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Enantio Separation of HMB (1-Hydroxy Methylthio Butanoic Acid) through Chymotrypsin Catalysed 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 alpha hydroxy acids. 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; 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 [3-5] 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 analogue 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 [9-11]. 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 [15]. 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 1 g 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 [16-19]. 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 catalysed 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) catalysed 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 catalysed kinetic resolution of numerous other substrates have been reported and an entire description is beyond the scope of this article [24-27]. We have reported procedures for the optical resolution of alpha hydroxy acids, especially HMB, through lipase catalysed hydrolysis and esterification [16,28].

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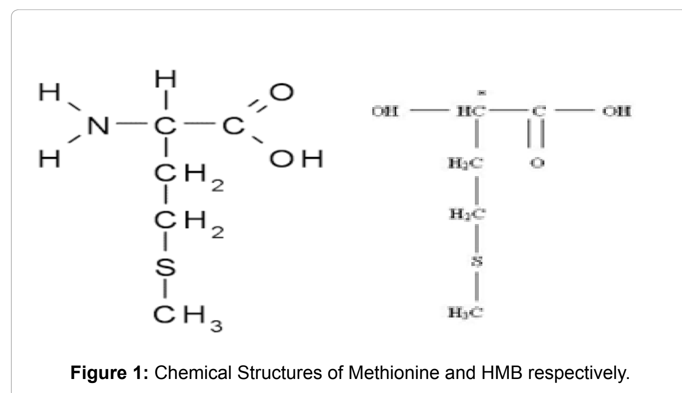


Figure 1: Chemical Structures of Methionine and HMB respectively.

Proteases belong to a class of enzymes that hydrolyse peptide bonds but under proper conditions they could be also used to catalyse 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 [16,29,30]. This property of proteases could be used to optically resolve racemic mixtures. For example, papain catalysed 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-catalysed 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, 28 mg 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 hydrolysates was carried out in a XPERCHROM C-18 column (250 mm × 4.6 mm) purchased from PJ Cobert Associates Inc., (St Louis, MO). The Chirobiotic -TAG column (250 mm × 4.6 mm) used for chiral separation of HMB was purchased from Advanced Separation Technologies Inc., (Whippany, NJ). The nanopure grade 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 Instruments Inc., 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 50 μL 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 Instruments Inc., 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 HMB capped co-oligomers.

Methods

Synthesis of DL-HMB ester: DL-HMB was esterified with either 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-Phe EE hydrochloride (0.5 mM) was added to a reaction flask containing 250 mL of DFP, 250 mL of n-octane, 50 mL of water, 10 mL of DIPEA, 2 g of cysteine, and 300 mg of papain. The reaction vial was incubated for 24 h in an incubator shaker. The reaction was stopped by heating the mixture to 80°C for 5 minutes. The reaction product was rotary evaporated to dryness. The dried product was washed with 10 mL of Nano pure 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.

Chymotrypsin catalysed HMB capping of Lys oligomers: 50 mg of Lys oligomer and 50 mg of HMB-ME were added to a reaction vial containing 5 mL of 50 mM sodium phosphate (dibasic) buffer (pH 7.8). To this vial, 250 μL (50 units) of enzyme suspension (3.33 mg/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 250 µL aliquot was taken for analysis. This aliquot was diluted proportionately, centrifuged, filtered and 5 µL 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 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 100 mL of nanopure water in a 500 mL flat-bottomed flask. 10 mM L-cysteine, 1.0 mM EDTA and 0.1 M sodium citrate were added after the ethyl ester of methionine was dissolved. The contents were stirred and the pH was adjusted to 5.5. 300 mg of crude papain was added to that mixture and the reaction mixture was kept at 37°C with constant shaking for 24 h. Then, the reaction was stopped by heating the reaction mixture at 80°C for 5 min. The mixture was transferred to 200 mL centrifuge vials and centrifuged at 10000 rpm 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: 500 mg of the purified Phe and Met oligomers were dissolved in 10 mL of 60% (v/v of acetonitrile/water) containing 1 mM L-cysteine, 0.1 mmole EDTA and 0.01 mmole sodium citrate. 500 mg of HMBEE and 30 mg of papain were added after the oligomers were dissolved. The reaction was allowed to proceed for 24 h. The reaction was stopped by heating it at 80°C for 5 min. 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 100 mL 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: 500 mg of the synthesized HMB-Phe and HMB-Met co-oligomers were placed in a 40 mL vial containing 10 mL of 6 N Hydrochloric acid. The contents were stirred and kept at 110°C on a sand bath for 48 h. A 1 mL aliquot of acid solution was after 8 h and transferred to a round bottom flask. The solution was dried with a rotary-evaporator and reconstituted with 5 mL 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.

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 5 mL of 6 N HCl. This mixture was placed in a heated sand bath maintained at 110°C for a period of 48 h. The hydrolysate was rotary evaporated to dryness under vacuum. The resultant product was reconstituted in 5 mL 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 was from the dried HMB-LysEE dimer and the corresponding monomer obtained from its acid hydrolysis was done in a reverse phase C-18 column (250 mm × 4.6 mm i.d.) and detected with a fixed wavelength UV detector (Hitachi Instruments). The separated analytes from the column were monitored at 210 nm. The mobile phase flow rate was maintained at 1 mL min^{-1} and 10 µL of the sample after filtration with a 0.22 µ 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 hydrolyzate; 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+10 mM HSA+0.1% O-Phosphoric acid) initial to 23% B (50% acetonitrile+10 mM HSA+0.1% O-Phosphoric acid) in 15 minutes for HMB-LysEE dimer acid hydrolyzate.

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 0.5 mg/mL solution. Synthesized Lys oligomers, HMB capped Lys co-oligomers and HMB-LysEE dimer was diluted to form 0.5 mg/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 0.2 mL/min into a Hitachi M-8000 ion trap mass spectrometry system using a syringe pump (Harvard Apparatus) at a flow rate of 1 mL/hr. An Electrospray Ionization interface was used. The operating parameters of the 3D Q-Ion Trap were as follows: Electrospray capillary voltage, +3.5 KV; detector voltage, 400 V; assistant gas heater temperature, 200°C; desolvator temperature and the aperture-1 temperature, 200°C and 150°C respectively. The 3D Q-Ion Trap mass analyzer was scanned from 50-1200 amu.

