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Enzymatic synthesis and characterization of oligopeptides, co-oligopeptides and hydroxy acid capped polypeptides in mixed aqueous organic media

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ENZYMATIC SYNTHESIS AND CHARACTERIZATION OF OLIGOPEPTIDES, CO-OLIGOPEPTIDES AND HYDROXY ACID CAPPED POLYPEPTIDES IN MIXED AQUEOUS ORGANIC MEDIA

by

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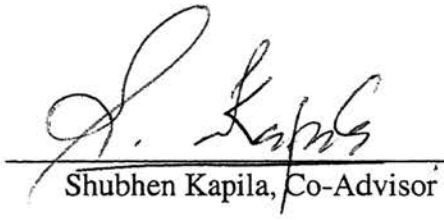

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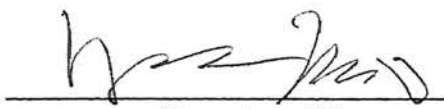

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PUBLICATION DISSERTATION OPTION

This dissertation consists of the following four articles that are being submitted for publication as follows:

Pages 14-44 are intended for submission to the JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Pages 45-79 are intended for submission to the BIOTECHNOLOGY PROGRESS

Pages 80-126 are intended for submission to the JOURNAL OF AMERICAN CHEMICAL SOCIETY

Pages 127-171 are intended for submission to the JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ABSTRACT

Proteolytic enzymes have been used for the synthesis of homo-oligomers and hydroxy acid capped peptides with amino acids and hydroxy acid esters as substrates in aqueous and aqueous organic biphasic and triphasic systems. However, successful incorporation of hydroxy acids in oligopeptide substrates has not been demonstrated. Use of monophasic media comprising of acetonitrile/water in which oligopeptide substrates were soluble was examined for the synthesis of oligomers of neutral and polar amino acids like Met, Tyr, Lys, Gly, Asp and Glu and tailored co-oligomers of Lys-Met and Arg-Met. Effect of reaction parameters such as starting substrate concentration, system composition, reaction time, temperature, and time of addition of second substrate on the peptide synthesis process was evaluated. The HPLC and ESI-MS characterization revealed the formation of oligomers and co-oligomers in yields of ~ 80%. Results also show that systematic change of reaction media composition and sequential addition of amino acid substrates would serve as the best approach for the synthesis of co-oligopeptides of Lys-Met and Arg-Met in acetonitrile/water system.

Use of anti-oxidants during oligomerization is essential. Traditionally mercaptoethanol has been used. However, because of their toxicity, such anti-oxidants pose a problem for utilization. To over come this, the effectiveness of L-Cysteine as an anti-oxidant for protease catalysis was also evaluated. Results show that L-Cysteine can be used as an effective replacement for mercaptoethanol in peptide synthesis. Finally, hydroxy acid capping of peptides was carried out in acetonitrile/water system. Results show that oligopeptides of Met, Phe and Insulin could be readily capped with a hydroxy acid in a monophasic system comprising of acetonitrile/water.

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1. INTRODUCTION

Supplementation of hydroxy analog of Met and amino acid oligomers has received widespread attention as efficient amino acid supplements in cattle and poultry (1, 2). Use of hydroxy acid capped peptides and tailored co-oligopeptides have also been suggested (1, 2). These strategies have been adopted to maximize amino acid availability to the animal for its optimal growth. Enzymatic synthesis of oligomers has been the most common technique for the synthesis of these oligomers and co-oligomers. Such synthesis has been carried in aqueous, bi-phasic and tri-phasic reaction media (1, 2). A brief overview of animal nutrition with emphasis on protein uptake, current approaches for dietary supplementation in farm animals and approaches for peptide synthesis are discussed in the following sections.

1.1 ANIMAL NUTRITION

Nutrition is defined as a series of interrelated steps by which a living organism assimilates and utilizes food for growth and maintenance (3). The discipline that deals with the process of food intake and utilization is called **"Nutrition Science"**. Plants are the primary source of biochemical energy needed for growth and maintenance of tissues and cells in farm animals. Fats and carbohydrates are the primary source of energy in animal feed (4, 5). Glucose, the end product of carbohydrate metabolism, is the most readily available source of energy. Excess carbohydrates are stored in the tissues as body fat (6). Diet also supplies the essential amino acids, minerals, vitamins and other micro nutrients required for the proper growth of animals. Proteins are essential for the structural growth of bones, tissues, tendons, muscles, etc. Lipoproteins are the most important constituents of cell membranes (7).

Protein requirements vary from species to species; requirements also change during different phases of life for the same species based on the growth, maintenance and

reproduction. The highest protein requirements are during growth stage of the animal (8, 9). Digestion of proteins in the diet supplies the amino acid requirements of the animals. Digestion is the process by which large proteins are broken down or hydrolyzed in the GI tract to the constituent amino acids (4). Amino acids are chiral molecules (except Glycine) and can occur in two enantiomeric or mirror image forms. However, only one form (L-Form) is synthesized in higher living forms. Absorption of amino acids occur through the intestinal brush border membrane. Any modification of the terminal ends of the amino acids could alter their absorption across this membrane (7). The absorbed amino acids are then utilized for the growth process or anabolic pathways of metabolism. Deamination and transamination are two important steps in the anabolic pathway (Figure 1.1). The absorbed amino acids undergo oxidative deamination in the presence of amino acid oxidase to form the corresponding α-keto acid analog. In the step of transamination, the amine group from one amino acid is transferred to the α-keto acid (7).

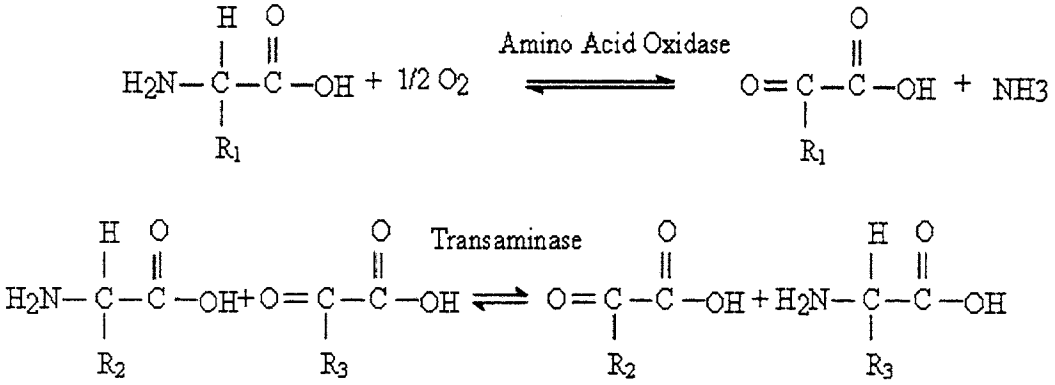

Figure 1.1: Post-absorption metabolism of amino acids

The digestive physiology varies from species to species. For example, in monogastrics species, digestion of ingested proteins takes place in the stomach while absorption of amino acids and short chain peptides take place in the intestine; not everything ingested is hydrolyzed. Monogastrics cannot utilize Non-Protein Nitrogen

(NPN) as a source of amino acids whereas ruminants can convert NPNs to amino acids to partially meet their protein requirements. In such animals, the pre-gut fermentation by rumen microbes converts free amino acids and peptides to short chain fatty acids and ammonia, thus making the free amino acids and peptides unavailable to the animal (10). This alters the amino acids homeostasis in the animal. Portion of proteins in the diet that survive through the rumen is *"High by-pass protein"*. Hence, the total amino acid requirement for ruminants is the sum of microbial degradable protein and ruminally unprotected protein or *"High by-pass protein"* (10). Amino acids are classified as non-essential and essential amino acids. Essential amino acids are those that are not synthesized in the living organism from the α-keto acid precursor (e.g. Lysine, Methionine, Phenyl alanine, Tryptophan). Essential amino acids should be supplemented in the diet in an available form. As a result, the nutritive value of a feed depends not only on its on its essential amino acid content but also on the digestibility, absorbability and composition of the diet (11). Certain essential amino acids are called Limiting amino acids. *"Limiting amino acids"* are the essential amino acids present in the least amount in the diet; the other essential amino acids are utilized only to the extent of these limiting amino acids (11). Lysine and Methionine are the primary limiting amino acids in dairy cattle whereas Arginine and Methionine are limiting in poultry feed (12, 13, 14).

Limiting amino acids may be supplemented through protein-enhanced diet as crystalline amino acids or as amino acid analog (14). Met can be supplemented as its hydroxy analog HMB. HMB is 2-hydroxy-4-(methylthio)butanoic acid. L-amino acids for supplementation are obtained through microbial fermentation, however chemical synthesis yields a racemic mixture containing 50% of L and D- forms. D-form must be converted to the L-form prior to utilization by the animal. It has been shown that supplementation of crystalline amino acids are of limited efficacy in ruminants and poultry due to microbial degradation in the rumen and crop, respectively (15). Some studies have shown that nutritive requirements are met optimally when the limiting amino acids are present in the diet in the required ratio (16).

Alpha hydroxy acids are low molecular weight organic acids that are analogous to amino acids (e.g. Lactic acid, HMB, Malic acid). These molecules contain a hydroxyl group (OH) attached to the α - carbon atom instead of an amine group of an amino acid.

α - hydroxy acids find applications as nutritive supplements, cosmetics and pharmaceuticals. Supplementation of analogs of L-amino acids like α - hydroxy acids is a potentially viable route to impart rumen degradation resistance to the feed. However, the bioavailability of these analogs is dependant on the stereospecific conversion of these analogs to the corresponding α - keto acid and then to the L-amino acid by enzymatic pathways (7). Inefficiencies in absorption and bioconversion limit the bioavailability in this case (17). Any absorbed analog that is not metabolized is excreted in the urine (7). HMB, 2-hydroxy-4-(methylthio)butanoic acid, which is one hydroxy analog that has been used as animal feed supplement is the α - hydroxy analog of methionine. HMB is reportedly more resistant to ruminal degradation than methionine and nearly 40% of HMB escapes as ruminally undegraded fraction and is therefore available for the animal (18). Only less than 10% of the initial Methionine escapes undegraded from the rumen (18). Researchers have shown that HMB is the most effective form of methionine supplement currently available (19, 20, 21). It is sold under the commercial name **ALIMET** by Novus International, St.Louis, MO. This commercial form is a racemic mixture comprising of D, L-HMB with 11% water (19). Synthesis of HMB capped limiting amino acid co-oligomers has been reported (1, 2). It is anticipated that the presence of HMB would increase the by-pass properties of such peptides. In a few studies, these HMB capped oligomers have been shown to be more resistant to enzymatic hydrolysis than the corresponding homo oligomers (22, 23).

As stated above, supplementation of HMB has been in the form of a racemic mixture and the effects of the individual enantiomers have not been studied. Since chemical synthesis yields a racemic mix, the effectiveness of individual enantiomers have not been studied. A few studies indicate that D-HMB is to a certain extent converted to L-Met (18). However, in the absence of studies with enantiopure HMB this claim remains open to debate. Studies with enantiopure HMB have been hampered due to the non-availability of these compounds.

The three most common resolution techniques have been direct crystallization, kinetic resolution and diastereomeric complex formation. A detailed description of these mechanisms and approaches is well-documented and available elsewhere (24, 25). Biological world is the richest source of chiral molecules and hence the most widespread

procedure for enantiomeric resolution has been biocatalyst (enzymes) based kinetic resolution. Enzymes have a specific reactivity towards one form of the enantiomer and the other unreacted enantiomer could be retrieved as enantiomeric excess from the leftover residue (25).

1.2 PEPTIDE SYNTHESIS

The three common techniques used for the synthesis of peptides are chemical synthesis (in bulk and in solid state), recombinant DNA technology and enzymatic peptide synthesis. Each method has its own advantages as well as pitfalls. Solution phase and solid-phase synthesis are the most common methodologies of chemical synthesis (26, 27). Chemical synthesis has advanced over the last decade and is the method of choice for bulk-scale production. However, the lack of stereo specificity of the synthesis step has been the main drawback of chemical synthesis (28). Recombinant DNA technology was mainly used for synthesis of peptides that are difficult to synthesize using chemical or enzymatic route (29, 30). Its main drawback is its high cost (31). Enzymatic peptide synthesis is attractive because of the specificity of enzyme-catalyzed reactions (32). Enzymes have structural, regio and stereo-specificity.

The most important advantages of enzymatic peptide synthesis are 1) Substrate specificity 2) Milder reaction conditions 3) Selectivity and 4) Formation of non-racemic products (33, 34). This specificity may also be disadvantageous at times because a specific enzyme can be used only for a specific reaction. Care must be taken to understand the properties and mechanism of enzymes in choosing an enzyme catalyzed synthetic approach.

Enzymes are biological catalysts essential for the normal functioning of the machinery of life. They catalyze numerous reactions that are too slow under physiological conditions (4, 5). Enzymes are proteins and their purification, crystallization and inactivation parallels those of other proteins. The three most significant and unequalled properties of enzymes are (35):

a. Efficiency: Because of enzymes numerous cellular reactions occur millions times faster than the normal rate

b. Specificity of action

c. Activity of enzyme is subject to regulation

A commission of the International union of Biochemistry on enzymes proposed classification of enzymes into six main divisions based on the reactions they catalyze. These six divisions are Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases and Ligases (36).

Proteases or peptidases belong to the hydrolases class of enzymes. These enzymes catalyze hydrolysis of the peptide bonds. Proteases are integral constituents of cell and nearly 4% of all genetic material in the cell is dedicated to the encoding of these enzymes (35). Proteases are further classified into exopeptidases and endopeptidases. Exopeptidases act on terminal (N or C) peptide linkages while endopeptidases act on internal peptide linkages. Endopeptidases are further subdivided into classes based on the catalytic active site constituents as Serine, Cysteine, Aspartic and Metallo peptidases (36). Under proper physiological conditions, proteases can catalyze the formation of peptide bonds instead of their hydrolysis. This property has been taken advantage of in the synthesis of numerous peptides (37).

Cysteine proteases like papain and serine proteases like chymotrypsin utilize a double addition-elimination reaction mechanism to hydrolyze a peptide bond (38). The two addition elimination steps are the acylation of the active site residue and subsequent deacylation through a nucleophilic attack on the acyl-complex by water molecule. The crucial step of the catalytic process involves formation of a reactive thiolate/imidazolium ion pair (Cys-S–/His-Im+), which results from proton transfer between Cys-25 and His-159 In the first acylation step, the thiolate anion attacks the carbonyl carbon of the scissile peptide bond, and the double bond between the carbon and the oxygen converts into a single one (Fig. 1A). The oxygen assumes a negative net charge allowing formation of the first tetrahedral transition state. The oxyanion is stabilized by hydrogen bonding to the NH groups of Gln-19 side chain and Cys-25 backbone. The tetrahedral intermediate then decomposes to an acyl-enzyme complex and a leaving group. In the second step, involves dissociation of the amine part of the substrate and its replacement with a water molecule. The imidazole nitrogen contributes to polarization of the water molecule that in turn attacks the carbonyl carbon of acyl enzyme. In the final step,

nucleophilic attack by water on the acyl-enzyme complex results in its breakdown and the release of the free carboxyl bearing part of the peptide bond being hydrolyzed, Figure 1.2 (38, 39, 40). The leaving group is represented as Me in the acylation step while Me represents the hydroxy group of water in the subsequent deacylation step.

Aspartic proteases like pepsin and renin utilize a single-addition elimination step for their catalytic action as opposed to the double-addition elimination step preferred by cysteine proteases. The mechanism is different in that there is no covalent attachment of the enzyme active site with the carbonyl group of the peptide (38).

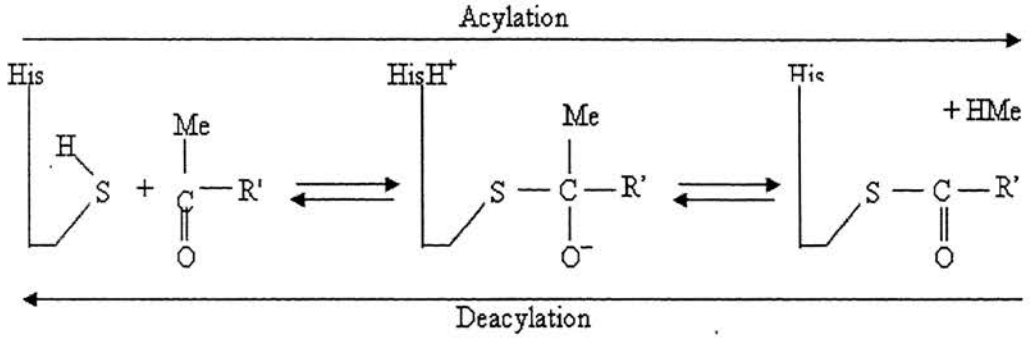

Figure 1.2: Schematic of peptide bond hydrolysis mechanism by papain (38)

An enzyme-substrate complex (held together by physical forces) is formed in this case (38). In the next step, the intermediate decomposes irreversibly to form the products of hydrolysis.

1.3 PROTEASE CATALYZED PEPTIDE SYNTHESIS

Protease catalyzed peptide synthesis has been investigated in aqueous medium for the past seventy years while biphasic system has been used for the past forty years (41, 42). Aqueous systems have been mainly used for the protease-catalyzed synthesis of neutral amino acid oligomers while biphasic systems are preferred for polar amino acids.

The advantages and disadvantages of each reaction medium is well documented and a complete description is available in numerous articles (41, 42, 43).

Enzyme catalyzed reactions were mainly carried out in aqueous or biphasic systems until 1980s because of the preconceived notion that organic solvents destroy the catalytic nature of enzymes (44). However, over the last two decades considerable interest has been shown monophasic aqueous organic solvent systems for esterification reactions and, to some extent, in peptide synthesis. Such systems offer distinct advantages over aqueous or biphasic systems such as

1. High solubility for apolar substrates
2. Little/No microbial contamination
3. Reduction of reverse hydrolysis
4. Easy downstream processing for product recovery
5. Enzymes are mostly insoluble in such systems, thus reducing the need for immobilization

This dissertation is focused on protease-catalyzed synthesis of high by-pass peptides in a monophasic reaction media (acetonitrile/water mixtures).

1.4 OBJECTIVES

The overall objective of this study was to exploit monophasic systems for the synthesis of oligopeptides, tailored co-oligopeptides and hydroxy acid capped polypeptides that find application as nutritive feed supplements. This dissertation is divided into four papers:

1. The first paper deals with papain-catalyzed synthesis of homo-oligopeptides of Met, Tyr, Lys and Gly in acetonitrile/water system. The system composition was optimized and the chiral specificity of the enzyme in the system was also evaluated.
2. The effectiveness of L-Cysteine as a suitable replacement for mercaptoethanol as an antioxidant in papain catalyzed oligopeptide synthesis organic solvent system was studied in the second paper.

3. The third paper is dedicated to studying papain-catalyzed synthesis of tailored co-oligopeptides of Lys-Met in a nominal composition of Lys: Met (3:1) using a sequential addition strategy. The effect of reaction parameters such as temperature, time of incubation, concentration of substrates, time of addition of substrates and system composition on the product composition and profile in an acetonitrile/water system was studied.
4. The fourth paper deals with the protease catalyzed HMB capping of Phe, Met and Lys oligomers. The process was evaluated to separate enantiopure forms of HMB from the commercial formulation ***ALIMET*** sold by Novus International, St.Louis, MO.

A complete survey of research carried out in such mixed aqueous organic solvent systems is described in detail in the next chapter.

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PAPER

I. PAPAIN CATALYZED OLIGOMERIZATION IN MONOPHASIC AQUEOUS ORGANIC MEDIA – SYNTHESIS AND CHARACTERIZATION OF NEUTRAL AND POLAR AMINO ACID OLIGOMERS

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Abstract. Synthesis of oligopeptides is generally carried out in aqueous or bi-phasic reaction media; use of monophasic reaction media is quite limited. In this study, homo-oligopeptides of lysine, glycine, methionine and tyrosine were synthesized through papain-catalyzed reaction in monophasic systems consisting of acetonitrile/water. Reaction conditions were optimized for peptide bond formation and to minimize enzyme denaturation. Such media are especially attractive because they can minimize the secondary and reverse hydrolysis of the acyl complex and the oligopeptide, respectively. The synthesized oligopeptides were purified and characterized by reversed phase liquid chromatography (RPLC) and Electrospray ionization mass spectrometry (ESI-MS). The yields of oligopeptides were approximately 80% for all amino acids. The stereo specificity of papain in acetonitrile/water media was also investigated. The separated enantiomers of methionine were characterized using Chiral HPLC. The results indicate the L-specificity of papain is maintained in monophasic media.

Keywords: Monophasic aqueous organic media; Amino acids; oligopeptides; Papain; Methionine; Tyrosine; Lysine; Glycine; Enantiomers; Liquid Chromatography; Mass Spectrometry

INTRODUCTION

Enzymatic peptide synthesis has gained importance and is a good alternative for chemical peptide synthesis because of its stereo-, regio-specificity and because it does not require side chain protection (*1, 2, 3*). Protease catalyzed synthesis of neutral amino acid homo-oligomers (Met, Tyr, and Leu) in aqueous systems have been reported (*4,5*). Synthesis of such amino acid oligomers in aqueous media is kinetically favored because precipitation of the hydrophobic products that shift the equilibrium towards peptide bond formation is conducive for higher yields (4, *5*). However, yields of polar amino acid homo-oligomers in such reaction systems are very low. Synthesis of polar amino acid homo-oligomers has been carried out more efficiently in low water bi-phasic systems, triphasic, or in nearly anhydrous systems (*4*). Efficient synthesis of di through penta peptides has been reported with modified, immobilized or free protease catalyzed reactions in biphasic systems consisting of water and water immiscible organic solvents such as toluene, trichloroethylene, cyclohexane and ethyl acetate have been reported (*6-12*).

Protease catalyzed synthesis of oligopeptides in monophasic aqueous organic solvent mixtures have received little attention. Such monophasic systems have mainly received attention for protease-catalyzed esterification of amino acids (*14, 15*). The major hindrance for the use of such systems is the denaturation and deactivation of enzymes because of the presence of organic solvent molecules (*16*). By contrast, denaturation is minimal in biphasic or tri-phasic solvent systems because of the absence of direct contact of the enzyme with organic solvents (*17*). Monophasic systems that do not contain denaturing protic cosolvents have a potential for oligopeptides synthesis. Their

advantages include very high solubility for various amino acid and their derivatives used as substrates that are insoluble in many polar solvents. The absence of two phases eliminates the mass transfer barrier encountered in Biphasic, triphasic system and hence leads to a higher reaction rate (*17, 18*). Leu and Met-Enkephalin derivatives have been synthesized in acetonitrile-Tris-HCl (pH 9) buffer (*13*). Organic solvent-stable protease PST-01 has been used for the synthesis of the tripeptide Cbz-Arg-Leu-NH_2; varying yields between ~70% to ~88% have been obtained in water and water-miscible organic solvents such as DMF and DMSO (*1*).

The enzymatic peptide synthesis is a typical two-step process (i.e., formation of an acyl-enzyme complex and nucleophilic attack of the second substrate or water on the complex to form a peptide or hydrolyzed product). Yield of the process is a function of the relative rates of hydrolysis and aminolysis (*19*). When the reaction is carried out in monophasic aqueous organic solvent systems, hydrolysis is greatly reduced; it is comparable to bi-phasic and tri-phasic solvent systems (*20*). The low water content media are highly attractive in kinetically controlled peptide synthesis because the secondary hydrolysis of the product peptide is minimized (*13*). However, in all monophasic systems, a certain minimum amount of water is essential for the catalytic activity of the enzyme (*21*). The effect of water in monophasic reaction mixtures has been quantified in terms of changes in product conversion upon changes in water content. (*22*).

Protease catalyzed peptide synthesis is reported to be stereo-specific (*1, 2, 23, 24*). It is a well-known fact that L and D enantiomers have different biological activity. Enzymatic resolution of L/D forms amino acids has been studied extensively (*23, 24, 25, 26*).

In this study, use of monophasic aqueous organic solvent systems was evaluated for papain-catalyzed oligomerization of both neutral and polar amino acid oligomers. Studies were directed at the synthesis of oligomers of Lys, Met and Tyr with potential application as high by-pass feed supplement in cattle feed and poultry. In addition, synthesis of Gly oligomers that have potential application as anti-bacterial agents was also carried out (*27*). Glycine and glycinate salts have been used as antibacterial agents in foods/drinks against Gram-negative pathogens like *Escherchia coli*, *Enterobacter Sakazakii*, *Salmonella* and *Campylobacter*. Mercaptoethanol was used as the anti-oxidant during the synthesis of polar amino acid oligomers while L-Cysteine was used for the synthesis of neutral amino acid oligomers.

MATERIALS AND METHODS

Materials. L-Methionine ethyl ester (MetEE) hydrochloride was purchased from Fluka Chemical Corp., (Milwaukee, WI). DL-Methionine, L-Tyrosine ethyl ester (TyrEE) hydrochloride, L-Lysine ethyl ester (LysEE) dihydrochloride, 2-Mercaptoethanol, N, N diisopropylethylamine (DIPEA), L-Cysteine, sodium citrate, acetic acid and trifluoroacetic acid were purchased from Sigma Chemical Co., (St.Louis, MO). Ethylenediaminetetraacetic acid (EDTA), sodium salt of Hexane sulfonic acid (HSA), acetonitrile (ACN), and O-Phosphoric acid were obtained from Fisher Scientific, (St.Louis, MO). Glycine ethyl ester (GlyEE) hydrochloride and Dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemical Co., (Milwaukee, WI). Decafluoropentane-1,1,1,2,3,4,4,5,5,5 (DFP) was purchased from Miller-Stephenson chemical company, (Danbury, CT). Papain (EC 3.4.22.2, 25 units activity/mg, 28mg protein/mL) was provided by Novus International Inc., (St.Louis, MO). RPLC

separations of amino acids, esters and oligomers were carried out with a XPERCHROM C-18 column (250mm x 4.6mm), purchased from P.J. Cobert Associates Inc., (St.Louis, MO). Separation of amino acid enantiomers was carried out with a CHIROBIOTIC-TAG (macrocyclic antibiotics) column (250mm x 4.6mm) obtained from Advanced Separation Technologies Inc.(ASTEC), (Whippany, NJ).

Evaluation of papain stability in monophasic solvent system. To evaluate the stability of papain in monophasic system, free papain was added to 7mL clear borosilicate glass vials along with 5mL of the acetonitrile/water mixture. The water content of the mixture was varied from 1% -15% (v/v); the contact period between the enzyme and solvent mix was varied from 0h to 24h. The enzyme was recovered by removing the solvent with a rotary evaporator. The recovered enzyme was evaluated for its activity of Met oligomerization in aqueous system (*4*). The residual ester and the synthesized oligomers were separated by HPLC.

Synthesis of Met oligomers from L-MetEE in ACN/water system. The oligomerization of Met was carried out in acetonitrile/water system; water content of ACN/Water mixture was varied from 15% to 100% (v/v). Three gram of L-Methionine ethyl ester hydrochloride was added to 10mL acetonitrile with varied water content. 1mmole L-cysteine, 0.1mmole EDTA, sodium citrate and 30mg of papain were also added to the reaction mixture. The amount of sodium citrate was varied till 0.01mM. The reaction mixture was incubated for 24h at room temperature. After 24h, the reaction was stopped by heating the mixture at 80^0C for 10 minutes. After deactivation of the enzyme, the reaction mixture was centrifuged and the supernatant was rotary evaporated to near

dryness. The precipitate was lyophilized. A small portion of the dry products was reconstituted in ACN/Water (70: 30) mixture for HPLC analysis.

Synthesis of Tyr oligomers in ACN/water system. The oligomerization of tyrosine was done in ACN/water system under the same conditions used for Met. Only 0.934g of L-Tyrosine ethyl ester hydrochloride was used as the substrate. The residual monomer, ester and oligomers were characterized with HPLC.

Synthesis of Met and Tyr oligomers with other co-solvents. The synthesis of Met and Tyr oligomers were also carried out in DMF/water and DMSO/water monophasic systems under the same conditions used with ACN/Water system

Assessment of relative solubility of Met and Tyr oligomers. The relative solubility of Met and Tyr oligomers was determined in ACN/water mixtures with varying water content (15% -100% (v/v)). 10mg of the oligomer was added to 5mL of the solvent mixture in a 7mL clear glass vial. The mixture was sonicated for 10minutes in a sonic bath. The mixture was then centrifuged and supernatant was filtered through a 0.22μ membrane filter and injected into HPLC.

Synthesis of DL-Met ethyl ester from DL-Met. The synthesis of DL-Met ethyl ester from DL-Met was carried out using a procedure similar to that described by Rajesh et.al. (*28*).

Synthesis of Met oligomers with DL-MetEE substrate in ACN/water system. Oligo- Met synthesis was carried out in a solvent system consisting of 40% water and 60% ACN (v/v). All other additives and reaction parameters were the same as that used for L-MetEE substrate. The separated precipitate from the reaction mixture was washed with nanopure water thrice to remove any residual monomers present in the precipitate

chain because the presence of these adsorbed un-reacted monomers will interfere in establishment of chiral purity. Once the precipitate was free of adsorbed monomers, it was lyophilized. This lyophilized product was hydrolyzed.

Acid hydrolysis of purified oligomers. 500mg of Met oligomers obtained from DL-MetEE substrate were placed in a 40mL vial containing 10mL of 6N HCl. The contents were stirred and kept at 110^0C on a sand bath. After 48h, the reaction mixture was cooled and a 1mL aliquot of the acid solution was taken and transferred to a 25mL round bottom flask. The solution was dried with a rotary-evaporator and reconstituted with 5mL of water. The sample was diluted and analyzed with RPLC to determine the completion of hydrolysis and chiral liquid chromatography to monitor the enantio-purity of methionine obtained from the oligomer hydrolysate.

Synthesis of Lys oligomers in ACN/water system. L-LysineEE dihydrochloride (123mg) was added to 7mL clear glass reaction vials containing 5mL of the solvent with varying water content (2, 4, 7, 10 and 15% (v/v)) in ACN. 100μL of DIPEA, 25μL of mercaptoethanol and 30mg of papain were added to the vials and these vials were then placed in a shaker at room temperature for 24h. The reaction was stopped by heating the reaction mixture to 80^0C for 10 minutes. The supernatant in each case was separated and rotary evaporated to dryness. The precipitate was lyophilized in all cases. Dried products from both the supernatants and precipitates were then reconstituted in 50% ethanol in water, centrifuged, filtered and analyzed with HPLC. Orthogonal information was obtained by analyzing the resultant products with ESI-MS

Synthesis of Gly oligomers in ACN/water system. Gly oligomerization was carried out in a system containing 10% (v/v) of water in ACN. 70mg of GlyEE was

added as the substrate. All other additives were added in the same condition as Lysine oligomerization.

HPLC analysis of oligomers and acid hydrolysate. A model L-7000 HPLC system (Hitachi systems Inc., San Jose, CA) was used for HPLC separations of oligomer product, monomers and their esters. The system consisted of a column oven, a reciprocating piston pump and an autosampler with a 50μL injection loop. The analytes were separated with a reverse phase C-18 column (250mm x 4.6mm i.d) and detected with a fixed wavelength UV detector set at 210nm. Separation of Met and Tyr monomers, esters and oligomers was achieved with a gradient elution, in which the mobile phase composition was changed from 100% A (Water + 0.1% TFA) initial to 80% B (Acetonitrile + 0.1%TFA) in 50 minutes for Met and to 57% B in 33 minutes for Tyr. Separation of residual monomers, esters, oligomers of Lys and Gly was also achieved with gradient elution, in this case the mobile phase gradient was changed from 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) initial to 75% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 50 minutes. DL-Met ethyl ester and Met oligomer acid hydrolysate were separated using a gradient from 100% A (Water + 0.1% TFA) initial to 32% B (Acetonitrile + 0.1%TFA) in 25 minutes. In all cases, the mobile phase flow rate was maintained at 1mL min^{-1} and 10μL of all solutions were filtered through a 0.22μ membrane filter prior to their injection into the column.

ESI (+) -MS characterization of Lys and Gly oligomers. Lys and Gly oligomers were also characterized with direct injection ESI-MS (Model M-8000, 3D-Q ion trap, Hitachi High Technologies, San Jose, CA). An Electrospray Ionization interface was used. The mass range of the 3D Q- Ion Trap mass analyzer was set from 50 –

1600amu. The electrospray capillary voltage was set at +3.5KV. The assistant gas heater temperature was set to 200^{0}C. The desolvator temperature and the aperture-1 temperature of the MS system were 200^{0}C and 150^{0}C, respectively. The 3D Q- Ion Trap mass analyzer was scanned from 50 – 1600amu. For such characterization, the oligomers were dissolved in ethanol/water mixture (50: 50) to form a nominal concentration of 0.5mg/mL solution. The solution was introduced into the MS with a syringe pump (Harvard Apparatus) at a flow rate of 1mL/hr. A make-up solution (50% acetonitrile in water with 0.1% acetic acid) was infused along with the sample at a flow rate of 0.2mL/min.

Analysis of Met enantiomers. The separation of amino acid enantiomers was carried out with a CHIROBIOTIC-TAG column (250mm x 4.6mm). The column was installed in a Model L-7000 HPLC system (Hitachi High Technologies Inc., San Jose, CA). The separation was achieved under isocratic elution with water-acetonitrile (50:50) mobile phase maintained at a flow rate of 0.2 mL min^{-1}. The effluent was monitored with a fixed wavelength UV detector set at 210nm.

RESULTS AND DISCUSSION

Stability of papain in monophasic system. The stability of papain in a 95% ACN/ 5% water solution was studied through its exposure for a period ranging between 2h to 24h. After each exposure period, enzyme was recovered from the solvent mix and introduced into a aqueous system optimized for synthesis of Met oligomers. Results obtained with virgin enzyme (not exposed to ACN) and enzyme exposed for 4h and 24h respectively in ACN/Water systems are shown in ***Figure 1(A, B, C)***. The chromatograms show no marked deactivation of papain after exposure to a high concentration of ACN. The percent yield of Met oligomers calculated using the formula mentioned below:

Percent yield = {(AA-EE)$_{initial}$ - (AA-EE + free AA)$_{final}$}/ (AA-EE)$_{initial}$ *100

remained the same (~80%) under all exposure conditions. Most of the peptide syntheses described in this article were carried out at 24hrs incubation times; thus, the effect of ACN on the activity of the enzyme was not monitored beyond this time. Thus the results clearly show the exposure of papain to ACN at this concentration and for no more than 24 hrs does not denature the enzyme.

Synthesis of Met and Tyr oligomers in ACN/water system. Once the stability of papain in ACN/water was established, it was used for the synthesis of Met and Tyr oligomers in ACN/water solvent mixtures with a water content varying from 15% to 100% (v/v). The oligomerization of Met and Tyr was also attempted in other water /organic solvent systems including water/DMSO and water/DMF. However, papain showed no activity towards Met and TyrEE in these systems. The concentration of residual substrate remained the same over a period of 24h indicating the absence of oligomerization. Such systems have been shown to be suitable for oligomerization of amino acids with subtilisin (*29, 30*). HPLC chromatograms of Met and Tyr oligomers synthesized in two ACN/water systems (15% (v/v) water/ 85% v/v ACN and 40% (v/v) water/60% ACN) are shown in ***Figures 2 and 3***. The chromatogram (***Figure 2***) clearly shows the presence of several peaks eluting after the retention time of Met and MetEE. These peaks correspond to oligomers of Met ranging from dimer through nanomer. The peak assignment for Met, MetEE and di-methionine were based on retention time matching while that of higher oligomers was in part based on the ESI-MS spectral information for each individual peak. Analogous results were obtained with Tyr. In this case, the oligomers ranged from dimer to decamer. The presence of oligomer peaks in

the chromatograms of the supernatant recovered from the reaction mixture in case of both Met and Tyr (***Figure 2 (i, ii)*** and ***Figure 3 (i, ii)***) indicate that they have a finite solubility in the ACN/Water. This solubility of peptides in the supernatant serves to distinguish such monophasic solvent systems from other systems, such as aqueous system where these neutral oligomers completely precipitate out. This is very significant if these hydrophobic peptides are chosen as substrates for further polymerization.

The relative distribution of Tyr oligomers obtained in ACN/water mixtures for different water content is shown in ***Figure 4***. The results show that the composition of oligomers with different ACN/water mixtures was nearly the same with 9-11 residues being dominant. Similar results were obtained with Met, however in this case the dominant oligomers were the hexamer through nanomer. These results show that ACN/water system bears some resemblance to aqueous systems (*4*), however higher oligomers were obtained in monophasic system for both Met and Tyr. This is most likely related to the higher solubility of oligomers in ACN/water system. Because of the increased solubility, higher oligomers are available to act as nucleophile in ACN/water systems whereas they completely precipitate out in aqueous systems. The obtained result correlates with the well-accepted acyl-intermediate mechanism for catalytic action of proteases (*19, 31*). As the size of the oligomer chain increases, it cannot fit into the active site of the enzyme to form acyl-intermediate complex. The ester group remains intact in all cases and hence they can act as substrates for further oligomerization. This is important because it is a well-established fact that ester or amide derivatives are thermodynamically more favorable (esters/amides have much higher energy) than the free carboxyl substrates in peptide synthesis (*30*).

The percent oligomer yield was calculated on the basis of the initial ester amount and the residual monomer and ester left in the reaction mixture after the completion of the reaction, using the formula mentioned above. The yield as a function of (%v/v) water content is shown in ***Figure 5***. The overall trend was similar in case of both Met and Tyr. Minor improvements were noticed in the yield when water concentration was increased beyond 60% (v/v). The percent yield for both Met and Tyr increases with increasing water content and reaches a maximum at 100% water. These results are in contrast with the results obtained during the trans-esterification of amino acid esters with immobilized papain in ACN/Water mixtures, where the yield increased with increasing water content and then decreased beyond a certain maximum value (*15*). It has been speculated that the decrease in yield at high water contents when immobilized enzymes are use is due to the increase in thickness of a water layer around the enzyme that acts as a diffusional barrier (*15, 29*). In the present case, the reaction is moved forward because of the enhanced precipitation of oligomers, which drives the kinetics of the reaction forward. Protease catalyzed synthesis in ACN/Water system shows a behavior that is similar to that observed in aqueous systems with increasing water content; where the higher availability of water increases the intrinsic reaction rate, through precipitation of the product. The absence of oligomerization when no water was added to the organic solvent is in good agreement with published results. This is due to the insolubility of the enzymes in completely anhydrous solvents (*21*). Results show that such systems should be amenable for addition of amino acid esters or other hydroxy acid moieties in reasonable yields.

Stevenson and Storer (*15*) found that the amino acid substrate must have its amine group deprotonated to participate in papain-catalyzed oligomerization reactions (*15*).

They based their conclusion on the fact that acidification of the reaction medium is eliminated by a buffer concentration that minimizes electrostatic forces. Therefore, we decided to study the effect of sodium citrate buffer concentration on the reaction rate and yield. Reactions carried out in the absence of sodium citrate or in amounts less than 0.01mmole did not show appreciable oligomerization.

The relative solubility of methionine and tyrosine oligomers in ACN/Water system was also studied. The result of relative solubility of Met oligomers as a function of water content is shown as a graphical representation in ***Figure 6***. Results showed a slight increase in solubility for when the water content was increased from 15% to 40% for the oligomers studied (2-8mers). Further increase in water content resulted in a dramatic decrease in solubility of these oligomers. Solubility in the absence of ACN was less than 1%. This data can be used for choosing a solvent composition that would be favorable for modification of oligopeptide substrates in monophasic solvent systems. The solubility of Tyr oligomers in ACN/water system was similar to Met oligomers (Plot not shown).

Enantio-specificity of papain in ACN/water mixture was assessed through the oligomerization of DL-MetEE as substrate in 40% water/60% acetonitrile (v/v) using the same reaction medium used for L-Met ethyl ester. After the enzyme was deactivated, oligomer yields were determined at set time periods by monitoring the residual esters and monomers. Results of the study show that oligomerization was complete by 24h and yield, based on the formula mentioned earlier was ~42%. These results are in agreement with results obtained with DL-MetEE substrate in aqueous systems (*24*). 42% yield vs 80% for L-MetEE is indicative of enantiospecificity of papain. Orthogonal HPLC

confirmation of enantio-specificity of papain was obtained by hydrolyzing the oligomer precipitate. The RPLC separation of acid hydrolysate obtained from purified oligomers is shown in ***Figure 7.*** The chromatogram shows the presence of a single peak corresponding to methionine indicating the completion of hydrolysis. The monomer from acid hydrolysate was characterized using a chiral HPLC to determine its enantio-purity. The chiral separation of oligo-methionine acid hydrolysate is shown in ***Figure 8.*** The chromatogram shows a clear separation of the enantiomers. The peak for L-Met was dominant with trace D-Met present in the oligomer precipitate. The enantiopurity of the hydrolysate was greater than ***95%*** L-Met; thus showing papain maintains its stereo specificity in monophasic reaction media.

Synthesis of Lys oligomers in ACN/water system. The yield of Lys oligomers determined on the basis of residual monomers as a function of water content is shown in ***Figure 9***. The plot shows that the maximum yield (~65%) is obtained at ~10% (v/v) of water. Once water content increases beyond 10%, the system behaves in a manner similar to aqueous systems and hydrolysis of the oligomers results in almost no oligomerization. The oligomer yields are lower in the case of hydrophilic amino acid oligomers when compared to hydrophobic amino acid oligomers because papain has a strong selectivity for peptides with hydrophobic side pockets than that of hydrophilic ones. This high specificity for a bulky hydrophobic group at the second position of the active site (P_1' position) is due to the presence of multiple flanking sub-sites in the binding sites of enzymes (*32*). Here again, the importance of water is clearly seen, with no oligomerization noticed under anhydrous conditions.

The RPLC separation of precipitated Lys oligomers synthesized in a monophasic system (7% water in acetonitrile) is shown in ***Figure 10***. The supernatant consisted of mainly the dipeptide. The chromatogram contains a series of peaks eluting after Lysine ethyl ester. These peaks correspond to the esterified residues of oligo-lysine. Similar results were obtained from 4% to 10% water. The ESI-mass spectrum of the synthesized oligomers is shown in ***Figure 11***. The spectrum contains series of ions that are 128 amu apart. This mass difference corresponds to the repeating Lys moiety. The dominant ions appeared at ***m/z 303, 431, 559, 687, 815 and 943.*** These ions correspond to the oligo-lysine residues with intact ester at the C-terminal (^{N}Lys – $(Lys)_n$ - Lys^{COOEt} + H^+). A tetramer of lysine, ^{N}Lys – $(Lys)_2$ - Lys^{C} + H^+ should appear at a ***m/z* 531** while a pentamer ^{N}Lys – $(Lys)_3$ - Lys^{C} + H^+ should appear at a ***m/z* 659.** These ions though present in the spectra were less dominant. This is in sharp contrast to the free acid intact oligomers resulting from synthesis in both bi-phasic (*6*) and triphasic system (*4*). The oligomers synthesized in ACN/water systems should be good substrates for further peptide synthesis, eliminating an additional esterification step. Hence in the case of polar amino acids like Lys, the oligomer yield increases, as the water concentration is decreases because of a reduction of hydrolysis and because of a decrease in product solubility, which drives the reaction forward. The distribution of Lys oligomer residues was estimated for varying amounts of water. From ***Figure 12***, it is quite evident that oligomers with 3-7 residues dominate the product.

