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SYNTHESIS AND EVALUATION OF VEGETABLE (SOYBEAN) OIL BASED EPOXY RESIN FOR BEVERAGE CAN COATING

AND

ENZYMATIC SYNTHESIS AND CHARACTERIZATION OF TAILORED N&C END PROTECTED OLIGOPEPTIDES

by

YING GAO

A DISSERTATION

Presented to the Faculty of the Graduate School of the

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

2008

Approved by

Shubhender Kapila, Advisor
Paul Nam, Co-Advisor
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David Westenberg
Monty Kerley

ABSTRACT

Research described in this dissertation covers two topics. Part-I is related to the development of a soybean oil based epoxy resin and its application in beverage can coatings. Interior surfaces of beverage can are invariably coated with polymer coating. The most commonly used coatings at present are derived from bisphenol A (BPA) based epoxy reins. However, due to perceived endocrine disrupting nature of BPA and consequential adverse effects on humans and wildlife concerns for the use of BPA based coatings and its leaching into beverages have been expressed. The research in the first part describes the synthesis and evaluation of an epoxidized ally soyate (EAS) based epoxy resin for can coating. The coating obtained with the resin was subjected to rigorous mechanical tests specific for beverage can coatings. In addition the beverage exposed to the coating was evaluated by an expert panel through blind organoleptic tests to evaluate its acceptance. Results of tests showed that EAS coatings possess mechanical properties that compare favorably to the beverages. Thus such coatings hold good potential for application in the beverage industry.

Part II of the dissertation deals with synthesis and characterization of tailored N & C end protected oligopeptides. Supplementation of limiting amino acids plays an indispensable role in improving the feed efficiency in animal production. However, direct supplementation with crystalline amino acids has been found to be inefficient due to extensive microbial degradation in the rumen, which converts the amino acids to short chain fatty acids and ammonia. To improve rumen bypass and increase nutritional efficiency research directed at synthesis of protected oligopeptides was undertaken. Oligopeptides comprised of methionine, lysine and arginine were synthesized through papain catalyzed reaction in an acetonitrile-water monophasic reaction medium. The oligopeptides with an ester moiety at the C-terminal were capped with a hydroxyl acid at the N-terminal end through chymotrypsin catalyzed reaction. The protected oligopeptides were characterized with RPLC and mass spectrometry. Results showed that protected oligopeptides with 3-6 amino acid residues can be synthesized with high yields. The oligopeptides exhibited low water solubility and low degradation in rumen fluid.

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Last but not least, I would further like to thank my parents and my husband along with other family members for their understanding, confidence, love and encouragement while I concentrated on my research work.

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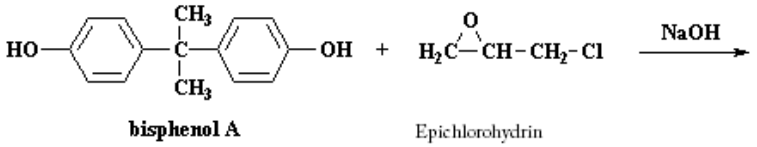
1. SYNTHESIS AND EVALUATION OF VEGETABLE (SOYBEAN) OIL BASED EPOXY RESIN FOR BEVERAGE CAN COATING

1.1. INTRODUCTION

1.1.1. Bisphenol A Based Epoxy Resin Coating. Interior surfaces of metallic food and beverage containers are invariably coated with polymeric films. Coatings prevent foods and beverages from becoming tainted with rusting metals. Additionally, the coatings enable better sealing and help preserving the taste and favor of foods and beverages (UK FSA, 2002). Of all the polymer films, epoxy resins are widely used in beverage or food industry because of their exceptional toughness, adhesion, formability, and chemical resistance (Lee and Neville, 1957). At present, nearly all epoxy resins (>99%) are obtained from bisphenol A (BPA) and epichlorohydrin. which cures (polymerizes and crosslinks) when mixed with a catalyzing agent or "hardener", as shown in Figure 1.1.



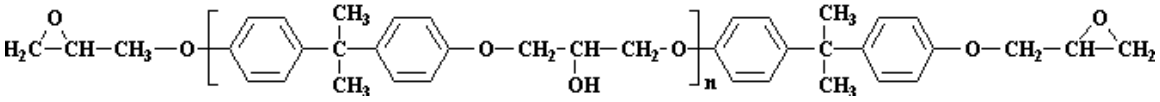


Figure 1.1: The formation of BPA based epoxy resin from BPA and epichlorohydrin

The epoxide group, which is also called an oxirane or ethoxyline group, consists of an oxygen atom joined by single bonds to two adjacent carbon atoms. Epoxy group is the functional group forming the three-membered epoxide ring, shown in Figure 1.2

(Skeist and Somerville, 1958). Epichlorohydrin is an epoxide and an organochloride. It is extremely reactive with substances containing active hydrogen atoms. The chemistry of epoxy resin and the range of commercially available variations allow cured polymers to be produced with a very broad range of properties. In general, epoxy resins are known for their excellent adhesion, chemical and heat resistance, good to excellent mechanical properties and very good electrical insulating properties. Coating is the one of the important applications for epoxy resin (Lee and Neville, 1957).

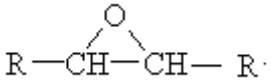


Figure 1.2: General structure of epoxide functionality

1.1.2. Health Concern of Bisphenol A Based Epoxy Resin. BPA is a commonly used name for 2, 2-(4, 4-dihydroxydiphenyl) propane. BPA is prepared by the reaction of two equivalents of phenol with one equivalent of acetone. Hydrochloric acid (HCl) or sulfonated polystyrene is used as a catalyst. It has two phenolic hydroxyl groups which are the difunctional building block to form several important polymers and polymer additives. More than 1 million metric tons of BPA have been produced annually throughout the world (NTP, 2007). Most of the BPA produced in United States is used in the production of polycarbonate plastic and epoxy resins (Fiege, 2002). It is also used as an antioxidant in plasticizers and as a polymerization inhibitor in PVC (Staples et al., 1998). Polycarbonates are widely used in many consumer products, including sunglasses, CDs, water and food containers, and shatter-resistant baby bottles (Lopez-Cervantes et al., 2003).

In recent years public concern about BPA migration into canned food has increased. The presence of BPA in foods or drinks from epoxy resins, phenol resins, polycarbonates, polyacrylates, polyesters, and lacquer coatings on food cans can adversely affect on human health and environment (Cooper and Bristow, 1996; Staples et al., 1998). Because canned foods are sterilized during production, the possibility of BPA migration from the can coating during the high temperature process is high. The European Commission's Scientific Committee on Food issued the opinion that the tolerable daily intake specification for BPA should be lowered to 0.01mg kg-1 (ECSC, 2002).