Liquid chromatography-mass spectrometry 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 1 mL min^{-1}. The column effluent was split and 80% was introduced into a fixed wavelength UV detector set at 210 nm. 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 mixtures of 30 mM ammonium acetate buffer (pH 4.0) and methanol in a Chirobiotic TAG (ASTEC, Inc.) column. The column effluent was monitored at 230 nm. The mobile phase flow rate was maintained at 0.2 mL min^{-1}. 10 µL of the sample after filtration with a 0.22 µ membrane filter was injected into the column.

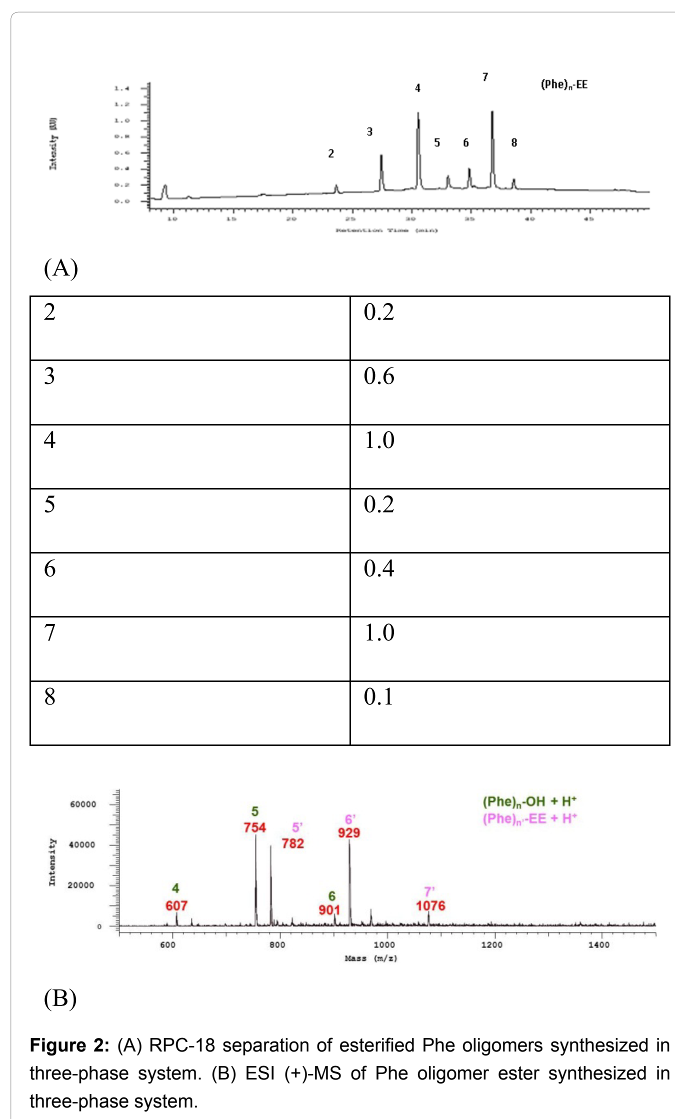
Results and Discussion

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 2A. 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 n 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 2B).

The spectrum consists of a series of peaks appearing at m/z 782, 929 and 1076 ; the mass difference is 147 amu. 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 (NPhe - (Phe)3 - PheC+H$^+$), a hexamer (NPhe - (Phe)4 - PheC+H$^+$) and a pentamer of Phe with ester intact at the C-terminal [NPhe - (Phe)3 - PheCOOEt+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 hydrophobic oligomers. Thus, the capping of Phe oligomers with HMBEE was catalized 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 3A. 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 coupled with a logarithmic progression of retention times. The comparison of Phe oligomer ester separation (Figure 2A) and HMB capped Phe co-oligomers (Figure 3A) 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 33 min while a HMB capped Phe tetramer {HMB- (Phe)4-EE} elutes at 34 min. 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 3A 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 3B 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.



2	0.2
3	0.6
4	1.0
5	0.2
6	0.4
7	1.0
8	0.1

Figure 2: (A) RPC-18 separation of esterified Phe oligomers synthesized in three-phase system. (B) ESI (+)-MS of Phe oligomer ester synthesized in three-phase system.

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 4A. 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 4B.

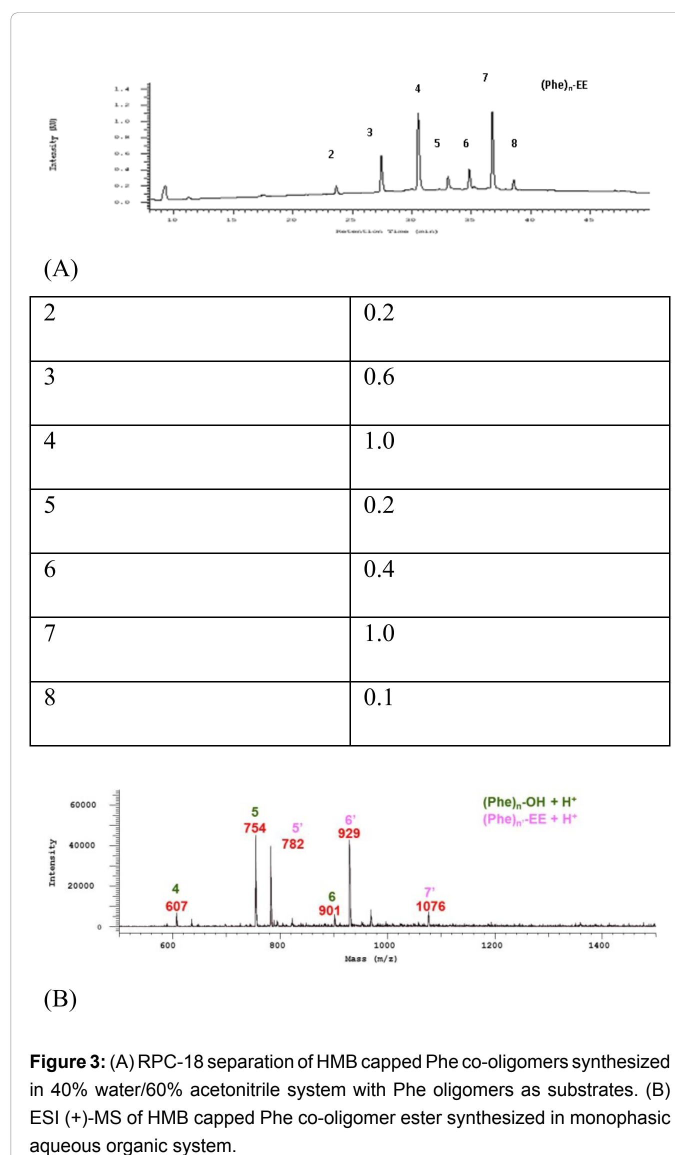
The spectrum is dominated by the heptamer and octamer of Met with an intact C-terminal ester appearing at m/z 964 and 1095. The spectrum also shows the presence of Met hexamer, heptamer and octamer (m/z 805, 936, 1067) with free acid present at the C-terminal. It is evident from the chromatogram and 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 5A. Comparison of this chromatogram with Figure 4A, 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 5B 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 1 amu 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 (Figures 3A and 5A) 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 6A and 6B 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 (NLys-(Lys)n-LysCOOH+H⁺). A tetramer of Lys, NLys-(Lys)2-LysCOOH+H⁺ should appear at a m/z 531 while a pentamer NLys-(Lys)3-LysCOOH+H⁺ should appear at a m/z 659. There is another peak that appears at m/z 303. This peak corresponds to a dimer (NLys-LysCOOH+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 15 min with HMBME as a substrate is shown in Figure 8A. The spectrum consists of a series of ions appearing at a mass difference of 128 amu. This m/z value is 4 amu 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 (NHMB-Lys-(Lys)2-LysCOOH+H⁺) it will appear



