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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 (Lys), glycine (Gly), methionine (Met) and tyrosine (Tyr) 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 High-Pressure Liquid Chromatography (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

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: Ethylene Diamine Tetra Acetic Acid; HAS: Hexane Sulfonic Acid; TFA: Trifluoroacetic Acid; UV/Vis: Ultraviolet/Visible; DMSO: Dimethyl Sulfoxide; DMF: Dimethyl Formamide; ACN: Acetonitrile

Introduction

Solid/solution phase chemical resolution dynamic/kinetic bioresolution and PCR and gene amplification techniques have been reported as preferred tools for peptide synthesis [1]. However, 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, [2-4]. Protease catalyzed synthesis of neutral amino acid homo-oligomers (Met, Tyr, and Leu) in aqueous systems have been reported [5,6]. 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 [5,6]. 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 [5]. 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 [7-13].

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 [14-16]. The major hindrance for the use of such systems is the denaturation and deactivation of enzymes because of the presence of organic solvent molecules [17]. 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 [18]. 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 [18,19]. Leu and Met-Enkephalin derivatives have been synthesized in acetonitrile-Tris-HCl (pH 9) buffer. Organic solvent-stable protease PST-01 has been used for the synthesis of the tripeptide Cbz-Arg-Leu-NH2; varying yields between ~70% to ~88% have been obtained in water and water-miscible organic solvents such as Dimethyl formamide (DMF) and Dimethyl sulfoxide (DMSO) [2].

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 [20]. 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

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[21]. The low water content media are highly attractive in kinetically controlled peptide synthesis because the secondary hydrolysis of the product peptide is minimized [14]. However, in all monophasic systems, a certain minimum amount of water is essential for the catalytic activity of the enzyme [20]. The effect of water in monophasic reaction mixtures has been quantified in terms of changes in product conversion upon changes in water content [21].

Protease catalyzed peptide synthesis is reported to be stereo-specific [2,3,22,23]. It is a well-known fact that L and D enantiomers have different biological activity. Enzymatic resolution of L/D forms of amino acids has been studied extensively [20-23].

Proteases catalyze the hydrolysis of peptide bonds and are involved in many physiological processes. Four mechanistic classes of proteases have been classified as aspartic, metallo, serine or cysteine proteases depending on the chemical nature of their catalytic sites [24-29]. Cysteine and serine proteases function by forming covalent acyl enzyme intermediates involving thioester or ester linkage to cysteine or serine residues at the active site. Metalloproteases lyse and break the peptide bond by noncovalent complexation of the carbonyl to an electrophilic metal ion, such as divalent zinc. Aspartate proteases, function by the concerted action of two active site aspartyl side chains and the activation barrier is overcome without formation of a covalent intermediate [30,31].

There are two groups of proteases: endopeptidases and exopeptidases. Endopeptidases cleave peptide bonds within the protein, but exopeptidases cleave only at the termini of polypeptides, therefore, they are generally chosen as possible catalysts for the oligomeriztion of long polypeptide substrates because they do not disturb internal peptide bonds [32,33].

In this study, use of monophasic aqueous organic solvent systems was evaluated for papain-catalyzed oligomerization of both neutral and polar amino acid oligomers. Papain (EC 3.4.22.2) [34] is one of the cysteine proteases present in papaya (*Carica papaya*) which is useful in tenderizing meat and other proteins. It is the best- studies member of this enzyme family and it consists of 212 amino acids stabilized by 3 disulfide bridges. Its 3D structure consists of 2 distinct structural domains with a cleft between them. This cleft contains the active site, which contains a catalytic triad that has been likened to that of chymotrypsin. Its catalytic triad is made up of 3 amino acids-cysteine-25 (from which it gets its classification), histidine-159, and asparagine-175 [35] (Figures 1 and 2). Papain exhibits broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine. It also hydrolyzes esters and amides. However, papain has also been used in the enzymatic synthesis of amino acids, peptides, and other molecules. The syntheis of oligomers of L-methionine (Met) and its hydroxy analogue, 2-hydroxy-4-(methylthio) butanoic acid (D, L-HMB) with papain as the enzymatic catalyst has already been reported [27].