Many studies have been carried out to ascertain BPA's endocrine disrupting properties that can lead to activation of estrogen receptors resulting in physiological effects similar to those of naturally occurring estrogens (O'Connor et al., 2003). The first evidence of BPA's estrogenicity was shown in experiments carried out in 1930s in which it was fed to ovariectomized rats (Dodds and Lawson, 1936; 1938 Through in vitro assays it has been shown that 10-25nM BPA exhibited estrogenic activity and induced progesterone receptors in cultured human mammary cancer cells (MCF-7) and increased the rate of proliferation of MCF-7, assessed by [3H]-estradiol for binding to estrogen receptors from rat uterus (Krishnan and Stathis, 1993). In an oral administration test for pregnant mice, a 20ng/g dose of BPA decreased sperm production efficiency by 20% relative to control males (Vom et.al, 1998).). BPA exhibits acute toxicity in aquatic organisms including microorganisms, invertebrates, and fish at concentrations 1-10ug mL-1 (Alexander and Dill, 1988). It has been reported that the endocrine disrupting effects of BPA lead to health problems such as lowered sperm count and infertile sperm

in men (Staples et al, 1998). Myrray et al. reported that exposure to 2.5, 25, 250 and 1000μg bisphenol A/kg body weight/day induces the development of ductal hyperplasias and carcinoma in situ at postnatal day 50 and 95 in rats. These highly proliferative lesions have an increased number of estrogen receptor-α positive cell. Thus, bisphenol A exposure has carcinogenic effects and is sufficient to induce the development of preneoplastic and neoplastic lesions in the mammary gland in the absence of any additional treatment aimed at increasing tumor development (Murray, 2007). It has been reported that exposure to BPA leads to developmental toxicity, carcinogenic effects, and possibly neurotoxicity (Lee, et al., 2007; Zsarnovszky et al., 2005). It has been suggested that exposure to BPA may also cause obesity by triggering fat-cell activity (Grossman, 2007).

In light of the potential adverse health related to BPA and the fact that the most exposure to BPA comes from food or beverage containers, development of a new coating materials for beverage container is required. It would be prudent to develop such coatings from naturally occurring renewable materials. .

1.1.3. Vegetable Oil Based Epoxy Resin. Vegetable oils are good potential sources for substituting BPA based epoxy resin. Commercially available vegetable oils are primarily obtained from oil seed plants through extraction of flaked oil seeds with non-polar solvents such as hexanes (Salunkhe et al., 1983). Triglycerides form the largest component of the extracted vegetable oils. Triglycerides are comprised of three fatty acid molecules attached to one molecule of glycerol through ester bonds. The structure of triglycerides is shown in Figure 1.3. . The groups R′, R″, and R‴ represent fatty acids, R’,

R'' and R''' can be the same or different. The functional properties, oxidative stability, and the nutritional value of oils are determined by fatty acid composition, geometric configuration, and positional distribution

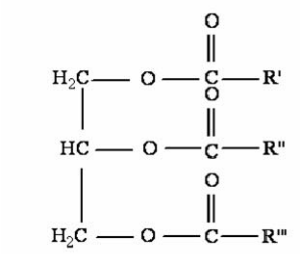


Figure 1.3: General structure of triglyceride

1.1.3.1. Soybean Oil. Most common vegetable oil available in United States is the soybean oil. This oil has attracted the greatest interest for industrial use due to their plentiful supply and relative lower cost. In the United States soybeans are one of the leading crops, grown in some 100 varieties. The reason soybeans has been one of the leading crops is chemical composition, it has the high protein content (around 40%) and good oil content (20%) (Salunkhe et al., 1983).

Minor components present in soybean include phospholipids, un-saponifiable material, free fatty acids, and trace metals. Refined soybean oil is more than 99% triglycerides. Fatty acid composition of soybean oil is given in Table 1.1. Soybean oil consists of approximately 15% saturated fatty acids that have no carbon-carbon double bond. Most fatty acids (85%) in soybean oil are unsaturated. The highest percentage of fatty acid in soybean oil is linoleic acid, followed by oleic, palmitic, linolenic, and stearic acids (Salunkhe et al., 1983). Linolenic acid contains three double bonds, linoleic acid contains two double bonds and oleic acid contains one double bond. The double bonds in

unsaturated fatty acid make these fatty acids susceptible to oxidation, which leads to the development of an off flavor. Thus, they are also the functional group for the following reaction.

Table 1.1: Fatty acid composition of soybean oil

Soy Fatty Acid	Structure			%
Unsaturated	Palmitic	$CH_3(CH_2)_{14}COOH$	C 16: 0	11
	Stearic	$CH_3(CH_2)_{16}COOCH_3$	C 18: 0	4
Saturated	Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$	C 18: 1	26
	Linoleic	$CH_3(CH_2CH=CH)_2(CH_2)_7COOCH_3$	C 18: 2	52
	Linolenic	$CH_3(CH_2CH=CH)_3(CH_2)_7COOCH_3$	C 18: 3	7

Soya proteins and soya lipids have been successfully in the development of biodegradable plastics and fuels. Transesterification of soybean and other vegetable/animal oils with methanol has been used for biodiesel production, a well accepted fuel that has been used by itself or in blended forms with the diesel fuel. Soyprotiens has been blended with glycerol to obtain low strength biodegradable plastics (Knothe, 2001). Epoxidized soybean oil has also been mixed with corn starch to produce biodegradable plastics (Jane et al., 1993). A low-cost, water-based paint using soy oil as the primary binder component based on more traditional latex technologies was reported by ICI, Strongsville, Ohio. A cost analysis between the common latex formulations and soyoil based latex showed that the cost of soyoil based latex can be 20% lower than the common latex formulations (Bernard, 1994).

1.1.3.2. Epoxidized Soybean Oil. In epoxidized soybean oil, the carbon-carbon double bonds of the unsaturated fatty acid esters are replaced with the oxiran/epoxide functionalities. These functionalities can react with moieties that contain active hydrogen atoms to produce a crosslinked polymer structure. This process of converting a liquid resin into a tough cross-linked polymer is called curing or hardening. Structures of epoxidized oleic, linoleic and linolenic acid methyl esters are shown in Figure1.4. These epoxidized esters can interact with appropriate hardeners to form rigid three dimentional structures. However, saturated fatty acid ester such as the palmitic acid and stearic acid methyl esters do not have any epoxy functionalities and therefore can not in polymer network formation.

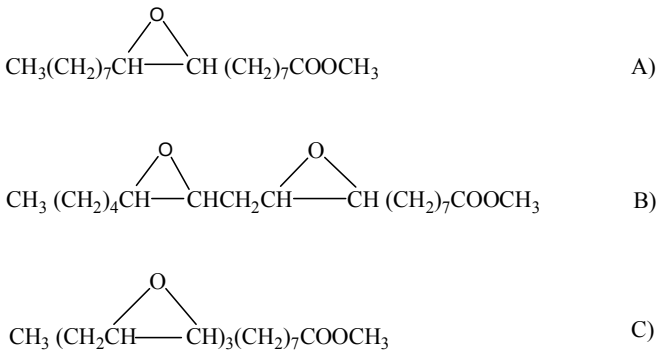


Figure 1.4: Structures of epoxidized fatty acid methyl esters; A) Epoxidized oleic methyl ester, B) Epoxidized linoleic methyl esters, C) Epoxidized linolenic methyl esters.

1.1.3.3. Epoxidized Allyl Soyate Resin. Since approximately 15% of the fatty acids present in soybean oil are saturated and do not yield epoxide functionalities and do

not participate in polymer formation. These fatty acids can be made to participate in the cross-linking process through transesterification with an appropriate alcohol. Transesterification of triglycerides is carried out with an alcohol in the presence of a strong acid or base, producing a mixture of fatty acids alkyl esters, and glycerol, as shown in Figure 1.5 (Wright et al., 1944; Freedman et al., 1986).

