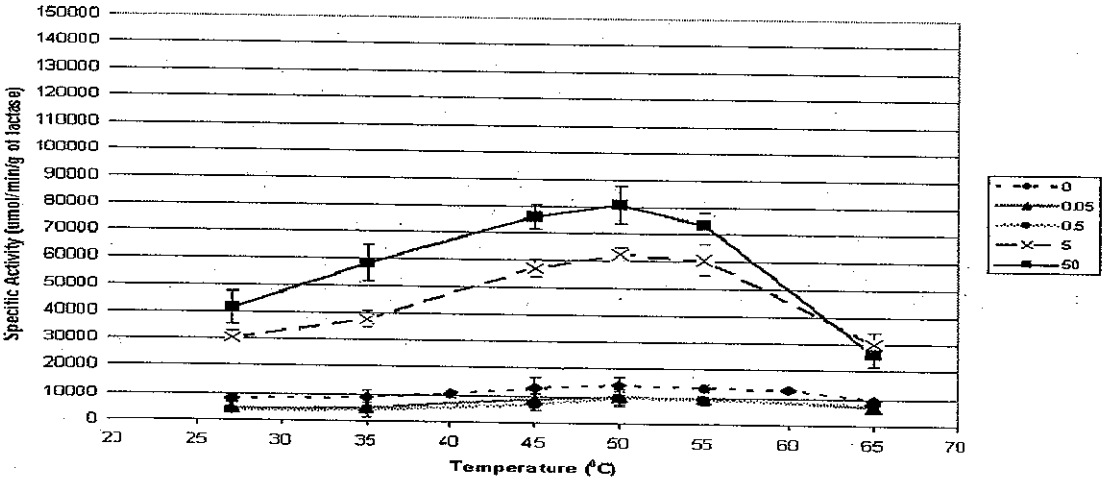

Figure 26. Effect of temperature on carbodiimide (EDC) modified lactase (*A. oryzae*), covalently immobilized on high carboxylic acid polystyrene-co-acrylic acid microspheres (1.1um; 6.2Å2 between carboxylic groups). Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Legend represents molar excess of EDC to enzyme carboxylic acid groups. Values represent mean values ± standard deviation (N=3).

Immobilization of lactase to low carboxylic acid polystyrene-co-acrylic acid microspheres displayed a similar trend, with significant activity increases corresponding to 5 and 50 molar excess EDC incubation (Figure 27 and Figure 28). The peak total specific activity of the immobilized enzyme corresponded to the 5 molar excess prep (111Kμmol/min/g of lactase) on the low carboxylic acid polystyrene co-acrylic acid microspheres. The activity accounted for 89% mean activity retention under optimum conditions. The highest activity retention was obtained with the 50 molar excess--having 92% mean activity retention compared to the corresponding 50 molar excess free lactase prep. This immobilized enzyme had lower total activity, though, because of the decrease in activity of the free enzyme after incubation with 50 molar excess EDC.

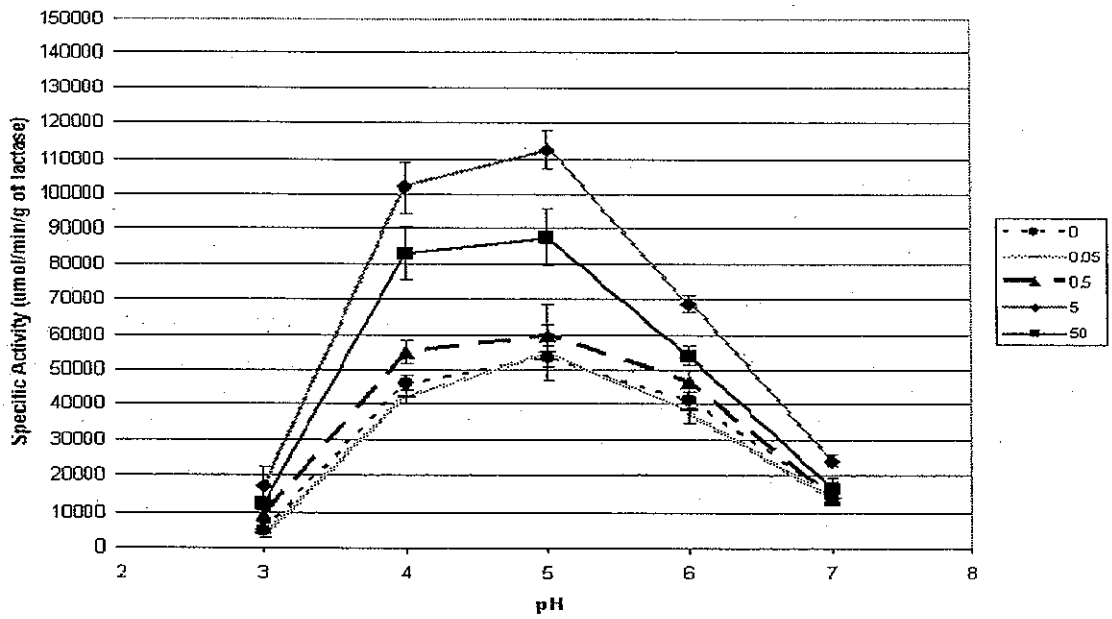

Figure 27. Effect of pH on carbodiimide (EDC) modified lactase (*A. oryzae*), covalently immobilized on low carboxylic acid polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups). Activity at 50^{0}C using ONPG as enzyme substrate. Legend represents molar excess of EDC to enzyme carboxylic acid groups. Values represent mean values ± standard deviation (N=3).

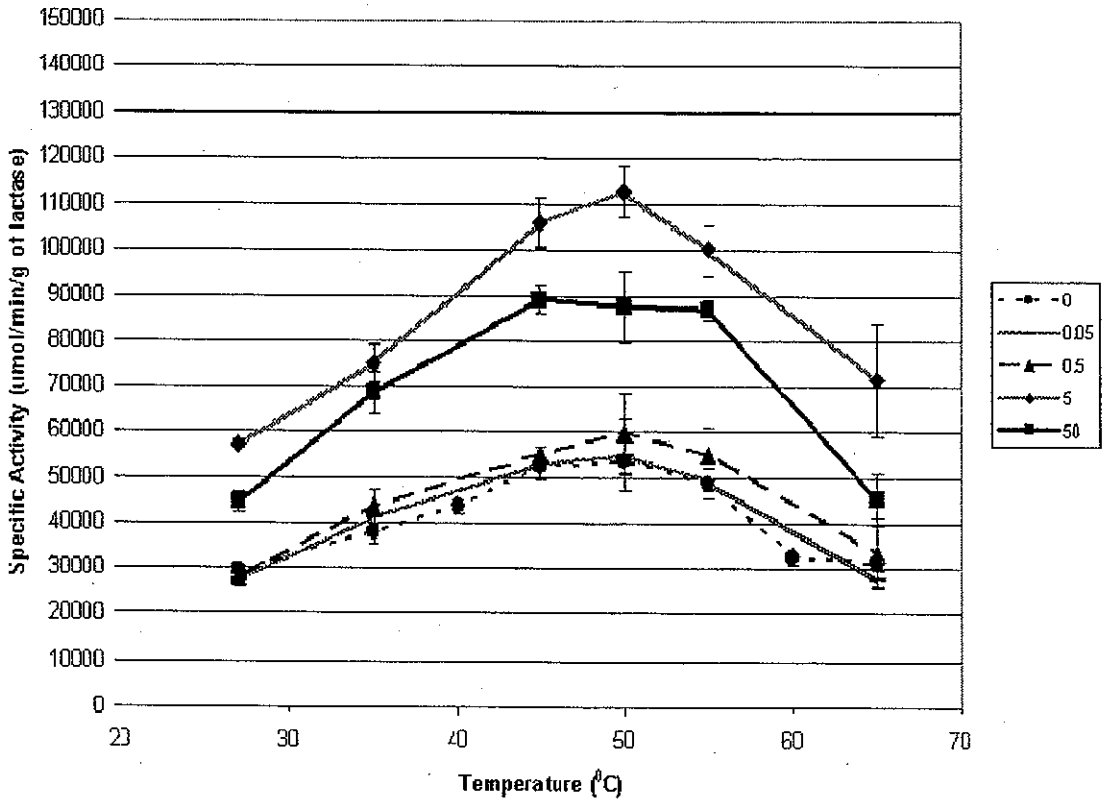

Figure 28. Effect of temperature on carbodiimide (EDC) modified lactase (*A. oryzae*), covalently immobilized on low carboxylic acid polystyrene-co-acrylic acid microspheres (1.2um; 87Å2 between carboxylic groups). Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Legend represents molar excess of EDC to enzyme carboxylic acid groups. Values represent mean values ± standard deviation (N=3).