2	0.2
3	0.6
4	1.0
5	0.2
6	0.4
7	1.0
8	0.1

Figure 3: (A) RPC-18 separation of HMB capped Phe co-oligomers synthesized in 40% water/60% acetonitrile system with Phe oligomers as substrates. (B) ESI (+)-MS of HMB capped Phe co-oligomer ester synthesized in monophasic aqueous organic system.

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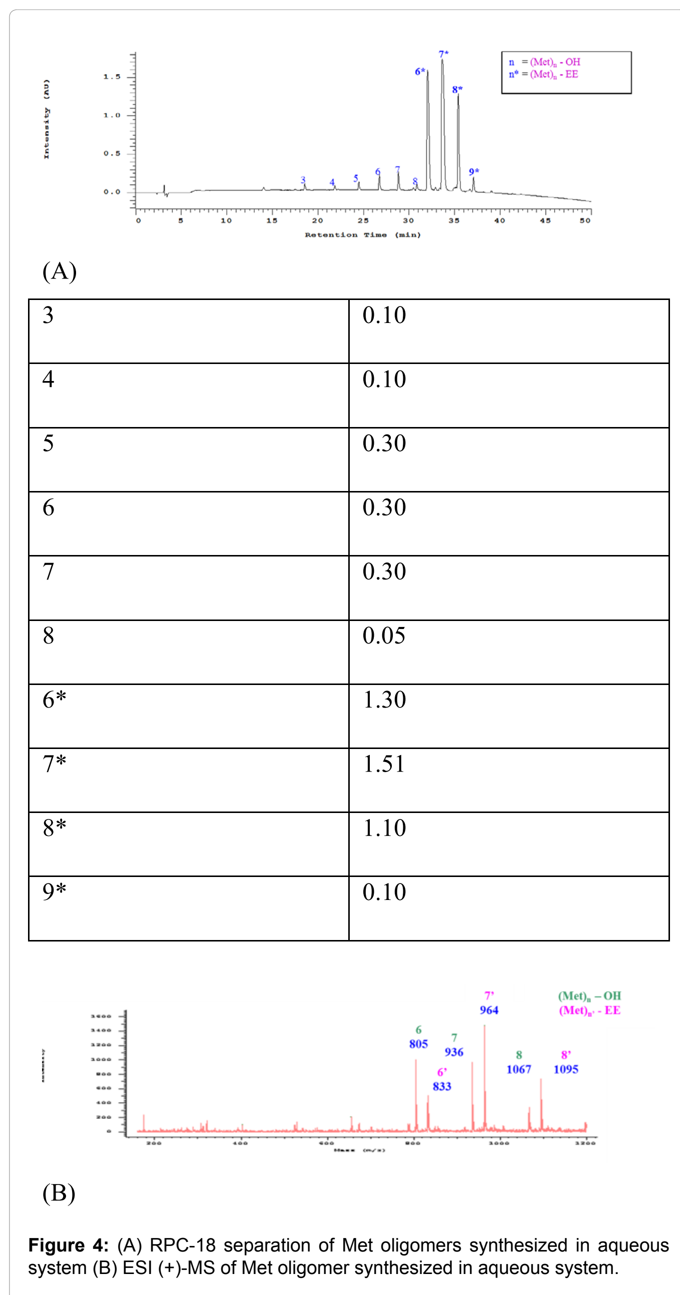


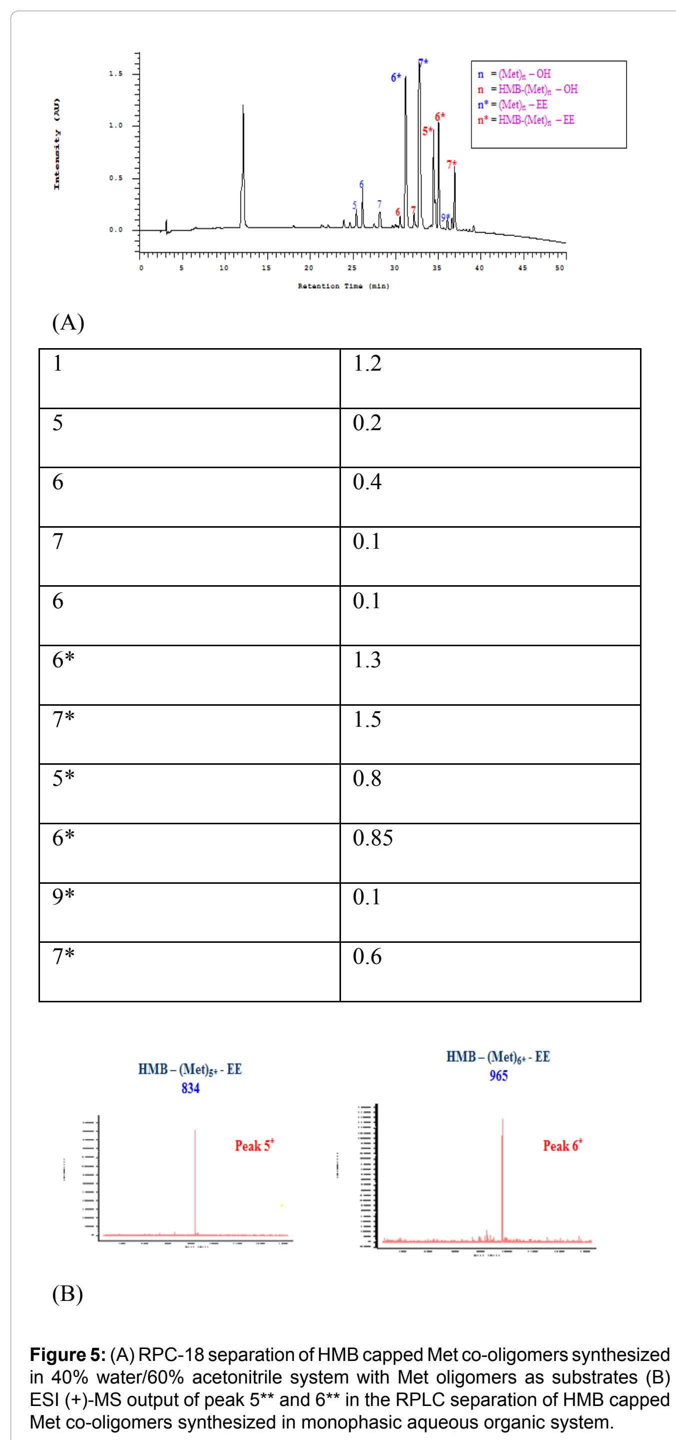
3	0.10
4	0.10
5	0.30
6	0.30
7	0.30
8	0.05
6*	1.30
7*	1.51
8*	1.10
9*	0.10