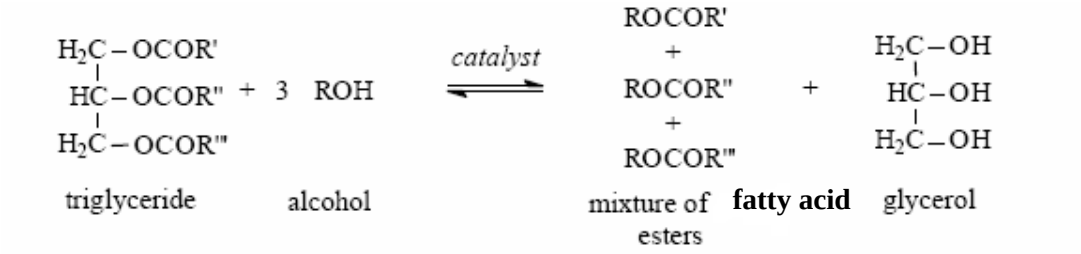


Figure 1.5: Transesterification of triglycerides with an alcohol

A monofunctional alcohol with one carbon – carbon double bond (Allyl alcohol, Figure 1.6) was selected for transesterification of epoxidized soybean oil to yield epoxidized fatty acid allyl esters termed epoxidized allyl soyate (EAS, Figure1.7). Epoxidation of the terminal carbon-carbon double bond is difficult and often yields are poor (≤10%). However, the terminal double bonds can interact in polymerization through free radical reactions with a free radical initiator, thus, increasing the feasibility of incorporating palmitic and stearic acids in the polymer matrix.

$$CH_2 = CH - CH_2 - OH$$

Figure 1.6: Structure of allyl alcohol

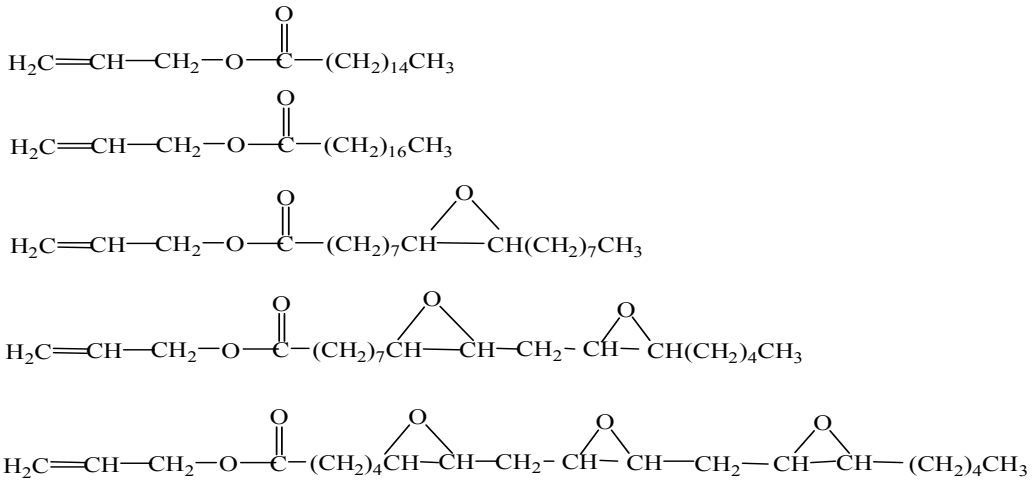


Figure1.7: Structures of epoxidized fatty acid allyl esters

Benzoyl peroxide (BPO) is a commonly used as free radical initiator and often added to promote free radical polymerization. Saturated fatty acid esters not incorporated in the polymer matrix increase tackiness of polymer (Rupali, 2005). Benzoyl peroxide is thermally unstable and decomposes into free radicals when heat is applied. Free radicals resulting from BPO are shown in Figure 1.8. The reaction mechanism for the free radical polymerization is depicted in Figure 1.9. Free radical species with unpaired electrons are very reactive and rapidly interact with any molecule that has either a radical site with a lone electron or a site that can readily donate one of its electrons. The instability of allyl group's carbon-carbon double bonds in the EAS polymer makes them susceptible to reaction with the unpaired electrons in the radical. The active center of the radical pairs with one of the electrons from the double bond of the monomer leaves an unpaired electron to appear as a new active center at the end of the chain. This type of polymerization is initiated by adding a small amount ~1% of benzoyl peroxide to the resin. The resin is then heated for 48 hours at 120 ºC.

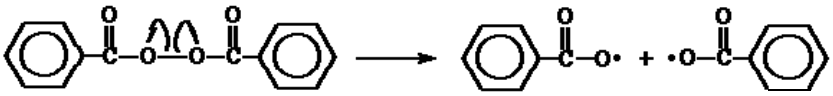


Figure 1.8: Free radicals formed through the decomposition of benzoyl peroxide.

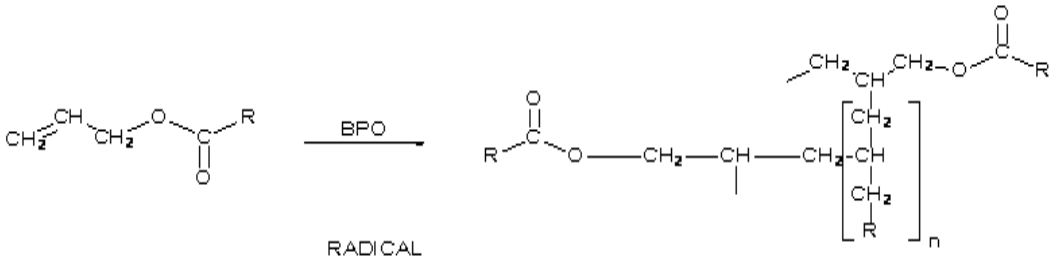


Figure 1.9: BPO initiated free radical interaction of EAS

1.1.4. Hardeners for Curing Epoxy Resins. "Epoxy" functionalities can react with other agents to produce a crosslinked polymer structure by opening the ring structure of the epoxy with aromatic or aliphatic anhydrides, carboxylic acids or amines. The chemical species that interact with epoxy resins to form rigid polymer matrices are known as the curing agents or hardeners. The rigidity of the material depends on the degree of cross linking. Most amine hardeners are liquids and cure at room temperature through exothermic reactions. However, most acid anhydrides are solids and need heat for curing, which allows for better reaction control, thus improving the mechanical and electrical properties of the cured resin (Venables, 1989). These systems are generally thermally and chemically stable, but are more prone to attack with a strong base than the amine-cured systems. A common anhydride, the phthalic anhydride is a solid at room temperature and requires heat to mix with the resin. Its melting point is 130.8ºC (267.4ºF) and sublimation temperature is 135 ºC (275 ºF). Hence, a lot of curing agent can be lost

while mixing and curing. Anhydride hardeners are generally hygroscopic and are less irritating to skin. When used as curing agents for epoxy resins, organic acids have been found to be useful primarily in surface coatings. These properties are preferred for curing epoxy resins in casting and laminating formulations. Anhydride-cured epoxy resins have low peak exothermic values and can be used in combination with amine accelerators to reduce curing time (Lee and Neville, 1957). With suitable curing agents epoxy resins including those derived from epoxidized soybean oil can form a variety of products suitable for different applications. A curing mechanism using tertiary amine and anhydrides are shown in Figure 1.10.