The enzyme preps incubated with a high mole excess of EDC (5 and 50 molar excess), when immobilized, display characteristics similar to that of carboxylic acid blocked lactase. To determine if carboxylic acid groups of the enzyme were modified, isoelectric focusing of lactase after incubation with 50 molar excess EDC was performed. IEF gel revealed an isoelectric range corresponding to that of carboxylic acid blocked lactase suggesting carboxlic acid groups of lactase had been modified (Figure 29).

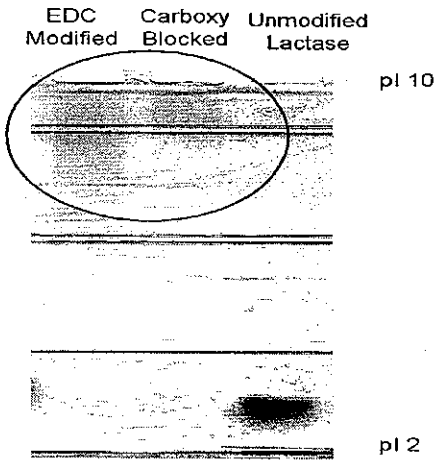

Figure 29. Isoelectric focusing gel of native, carboxylic acid-blocked, and carbodiimide modified (50X) lactase.

Nakajima and Ikada observed that excess carbodiimide, relative to mole quantity of carboxylic acids, can form stable N-acylurea products in the absence of amines—noting that no change in carboxylic groups (ie N-acylurea formation) occurred at less than molar excess quantities because of hydrolysis of the carbodiimide activated carboxylic acid groups back to free carboxylic acid (Nakajima and Ikada 1995) (Figure 30). A stable N-acylurea product being formed when EDC is in excess of lactase carboxylic acid groups (ie blocking of the carboxylic acid groups) corresponds with isoelectric focusing and immobilized stability results. The results suggest that under assay conditions, N-acylurea formation from reaction of excess EDC with carbodiimide activated carboxylic acid groups of lactase can occur without the formation of intra and intermolecular crosslinks. This data verifies the negative-negative electrostatic effect between the enzyme and carrier, and suggest that, for *A. oryzae* lactase, a "one pot" attachment to a reactive amine surface may be used without concern for intra and intermolecular crosslinking or carboxylic group modification of the free enzyme, provided EDC is not used in excess.

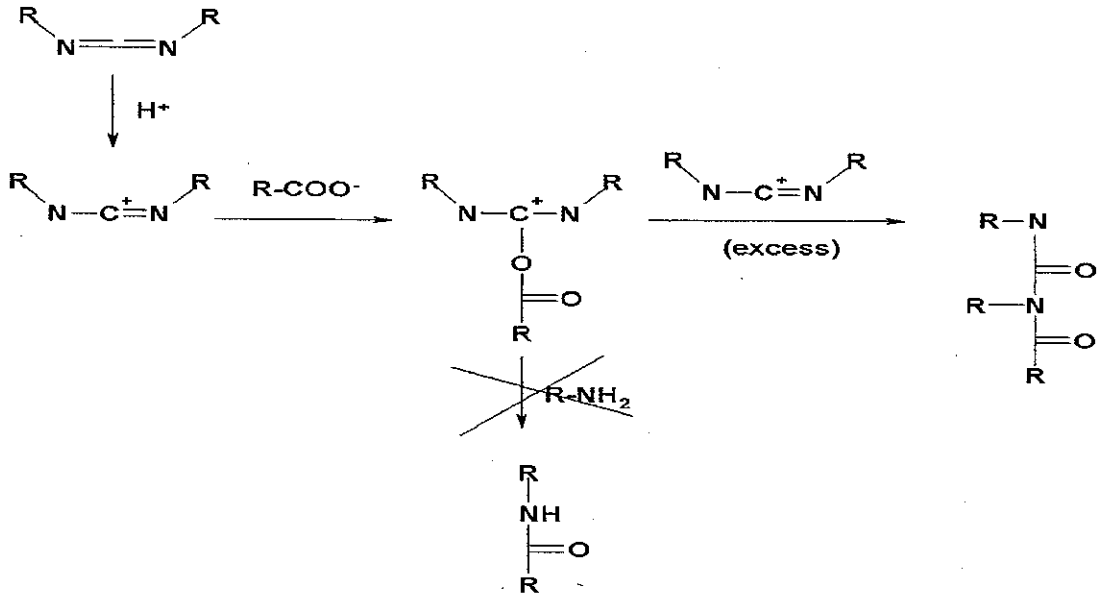

Figure 30. N-acylurea formation in the presence of excess carbodiimide and absence of a reactive nucleophile (R-NH_2). R-COO^- represents aspartic acid and glutamic acid residues of lactase.

Lactase Immobilization to Polystyrene-Acrylic acid Microspheres via a Tethered Chitosan Intermediate

Loss of enzyme activity after attaching lactase to carboxylic-acid activated polystyrene-co-acrylic acid microspheres and low protein loading (<10mg of enzyme/g of microspheres) on such supports are limitations to application of these lactase-immobilized systems. Investigations were made into the covalent immobilization of lactase to polystyrene-co-acrylic acid microspheres via a chitosan-tethered intermediate using carbodiimide-mediated chemistry, given that:

1. loss of enzyme activity on polystyrene-co-acrylic acid microspheres can be attributed to electrostatic interactions between carboxylic acid groups on the enzyme and carrier surface
2. glucosamine can be conjugated to lactase carboxylic acid groups without a significant loss of activity

3. polymers, tethered to a solid support, have been shown to increase surface loading on a carrier (Matoba, Tsuneda et al. 1995; Ye, Xu et al. 2005; Ye, Xu et al. 2006; Ye, Xu et al. 2006)
4. lactase (*A. oryzae)* has been shown to retain activity when conjugated to chitosan—an FDA approved natural polymer composed of repeating glucosamine monomer units (Rejikumar and Devi 1995)
5. carbodiimide chemistry can be used to conjugate lactase to glucosamine without concern of intra or intermolecular crosslinking

Chitosan was covalently grafted to high carboxylic acid polystyrene-co-acrylic acid microspheres using EDC—linking the amine groups of chitosan to the carboxylic acid groups of the microspheres and leaving free amine groups on the surface for further conjugation (Figure 31).