Figure 4: (A) RPC-18 separation of Met oligomers synthesized in aqueous system (B) ESI (+)-MS of Met oligomer synthesized in aqueous system.

at m/z 663, which is 4 amu higher than the pentamer of Lys that will appear at m/z 659.

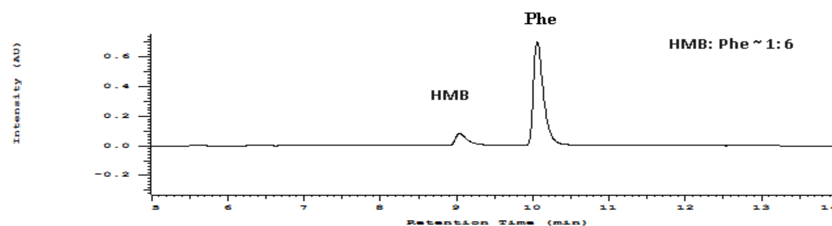
The spectrum had a series of peaks appearing at m/z 435, 535, 663, 791 and 919 corresponding to HMB capped poly Lys residues ranging from 2 to 6. The ion appearing at m/z 435 corresponds to HMB capped dimer of Lys with intact ester at the C-terminal end (NHMB-Lys-LysCOOEt+H$^+$). The spectrum also shows a small peak corresponding to an unreacted dimer of Lys. The ESI (+)-MS of the sample incubated for a period of 30 min with HMBME as substrate (Figure 8B) shows the absence of any Lys oligomer residues indicating that the process of capping was complete in 30 min. 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 30 min, 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 10A and 10B). However, in this case only after an incubation period of 60 min 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.

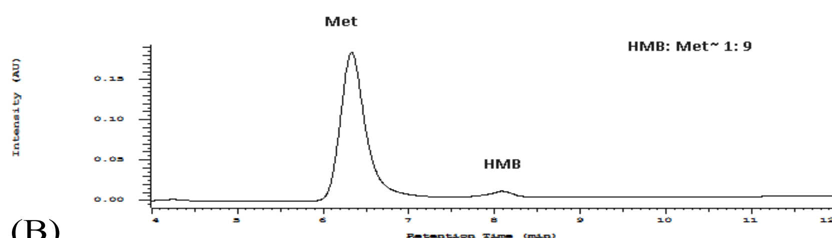


1	1.2
5	0.2
6	0.4
7	0.1
6	0.1
6*	1.3
7*	1.5
5*	0.8
6*	0.85
9*	0.1
7*	0.6

Figure 5: (A) RPC-18 separation of HMB capped Met co-oligomers synthesized in 40% water/60% acetonitrile system with Met oligomers as substrates (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.



(A)



(B)

Figure 6: RPLC separation of (A) HMB-Phe co-oligomer acid hydrolysate and (B) HMB-Met co-oligomer acid hydrolysate.

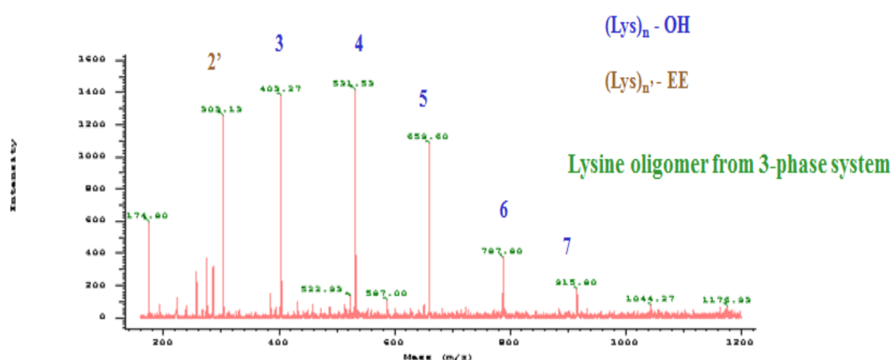
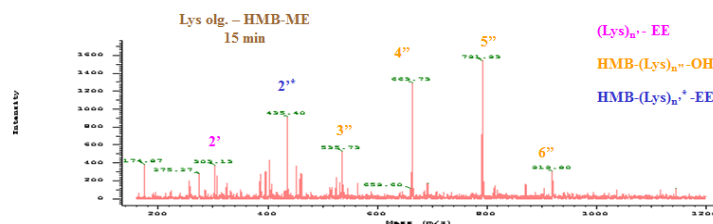
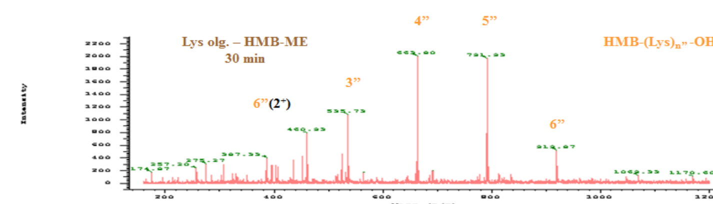


Figure 7: ESI (+)-MS Spectrum of Lys oligomers synthesized in three-phase system with LysEE substrate.