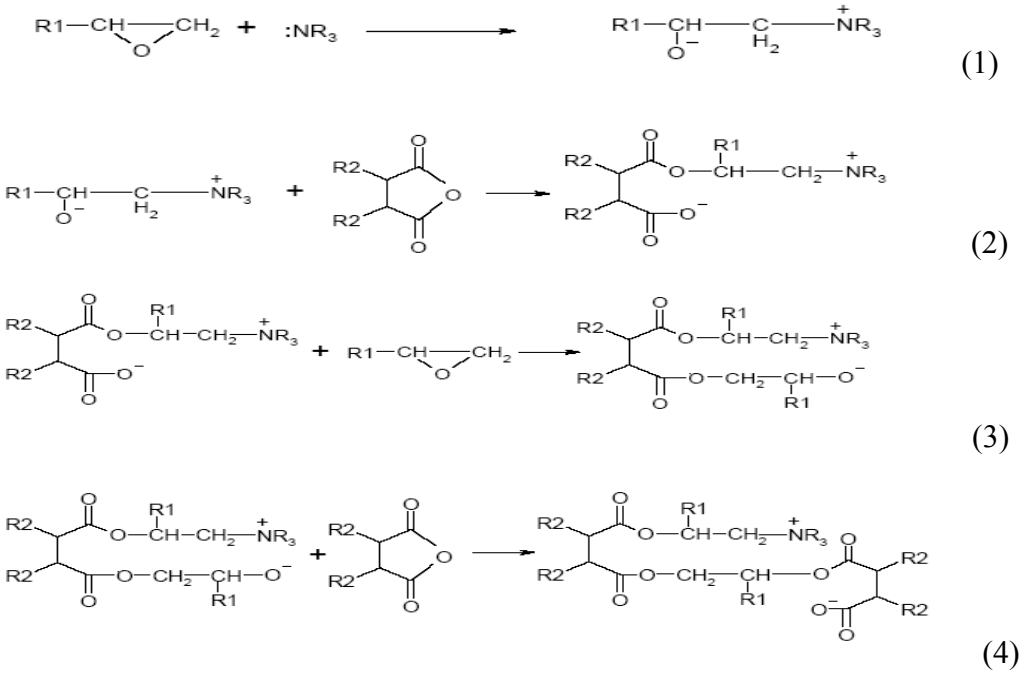
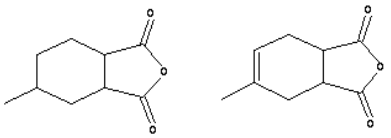


Figure 1.10: Curing mechanism of epoxy resins with t-amines and anhydrides (Rock et.al.)

Rock and co-workers has proposed that the tertiary amine reacts with the epoxy monomer and forms a zwitterion that contains a quaternary nitrogen atom and an alkoxide, reaction (1), Figure10. The alkoxide rapidly reacts further with an anhydride group leading to a carboxylate anion, reaction (2). This carboxylate anion can act as an active center for co-polymerization. Propagation occurs through the reaction of the carboxylate with an epoxy group and the accompanied formation of new alkoxide anion, reaction (3), Which in turn reacts at a very fast rate with an anhydride group, regenerating the carboxylate anion, reaction (4) (Rock, et. al., 2004).

1.1.4.1. Organic Acid Based Curing Hardener. Methylated alicyclic anhydrides are increasingly being used as curing agents because they are liquids and are, therefore, easy to work with especially in continuous automated production (Jonsson, et al., 1996). A commercially available mixture (LS-682K) of methylhexahydrophthalic anhydride (MHHPA) and methyltetrahydrophthalic anhydride (MTHPA) is often employed in epoxy resins. The structure of MHHPA and MTHPA are shown in Figure 1.11.



A) B)

Figure 1.11: The structures of two methyl phthalic anhydrides;
MHHPA (A) and MTHPA (B)

MHHPA and MTHPA are skin sensitizing agents and can induce Immunoglobulin E mediated respiratory allergies, including asthma, allergic rhinitis, and hypersensitivity pneumonitis (Nielsen et al., 1992; Venables, 1989; Keskinen et al., 1988). Anhydrides also affect the mucous membranes and induce irritation and conjunctivitis in the eyes (Yokota et al., 1999). Additionally, contact urticaria can be caused by exposure to these two anhydrides (Tarvainen et al., 1995).

1.1.4.2. Analytical Methods for Determination of Phthalic Anhydrides and Acids. Anhydrites can be determined with different methods. Phthalic anhydride particulates in air can be determined with a U.S. National Institute for Occupational Safety and Health (NIOSH, 1977) recommended method that involves collection of particulates from air on a filter, followed by extraction, hydrolysis by aqueous ammonia, and acidification, the phthalic acids in the solution are analyzed with normal phase high-pressure liquid chromatography (HPLC) interfaced to a UV absorption detector. RPLC with a cationic ion-paring reagent (tetrabutylammonium hydrogen sulphate) has been used for the determination of phthalic acids (Geyer and Saunders, 1986; Nielsen et al., 1988).

Organic (or Phthalic) anhydrides in air have been determined with a gas chromatograph interfaced with an electron-capture detection (GC-ECD), the method involved collection of anhydrides from air with sampling tubes filled with amberlite resin, silica-gel, TenaxTA or Teflon filter. Anhydrides are desorbed with (methanol) analyzed with GC interfaced to a flame ionization detector (FID) or GC interfaced to mass spectrometric detection (MS) (Welinder et al., 1992; Jonsson et al., 1996; Yokota et

al., 2002; Pfaffli et al., 2004). GC-FID, GC-ECD and GC-MS based methods for the determination of MHHPA and MTHPA in urine and plasma have been reported (Lindh et al., 1997; Yokota et al., 2005; Rosqvist et al., 2000). However, experiments to examine the extent of phthalic acids (MHHPA and MTHPA) leaching from the Epoxy can coatings into food or beverages have been reported in the open literature.

1.1.5. Analytical Methods for Determination of Bisphenol A. Various methods for the determination of BPA in foods, beverages or simulated foods and beverages have reported. A HPLC-UV detection method for determination of BPA in canned fruit and vegetables was reported by Yoshida et al., the quantification limits were 10ng/g for the solid portion and 5ng/ml for the aqueous portion, respectively(Yoshida et al., 2001). HPLC with fluorescence detention was also used to determine low level of BPA in real or simulated food or beverages (Biles et al., 1997; Nerin et al., 2002; Kang 2002; Inoue et al., 2003; Kang and Kondo, 2003). Sun and his colleague in 2004 determined BPA in human breast milk with HPLC and Fluorescence intensity was monitored at 475 nm with the excitation of 350 nm. A good linearity ($r = 0.994$) was observed of BPA in the concentration range of 0.2-5.0 ng mL^{-1} in breast milk, and the detection limit was 0.11 ng mL^{-1} at a signal-to-noise ratio of 3. BPA was derivatized with fluorescent labeling reagent, 4-(4, 5-diphenyl-1H-imidazol-2-yl) benzoyl chloride (DIB-Cl) before separation of DIB-BPA from endogenous materials in milk by HPLC system (Sun et al., 2004). UV absorbance and fluorescence detection usually demand a tedious sample preparation due to their limited selectivity and sensitivity to complex matrices. Moreover, derivatization of BPA has been proposed to increase sensitivity with fluorescence detection, adding further extraction and reaction steps. Furthermore, in these techniques the identification

of the target compound has a high degree of uncertainty, because it is based only on the comparison of the retention time of the unknown to that of a standard solution, except for the case that a diode array detector is used.