Synthesis of Gly oligomers in ACN/water system. Oligomers of Gly were synthesized in a 10% (v/v) Water/Acetonitrile system optimized previously for oligo-lysine. The supernatant and the precipitate separated from the reaction mixture were

characterized using HPLC and ESI (+)-MS. Quantification of Gly and Gly ethyl ester left in the supernatant showed that the ~ 73% of the substrate was oligomerized. The RPLC separation of the precipitate obtained from the Gly oligomerization reaction is shown in ***Figure 13.*** The chromatogram shows a series of peaks at retention times longer than Gly ethyl ester, indicating the formation of oligomers of glycine. The precipitate was also devoid of residual Gly and Gly ethyl ester. The glycine oligomers were identified through ESI-MS spectra of the precipitate solution in water: ethanol (50:50) mixture. The positive ion ESI-MS spectrum is shown in ***Figure 14***. The spectrum shows the presence of ions at ***m/z 275, 297, 332,389, 446, 503, 582, 582, 617, 674 and731***. The ions in series **275 – 731**show a mass difference of 57, which corresponds to the Gly residue [NH- CH_2 – CO]. Mass calculation shows that ions correspond to $(Gly)_4$ – EE to $(Gly)_{12}$-EE. Sodiated ions $(Gly)_4$-EE Na^+ [m/z 297] and $(Gly)_9$-EE Na^+ [m/z 582] are also observed in the spectrum.

Table 1 summarizes the yield and relative distribution for polar and neutral amino acid oligomers in ACN/Water system and compares it with the results obtained for Met oligomerization in aqueous system (*5*) previously reported. There was no oligomerization of Met and Tyr when DMF and DMSO were used in the solvent system. The yields and solubility of hydrophobic amino acids do indicate that 40% (v/v) of water in acetonitrile should serve as an optimum condition for peptide synthesis. In the case of Lys and Gly, 5 to 10% (v/v) of water was found to be optimal. These results show that by manipulation of water content, one single system could be used for the oligomerization of both polar and neutral amino acids. Another important aspect is the presence of ester intact oligomer residues in ACN/water system as compared to biphasic system, where the free acid form

of the oligomer is the dominant product. As mentioned earlier, esters are more amenable substrates than free acids if these oligomers are chosen for further modification.

Conclusions. Papain catalyzed oligomerization of hydrophobic and hydrophilic amino acids in monophasic aqueous organic media were successfully carried out. This proves the potential of papain to catalyze peptide formation in organic solvents. Our results also prove that papain maintains its activity in ACN/Water monophasic systems contrary to the belief that they lose their activity (*16, 17*). The studies on enantio-selectivity also show that papain maintains its stereo specificity in monophasic system. Studies on enantio-selectivity of papain for polar amino acids should aid in obtaining pure D-form of amino acids, which is also important. Our results also show the utility of monophasic system for synthesis of co-oligopeptides tailored for specific amino acid composition. The synthesis could be carried out with water content favoring the oligomerization of one substrate and then altered to favor the incorporation of the second substrate.

LIST OF ABBREVIATIONS

Met, Methionine; Tyr, Tyrosine; Lys, Lysine; Gly, Glycine; HPLC, High Pressure Liquid Chromatography; RPLC, Reverse Phase Liquid Chromatography; ESI-MS, Electrospray Ionization Mass Spectrometry; EDTA, Ethylenediaminetetraacetic acid; HSA, Hexane sulfonic acid; TFA, Trifluoroacetic acid; UV/Vis, Ultraviolet/Visible; DMSO, Dimethyl sulfoxide; DMF, Dimethyl formamide; ACN, Acetonitrile.

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FIGURES

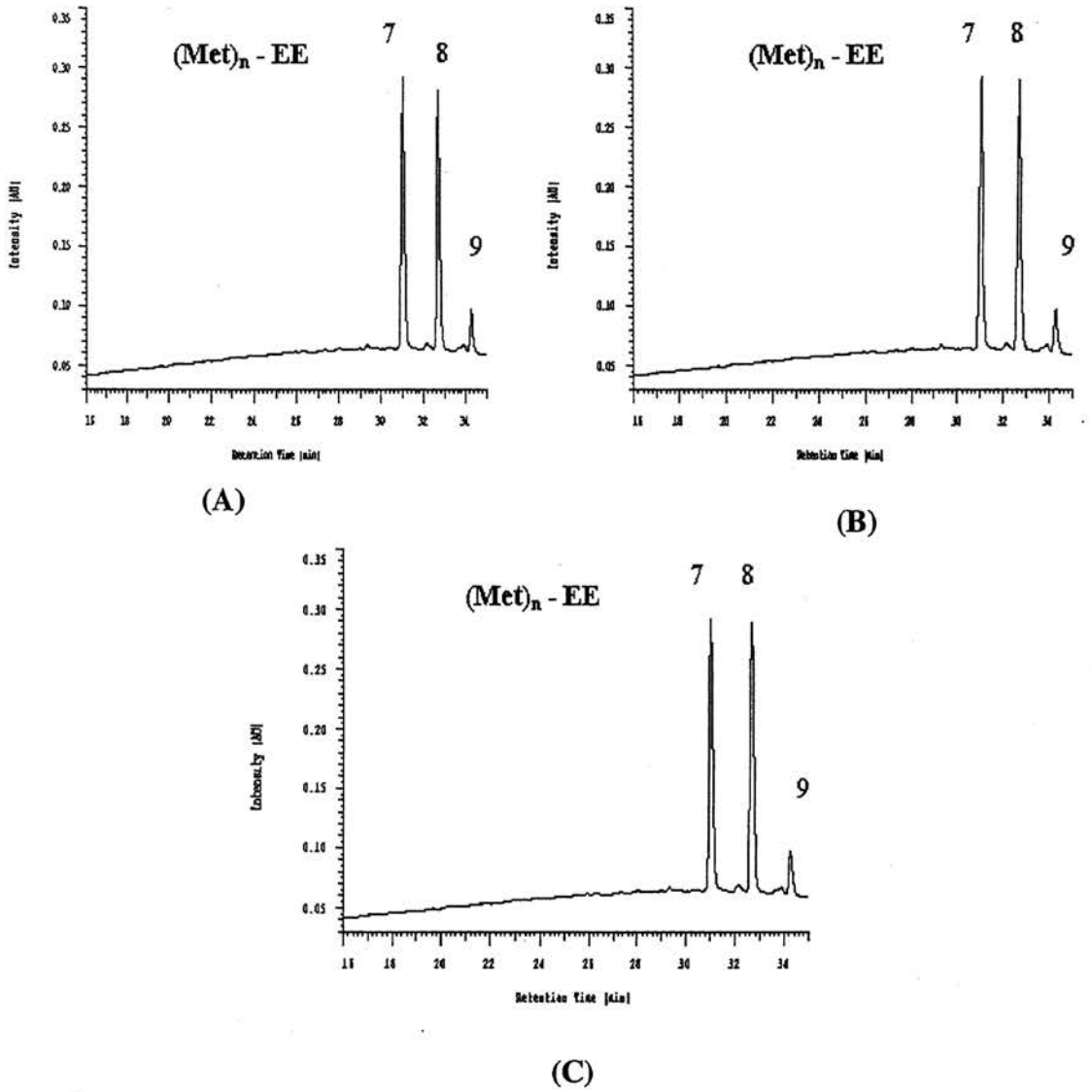

Figure 1: Chromatogram of methionine oligomers synthesized in aqueous system with enzyme recovered from 95% acetonitrile/ 5% water system incubated for (A) 4h and (B) 24h. C) Chromatogram of Met oligomers synthesized with enzyme recovered from 100% water system. Separation was achieved with a RPLC C-18 column using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 80% B (Acetonitrile + 0.1%TFA) in 50 minutes.

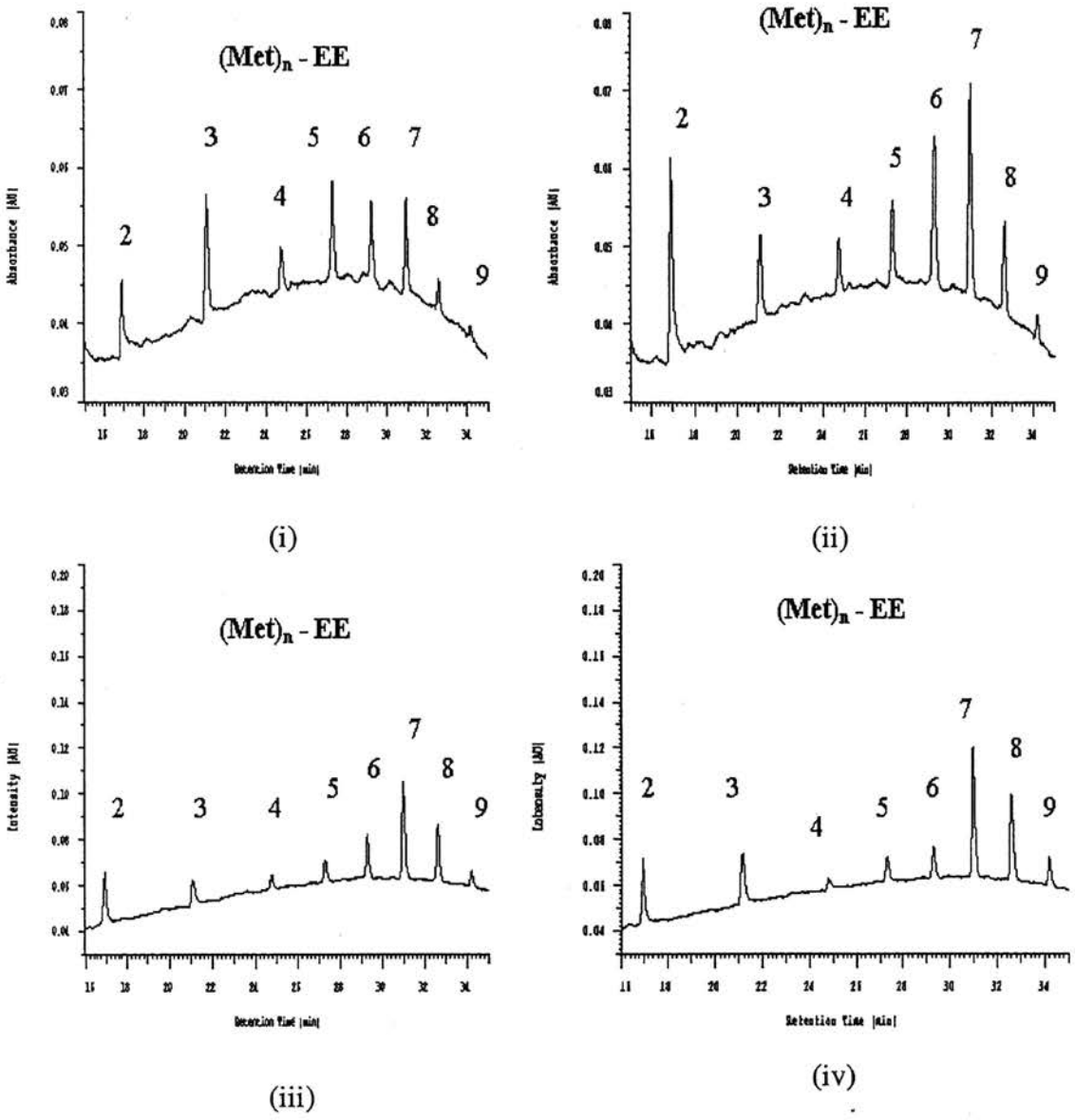

Figure 2: Chromatogram of methionine oligomers synthesized in acetonitrile/water system. i) Supernatant of 15(%v/v) water/acetonitrile ii) Supernatant of 40(%v/v) water/acetonitrile iii) Precipitate of 15(%v/v) water/acetonitrile iv) Precipitate of 15(%v/v) water/acetonitrile incubated for 24hours. Separation was achieved with a RPLC C-18 column using a mobile phase gradient mentioned in Figure 1.

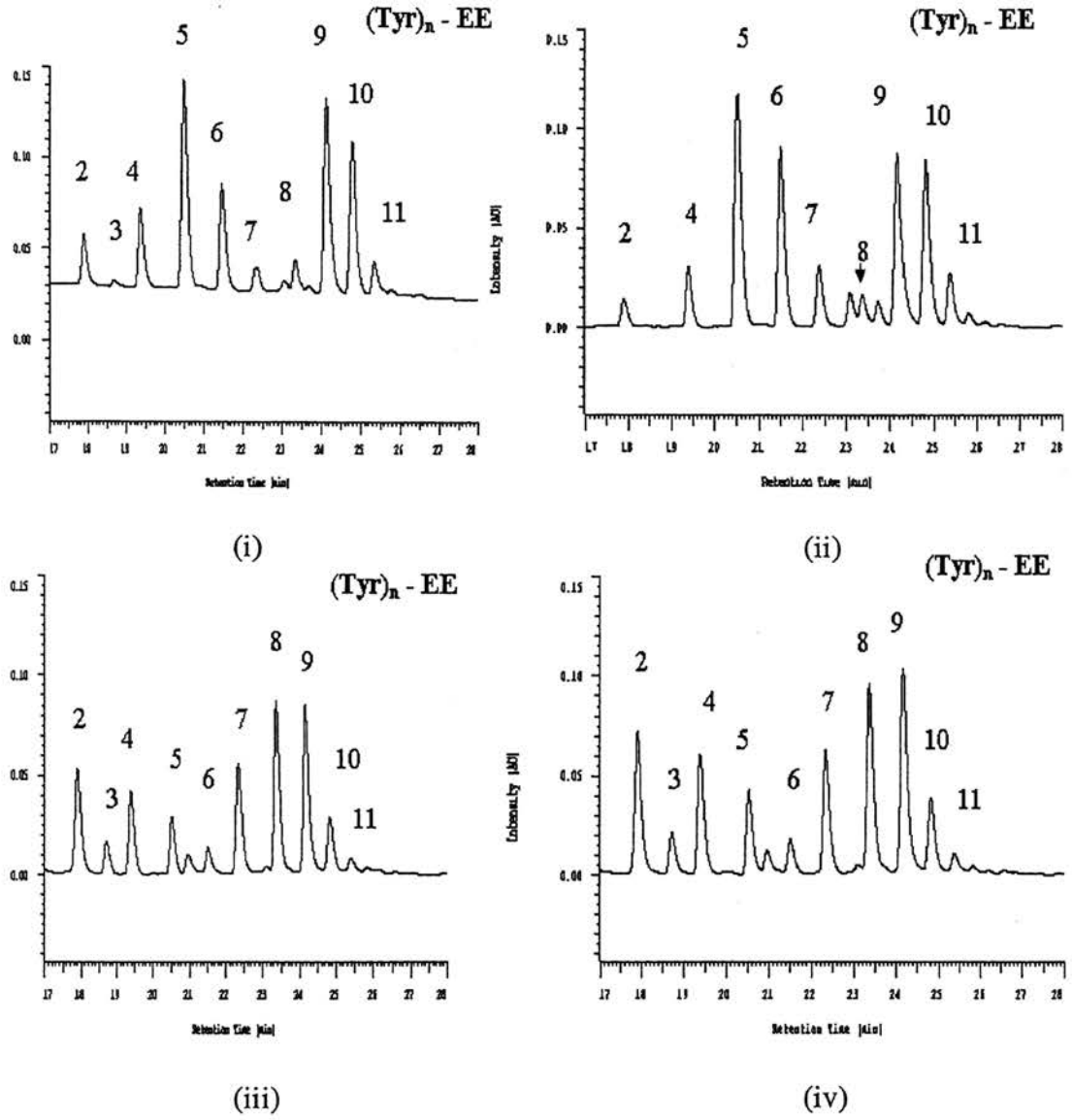

Figure 3: Chromatogram of tyrosine oligomers synthesized in acetonitrile/water system. i) Supernatant of 15 (%v/v) water/acetonitrile ii) Supernatant of 40 (%v/v) water/acetonitrile iii) Precipitate of 15 (%v/v) water/acetonitrile iv) Precipitate of 40 (%v/v) water/acetonitrile incubated for 24hours. Separation was achieved with a RPLC C-18 column using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 57% B (Acetonitrile + 0.1%TFA) in 33 minutes.

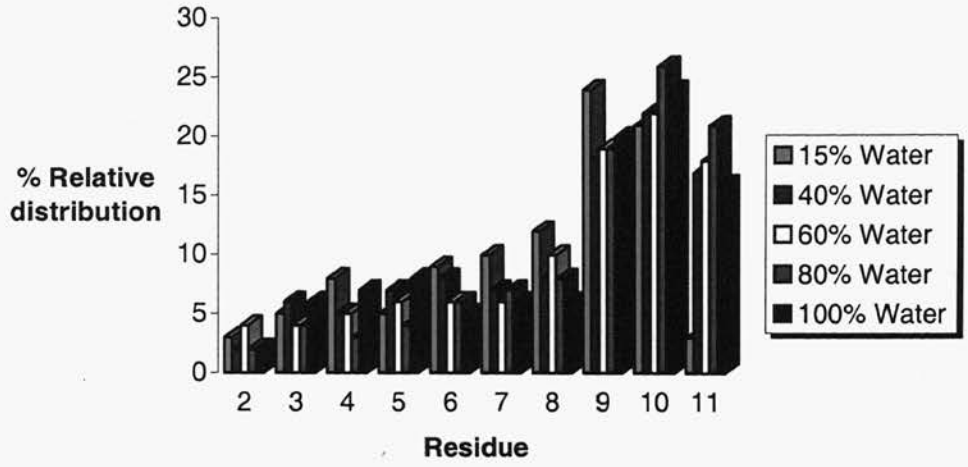

Figure 4: Relative distribution of tyrosine oligomer residues synthesized in acetonitrile/water solvent system as a function of varying water content.

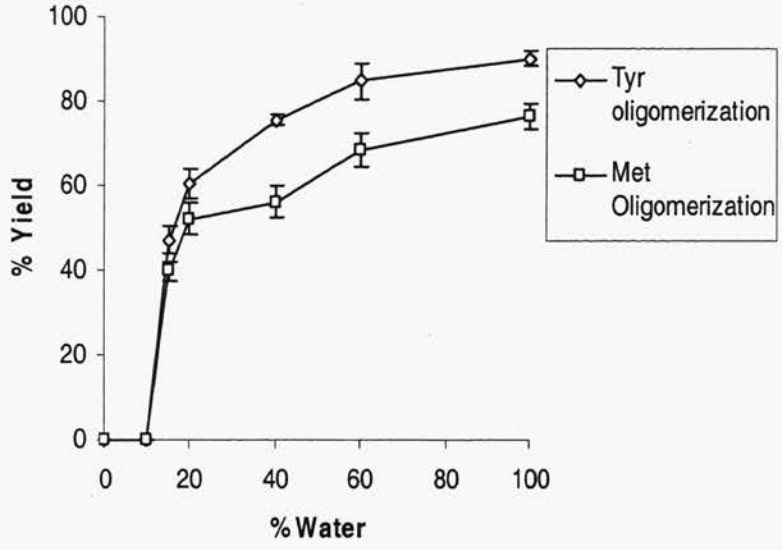

Figure 5: Percent yield of Met and Tyr oligomers synthesized in acetonitrile/water solvent system as a function of varying water content.

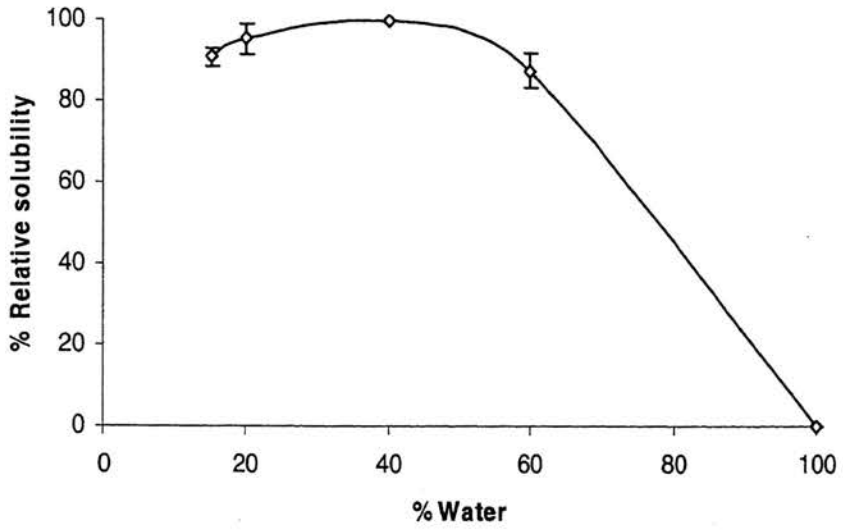

Figure 6: Relative solubility of Met oligomers in acetonitrile/water solvent system with varying water content.

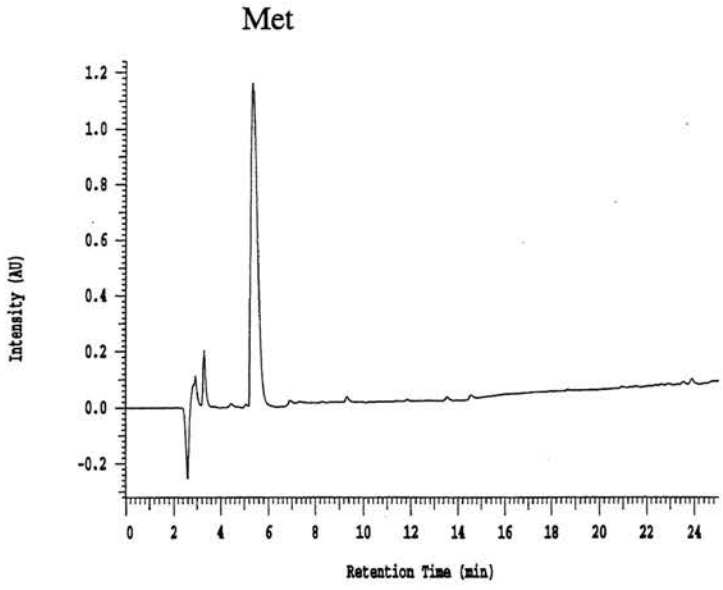

Figure 7: RPLC-18 separation of acid hydrolysate obtained from purified oligo-methionine precipitate synthesized in 40 (% v/v) water/acetonitrile system. The chromatogram shows a peak at a retention time of 6 minutes corresponding to Methionine indicating complete hydrolysis of the oligomers. Separation was achieved with a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 32% B (Acetonitrile + 0.1%TFA) in 25 minutes.

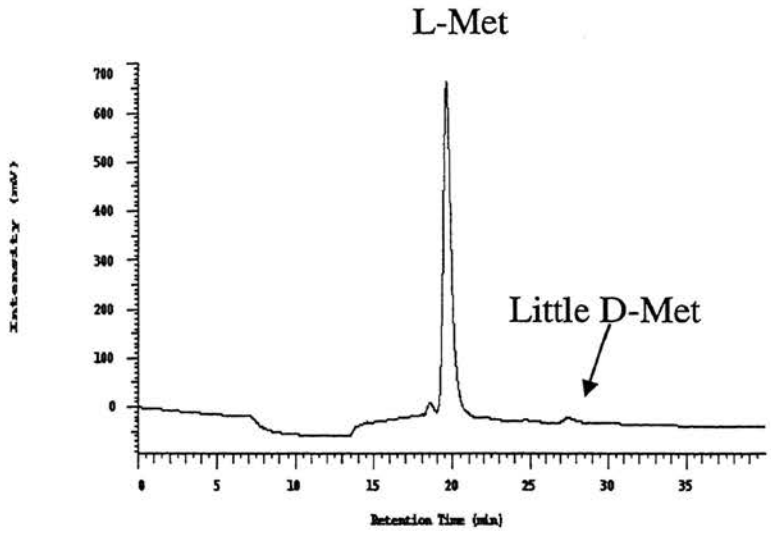

Figure 8: Chiral separation of acid hydrolysate obtained from purified oligo-methionine precipitate synthesized in 40 (% v/v) water/acetonitrile solvent system. The chromatogram shows a peak at 20minutes retention time corresponding to L-Methionine and a small peak at 27minutes corresponding to D-Methionine. The separation was achieved using a CHIROBIOTIC-TAG (Teicoplanin) column with an isocratic elution of 50:50 ACN/water. The enantiomeric excess (e.e) was calculated to be >95%.

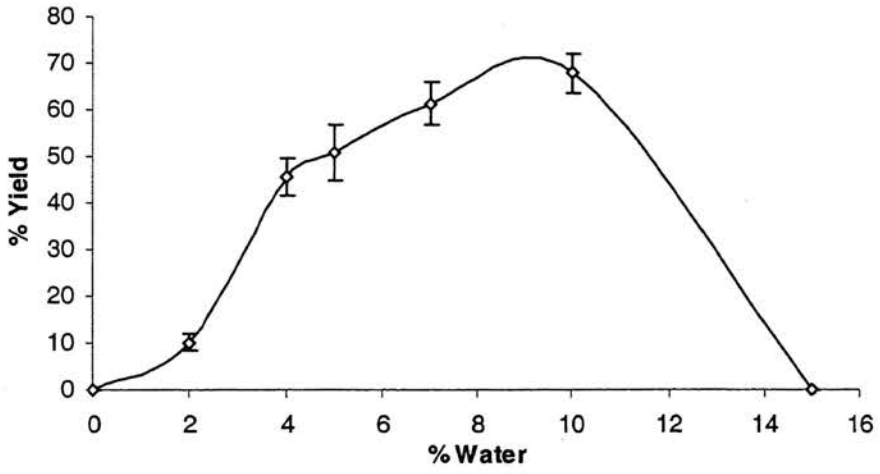

Figure 9: Percent yield plot for lysine oligomers synthesized in acetonitrile/water solvent system as a function of varying water content.

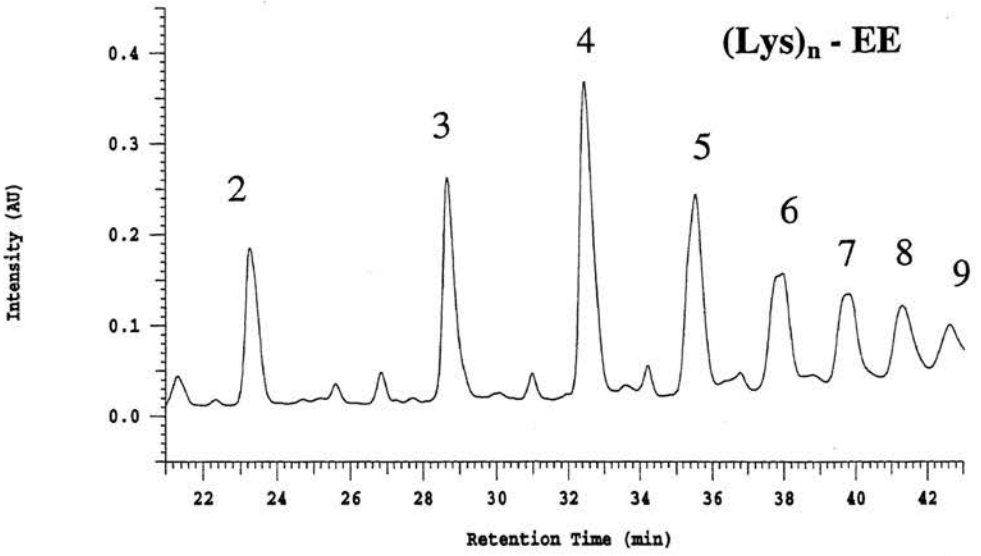

Figure 10: Chromatogram of precipitated lysine oligomers synthesized in 7 (%v/v) of water/acetonitrile system for 24h incubation. Separation was achieved in a RPLC C-18 column with a mobile phase gradient comprising of 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) initial to 75% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 50 minutes.

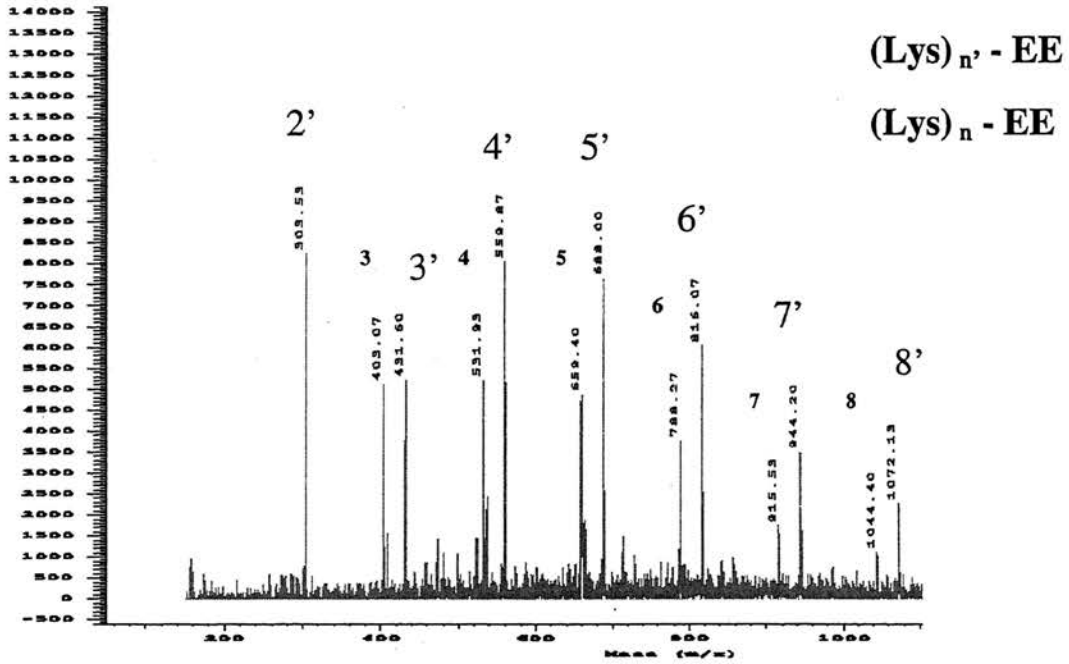

Figure 11: Mass spectra of precipitated lysine oligomers synthesized in 10 (%v/v) of water/acetonitrile system after 24h incubation obtained through direct injection ESI (+) – MS. The spectrum shows the presence of series of peaks corresponding to oligo-Lysine.

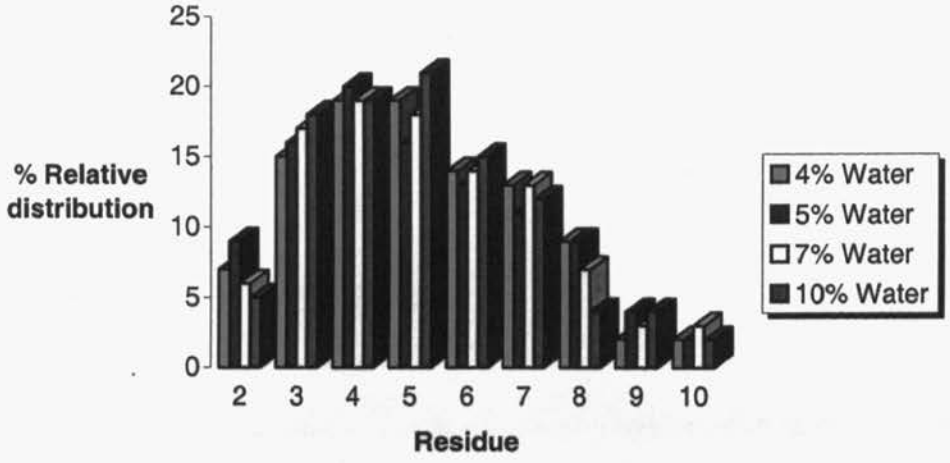

Figure 12: Relative distribution of lysine oligomer residues synthesized in acetonitrile/water solvent system as a function of varying water.

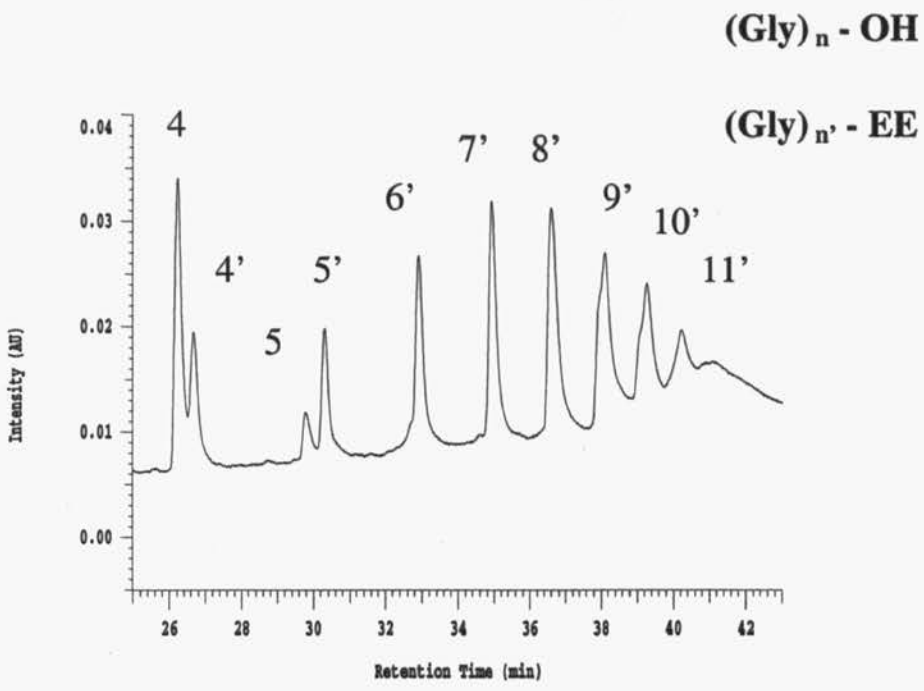

Figure 13: Chromatogram of glycine oligomers synthesized in 10(%v/v) of water/acetonitrile system after 24h incubation. Separation was achieved with a RPLC C-18 column using a mobile phase gradient mentioned in Figure 10.

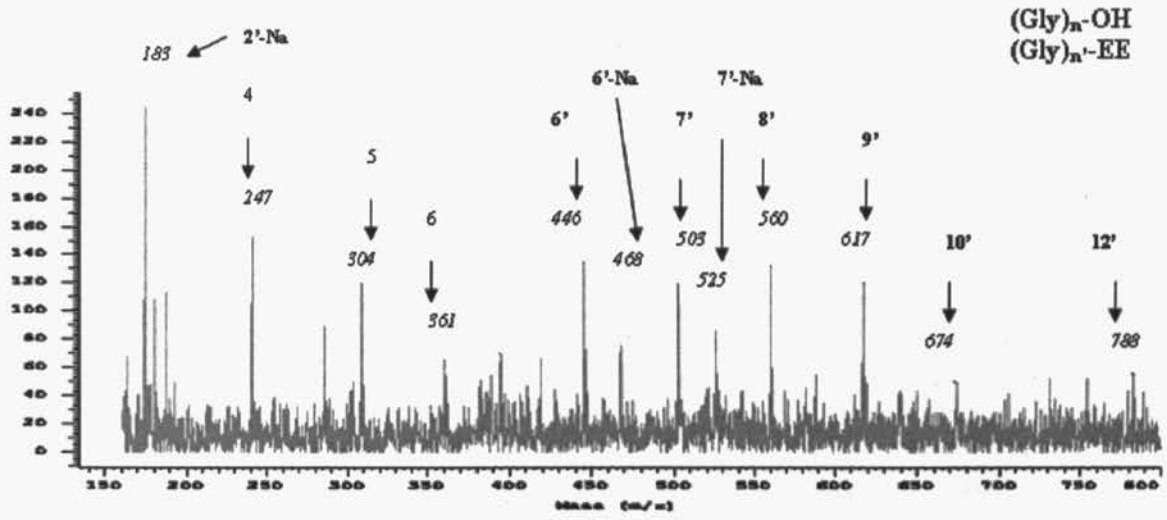

Figure 14: Mass spectra of precipitated glycine oligomers synthesized in 10 (%v/v) of water/acetonitrile system after 24h incubation obtained through direct injection ESI (+) – MS. The spectrum shows the presence of series of peaks corresponding to oligo-glycine.

TABLES

Substrate	Optimal water composition (%)	Yield at optimal composition (%)	Dominant residues
Met	> 40	75	6-8
Tyr	> 40	85	9-11
Lys	10	65	3-6
Gly	10	70	4-8
Met (**Jost et.al**) – (*5*)	100	80	6-9

Table 1: Comparison of oligomerization of neutral and polar amino acids in ACN/water system.

II. EFFICACY OF L-CYSTEINE AS AN ANTI-OXIDANT IN PAPAIN CATALYZED SYNTHESIS OF OLIGOPEPTIDES IN ORGANIC SOLVENT SYSTEM

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Abstract. Enzymatic peptide synthesis has drawn considerable attention for synthesis of high by-pass oligopeptide feed supplements in animal nutrition. A hardy protease, (papain) with a cysteine moiety at the active site requires the presence of an anti-oxidant in the reaction medium to ensure that the thiol group remains intact. Free cysteine has been the antioxidant of choice for papain-catalyzed synthesis of oligopeptides in aqueous systems. However, due to limited solubility of cysteine in organic solvents, it is generally not a suitable antioxidant for the synthesis of oligopeptides in biphasic solvent systems; instead mercaptoethanol is often used. The inherent toxicity of mercaptoethanol when present even at trace amounts would make its use undesirable during synthesis of oligopeptides to be used as feed supplements. Use of non-toxic antioxidants or anoxic condition should be more prudent for such synthesis.

Therefore efficacy of L-cysteine as an anti-oxidant was investigated during papain catalyzed oligomerization of Lys, Arg, Glu and Asp in two organic systems: a three phase micro-aqueous media consisting of n-octane, DFP and water; as well as homogeneous ACN / water mixtures. Reactions were also carried out under an argon atmosphere in the presence and absence of anti-oxidants. The results of the experiments showed that L-cysteine facilitated oligomer synthesis in both the three phase system and the ACN / water mixture. The overall oligomer yields were found to be better than 75% in the presence of L-Cysteine. Oligopeptide yields obtained through reactions carried out under the argon atmosphere were less than 20%.

Keywords: Lys, Met, Arg, Asp, Glu, Papain, L-Cysteine, mercaptoethanol, Reverse-Phase Liquid Chromatography, Electro-spray Ionization Mass Spectrometry.

INTRODUCTION

Primary function of proteolytic enzymes (proteases) is to catalyze hydrolysis of the peptide bond, however, under the right conditions these enzymes can also catalyze the synthesis of the peptide bond (*1, 2, 3, 4, 5*). Papain belongs to a class of thiol proteases that have a cysteine residue at their active center. The activity of these proteases is based on a catalytic diad of cysteine and histidine (*6, 7*).

Oligomers of Lys and Arg have potential application as high by-pass feed supplements in cattle-feed and poultry nutrition. Several studies have shown the optimal requirements of these amino acids in the diet and a complete discussion of the same is beyond the scope of this article and is available elsewhere (*8, 9, 10, 11*). Glu and Asp are di-carboxylic amino acids that are acidic at physiological pH (*12*). Glu is one of the key molecules in cellular metabolism and can serve as metabolic fuel in the body when released as a consequence of hydrolysis of dietary proteins (*13, 14, 15*). It is present in high amounts in the blood and a small amount of it can easily permeate through the blood-brain barrier providing a fuel source for the brain (*16*). Glu is also the most abundant excitatory neurotransmitter in the mammalian nervous system (*17, 18*). It helps to treat mental retardation, muscular dystrophy and ulcers also (*19, 20*). The sodium salt of Glu has a brothy taste at neutral pH and is used as a food additive to enhance the flavor of foods. Glu oligomers can be used in the fields of food and medicinal chemistry as a masking additive for bitter compounds in many foods (*21*). These highly ionic peptides can also find applications as functional property modifiers. Asp also plays a crucial role in generating cellular energy (*22, 23*). Asp may provide resistance to fatigue and thus lead to endurance as a neuro-transmitter (*24, 25*), although the evidence to support this

idea is not strong. Asp is found in dairy, beef, poultry, sugar cane and molasses (the artificial sweetener aspartame is made from Asp and Phe). People with low proteinaceous diet or with eating disorders or malnutrition may develop an Asp deficiency and experience extreme fatigue or depression. Asp also helps in the removal of harmful ammonia from the body (*26*). Glu and Asp oligomers have a potential use as slow release source in the food and medicinal chemistry (*21*). Glu and Asp oligomers can serve as slow release source of Glu and Asp. Aqueous solvents are not suitable for enzymatic synthesis of these peptides because of the increased solubility of these hydrophilic oligomers in water (*27*).

Peptide synthesis catalyzed by cysteine proteases requires the presence of an antioxidant in the reaction medium to maintain enzymatic activity (*28, 29*). Free L-Cysteine has often been used as anti-oxidant during papain catalyzed oligopeptide synthesis in aqueous media, however, due to its limited solubility in organic solvents employed in the two phase systems during synthesis of polar amino acid oligopeptides mercaptoethanol has been used as antioxidant instead. However, mercaptoethanol is unsuitable for the synthesis of oligopeptides that are used in feed due to its toxicity. Mercaptoethanol is toxic (LD-50 190 mg kg^{-1} in mice) and its presence in oligopeptides even at trace level can render oligopeptides unsuitable as feed supplements (*30*). Hence, it is desirable to evaluate a non-toxic anti-oxidant in organic solvent systems for peptide synthesis. We studied the use of L-Cysteine as a replacement for mercaptoethanol for papain catalyzed peptide synthesis in two organic solvent systems: a micro aqueous tri-phasic media and a monophasic media.

Papain catalyzed synthesis of Leu, Met, Phe and Tyr oligomers in citric acid buffer in the presence of L-Cysteine has been reported, and oligomer yields range between 51 to 96% (*31, 32*). It has been shown that papain can catalyze oligomerization of Met and Tyr in a monophasic system comprising of acetonitrile and citrate buffer with L-Cysteine as the reducing agent (*33*). Papain catalyzed synthesis of a protected dipeptide BocGly-PheOMe in the presence of L-Cysteine has been carried out in an aqueous organic two-phase systems consisting of different solvents like carbon tetrachloride, trichloroethylene, toluene and benzene (*34*). Mercaptoethanol was used as an antioxidant in papain catalyzed hydrolysis and amino acid incorporation into BSA and Zein (a protein from corn) in low water organic media (*35*). Mercaptoethanol has also been used for papain stabilization during the synthesis of Leu-Enkaphalin precursors in ethyl acetate saturated with Mes/NaOH buffer and Tris/HCl buffer (*36, 37*). Dithiothreithol has also been used as an anti-oxidant during papain catalyzed oligopeptide synthesis in water/water immiscible organic solvents such as hexane, toluene and chloroform (*38*).