(A)



(B)

Figure 8: ESI (+)-MS spectrum of HMB capped Lys oligomers synthesized through chymotrypsin catalysis with HMBME substrate for A) 15 min and B) 30 min incubation period.

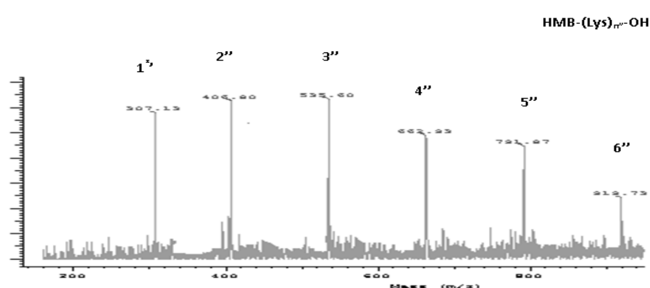


Figure 9: ESI (+)-MS spectrum of HMB capped Lys oligomers synthesized through chymotrypsin catalysis with HMBME substrate for an incubation period of 60 min.

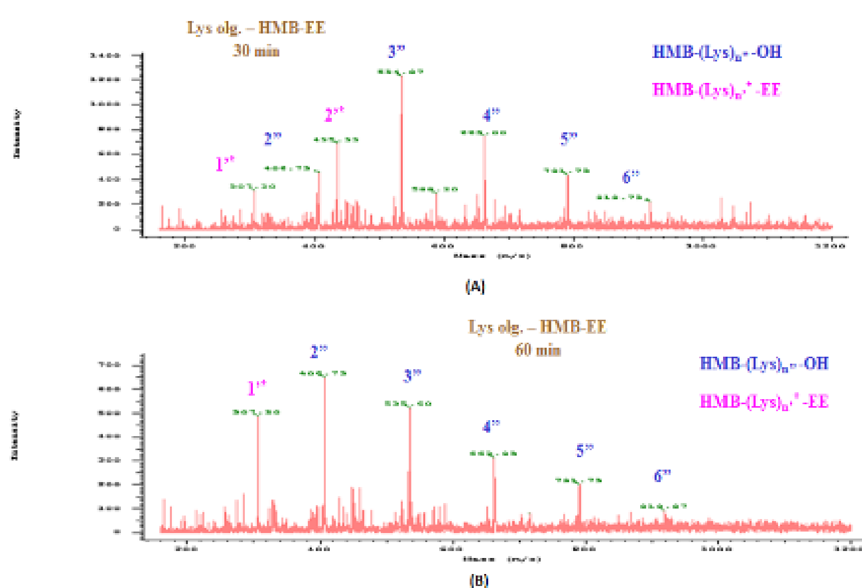


Figure 10: ESI (+)-MS spectrum of HMB capped Lys oligomers synthesized through chymotrypsin catalysis with HMBEE substrate for A) 30 min and B) 60 min incubation period.

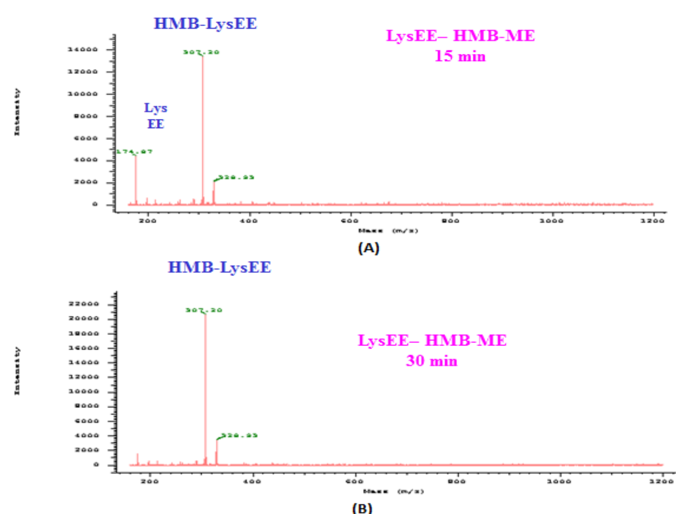


Figure 11: ESI (+)-MS spectrum of HMB-LysEE dimer synthesized through chymotrypsin catalysis with HMBME substrate for A) 15 min and B) 30 min incubation period.

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 15 min is shown in Figure 11A. 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 30 min, there was no residual LysEE left (Figure 11B). 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 [30]. 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 12A and 12B) and HMBPE (Results not shown), but the reaction was slower. The reaction went to completion in 60 min with HMBEE and it was complete in 120 min 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 [29,30]. 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 15 min to 180 min. 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 30 min is shown in Figure 13A. 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 13B). 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 30 min 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 60 min incubation 46% of the initial HMB was bound while for HMBPE it took 120 min 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 30 min is shown in Figure 14A. 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 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 14B). purity of L-HMB incorporated into the dimer was greater than However, the enantio-purity of L-enantiomer incorporated into the dimer when HMBPE was used (30 min incubation time) was close to 85% (Figure 14C). Capping was much slower with HMBPE. This resulted in a higher amount of residual DL HMBPE after 30 min 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 15A. The identification of the separated peaks was done by comparing them against MB 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 15B shows the RPLC separation of the residual HMBME acid hydrolysate.

The chromatogram shows the complete hydrolysis of HMBME to HMB. Quantification of HMBEE (Figure 16A and 16B) and HMBPE (Figure 17A and 17B) recovered from the reaction medium for an incubation period of 30 min 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 60 min and 120 min, 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 30 min incubation shows an enantiomeric excess e.e (D/L ratio) of 92% (Figure 18A). 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 18B) while it was only 65% with HMBPE (Figure 18C).

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 60 min, the e.e for HMBEE was 94% while it was 91% with HMBPE for a reaction time of 120 min. 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.