GC–MS allows identification with a high degree of confidence and it has been a alternative method to determine the low trace amount of BPA. (Wingender et al., 1998; Goodson et al., 2002; Kang et al., 2002; Kuo et al., 2004; Otaka et al., 2003). Goodson and his colleague determined BPA in sixty two different canned foods by GC-MS. The detection limit of was 2 μg Kg-1. But the complex and time-consuming sample treatments are required for GC-MS methods, which comprise at least three steps: extraction of the target analytes, evaporation of the extract to dryness and derivatization.

Linking HPLC with mass spectrometry can overcome these limitations. LC–MS does not require derivatization of the compounds of interest and offers more definitive confirmation of the presence of the analyte in an unknown sample, comparing to UV absorbance and fluorescence detection. Additionally, it allows quantitative determination of compounds that are not fully resolved chromatographically using single ion monitoring and it offers higher specificity and sensitivity. LC-MS with electrospray ionization (ESI) has been used to determine BPA in environmental samples and biological fluids (Rodriguez-Mozaz et al., 2004; Motoyama et al., 1999; Inoue et al., 2003). LC-MS with atmospheric pressure chemical ionization (APCI) has been used to determine BPA in fish tissue (Pedersen et al., 1999).

Due to trace amounts of chemicals in the food or drink, sample extraction methods have been reported for extracting of BPA from those complicated matrices. Liquid-Liquid extraction is applied to extract BPA from aquatic samples such as river

water (Jeannot et al., 2002). Off-line or on-line Solid-phase extraction (SPE) is the technique commonly used for bisphenols extraction from environmental aqueous samples prior to LC analysis because of its advantages over liquid–liquid extraction: high pre-concentration factors, low consumption of organic solvents, elimination of emulsions and easy automatization. SPE has been widely used as a method for extracting of BPA from food (Inoue et al., 2003; Chang et al., 2005; Goodson et al., 2002; Kang et al., 2003).

1.1.6. Leaching Study using Food and Drink Simulants. Epoxy can coating may come into contact with all kinds of foods and drink. Water, 3% aqueous acetic acid, 15% ethanol, 50% ethanol, vodka and olive oil were used as food simulants for migration from polyethylene tetraphthalate (Ashby, 1988). Vegetable oils are not widely used as simulants because they cause a number of difficulties and are reported to require time consuming procedure for extraction of the analyte (Van Battum et al., 1982; Figge 1996). Isooctane or heptane and ethanol-water were used as alternative fatty food simulants to overcome the laborious problem (Kontominaa et al, 2006; Baner et al., 1992; De Kruijf and Rijk, 1996; Hamdani and Feigenbaum, 1996). The Council of the European communities laid down the list of simulants to be used for testing migration of constituents of plastic materials and articles intended to come into contact with foodstuffs in 1985. In the list, Distilled water, 3% acetic acid (w/v) in aqueous solution, 15% ethanol (v/v) in aqueous solution, and rectified olive oil were brought out for the simulants (EEC 1985). 3% acetic acid was used as a stimulant for aqueous and acidic drinks such as soft drinks. 95% Ethanol was a stimulant for fatty foods (Widen et al., 2004; Begley et al., 2004).

The potentially adverse health concerns about the BPA and the fact that the most exposure to BPA comes from coatings in food or beverage cans. In sight of trace amount leaching concentration from the can coating, it is necessary to develop a sensitive robust and less laborious method for determination of BPA and phthalic acids in simulated foods and beverages.

1.2. OBJECTIVES.

The overall objective of this research was to develop a soybean oil based epoxy resin system. The overall objective was met through a series of experiments designed to meet following specific sub objectives.

1. Synthesis of allyl esters of epoxidized soybean oil (EAS).
2. Evaluation of hydrophthalic anhydride hardeners with the EAS.
3. Determination of curing characteristics of selected hardener – EAS systems.
4. Evaluation of mechanical properties of EAS – hydrophthalic anhydride coatings and comparison with bisphenol A based epoxy coatings.
5. Evaluation of EAS and bisphenol A based coatings through blind organolaptic tests
6. Determination of the extent of bisphenol A leaching in aqueous and alcohol – aqueous mixtures.

1.3. EXPERIMENTAL

1.3.1. Materials. Bisphenol A (BPA), Bisphenol F (BPF), and Dimethyl Phthalate (DIMP) were obtained from Aldrich Chemical Co. (Milwaukee, WI). The stock solution

(1mg/mL) of these two compounds were prepared in methanol and used as calibration standard after further dilutions. The solutions were stored in a refrigerator at 4 °C. HPLC grade acetonitrile, methanol, ethanol, hexane, and isooctane were purchased from Fisher Scientific (St. Louis, MO). The purified water "nanopure water" used in the experiments was obtained from a Synergy 185 filtration unit manufactured by Millipore Corporation (Billerica, MA). The test aluminum panels were prepared from aluminum sheets provided by Anheuser Busch (St. Louis, MO). EPON 9500, a bisphenol-A based epoxy, was purchased from Shell Chemicals (Deer Park, TX). Another bisphenol a based coating resin was obtained from Metal Container Corporation (MCC), Anheuser Busch (St. Louis, MO). Epoxidized allyl soyate (EAS) was synthesized from epoxidized soybean oils. Liquid hardeners LS-682K, a mixture of methylhexahydrophthalic anhydride (MHHPA), and methyltetrahydrophthalic anhydride (MTHPA) were obtained from the Lindau Chemicals (Columbia, SC).

1.3.2. Synthesis of EAS Resin. 1000g of epoxidized soybean oil (ESO) were added into a 2L three neck round bottomed flask. The flask with content was heated to 110 °C at the heating mandrel with the magnetic stirrer. Preheating ESO before adding allyl alcohol is to make the reaction faster. 350g of allyl alcohol was weighed out and added to another conical flask with an aluminum foil cover on it. To the same conical flask, 3.5g of sodium (potassium hydroxide) were added slowly. The conical flask were allowed to cool down to room temperature, then the mixture of allyl alcohol and sodium were added into the round bottom flask which already contains heated ESO. A reflux condenser was connected to the middle opening of the round bottom flask. The reaction mixture was

stirred by a magnetic bar and refluxed for 6 hours at 110 °C. After the flask was allowed to cool down, the mixture was separated into 2 1L flasks. Each flask containing the reaction mixture was heated at 120 °C with stirring to remove the excess allyl alcohol (The boiling point of allyl alcohol is 97 °C). After heated for 72 hours, the flasks were allowed to cool down to the room temperature. The glycerol were removed through centrifuged the mixture at a speed of 4000rpm for 45 minutes.

After centrifuging, the residues were collected together into conical flask. For every 1 ml of resin in the flask, 1% of benzoyl peroxide was added to increase the polymerization of EAS. Then, the flask with mixture was heated at 120 °C with stirring. After heating for 48 hours, the flask was allowed to cool down and stored at room temperature.

1.3.3. Epoxy Resin System. Three resins were prepared:

i. EPON - Epon 9500, a commercial BPA based general purpose epoxy resin with LS682K anhydride hardeners consisting of a mixture of MHHPA and MTHPA.

ii. AB - A proprietary BPA based epoxy resin formulation used by Metal Container Corporation, St. Louis [subsidiary of Anheuser Busch for beverage can coating].

iii. EAS - epoxidized allyl soyate resin with LS682K anhydride hardeners. Three different formulation of EAS resin and hardener were evaluated (1 part resin to 0.70, 0.68, and 0.65 parts LS682).