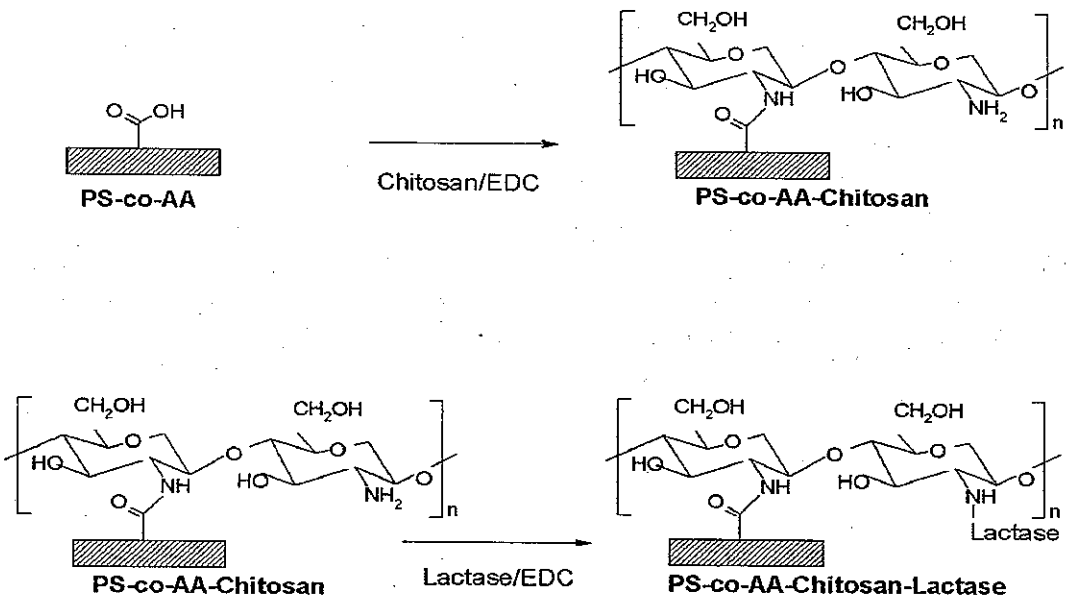

Figure 31. Lactase immobilization by a chitosan tethered intermediate

The pKa of the amino groups of chitosan (pH 6.5), as with glucosamine, promotes reactivity (i.e. deprotonation of the amine to induce nucelophilic attack under the conditions of the assay) of the aminated polymer with carboxylic acid groups in the presence of EDC. Here, too, ester formation associated with hydroxyl groups of the polymer would not be expected to compete because of dominate reactivity of the amine groups. Though acetic acid is most often used to dissolve chitosan, diluted hydrochloric acid was employed to prevent non-specific reaction with EDC. Evidence of chitosan attachment is shown in Figure 32 by the change in carboxyl and amino groups using dye adsorption. There is a significant increase ($p<0.05$) in the surface amines (131 ± 59nmol/cm^2 to 54928 ± 6740nmol/cm^2) and decrease in carboxylic groups (97816 ± 4228nmol/cm^2 to 26774 ± 1629nmol/cm^2) upon conjugation of chitosan. Coverage of surface carboxylic acid groups on the microspheres was incomplete, which can be attributed to steric hindrance of the polymer chains during conjugation or amine accessibility to available carboxylic acid groups (Ye, Xu et al. 2006).

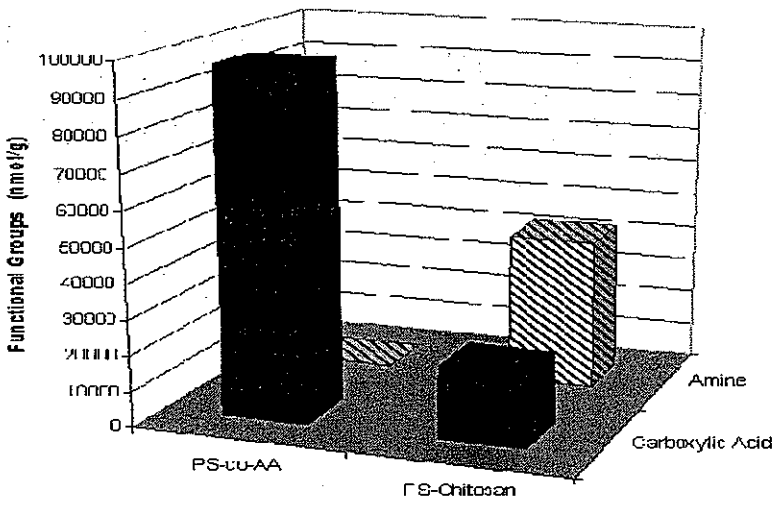

Figure 32. Functionality of microspheres determined by dye binding assays (N=3).

Zeta potential testing was utilized to determine the electrokinetic potential of the supports at lactase conjugation pH (5.3). Though not a measure surface charge, zeta potential does reflect the nature of the particle surface by determining the potential between the medium and stationary layer attached to the microspheres. Analysis verified conjugation of chitosan (Figure 33 and Figure 34). Zeta potential increased from -42mV for the carboxylated microspheres to 4mV for the chitosan-tethered microspheres at pH 5.3. As amines are introduced to the surface and carboxylic acid groups are blocked, the charge increases. As can be seen from Figure 33, the analysis also revealed that, at pH 5.3, the zeta potential of the chitosan-tethered molecules is approaching zero--indicative of the isoelectric point of the carrier system. The potential is reflective of not only the available carboxylic acid and amine groups being present but also the isoelectric points of the carboxylic acid and amine molecules. Stability of colloids, with respect to zeta potential, increases as the potential moves away from zero in the positive or negative direction. This result is significant in that it suggests pH conditions used for lactase conjugation to the chitosan-tethered supports promotes flocculation of the support. This measurement; however, may be skewed given that the microspheres would be settling under gravity during the assay (i.e. moving out of the field of measurement), which may alter the absolute value of the corresponding zeta potential.

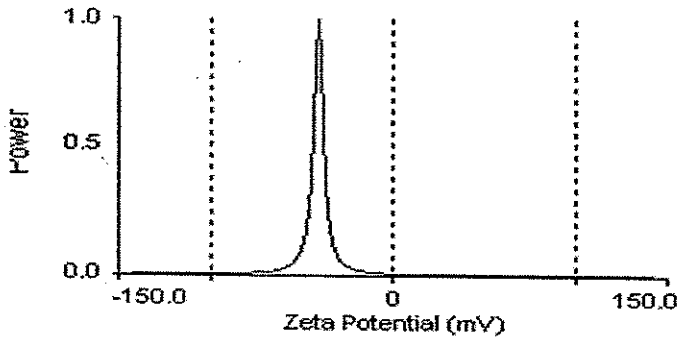

Figure 33. Zeta potential of carboxylated microspheres (pH 5.3)

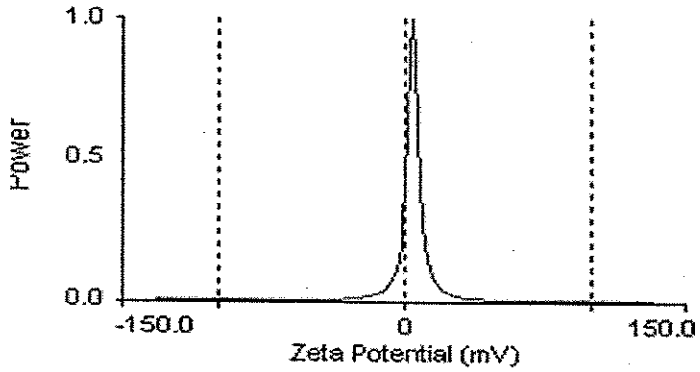

Figure 34. Zeta potential of chitosan-tethered microspheres (pH 5.3)

Though the microspheres appeared suspended in the solution to the naked eye, the flocculation phenomenon was observed by scanning electron micrographs (Figure 35 and Figure 36). Aggregation appears to be between chitosan chains—suggesting surface interaction as the pH of the system (5.3) is nearing the isoelectric point of chitosan (6.5). Association is evident for solubilized chitosan in solution as it moves from acidic to neutral and basic conditions.