Fukuoka et al. studied the polymerization of tyrosine ester hydrochlorides in the presence of papain catalyst to give a polymer of alpha-peptide structure [36]. Xiang et al. described the enzymatic

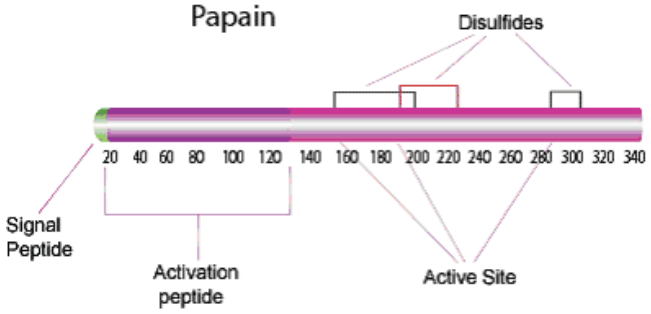

Figure 1: The structure of papain.

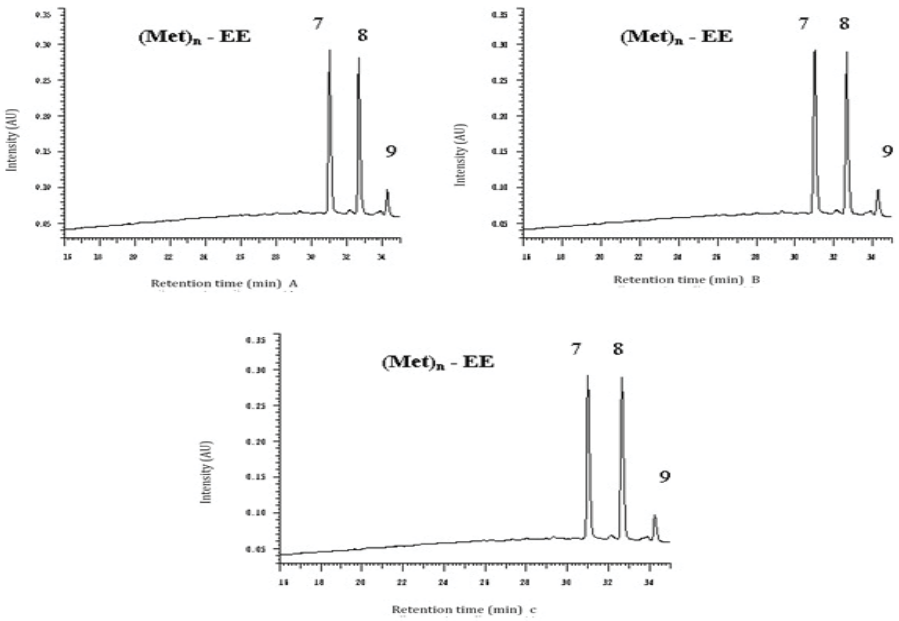

Figure 2: Chromatogram of methionine oligomers synthesized in aqueous system with enzyme recovered from 95% acetonitrile/ 5% water system incubated for (A) 4 h and (B) 24 h. C) Chromatogram of Met oligomers synthesized with enzyme recovered from 100% water system. Separation was achieved with a RPLC 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.

synthesis of the C-terminal fragment H-Gly-Trp-Met-Asp-Phe-NH2 of cholecystokinin using immobilized papain as biocatalyst in buffered ethyl acetate [37].

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 [26]. 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

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, 28 mg 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 (250 mm × 4.6 mm), 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 (250 mm × 4.6 mm) 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 7 mL clear borosilicate glass vials along with 5 mL of the acetonitrile/water mixture. The water content of the mixture was varied from 1% to 15% (v/v); the contact period between the enzyme and solvent mix was varied from 0 h to 24 h. 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 [5]. 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 10 mL acetonitrile with varied water content. 1 mmole L-cysteine, 0.1 mmole EDTA, sodium citrate and 30 mg of papain were also added to the reaction mixture. The amount of sodium citrate was varied till 0.01 mM. The reaction mixture was incubated for 24 h at room temperature. After 24 h, the reaction was stopped by heating the mixture at 80°C 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 0.934 g 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% to 100% (v/v)). 10 mg of the oligomer was added to 5 mL of the solvent mixture in a 7-mL clear glass vial. The mixture was sonicated for 10 minutes in a sonic bath. The mixture was then centrifuged and supernatant was filtered through a 0.22 μ 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. [27].