1.3.3.1. Coating of Panels with EAS. The aluminum sheets obtained from Anheuser Busch were cut into 5x5 cm square panels. A 1/16" whole was drilled at the top of each panel. The panels were rinsed with water and acetone and allowed to dry in a fume hood. The dried panels were weighed with semimicro balance. The resin hardener mixtures were mixed with a high shear mixer. The mixed resins were placed in 100mL borosilicate glass beakers. The dried aluminum panels were strung on thin stainless steel wires and dipped in the resin hardener mixture for 30 seconds. The panels were pulled out of the beaker and the excess resin mixture was allowed to drip from the panels for 5 minutes. The panels were then placed in oven for curing at selected temperatures for specific time periods (Table 1.2). After curing, the wires were removed from the panels and each panel was reweighed and the weight of the coating on each panel was determined.

Table 1.2: EAS curing time and temperature with LS-682K

Temp. ^{o}F	Time (mins)
410	8
400	5
405	3
410	3

1.3.2.2. Coating of Panels with BPA Based Epoxy Resins. One part Epon 9500(EPON) was added to 0.7 parts of LS-682K hardener and was mixed for 5 minutes with a high shear mixer at 200 rpm. The epoxy resin formulation obtained from MCC (AB) was also prepared for coating. Panels were dipped in the BPA resins in manner

analogous to the one described for the EAS resin. The panels were cured in the oven for 3 min at 204ºC (410ºF). Each panel with cured epoxy coating was reweighed and the weight of the coating on each panel was determined.

1.3.3. Mechanical Property Tests. To test the applicability of EAS resin derived coating for food and drink containers, the coating was subjected to series of tests to assess relevant mechanical properties. Tests were carried in accordance with the American Society for Testing and Materials (ASTM) standard test procedures. The coatings obtained with the commercial epoxy resin Epon 9500 and the resins used by MCC were also subjected to the same tests to obtain a relative measure of their relevant mechanical properties.

Impact resistance

The impact resistance of the coatings was assessed with a standard test method ASTM D2794. The test method measures a coating's resistance of organic coatings to rapid deformation from an impact. Five aluminum test panels (10 x 15 cm) with coatings obtained from each formulation were prepared. The test panels with the coated side facing down were placed on an impacter with a 0.5 inch diameter indenter. The indenter with different weight was dropped from different heights. After each impact, the impact area was observed for appearance of cracks. Weight of the indenter was increased until the cracks appeared on the coatings. The presence of cracks in the impacted area was established by placing a flannel-type cloth saturated with an acidified copper sulfate

(CuSO4) solution over the impacted area for 15 minutes. The cloth was then removed and both the test areas and cloth were examined for evidence of copper deposition.

Flexibility

A flexibility of the coatings was examined with a standard test method ASTM D 522. The test method involves bending of test specimens with organic coatings with a mandrel. The percent elongation of coatings without rupture is measured to assess the flexibility of the coatings. Five aluminum test panels (10 x 15cm) each with a coating obtained from different formulation were prepared. A photograph of the test apparatus with a conical mandrel is shown in Figure 1.12. Test specimens were mounted on the apparatus and the bend in accordance with the procedure prescribed in the standard test method.

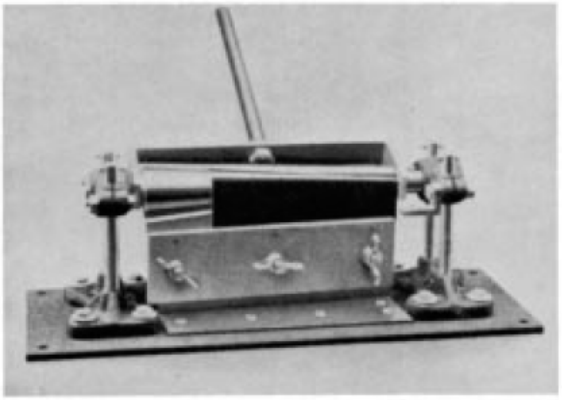


Figure 1.12: Conical mandrel test apparatus

Adhesion

The adhesion of coating to the metal surface was evaluated with a standard test. The ASTM D3359, a test method for measuring adhesion of coatings. Three 10 x 15 cm

aluminum test panels with the coatings obtained from each resin formulation were prepared. An aqueous solution containing 0.5% detergent, 0.025% $MgSO_4$ and $CaCl_2$, and 0.013% $CaCO_3$ was prepared. The solution was brought to a boil and then allowed to cool down to room temperature. Test samples were introduced into the solution and allowed to equilibrate in the solution for 15 minutes. The test panels were removed from the solution, rinsed with hot water, dried, and visually inspected for blush. Each sample panel was marked with eleven crosshatch marks 1 mm apart in each direction on the treated panels. Pieces of Scotch Tape (3M #610) were cut and uniformly applied to the crosshair patterns immediately after the pattern were marked on the panels, the tape pieces was then peeled of from the panel in one quick motion.

Abrasion Resistance

An abrasion resistance of the coatings was determined with a standard test, ASTM D4060. In this test method abrasion resistance of organic coatings is determined with the Taber Abraser. Five (10x10 cm) aluminum panels were cut and coated the resins as described earlier. The test samples were weighed and mounted on the Taber Abraser apparatus, Figure 1.13. An abrasive wheel (Resilient calibrase wheels no. CS-10) was run across the surface in prescribed manner for 5 cycles, the weight on the wheel was set at 500 grams. The test sample was removed from the apparatus lightly brushed and weighed again.

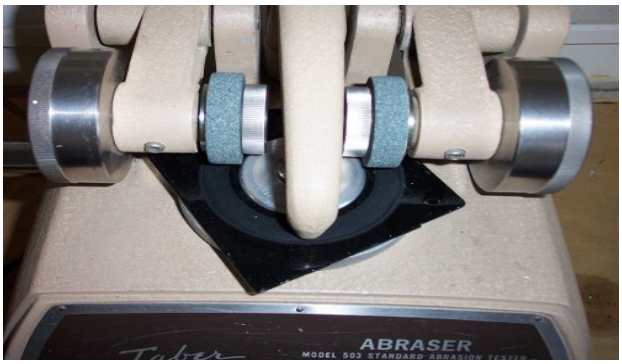


Figure 1.13: Sample mounted on Taber Abraser

Water Resistance

A water resistance of the coatings was determined in accordance with the ASTM D 870. The test permits an assessment of the water resistance of coatings after water immersion. The sample size was 5 x 10 cm. Three samples were prepared for each formulation. The test samples were fully immersed in distilled water for 3 days at a temperature of 30^{o} C in a corrosion-resistant tank. The water in the tank was aerated to prevent stagnation and oxygen depletion. The samples were then removed, dried, examined for the sign of blistering, softening, loss of adhesion, etc

Organoleptic Test

A blind organoleptic test was carried out to determine the extent of off-flavor introduced into beverages after coming in contact with the coatings obtained with different formulations. The test was carried out at Anheuser Busch facilities in St. Louis, MO. Five aluminum panels with coatings obtained from different formulations; representing control 1 (blank aluminum, not heated), control 2 (aluminum heated), and EAS + LS-682K anhydride hardener were prepared for the test and placed identical glass

containers. The containers were uniquely numbered and a record of number and the coating was made. The containers were filled with beer and aged for 15 days at 25 ^{o}C. After 15 day "aging" the containers were opened in the Anheuser Busch beer tasting room and the beer in the containers was evaluated by a panel of experts for the any off-flavor and bad taste imparted by the coatings.