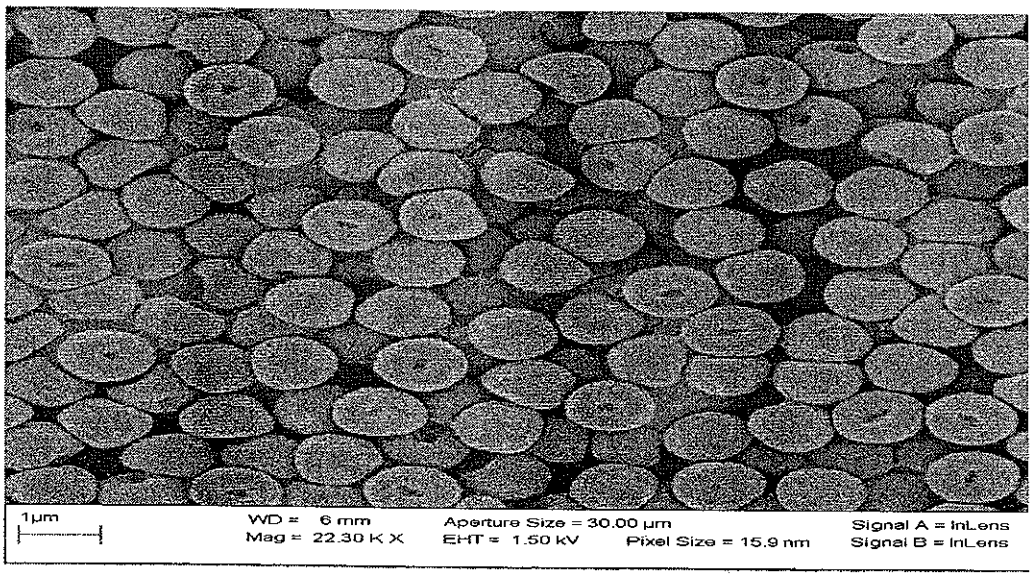

Figure 35. Scanning electron micrograph of high carboxylic acid polystyrene-co-acrylic acid microspheres.

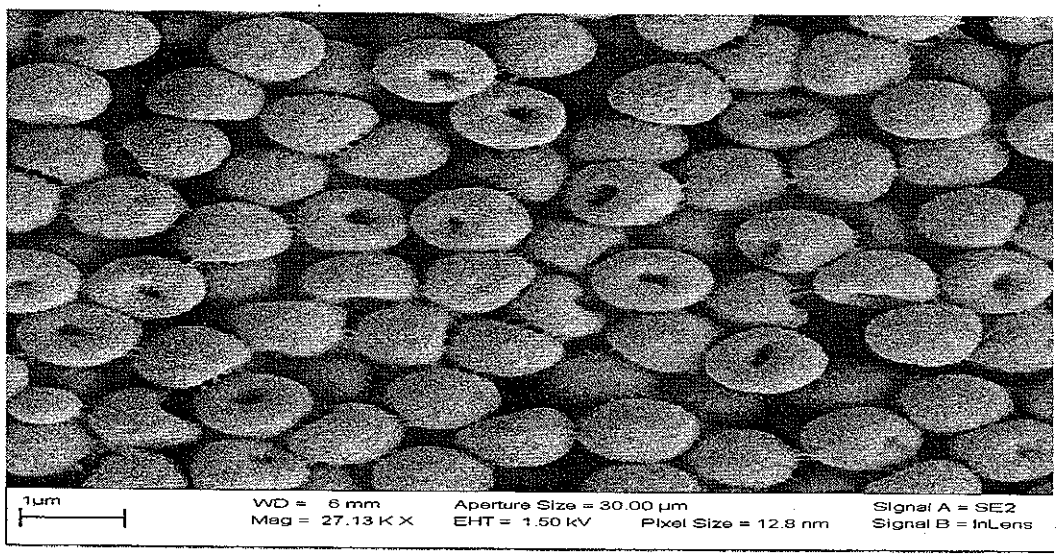

Figure 36. Scanning electron micrograph of chitosan-tethered, high carboxylic acid polystyrene-co-acrylic acid microspheres.

Lactase (*A. oryzae*) was conjugated to the chitosan-tethered microspheres via the addition of EDC in a 0.9:1 molar ratio of EDC to carboxylic acid groups to alleviate concerns of carboxylic blocking from N-acylurea formation. The reaction scheme for lactase conjugation to chitosan-tethered supports is shown in Figure 31. Conjugation of lactase (500mg of lactase offered per gram of carrier) to chitosan-tethered microspheres resulted in a significantly higher ($p<0.05$) protein loading compared to loading on microspheres without a chitosan-tethered intermediate (Figure 37). The increase in loading can be explained by the available surface area associated with the microspheres being enhanced with chitosan tethering and resulting polymer brushing/extension from the carrier surface, or physical/chemical interactions of the enzyme with the surface that promotes loading (Matoba, Tsuneda et al. 1995; Ye, Xu et al. 2005; Ye, Xu et al. 2006; Ye, Xu et al. 2006). Though no intermolecular crosslinking was observed with the addition of EDC to free lactase, changing the surface microenvironment may drive the protein to the surface, promoting enzyme

interaction, aggregation, and crosslinking and increased protein loading. A similar phenomenon is observed in the formation of crosslinked enzyme aggregates (CLEAs).

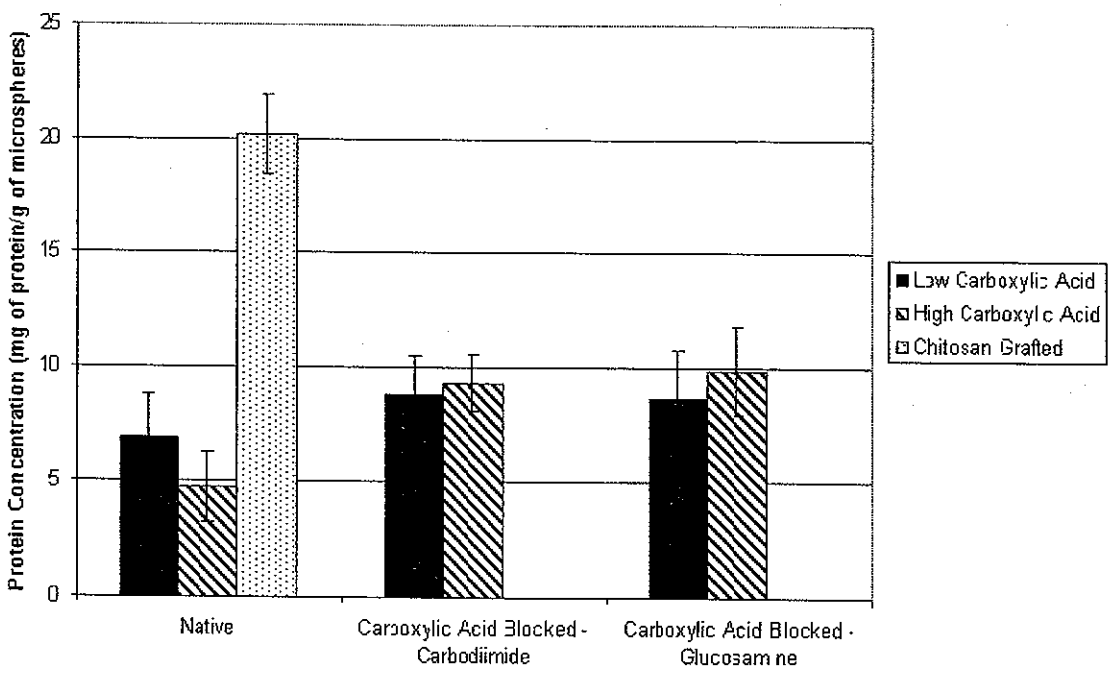

Figure 37. Protein loading (*A. oryzae* lactase) on microspheres. Values represent mean values ± standard deviation (N=3).

The effect of offered enzyme (i.e. free enzyme available in solution) on chitosan-tethered microspheres protein loading and specific activity was investigated to determine the optimal condition for conjugation. Protein was offered from a theoretical value of 5mg/g to 500mg/g. At low protein concentrations (ie 5mg/g), the enzyme taken-up approaches 100% (Figure 38). Beyond the offered 100mg/g, activity decreases, as well as the rate of loading. Loss of activity with increased loading has been observed on other supports and has been attributed to crowding of the enzyme at the surface which leads to spatial restrictions, limited active site accessibility, or denaturing of the protein (Ganapathi, Butterfield et al. 1995; Yamada, Iizawa et al.