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

500 mg of Met oligomers obtained from DL-MetEE substrate were placed in a 40 mL vial containing 10 mL of 6 N HCl. The contents were stirred and kept at 110°C on a sand bath. After 48 h, the reaction mixture was cooled and a 1 mL aliquot of the acid solution was taken and transferred to a 25-mL round bottom flask. The solution was dried with a rotary-evaporator and reconstituted with 5 mL 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 (123 mg) was added to 7 mL clear glass reaction vials containing 5 mL of the solvent with varying water content (2%, 4%, 7%, 10% and 15% (v/v)) in ACN. 100 μL of DIPEA, 25 μL of mercaptoethanol and 30 mg of papain were added to the vials and these vials were then placed in a shaker at room temperature for 24 h. The reaction was stopped by heating the reaction mixture to 80°C 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 Electrospray Ionization Mass Spectrometry (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. 70 mg 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 50 µL injection loop. The analytes were separated with a reverse phase C-18 column (250 mm × 4.6 mm i.d.) and detected with a fixed wavelength UV detector set at 210 nm. 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% Trifluoroacetic acid (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+10 mM HSA+0.1% O-Phosphoric acid) initial to 75% B (50% acetonitrile+10 mM 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 1 mL min-1 and 10 µL of all solutions were filtered through a 0.22 µ 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 amu to 1600 amu. The electrospray capillary voltage was set at +3.5 KV. The assistant gas heater temperature was set to 200°C. The desolvator temperature and the aperture-1 temperature of the MS system were 200°C and 150°C, respectively. The 3D Q- Ion Trap mass analyzer was scanned from 50 amu to 1600 amu. For such characterization, the oligomers were dissolved in ethanol/water mixture (50:50) to form a nominal concentration of 0.5 mg/mL solution. The solution was introduced into the MS with a syringe pump (Harvard Apparatus) at a flow rate of 1 mL/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 0.2 mL/min.

Analysis of Met enantiomers

The separation of amino acid enantiomers was carried out with a CHIROBIOTIC-TAG column (250 mm × 4.6 mm). 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 210 nm.

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 2 h to 24 h. After each exposure period, enzyme was recovered from the solvent mix and introduced into an aqueous system optimized for synthesis of Met oligomers. Results obtained with virgin enzyme (not exposed to ACN) and enzyme exposed for 4 h and 24 h respectively in ACN/Water systems are shown in Figures 2A to 2C. 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,