Oligomers of Lys, Arg, Glu and Asp were synthesized in a three-phase system consisting of n-octane, DFP and water and also in ACN / water mixture. The efficacy of antioxidants (mercaptoethanol and L-Cysteine) and use of anoxic conditions on the oligopeptide yield and degree of oligomerization was evaluated. Concentration of L-Cysteine required for maintaining enzyme activity was optimized.

MATERIALS AND METHODS

Materials. L-Lysine ethyl ester (LysEE) dihydrochloride, L-Arginine ethyl ester (ArgEE) dihydrochloride, L-Aspartic acid (Asp), L-Glutamic acid (Glu), L-Cysteine hydrochloride monohydrate (Cys), n-Octane, 2-Mercaptoethanol, N, N

diisopropylethylamine (DIPEA) and acetic acid were purchased from Sigma Chemical Co., (St.Louis, MO). Sodium salt of Hexane sulfonic acid (HSA), anhydrous ethanol (200 proof), O-Phosphoric acid, acetonitrile and ethanol (HPLC grade) were obtained from Fisher Scientific, (St.Louis, MO). 1,1,1,2,3,4,4,5,5,5-decafluorpentane (DFP) was purchased from Miller-Stephenson Chemical Company (Danbury, CT). Papain (EC 3.4.22.2, 25 units activity/mg, 28mg protein/mL) was provided by Novus International Inc., (St.Louis, MO). Argon (Grade 336) was purchased from Oz-Arc/gas, Rolla, MO. RPLC separation of oligomers was carried out with a XPERCHROM C-18 column (250mm x 4.6mm) obtained from P.J. Cobert Associates Inc., (St.Louis, MO). Nanopure water used in the experiments was obtained after filtration through a Synergy 185 filtration system purchased from Millipore Corp. (Billerica, MA).

LC with UV detection. A model L-7000 HPLC system (Hitachi High Technologies America, San Jose, CA) was used to carry out the HPLC separations. The system consisted of a reciprocating piston pump (L-7100) fitted with a column oven (L-7300), autosampler (L-7200) and with a 50μL injection loop. The analytes separated on reverse phase columns were then introduced into a UV-Vis absorbance detector (L-7420).

ESI-Mass Spectrometer. An Ion Trap Mass Spectrometer equipped with an Electrospray ionization interface (Model M-8000) purchased from Hitachi High Technologies America, San Jose, CA was used for the mass analysis of the synthesized oligomers.

Synthesis of basic amino acid oligomers under anoxic conditions. The synthesis of Lys and Arg oligomers was carried out in an anoxic condition using argon as the degassing agent. 25mL of DFP, n-octane and water were taken in separate 100mL

round bottom flasks and purged with argon for 15 minutes. From these degassed solvents, 2.5mL of DFP, n-octane and 0.5mL of water were added to another 25mL round bottom flask that was already purged with argon for 15 minutes. To this, 123mg of L-LysEE was added. 100μL of degassed DIPEA was added to this reaction mixture. Purging was continued for another 15 minutes. After purging, 30mg of papain was added and the reaction flask was sealed with stopper cock-Para film. The reaction media was incubated in a shaker for a 24h. After 24h, the reaction was stopped by heating the contents at 80^0C for 5 minutes. The reaction product was rotary evaporated to dryness. The resulting product was reconstituted in 50% ethanol, diluted, centrifuged, filtered and injected into HPLC for product characterization. Yield was calculated based on the amount of residual monomer left in the reaction product after the incubation period. Similar procedure was used for Arg oligomerization except that 137 mg of L-ArgEE was added as the substrate. A similar approach was used for the oligomerization of Arg and Lys in a 5mL ACN/water system consisting of 10% water. In this case, the supernatant and precipitate were separated, dried and reconstituted in 50% ethanol/water solution for HPLC characterization.

Synthesis of basic amino acid oligomers in the presence of L-Cysteine. L-LysEE dihydrochloride (123mg – 0.5mM) was added to a 7mL clear glass reaction vial containing 2.5mL of DFP, 2.5mL of n-octane and 0.5mL of water. 100μL of DIPEA, 30mg of papain, and. 20mg of L-Cysteine were also added. The reaction vial was placed in an incubator shaker for a period of 24h. The reaction was stopped by heating the reaction mixture to 80^0C for 5 minutes. The reaction product was rotary evaporated to dryness. The dried product was reconstituted in 50% ethanol in water, centrifuged,

filtered and analyzed in HPLC, to determine the amount of residual monomers and characterize the oligomers. Arg oligomerization was also carried out in the same procedure except that 0.5mM ArgEE was used as a substrate in that case. Lys and Arg oligomerization was also attempted in 5mL of ACN/water mixture containing 10% water in the presence of L-Cys anti-oxidant. Other reaction conditions and additives were the same as used for three-phase system.

Synthesis of Asp and Glu esters. Asp and Glu were esterified with anhydrous ethanol in the presence of HCl gas using a procedure described by Rajesh (*28*). The synthesized ester was rotary evaporated to dryness. The dried ester was reconstituted in water, centrifuged, filtered and injected into RPLC for characterization. The percent conversion of monomers to ester was calculated based on the amount of residual monomers left in the product after the esterification reaction.

Synthesis of acidic amino acid oligomers in organic solvent system. Glu and Asp oligomers were synthesized from their corresponding esters in three-phase and monophasic solvent systems. The synthesis followed the same procedure as in Lys and Arg oligomerization. In this case, either 0.5mM Asp-diester or Glu-diester was added as the substrate. For oligomerization in monophasic system, 0.5mM Glu-diester/Asp-diester substrate was added to a 7mL clear glass vial containing 0.5mL of water and 4.5mL of acetonitrile along with 100μL DIPEA and 30mg of papain. The reaction mixture was incubated in a shaker for 24h. After the incubation period, the mixture was heated at 80^{0}C for five minutes to deactivate the enzyme. The supernatant was separated and rotary evaporated to dryness and the precipitate was lyophilized. The dried products were reconstituted in 50% ethanol, centrifuged, filtered and injected into HPLC for

determining reaction yields based on the residual monomer. Product distribution information was obtained by injecting the dried precipitate into ESI (+)-MS. In both solvent systems, either 20mg of L-Cysteine or 25μL of 2-mercaptoethanol was added as the reducing agent to maintain papain stability.

HPLC analysis of synthesized oligomers. The separation of residual Lys, Arg, Asp, Glu monomers and Lys, Arg oligomers was carried out with a reverse phase C-18 column (250mm x 4.6mm i.d, 5μ) and detected with a fixed wavelength UV-Vis detector maintained at 210nm (Hitachi High Technologies, San Jose, CA). A gradient elution was used, the mobile phase gradient was changed from 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) to 75% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 50 minutes for the separation of residual monomers of Lys, Arg and the corresponding oligomers. In case of Asp and Glu oligomers, the separation of residual monomers was again carried out using a gradient elution with a mobile phase gradient from 100% A (Water + 5mM HSA +0.1% O-Phosphoric acid) to 60% B (50% acetonitrile + 5mM HSA +0.1% O-Phosphoric acid) in 30 minutes. The mobile phase flow rate was maintained at 1mL min^{-1} and 10μL of the sample after filtration with a 0.22μ membrane filter was injected into the column in all the cases.

ESI (+)-Mass Spectrometric characterization of acidic amino acid oligomers. Asp and Glu oligomers were characterized by injecting them directly into an ESI-MS (M-8000, Model 3D-Q ion trap, Hitachi high Technologies, San Jose, CA). An electrospray ionization interface was used. The mass analyzer was scanned from 50 – 1200amu. The operating parameters of the MS were as follows: Electrospray capillary voltage, +3.5KV; detector voltage, 400V; assistant gas heater temperature, 200^{0}C; desolvator temperature

and the aperture-1 temperature, 200^0C and 150^0C respectively. Asp and Glu oligomers were dissolved in ethanol/water (50:50) mixture to form a nominal concentration of 0.5mg/mL solution. The solution was injected into the MS using a syringe pump (Harvard Apparatus) at a flow rate of 1mL/hr. A make-up solution (50% acetonitrile in water with 0.1% acetic acid) was infused along with the sample at a flow rate of 0.2mL/min.

RESULTS AND DISCUSSION

Oligomer synthesis under an argon atmosphere. The oligomerization of Lys and Arg in three-phase system was carried out under an argon atmosphere. The oligomerization was evaluated with and without antioxidants (L-Cysteine and mercaptoethanol). Oligomer yield was calculated on the basis of initial concentration of monomer (AA-EE) in the reaction and concentration of residual monomer (AA-EE + free AA) in the reaction mixture after the set incubation period.

$$\textbf{Percent yield} = \{(\textbf{AA-EE})_{\textbf{initial}} - (\textbf{AA-EE + free AA})_{\textbf{final}}\}/ (\textbf{AA-EE})_{\textbf{initial}} * 100$$

The residual amount of monomer and ester left in the reaction mixture was calculated based on the calibration curve obtained by determining the response of standards of the monomer and ester with HPLC-UV.

The RPLC separation of Lys oligomers synthesized under anoxic conditions with argon purging in three-phase system for a 24h incubation period is shown in ***Figure 1.*** The chromatogram contains a series of peaks that appear after the Lys and LysEE peaks. Percent yield for $[Lys]_n$ based on the formula mentioned above was determined to be approximately 30%. Tentative identification of the oligomer peaks was based on ESI-MS results and expected increase in retention time with increased molecular weight within

the homologous oligomers. The degree of oligomerization ranged from 2-7 in case of [Lys] $_n$ in three-phase system. Similar yield (~30%) was obtained for Arg oligomerization also in three-phase system. In this case, the degree of polymerization ranged from tetramer to heptamer. The percent yield for Lys oligomerization based on residual monomers left in the supernatant from a 10% water/90% ACN mixture was determined to be ~ 25% (***Table 1***). The degree of oligomerization for Lys in case of a monophasic system ranged from 2-7. The results obtained for Arg oligomerization in ACN/water system was similar to that obtained for Lys oligomerization (***Table 1***).

Basic amino acid oligomerization in the presence of free Cysteine. The synthesis of Lys oligomers in a three-phase system with free L-Cys as the anti-oxidant was also evaluated. The RPLC separation of Lys oligomers synthesized in three-phase system with L-Cysteine anti-oxidant for a 24h incubation period is shown in ***Figure 2***. The oligomer yield was calculated on the basis of residual monomer found to be present in the reaction after the specific incubation period, as outlined earlier. The yield for $[Lys]_n$ was found to be ~ 80% . The chromatogram consists of series of peaks eluting after the retention time of LysEE. These peaks correspond to oligomers of Lysine. Tentative assignment of oligomer peaks was carried on the basis of ESI-MS data and expected retention increase in a homologous series. Similar oligomer yield (~80%) was obtained in the case of [Arg] $_n$ in three-phase system (***Figure 3***). The Lys and Arg oligomers synthesized are mostly 2 to 7 residues long. The percent conversion for both Lys and Arg in 10% water/90% ACN mixture in the presence of L-Cysteine anti-oxidant was ~ 75% (***Table 1***). The degree of polymerization in this case ranged from 2-7 for both Lys and Arg. These results compare well with the yield and degree of polymerization

obtained for basic amino acid oligomerization in both three-phase and monophasic system with mercaptoethanol as the anti-oxidant (*28, 33*). The results in the presence of mercaptoethanol are also given in ***Table 1***. With mercaptoethanol anti-oxidant, the yield in three-phase system was close to 80% while it was close to 75% in case of ACN/water system (*28, 33*). This is significantly different from the results obtained in the absence of mercaptoethanol or L-Cysteine or argon purging (***Table 1***). In the latter case, the percent conversion was only 15% and the product mainly comprised of the dimer with very little trimer and tetramer.

These results confirm the need for an anti-oxidant in the reaction medium to maintain the activity of a thiol protease like papain. The anti-oxidants maintain the active site cysteine residue in its native thiol form and avoid the formation of disulfide bridges between the active site cysteine residue with another cysteine residue (Cys-22, 63) that denature the enzyme (*39, 40*).

Synthesis of Asp and Glu acid oligomers. The synthesis of di-carboxylic amino acids Asp and Glu oligomers was also evaluated in three-phase and monophasic systems. The synthesized ester of Asp and Glu (***Figure 4 A*** **and** ***Figure 4 B***) was used as the substrate for oligomerization of Asp and Glu in three-phase and monophasic system in the presence of L-Cysteine and mercaptoethanol. The RPLC chromatogram of the residual monomers left in the Asp oligomers synthesized in three-phase system with L-Cysteine and mercaptoethanol for a 24h incubation is shown in ***Figure 5 (A, B)***. The chromatogram shows a clear separation of Asp, Asp monoester and Asp diester. The percent conversion of the reaction was calculated from the residual monomer left in the reaction mixture as mentioned above. The separation of residual monomers in the Glu

oligomerization product obtained from three-phase system for a 24h incubation is shown in ***Figure 6 (A, B)***. Conversion was close to 80% in the presence of L-Cysteine while it was close to 40% in the presence of mercaptoethanol. Similar conversion was obtained for Glu oligomerization. The ESI (+)-MS analysis of the synthesized oligomer product was carried out to determine the product distribution in the oligomer chain. ***Figure 7 (A)*** shows the ESI-MS spectrum of Asp oligomer synthesized in three-phase system with L-cysteine as the anti-oxidant, for a 24h incubation period. The spectrum consists of a series of peaks appearing at ***m/z 305, 448, 591, 734, 877 and 1020***. These peaks appear at a mass difference of 143 amu corresponding to an Asp residue backbone with an intact ester group. These peaks correspond to 2 to 7 residues of Asp. A simple Asp dimer is shown in ***Figure 8***. There are three possible sites for the presence of ester intact residues. The oligomer residues of Lys, Met, Arg and other amino acids obtained in a three-phase system typically has the α - carbon in a free acid form. This coupled with the dominant presence of diester in the starting substrate is an indication of the presence of intact ester residue in the side chain carboxylic acid group. When mercaptoethanol was used as the anti-oxidant in three-phase system, the mass spectrum of Asp oligomers showed a product consisting of only Asp dimer (***Figure 7 B***). Oligomerization is more complete in the presence of L-Cysteine than in the presence of mercaptoethanol. Only the dimer is formed in mercaptoethanol whereas higher oligomers are formed in L-Cysteine. The ESI – MS spectrum of Glu oligomers synthesized with L-Cysteine and mercaptoethanol in a three-phase system, for a 24h incubation is shown in ***Figure 9 (A, B)***. ***Figure 9 A*** consists of series of peaks appearing at ***m/z 490, 647, 804, 961 and 1118*** with 157 amu difference indicated the formation of Glu oligomer products with residue numbers (n)

3,4,5,6 and 7 respectively. The mass difference of 157amu corresponds to the addition of Glu backbone with intact ester residue. The ion at ***m/z 316*** corresponds to a fragment ion of Glu dimer arising from the loss of ammonia as a neutral from the actual dimer ion. Only Glu dimer is formed in the presence of mercaptoethanol as the anti-oxidant (***Figure 9 B***).

Asp and Glu oligomerization was also carried out in a monophasic system (acetonitrile/water (9:1)). The equilibrium was shifted towards peptide synthesis by the precipitation of the formed Asp and Glu oligomers from the reaction medium because of the presence of very little water. The percent conversion was calculated based on the residual amount of monomer left in the supernatant of the reaction mixture. The conversion was close to 80% for Glu oligomerization in the presence of L-Cysteine while it was only 50% in the presence of mercaptoethanol (***Table 1***), for a 24h incubation of the reaction mixture. The ESI (+)-MS spectrum of the precipitated Glu oligomers synthesized in acetonitrile/water system in the presence of L-Cysteine, for a 24h incubation is shown in ***Figure 10 A***. The product profile though close to three-phase system, shows only the formation up to pentamer. The tetramer and pentamer were the dominant products. As the product precipitates out, the solubility of the higher oligomer residues decrease thereby reducing their availability as substrates for further oligomerization. This resulted in the formation of shorter chain oligopeptides in monophasic solvent system when compared to a three-phase system. The ion appearing at ***m/z 316*** corresponds to the ion resulting from the fragmentation of Glu dimer in the MS with the loss of ammonia as a neutral. The precipitated product mainly comprised of an ion appearing at ***m/z 333*** corresponding

to a Glu dimer residue with two intact ester groups when mercaptoethanol was used as the reducing agent (***Figure 10 B***).

The percent conversion for the Asp oligomerization reaction, based on the amount of monomer left in the supernatant in an acetonitrile/water (9:1) solvent system with L-Cysteine as anti-oxidant for a 24h incubation was only 45% (***Table 1***). The reduction in oligomerization efficiency in case of Asp is due to the highly polar nature of Asp that results in the chemical hydrolysis of the synthesized oligomers even when only 10% water is present. The percent conversion dropped to 35% when mercaptoethanol was used as the reducing agent (***Table 1***). The ESI-MS analysis of the precipitated product synthesized in the presence of L-Cysteine and mercaptoethanol in a 10% water/ACN mixture for a 24h incubation (***Figure 11 A, B***) revealed only the presence of a dimer with three intact ester groups appearing at ***m/z 333.***

In order to increase the percent oligomerization of Asp, the solvent composition was changed to acetonitrile (95%) / water (5%). The RPLC separation of the supernatant from the reaction product in the presence of L-Cysteine, for a 24h incubation (***Figure 12***) shows that the percent conversion of monomer was ~80%. The percent conversion in the presence of mercaptoethanol was only 35% (***Table 1***). When the precipitated Asp oligomers synthesized in the presence of L-Cysteine was analyzed using ESI (+) – MS, the spectrum shows the presence of peaks appearing at ***m/z 305, 448, 591, 734, 877*** corresponding to Asp oligomer residues ranging from 2 to 6 respectively (***Figure 13 A***). Asp dimer dominated the product synthesized under the same solvent composition in the presence of mercaptoethanol (***Figure 13 B***). The dimer in this case had only two ester intact residues.

Table 1 is a summation of results obtained for different amino acids under different conditions. The higher amounts of residual monomers left in the reaction medium incubated under anoxic conditions and in the absence of anti-oxidants show their importance in maintaining the activity of thiol proteases in reaction medium. The anoxic conditions used are not sufficient to maintain the cysteine residue of the active site in its reduced form (-SH) and it oxidizes and forms a disulfide bridge with another cysteine residue. Rzychon and Chmiel (39) reported that the catalytic activity of Cysteine proteases is mainly dependant on the formation of a thiolate/imidazolium pair resulting from the proton transfer between Cys-25 and His-159 residue. Hussain and Lowe (40) have reported the amino acid sequence of Papain. The sequence shows that the Cys residues (Cys – 22, 63) that are adjacent to the active site Cys-25 can form a disulfide bridge, thereby preventing the proton transfer to the Histidine group during the catalytic process. L-Cysteine served as an effective anti-oxidant for oligomerization reactions in three-phase and monophasic solvent systems. Oligomers of Lys, Arg, Asp and Glu were successfully synthesized in the presence of L-Cysteine. The oligomerization efficiency in the presence of L-Cysteine was close to 80% and there was no change in product distribution for basic amino acid oligomers when mercaptoethanol was replaced with L-Cysteine. However, in case of acidic amino acid oligomers L-Cysteine provided better conversion than mercaptoethanol. The elimination of mercaptoethanol by L-Cysteine will result in reduction of product toxicity.

Conclusions. We have shown that anti-oxidants in the reaction medium are needed for papain-catalyzed oligomerization of basic and acid amino acids. Our results show that L-cysteine may be used efficiently to replace mercaptoethanol as the anti-

oxidant. The elimination of mercaptoethanol from the reaction mixture would allow the use of the synthesized oligopeptides as dietary supplements.

LIST OF ABBREVIATIONS

Met, Methionine; Lys, Lysine; K, Lysine; M, Methionine; R, Arginine; LysEE, Lysine Ethyl Ester di-hydrochloride; MetEE, Methionine Ethyl Ester Hydrochloride; HPLC, High Pressure Liquid Chromatography; RPLC, Reverse Phase Liquid Chromatography; ESI-MS, Electrospray Ionization-Mass Spectrometry; HSA, Hexane sulfonic acid; UV/Vis, Ultraviolet/Visible; ACN, Acetonitrile; MS, Mass Spectrometry; MS/MS, Tandem Mass Spectrometry; Arg, Arginine; Asp, Aspartate; Glu, Glutamate; Cys, Cysteine; E, Glutamate; D, Aspartate; DFP, 1,1,1,2,3,4,4,5,5,5-decafluorpentane; DIPEA, N, N-di-isopropyl ethyl amine.

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FIGURES AND TABLES

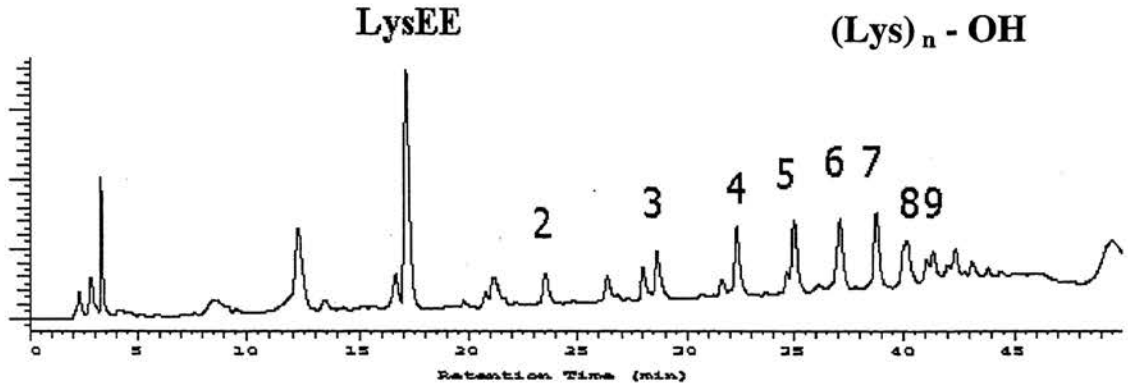


Figure 1: RPLC separation of Lys oligomers synthesized under anoxic conditions (argon atmosphere) in three-phase system. Separation was achieved with a RPLC C-18 column using a mobile phase gradient 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) to 75% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 50 minutes. The Chromatogram shows the presence of peaks corresponding to oligomers of Lysine.

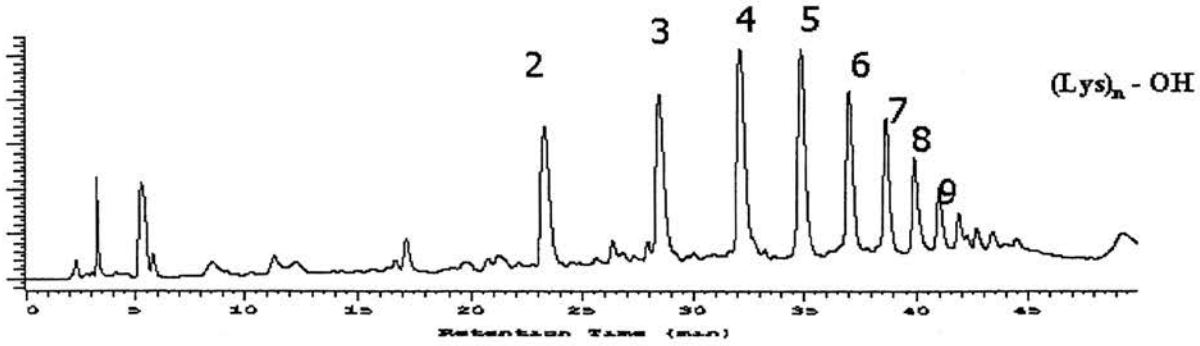


Figure 2: RPLC separation of Lys oligomers synthesized in three-phase system with L-Cysteine as the anti-oxidant. Separation was achieved in a RPLC C-18 column with HSA as the ion-pairing agent in the mobile phase using a mobile phase gradient mentioned in Figure 1. The separation shows the presence of peaks corresponding to oligomers of Lysine.

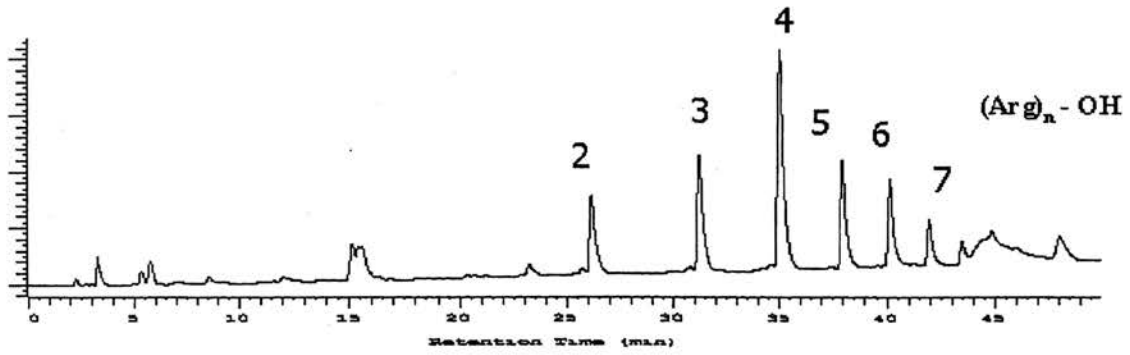
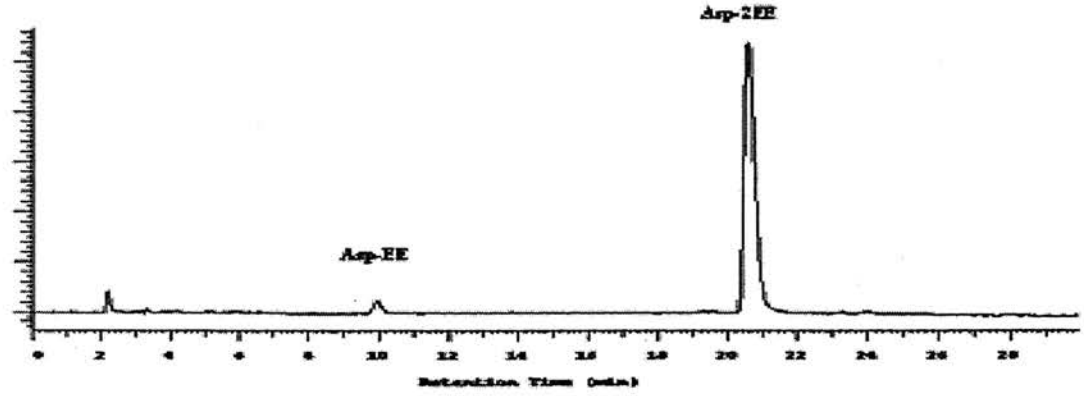


Figure 3: RPLC separation of Arg oligomers synthesized in three-phase system with L-Cysteine as the anti-oxidant. Separation was achieved in a RPLC C-18 column with HSA as the ion-pairing agent in the mobile phase using a mobile phase gradient mentioned in Figure 1. The separation shows the presence of peaks corresponding to oligomers of Arginine.



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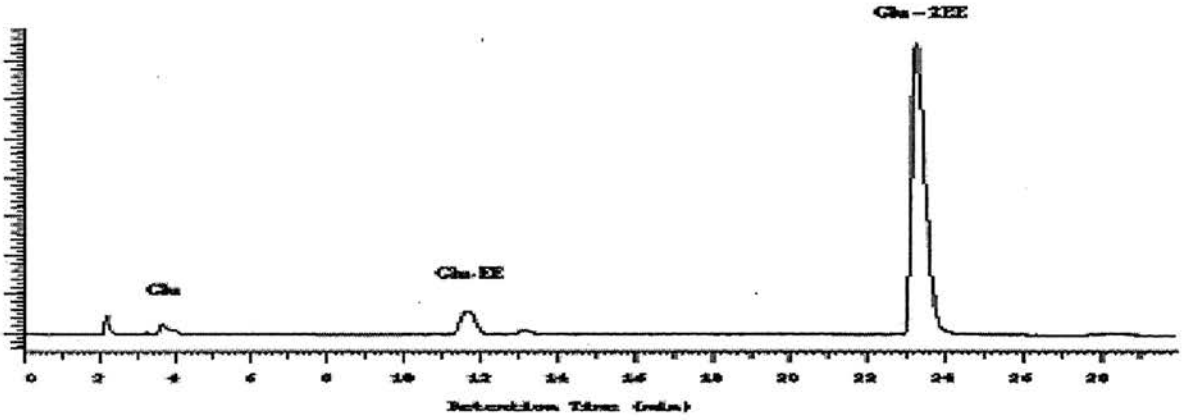
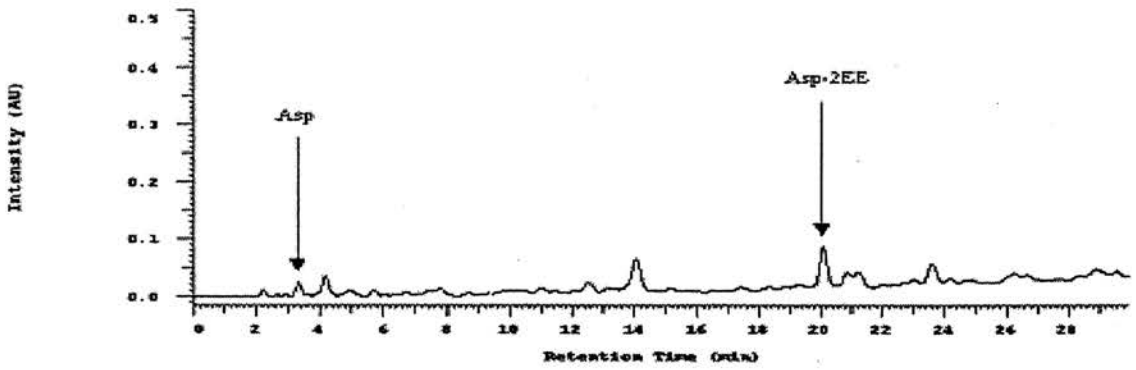


(B)

Figure 4: HPLC separation of acidic amino acid esters (A) Asp and (B) Glu synthesized with absolute ethanol. Separation was achieved with a RPC-18 column using a mobile phase gradient comprising of 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) to 60% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 30 minutes. The chromatograms show the presence of two peaks eluting after the retention time of Asp and Glu corresponding to their mono and di-esters, respectively.



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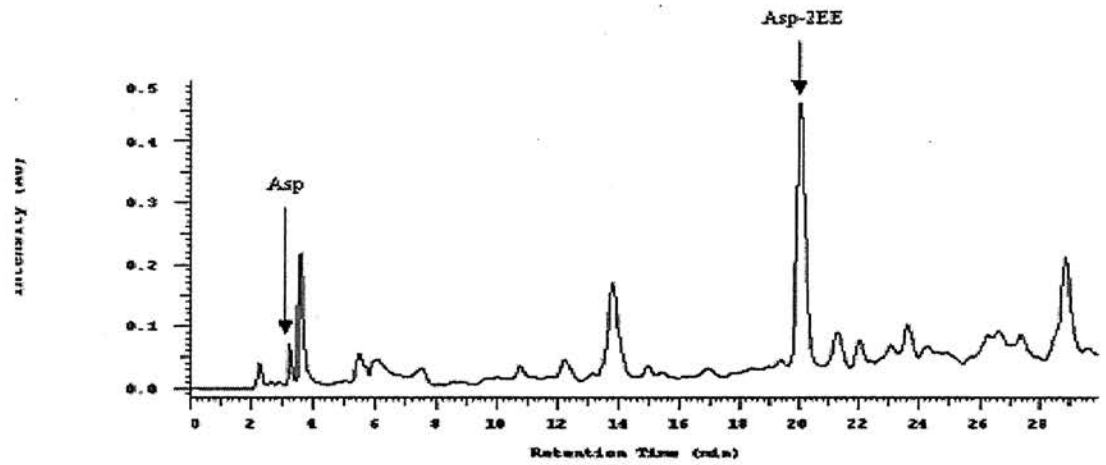
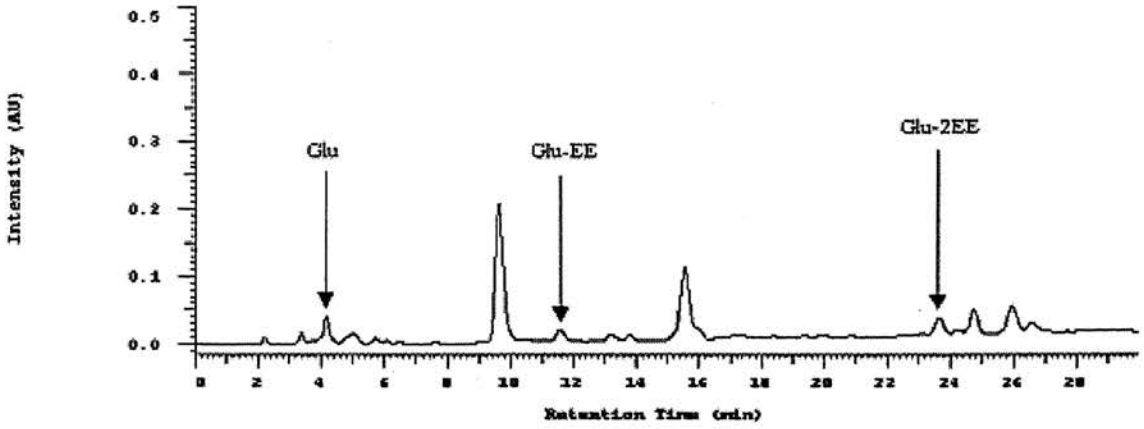


(B)

Figure 5: RPLC separation of residual monomers left in the Asp oligomer product synthesized in three-phase system with (A) L-Cysteine and (B) 2-mercaptoethanol as anti-oxidant. Separation was achieved in a RPLC C-18 column with HSA as the ion-pairing agent in the mobile phase using a mobile phase gradient mentioned in Figure 4. The chromatograms show the clear separation of residual Asp ester and monomer.



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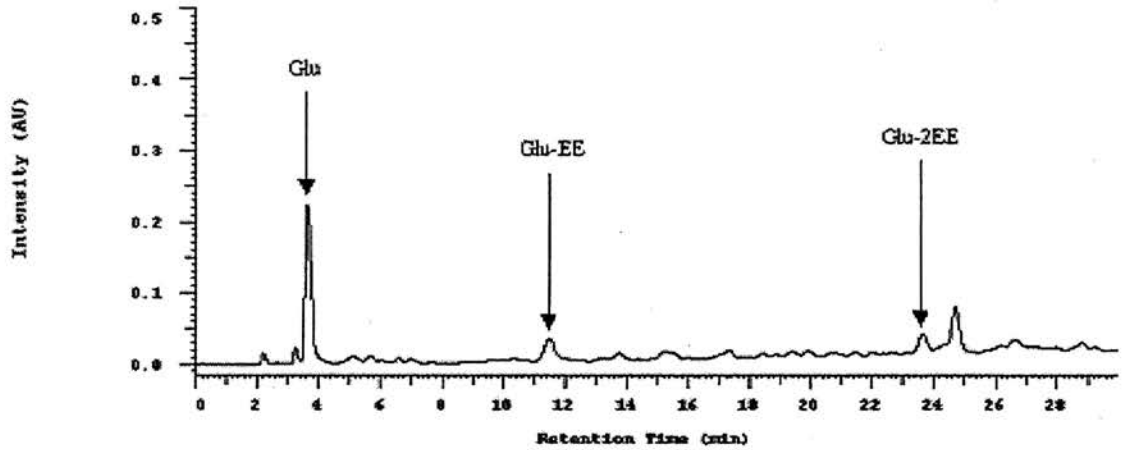
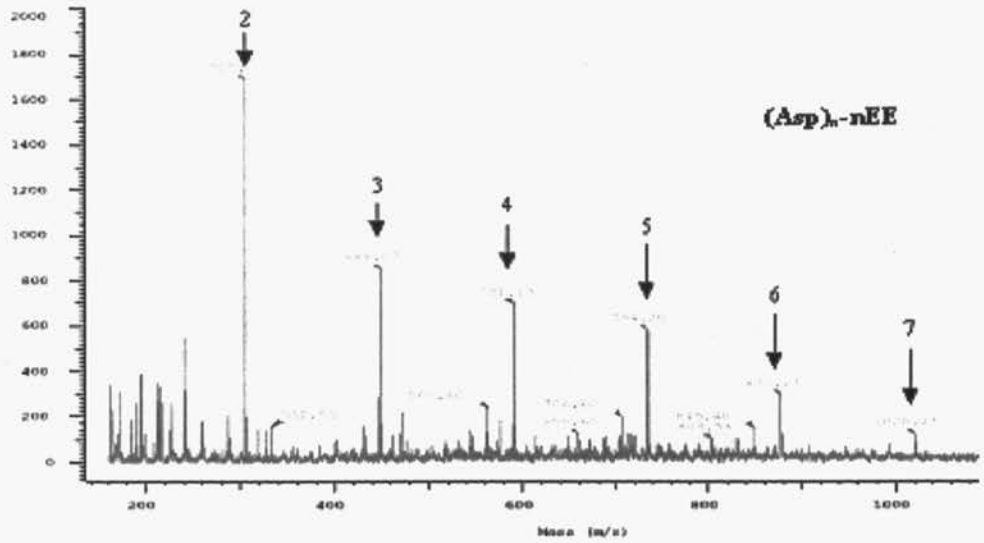
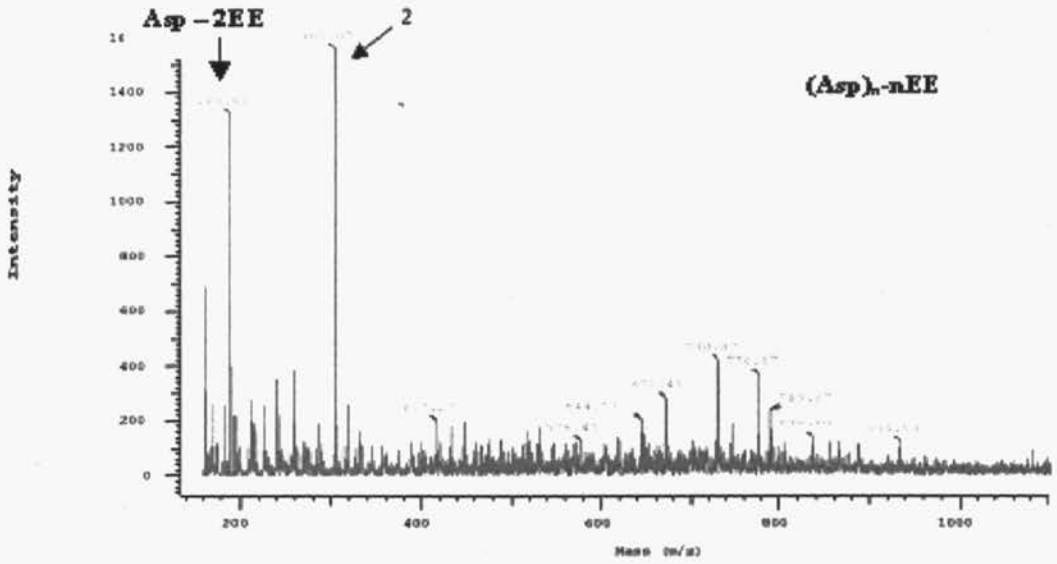


(B)

Figure 6: RPLC separation of residual monomers left in the Glu oligomer product synthesized in three-phase system with (A) L-Cysteine and (B) 2-mercaptoethanol as anti-oxidant. Separation was achieved in a RPLC C-18 column with HSA as the ion-pairing agent in the mobile phase using a mobile phase gradient mentioned in Figure 4. The chromatograms show the clear separation of residual Glu ester and monomer.



(A)



(B)

Figure 7: Direct injection ESI (+)–MS spectra of Asp oligomers synthesized with (A) L-Cysteine and (B) 2-mercaptoethanol as the anti-oxidant in three-phase system. The spectrum shows the presence of series of peaks corresponding to oligomers of Asp.

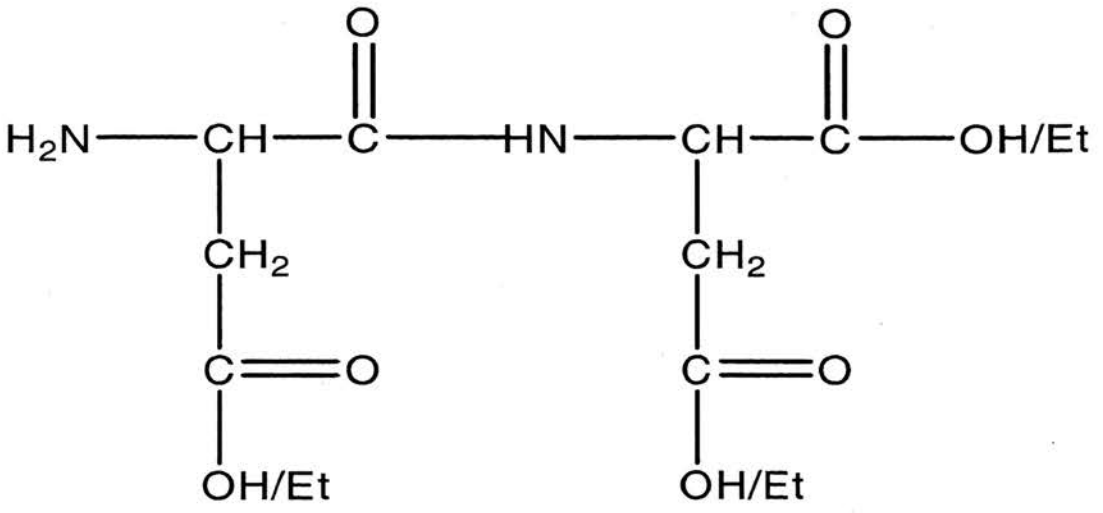
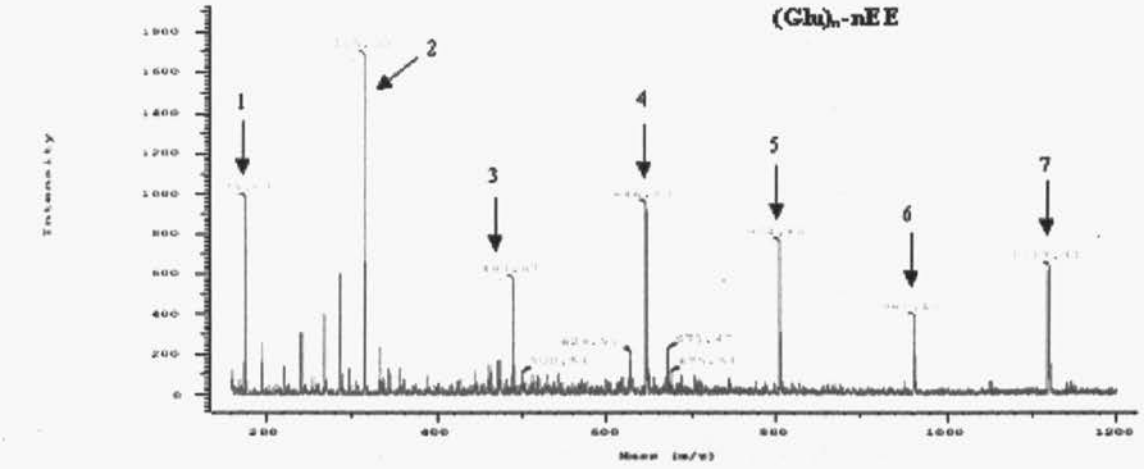
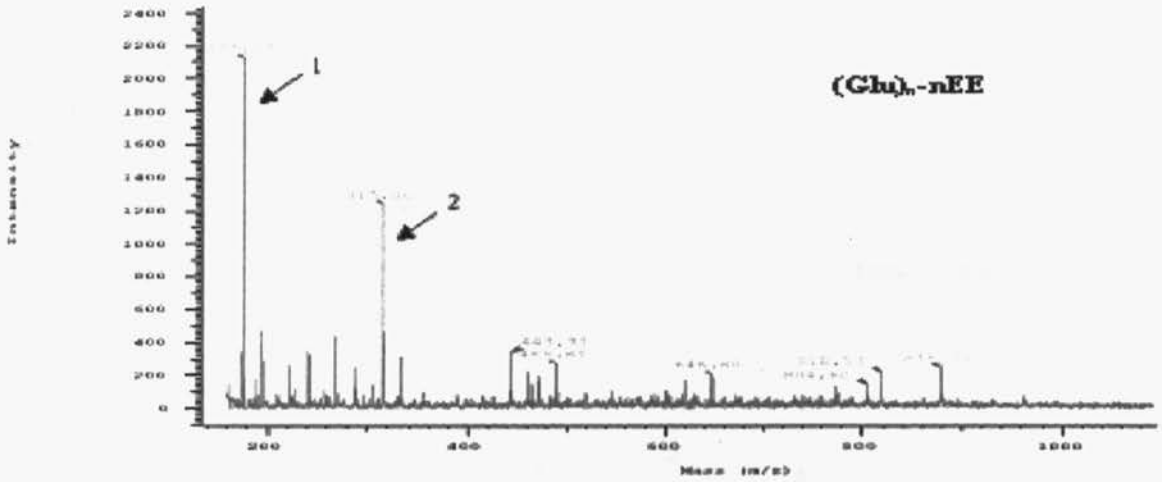


Figure 8: Structure of an Asp dimer indicating the possible sites for the presence of intact ester groups. Both the α- carboxyl group and the side chain carboxyl group can have intact ester moiety.