2006). The loading was fitted by nonlinear regression using a one site total binding model (Graphpad Prism software) (Figure 39). The equation fitting the model is described in Equation 7:

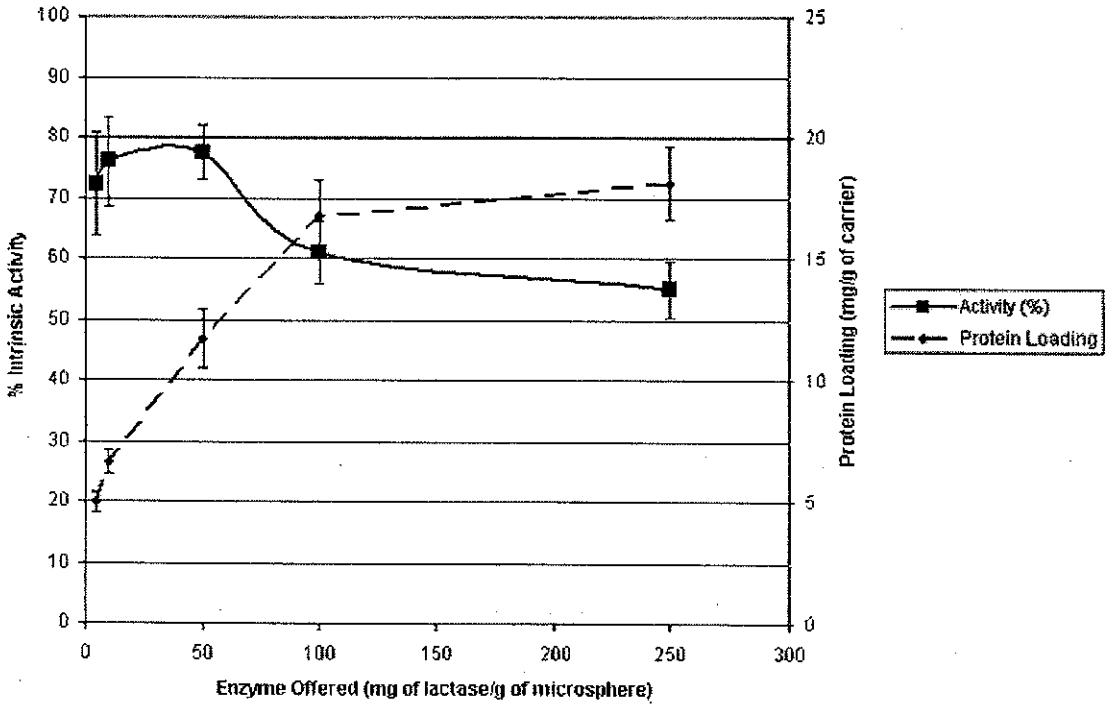

Figure 38. The effect of protein offered on loading and activity of (*A. oryzae* lactase) on chitosan-tethered microspheres. Activity at pH 5.0 and 50^{0}C. Values represent mean values ± standard deviation (N=3).

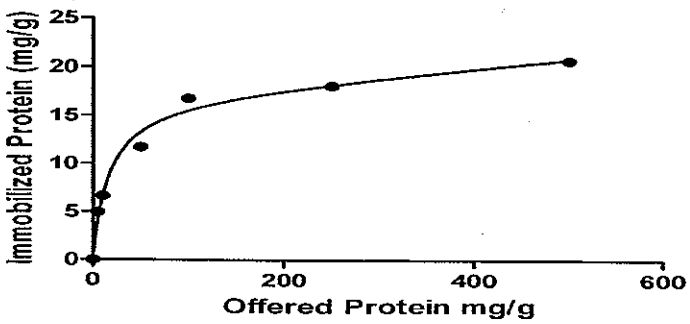

Figure 39. Nonlinear fit to mean loading of lactase (*A.oryzae*) on chitosan-tethered microspheres using a one-site total binding model. $R^2 = 0.98$

Equation 7:

$Y=Bmax*X/(Kd+X) + NS*X + Background$

Where:

Bmax is the maximum specific binding in the same units as Y.

Kd is the protein offered needed to achieve a half-maximum binding at equilibrium (same units as X).

NS is the slope of nonspecific binding in Y units divided by X units.

Giving:

$Y=16.7*X/(17.14+X) + 0.0085*X + 0.393$

Comparing the model with Figure 37, lactase binds specifically (ie in a noncrowded fashion) until reaching a loading of 16.7mg/ml (B_{max}). Beyond the B_{max}, the protein associates in a nonspecific way (ie crowded). This explanation appears to correlates well with the specific activity of the enzyme under the noncrowded/crowded conditions. The curve may also be described as a Langmuir adsorption isotherm--suggest that, as more enzyme is offered, the fraction of available binding sites decreases until the fixed number of sites are covered.

The specific activity of lactase on chitosan-tethered microspheres was not significantly different ($p<0.05$) compared to the free enzyme under optimum conditions (Figure 40 and Figure 41). Optimum pH was shifted to a more acidic condition upon conjugation of the enzyme to the chitosan-tethered support, most likely due to the microenvironmental effects associated with protonated amines near the support surface which results in a more basic localized pH and apparent optimal pH shift associated with the bulk. The effect of pH and temperature on lactase activity indicates that increasing temperature and decreasing pH causes the conjugated lactase to behave more like the free enzyme. This result would be expected if mass transfer limitations exist when moving from extended, solubilized chitosan polymers and high molecular interaction at low pH and high temperature to collapsing, insoluble chitosan (deprotonated amines) and low molecular interactions at increasing pH and decreasing temperature. Though surface carboxylic acid groups are still available after tethering of chitosan (at levels greater than low carboxylic acid polystyrene-co-acrylic acid

microspheres), the negative protein-surface interactions, as determined from specific activity, do not seem to occur. Both spacing between the protein and surface accompanying the chitosan tether, and preferential interaction between the negatively net charged enzyme and positively charged polyglucosamine surface may prevent these negative associations. Both of these explanations are in agreement with previous work (De Maio, El-Masry et al. 2003; Mateo, Torres et al. 2003; Ye, Xu et al. 2005; Ye, Xu et al. 2006; Hamlin, Dayton et al. 2007) These results indicate that a chitosan-tethered surface can promote loading and stability of lactase on carboxylic acid activated supports. Though the system may serve as an alternative to blocking of the enzyme for conjugation to the carboxylic acid activated supports, flocculation of the carrier may present a limitation to the use of the system for some applications that require high dispersability of the support under low mixing force.