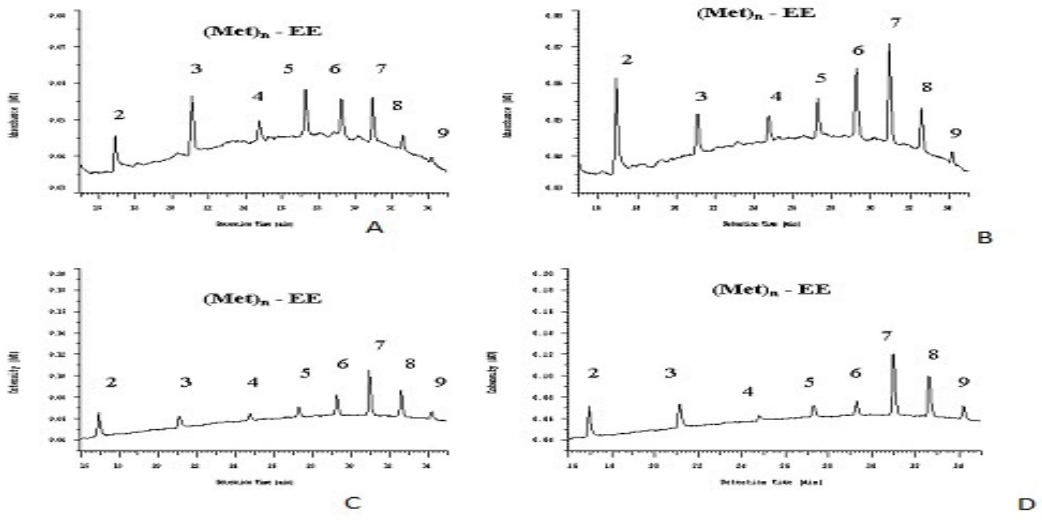

Figure 3: Chromatogram of methionine oligomers synthesized in acetonitrile/water system. A) Supernatant of 15(%v/v) water/acetonitrile B) Supernatant of 40(%v/v) water/acetonitrile C) Precipitate of 15(%v/v) water/acetonitrile D) Precipitate of 15(%v/v) water/acetonitrile incubated for 24 hours. Separation was achieved with a RPLC C-18 column using a mobile phase gradient mentioned in Figure 2.

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 synthesis described in this article were carried out at 24 hrs 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 24 h indicating the absence of oligomerization. Such systems have been shown to be suitable for oligomerization of amino acids with subtilisin [28,29]. 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 3 and 4. The chromatogram (Figure 3) 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 (Figures 3 and 4) 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 systems where these neutral oligomers completely precipitate out. This is very significant if these hydrophobic peptides are chosen as substrates for further polymerization. The precipitate obtained from the reaction mixture of both Met and Tyr (Figures 3 and 4) also showed the presence of oligomer peaks. A careful examination of (Figures 3 and 4) also shows that the relative distribution of oligomers in both the supernatant and precipitate was nearly the same for both Met and Tyr reaction systems respectively. Further optimization work on the reaction system needs to be undertaken to enhance the partitioning of the oligomer residues into the supernatant to reduce work-up requirements. Such a detailed optimization study was not the main objective of the study and was not pursued further.

The relative distribution of Tyr oligomers obtained in ACN/water mixtures for different water content is shown in Figure 5. 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 [5]; however, higher oligomers were obtained in monophasic systems for both Met and Tyr. This is most likely related to the higher solubility of oligomers in ACN/water systems. 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 [20,30]. 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 [31].