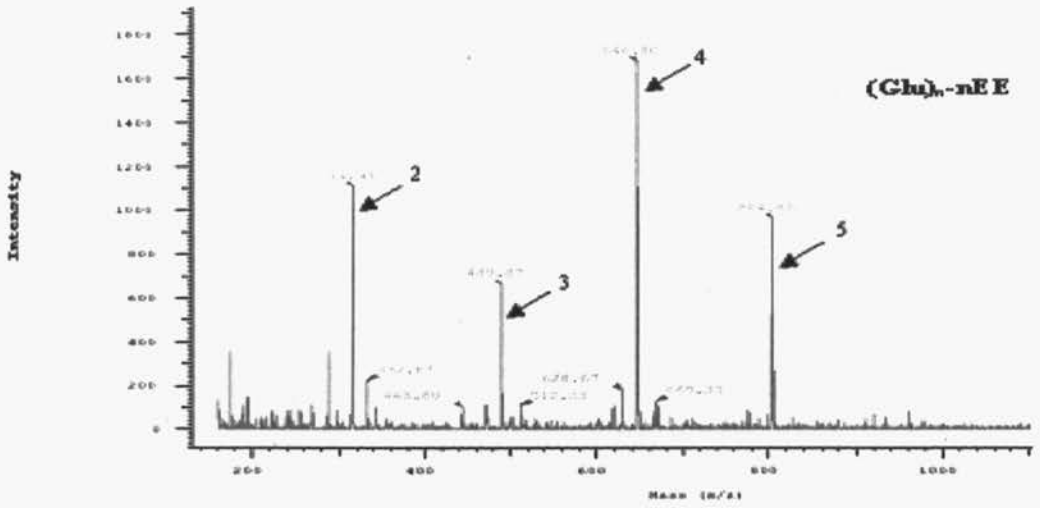


(A)

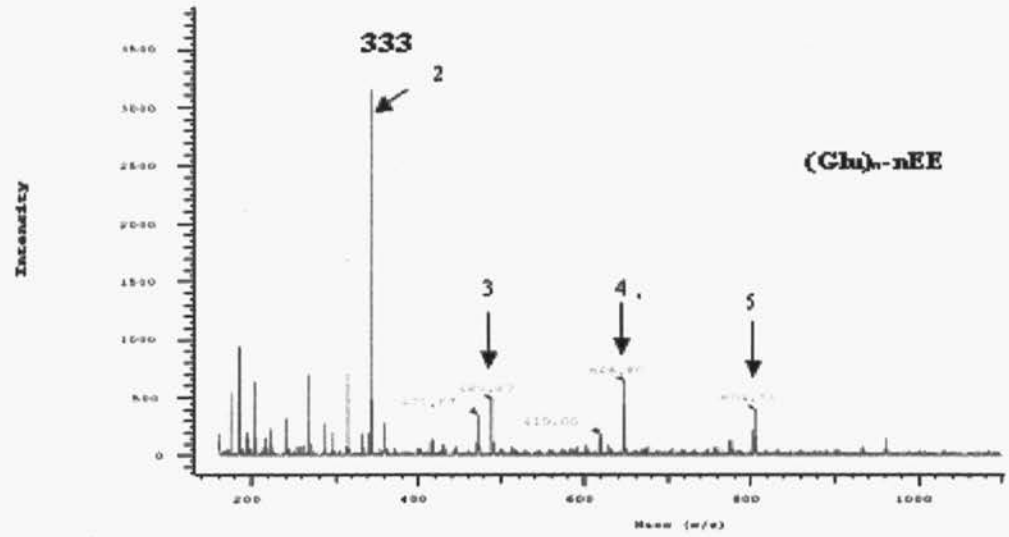


(B)

Figure 9: Direct injection ESI (+)–MS spectra of Glu oligomers synthesized with (A) L-Cysteine and (B) 2-mercaptoethanol as anti-oxidant in three-phase system. The spectrum shows the presence of peaks corresponding to oligomers of Glu.

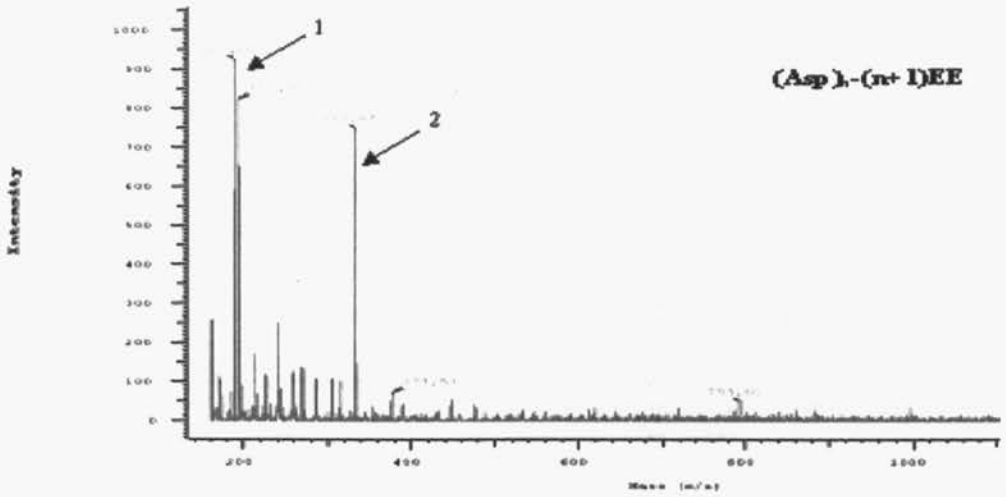


(A)

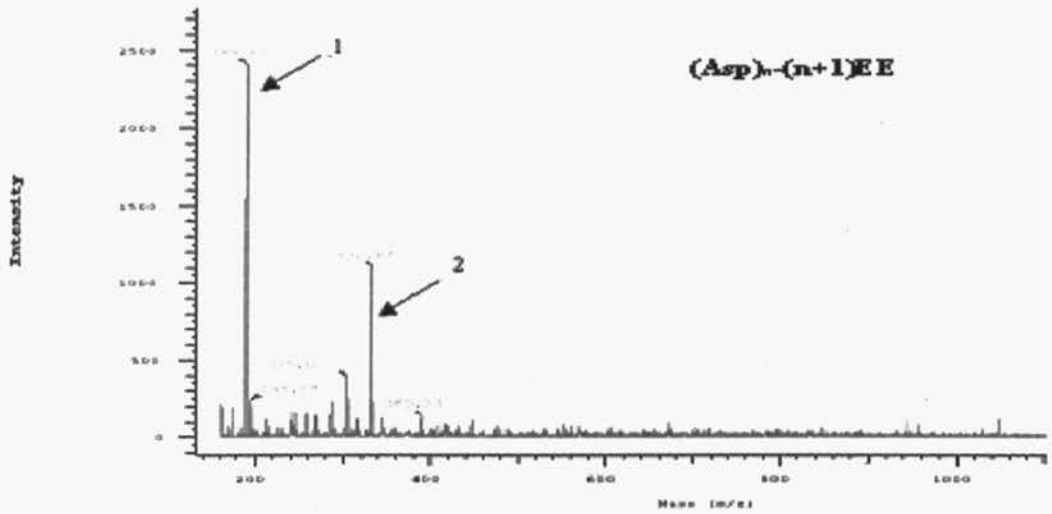


(B)

Figure 10: Direct injection ESI (+)–MS spectra of Glu oligomers synthesized with (A) L-Cysteine and (B) 2-mercaptoethanol as anti-oxidant in monophasic solvent system comprising of 90% acetonitrile/ 10% water. The spectrum consists of a series of peaks corresponding to Glu oligomers.



(A)



(B)

Figure 11: Direct injection ESI (+)–MS spectra of Asp oligomers synthesized with (A) L-Cysteine and (B) 2-mercaptoethanol as anti-oxidant in monophasic solvent system comprising of 90% acetonitrile/ 10% water. The spectrum shows the presence of only Asp dimer.

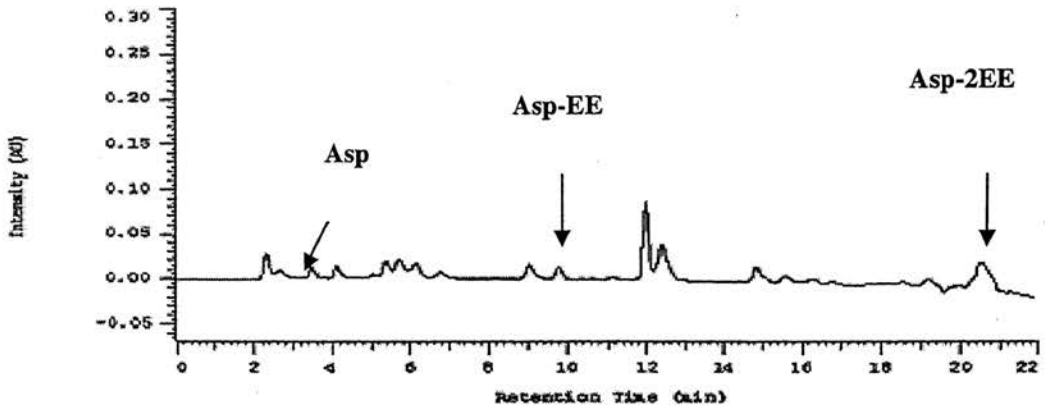
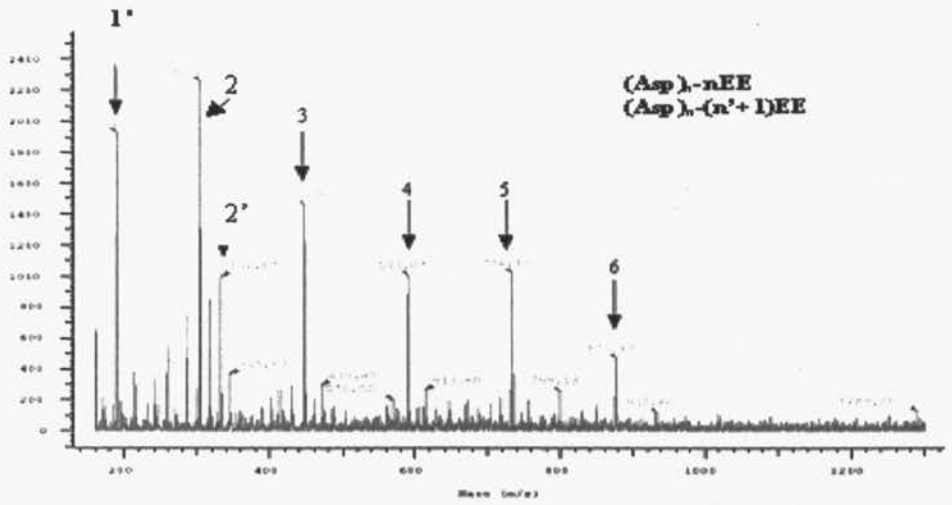
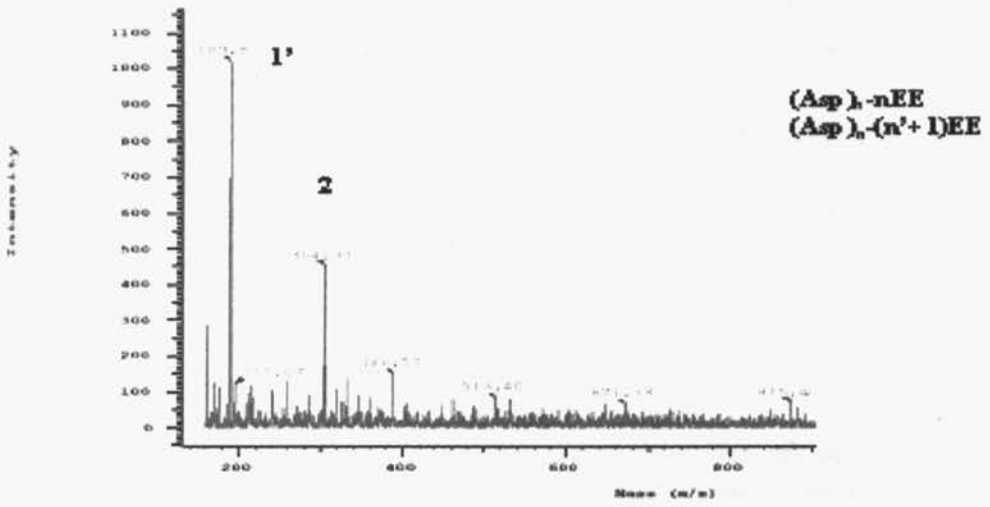


Figure 12: RPLC separation of residual monomers left in the Asp oligomer product synthesized in monophasic system with 95% acetonitrile and 5% water in the presence of L-Cysteine as an anti-oxidant for papain stabilization. Separation was achieved in a RPLC C-18 column with HSA as the ion-pairing agent in the mobile phase using a mobile phase gradient mentioned in Figure 4. The chromatogram shows the clear separation of residual Asp ester and monomer.



(A)



(B)

Figure 13: Direct injection ESI (+)–MS spectra of Asp oligomers synthesized with (A) L-Cysteine and (B) 2-mercaptoethanol as anti-oxidant in monophasic solvent system comprising of 95% acetonitrile/ 5% water. The top spectrum consists of a series of peaks corresponding to oligomers of Asp, while the bottom spectrum shows only the formation of Asp dimer.

Substrate	Conversion (%)				Dominant species			
	Anoxic	L-Cys	M.E	No A.O	Anoxic	L-Cys	M.E	No A.O
Lys (K)	30	80	80	15	4 – 7	2 – 7	2 - 7	2, 3
Arg (R)	28	82	78	17	4 – 7	2 – 7	2 – 6	2, 3
Asp (D)	20	80	40	12	2	2 – 6	2	2
Glu (E)	24	83	38	15	2	2 - 7	2	2

(A)

Substrate		Conversion (%)				Dominant species			
		Anoxic	L-Cys	M.E	No A.O	Anoxic	L-Cys	M.E	No A.O
Lys (K)		25	73	70	18	2	2 – 7	2 - 7	2, 3
Arg (R)		27	70	65	20	2	2 – 7	2 - 7	2
Asp (D)	5% water	30	80	35	15	2	2 - 5	2	2
	10% water	20	40	30	22	2	2	2	2
Glu (E)		30	80	35	20	2	2 - 5	2	2

(B)

Table 1: Comparison of amino acid oligomerization efficiency of L-Cys, mercaptoethanol and argon purge as anti-oxidant conditions for papain stabilization in (A) three-phase system and (B) monophasic (acetonitrile/water) system.

III. SYNTHESIS AND CHARACTERIZATION OF TAILORED PEPTIDES SYNTHESIZED THROUGH PROTEASE CATALYSIS IN MIXED AQUEOUS ORGANIC MEDIA

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Abstract. Supplementation of tailored peptides has potential for catering to the nutritional requirements of the animal. Protease catalyzed synthesis of tailored oligopeptides in aqueous, biphasic and triphasic media have been reported in literature. Use of monophasic aqueous organic system in which polypeptide substrates are soluble was examined for the synthesis of oligopeptides and tailored high by-pass co-oligopeptides under different elevated temperature conditions using a simple sequential addition approach coupled with change in solvent composition. The synthesis of oligomers of Lys and co-oligomers of Lys and Met was carried out in a monophasic organic system consisting of water in acetonitrile under elevated temperatures using papain as a catalyst. The effect of temperature and time of incubation on the yield of Lys oligomers was studied. Yields as high as 70% were obtained even at 60^0C. For Lys-Met co-oligopeptides, the reaction was started with a water content favoring the oligomerization of the first substrate, and then the water content was changed to favor the addition of the second substrate. The effect of temperature, time of incubation, time of addition of the second substrate, concentration of the substrates and solvent composition on the yield of tailored peptides was studied. The synthesized tailored peptides were then characterized by Liquid chromatography, Electro-spray Ionization Mass Spectrometry and Tandem Mass Spectrometry. The results show that sequential addition of the second substrate with changing water composition is a simple and rapid approach for synthesizing tailored co-oligopeptides.

Keywords: Amino acids, tailored peptides, Temperature, Monophasic organic system, High Performance Liquid Chromatography, Electrospray Ionization Mass Spectrometry (ESI-MS), Tandem Mass Spectrometry.

INTRODUCTION

Lysine (K), Methionine (M) and Arginine (R) are considered the primary limiting amino acids in cattle feed and poultry (*1-4*). For example, in dairy cows, milk production accounts for nearly 95% of this amino acid requirement (*5*). Supplementation of crystalline amino acids is not feasible because the pregut fermentative step converts these amino acids into short chain fatty acids and ammonia (*6, 7*). Studies have proven that ruminally protected forms of these amino acids help in improving milk production (*3, 5, 8, 9, 10*). Different strategies have been adopted to supplement these amino acids in the ration. Methionine has been supplemented as a 50% mixture (by moles) of Methionine Hydroxy Analogue (HMB, 2-Hydroxy-4-(methylthio) butanoic acid) and DL-Met (*2, 6, 11, 12*). The efficacy of this supplementation procedure depends on the resistance of MHA to ruminal microbial degradation and the subsequent absorption and metabolism of Met in the tissues (*13*). Abomasal (Post-rumen) infusion of these amino acids has also received widespread attention for their supplementation (*14, 15, 16*), especially in the case of Lys. Studies have shown that supplementation of Met or Lys individually has little effect on milk production when compared to their addition together in the ration in a 1:3 ratio (*15, 17*). An alternative supplementation protocol may be the use of oligomers and peptides of amino acids (*6, 18, 19, 20*). Research has been mainly focused on the synthesis of homo-oligomers of Met, Lys and capped co-oligomers of MHA with Met (*6*). Very little work has been reported on the synthesis of poly-Lys-met co-oligopeptides. One such study has been reported on the synthesis of a branched chain polypeptide multioligo (L-methionyl) poly-L-Lys (*18*) with nutritional value and assessment of its bioavailability. Such peptides are not readily available because they are expensive and

difficult to synthesize. In this study we propose a simple enzymatic protocol for the synthesis of Lys-Met co-oligopeptides with their composition tailored in the ratio of ~3:1 (Lys: Met) to meet nutritional requirements.

Remarkable advancement has been made in the field of peptide synthesis in the past century. The common techniques adopted for peptide synthesis are chemical synthesis, recombinant DNA technology and enzymatic synthesis (*21, 22, 23*). The stereo- and regio-specificity of enzymatic synthesis has made it an attractive technique for peptide synthesis (*24, 25*). Enzymatic peptide synthesis has long been carried out in aqueous, bi-phasic and tri-phasic reaction media (*6*). The use of monophasic organic solvent systems has been limited in peptide synthesis because enzymes loose their catalytic power in some protic solvents (*26, 27, 28*).

Proteolytic enzymes maintain their catalytic activity in monophasic medium, thereby preventing them from undergoing inactivation (*29*). Direct contact of organic solvents with the enzyme may affect the catalytic efficiency of the enzyme (*30, 31*). Monophasic aqueous organic media offer numerous advantages for enzymatic peptides synthesis such as higher solubility for non-polar substrates, thermodynamic equilibrium shift towards peptide bond synthesis, ease of enzyme recovery eliminating/reducing the need for immobilization, high thermal stability of the enzymes and very little microbial contamination (*28*). Higher reaction rates have been reported in some cases with monophasic systems because of the reduction in mass-transfer resistance/barrier existing in bi-phasic systems (*32*).

Another added advantage of monophasic solvent systems is the increased stability of enzyme to thermo-inactivation (33). Covalent modifications in the primary molecular

structure coupled with the partial unfolding of the enzyme leads to its inactivation at high temperatures in aqueous media (*33, 34, 35*). Porcine pancreatic lipase was shown to have a 5 times enhanced transesterification rate at 100^0C when compared to 20^0C with a half-life time of 12h in dry tributyrin containing heptanol (*36*). Enzymatic catalysis in organic solvent system thus obeys the conventional chemical principle of enhanced reaction rate at higher temperatures. This increased thermo stability of enzymes in non-aqueous media could be utilized for biocatalysis in supercritical systems as the enzyme remains viable at high temperatures (26, *37-40*).

Acetonitrile containing pH 9.0 Tris/HCl buffer has been used for the synthesis of Leu and Met Enkephalin derivatives (*41*). The synthesis of the aspartame precursor N-(benzyloxycarbonyl)-L-aspartyl-L-Phenyl-alanine methyl ester at high yield was reported in a homogeneous reaction medium consisting of DMSO with boilysin and thermolysin as catalysts. The enzymes were enantio-selective (*42*). Papain catalyzed synthesis of chemotactic peptides in a mixture of McIlvaine buffer and ethanol with high yields has been reported (*43*). Trypsin and chymopapain catalyzed synthesis of RGD tripeptide has been reported in Ethanol-Tris-Hcl buffer and CHES/NaOH buffer in high yields (~80%) (*44*). Corrine (*45*) reported the subtilisin- Carlsberg catalyzed synthesis of a tetrapeptide ester containing the unnatural amino acid allylglycine exhibiting β-sheet structure in several miscible aqueous/organic solvent systems.

The solubility of peptides in monophasic solvent systems coupled with the enhanced thermal stability of enzymes in such media makes them an ideal choice for synthesis of homo-oligopeptides and targeted/tailored co-oligopeptides. Papain-catalyzed oligomerization of Met, Tyr, Lys and Gly in monophasic aqueous organic media

consisting of varying amounts of acetonitrile/water has already been reported (*46*). The synthesis of both polar and non-polar amino acid oligomers could be achieved in such solvent systems with proper manipulation of the water content of the reaction medium. The optimal condition for Lys oligomerization was around 10 (%v/v) of water in acetonitrile while Met was polymerized at water contents greater than 15 (%v/v) in ACN/water systems. This unique property of monophasic solvent systems could be advantageous in the synthesis of peptides of Lys-Met with tailored composition.

Papain catalyzed synthesis of co-oligopeptides of Lys and Met with tailored amino acid composition was studied in this work. The reaction was started with the water content required for oligomerization of the first substrate. After the first substrate was incubated for a specific period of time, the second substrate was added and the system composition was altered to favor its incorporation into the oligopeptide chain. The effect of temperature of the reaction media, time of incubation, time of addition of the second substrate, concentration of the substrates, system composition and type of anti-oxidant (Mercaptoethanol or L-Cysteine) on the co-oligomerization process was studied. The amino acid sequence of some of the oligomers synthesized was determined using Tandem Mass Spectrometry. A comparative study was done by carrying the synthesis of the same peptides in three-phase system with L-Cysteine as the reducing agent.

MATERIALS AND METHODS

Materials. L-Methionine Ethyl ester (MetEE) hydrochloride was purchased from Fluka Chemical Corp., (Milwaukee, WI). L-Lysine ethyl ester (LysEE) dihydrochloride, L-Cysteine hydrochloride monohydrate (Cys), n-Octane, 2-Mercaptoethanol, N, N diisopropylethylamine (DIPEA), acetic acid and trifluoroacetic acid were purchased from

Sigma Chemical Co., (St.Louis, MO). 1,1,1,2,3,4,4,5,5,5-decafluorpentane (DFP) was purchased from Miller-Stephenson Chemical Company (Danbury, CT). Sodium salt of Hexane sulfonic acid (HSA), O-Phosphoric acid, acetonitrile and ethanol (HPLC grade) were obtained from Fisher Scientific, (St.Louis, MO). Papain (EC 3.4.22.2, 25 units activity/mg, 28mg protein/mL) was provided by Novus International Inc., (St.Louis, MO). RPLC separation of the synthesized oligomers and co-oligomers was carried out with a XPERCHROM C-18 column (250mm x 4.6mm) obtained from P.J. Cobert Associates Inc., (St.Louis, MO). Nanopure water used in the experiments was obtained after filtration through a Synergy 185 filtration system purchased from Millipore Corp. (Billerica, MA).

LC with UV detection. A model L-7000 HPLC system (Hitachi High Technologies America, San Jose, CA) was used to carry out the HPLC separations. The system consisted of a reciprocating piston pump (L-7100) fitted with a column oven (L-7300), autosampler (L-7200) and with a 50μL injection loop. The analytes separated on reverse phase columns were then introduced into a UV-Vis absorbance detector (L-7420).

ESI-Mass Spectrometer. An Ion Trap Mass Spectrometer equipped with an electrospray ionization interface (Model M-8000) purchased from Hitachi High Technologies America, San Jose, CA and a Triple Quadrupole Mass Spectrometer fitted with an electrospray ionization source (Model 1200) purchased from Varian Inc., Walnut Creek, CA were used for the mass analysis of the synthesized oligomers and co-oligomers.

Synthesis of Lys oligomers at elevated temperatures. L-LysEE dihydrochloride (123mg) was added to a 7mL clear glass reaction vial containing 5mL of the solvent

consisting of 10% (v/v) water in acetonitrile. 100μL of DIPEA, 25μL of mercaptoethanol and 30mg of papain was added and the sealed vial was then placed in an incubator shaker. The incubation temperature was varied from 25^0C to 60^0C and the incubation period was varied from 2h to 24h. The reaction was stopped by heating the reaction mixture to 80^0C for 10 minutes. The supernatant was separated and rotary evaporated to near dryness. The precipitate was lyophilized. Dried products from both the supernatant and the precipitate were then reconstituted in 50% ethanol in water, centrifuged, filtered and analyzed in HPLC.

Synthesis of Lys-Met co-oligomers in monophasic organic solvent system. A simple sequential addition strategy was adopted for the synthesis of Lys-Met co-oligomers in acetonitrile/water solvent system. The reaction was started at a water content favoring the oligomerization of Lys. After a specific period of incubation, MetEE was added and the water content of the solvent system was changed to favor the incorporation of the second substrate into the Lys oligomer. A simple schematic of the strategy is shown in ***Figure 1***. 0.5mM LysEE.2HCl and 30 mg of papain were dissolved in 10 %(v/v) of water in acetonitrile. 25µL of mercaptoethanol (or 20mg of L-Cysteine) and 100µL DIPEA were also added to the reaction mixture. After a pre-established period of time, MetEE.HCl (from 0.1 to 0.5 mM) was added as the second substrate to favor the formation of Lys-Met co-oligomers. Papain prefers hydrophobic amino acids to hydrophilic amino acids as substrates for polymerization. To achieve an average composition of 3:1 (Lys: Met), it becomes clear that steps have to be taken to slow down the incorporation of Methionine. We previously reported that methionine oligomerization in acetonitrile/water systems increases with increasing water content while Lys

oligomerization is the highest at 10 (%v/v) of water in acetonitrile (*46*). With this in mind and for initial studies, the composition of the reaction medium was kept initially at 10 (%v/v) of water in acetonitrile and at the time of addition of MetEE.HCl, the composition was changed to 15 (%v/v) of water in acetonitrile. This increase in water content serves two purposes: 1) it slows down the polymerization of Lys and 2) facilitates the incorporation of Met at a slow rate.

After the reaction was completed, the mixture was rotary evaporated to dryness. The product was reconstituted in 50% ethanol, centrifuged, filtered and then injected into HPLC for product characterization. Orthogonal confirmation was obtained by the analysis of the synthesized co-oligomers using ESI (+)-MS. The incubation temperature was varied from 25^0C to 60^0C. The overall incubation period was also varied from 2h to 12 h. The yield of oligomers synthesized was determined based on the residual substrates left in the oligomer product.

Acid hydrolysis of purified oligomers. 100mg of the synthesized Lys-Met oligomers were placed in a 40mL vial containing 5mL of 6N Hydrochloric acid. The contents were stirred and kept at 110^0C on a sand bath for 48h. A 1mL aliquot of the solution was taken at 48h and transferred to a round bottom flask. The solution was dried with a rotary-evaporator and reconstituted with 5mL of water. The sample was diluted appropriately and analyzed with ESI (+) – MS to establish the relative concentrations of Lys and Met present in the oligomer product.

Synthesis of Lys-Met tailored peptides in organic solvent systems in the presence of L-Cysteine. The synthesis of Lys-Met tailored peptides was carried out in three-phase system in the presence of L-Cysteine. 0.5mM LysEE was added initially to

the reaction medium that consisted of 2.5mL of DFP, 2.5mL of n-octane and 0.5mL of water. The second substrate (MetEE) was added initially or after 0.5 or 1h incubation period. The concentration of MetEE was either 0.1mM or 0.17mM. 30mg of papain and 100μL of DIPEA were added to the reaction vial. For initial studies, 20mg of L-Cysteine was added as the reducing agent in place of 2-mercaptoethanol. The reaction mixture was placed in an incubator shaker for a period of 24h. Then, the reaction mixture was heated at 80^0C for 5 minutes to stop the reaction. The product was rotary evaporated to dryness. The dried product was reconstituted in 50% ethanol, centrifuged, filtered and injected to HPLC. Orthogonal confirmation was obtained by analysis of the synthesized co-oligopeptides using ESI (+) – MS. Once the starting substrate concentration ratios and time of addition of second substrate was established to obtain a nominal composition of Lys-Met peptides in the ratio of 3:1, the minimum amount of L-Cysteine required for enzyme stabilization was determined.

HPLC analysis of synthesized oligomers and co-oligomers. The separation of residual monomers of Lys, Met, Lys oligomers and Lys-Met co-oligomers was carried out with a reverse phase C-18 column (250mm x 4.6mm i.d, 5μ) and then detected with a fixed wavelength UV-Vis detector maintained at 210nm (Hitachi High Technologies America, San Jose, CA.). A gradient elution program was used and the mobile phase gradient was changed from 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) to 75% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 50 minutes. In all cases, the mobile phase flow rate was maintained at 1mL min^{-1} and 10μL of the sample after filtration with a 0.22μ membrane filter was injected into the column.

ESI (+)-MS characterization of Lys-Met co-oligomers and acid hydrolysate. The distribution of Lys-Met co-oligomers was obtained by injecting the co-oligomers directly into a Hitachi M-8000 ion trap (3DQ- Ion Trap) /Varian 1200 (Triple-Quadrupole) ESI-MS system. An electrospray ionization interface was used in both the cases. The operating parameters of the trap were as follows: Electrospray capillary voltage, +3.5KV; detector voltage, 400V; assistant gas heater temperature, 200^0C; desolvator temperature and the aperture-1 temperature, 200^0C and 150^0C respectively. The trap mass analyzer was scanned from 50 – 1200amu. The operating parameters of the Triple-Quadrupole (operated in MS mode) were as follows: capillary voltage, +5.0 KV; desolvator temperature: 180^0C; assistant gas heater temperature, 180^0C; detector voltage, 1600V. The mass range of the Quadrupole mass filter (Q_1) was set from 100 – 1300amu. Lys-Met co-oligomers were dissolved in 50% ethanol in water solution to form 0.5mg/mL solution. The solution was injected into the ESI-MS using a syringe pump (Harvard Apparatus) at a flow rate of 1mL/h. A make-up solution consisting of 50% acetonitrile in water with 0.1% acetic acid was infused along with the sample at a flow rate of 0.2mL/min.

MS/MS analysis of Lys-Met co-oligomers. The amino acid sequence of specific oligomer residues in the synthesized product was carried out with the Varian 1200 model Triple Quadrupole mass spectrometer operated in the MS-MS mode. Q_1 was used to separate the targeted oligopeptide parent ions, which were then subjected to Collisionally Induced Dissociation (CID) in Q_2. The fragment ions were then separated and monitored in Q_3. All other analysis conditions were exactly the same as that mentioned in the

previous paragraph for characterization of co-oligomers in MS mode using the same model instrument.

RESULTS AND DISCUSSION

The synthesis of co-oligomers of Lys and Met was pursued using two different solvent systems: ACN/water and a three phase systems. The ideal product from the viewpoint of animal nutrition should have a 3:1 ratio of Lys to Met. To achieve this, the following strategy was adopted. Lys was allowed to polymerize but before reaching completion Met was added to the reaction mixture. Therefore it is important to determine the effect of reaction parameters like temperature and incubation period on the oligomerization of Lys. The time-temperature data of Lys oligomerization provides information on the time taken for the completion of Lys oligomerization and also for the effectiveness of papain at high temperatures. Once the time taken for the completion of Lys oligomerization is determined, the second substrate MetEE could be added at a time before the oligomerization of Lys is complete, so that the co-oligomerization process may occur. The percent conversion of Lys and Met was determined for each case and the average composition ratio of the amino acids in the co-oligomer was obtained using the formula shown below:

$$\text{Average Lys : Met composition} = \frac{Lys_0 * X_{Lys}}{Met_0 * X_{Met}}$$

where Lys_0 and Met_0 are the initial concentrations of Lys and Met and X_{Lys} and X_{Met}, their conversion. The results obtained in an acetonitrile/water system were then

compared to that obtained in a three-phase system with L-Cysteine as the anti-oxidant in place of mercaptoethanol.

Effect of temperature on Lys oligomerization in ACN/water system. The effect of temperature on the protease activity in monophasic organic solvent systems was studied first. Lys oligomerization was carried out in 10 (%v/v) water/ACN with the reaction temperature varying from 25^0C to 60^0C. The extent of monomer consumption was determined by assessing the residual Lys and LysEE left in the supernatant after different incubation periods and for each reaction temperature. The chromatogram of the precipitated Lys oligomers synthesized at 25^0C, 37^0C, 50^0C and 60^0C for an incubation period of 2h is shown in ***Figure 2(A-D).*** The chromatogram contains a series of peaks eluting after the retention time of LysEE corresponding to esterified residues of poly-Lys. The degree of polymerization and extent of monomer consumption remain constant after 2h.. The poly-Lys species observed after 24h incubation period at 25^0C were the same as those obtained after 2h. Similar results were obtained at the other temperatures. The ESI (+)-MS spectrum of the precipitated lysine oligomers for an incubation time of 2h at 25^0C is shown in ***Figure 3.*** The oligo-lysine residues with intact ester at the C-terminal (^{N}Lys – $(Lys)_3$ - Lys^{COOEt} + H^+) corresponding to ***m/z 303, 431, 559, 687, 815 and 943*** were the dominant ions in the spectra. The ions were 128amu apart, representing a repeating Lys moiety. ^{N}Lys – $(Lys)_2$ - Lys^C + H^+, a tetramer of lysine should appear at a ***m/z* 531** while ^{N}Lys – $(Lys)_3$ - Lys^C + H^+, a pentamer should appear at a ***m/z* 659.** These ions are less dominant in the spectra.

The extent of Lys oligomerization obtained at different incubation times for various reaction temperatures was studied (***Figure 4***). The oligomerization yield was

monitored by determining the amount of residual amount of monomer and ester left in the reaction mixture. The following formula was used:

Percent yield = $\{(AA\text{-}EE)_{initial} - (AA\text{-}EE + free\ AA)_{final}\}/ (AA\text{-}EE)_{initial} * 100$

The results show that the concentration of free Lys and LysEE in the reaction mixture decreased rapidly in the presence of the enzyme. At an incubation temperature of 50^0C the yield of the oligomerization process was the highest; more than 80% of the initial LysEE added to the reaction medium was incorporated into the oligomer chain. The results also show that the reaction reaches completion in about 2h. There is a decrease in the percent incorporation of Lys in the oligomer chain at 60^0C. We speculate that this drop in monomer incorporation could be due to the drop in activity of papain at this temperature for a prolonged incubation period.

Synthesis of Lys-Met tailored peptides in ACN/water system. The results discussed in the previous paragraphs show that lysine oligomerization goes to completion in two hours; hence MetEE should be added before 2h incubation of the initial reaction mixture. Starting substrate concentration ratio of Lys and Met, time of addition of the second substrate, incubation period, anti-oxidants, temperature of incubation and solvent composition of the reaction medium were varied to achieve a Lys to Met ratio of 3:1.

The synthesis of tailored peptides was carried out with a starting substrate composition of Lys: Met at 1: 1. MetEE was added after an incubation time of 1h. The ESI-MS spectrum of the synthesized oligomers is shown in ***Figure 5.*** The product profile shows a series of peaks corresponding to Lys oligomers and Lys-Met co-oligomers. Lys_3-Met oligomers were almost absent. The product of the reaction was injected into a HPLC column to determine the residual amounts of Lys, Met, LysEE and MetEE present

in the product. The results indicated that percent conversion of both Lys and Met was about 70%. The average composition of the synthesized co-oligomer was determined using the formula mentioned above and it was determined to be ~1: 1 of Lys and Met, which is not the desired result. Because of the higher affinity of the enzyme for Met, more "unfavorable" conditions for the incorporation of Met are obviously needed.

The synthesis of Lys-Met co-oligomers was also carried out with a starting substrate composition of 3: 1 (Lys: Met) in acetonitrile/water system to enrich the final reaction mixture in Lys. MetEE.HCl was added after 0.5h has elapsed and the water concentration was changed from 10% to 15% at the time of addition. The synthesis was carried out at 25^0C, 37^0C, 50^0C and 60^0C and the total incubation time was varied from 2h to 12h. The chromatographic separation of the product obtained at 25^0C for a period of 2h is shown in ***Figure 6A.*** The chromatogram consists of a series peaks eluting after the retention time of LysEE and MetEE. These peaks correspond to oligomers and co-oligomers of Lys and Met. The peaks were co-eluting in some cases and individual peak characterization was not feasible because of the use of the ion-pairing agent hexane-sulfonic acid in the separation. The use of HSA in ESI-MS will lead to salt formation at the capillary leading to increased background (*23*). The residual amount of Lys, Met, LysEE and MetEE present in the product was determined to estimate the percent incorporation of the both amino acids into the oligomers. There was no marked change in the chromatographic output obtained when the incubation period was increased to 6h while all other reaction conditions remained the same ***(Figure 6B)***. The concentrations of free Lys and Met present in the product also remained essentially the same after 2h incubation of the reaction mixture. The product profile was determined by analyzing the

synthesized oligomer product in ESI (+)-MS. The spectrum of Lys-Met co-oligomers is shown in ***Figure 7A.*** The spectrum consists of a series of peaks corresponding to homo-oligomers of lysine and hetero-oligomers of Lys-Met. The homo-oligomers, though present, were less dominant than the hetero-oligomers. The spectral output of the product synthesized when the incubation time was increased to 6h without any change in other reaction parameters showed no differences ***(Figure 7B)***. The product has nearly the same distribution as the one obtained at 2h with the exception of an increase in the intensity of Lys_2-Met.

The ESI (+)-MS spectral output of Lys-Met co-oligopeptides synthesized at incubation temperatures of 37^0C and 50^0C showed a series of peaks appearing at a mass difference of 128 amu corresponding to oligo-Lysine residues. Small amounts of hetero-oligomers of Lys-Met were also present in the product. The relative abundance of oligomers of Lys, Met and Lys-Met co-oligomers synthesized under these conditions is shown in ***Table 2***. The higher abundance of Lys oligomers when compared to Met oligomers and Lys-Met co-oligomers clearly indicates that the incorporation of Met in the oligomer chain was much lower to that of Lys. This was further confirmed by assessing the amount of residual Lys and Met present in the oligomerization product. These results indicated that more than 55% of the initial MetEE remained as free monomer even after 12h incubation. This could be explained to some extent based on the oligomerization profile of Lys at these temperatures. The yield of Lys oligomerization is the highest at 50^0C. At these high temperatures a change in the selectivity of papain can occur in such monophasic solvent systems (*23*) making Lys a more favorable substrate than Met. However, when the reaction temperature was elevated to 60^0C, there was a significant

change in the product profile. The spectrum consists of a series of peaks corresponding to hetero-oligomers of Lys-Met along with homo-oligomers of Lys. The product distribution though similar to that obtained at 25^0C, shows a slightly higher abundance for homo-oligomers of Lys (***Table 2***). The efficiency of Lys oligomerization was much lower at 60^0C, than at other temperatures and this could be the possible reason for the increased formation of hetero-oligomers when compared to 37^0C and 50^0C. Although the product distribution favored the formation of co-oligomers, determination of residual Lys and Met shows that the incorporation of Lys and Met in the oligomer chain was only around 50%. Similar results were obtained when mercaptoethanol was replaced with 20mg of L-Cysteine as the reducing agent in the reaction medium. This indicates that L-cysteine could be an effective replacement for mercaptoethanol, which is very toxic.