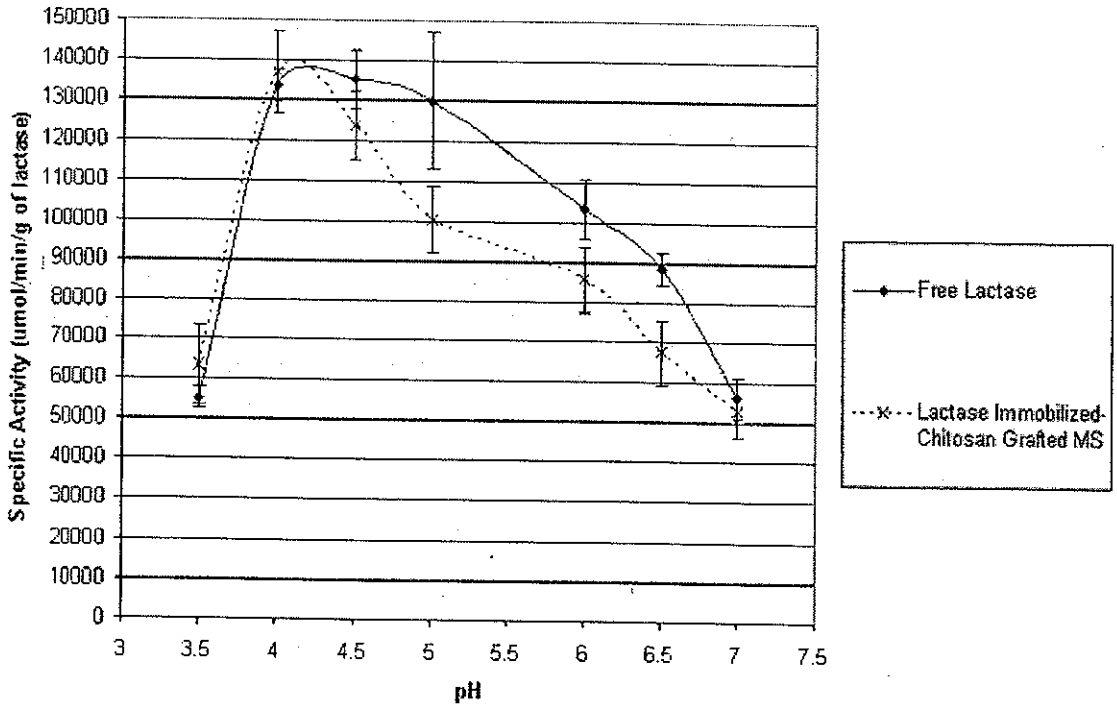

Figure 40. Effect of pH on native and covalently immobilized lactase (*A. oryzae*) on chitosan-tethered microspheres. Activity at 50^{0}C using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

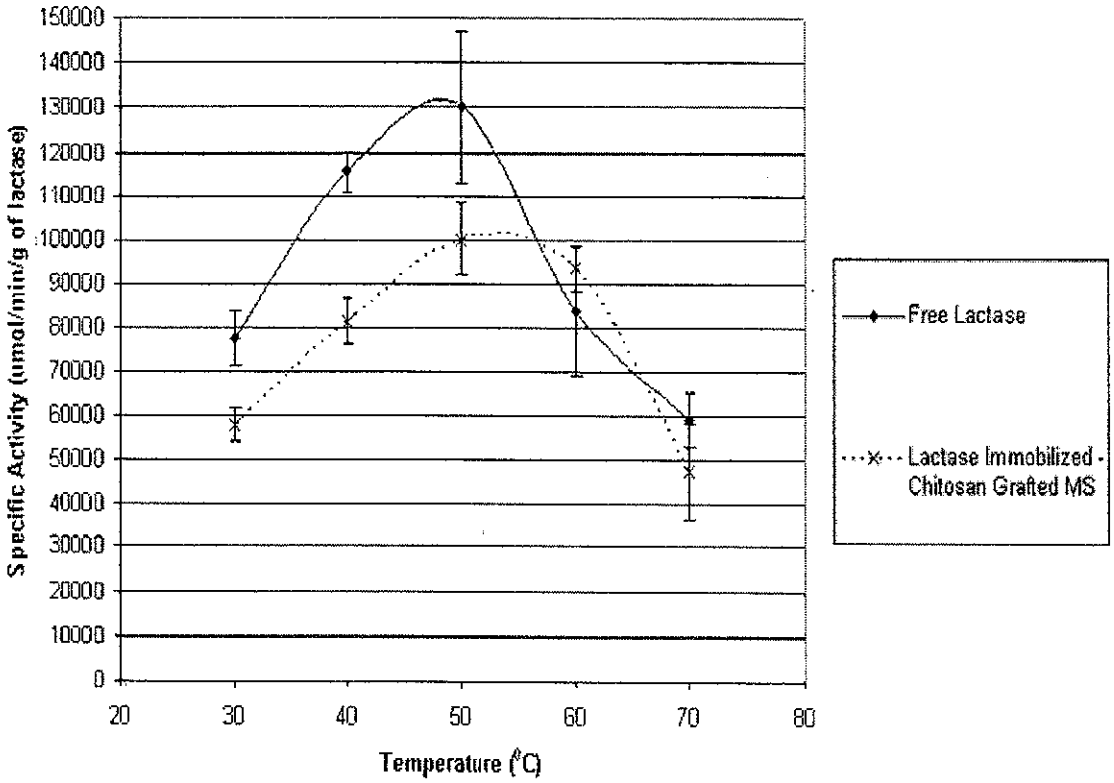

Figure 41. Effect of temperature on native lactase (*A. oryzae*) and covalently immobilized lactase on chitosan-tethered microspheres . Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Values represent mean values ± standard deviation (N=3).

Immobilization of Lactase on Low Density Poly(ethylene) Films

A package that is able to reduce lactose is an area of interest in new product development for dairy applications. Potential markets exist for lactose reduced products though advancements into these markets are limited for small producers because of capital investment and technical input. Employing the package itself as a reactor could produce lactose-reduced fluid milk that is independent of processing variability—requiring only a packaging change at the end of the production line that, during shipping, would produce a lactose-reduced product (Figure 42).

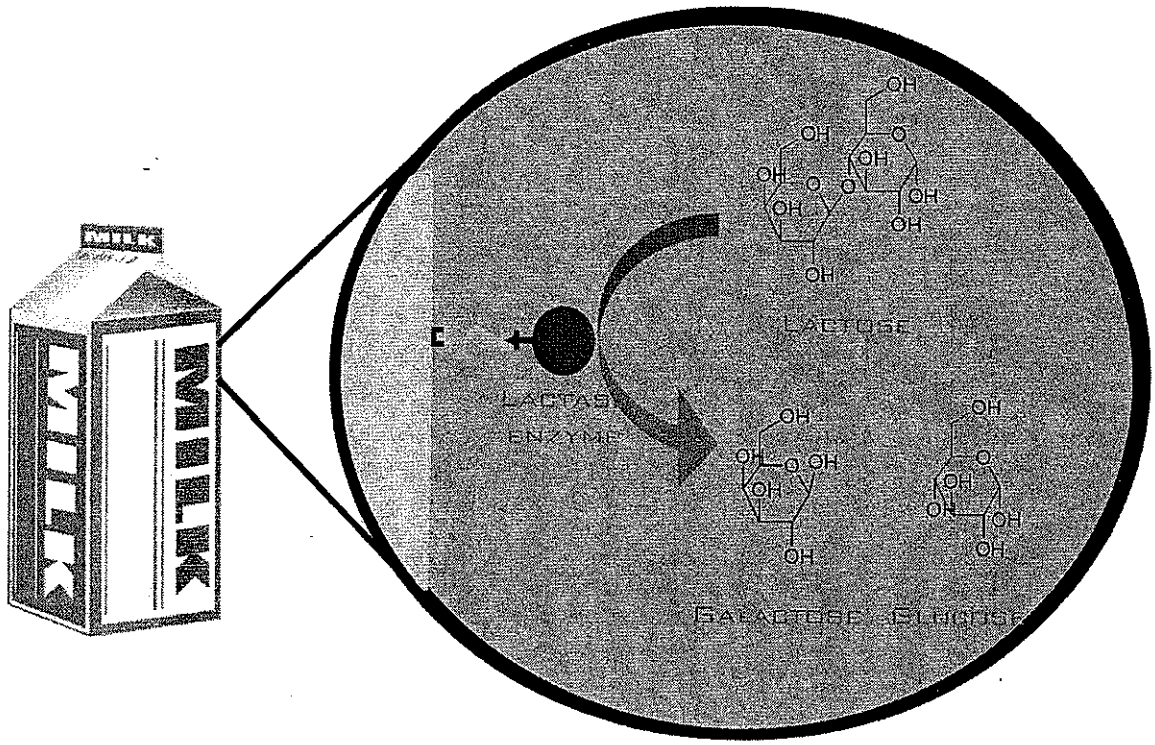

Figure 42. Lactose-reducing milk carton. Adapted from Brody and Bundy, 1995.