Conclusion

Protease catalysed capping of Phe, Met and Lys oligomers with HMB was carried out successfully. Our results show that chymotrypsin catalysed hydroxy-acid capping reactions are very rapid and completion is reached in less than 30 min. The reaction of HMBME

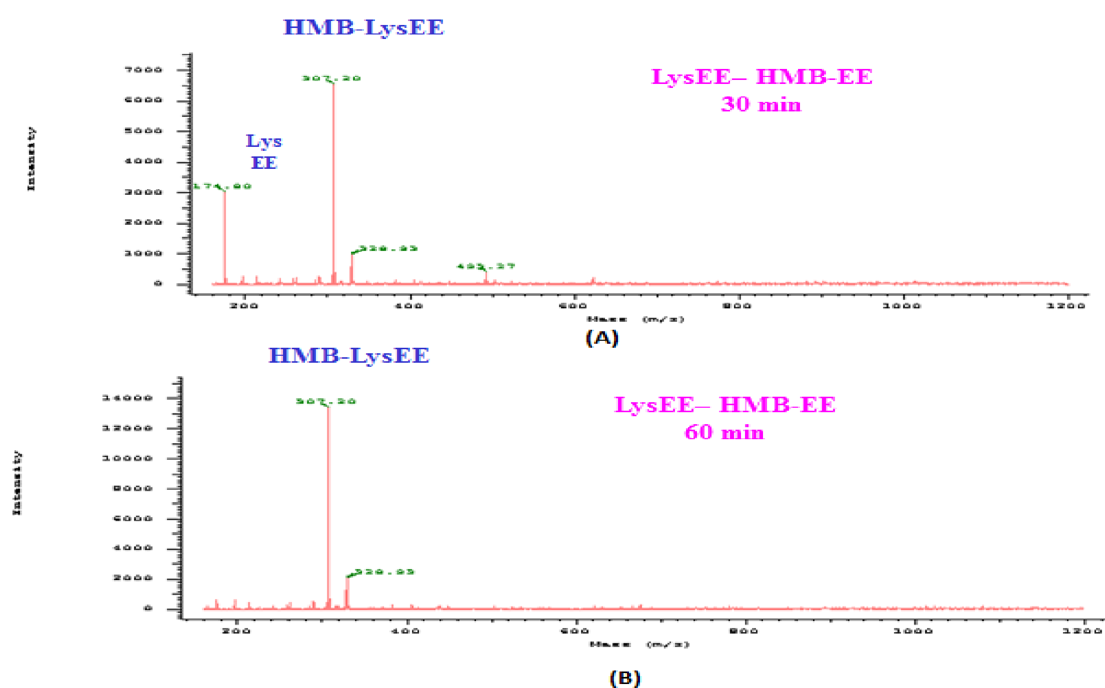


Figure 12: ESI (+)-MS spectrum of HMB-LysEE dimer synthesized through chymotrypsin catalysis with HMBEE substrate for A) 30 min and B) 60 min incubation period.

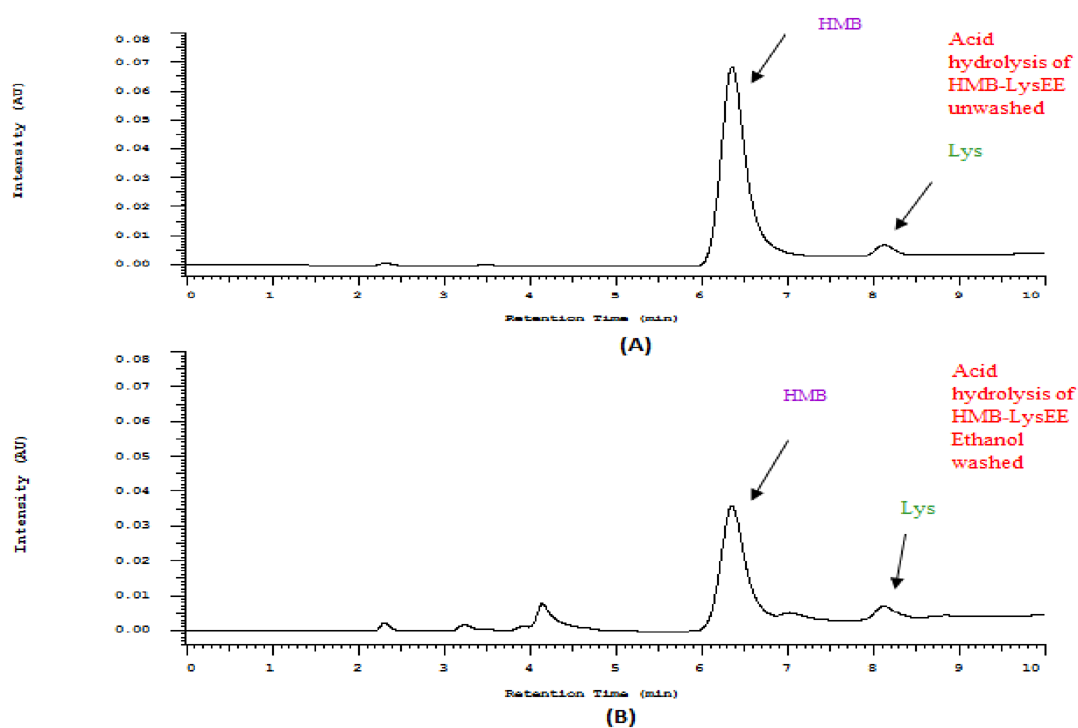


Figure 13: RPLC separation of acid hydrolysate obtained from HMB-LysEE dimer synthesized with HMBME substrate A) Un-washed and B) Washed with absolute ethanol. The separation was achieved in a C-18 column with HAS added to the mobile phase as ion-pairing agent.

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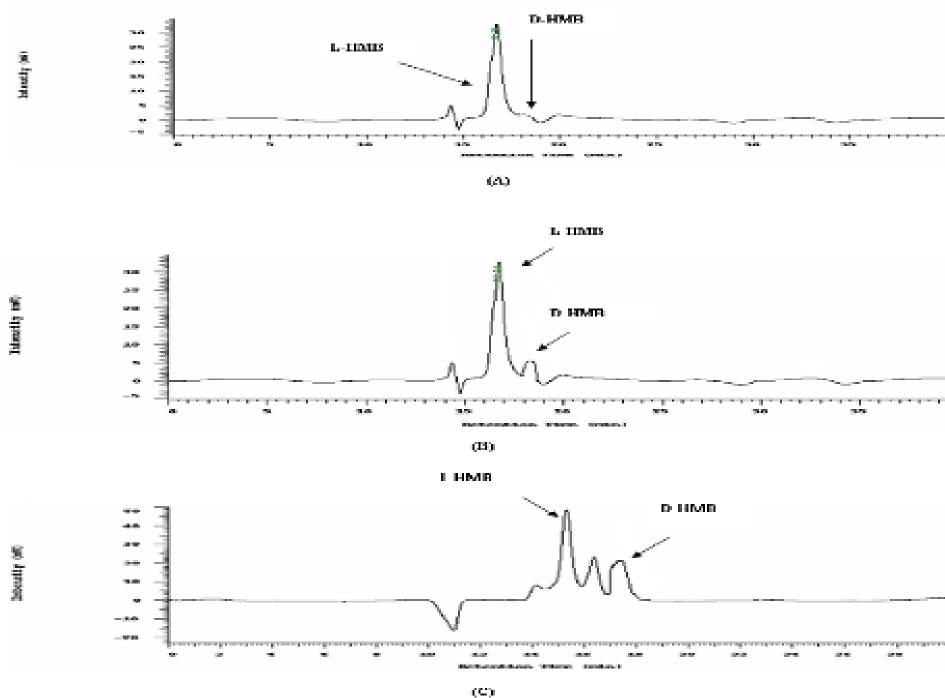