The effect of the time of addition of the second substrate on the oligomerization process was studied next by adding MetEE.HCl after an incubation period of 1h. The ESI (+)-MS spectrum of the product (Spectrum not shown) showed a product profile that closely resembles the profile obtained for the oligomer product with 0.5h addition of MetEE.HCl (***Figure 7A***). The only difference is a slight increase in the ratio between homo-oligomers and co-oligomers (***Table 2***). The residual monomer analysis shows a decrease in the incorporation of Met (~55%) in the oligomer chain, down to 55% from the 70% observed when MetEE.HCl was added after 0.5h. Similar results were obtained for 37^0C, 50^0C, and 60^0C for all incubation times. When MetEE.HCl is added after a period of 1h, most of the Lys monomers have been already converted into oligo-Lys and thus very little of it is available for co-oligomerization with Met.

The percent conversion of starting substrates into oligomers was determined in each case by assessing the residual amounts of Lys, LysEE, Met and MetEE present in the product. Percent conversion of Lys and Met substrates into oligomers for different reaction conditions with a starting substrate ratio of 3: 1 (Lys: Met), the addition of second substrate MetEE after 0.5h and 1h incubation of the initial reaction mixture is given in ***Table 1***. Only when the incubation temperature is 25^0C or 60^0C with a starting substrate concentration of 3:1 (Lys: Met) and 0.5h addition of MetEE.HCl, the conversion of Lys and Met in the oligomer chain is nearly the same (~ 70% and 50% respectively) indicating their presence in the oligomer product in the molar ratio of 3:1 (Lys: Met). This was further confirmed by determining the average composition of the product (***Table 1***). It is clear from this table that for a starting substrate ratio of 3:1, with 0.5h addition of MetEE.HCl the nominal composition value is close to 3:1 at 25^0C and 60^0C. There is slight reduction in the incorporation of Met in the oligomer chain when the second substrate MetEE.HCl is added after an incubation period of 1h (***Table 1***). As a result, there is a net increase in the molar ratio of Lys: Met; close to 4:1 in the oligomer product, as is shown in ***Table 1***.

The effect of water content in the reaction medium on co-oligomerization was studied to maximize the distribution of tailored co-oligopeptides in the final product. The water content at the time of addition was changed from 15, 20, 25 and 30% (v/v) of water in acetonitrile. The starting substrate ratio was maintained at 3:1 (Lys: Met); the second substrate MetEE.HCl was added after 0.5h incubation; reaction temperature was maintained at 25^0C and the mixture was incubated for 6h. ***Figure 8*** shows the ESI (+)-MS spectrum of the product synthesized when the water composition of the reaction

medium was changed to 20%. The spectrum shows an increase in the formation of Lys-Met co-oligopeptides with very little formation of homo-oligomers of Lys and Met. The relatively large amounts of homo-oligomers of Lys present when the water content of the reaction medium was changed to 15% (***Figure 7A***) is now absent or present in relatively lower amounts. The assessment of residual amounts of Lys and Met present in the product also shows that the incorporation of Lys and Met in the oligomer chain did not change considerably with the change in water content from 15% to 20%. ESI (+)-MS spectrum of purified Lys-Met co-oligomer acid hydrolysate is shown in ***Figure 9***. The spectrum contains ions appearing at ***m/z 147 and 150*** corresponding to Lys and Met. However, the dominant ions in the spectrum appeared at ***m/z 130 and 133*** corresponding to Lys and Met fragment ions with the loss of NH_3. The relative intensities of these ions were measured to obtain the molar concentration of Lys and Met in the product. The analysis revealed that the actual molar composition was 3.25:1. The ESI (+)-MS spectrum of the oligomers synthesized when the water composition was changed to 30% is shown in ***Figure 10***. The spectrum shows the presence of a series of peaks corresponding to homo-oligomers of Met with lower amounts of co-oligomers of Lys-Met. Therefore, by manipulating the reaction medium composition product profile can be tailored without changing the molar concentration of the amino acids in the oligomers. The effect of change in composition of the reaction medium on the percent conversion of monomers is shown in ***Figure 11***. When the water composition was increased beyond 20%, there was a noticeable drop in the conversion of Lys while the conversion of Met showed a considerable increase. As the water composition increases beyond 20% (v/v) of water in acetonitrile the system tends to behave in a manner similar to 100% aqueous

systems. This results in an increased hydrolysis of preformed Lys oligomers dropping the net conversion of Lys while it favors the formation of Met oligomers.

Tandem MS analysis of specific co-oligomer residues in the synthesized product was carried out to determine the sequence of the amino acids in the residues. MS/MS analysis of different oligomer residues such as Lys_2-Met, Lys_3-Met and Lys_4-Met in the product synthesized with a starting concentration of 3: 1 (Lys: Met) for 0.5h addition of MetEE was performed using a Varian Triple Quadrupole MS (Model 1200). The daughter ion spectrum obtained from the CID of Lys_3-Met (***m/z – 534amu***) parent ion is shown in ***Figure 12***. A detailed explanation of peptide fragmentation and possible daughter ions is well documented in textbooks and review articles (*26*). From the fragment daughter ions in the spectrum we speculate the possible sequence of the Lys_3-Met residue analyzed was Lys-Met-Lys-Lys. From the fragment daughter ions obtained for different residues, we speculate that Met is the second residue in all cases. It seems like before the addition of Met, the partial consumption of Lys (as it forms dimers and higher oligomers) leaves the necessary amount of Lys to react immediately with Met. After that initial addition the dimer Lys-Met becomes the substrate for further additions of Lys (either as monomers, dimers, or higher oligomers).

The above results suggest that a large excess of Lys would decrease the incorporation of Met in the product. This was confirmed by performing the synthesis with a Lys to Met ratio of 5 to 1. The product distribution in this case shows an increase in homo-oligomers of Lys and a decrease in Lys-Met co-oligomers (***Table 3***). The same was true even when MetEE.HCl was added after 1h incubation. No attempts were done beyond that time because the polymerization of Lys is expected to be complete after

approximately two hours. HPLC analysis showed that less than 35% of the initial MetEE added to the reaction mixture was incorporated in the oligomer chain in most cases. Percent conversion of Lys and Met substrates into oligomer product for different reaction conditions with a starting substrate ratio of 5: 1 (Lys: Met), the addition of second substrate MetEE after 0.5h and 1h incubation of the initial reaction mixture is given in ***Table 1***. The percent incorporation of Lys into the product is much higher than the incorporation of Met independently of its time of addition (either 0.5 or 1 h). ***Table1*** shows that the average composition of Lys: Met in the co-oligomer was higher than 10:1 in most cases. In all cases, the reaction was completed in 2 h; there was no observable difference in the residual amounts of Lys and Met left in the oligomer product after this time period.

These results show that a starting substrate concentration of 3:1 (Lys: Met) when incubated at 25^0C for a time period of 2h with MetEE added after 0.5h should result in the desired composition of Lys-Met (3-1) in the tailored peptide.

Synthesis of Lys-Met tailored peptides in three-phase system with L-cysteine. The ESI (+) – MS spectra of co-oligomers synthesized in a three-phase system with a starting substrate ratio of 3:1 (Lys: Met) with simultaneous addition of Met along with Lys for a 24h incubation period is shown in ***Figure 13***. The homo-oligomers present in the spectrum were less dominant than the hetero-oligomers. ***Figure 14*** shows the ESI (+)-MS spectra of Lys-Met co-oligomers synthesized in three-phase system with a starting substrate ratio of 3:1 (Lys: Met) with 0.5h addition of Met along with Lys for a 24h incubation period. Hetero-oligomers of Lys-Met though present in the spectrum were less dominant than homo-oligomers of Lys. Finally, tandem MS analysis shows that the

possible sequence of the Lys_3-Met residue is ***Lys-Met-Lys-Lys***, which is the same as the sequence of the residues obtained in monophasic system. A similar sequence with Met as the second residue was obtained in all the co-oligomer residues analyzed.

The residual amount of Lys, LysEE, Met and MetEE left after the reaction was completed was determined to estimate the percent monomer incorporation in the oligomer chain. The plot of percent conversion as a function of time of addition of second substrate Met is shown in ***Figure 15*** for starting substrate concentrations of 3:1 and 5:1. There is a slight decrease in the percent incorporation of Met with a delayed addition of Met to the reaction medium. The conversion of both Lys and Met is nearly the same (~70%). Therefore, when the substrates are added simultaneously, the average composition of the product is approximately the same as the starting substrate composition.

The minimum amount of L-Cysteine required for maintaining the activity of papain in three phase system was also studied. The synthesis of Lys-Met co-oligomers was carried out with a starting substrate ratio of 3:1 (Lys: Met) with simultaneous addition of Met along with Lys. The amount of L-Cysteine added to the reaction medium was varied from 0.5mmole to 3.3mmole. Percent conversion in each case was determined based on the residual amount of monomers left in the reaction medium. ***Figure 16*** shows that beyond 0.5 mM of L-cysteine added the conversion becomes independent of cysteine concentration. The mass spectrum of the synthesized product shows no change in product distribution upon changes in the amount of L-cysteine added. Finally, the results obtained with L-Cysteine are similar to that obtained with mercaptoethanol as the reducing agent in three phase system (*48*).

Simultaneous addition of Lys and Met is the best approach to synthesize co-oligopeptides of the required average composition in three phase systems because there is no appreciable change in the amount of monomer incorporated into the co-oligopeptide with sequential addition of the substrates. This must be contrasted with the results obtained when the synthesis was done in ACN/water systems where the sequential addition of substrates was needed.

Conclusions. Papain catalyzed oligomerization of Lys at elevated temperatures was successfully carried out. The synthesis of Lys-Met co-oligopeptides with the desired final average composition of 3:1 (Lys: Met) was demonstrated successfully in acetonitrile/water solvent and in three-phase systems. Whereas it was necessary to implement a sequential addition approach in ACN/systems, the synthesis using three phase systems was successful when both substrates were added simultaneously. The reaction parameters were successfully optimized in monophasic system as follows: starting substrate concentration ratio 3:1 (Lys: Met); Time of incubation 2h; Time of addition of second substrate 0.5h; temperature of incubation 25^0C; final reaction medium composition 20 (%v/v) of water in acetonitrile. Manipulating the solvent composition of the system could alter the co-oligomer distribution. The results also indicate that L-Cysteine could be used as an effective anti-oxidant for peptide synthesis. The MS/MS analysis of synthesized peptide residues in both solvent systems indicates the possible presence of Met as the second amino acid in the sequence of some co-oligomers.

LIST OF ABBREVIATIONS

Met, Methionine; Lys, Lysine; K, Lysine; M, Methionine; R, Arginine; LysEE.2Hcl, Lysine Ethyl Ester di-hydrochloride; MetEE.Hcl, Methionine Ethyl Ester Hydrochloride;

MHA, Methionine Hydroxy Analogue; CHES, 2-(N-Cyclohexylamino) ethane Sulfonic Acid; HPLC, High Pressure Liquid Chromatography; RPLC, Reverse Phase Liquid Chromatography; ESI-MS, Electrospray Ionization-Mass Spectrometry; HSA, Hexane sulfonic acid; UV/Vis, Ultraviolet/Visible; ACN, Acetonitrile; MS, Mass Spectrometry; MS/MS, Tandem Mass Spectrometry; RGD peptide, Arg-Gly-Asp peptide; Arg, Arginine; Asp, Aspartate; Gly, Glycine

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FIGURES AND TABLES

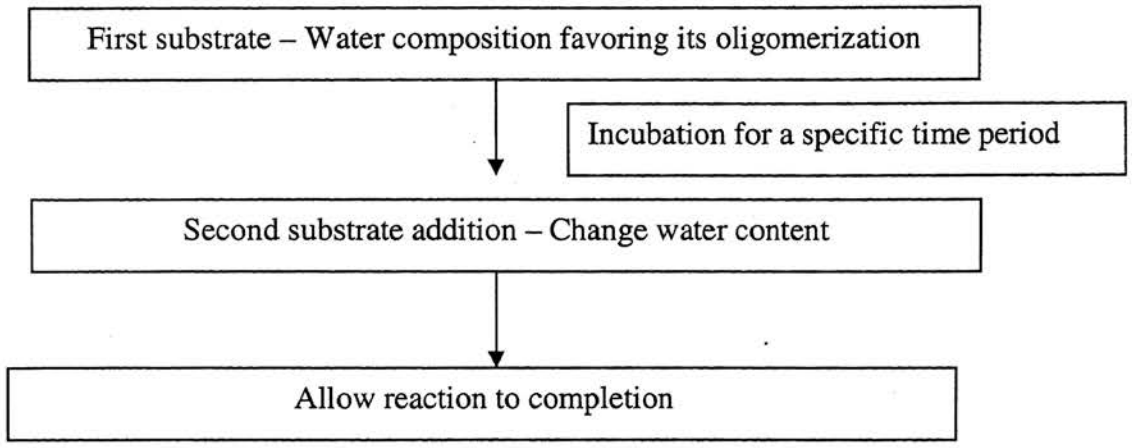


Figure 1: A simple schematic of the strategy adapted for enzymatic synthesis of tailored peptides in monophasic system.

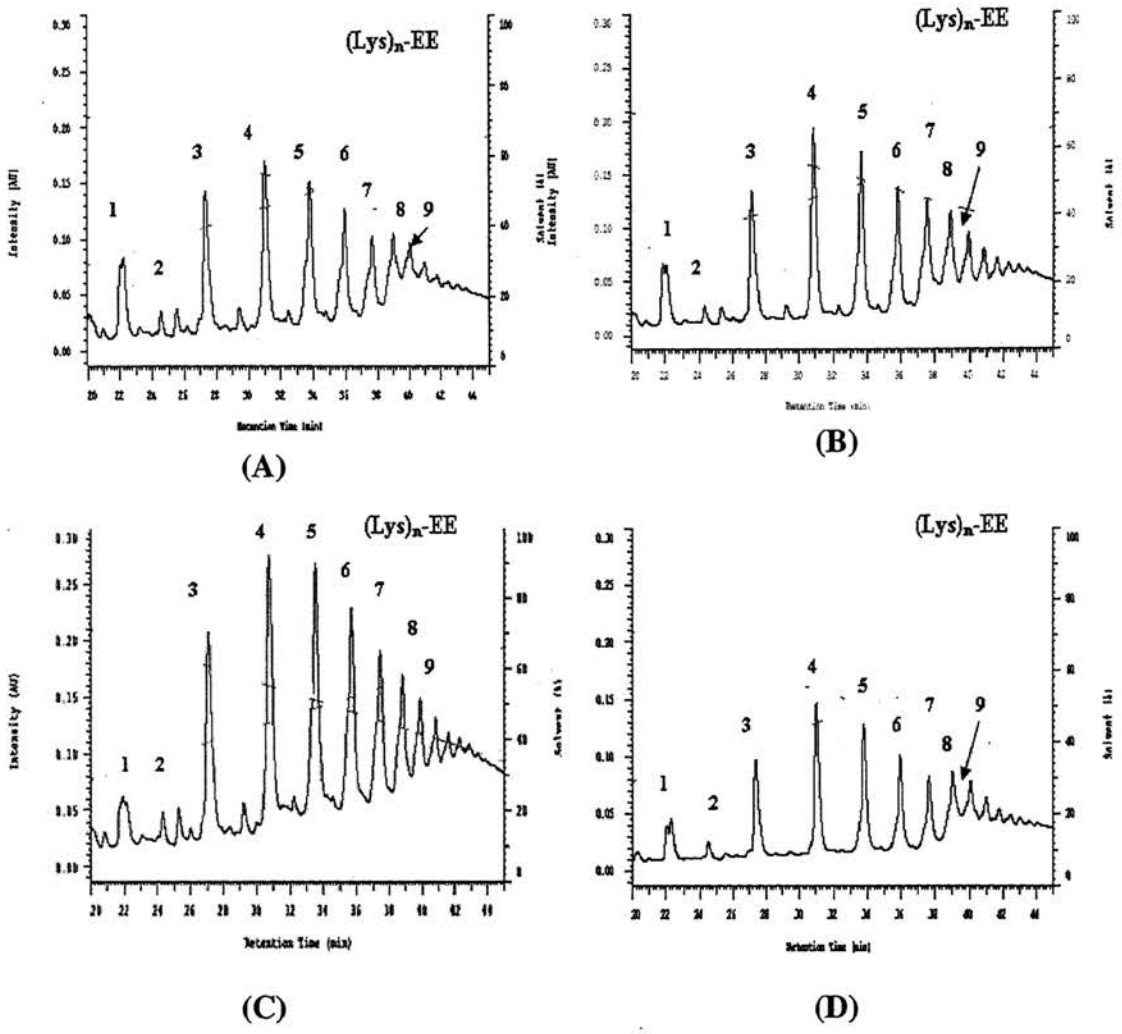


Figure 2: Chromatogram of precipitated Lys oligomers synthesized in 10 (%v/v) of water in acetonitrile system for an incubation period of 2h at A) 25^{0}C B) 37^{0}C C) 50^{0}C and D) 60^{0}C. Separation was achieved in a RPLC C-18 column with HSA as the ion-pairing agent in the mobile phase. Separation was achieved in a RPLC C-18 column with a mobile phase gradient comprising of 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) initial to 75% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 50 minutes.

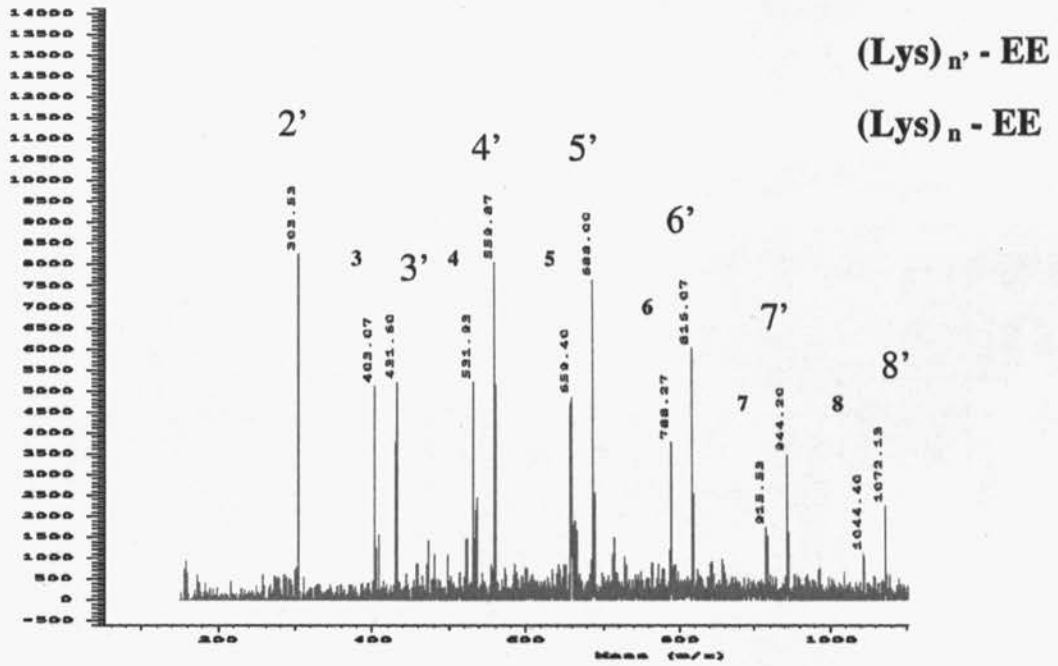


Figure 3: Mass spectrum of precipitated lysine oligomers synthesized in 10 (%v/v) of water/acetonitrile system after 2h incubation at 25^{0}C obtained through direct injection ESI (+) – MS. The spectrum shows the presence of series of peaks corresponding to oligo-lysine.

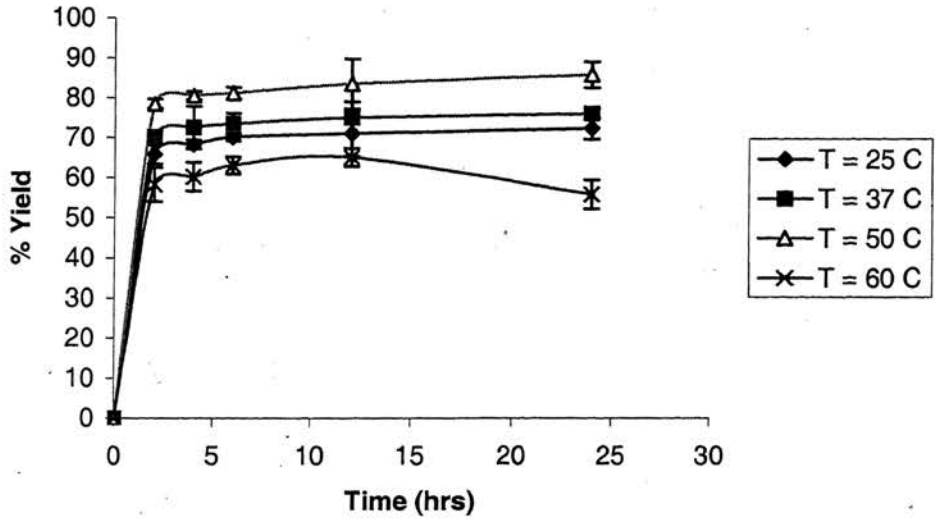


Figure 4: Effect of incubation period on the yield of Lys oligomers synthesized in 10 (%v/v) of water/acetonitrile system at different incubation temperatures.

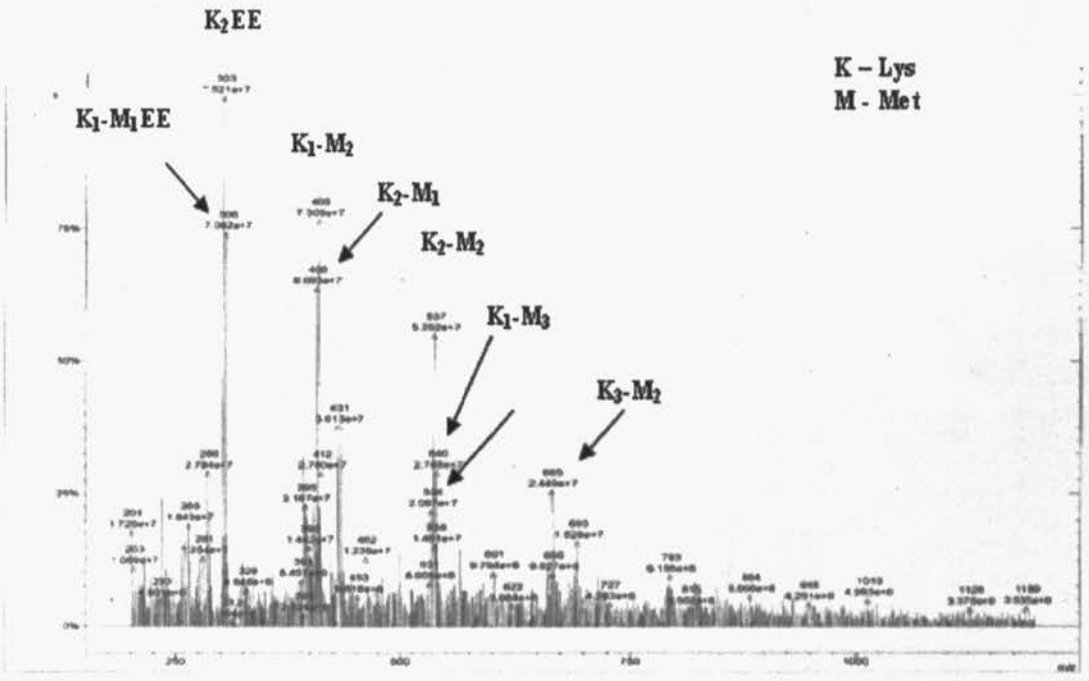


Figure 5: Direct injection ESI (+)-MS spectrum of Lys-Met co-oligomers synthesized in water/acetonitrile system after 24h incubation period for a starting substrate ratio of Lys: Met (1:1). The second substrate MetEE.HCl was added after 1h incubation of the reaction mixture and the system composition was changed to 15 (%v/v) of water/acetonitrile. The spectrum shows the presence of series of peaks corresponding to oligomers of Lys, Met and co-oligomers of Lys-Met.

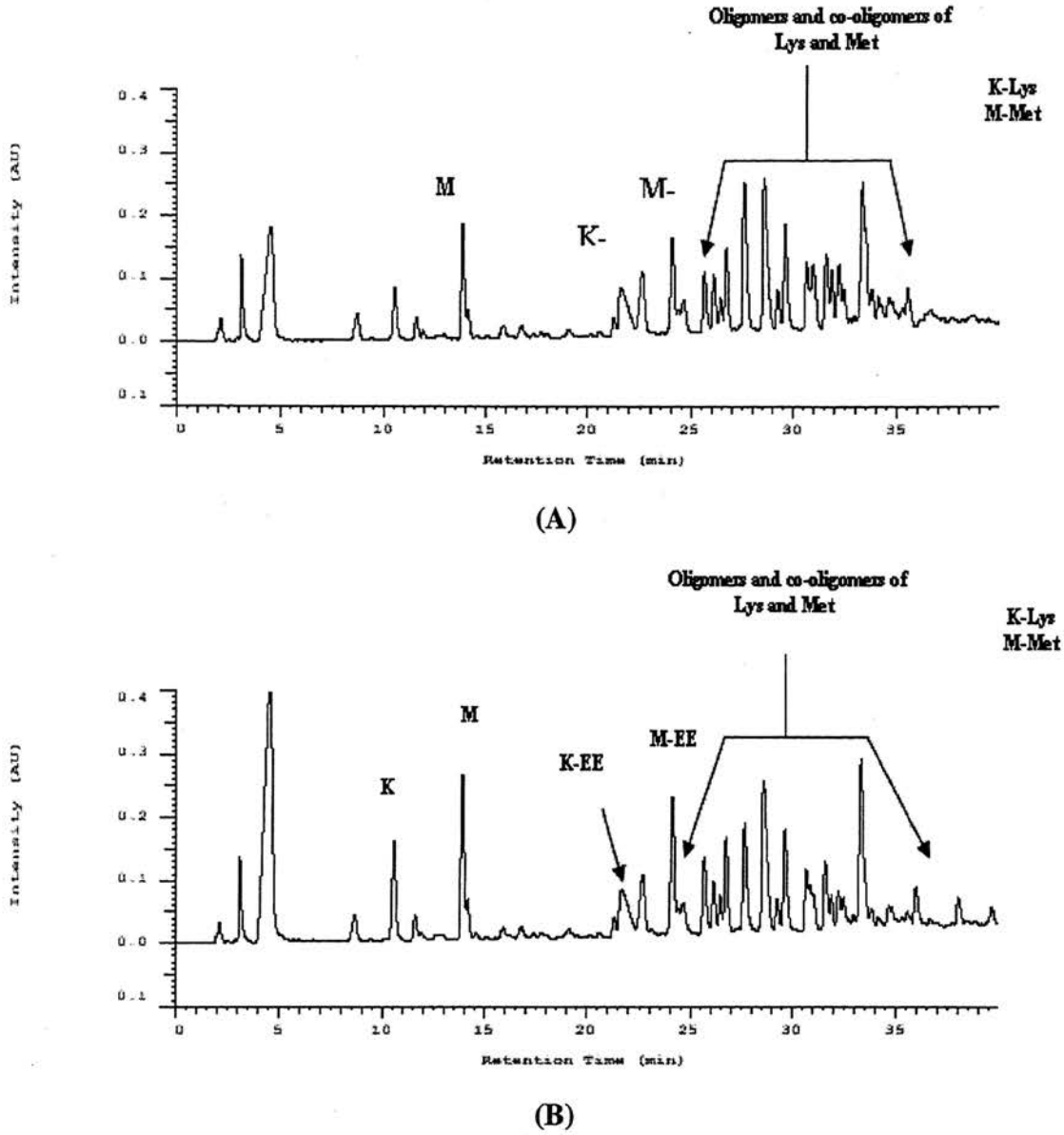


Figure 6: Chromatogram of Lys-Met co-oligomers synthesized in water/ acetonitrile system at an incubation temperature of 25^{0}C with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl for an incubation period of A) 2h and B) 6h. The system composition was changed to 15 (%v/v) of water in acetonitrile at the time of addition of MetEE.HCl. Separation was achieved with a RPLC C-18 column using a mobile phase gradient mentioned in Figure 2. The oligomers co-eluted with one another making their identification difficult.

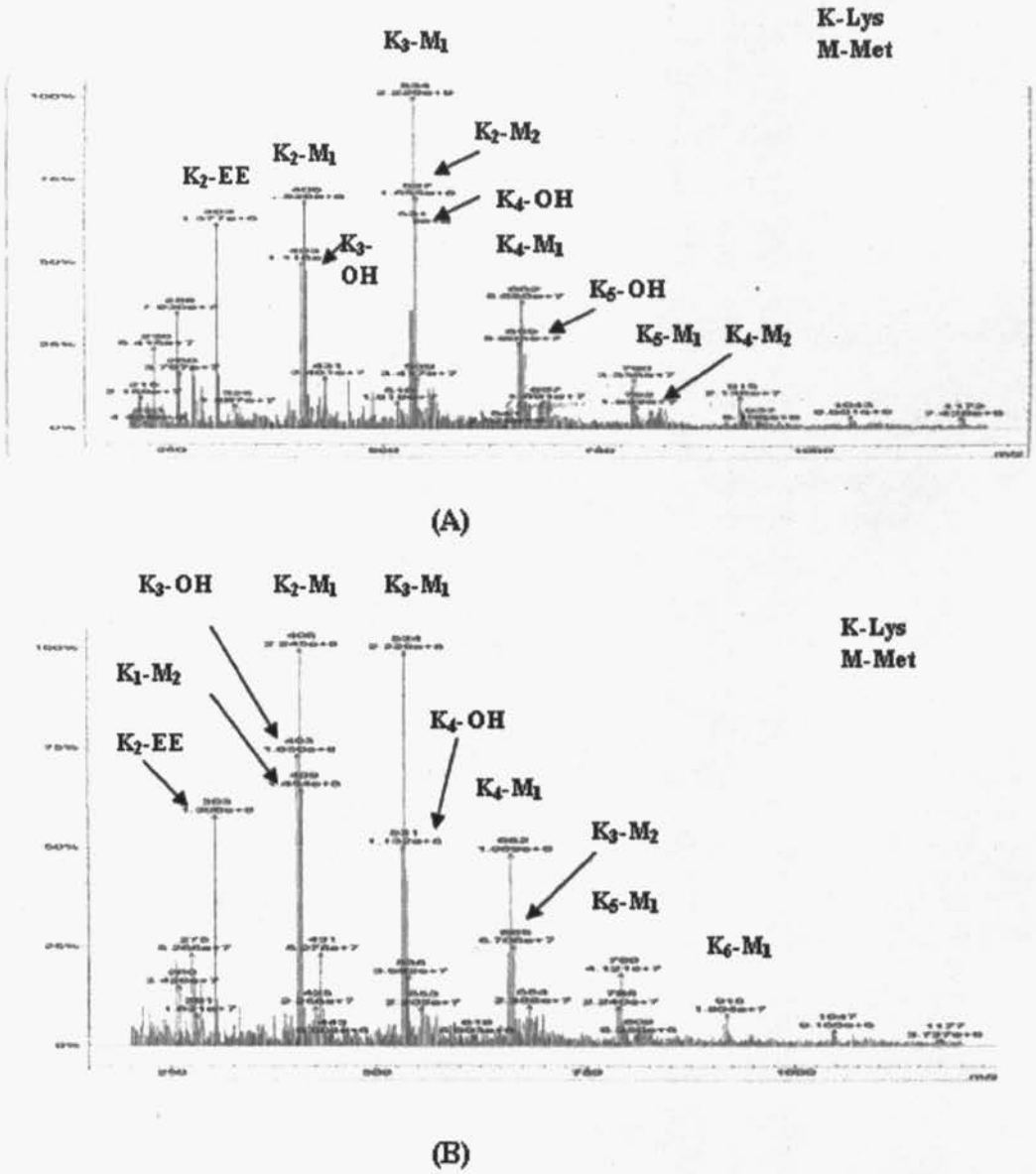


Figure 7: Direct injection ESI (+)-MS spectra of Lys-Met co-oligomers synthesized in water/ acetonitrile system at an incubation temperature of 25^{0}C with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl for an incubation period of A) 2h and B) 6h. The system composition was changed to 15 (%v/v) of water in acetonitrile at the time of addition of MetEE.HCl. The spectrum shows the presence of series of peaks corresponding to oligomers of Lys, Met and co-oligomers of Lys-Met.

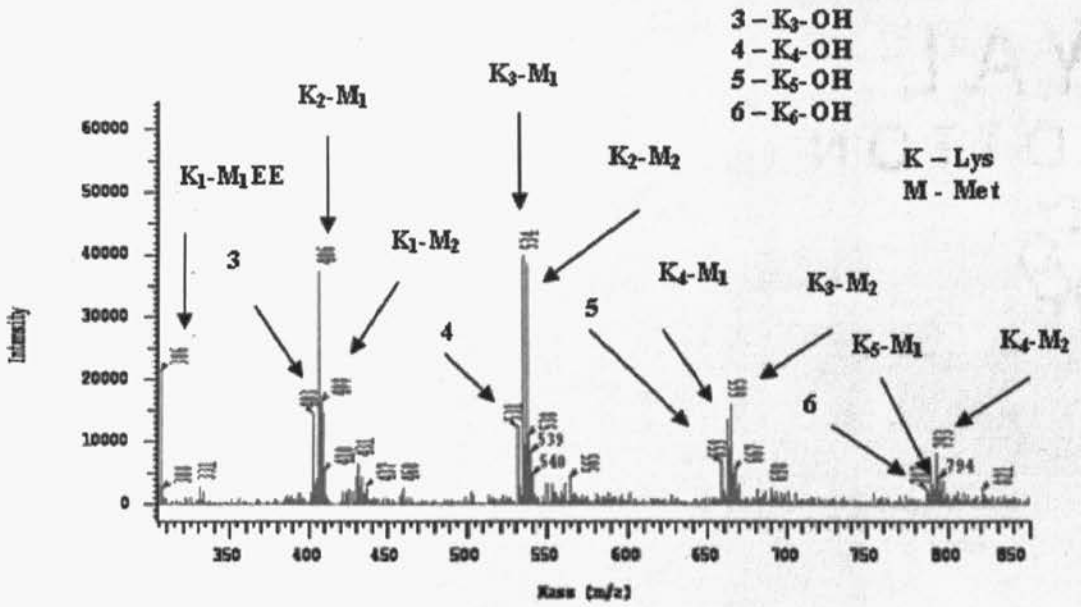


Figure 8: Direct injection ESI (+)-MS spectrum of Lys-Met co-oligomers synthesized in water/ acetonitrile system with a composition change to 20 (%v/v) of water in acetonitrile at the time of addition of second substrate MetEE.HCl for an incubation period of 6h at 25^{0}C with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl. The spectrum shows the presence of series of peaks corresponding to oligomers of Lys, Met and co-oligomers of Lys-Met.

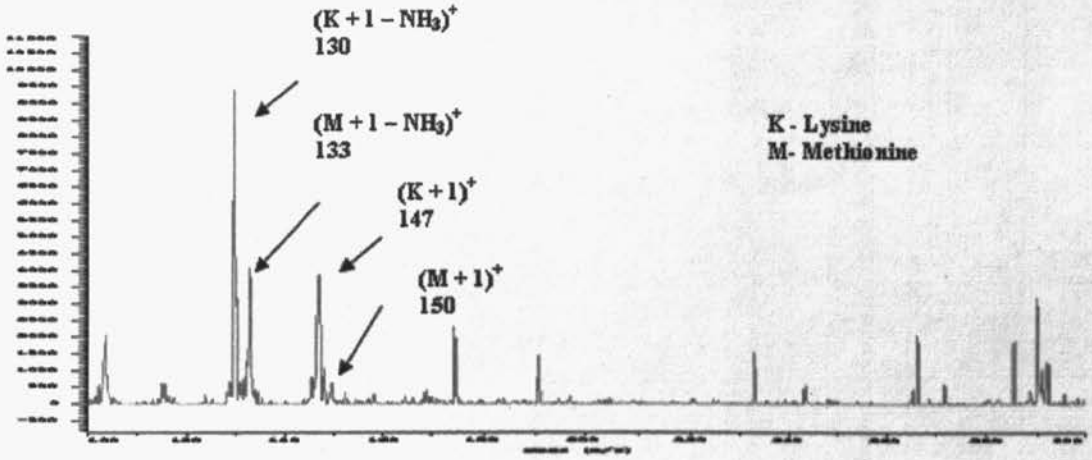


Figure 9: Direct injection ESI (+)-MS of acid hydrolysate obtained from Lys-Met co-oligomers. The co-oligopeptides were synthesized in water/ acetonitrile system at an incubation temperature of 25^{0}C with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl for an incubation period of 6h. The system composition was changed to 20 (%v/v) of water in acetonitrile at the time of addition of MetEE.HCl. The spectrum shows the presence of ions corresponding to Lys and Met.

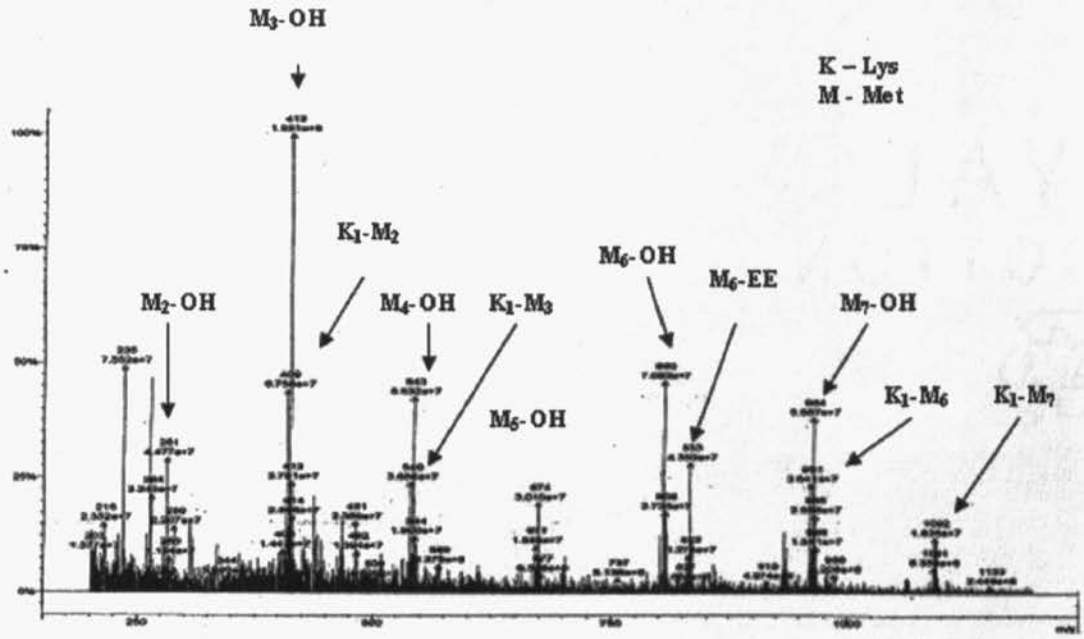


Figure 10: Direct injection ESI (+)-MS spectrum of Lys-Met co-oligomers synthesized in water/ acetonitrile system with a composition change to 30 (%v/v) of water in acetonitrile at the time of addition of second substrate MetEE.HCl for an incubation period of 6h at 25^{0}C with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl. The spectrum shows the presence of series of peaks corresponding to oligomers of Lys, Met and co-oligomers of Lys-Met.

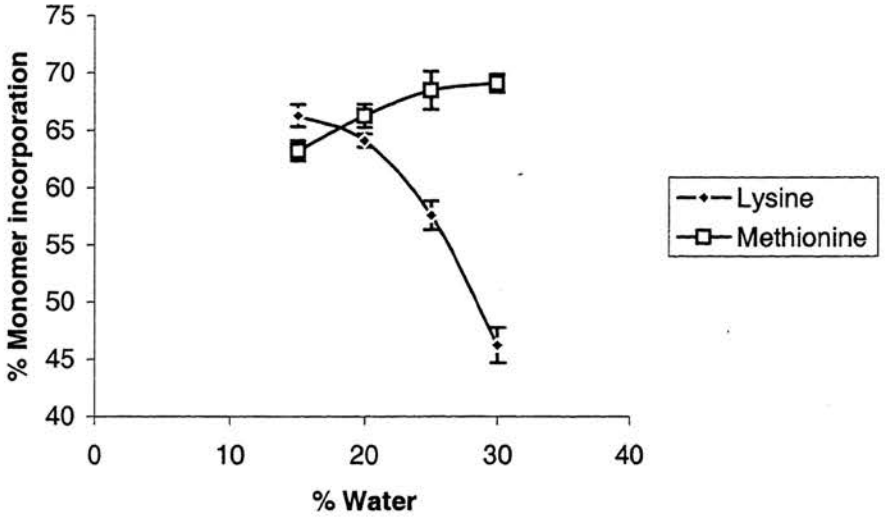


Figure 11: Effect of change in water content on the percent incorporation of Lys and Met in the oligomers synthesized in monophasic water/acetonitrile system. The synthesis was carried out with a starting substrate ratio of 3:1 (Lys: Met) at 25^{0}C for an incubation period of 6h. The second substrate MetEE.HCl was added after 0.5h incubation of the reaction mixture.

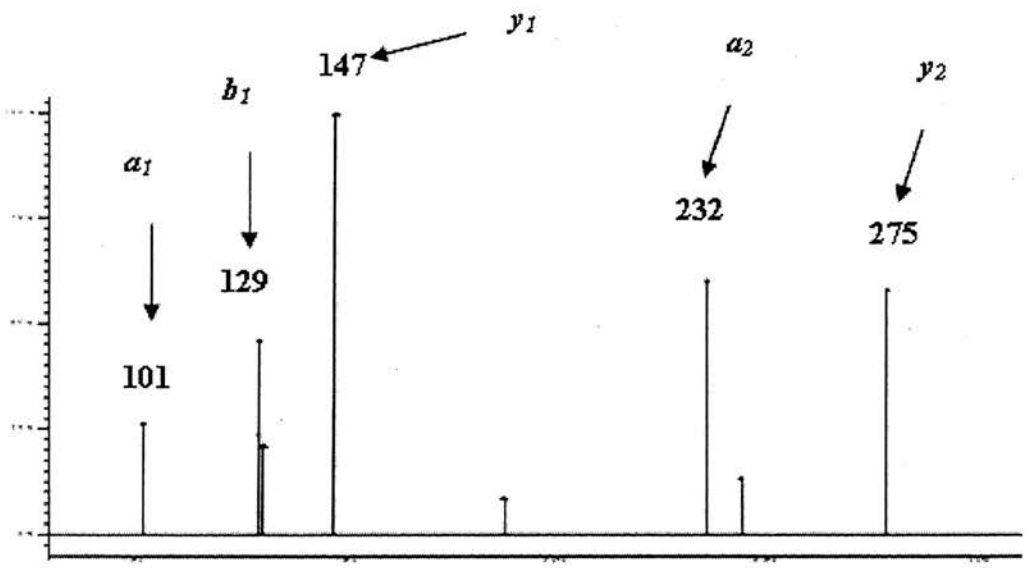


Figure 12: MS/MS spectrum of Lys_3-Met-OH (m/z – 534) co-oligopeptide synthesized in water/ acetonitrile system at an incubation temperature of 25^0C with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl for an incubation period of 6h. The system composition was changed to 20 (%v/v) of water in acetonitrile at the time of addition of MetEE.HCl. The m/z values of the fragment ions labeled in the spectrum indicate the possible sequence of the tetra-peptide to be Lys-Met-Ly-Lys.