Low density poly(ethylene) and poly(ethylene vinyl acetate) make attractive carrier foundations for lactose-reducing films because of the low cost of materials and the inherent use of the films as heat seal layers in such packages—assuring contact of the innermost film surface with the product. Immobilization of proteins to these films; however, is limited because of the physical nature of the polymer, processing conditions that are not amendable to enzyme entrapment, and the inert surface of the films which restricts covalent conjugation chemistries. Oxidation of the surface of the film by plasma oxidation and wet chemical methods has proven to be an effective means of creating functional groups of the surface of the material (Goddard and Hotchkiss 2007); (Bag, Kumar et al. 1999; Aouinti, Bertrand et al. 2003; Steven 2004). Previous attempts have been made to immobilize lactase to carboxylic acid-activated poly(ethylene) for producing a lactose-reducing film (Steven 2004; Goddard,

Talbert et al. 2007) and entrapped within poly(ethylene vinyl acetate) films (Talbert 2004). These methods; however, retain little or no intrinsic enzyme activity. With the knowledge that microenvironmental influences, when conjugated carboxylic-activated hydrophobic microspheres, can be minimized and lactase activity preserved by blocking enzyme carboxylic acid groups, investigations were made into extending this approach to the immobilization of lactase on carboxylic acid activated low density poly(ethylene) packaging films.

Low density poly(ethylene) (LDPE) films were activated using chromic acid to oxidize the surface of the film and generate carboxylic acid groups. X-ray photoelectron spectroscopy verified the presence of oxidative products as indicated by the presence of oxygen in the surface elemental analysis (Figure 43)

	%C	%O
PE	99.6	0.4
PE-COOH	94.4	5.6

Figure 43. XPS composition of polyethylene films.

Contact angle measurement provided qualitative evidence for surface activation (Figure 44). Oxidation of the methyl groups on the surface of the film lowers the surface energy, yielding a more hydrophilic interface and lower surface contact angle. As seen in Figure 44, oxidation of LDPE films reduces the water contact angle of the surface from a mean $99^0$to 52^0—verifying surface modification of the film.

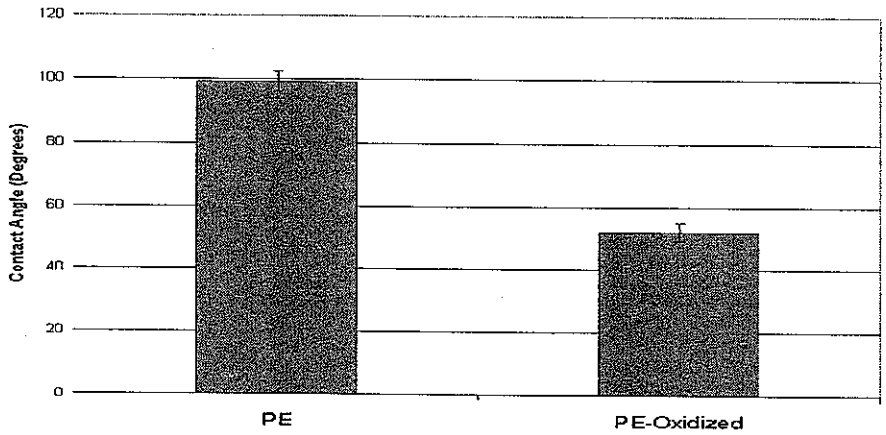

Figure 44. Water contact angle measurements on virgin and oxidized low density polyethylene films. Values represent mean values ± standard deviation (N=3).

Oxidation of the LDPE surface to produce carboxylic acids was quantified using Toluidine Blue O dye adsorption/desorption with the charged surface (Figure 45). As seen in Figure 45, there was a significant increase ($p<0.05$) in the carboxylic acid on the surface of the film, resulting in 2.4 (±0.32)nmol of carboxylic acid groups/cm^2 of film. The amount of carboxylic acid groups indicates more than a monolayer of surface carboxylic groups—indicating etching of the surface.

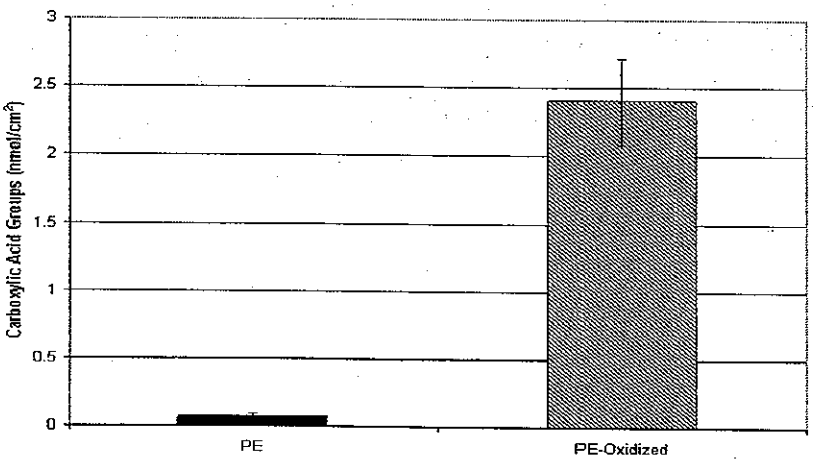

Figure 45. Functionality of polyethylene films. Values represent mean values ± standard deviation (N=3).

Lactase and carboxylic-acid blocked lactase (*A. oryzae*) were conjugated to carboxylic acid-activated LDPE films using carbodiimide chemistry. Protein loading on the carboxylic acid-activated surface ($0.67 \pm 1.7 \mu g/cm^2$) was higher than a monolayer verifying etching of the surface. The specific activity of lactase-immobilized LDPE films was accessed as a function of pH and temperature (Figure 46 and Figure 47).

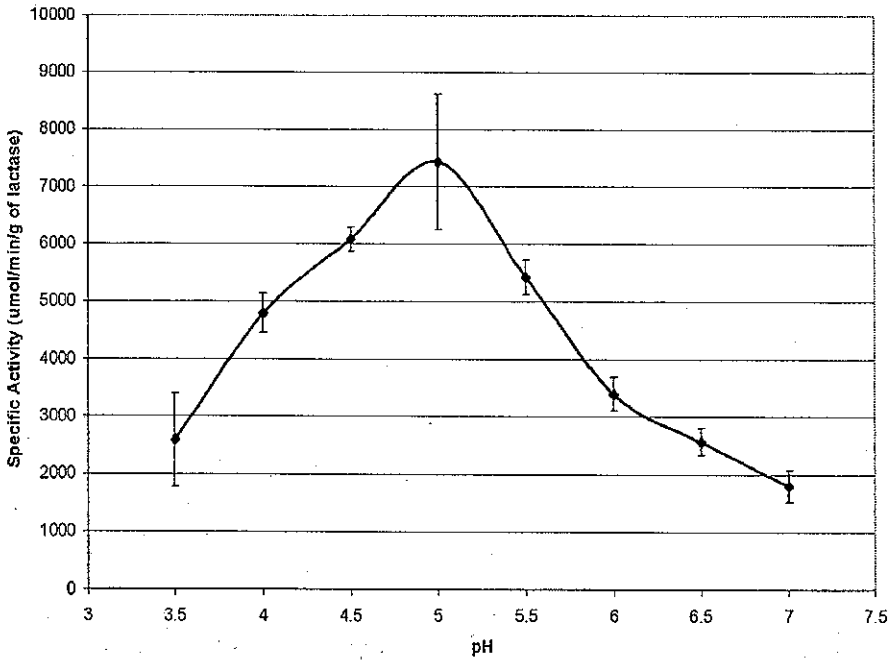

Figure 46. Effect of pH on covalently immobilized, carboxylic acid -blocked lactase (*A. oryzae*) on polyethylene films. Activity at 50^0C using ONPG as enzyme substrate. Unblocked lactase exhibited no measurable activity when conjugated. Values represent mean values ± standard deviation (N=3).