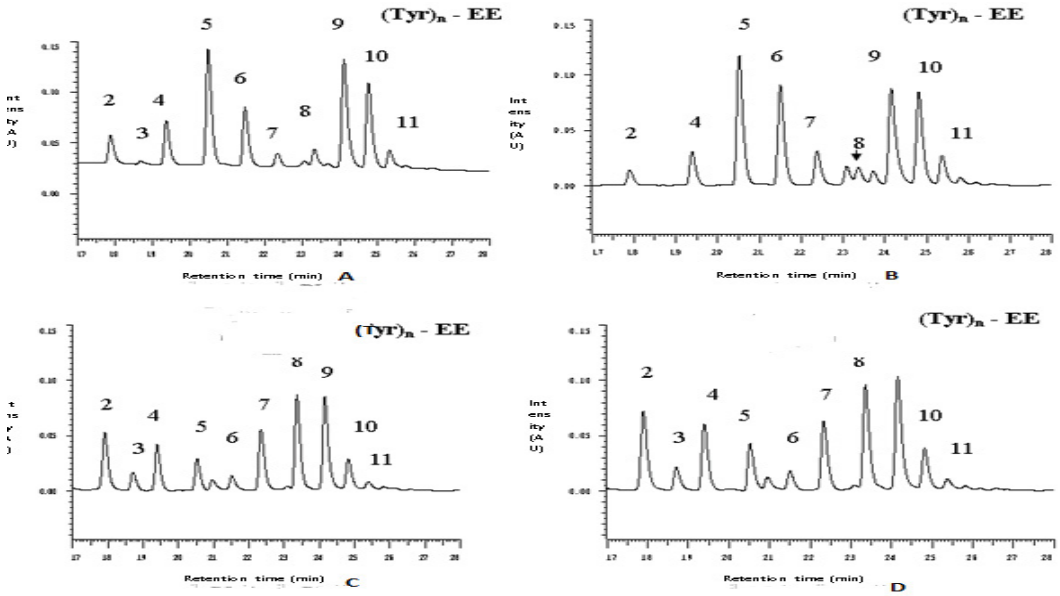

Figure 4: Chromatogram of tyrosine oligomers synthesized in acetonitrile/water system. A) Supernatant of 15 (%v/v) water/acetonitrile B) Supernatant of 40 (%v/v) water/acetonitrile C) Precipitate of 15 (%v/v) water/acetonitrile D) Precipitate of 40 (%v/v) water/acetonitrile incubated for 24hours. Separation 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.

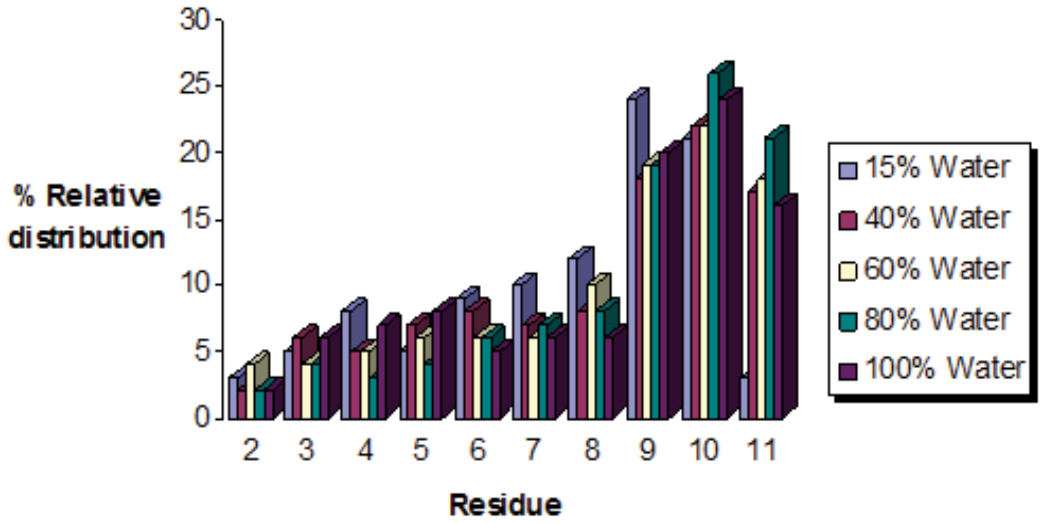

Figure 5: Relative distribution of tyrosine oligomer residues synthesized in acetonitrile/water solvent system as a function of varying water content.

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 6. 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 [16]. It has been speculated that the decrease in yield at high water contents when immobilized enzymes are used is due to the increase in thickness of a water layer around the enzyme that acts as a diffusional barrier [16,28]. 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 systems 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 [22]. Results show that such systems should be amenable for addition of amino acid esters or other hydroxy acid moieties in reasonable yields.

The amino acid substrate must have its amine group deprotonated to participate in papain-catalyzed oligomerization reactions [16]. 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 0.01 mmole did not show appreciable oligomerization. The relevant illustrations for this study have not been presented here since it is not within the critical scope of work alluded to in this article.

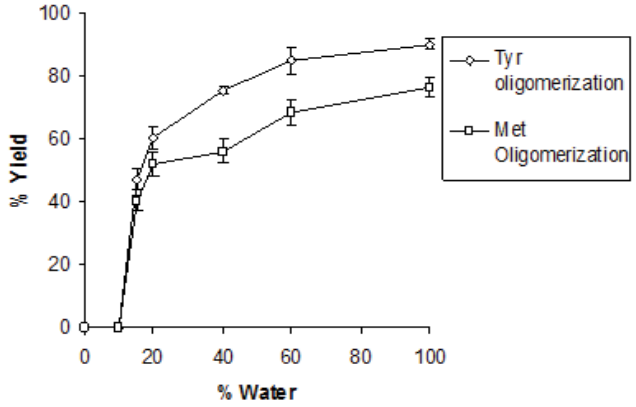

Figure 6: Percent yield of Met and Tyr oligomers synthesized in acetonitrile/water solvent system as a function of varying water content. The yield is calculated based on the measurement of the residual amount of the starting substrate left in the reaction mixture. The error bars represent the range obtained from three independent oligomerization experiments for each % water content mentioned in the X-axis.