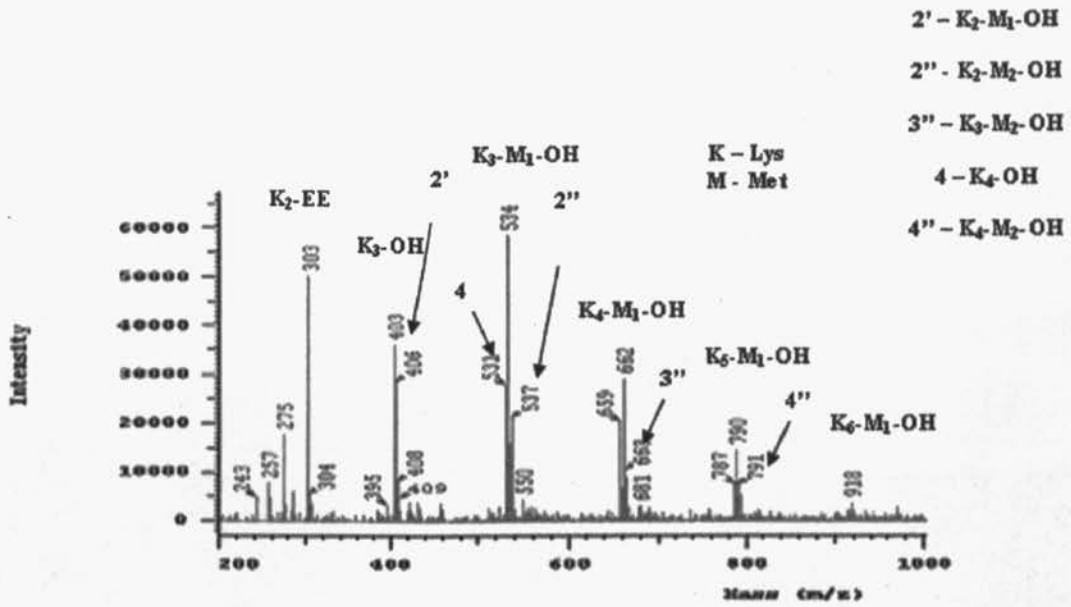


Figure 13: Direct injection ESI (+)-MS spectrum of Lys-Met co-oligomers synthesized in three-phase system with a starting substrate ratio of 3:1 (Lys: Met) and simultaneous addition of second substrate MetEE.HCl for an incubation period of 24h. The spectrum shows the presence of series of peaks corresponding to oligomers of Lys, Met and co-oligomers of Lys-Met.

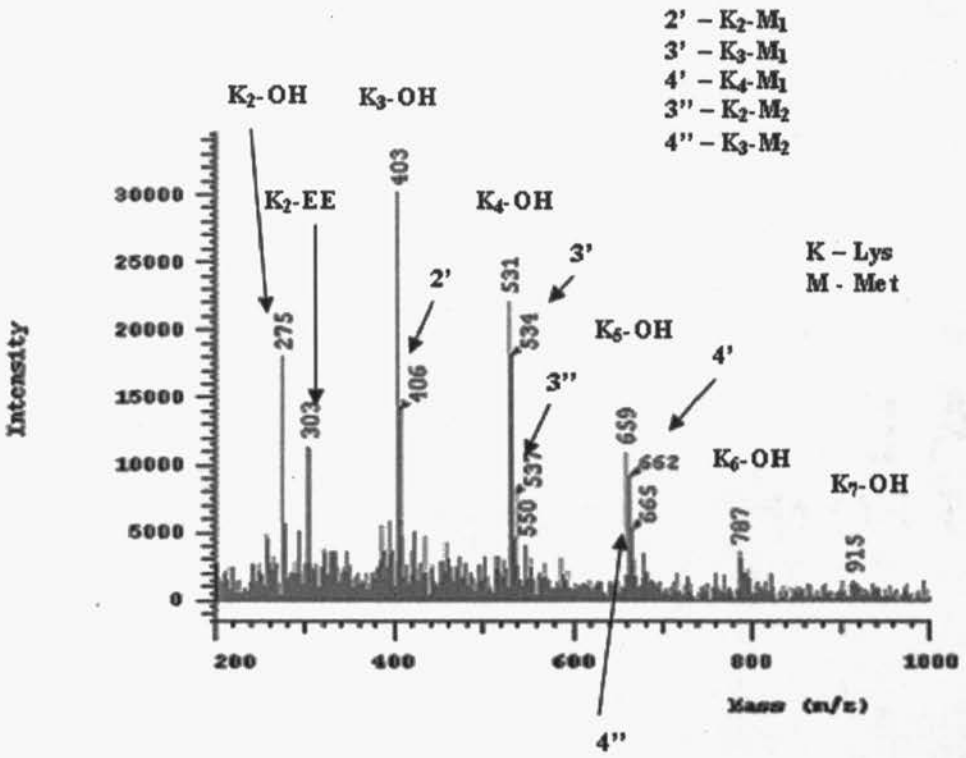
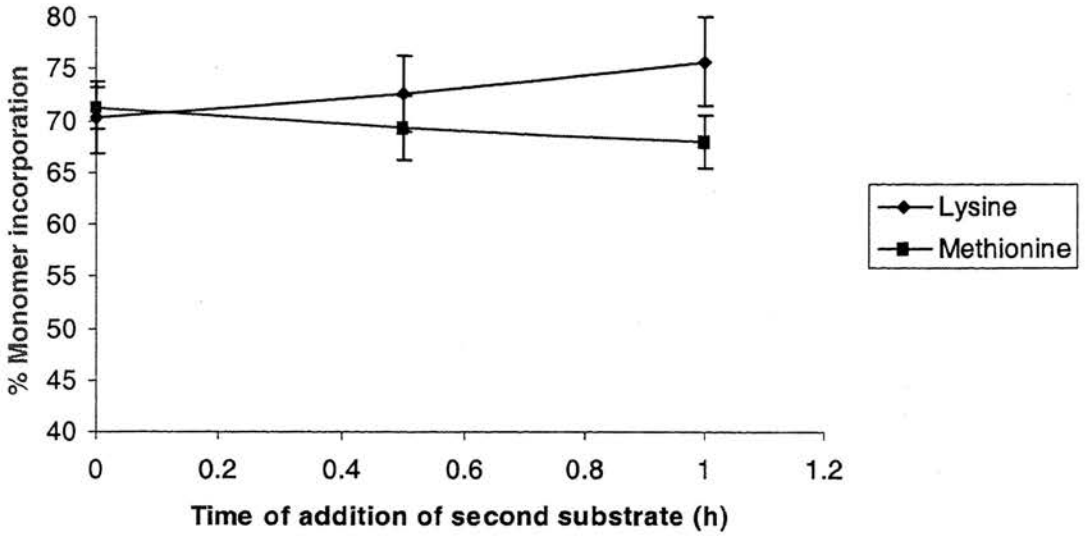
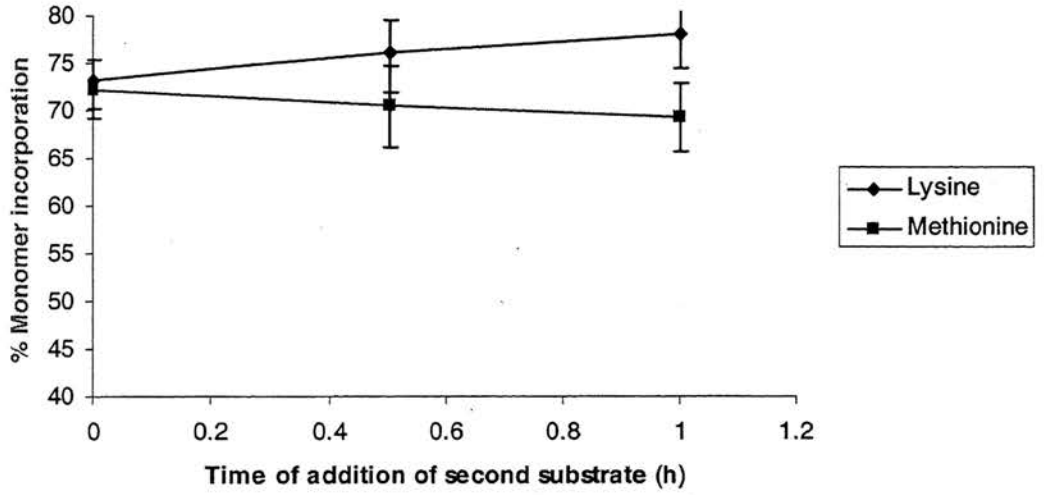


Figure 14: Direct injection ESI (+)-MS spectrum of Lys-Met co-oligomers synthesized in three-phase system with a starting substrate ratio of 3:1 (Lys: Met) and 0.5h addition of second substrate MetEE.HCl for an incubation period of 24h. The spectrum shows the presence of series of peaks corresponding to oligomers of Lys, Met and co-oligomers of Lys-Met.



(A)



(B)

Figure 15: Percent incorporation of Lys and Met into oligomers and co-oligomers synthesized in three-phase system with 20mg of L-Cysteine as the anti-oxidant in the reaction medium as a function of time of addition of second substrate MetEE.HCl for starting substrate concentrations of (A) 3:1 {Lys: Met} and (B) 5:1 {Lys: Met}.

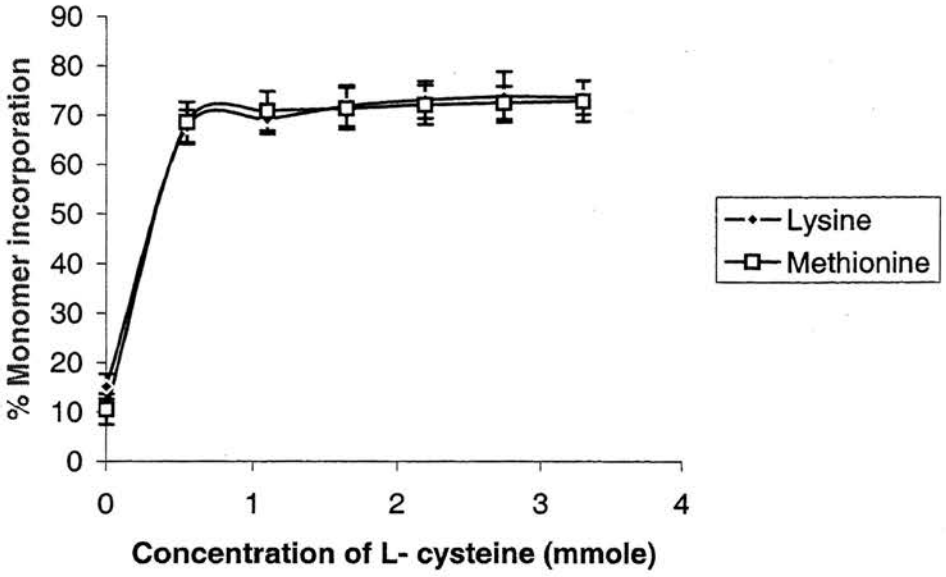


Figure 16: Percent incorporation of Lys and Met into oligomers and co-oligomers synthesized in three-phase system for a starting substrate concentrations of 3:1 (Lys: Met) for varying amounts of L-Cysteine added as the anti-oxidant for papain stabilization in three phase system.

Starting substrate ratio	Time of addition of MetEE.HCl (h)	Temp (°C)	Incubation period (h)											
			2			2			6			12		
			% Con		Nom. comp	% Con		Nom. comp	% Con		Nom. comp	% Con		Nom. comp
			K	M		K	M		K	M		K	M	
3:1	0.5	25	59	55	3.33	66	45	4.13	72	50	4	50	50	2.78
		37	61	61	3.1	69	49	4.4	75	52	4.22	53	52	3
		50	66	66	3	70	50	3.9	77	56	3.9	57	55	3.2
		60	68	70	3.4	72	54	4	80	59	4	60	60	3
	1	25	62	44	3.9	70	40	5	74	39	5.3	57	46	3.6
		37	66	50	3.7	73	43	5.3	78	43	5.6	59	49	3.8
		50	70	53	3.9	75	49	4.8	80	45	5	63	50	3.6
		60	72	59	3.6	78	51	4.3	82	49	5.1	67	52	3.8

Starting substrate ratio	Time of addition of MetEE.HCl (h)	Temp (°C)	Incubation period (h)											
			2			4			6			12		
			% Con		Nom. comp	% Con		Nom. comp	% Con		Nom. comp	% Con		Nom. comp
			K	M		K	M		K	M		K	M	
5:1	0.5	25	62	29	10.3	69	21	17.5	74	19	18.5	52	35	6.5
		37	65	32	11	71	25	12	79	22	20	56	39	7
		50	67	33	11.3	74	30	12.3	80	25	13.3	58	42	7.3
		60	70	36	8.8	76	31	12.7	82	26	13.3	61	45	6.2
	1	25	64	34	10.7	72	26	12	76	20	19	57	40	7.3
		37	68	37	8.5	76	30	12.7	80	25	13.3	59	44	7.5
		50	72	38	9	79	35	10	83	26	14	63	48	6.4
		60	74	40	9.3	80	37	10	85	29	14.3	67	50	6.8

Table 1: Nominal composition of Lys-Met peptides synthesized under different conditions in monophasic system (K-Lys; M-Met; Nom. Comp – ratio of K: M).

Time of addition of MetEE.HCl (h)	Temperature (^{0}C)	Incubation time (h)											
		2			4			6			12		
		K	M	K-M	K	M	K-M	K	M	K-M	K	M	K-M
0.5	25	30	5	65	28	5	67	29	4	67	28	5	67
	37	65	2	33	67	3	30	68	3	29	67	3	30
	50	75	2	23	77	3	20	77	2	21	78	2	20
	60	52	3	45	51	3	46	52	2	46	53	2	45
1	25	58	2	40	57	2	41	57	3	40	58	3	39
	37	69	3	28	70	3	27	70	2	28	71	1	28
	50	78	2	20	79	3	18	78	3	19	79	3	18
	60	62	3	35	63	3	34	62	2	36	63	2	35

Table 2: Relative abundance of homo-oligomers of Lys (K), Met (M) and Lys-Met co-oligomers (K-M) synthesized in ACN/water system for different incubation periods and temperature with a starting substrate ratio of 3:1 (Lys: Met). The second substrate MetEE.HCl was added after 0.5h and 1h, respectively. The system composition was changed to 15 (%v/v) of water in acetonitrile at the time of addition of MetEE.HCl.

Time of addition of MetEE.HCl (h)	Temperature (^{0}C)	Incubation time (h)											
		2			4			6			12		
		K	M	K-M	K	M	K-M	K	M	K-M	K	M	K-M
0.5	25	70	2	28	71	3	26	70	3	27	73	2	25
	37	78	1	21	79	2	19	79	1	20	79	2	19
	50	81	2	17	82	2	16	81	2	17	82	2	16
	60	65	3	32	64	3	33	66	2	32	66	3	31
1	25	73	3	24	74	2	24	75	3	22	74	3	23
	37	80	1	19	81	2	17	81	1	18	82	1	17
	50	85	2	13	84	2	14	85	2	13	84	2	14
	60	68	3	29	67	3	30	68	2	30	69	3	28

Table 3: Relative abundance of homo-oligomers of Lys (K), Met (M) and Lys-Met co-oligomers (K-M) synthesized in ACN/water system for different incubation periods and temperature with a starting substrate ratio of 5:1 (Lys: Met). The second substrate MetEE.HCl was added after 0.5h and 1h, respectively. The system composition was changed to 15 (%v/v) of water in acetonitrile at the time of addition of MetEE.HCl.

IV. ENANTIO SEPARATION OF HMB THROUGH PROTEASE CATALYZED PEPTIDE SYNTHESIS AND CHIRAL LC-MS ASSESSMENT

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Abstract. Alpha hydroxy acids (AHAs) have received wide attention in cosmetic and animal-feed industries. Lactic acid, malic acid and the hydroxy analog of methionine (HMB) are important members of the AHA group. The chemically synthesized formulations of alpha hydroxy acids are a racemic mixture comprising of 50% L and 50% D forms. It is a well-documented fact that enantiomeric forms of chemicals can possess different activities. Enantio purity of chemicals is important in their use in pharmaceutical applications and it may play a significant role in nutritional and cosmetic applications as well. However, this role has not been fully documented because of the lack of enantio pure HMB and other AHAs. In this work, papain and chymotrypsin catalyzed peptide capping was evaluated for obtaining enantio pure HMB. Chymotrypsin catalyzed peptide capping was found to be a very fast and efficient tool to obtain enantiopure HMB. The enantio purity of products was evaluated with Reverse Phase liquid Chromatography and Chiral Liquid Chromatography-Mass Spectrometry. The results indicate that enantio-purity of greater than 95% was obtained in less than ~ 30 minutes with chymotrypsin catalyzed capping HMB capping reaction.

Keywords: Alpha hydroxy acids (AHAs), HMB, Lactic acid, Malic acid, enantiomers, enantio-enrichment, Chymotrypsin, Papain, Chiral Liquid chromatography-Mass Spectrometry

INTRODUCTION

Met and Lys are the primary limiting amino acids in a number of animal species. Therefore, supplementation of these amino acids in the feed is required for their proper growth (*1, 2*). Studies indicate that 40 to 80% of Met in the feed proteins is used by microbial colonies of the digestive system (*3, 4, 5*) and is not available for the animal. When crystalline forms of these amino acids are supplemented, the pre-gastric fermentative step converts these amino acids to short chain fatty acids and ammonia (6, *7*). Structural manipulation of these amino acids to provide effective resistance to microbial degradation is one method to achieving high by-pass of these amino acids in the digestive system (*8*). One of those strategies involves the supplementation of amino acid analogs. HMB (1-hydroxy methylthio butanoic acid), also known as MHA is an analog of Met (The structures of Met (***A***) and HMB (***B***) are shown in ***Figure 1***). Studies have proven the effectiveness of HMB as a high by-pass Met feed supplement for poultry, swine, lactating cows, lambs and heifers (*9, 10, 11*). The rumen microorganisms do not recognize the terminal hydroxy group and hence HMB escapes the rumen intact (*12, 13*). HMB is converted to Met in the animal by a two-step reaction: 1) oxidation of the hydroxyl group and 2) subsequent trans-amination (*14*). A racemic mixture of HMB (89% DL-HMB and 11% water) is produced chemically in commercial scale and is sold under the trade name ***ALIMET*** by Novus International, St. Louis, MO.

It is a well-established fact that different enantiomers have different biological activities. For example, only the L-form of Met is absorbed in the brush border membrane of the intestines (*16*). Supplementation of HMB till date has been in the form of a racemic mixture and the long-term effects of the individual enantiomers have not

been determined. Enantiopure forms of HMB have to be synthesized to evaluate their difference in activities and properties. Novus International reports have mentioned that 1g of enantiopure HMB costs around $1000 and these high costs have prevented a detailed assessment of the properties of these enantiomers. It becomes necessary to develop an economically viable process to obtain enantiopure HMB.

The three most common methods to resolve optical isomers are direct crystallization, kinetic resolution and diastereomeric complex formation. A detailed description of these mechanisms and approaches is well documented and available elsewhere (*16, 17, 18, 19*). The most widespread procedure for enantiomeric resolution has been biocatalyst (enzymes) based kinetic resolution. Most enzymes act only on one form of the enantiomer while the other enantiomer could be recovered in excess from the unreacted residue (*20*). Lipases (EC 3.1.1.3) have been the preferred enzymes of choice for chiral resolution studies because of their high selectivity and specificity for one of the enantiomers. Lipase catalyzed resolution of racemic acids in bi-phasic medium by asymmetric esterification and trans-esterification was first demonstrated twenty years ago (*21, 22*). Lipase (***Aspergillus Niger***) catalyzed resolution of N-protected non-protein amino acids (e.g. Homocysteine, Ornithine, Citrulline), which are used as toxins and hormones through ester hydrolysis, have been reported (*23*). Lipase catalyzed kinetic resolution of numerous other substrates have been reported and an entire description is beyond the scope of this article (*24, 25, 26, 27*). We have reported procedures for the optical resolution of alpha hydroxy acids, especially HMB, through lipase catalyzed hydrolysis and esterification (*16, 28*).

Proteases belong to a class of enzymes that hydrolyze peptide bonds but under proper conditions they could be also used to catalyze highly enantio-specific peptide bond synthesis. Proteases like papain and chymotrypsin show an enhanced specificity towards L- amino acids while the D-amino acids remain unreacted (*16, 29, 31*). This property of proteases could be used to optically resolve racemic mixtures. For example, papain catalyzed enantio-enrichment of Met through the formation of oligomers of Met in aqueous system has been reported (*29*). L-Met was oligomerized while D-Met was not. We have also reported chiral resolution of Met using the same procedure in a monophasic aqueous organic system consisting of 40% water/60% acetonitrile (*30*). Proteases could also be used to obtain optically pure alpha hydroxy acids. When hydroxy acid capped co-oligomers are synthesized, only one form of the enantiomer is incorporated into the oligomer chain. Thus, the unreacted mixture is enriched in the other enantiomer.

HMB capped co-oligomers of Met. Tyr, Phe, Lys, Arg and numerous other amino acids were synthesized in different solvent systems using papain as the catalyst (*16, 29*). Hydrolysis and the subsequent chiral LC-MS studies proved the enantio-selective incorporation of L-HMB in the peptide chain (*16, 29*). In this study, we present a simple and rapid approach for enantio-enrichment of HMB using a protease-catalyzed peptide capping reaction. Two approaches were followed. In the first one, HMB was incorporated into Phe, Met and Lys oligomer chain using papain and chymotrypsin as the catalyst. In the second, HMB-LysEE adducts were synthesized using chymotrypsin as the catalyst. After the reactions were completed, residual HMB ester was recovered, hydrolyzed and its enantio purity was assessed with chiral chromatography.

MATERIALS AND METHODS

Materials. L-Lysine ethyl ester (LysEE) dihydrochloride, L-Cysteine hydrochloride monohydrate (Cys), n-Octane, L-Phenyl alanine ethyl ester (PheEE) hydrochloride, L-Methionine ethyl ester (MetEE) hydrochloride, N, N diisopropylethylamine (DIPEA), acetic acid, ammonium acetate and trifluoroacetic acid were purchased from Sigma Chemical Co., (St.Louis, MO). Anhydrous methanol, ethanol and propanol (200 proof), sodium phosphate (dibasic, anhydrous), sodium salt of Hexane sulfonic acid (HSA), O-Phosphoric acid, acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific, (St.Louis, MO). 1,1,1,2,3,4,4,5,5,5-decafluorpentane (DFP) was purchased from Miller-Stephenson Chemical Company (Danbury, CT). Papain (EC 3.4.22.2, 25 units activity/mg, 28mg protein/mL) was provided by Novus International Inc., (St.Louis, MO). DL-Hydroxy methylthio butanoic acid (HMB-Alimet) was procured from Novus International, (St. Louis, MO). α - Chymotrypsin (EC 3.4.21.1, 66 units /mg of solid, From Type II: Bovine Pancreas) crystallized thrice from chymotrypsinogen was purchased from Sigma Aldrich Co., (St. Louis, MO). RPLC separation of hydrolysate was carried out with a XPERCHROM C-18 column (250mm x 4.6mm) purchased from P.J. Cobert Associates Inc., (St.Louis, MO). The Chirobiotic - TAG column (250mm X 4.6mm) used for chiral separation of HMB was purchased from Advanced Separation Technologies Inc., (Whippany, NJ). The nanopure water used in the experiments was obtained after filtration through a Synergy 185 filtration system purchased from Millipore Corp. (Billerica, MA).

Liquid Chromatography with UV detection. A model L-7000 HPLC system (Hitachi High Technologies America, San Jose, CA) was used to carry out the HPLC

separations. The system consisted of a reciprocating piston pump (L-7100) fitted with a column oven (L-7300), autosampler (L-7200) and with a 50μL injection loop. The analytes separated on the reverse phase columns were introduced into a UV-Vis absorbance detector (L-7420).

ESI-Mass Spectrometer. An Ion Trap Mass Spectrometer equipped with an Electrospray ionization interface (Model M-8000) purchased from Hitachi high Technologies America, San Jose, CA was used for the mass analysis of the synthesized oligomers and HMB capped co-oligomers.

Synthesis of DL-HMB ester. DL-HMB was esterified with anhydrous methanol, ethanol or propanol in the presence of HCl gas using a procedure described elsewhere 16). The synthesized ester was rotary evaporated to dryness. The dried ester was reconstituted in water, centrifuged, filtered and injected into RPLC for characterization. The synthesized esters had a purity of ~98%.

Synthesis of Phe and Lys oligomers in three-phase system. L-PheEE hydrochloride (0.5mM) was added to a reaction flask containing 250 mL of DFP, 250 mL of n-octane, 50 mL of water, 10mL of DIPEA, 2 g of cysteine, and 300mg of papain. The reaction vial was incubated for 24h in an incubator shaker. The reaction was stopped by heating the mixture to 80^0C for 5 minutes. The reaction product was rotary evaporated to dryness. The dried product was washed with 10mL of Nanopure water three times. The water washed oligomer product was lyophilized. The dried product was esterified with absolute ethanol in the presence of HCl gas. The esterification mixture was refluxed overnight. The reaction product was recovered by rotary evaporating the excess ethanol under vacuum. An aliquot of the dried Phe oligomer ester was made by dissolving it in

70% acetonitrile/30% water, centrifuged, filtered and injected into HPLC for characterization. Another aliquot of the dried oligomer ester was dissolved in DMSO, centrifuged, filtered and injected into ESI – MS for determining the oligomer distribution. A similar approach was used for the synthesis of Lys oligomers with LysEE as the substrate. In this case, the dried oligomers were dissolved in 50% ethanol/water for ESI-MS characterization.

Synthesis of Met oligomers in aqueous system. Methionine oligomers were synthesized in an aqueous reaction media at pH 5.5 using papain as catalyst. 30.0 grams of methionine ethyl ester was dissolved in 100ml of nanopure water in a 500mL flat-bottomed flask. 10mM L-cysteine, 1.0mM EDTA and 0.1mole sodium citrate were added after the ethyl ester of methionine was dissolved. The contents were stirred and the pH was adjusted to 5.5. 300mg of crude papain was added to that mixture and the reaction mixture was kept at 37^0C with constant shaking for 24h. Then, the reaction was stopped by heating the reaction mixture at 80^0C for 5min. The mixture was transferred to 200mL centrifuge vials and centrifuged at 10000rpm for 10 minutes to remove residual monomers and salts. The precipitate was washed thrice with 100 mL deionized water to remove residual monomers. The supernatant after the third wash was analyzed with HPLC to check for the presence of any monomers. The washed precipitate was dried by lyophilization. A known aliquot of the dried oligomer was made by dissolving it in 70% acetonitrile/30% water, centrifuged, filtered and injected into HPLC for characterization. Another aliquot of the dried oligomer was dissolved in DMSO, centrifuged, filtered and injected into ESI – MS for determining the oligomer distribution.

Synthesis and purification of HMB capped poly-Phe and poly-Met. 500mg of the purified Phe and Met oligomers were dissolved in 10mL of 60 % (v/v of acetonitrile/water) containing 1mmole L-cysteine, 0.1mmole EDTA and 0.01mmole sodium citrate. 500mg of HMBEE and 30mg of papain were added after the oligomers were dissolved. The reaction was allowed to proceed for 24h. The reaction was stopped by heating it at 80^0C for 5min. The resultant mixture was centrifuged to separate the supernatant (residual Met and Phe oligomers and HMB monomers) from the precipitate (co-oligomers).

The precipitate was lyophilized. The dried precipitate was injected into HPLC-ESI (+)-MS to obtain the distribution of the synthesized co-oligomers. The freeze-dried oligomers were washed three times with 100mL of water to remove any residual monomers and smaller oligomers of Met and Phe. The precipitate from this washing was dissolved thrice in DMSO and reprecipitated with water to remove residual HMB monomers. This washed precipitate was lyophilized to obtain pure co-oligomers.

Acid hydrolysis of HMB-$(Phe)_n$ and HMB-$(Met)_n$ co-oligomers. 500mg of the synthesized HMB-Phe and HMB-Met co-oligomers were placed in a 40mL vial containing 10mL of 6N Hydrochloric acid. The contents were stirred and kept at 110^0C on a sand bath for 48 h. A 1mL aliquot of acid solution was after 8h and transferred to a round bottom flask. The solution was dried with a rotary-evaporator and reconstituted with 5mL of water. The sample was diluted and analyzed by reverse phase liquid chromatography to determine the percent incorporation of HMB in to the oligomer chain.

Chymotrypsin catalyzed HMB capping of Lys oligomers. 50mg of Lys oligomer and 50 mg of HMB-ME were added to a reaction vial containing 5mL of 50mM

sodium phosphate (dibasic) buffer (pH 7.8). To this vial, 250μL (50units) of enzyme suspension (3.33mg/mL of sodium phosphate-dibasic) was also added. The reaction mixture was incubated in a shaker for 15, 30, 45, 60, 120 and 180 min. After each time period, a 250μL aliquot was taken for analysis. This aliquot was diluted proportionately, centrifuged, filtered and 5μL aliquot of the filtered product was injected directly into ESI (+) -MS for determining the product profile. The same procedure was repeated with HMBEE as substrate.

Synthesis and recovery of HMB-LysEE dimer. HMB capped LysEE dimer was synthesized using the same procedure mentioned above for the capping of oligomers of Lys with HMB with the addition of HMBME, HMBEE and HMBPE substrate along with LysEE. The product was characterized by injecting it into ESI (+)-MS for characterization.

The product (HMB-LysEE dimer plus unreacted substrates) was rotary evaporated to dryness. The dried product was washed with absolute ethanol thrice to remove residual unreacted HMB ester and residual HMB. The ethanol washings were then pooled, diluted to 50% ethanol/water mix, centrifuged, filtered and injected to RPLC for characterization.

Acid hydrolysis of residual HMB ester. The pooled ethanol washing was rotary evaporated to dryness to recover the residual HMB ester and HMB. The recovered HMB ester was hydrolyzed with 5mL of 6N HCl. This mixture was placed in a heated sand bath maintained at 110^{0}C for a period of 48h. The hydrolysate was rotary evaporated to dryness under vacuum. The resultant product was reconstituted in 5mL of water, centrifuged, filtered, characterized and quantified by HPLC. The enantiopurity of the

hydrolysate was determined by characterizing it using Chiral Liquid chromatography equipped with UV detection.

Acid hydrolysis of HMB-LysEE dimer. The dried HMB-LysEE dimer product (ethanol washed and unwashed) was hydrolyzed using the same procedure mentioned above. The hydrolysate was dissolved in water, centrifuged, filtered and then injected into HPLC for characterization and quantification. The enantiopurity of HMB incorporated into the dimer was also established by characterizing the hydrolysate using chiral liquid chromatography equipped with UV detection.

Reverse Phase Liquid Chromatography. The separation of A) Phe oligomers and HMB capped Phe co-oligomers; B) Phe, Met and HMB present in the co-oligomer acid hydrolysate and C) the residual HMB ester recovered by ethanol from the dried HMB-LysEE dimer and the corresponding monomer obtained from its acid hydrolysis was carried out with a reverse phase C-18 column (250mm x 4.6mm i.d) and detected with a fixed wavelength UV detector (Hitachi high Technologies America, San Jose, CA.). The separated analytes from the column were monitored at 210nm. The mobile phase flow rate was maintained at 1mL min^{-1} and 10μL of the sample after filtration with a 0.22μ membrane filter was injected into the column.

The following gradients were used: 100% A (Water + 0.1% TFA) to 80% B (Acetonitrile + 0.1%TFA) in 50 minutes for the separation of Phe oligomers and HMB capped Phe co-oligomers; 100% A (Water + 0.1% TFA) initial to 45% B (Acetonitrile + 0.1% TFA) in 20 minutes for the residual Phe, Met, and HMB in the co-oligomer acid hydrolysate; 100% A (Water + 0.1% TFA) initial to 45% B (Acetonitrile + 0.1% TFA) in 30 minutes for residual HMB ester recovered by ethanol wash and the monomer obtained

from its acid hydrolysis, and 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) initial to 23% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 15 minutes for HMB-LysEE dimer acid hydrolysate.

ESI (+)-MS characterization of oligomers and co-oligomers. Synthesized Phe oligomers, Phe oligomer ester and HMB capped Phe co-oligomers were dissolved in DMSO to form 0.5mg/mL solution. Synthesized Lys oligomers, HMB capped Lys co-oligomers and HMB-LysEE dimer was diluted to form 0.5mg/mL solution in water. A make-up solution comprising of 50% acetonitrile in water with 0.1% acetic acid was infused along with the sample at a flow rate of 0.2mL/min into a Hitachi M-8000 ion trap mass spectrometry system using a syringe pump (Harvard Apparatus) at a flow rate of 1mL/hr. An Electrospray Ionization interface was used. The operating parameters of the 3D Q-Ion Trap were as follows: Electrospray capillary voltage, +3.5KV; detector voltage, 400V; assistant gas heater temperature, 200^0C; desolvator temperature and the aperture-1 temperature, 200^0C and 150^0C respectively. The 3D Q- Ion Trap mass analyzer was scanned from 50 – 1200amu.

LC-MS characterization of Met oligomers and co-oligomers. The LC-ESI-MS separation of Met oligomers and HMB-Met co-oligomers were carried out with a mobile phase gradient comprising of 100% A (Water + 0.1% acetic acid) initial to 80% B (Acetonitrile + 0.1% acetic acid) in 50 minutes. The separation was done in a RP C-18 column. The mobile phase flow rate was maintained at 1mL min^{-1}. The column effluent was split and 80% was introduced into a fixed wavelength UV detector set at 210nm. The remaining 20% was introduced into the ESI (+) – MS ion trap mentioned in the previous

paragraph. The LC system used for this analysis was similar to the one mentioned for the analysis of Phe oligomers and co-oligomers.

Chiral LC analysis of HMB enantiomers. The chiral separation of HMB enantiomers was done with an isocratic mobile phase gradient comprising of 70: 30 mixture of 30mM ammonium acetate buffer (pH 4.0) and methanol with a Chirobiotic TAG (ASTEC, Inc.) column. The column effluent was monitored at 230nm. The mobile phase flow rate was maintained at 0.2mL min^{-1}. 10μL of the sample after filtration with a 0.22μ membrane filter was injected into the column.

RESULTS AND DISCUSSION

The enantiomeric resolution of HMB was attempted following two different approaches. In one approach, HMB was incorporated into oligomers of Phe, Met or Lys using either papain (Phe, Met) or chymotrypsin (Lys). A second approach consisted in the synthesis of dimers of Lys-HMB.

Phe oligomers were synthesized in three-phase systems. The oligomers were washed with nanopure water thrice to remove any residual Phe and PheEE left. This was done to eliminate co-oligomerization of HMB with residual PheEE that might significantly increase the amount of HMB capped Phe co-oligomers. The analysis of the supernatant obtained after three water washes showed no residual monomers. This was confirmed by the RPLC analysis of Phe oligomers. The Phe oligomers were esterified for their HMB capping activation. The RPLC separation of the Phe oligomer ester is shown in ***Figure 2 A***. The identification of peaks was made by comparing them against the retention time of Phe, PheEE, Phe-Phe dimer and Phe oligomers with intact free acid at the C-terminal. The chromatogram shows the presence of Phe oligomer residues

consisting of 2 to 7 amino acids. All the oligomers have an ester group at the C-terminal. Further orthogonal confirmation was obtained by injecting the esterified Phe oligomers in an ESI (+)-MS ion trap mass spectrometer (***Figure 2 B***). The spectrum consists of a series of peaks appearing at ***m/z 782, 929 and 1076***; the mass difference is 147amu.. This difference corresponds to a repeating Phe group. The spectrum also consists of another series of less dominant peaks at ***m/z 607, 754 and 901*** that also correspond to the addition of a Phe group. The peaks at m/z 754, 901, and 782 correspond to a pentamer of Phe (^{N}Phe – $(Phe)_3$ - Phe^{C} + H^{+}) , a hexamer (^{N}Phe – $(Phe)_4$ - Phe^{C} + H^{+}) and a pentamer of Phe with ester intact at the C-terminal [^{N}Phe – $(Phe)_3$ - Phe^{COOEt} + H^{+}] respectively. The ions at 782 are dominant in the spectrum because of the esterification reaction. Higher or lower oligomers of Phe are absent because of problems in solubilizing Phe oligomers with DMSO.

Previous results (*28*) showed that a monophasic system consisting of 40% water/60% acetonitrile is optimal for neutral oligomers. Thus, the capping of Phe oligomers with HMBEE was catalyzed with papain in 40% water/ 60% acetonitrile systems. The supernatant and precipitate were separated and characterized in RPLC and ESI-MS. HMB capped Phe oligomers precipitated out. The RPLC separation of HMB capped Phe oligomers is shown in ***Figure 3 A***. The chromatogram consists of a series of peaks eluting after the retention time of PheEE and HMBEE. The tentative peak identification was based on the comparison of retention time against standards of Phe, PheEE, HMB, HMBEE and Phe oligomer substrates used as substrates. The comparison of Phe oligomer ester separation (***Figure 2 A***) and HMB capped Phe co-oligomers (***Figure 3 A***) shows a shift in retention time of separated peaks in the latter. This shift is

due to the reduced polarity of HMB capped Phe oligomer ester when compared to Phe oligomer ester. For example a Phe pentamer {$(Phe)_5$-EE} elutes at a retention time of 33min while a HMB capped Phe tetramer {HMB- $(Phe)_4$-EE} elutes at 34min. The difference in retention times is due to the addition of a HMB residue in place of Phe. This difference becomes more pronounced for higher oligomers and corresponding co-oligomers. ***Figure 3 A*** also shows a significant amount of Phe monomer formed by the chemical hydrolysis of oligomers that occur in the presence of water. Further orthogonal confirmation of capping was obtained by analyzing the synthesized co-oligomers in ESI-MS. ***Figure 3 B*** shows the ESI (+)-MS spectrum of HMB-Phe co-oligomers. The spectrum consists of a series of peaks separated by 147 amu appearing at ***m/z 620, 767 and 914***. This difference in m/z corresponds to the addition of a recurring Phe group. However, these ions do not correspond to Phe oligomers but rather to the addition of a HMB residue to Phe oligomers. The ions appear at an m/z value corresponding to HMB capped Phe oligomers with intact ester at the C-terminal end. The absence of other ions in the spectrum is due to solubility problems encountered with DMSO, as is the case with Phe oligomers.

The precipitated Met oligomers synthesized in aqueous systems were washed thrice with water to remove any residual monomers. The RPLC analysis of the washing fluid shows also the removal of smaller chain oligomers (dimer, trimer, tetramer and pentamer) of Met. The RPLC separation of the Met oligomers is shown in ***Figure 4 A***. The peak identification was carried out by obtaining the ESI-spectrum of the individual peaks eluting out of the column. The chromatogram shows the complete absence of monomer of Met but trace amounts of lower oligomers. The ESI-MS spectrum of washed

Met oligomers is shown in ***Figure 4 B***. The spectrum is dominated by the heptamer and octamer of Met with an intact C-terminal ester appearing at ***m/z 964 and 1095***. The spectrum also shows the presence of Met hexamer, heptamer and octamer (***m/z 805, 936, 1067***) with free acid present at the C-terminal. It is evident from the chromatogram and ESI-MS spectrum that ester intact oligomers are the dominant products. This eliminated the need for an additional esterification reaction in preparation for HMB capping in this case.

Met oligomers were capped with HMBEE under conditions similar to that of the Phe oligomer capping reaction (40% water/60% acetonitrile) The LC-UV output of the purified HMB-Met co-oligomers is shown in ***Figure 5 A***. Comparison of this chromatogram with ***Figure 4 A***, shows the presence of additional peaks. These additional peaks were identified as HMB capped Met co-oligomers. The peak identification was done by obtaining the ESI-MS spectral output of each separated peak. ***Figure 5 B*** shows the ESI-MS output of peak labeled 5^{**} ***and*** 6^{**} in the LC-UV output. The spectrum shows an ion at ***m/z 834 and 965***. These ions appear at a mass value ***1amu*** higher than the corresponding Met oligomers. This corresponds to HMB capped Met pentamer (HMB-$(Met)_5$-EE) and hexamer (HMB- $(Met)_6$-EE) respectively.

When the RPLC separation of HMB capped Phe oligomers and HMB capped Met oligomers (***Figure 3 A and Figure 5 A***) are compared, it is evident that capping is more complete with poly-Phe than with poly-Met. More than 90% of the initial Phe oligomers were capped while only 65% of the initial Met oligomers were capped. Phe oligomers have a higher solubility than Met oligomers in monophasic system and hence were capped more efficiently than Met oligomers.

The amount of HMB incorporated into Met and Phe oligomers was determined by hydrolyzing the synthesized HMB capped co-oligomers under acidic conditions. ***Figure 6 A*** and ***6 B*** shows the RPLC separation of acid hydrolysate of HMB capped Phe and HMB capped Met co-oligomers. The separated peaks were identified and quantified by comparing them against standards of HMB, Phe and Met. Approximately 16% of the initial HMB was incorporated into Phe oligomers while only 11% was incorporated into Met oligomers. The percent HMB incorporated is less than half the initial amount and hence the unreacted HMBEE is not highly enantioenriched. Hence a different approach was utilized for obtaining enantiopure HMB with Lys oligomer as substrate.

Lys oligomerization was carried out in a three-phase system (DFP/n-octane/water). The dried oligomer was injected into ESI (+)-MS for characterization. The positive ion ESI-MS spectrum of synthesized Lys oligomers is shown in ***Figure 7***. The spectrum contains a series of ions, which are 128 amu apart. This mass difference corresponds to the repeating Lys moiety. The dominant ions appeared at ***m/z 403, 531, 659, 787 and 915*** which correspond to the oligo-Lys residues with a free acid group at the C-terminal (^{N}Lys – $(Lys)_n$ - Lys^{COOH} + H^+). A tetramer of Lys, ^{N}Lys – $(Lys)_2$ - Lys^{COOH} + H^+ should appear at a ***m/z 531*** while a pentamer ^{N}Lys – $(Lys)_3$ - Lys^{COOH} + H^+ should appear at a ***m/z 659***. There is another peak that appears at ***m/z 303***. This peak corresponds to a dimer (^{N}Lys – Lys^{COOH} + H^+).