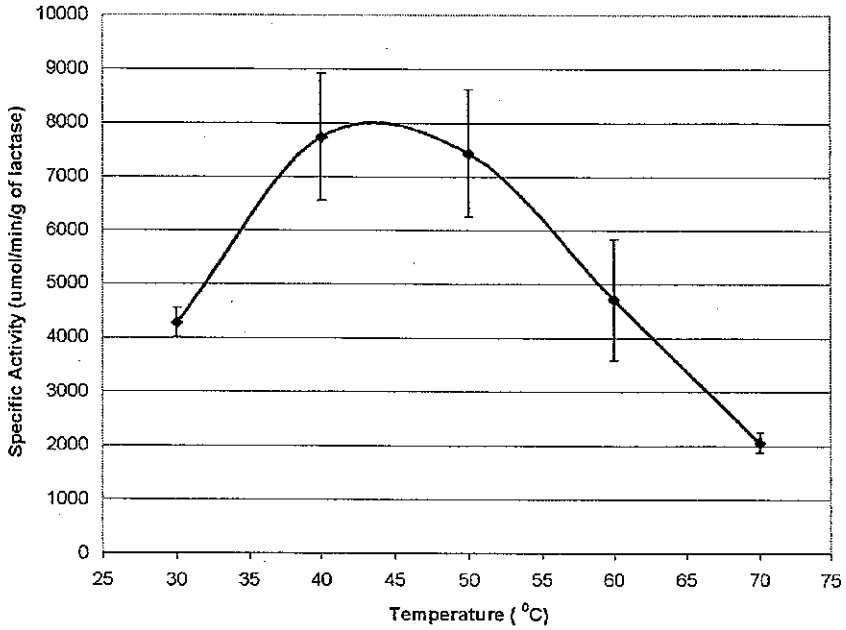

Figure 47. Effect of temperature on covalently immobilized, carboxylic acid-blocked lactase lactase (*A. oryzae*) on polyethylene films . Activity at pH 5.0 (0.1M acetate buffer) using ONPG as enzyme substrate. Unblocked lactase exhibited no measurable activity when conjugated. Values represent mean values ± standard deviation (N=3).

Unmodified lactase on oxidized LDPE films demonstrated no measurable specific activity under conditions of low mass transfer limitations (ie high stirring and excessive bulk substrate concentration). The activity of the enzyme on oxidized polyethylene films is dependent upon the surface of the enzyme and the surface of the film. Blocking the carboxylic acid groups of lactase with glucosamine, as with conjugated polystyrene-co-acrylic acid microspheres, aided in the retention of activity when the enzyme was conjugated to carboxylic acid-activated LDPE—indicating that electrostatic interactions between the carboxylic groups of the oxidized surface and enzyme influences structural and activity stability. For a typical one liter package, immobilization-lactase, under optimum conditions (and assuming unaltered kinetics

when interchanging lactose for ONPG), would take 24 days to reduce lactose in the package by 70%.

Assuming microenvironmental influences were managed, as demonstrated on carboxylic-acid activated microspheres, the dramatic loss of activity must be explained by additional variables. The regularity of a surface as well as the curvature of a carrier as it relates to protein-protein lateral interaction, have been shown to contribute to enzyme activity (Asuri, Karajanagi et al. 2006; Kane and Stroock 2007). Michaelis-Menton kinetics, and, subsequently, an enzyme's intrinsic activity, applies to systems that succumb to Brownian motion and are freely allowed to randomly move and collide with substrate molecules and visa versa (Kawaguchi 2000). The apparent activity of "immobilized" enzymes, when mass transfer limitations are removed (i.e. system is under high stirring), bulk substrate concentration is much greater than enzyme concentration, there are no electrostatic interactions driving substrate interaction, and the intrinsic activity of an enzyme is unchanged upon conjugation, may be owed to the mobility of the carrier (and the enzyme). The Stokes-Einstein equation, which is used to describe molecules in solutions, has been shown to apply to larger particles—including microspheres (Crocker 1997; Norris and Sinko 1997; Bremmell, Wissenden et al. 2001). Jia and colleagues have demonstrated that kinetic parameters are only slightly changed between free alpha-chymotrypsin and the enzyme immobilized to polystyrene nanoparticles with diameters between 110-1000nm (Jia, Zhu et al. 2003). The nanoparticle biocatalysts behaved according to the Stokes-Einstein equation and collision theory. However, when the enzyme was attached to a thin film (free from mass transfer effects) and the Stokes-Einstein equation no longer held, the activity was 1% of the enzyme activity when attached to the 1000nm particles. The authors believed the change in activity was due to hindered mobility of the enzyme. If this is the case, then not only microenvironment but also

mobility/size of the carrier will influence conjugated enzyme activity, which may hinder active packaging applications. As indicated before, the system was conducted under high stirring--limiting mass transfer limitations. The Michaelis constant (K_m) of the immobilized system was evaluated to determine if substrate accessibility influenced perceived activity. A significant increase in K_m was observed when immobilized to the polyethylene films (2.05±0.28mM vs 0.83±0.22mM). Though an increase in Michaelis constant was observed, and can be attributed to the mobility of the carrier as described, previously, the K_m was below the substrate concentration used to determine specific activity (12mM). Because the activity, under the conditions of the assay, is not influenced the substrate concentration of the system, loss of activity must be attributed to alternative factors such as surface morphology caused by etching, surface irregularity, dehydration near the film surface, or oxidative byproducts (ie hydroxyl or aldehyde) groups interacting with the enzyme (Asuri, Karajanagi et al. 2006; Matsuno, Nagasaka et al. 2007).

CHAPTER 5

CONCLUSIONS

- Lactase (*A. oryzae)* could be covalently bound to carboxylic acid, hydrophobic supports by "zero length" carbodiimide-mediated chemistry

- Loss of lactase activity when conjugated to polystyrene-co-acrylic acid microspheres is, predominately, a protein-carrier, surface-surface phenomenon and dependent upon the interfacial density of surface carboxylic acid groups on the support

- Increasing the density of surface carboxylic acid from 87$Å^2$ between surface carboxylic acid groups to 6.2 $Å^2$ between surface carboxylic acid groups results in a 67% and 89% decrease, respectiviely, in enzymatic specific activity relative to free lactase under optimum conditions

- Blocking carboxylic acid groups of lactase with glucosamine ("carboxylic acid blocked-lactase") does not significantly alter specific enzyme activity

- On a per protein basis, conjugation of carboxylic acid-blocked lactase to polystyrene-co-acrylic acid microspheres significantly increases enzymatic activity compared to the conjugated enzyme that was not blocked without significantly effecting the amount of enzyme loaded on the carrier.

- Negative surface-negative surface enzyme-carrier electrostatic interfacial interactions are responsible for loss of enzyme activity upon conjugation

- The addition of excess carbodiimide (EDC), relative to enzyme carboxylic acid groups and in the absence of a nucleophile, results in blocking of enzyme carboxylic acid groups by N-acylurea formation

- On a per protein basis, modification of enzyme carboxylic acid groups with excess EDC prior to attachment yields a significant decrease in free enzyme activity, but a significant increase in enzymatic activity upon conjugation to polystyrene-co-acrylic acid microspheres—verifying negative surface-negative surface enzyme-carrier electrostatic interfacial interactions are responsible for loss of enzyme activity upon conjugation

- Chitosan can be tethered to polystyrene-co-acrylic acid microspheres with amine groups available for the attachment of lactase

- On a per protein basis and under optimum conditions, attachment of lactase to chitosan-tethered polystyrene-co-acrylic acid microspheres yields no significant change in specific activity compared to the free lactase

- Blocking enzyme carboxylic acid groups with glucosamine prior to covalent immobilization of lactase to oxidized low density polyethylene yields an active packaging film with lactase activity.

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