Figure 14: Chiral LC separation of acid hydrolysate of washed HMB-LysEE dimer synthesized with A) HMBME B) HMBEE and C) HMBPE substrate.

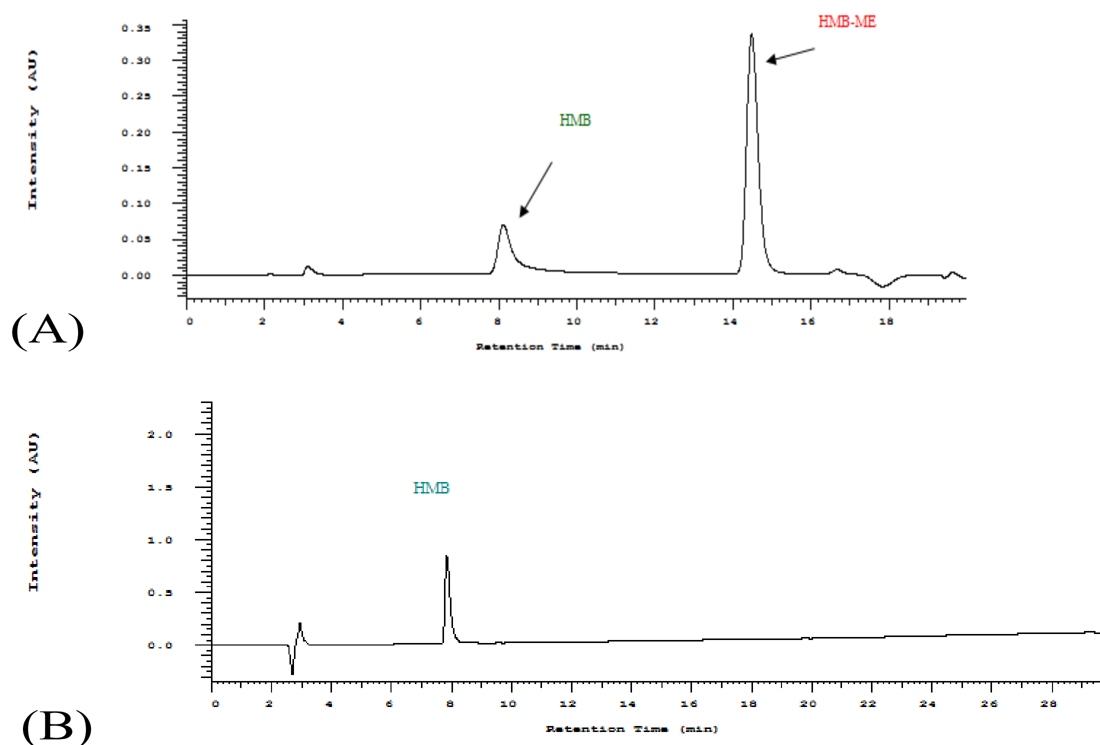

Figure 15: RPLC separation of (A) Residual HMBME and HMB and (B) Acid hydrolysate of residual HMBME recovered by absolute ethanol washing of HMB-LysEE dimer synthesized with HMBME substrate. Separation was achieved with a C-18 column using a mobile phase gradient comprising of 100% A (Water+0.1% TFA)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

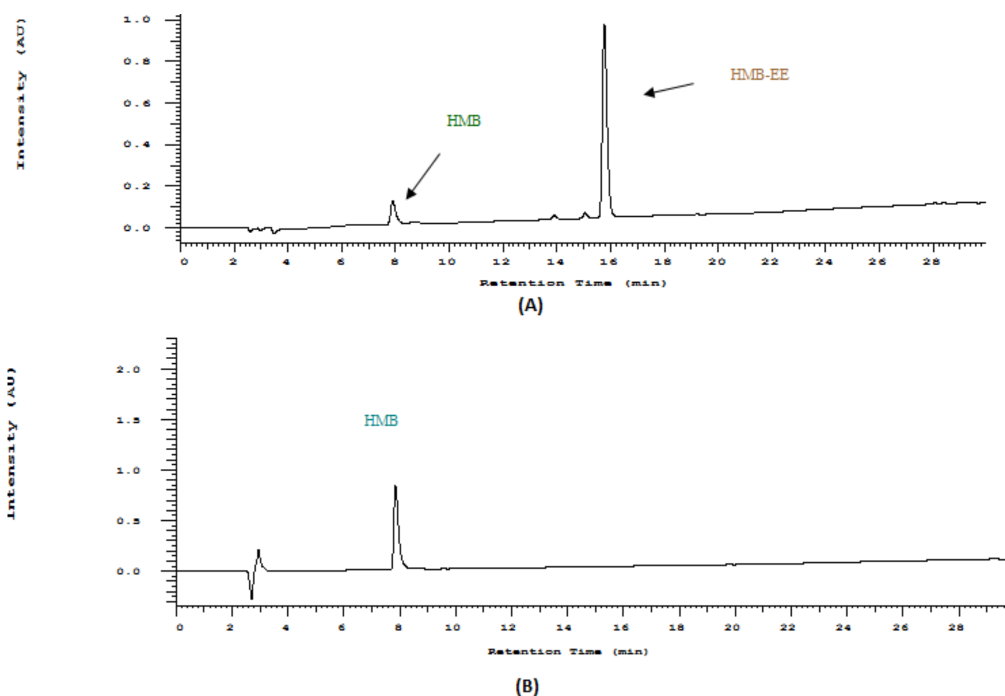

Figure 16: RPLC separation of (A) Residual HMBEE and HMB and (B) Acid hydrolysate of residual HMBEE recovered by absolute ethanol washing of HMB-LysEE dimer synthesized with HMBEE substrate. Separation was achieved with a C-18 column using a mobile phase gradient comprising of 100% A (Water+0.1% TFA) initial to 45% B (Acetonitrile+0.1% TFA) in 30 minutes. Qualification 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.