1.3.4. Leaching Tests from Can Coating. Epoxy can coatings come into contact with many types of food and drink. Following the FDA's recommendations regarding food simulates, aqueous solutions containing 10, 20, 55 and 95% ethanol were used for the leaching test. Aqueous solution of 10% ethanol is an appropriate simulant for aqueous, acidic, and low alcohol content (up to 10% ethanol) foods, including beer. Aqueous solution of 95% ethanol is recommended as the simulant for fatty food. For our study, aqueous solutions of 20 and 55% ethanol were also used as simulants for fatty food. Solutions with pH 2, 7, and 10 were used to simulate the acidic and basic foods or beverages. Acetic acid and sodium hydroxide were used to prepare the simulants of pH 2 and 10, respectively. The distilled water was used as the neutral simulant. Sodium chloride solutions of 0, 0.5, 0.9, 5.0 and 10.0 % were prepared to simulate food and drink of different salt content. The coating panel was soaked in each of food and drink stimulant at room temperature. Each exposure test was performed in duplicate. Figure 1.14 illustrates the overall procedure. The length of exposure in the stimulant was up to one month. Leachate samples were collected after days 1, 7, 14, and 30.

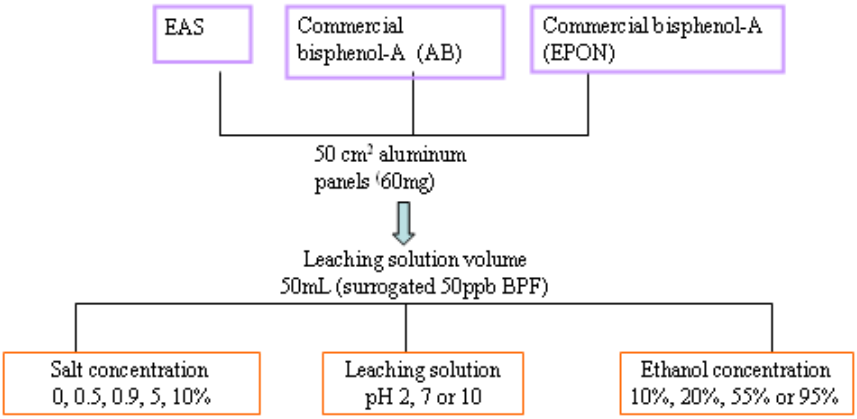


Figure 1.14: Epoxy resin coatings cured on aluminum coupons were subjected to foods and beverages simulants.

1.3.5. Analysis of Bisphenol A Leachate by LC-MS. The amount of BPA leached from the three types of coating was determined with a LC-MS. Due to the matrix of sodium chloride, the leachate samples from the brine test were passed through the C18 cartridge before analysis by LCMS. The C18 cartridge was conditioned with 5mL methanol and 5mL acetonitrile, and equilibrated with 5mL water. Each 5mL leachate sample was loaded onto the cartridge. Then, a 5mL mixture of acetonitrile and water eluted the BPA out. Prior to LC-MS measurement, each 1mL leaching sample was filtered by a 0.22um syringe driven micron filter. Standard solution of 100ppb BPA and 100ppb BPF was spiked in the sodium chloride solutions of 0.9% and 5.0% for the recovery test.

A Hitachi L-2100 HPLC system and a Varian 1200 quadrupole mass spectrometer with an electrospray ionization interface and atmospheric pressure chemical ionization (APCI) interface were used to carry out the liquid chromatographic separation and characterization of BPA and BPF from the substances of the leaching samples. The HPLC system was comprised of a column oven, a reciprocating piston pump, and an autosampler with a 50µL injection loop. The analytes were then separated on a reverse

phase C-8 column (150mm x 3.0mm i.d.) and then detected with a fixed wavelength UV detector. Proprietary HPLC software supplied by Hitachi instruments was used to record the data. The composition and flow rate of mobile phase and the MS operating parameters (position and the potential of the capillary, cone voltage, temperature and flow rate of the drying gas, and detector voltage) were optimized. Separation was achieved using an isocratic mobile phase system of 50% A (water) and 50% B (acetonitrile) with the flow rate maintained at 0.3mL min-1. The wavelength of the UV detector was monitored at 210nm. After filtration with a 0.22μ membrane filter, 10μL of the sample was injected into the column.

Both APCI and ESI were applied in the negative ionization mode. The assistant gas heater temperature was set to 200 ^{o}C. The APCI torch temperature and the housing temperature of the MS system were 500 ^{o}C and 50 ^{o}C, respectively. The quadrupole mass analyzer was scanned from 120-300amu. Drying gas temperature was set at 150 ^{o}C with a flow rate of 13 PSI. The electrospray capillary voltage was set at -4.5 KV with an ESI-negative mode. Drying gas temperature was set at 300 ^{o}C for the ESI mode. The separation was achieved using an isocratic mobile phase system of 50% A (water) and 50% B (Acetonitrile) with the flow rate maintained at 0.2mL min^{-1} for the ESI mode.

1.3.6. Analysis of Organic Acid Leachate by GC/MS. Cyclic anhydrides are compounds that are so reactive and hygroscopic that water hydrolyses them to corresponding acids. The dicarboxylic acid need to be derivatized to dicarboxylic ester prior to their analaysis with GCMS. Esterification is carried out with a suitable alcohol (Methanol – CH3OH) in the presence of an acid (sulfuric acid – H2SO4) to yield

corresponding ester. Esterification of Methyl hexahydrophthalic anhydride and Methyl tetrahydrophthalic anhydrides to methyl hexahydro phthalic dimethyl ester and methyl tetrahydro phthalic dimethyl ester is shown in Figure 1.15.

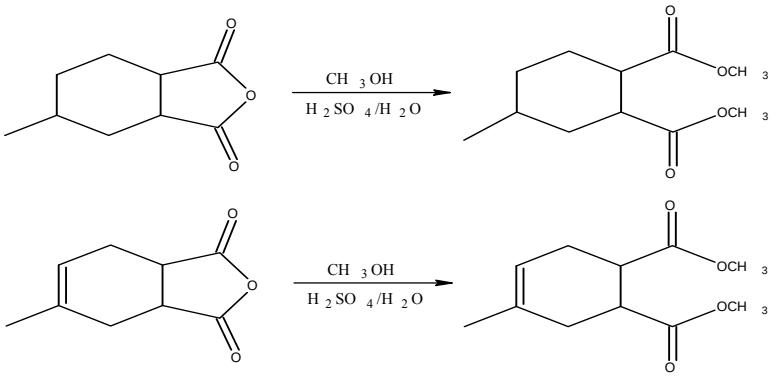


Figure 1.15: Synthesis of MHHPA and MTHPA methyl esters

The aqueous solutions with varying amounts of ethanol (0, 10, 20, 55, and 95%) were used to determine the extent of phthalic acids leaching. One mL aliquots of solutions were collected after emersion of coated panels in the solutions for 1 – 30 days. The leachate aliquots were transferred to 7mL borosilicate glass vials. The ethanol in the leachate samples was removed with dry nitrogen, 1mL H_2O was added to each vial along with 0.6 mL concentrated H_2SO_4. Vials were allowed to cool down to room temperature then 0.4mL methanol was added to each vial. Vials were closed loosely with Teflon lined screw caps and placed in sand bath maintained at 75ºC. The phthalic acids in the vials were allowed to react with methanol for 8 hours. Vials were then removed from the sand bath and allowed to cool down to room temperature, 2mL of iso-octane was added to the vial and the contents in the vial were shaken to transfer phthalic acid esters into the iso-octane layer (top layer). This layer was carefully transferred to a separate vial. The procedure was repeated three times and iso-octane layers were pooled. The pooled iso-