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 7. Results showed a slight increase in solubility for when the water content was increased from 15% to 40% for the oligomers studied (2-8 mers). 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 and hence the graphical solubility profile for the same is not presented separately in this article.

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

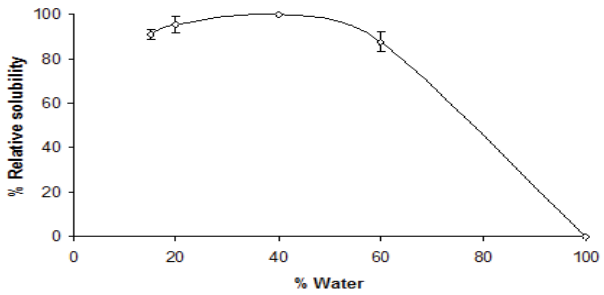

Figure 7: Relative solubility of Met oligomers in acetonitrile/water solvent system with varying water content. The error bars represent the range obtained from three independent oligomerization experiments for each % water content mentioned in the X-axis.

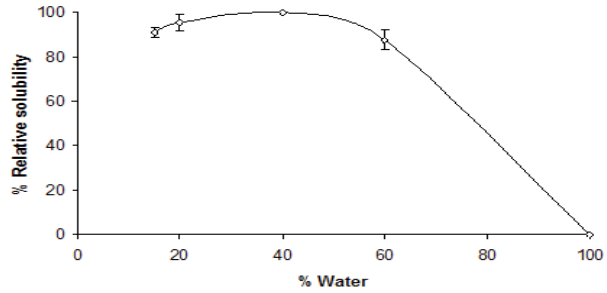

Figure 8: RPLC-18 separation of acid hydrolysate obtained from purified oligo-methionine precipitate synthesized in 40 (% v/v) water/acetonitrile system. The chromatogram shows a peak at a retention time of 6 minutes corresponding to Methionine indicating complete hydrolysis of the oligomers. Separation 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.

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 24 h 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 [25]. 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 8. 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 9. 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 10. 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 (P1' 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 11. 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 12. The spectrum contains series of ions that are 128 amu apart. This mass difference corresponds