The capping of Lys oligomers with HMB was done by incubating equal amounts of HMB methyl ester with Lys oligomers (molar ratio- 3:1) in a sodium phosphate dibasic buffer with chymotrypsin catalyst. The positive ion ESI-MS spectra of an aliquot taken from the reaction mixture incubated for a period of 15min with HMBME as a

substrate is shown in ***Figure 8 A.*** The spectrum consists of a series of ions appearing at a mass difference of 128amu. This m/z value is 4amu higher than poly Lys residues, which corresponds to the addition of one HMB moiety to the oligo-Lys residues. For example, when a HMB residue is added to a tetramer of Lys (^{N}HMB – Lys - $(Lys)_2$ - Lys^{COOH} + H^+) it will appear at ***m/z 663,*** which is 4amu higher than the pentamer of Lys that will appear at ***m/z 659.*** The spectrum had a series of peaks appearing at ***m/z 435, 535, 663, 791 and 919*** corresponding to HMB capped poly Lys residues ranging from 2 to 6. The ion appearing at ***m/z 435*** corresponds to HMB capped dimer of Lys with intact ester at the C-terminal end (^{N}HMB-Lys-Lys^{COOEt} + H^+). The spectrum also shows a small peak corresponding to an unreacted dimer of Lys. The ESI (+)-MS of the sample incubated for a period of 30min with HMBME as substrate (***Figure 8 B***) shows the absence of any Lys oligomer residues indicating that the process of capping was complete in 30min. The only peaks appearing in the spectrum at ***m/z 535, 663, 791 and 919*** were that of HMB capped poly Lys residues. An ion corresponding to doubly protonated HMB capped Lys hexamer also appeared in the spectrum (***m/z 460***). When the reaction period was increased beyond 30min, there was a significant hydrolysis of higher oligomers to HMB capped dimer and trimer of Lys (***Figure 9***). Similar results were observed when HMBEE was used as the substrate (***Figure 10 A, B***). However, in this case only after an incubation period of 60min the reaction went to completion. These results show that HMB is capped to the N-terminal end of the peptide and only one residue is attached. To simplify the processing steps, enantioenrichment studies of HMB were carried out by synthesizing HMB-LysEE dimer starting with HMB ester and LysEE.

The synthesis of HMB-LysEE dimer was carried out in the presence of chymotrypsin with HMB methyl ester and LysEE as substrates. The ESI (+)-MS spectra of the product synthesized for an incubation period of 15min is shown in ***Figure 11 A.*** The spectrum shows a single residue appearing at ***m/z 307*** corresponding to a dimer HMB-LysEE. The spectrum also shows the presence of residual LysEE. However, when the reaction medium was incubated for 30min, there was no residual LysEE left (***Figure 11 B***). Another important aspect of this capping process is the exclusive formation of HMB-LysEE when HMB ester and LysEE were used as substrates. This result is quite different from the mixture of oligomers and co-oligomers obtained when the same substrates were used with papain (31). This also implies that the acyl-enzyme complex is formed with HMBME and LysEE acts as the nucleophile. If LysEE formed the acyl-complex with the enzyme, then oligomerization of Lys should also occur with the formation of HMB-LysEE dimer. Similar results though were obtained with HMBEE (***Figure 12 A, B***) and HMBPE (Results not shown), but the reaction was slower. The reaction went to completion in 60min with HMBEE and it was complete in 120min with HMBPE. The formation of only dimers is significant in terms of enantio-specificity of chymotrypsin. Only one HMB residue is incorporated for each LysEE residue. It is a well-established fact that proteases are specific towards L-form of the enantiomer (29, 31). With this in mind, if we start with twice the stoichiometric amounts of HMB ester and the reaction is allowed to proceed to completion, then the residual HMB ester left in the reaction medium should be D-enriched. This hypothesis was validated by recovering the residual HMB ester, subjecting it to hydrolysis and characterizing it with Chiral LC.

The enantio-enrichment of HMB was determined with each ester substrate for incubation times from 15min to 180min. After each incubation period, the reaction mixture was dried by rotary evaporation to recover the synthesized HMB-LysEE dime and residual monomers. The dried dimer was washed with ethanol thrice to recover any residual HMB ester present. Ethanol washing removed only residual HMB ester and HMB while it did not remove any residual LysEE.

The synthesized HMB-LysEE dimer was hydrolyzed (Unwashed and washed with ethanol). The difference in the amount of HMB present in the hydrolysate in both cases is a good estimate of the amount of HMB incorporated. Ethanol washing removes the free or unreacted HMB and hence the amount of HMB determined from the washed residue will correspond directly to the amount of HMB bound as a dimer. The RPLC separation of acid hydrolysate obtained from HMB-LysEE dimer synthesized with HMBME for an incubation period of 30min is shown in ***Figure 13 A***. The hydrolysate consists of two peaks eluting at the retention times of HMB and Lys. The response for HMB in this chromatogram was nearly twice that obtained with the dimer hydrolysate after ethanol washing (***Figure 13 B***). The amount of HMB present in both cases was quantified by comparing their response with HMB standards. Such a comparison shows that the amount of HMB incorporated into HMB-LysEE with HMBME for 30min incubation time was approximately 45%. The amount of bound HMB was determined for each incubation period with each substrate. In case of HMBEE, only after 60min incubation 46% of the initial HMB was bound while for HMBPE it took 120min for 44% of initial the HMB to be incorporated into the dimer. The optical purity of HMB present in the dimer washed with ethanol was determined by chiral liquid chromatography. The chiral

LC separation of HMB enantiomers present in the hydrolysate of washed HMB-LysEE dimer synthesized with HMBME substrate for an incubation period of 30min is shown in ***Figure 14 A***. The chromatogram shows two peaks, which were identified by comparing them against the retention time of DL-HMB standard under the same conditions. It is evident that chymotrypsin acts only on the L-form HMB, which was incorporated in excess when compared to the D-form. The purity of L-HMB incorporated into the dimer was greater than 98%. When HMBEE was used as the substrate with an incubation time of 30 min, the chiral separation shoes that the enantiopurity of L-HMB incorporated in this case was also close to 98% (***Figure 14 B***). However, the enantio-purity of L-enantiomer incorporated into the dimer when HMBPE was used (30min incubation time) was close to 85% (***Figure 14 C***). Capping was much slower with HMBPE. This resulted in a higher amount of residual DL HMBPE after 30min incubation and washing the dimer with ethanol thrice did not remove all the residual HMBPE. Therefore, this residual DL HMBPE contributed to the low enantio-purity with HMBPE. This was resolved by extending the incubation to 120 min; in this case the amount of L-HMB incorporated into the dimer was approximately 98%.

HMB ester cannot be separated in the Chirobiotic TAG column used for chiral separation and hence it has to be hydrolyzed to the corresponding free acid. The residual HMB ester left in the HMB-LysEE dimer was recovered by washing it with absolute ethanol. The RPLC separation of residual HMBME and HMB recovered from HMB-LysEE synthesized with methyl ester as substrate is shown in ***Figure 15 A***. The identification of the separated peaks was done by comparing them against HMB and HMBME standards. The chromatogram shows that 57% of the initial HMBME added

was recovered. This correlates well with the amount of bound HMB (**45%**) determined by acid hydrolysis of washed and unwashed HMB-LysEE dimer. ***Figure 15 B*** shows the RPLC separation of the residual HMBME acid hydrolysate. The chromatogram shows the complete hydrolysis of HMBME to HMB. Quantification of HMBEE (***Figure 16 A, B***) and HMBPE (***Figure 17 A, B***) recovered from the reaction medium for an incubation period of 30min showed that 65% and 82% of initial HMB was recovered in each case, respectively. However, the amount of residual HMBEE was 55% and 85% for an incubation time of 60min and 120min, respectively. The chiral separation of the residual HMB ester acid hydrolysate confirmed our initial hypothesis that it should be enantio-enriched. The chiral LC separation of the hydrolysate of residual HMBME recovered from HMB-LysEE for 30min incubation shows an ***enantiomeric excess e.e*** (D/L ratio) of ***92%*** (***Figure 18 A***). This correlates well with the amount of bound HMB (**45%**) determined by the hydrolysis of washed and unwashed dimer and the RPLC separation of HMBME recovered by ethanol. For the same incubation period, e.e was determined to be 85% for HMBEE as a substrate (***Figure 18 B***) while it was only 65% with HMBPE. (***Figure 18 C***). ***Figure 19*** shows the effect of reaction time on the ***e.e*** for different starting HMB ester substrates. When the reaction time was increased to 60min, the ***e.e*** for HMBEE was 94% while it was 91% with HMBPE for a reaction time of 120min. The ***e.e*** was the same (nearly 90%) for all HMB ester substrates when the synthesis extended beyond the time required for the completion of the reaction.

Conclusions. Protease catalyzed capping of Phe, Met and Lys oligomers with HMB was carried out successfully. Our results show that chymotrypsin catalyzed hydroxy-acid capping reactions are very rapid and completion is reached in less than

30min. The reaction of HMBME with LysEE yields the HMB-LysEE dimer exclusively as opposed to the co-oligomers formed with papain (*29*). In case of HMB, only the L-form of the hydroxy acid was incorporated into the dimer. The chiral separation of residual HMB show that it was D-enriched and a ***e.e*** of 90% could be achieved in 30min. There was no change in the Chirality of HMB incorporated once the capping reaction was complete indicating that chymotrypsin is enantio-specific towards HMB. Similar enantio enrichment studies with other hydroxy acids should be carried out to get a much broader picture about the specificity of chymotrypsin towards other hydroxy acids and also obtaining optically pure form of these compounds for evaluating their properties.

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FIGURES

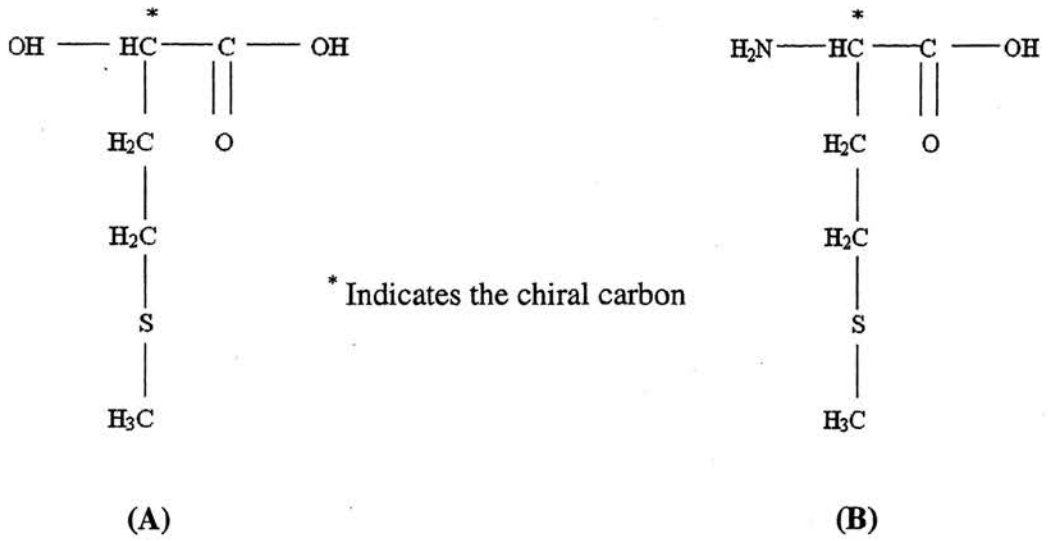


Figure 1: Chemical structures of A) HMB and B) Methionine indicating the chiral center.

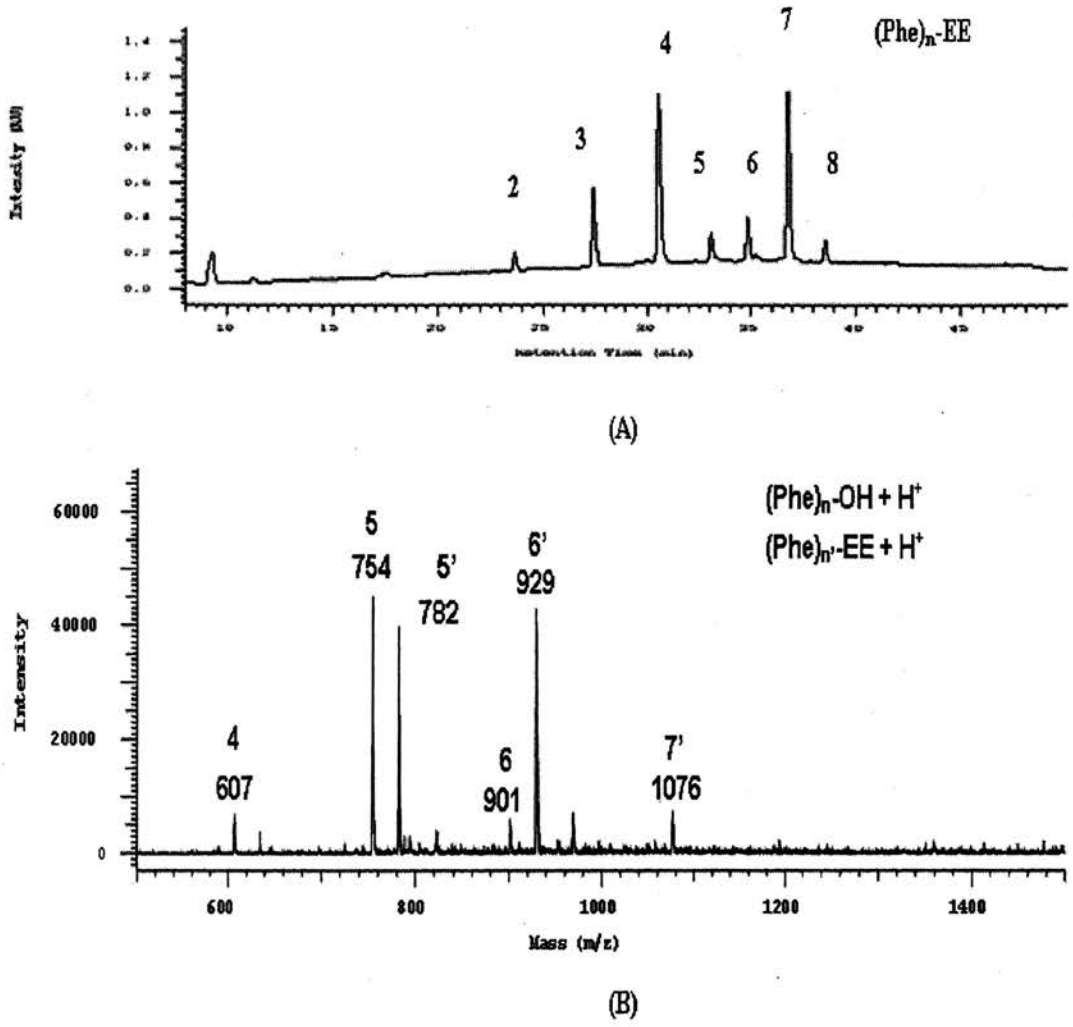
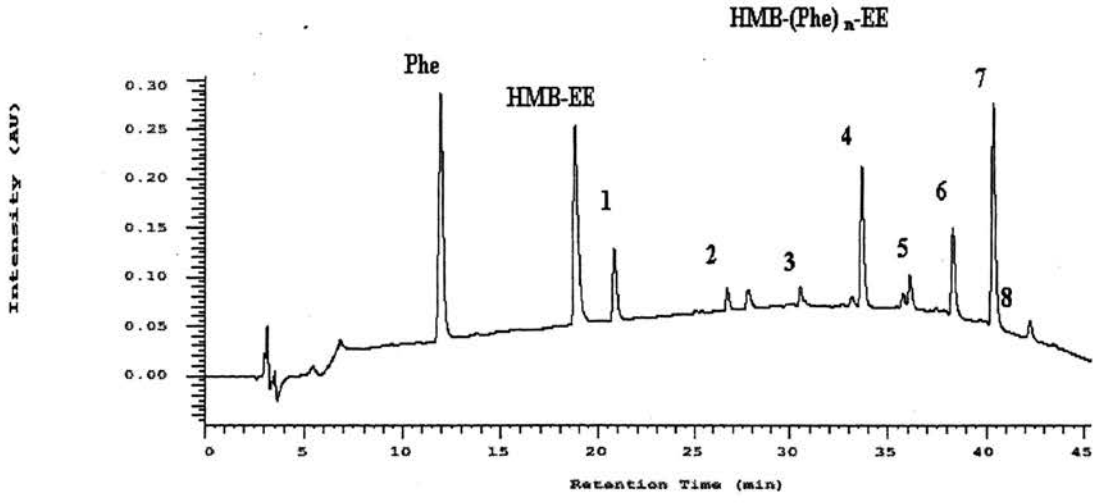
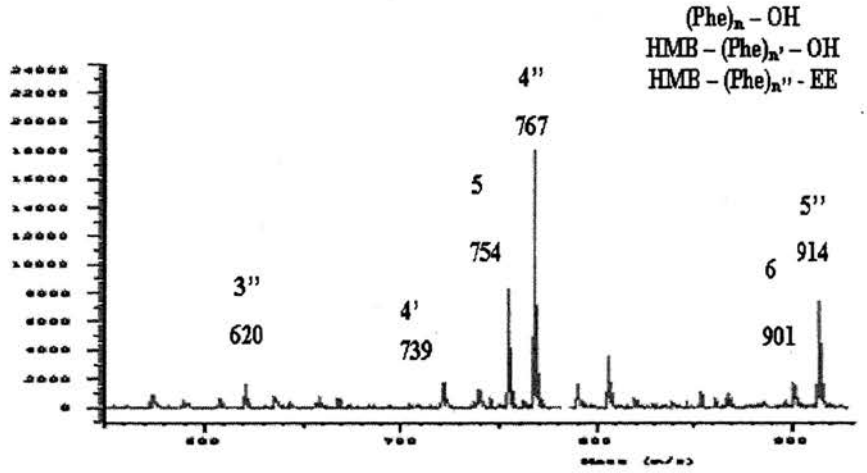


Figure 2: (A) RPC-18 separation of esterified Phe oligomers synthesized in three-phase system using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 80% B (Acetonitrile + 0.1%TFA) in 50 minutes. The chromatogram shows the clear separation of Phe oligomer esters. (B) ESI (+)-MS of Phe oligomer ester synthesized in three-phase system. The spectrum shows the presence of series of peaks corresponding to oligomers of Phe.



(A)



(B)

Figure 3: (A) RPC-18 separation of HMB capped Phe co-oligomers synthesized in 40% water/60%acetonitrile system with Phe oligomers as substrates. The separation was carried out using the same mobile phase gradient mentioned in Figure 2. The chromatogram shows the shift in retention of time of the eluting peaks, corresponding to the incorporation of HMB. (B) ESI (+)-MS of HMB capped Phe co-oligomer ester synthesized in monophasic aqueous organic system. HMB incorporation is confirmed by the presence of HMB-(Phe)$_n$ oligomer peaks in the spectrum.

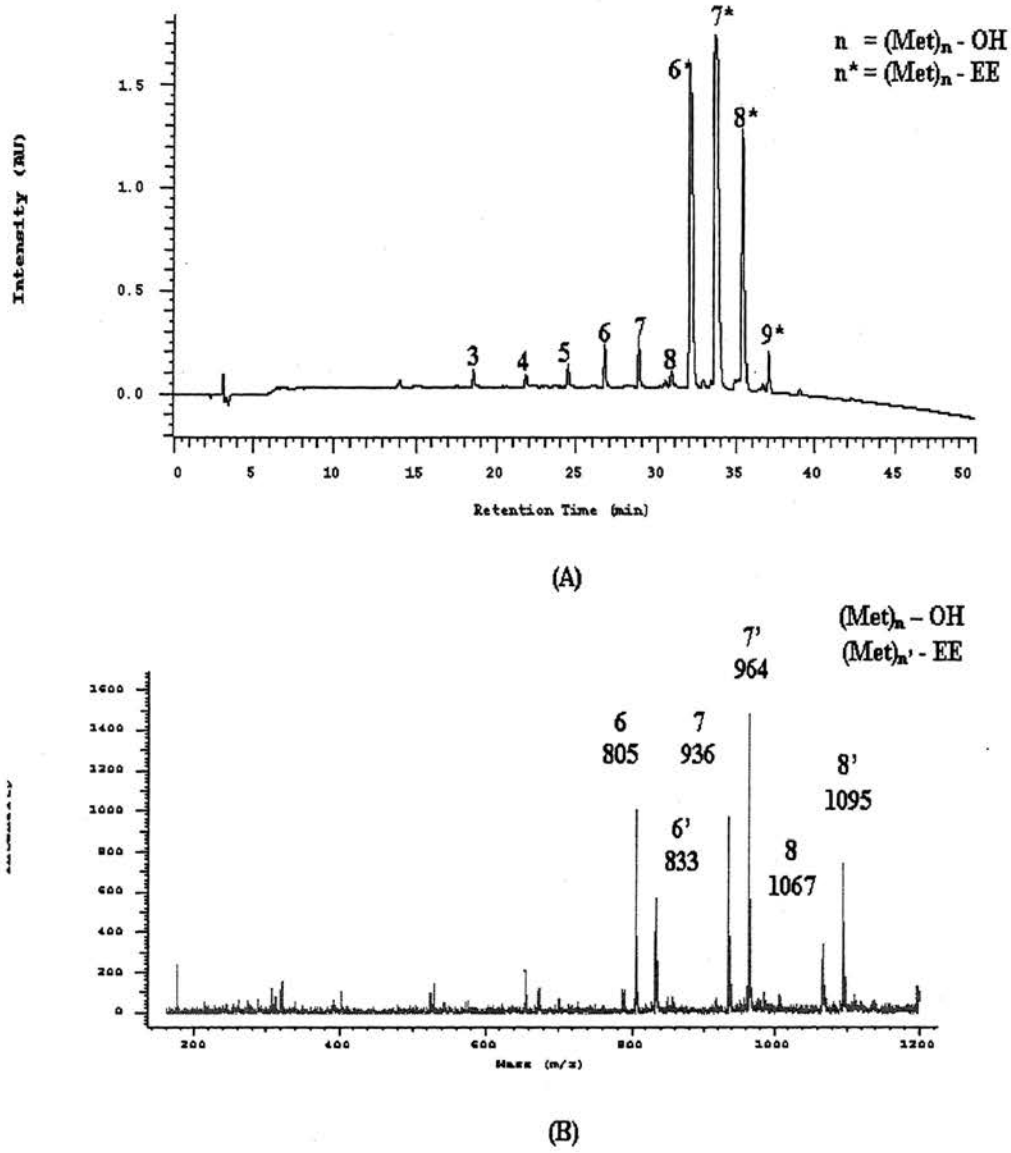


Figure 4: (A) RPC-18 separation of Met oligomers synthesized in aqueous system. The separation was carried out using the same mobile phase gradient mentioned in Figure 2. The chromatogram shows the clear separation of Met oligomers. (B) ESI (+)-MS of Met oligomer synthesized in aqueous system. The spectrum shows the presence of series of ions appearing at m/z values corresponding to Met oligomers.

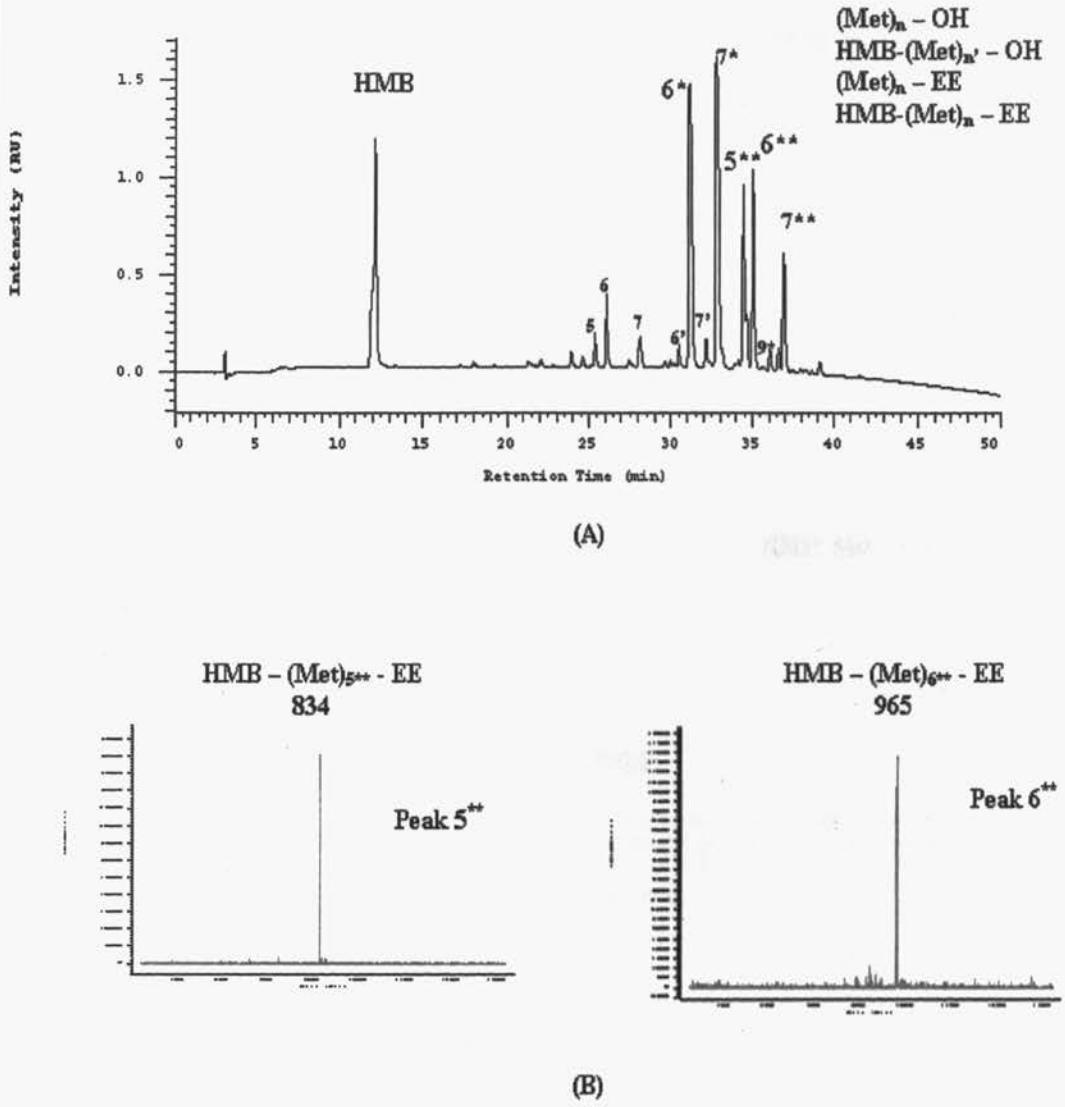


Figure 5: (A) RPC-18 separation of HMB capped Met co-oligomers synthesized in 40% water/60%acetonitrile system with Met oligomers as substrates. The separation was carried out using the same mobile phase gradient mentioned in Figure 2. The chromatogram shows the presence of additional peaks identified as HMB-$(Met)_n$ co-oligomers. (B) ESI (+)-MS output of peak 5^{**} and 6^{**} in the RPLC separation of HMB capped Met co-oligomers synthesized in monophasic aqueous organic system. The m/z values correspond to HMB- $(Met)_5$-EE and HMB-$(Met)_6$-EE.

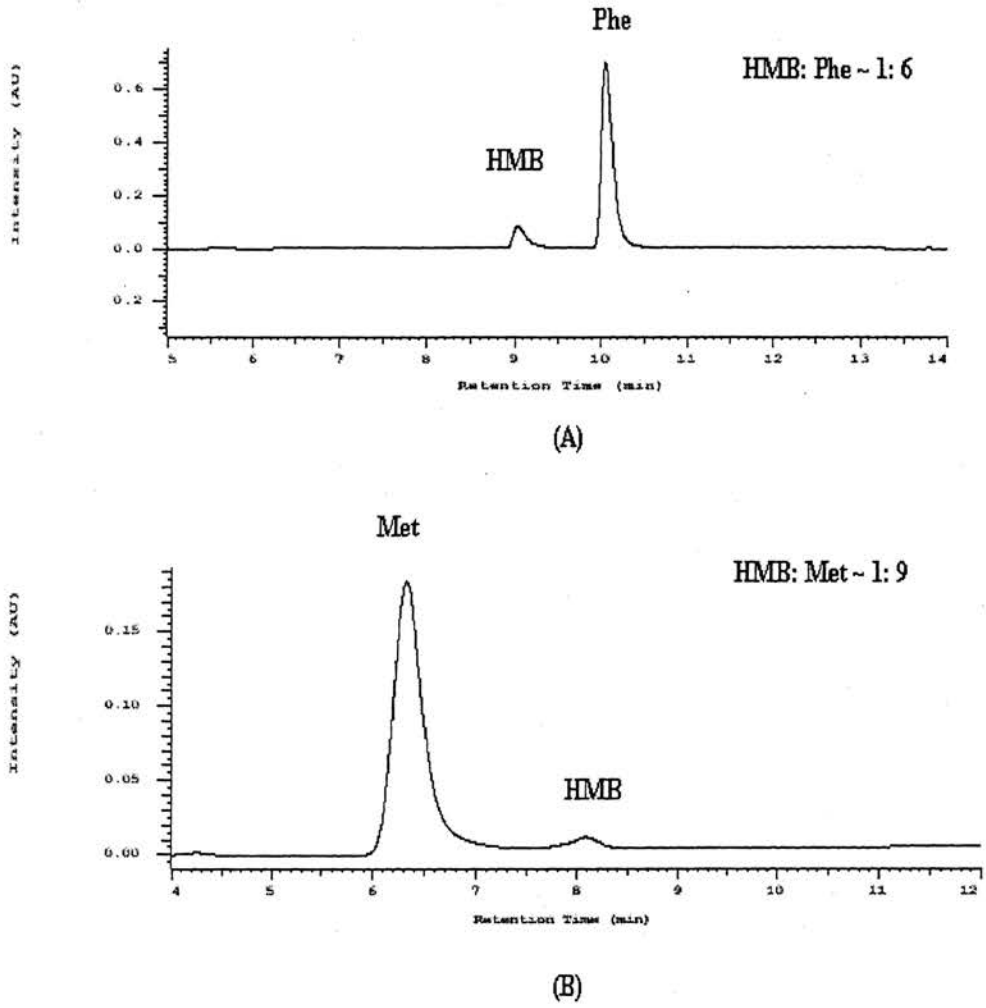


Figure 6: RPLC separation of (A) HMB-Phe co-oligomer acid hydrolysate and (B) HMB-Met co-oligomer acid hydrolysate. The separation was obtained with a C-18 column using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 45% B (Acetonitrile + 0.1% TFA) in 20 minutes. The chromatogram shows the complete hydrolysis of co-oligomers to Phe, Met and HMB. The percent HMB incorporation was ~16% for Phe oligomers and ~ 11% for Met oligomers.

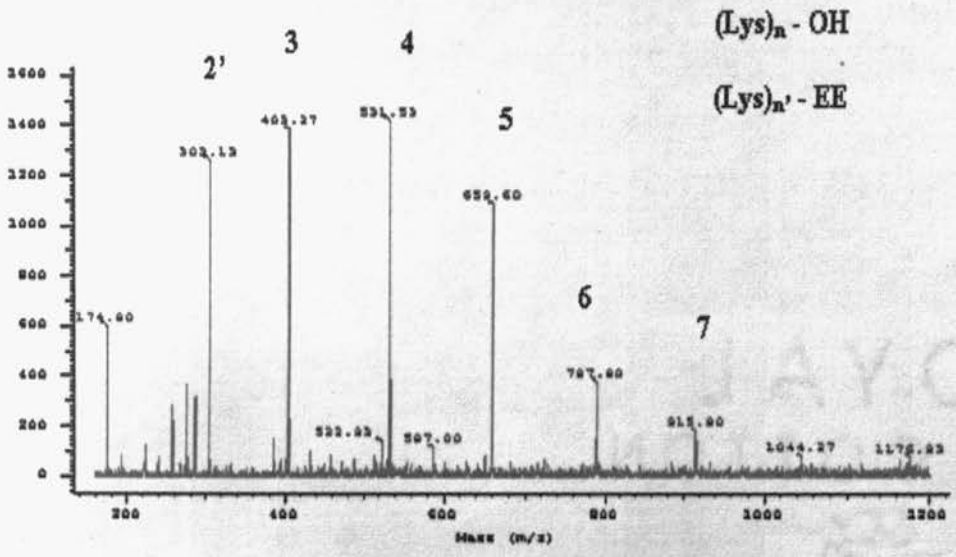


Figure 7: ESI (+)-MS Spectrum of Lys oligomers synthesized in three-phase system with LysEE substrate. The spectrum shows the presence of series of peaks corresponding to oligomers of Lysine.

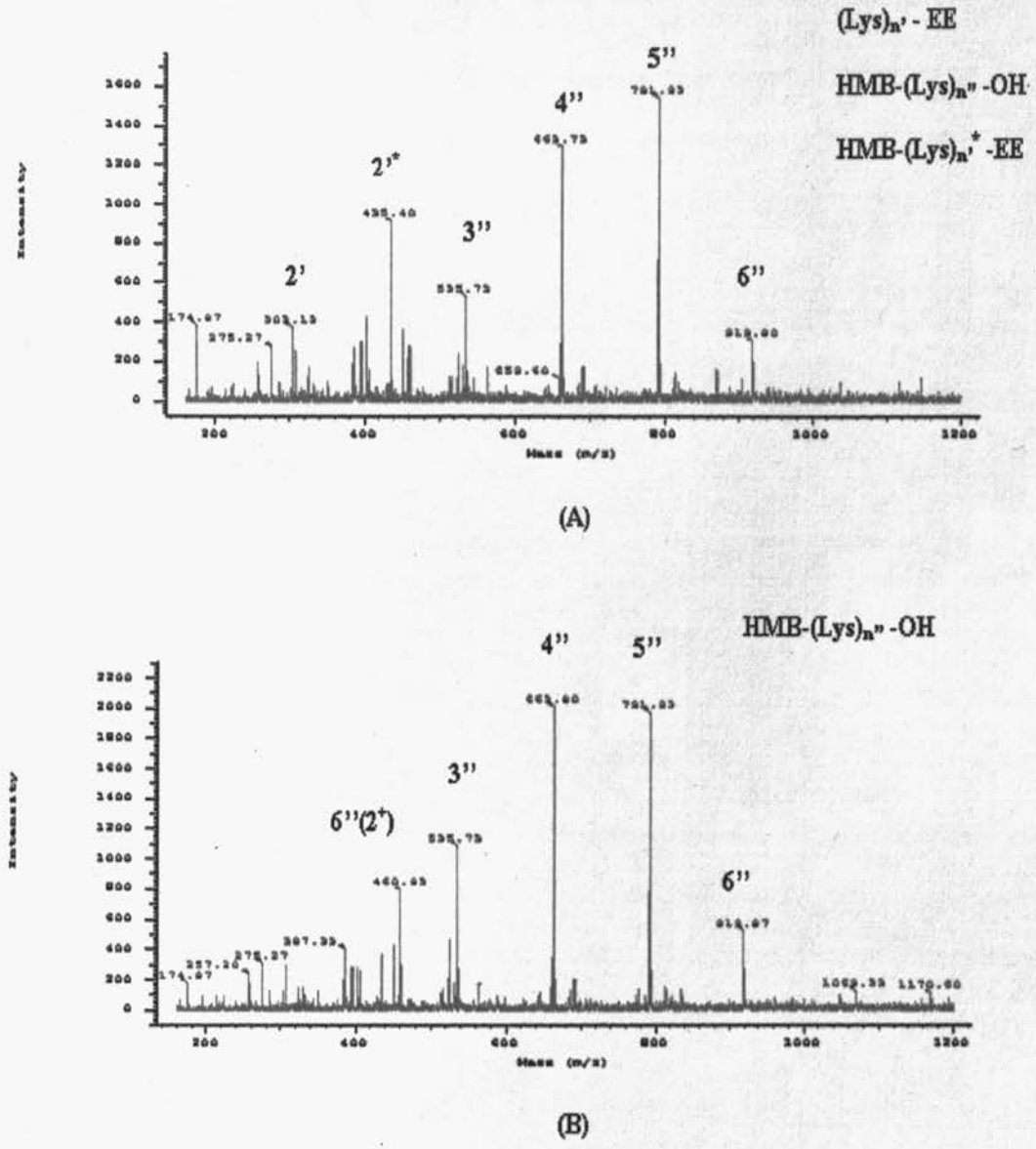


Figure 8: ESI (+)-MS spectrum of HMB capped Lys oligomers synthesized through chymotrypsin catalysis with HMBME substrate for A) 15min and B) 30min incubation period. The spectra show the presence of additional peaks appearing at 4amu higher than Lysine oligomers. These peaks correspond to HMB-(Lys)$_n$ co-oligomers.

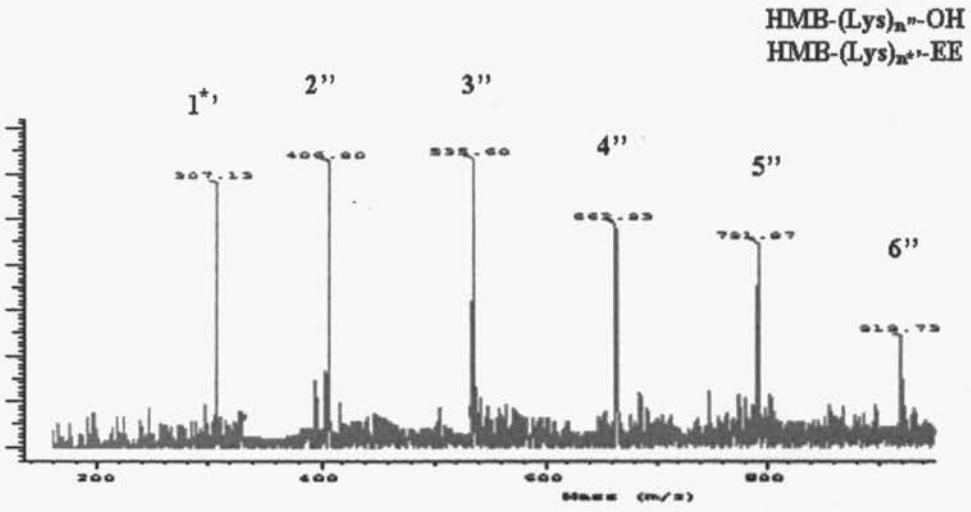


Figure 9: ESI (+)-MS spectrum of HMB capped Lys oligomers synthesized through chymotrypsin catalysis with HMBME substrate for an incubation period of 60min. the spectrum shows the complete absence of peaks corresponding to Lys oligomers and only the presence of peaks corresponding to HMB-$(Lys)_n$ co-oligomers.

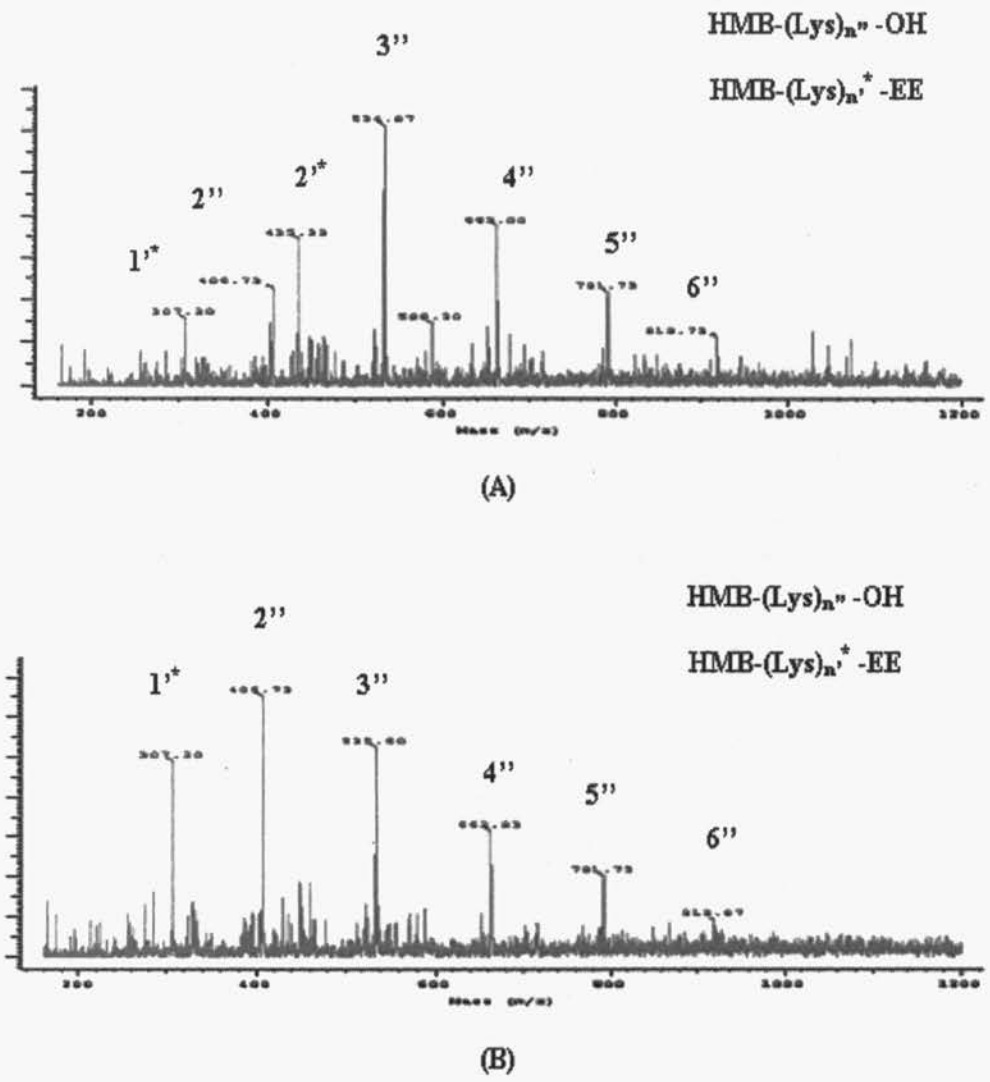


Figure 10: ESI (+)-MS spectrum of HMB capped Lys oligomers synthesized through chymotrypsin catalysis with HMBEE substrate for A) 30min and B) 60min incubation period. The spectra show the presence of additional peaks appearing at 4amu higher than Lysine oligomers. These peaks correspond to HMB-(Lys)$_n$ co-oligomers.