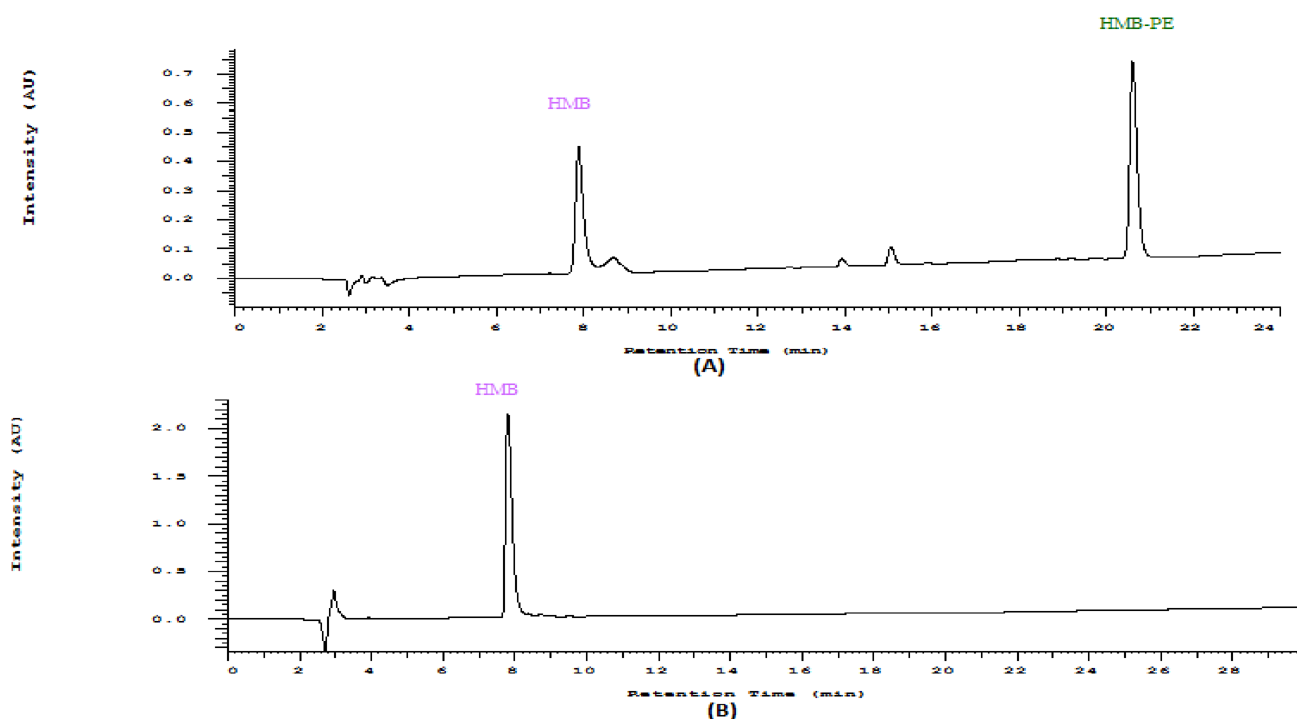

Figure 17: RPLC separation of (A) Residual HMBPE and HMB and (B) Acid hydrolysate of residual HMBPE recovered by absolute ethanol washing of HMB-LysEE dimer synthesized with HMBPE substrate. Separation was achieved with a C-18 column using a mobile phase gradient comprising od 100% A (Water+0.1% TFA) initial to 45% B (Acetonitrile+0.1% TFA) in 30 minutes. Qualification 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 30 mM ammonium acetate buffer (pH - 4.0) with methanol. The chiral separations show the D-enrichment of residual HMB left in the reaction mixture.

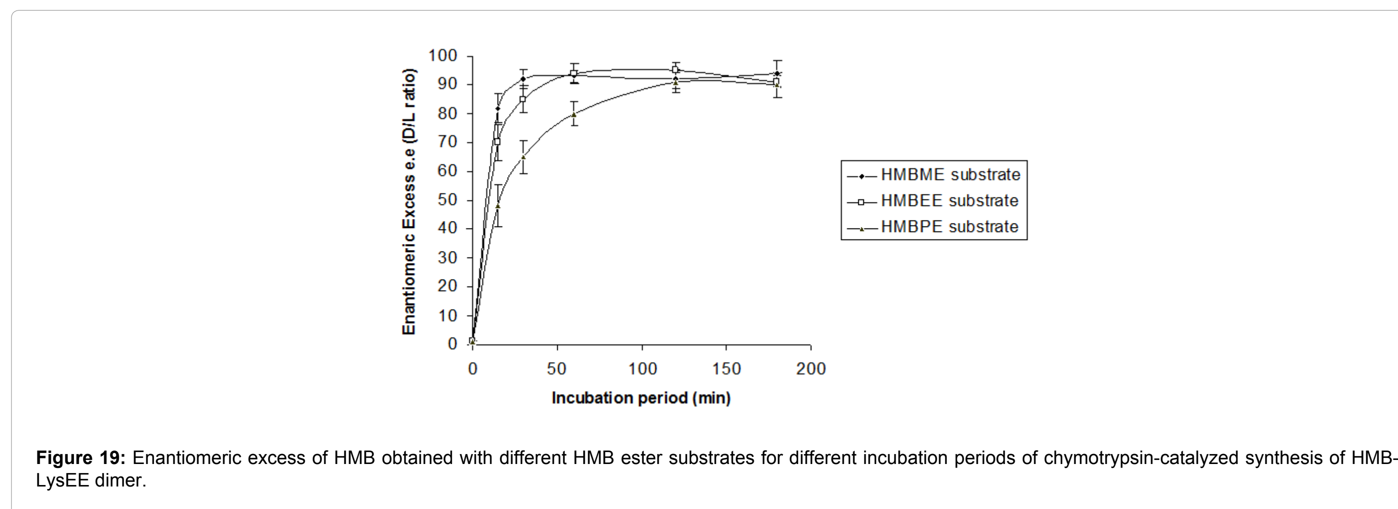

Figure 19: Enantiomeric excess of HMB obtained with different HMB ester substrates for different incubation periods of chymotrypsin-catalyzed synthesis of HMB-LysEE dimer.

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 30 min. 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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