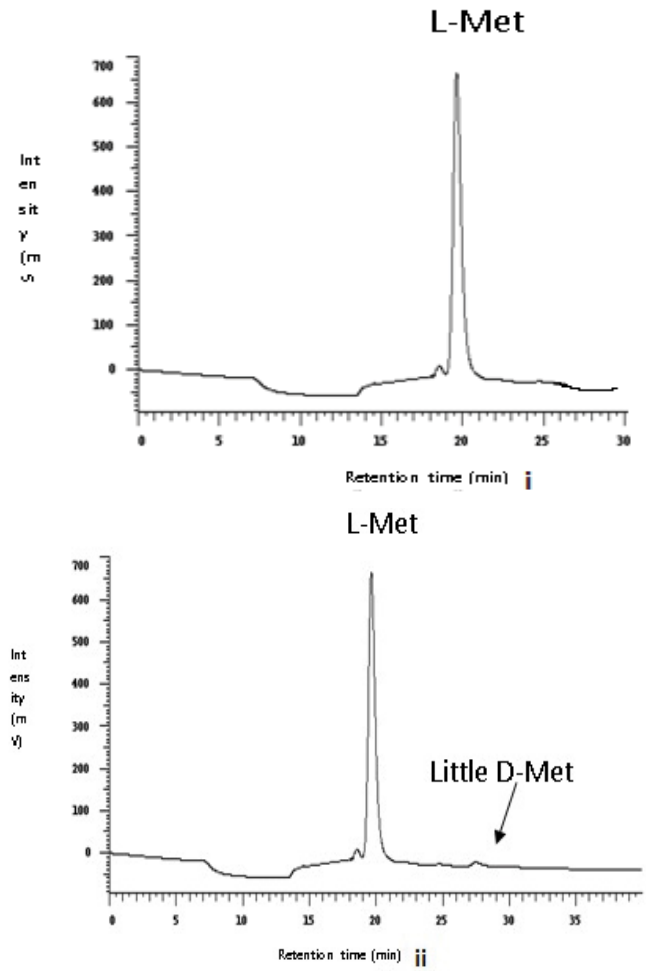

Figure 9: Chiral separation of acid hydrolysate obtained from purified oligo-methionine precipitate synthesized in 40 (% v/v) water/acetonitrile solvent system. (i) The chromatogram shows the elution of L-Methionine standard at ~ 20 min (ii) The chromatogram shows a peak at 20minutes retention time corresponding 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%.

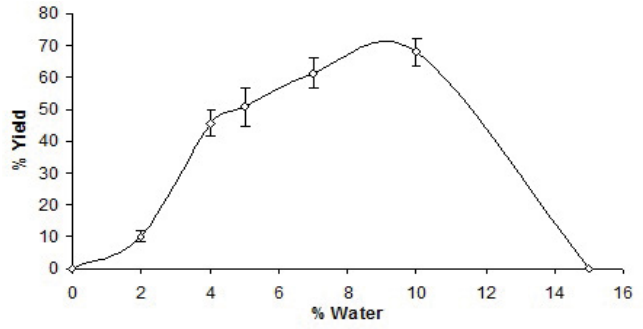

Figure 10: Percent yield plot for lysine oligomers synthesized in acetonitrile/water solvent system as a function of varying water content. The yield is calculated based on the measurement of the residual amount of the starting substrate left in the reaction mixture. The error bars represent the range obtained from three independent oligomerization experiments for each % water content mentioned in the X-axis.

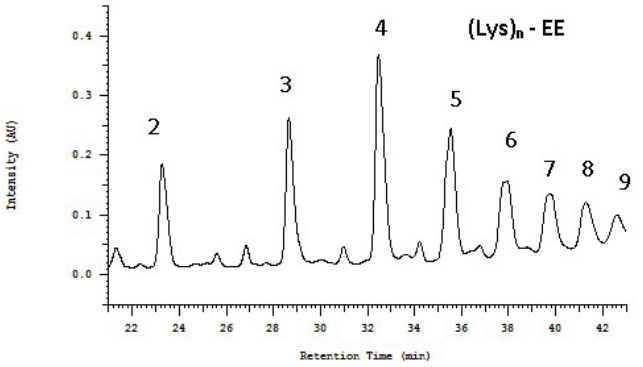

Figure 11: Chromatogram of precipitated lysine oligomers synthesized in 7 (%v/v) of water/acetonitrile system for 24 h incubation. Separation was achieved in a RPLC C-18 column with a mobile phase gradient comprising of 100% A (Water+10 mM HSA+0.1% O-Phosphoric acid) initial to 75% B (50% acetonitrile+10 mM HSA +0.1% O-Phosphoric acid) in 50 minutes.

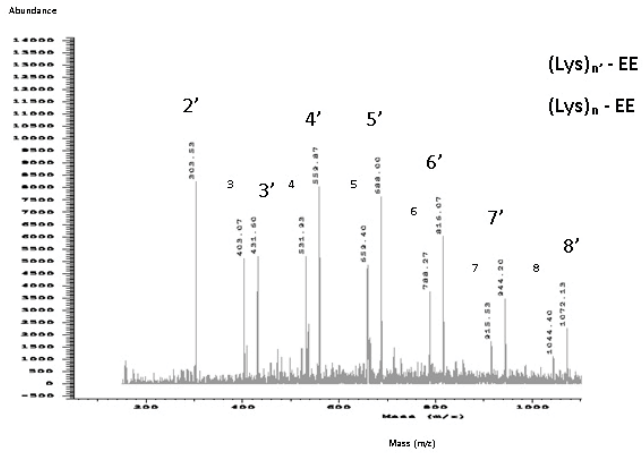

Figure 12: Mass spectra of precipitated lysine oligomers synthesized in 10 (%v/v) of water/acetonitrile system after 24 h incubation obtained through direct injection ESI (+) – MS. The spectrum shows the presence of series of peaks corresponding to oligo-Lysine.