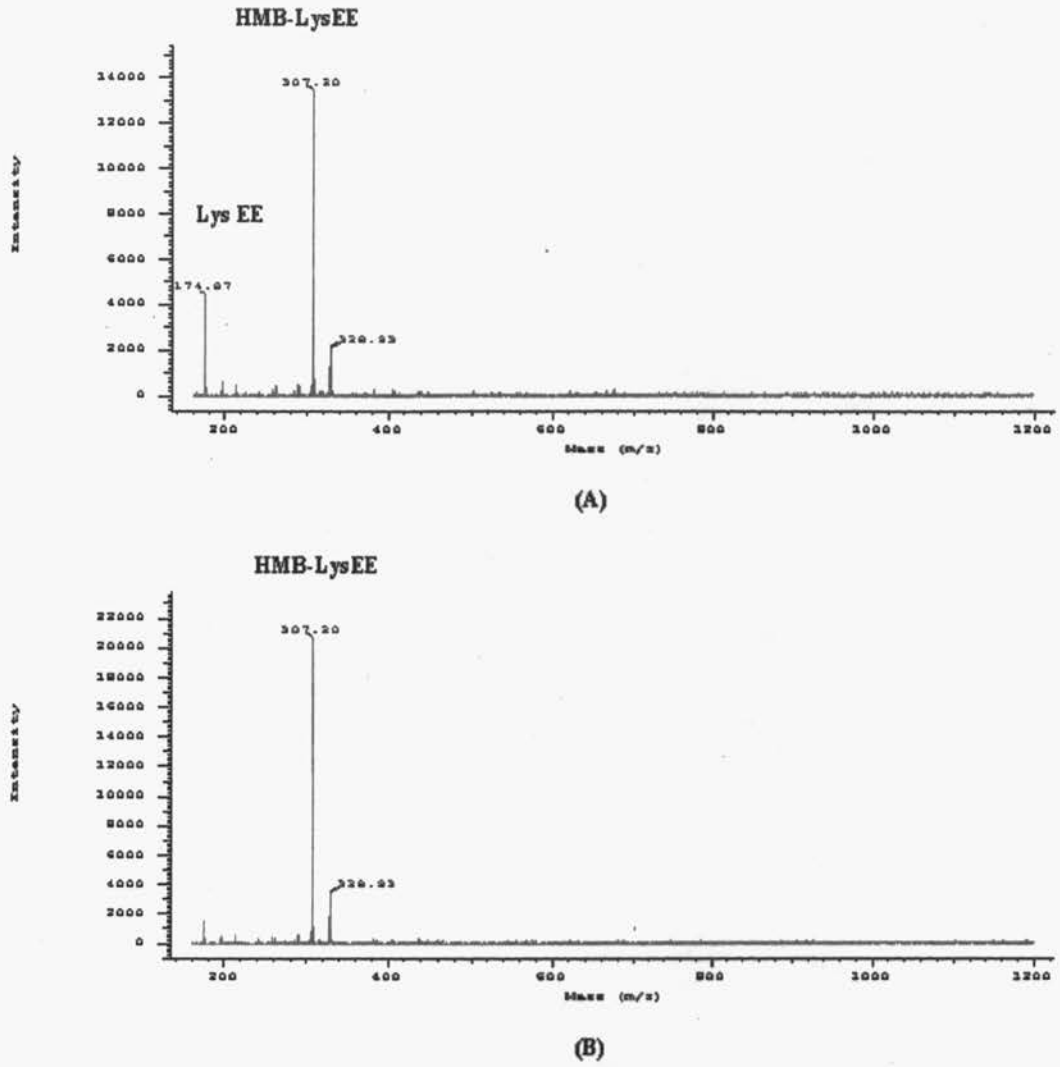


Figure 11: ESI (+)-MS spectrum of HMB-LysEE dimer synthesized through chymotrypsin catalysis with HMBME substrate for A) 15min and B) 30min incubation period. The spectra show the exclusive formation of an HMB-LysEE dimer.

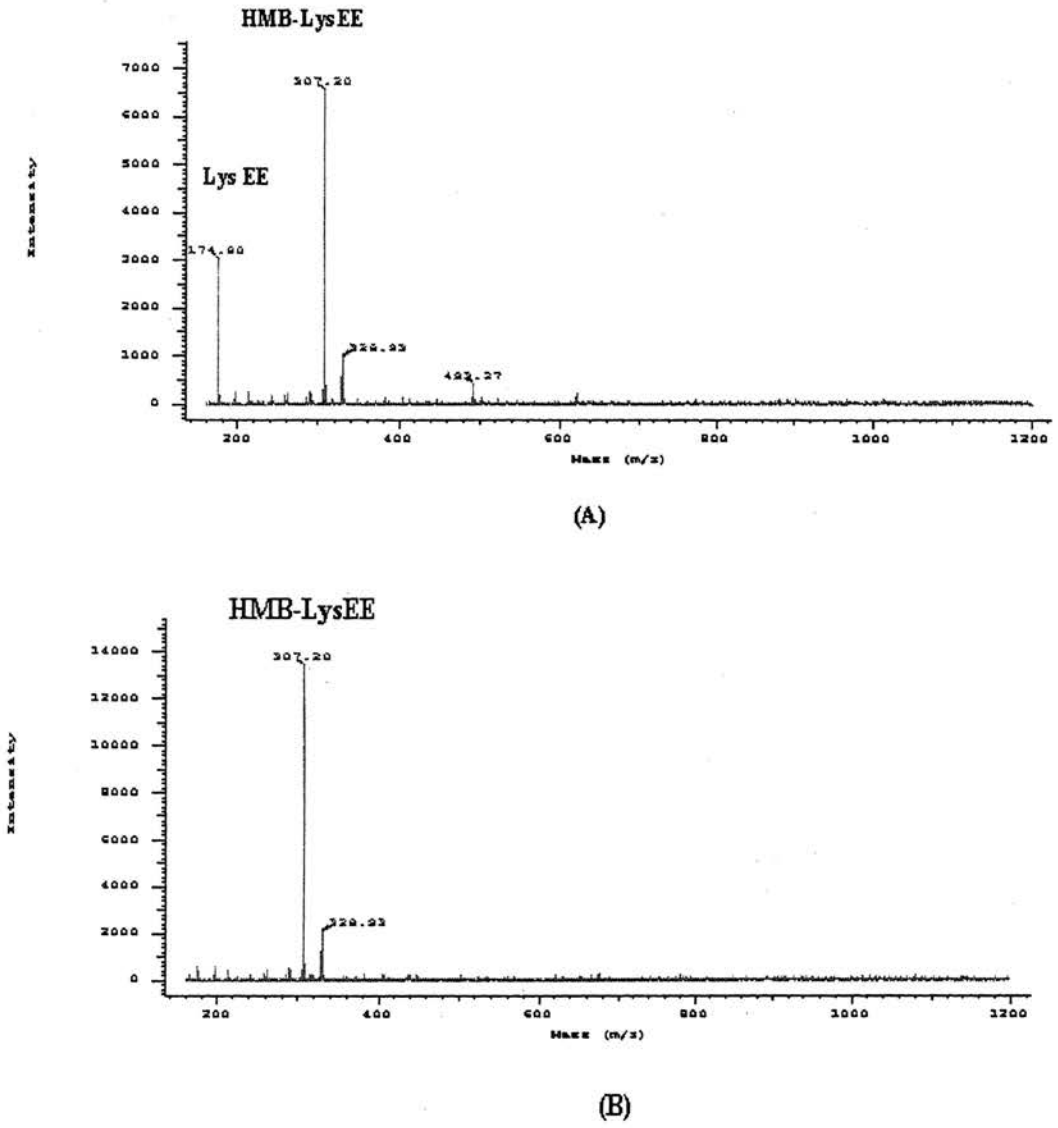


Figure 12: ESI (+)-MS spectrum of HMB-LysEE dimer synthesized through chymotrypsin catalysis with HMBEE substrate for A) 30min and B) 60min incubation period. The spectra show the exclusive formation of an HMB-LysEE dimer.

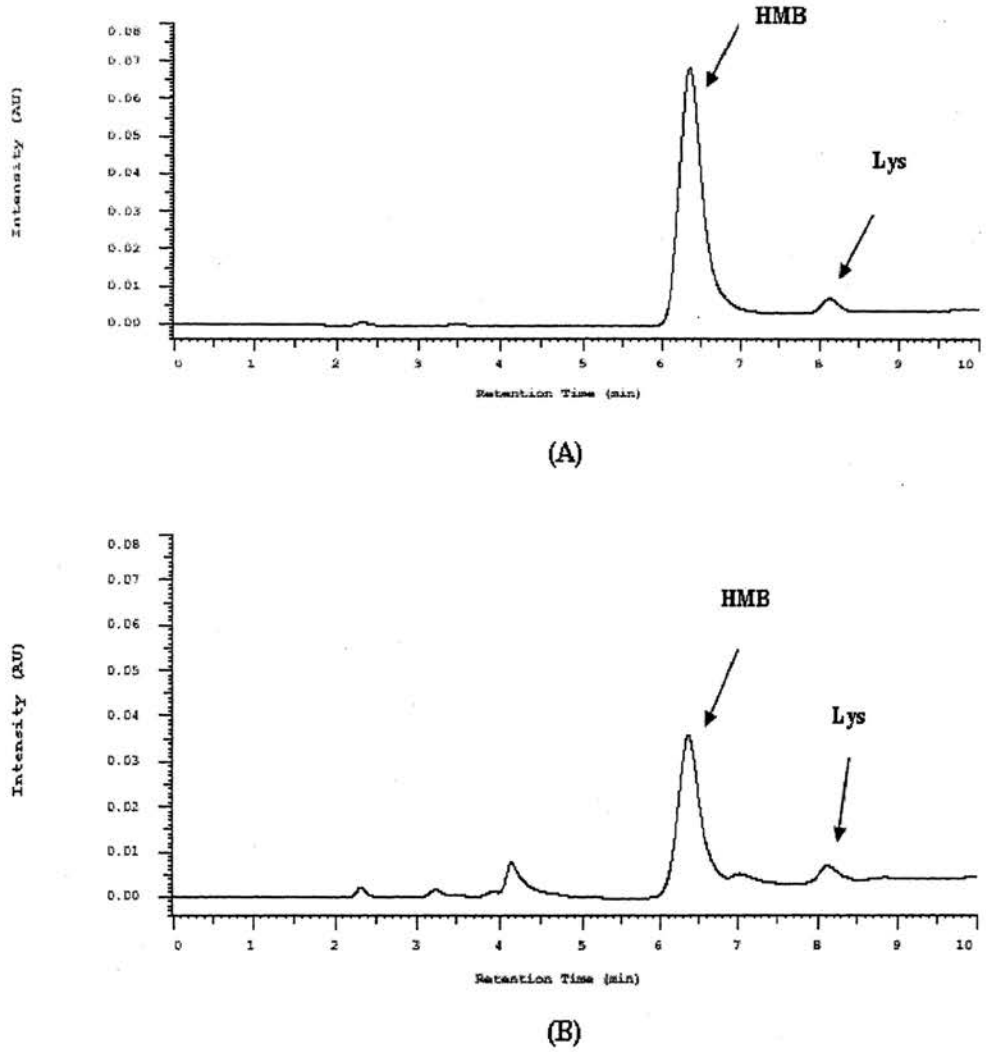


Figure 13: RPLC separation of acid hydrolysate obtained from HMB-LysEE dimer synthesized with HMBME substrate A) Un-washed and B) Washed with absolute ethanol. The separation was achieved with a C-18 column using a mobile phase gradient comprising of 100% A (Water + 10mM HSA +0.1% O-Phosphoric acid) initial to 23% B (50% acetonitrile + 10mM HSA +0.1% O-Phosphoric acid) in 15 minutes. The chromatogram shows the clear separation of HMB and Lys present in the hydrolysate. The difference in the response of HMB from the two chromatograms revealed that 45% of the initial HMB was bound in the dimer.

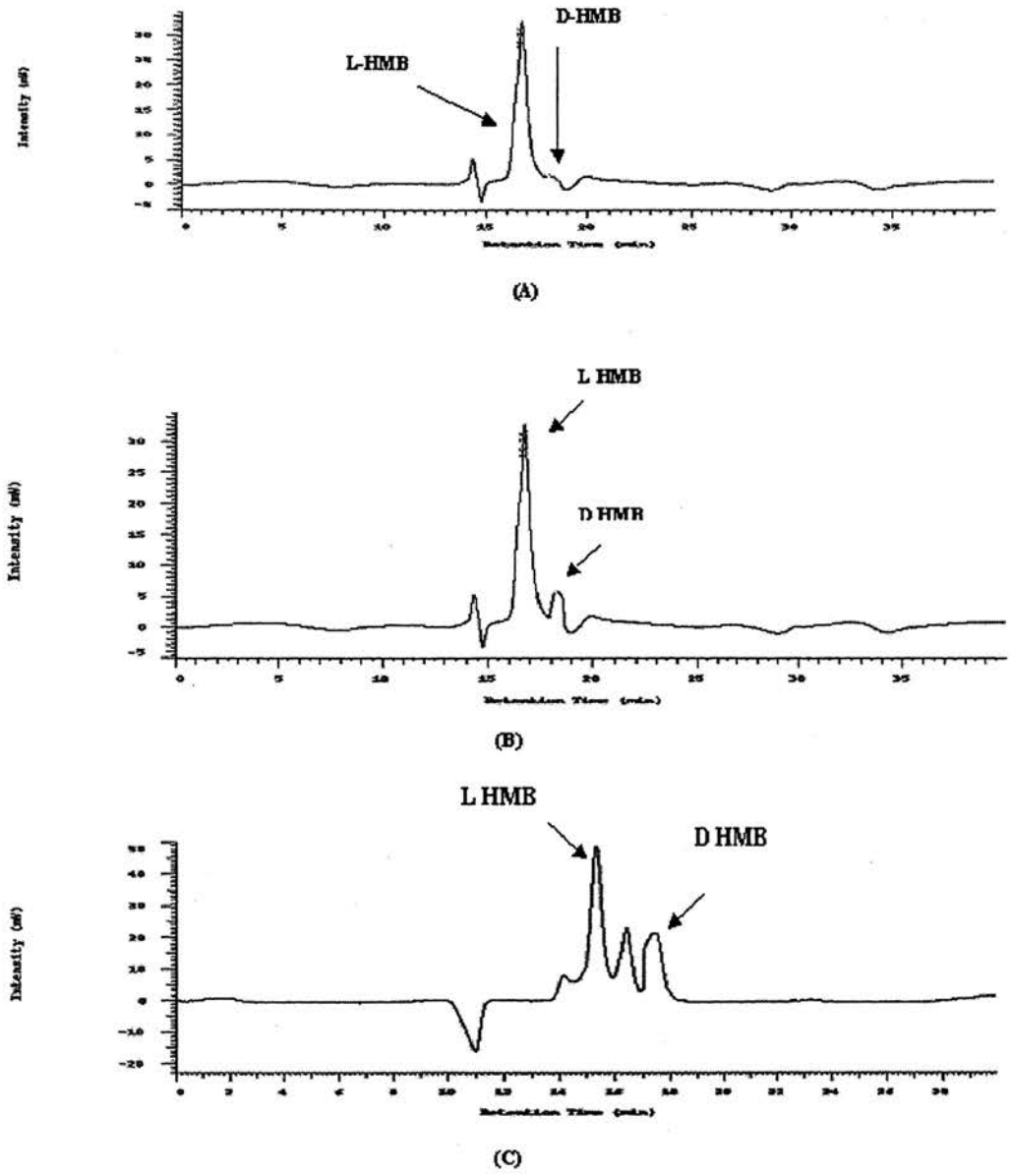


Figure 14: Chiral LC separation of acid hydrolysate of washed HMB-LysEE dimer synthesized with A) HMBME B) HMBEE and C) HMBPE substrate. Separation was achieved with a Chirobiotic TAG column using an isocratic gradient of 70: 30 mixture of 30mM ammonium acetate buffer (pH –4.0) with methanol. The chiral separations show the enantio-selective incorporation of L-HMB into the dimer by chymotrypsin.

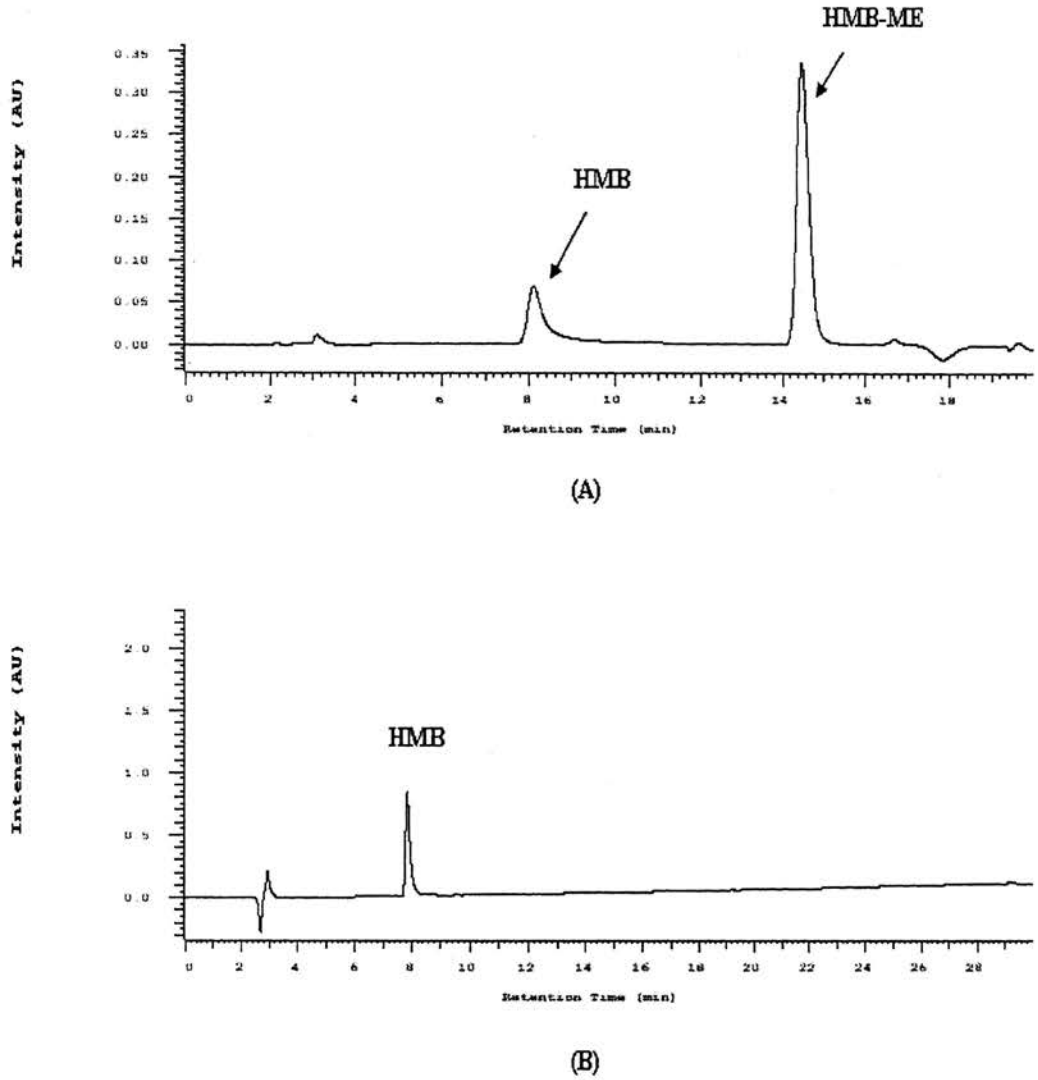
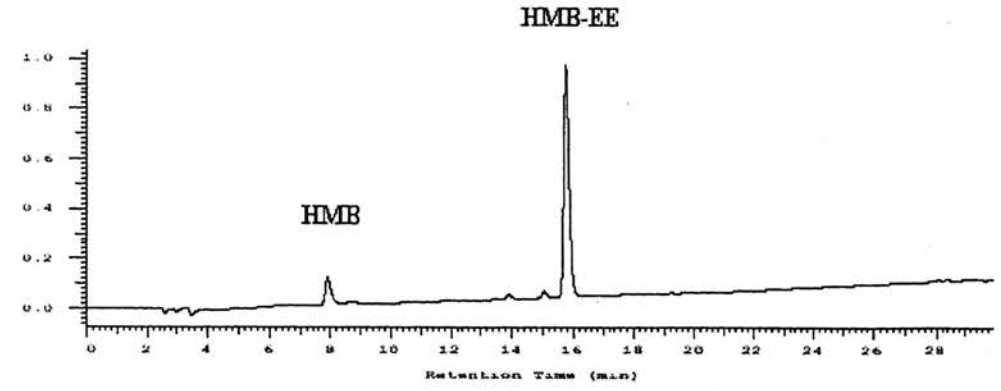
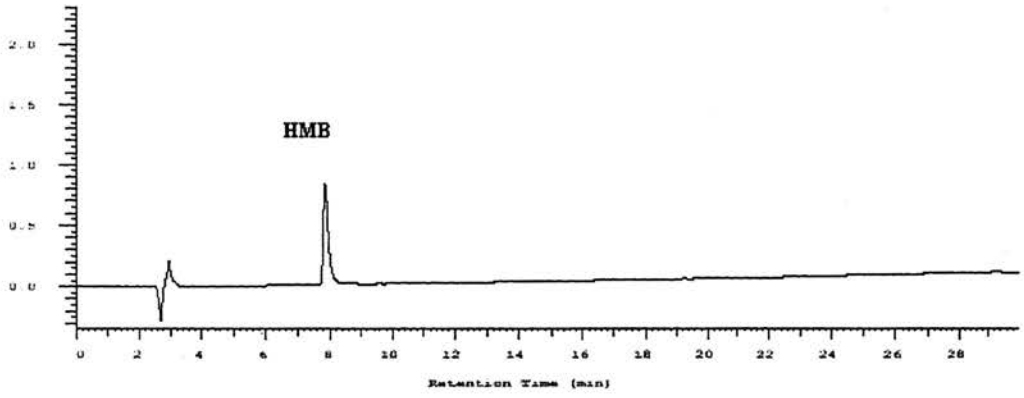


Figure 15: RPLC separation of A) Residual HMBME and HMB and B) Acid hydrolysate of residual HMBME recovered by absolute ethanol washing of HMB-LysEE dimer synthesized with HMBME substrate. Separation was achieved with a C-18 column using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 45% B (Acetonitrile + 0.1% TFA) in 30 minutes. Quantification revealed 57% of the initial HMB-ME was recovered. The presence of only a HMB peak in the hydrolysate indicates the completion of acid hydrolysis.



(A)



(B)

Figure 16: RPLC separation of A) Residual HMBEE and HMB and B) Acid hydrolysate of residual HMBEE recovered by absolute ethanol washing of HMB-LysEE dimer synthesized with HMBEE substrate. Separation was achieved with a C-18 column using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 45% B (Acetonitrile + 0.1% TFA) in 30 minutes. Quantification revealed 65% of the initial HMB-ME was recovered. The presence of only a HMB peak in the hydrolysate indicates the completion of acid hydrolysis.

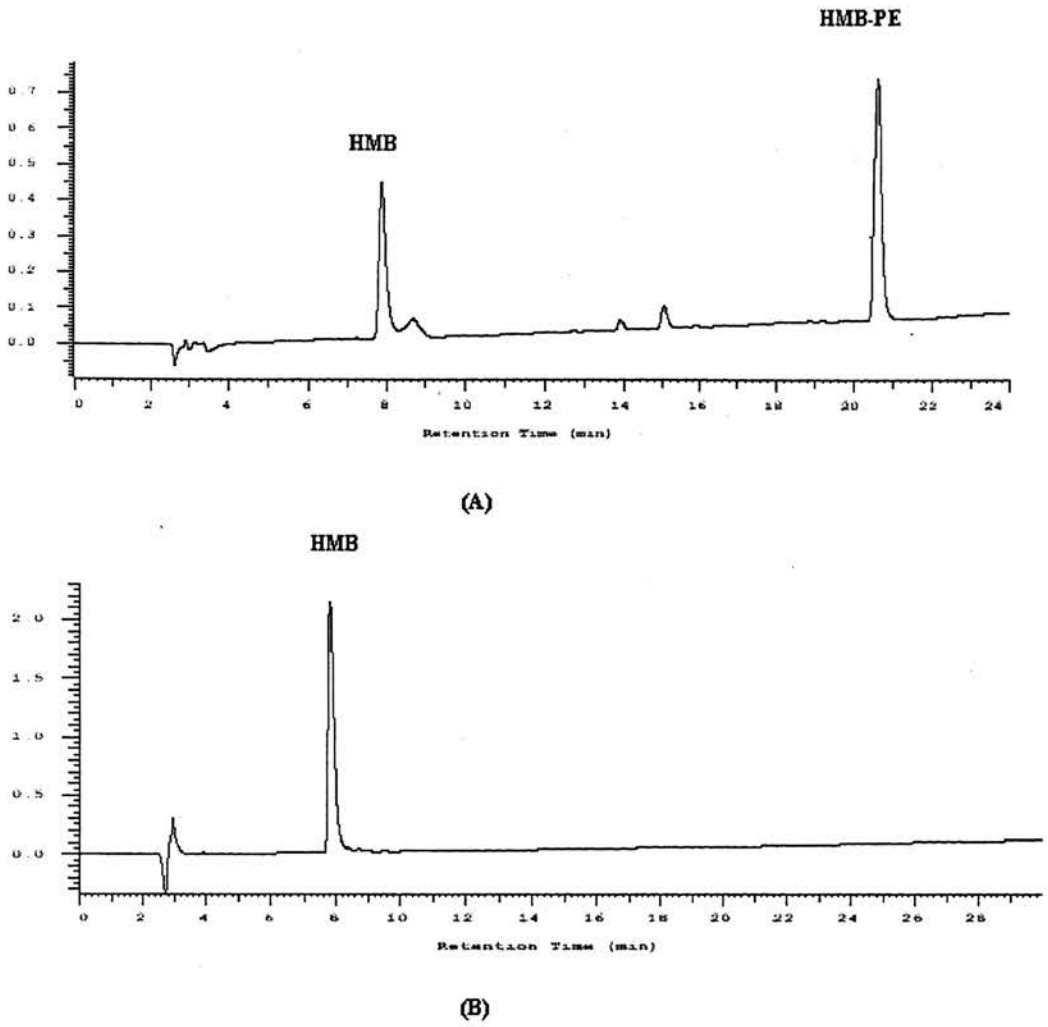


Figure 17: RPLC separation of A) Residual HMBPE and HMB and B) Acid hydrolysate of residual HMBPE recovered by absolute ethanol washing of HMB-LysEE dimer synthesized with HMBPE substrate. Separation was achieved with a C-18 column using a mobile phase gradient comprising of 100% A (Water + 0.1% TFA) initial to 45% B (Acetonitrile + 0.1% TFA) in 30 minutes. Quantification revealed 82% of the initial HMB-ME was recovered. The presence of only a HMB peak in the hydrolysate indicates the completion of acid hydrolysis.

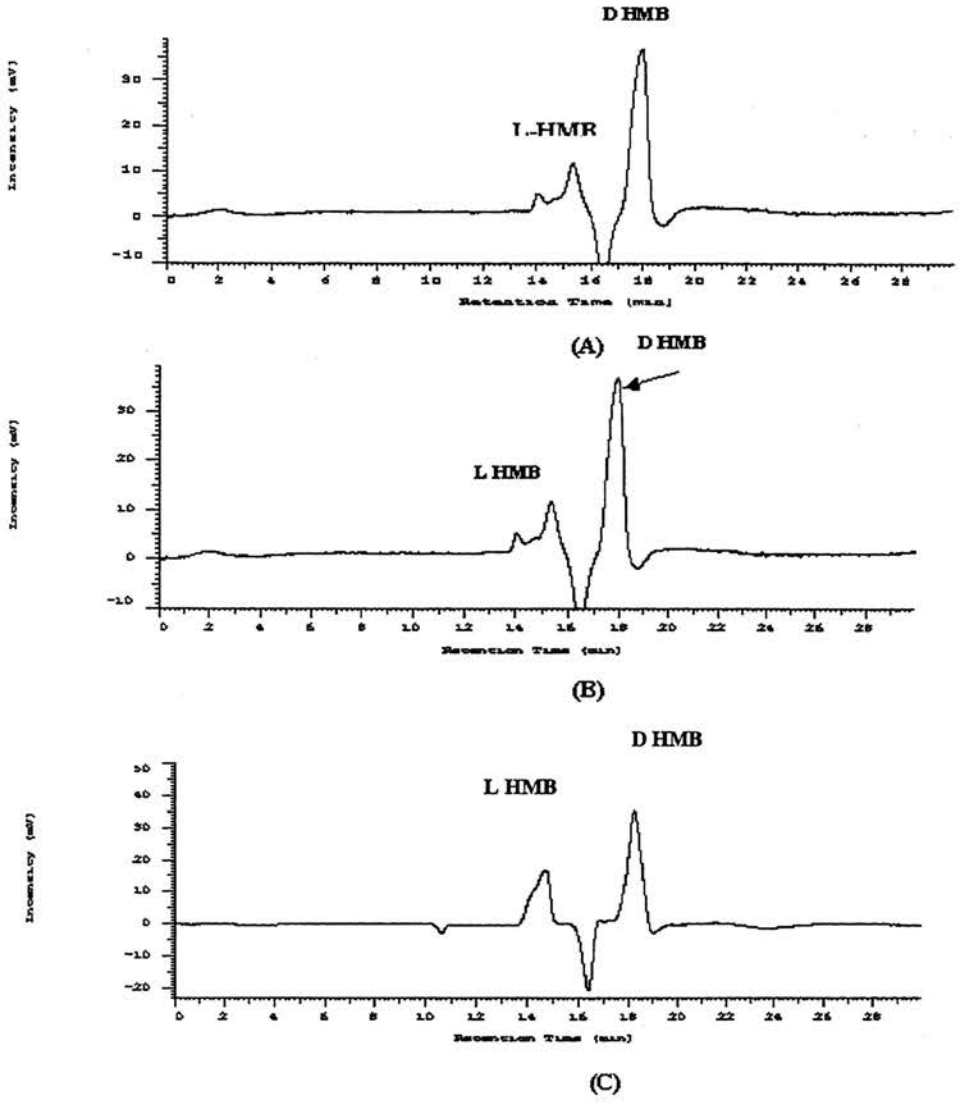


Figure 18: Chiral LC separation of acid hydrolysis of residual HMB ester recovered from HMB-LysEE dimer synthesized with A) HMBME B) HMBEE and C) HMBPE substrate. Separation was achieved with a Chirobiotic TAG column using an isocratic gradient of 70: 30 mixture of 30mM ammonium acetate buffer (pH –4.0) with methanol. The chiral separations show the D-enrichment of residual HMB left in the reaction mixture.

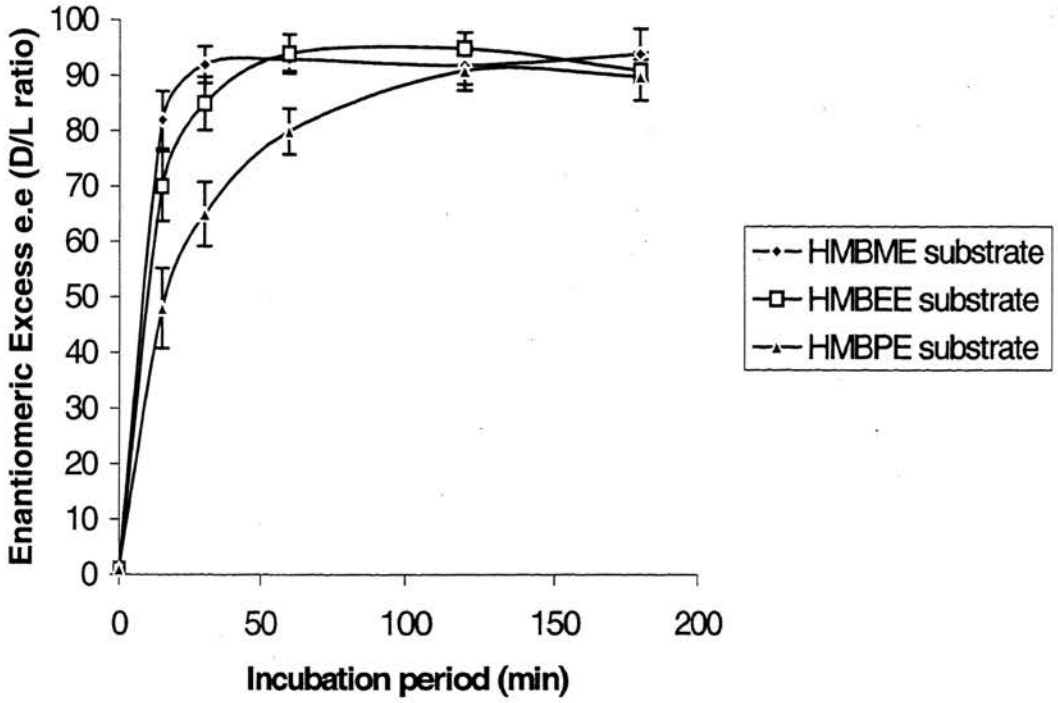


Figure 19: Enantiomeric excess of HMB obtained with different HMB ester substrates for different incubation periods of chymotrypsin-catalyzed synthesis of HMB-LysEE dimer.

APPENDIX

Synthesis of HMB capped bovine insulin. 150mg of Insulin was added to a reaction vial containing 30mL of solvent containing 40% (v/v) of 125mM sodium bicarbonate buffer in acetonitrile. To this 90mg of EDTA, 528mg of L-Cysteine was also added. 150mg of HMBEE was added to this reaction medium as the capping substrate. 50mg of papain was added to catalyze the capping reaction. The reaction mixture was incubated for a period of 24h. After 24h, the reaction was stopped by heating the mixture at 80^0C for a period of 5min. The reaction product was separated into a supernatant and precipitate. The supernatant was rotary evaporated to dryness while the precipitate was lyophilized to dryness. The dried supernatant and precipitate were then reconstituted in 10mL of 1% acetic acid solution, centrifuged, filtered and then injected into ESI-MS for characterization.

Bovine Insulin standard and synthesized HMB-Insulin was diluted to form 0.5mg/mL solution in 1% acetic acid. A make-up solution comprising of 50% acetonitrile in water with 0.1% acetic acid was infused along with the sample at a flow rate of 0.2mL/min into a Hitachi M-8000 ion trap mass spectrometry system using a syringe pump (Harvard Apparatus) at a flow rate of 1mL/hr. An Electrospray Ionization interface was used. The operating parameters of the 3D Q-Ion Trap were as follows: Electrospray capillary voltage, +3.5KV; detector voltage, 400V; assistant gas heater temperature, 200^0C; desolvator temperature and the aperture-1 temperature, 200^0C and 150^0C respectively. The 3D Q- Ion Trap mass analyzer was scanned from 500 – 3900amu.

The procured insulin was first characterized using ESI (+)-MS. The ESI-MS spectrum of the procured insulin standard is shown in ***Figure A.1***. The spectrum consists of a single peak appearing at ***m/z 979***. This corresponds to a hexa-sodiated ion of insulin (Insulin + $6Na^+$). The mass of bovine insulin used is 5734amu. There are a total of four basic amino acid residues in insulin that could stay protonated under the analysis conditions. This along with the terminal amine groups in the two chains leads to a total of six ionization sites. The sample was prepared in 1% acetic acid solution. Insulin has limited solubility in water, ethanol or acetonitrile and has high solubility in 1% acetic or hydrochloric acid and moderate solubility in 125mM sodium bicarbonate solution. Hence

for analytical characterization, insulin samples were prepared in 1% acetic acid solution while capping was done in a monophasic solvent system comprising of 40% 125mM sodium bicarbonate buffer and 60% water. Because of the highly acidic nature of the sample, all the possible ionization sites were sodiated resulting in a multiply charged ion of insulin.

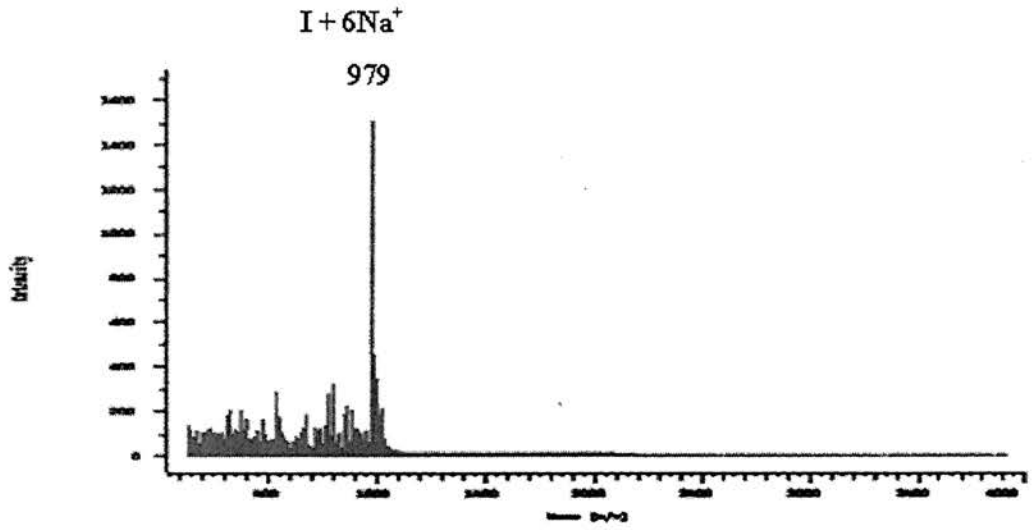


Figure A.1: ESI (+)-MS of Bovine Insulin in an ion trap mass spectrometer. The trap was scanned from 500-3900amu.

Bovine Insulin capping with HMB was attempted in monophasic system. Phe is the N-terminal residue in one of the insulin chains. Earlier results show that Phe oligomers acted as a good substrate for capping with HMB in monophasic system. Hence, we attempted the capping of insulin with HMB under the same conditions replacing sodium citrate with sodium bicarbonate. After incubation, supernatant was separated from the precipitate. The ESI-MS spectrum of dried (rotary-evaporation) supernatant is shown in ***Figure A.2 (i)***. The spectrum shows the presence of two ions appearing at ***m/z 957 and 1468***. The ion at ***m/z 957*** corresponds to hexa charged insulin (Insulin + $6H^+$) while the ion at ***m/z 1468*** corresponds to tetra-charged ion of HMB

capped insulin (HMB-Insulin + $4H^+$). The ESI-MS spectrum of the precipitate (***Figure A.2 (ii)***) revealed the presence of peaks appearing at ***m/z 1468, 1897, 2469 and 3405***.

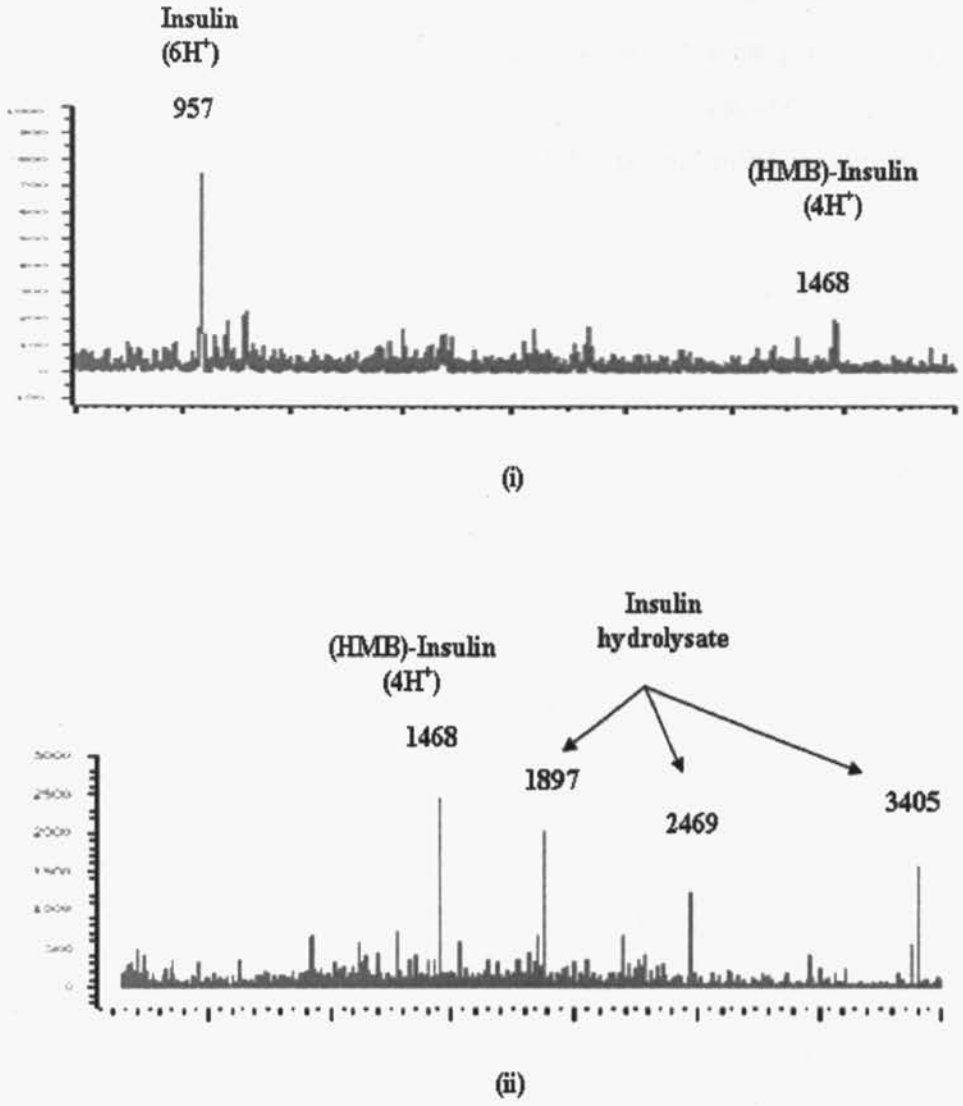


Figure A.2: ESI (+)-MS output of dried (i) Supernatant and (ii) Precipitate of HMB-Insulin synthesized in monophasic aqueous organic system.

Of these peaks only the ion at ***m/z 1468*** corresponds to HMB-Insulin ($4H^+$). The ESI-MS spectrum of the precipitate obtained from incubation of insulin under the reaction conditions with out any HMBEE showed the presence of the additional peaks appearing in ***Figure A.2 (ii)*** indicating that these additional peaks could be possible insulin hydrolysate. The MS data also shows the addition of only one HMB residue to the peptide. This shows that only one of the chains is capped in the protein. This is due to the stearic hindrance arising when both the terminal amino acid residues (Phe and Gly) are capped. Quantification revealed that nearly 50% of the initial insulin was capped with HMB.

VITA

Santhana Krishnan Srinivasan was born in Tuticorin, India on June 13, 1982. He completed his secondary school education at St. Thomas Matriculation Higher Secondary School in Tuticorin, India. He obtained his Bachelor's degree in Chemical Engineering as a Gold medallist from Shanmugha College of Engineering, Bharathidasan University, India in June 2003. In August 2003, he joined the Chemical Engineering Ph.D. program at the University of Missouri-Rolla under the guidance of Prof. Daniel Forciniti. During the course of his graduate program, he carried out his research as a Graduate Research Assistant under the guidance of Prof. Shubhen Kapila. He received his PhD in August 2007.