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 (NLys – (Lys)n - LysCOOEt + H+). A tetramer of lysine, NLys – (Lys)2 - LysC + H+ should appear at a m/z 531 while a pentamer NLys – (Lys)3 - LysC + 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-phasic6 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 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 13, 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 14. 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 15. The spectrum shows the presence of ions at m/z 275, 297, 332, 389, 446, 503, 582, 582, 617, 674 and 731. 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 [6] 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

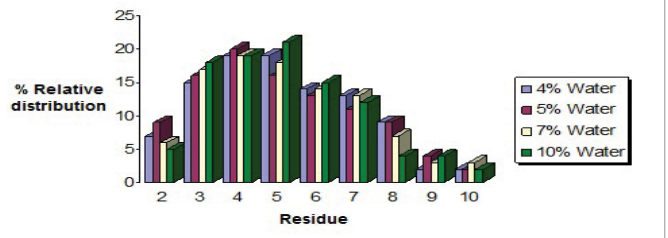

Figure 13: Relative distribution of lysine oligomer residues synthesized in acetonitrile/water solvent system as a function of varying water.

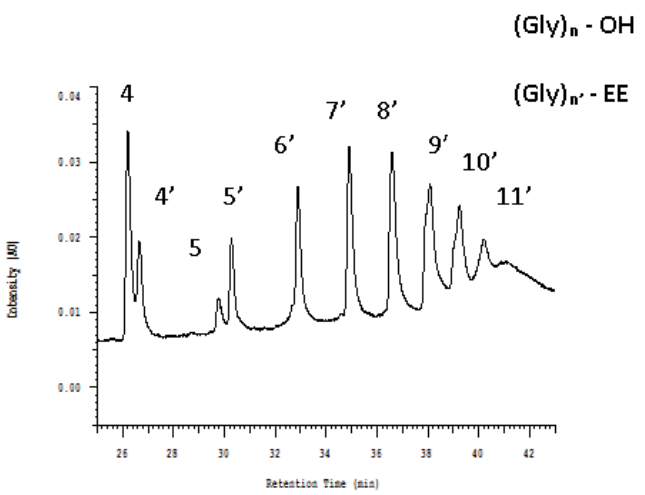

Figure 14: Chromatogram of glycine oligomers synthesized in 10 (%v/v) of water/acetonitrile system after 24 h incubation. Separation was achieved with a RPLC C-18 column using a mobile phase gradient mentioned in Figure 11.

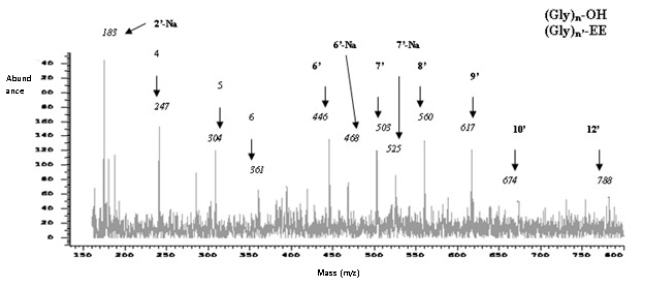

Figure 15: Mass spectra of precipitated glycine oligomers synthesized in 10 (%v/v) of water/acetonitrile system after 24h incubation obtained through direct injection ESI (+) – MS. The spectrum shows the presence of series of peaks corresponding to oligo-glycine.

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 [5]	100	80	6-9

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

and neutral amino acids. Another important aspect is the presence of ester intact oligomer residues in ACN/water systems as compared to biphasic systems, 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.

Conclusion

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 [17,18]. The studies on enantio-selectivity also show that papain maintains its stereo specificity in monophasic systems. 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 systems 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.

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