octane was rinsed twice with 5mL D.I water to remove acid. The acid free iso-octane layer was made to pass through a funnel containing a bed of anhydrous Na_2SO_4 (~2g). The Na_2SO_4 bed was rinsed with 5mL of hexane, the resin hexane was added to the iso-octane in a 10mL borosilicate glass vial. The solution volume was brought down to ~1mL under a gentle nitrogen stream. The final volume of the phthalic acid ester solution was adjusted to 2mL with iso-octane. A 2μL aliquot of the ester solution was injected into a calibrated GC-MS system, the overall analysis methodology is shown in Figure 1.16.

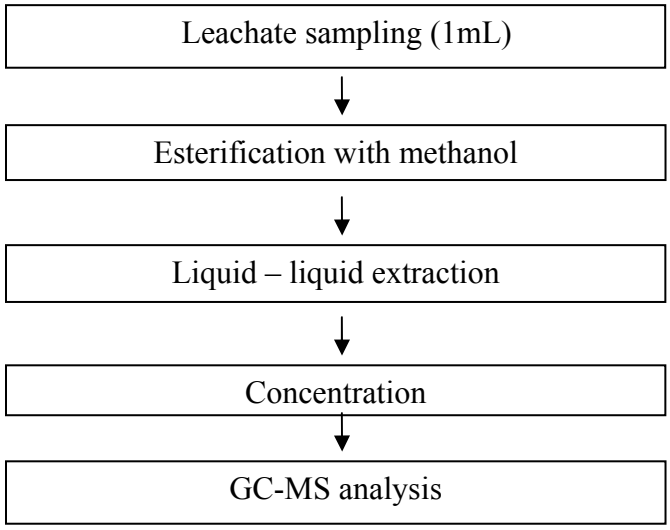


Figure 1.16: The flow schematic the analytical methodology to assess leaching of phthalic acid from the Epoxy resin coated panels.

The GC-MS measurements were carried out with a bench-top GC-MS system comprised of Model Trace GC, Model Polarise Q MS and a Model Triplus autosampler

(Thermo Scientific Corporation, Waltham, MA). The gas chromatographic separations were performed with a 30 m x 0.25mm capillary column. The stationary phase in the column was 5% poly phenyl – 95% methyl siloxane (Agilent Technologies, Santa Clara, CA). The stationary film thickness was 0.1 μm. Helium was used as carrier gas, its flow rate was maintained at 1.0 mL/min. Separations were achieved with a column oven temperature program under which the initial column oven was set a 60 ºC and after a 2 minute initial hold it was linearly ramped to 270 ºC at 10 ºC per minute, column oven temperature was maintained at 270 ºC for 6 minutes. The analyte solution was introduced into the GC-MS through the splitless mode. The splitless time was set to 1.5 minutes. The injector temperature was set at 250 ºC, the transfer line was set at 250 ºC, and the trap temperature was set at 150 ºC.

The ion trap mass analyzer was operated with an electron ionization source in the scan mode. Electron energy was set at 70 eV. The mass range was set between 50 to 400 amu. Quantification of dimethyl phthalate was carried out by integrating the area of the characteristic ion at m/z 163 in the extracted ion chromatogram, quantification of dimethylhexahydro phthalate was carried out by integrating the area of the characteristic ion at m/z 183 in the extracted ion chromatogram, while quantification of dimethyltetrahydro phthalate was carried out by integrating the area of the characteristic ion at m/z 181.

1.4. RESULTS AND DISCUSSION

1.4.1. Property of EAS, EPON and a Proprietary Resin Coating. Desired properties a resin for can coating applications are:

A short cure time preferably three minutes or less and a moderate curing temperature ~ 200 ºC or ~ 400 ºF. The film should show good adhesion, low cracking, flexibility, good scratch resistance, good impact resistance and low film mass. These properties were evaluated through standard tests (Rupali, 2005).

The EAS resin with the phthalic anhydride curing agents cured fully in less than 2 minutes at 200 ºC thus readily the first requirement for a beverage can coating. The EAS coating on test aluminum panels performed satisfactorily in all standard mechanical tests.

Impact Resistance

Impact resistance of the films was evaluated with drop weight test (ASTM D2794). The drop weight in the test was varied from of 4 inch-pounds to 18 inch-pounds. The panels were examined for copper deposition. Examinations showed that the copper deposition was more pronounced on the panels coated with the Epon resins and the resin supplied by Anhueser Busch. However, cracks were not directly observed in any of the coatings at low test weights. The cracks appeared in all panels when the test weight was increased to beyond 8 inch-pounds because high weight caused the aluminum sheets to rupture. Test was very reproducible. Results showed that EAS coating has better impact resistant than the coatings obtained with both the Epon and proprietary (Anhueser Busch) resins

Flexibility

The flexibility of coating was determined in accordance with ASTM D 522. The flexibility test is to test the property of a material which allows it to be flexed and bowed

repeatedly without undergoing rupture Coatings obtained with the EAS resin and the proprietary resin obtained from Anhueser Busch showed excellent flexibility, neither coating showed any cracking.

Abrasion Resistance

Abrasion resistance of the coatings was examined with ASTM D4060. The abrasion resistance text is to test the ability of a material to withstand mechanical action such as rubbing, scraping, or erosion that tends progressively to remove material from its surface. Such ability helps to maintain the material's original appearance and structure. The wear index is to indicate the abrasion resistance property. The wear index is the loss of weight in milligrams per 1,000 cycles of abrasion under a specific set of test conditions. The wear index value for the coatings was calculated with the following expression

$$I = (A - B)/ C * 1000$$

Where,

A = weight of test specimen before abrasion, mg,

B = weight of test specimen after abrasion, mg, and

C = number of cycles of abrasion recorded.

An average wear index value (“I”) of 0.053 was obtained for the EAS coating, the index value for Epon resin was found to be 0.038 indicating that EAS coating is more wear resistant.

Adhesion

ASTM Adhesion test (ASTM D3359) was carried out to test the adhesion property of coatings. Adhesion test is to test how well the two surfaces of coating and panel held together. Normally two surfaces held together by interfacial forces which may consist of valence forces or interlocking action or both. Results of the ASTM adhesion test (ASTM D3359) conducted with the EAS coating showed that none of the crosshatch marks on the coating that has been treated with a water containing 0.5% detergent, .025% $MgSO_4$ and $CaCl_2$ and .013% $CaCO_3$ peeled off from the surface with the tape (0% present removal of the coating). Thus the coating fell in category 5B, results for the EPON coating and the coating obtained with the Anhueser Busch supplied resin. Thus proving that the adhesion of the EAS coating to the aluminum surface is a comparable to the commercially bisphenol based epoxy resins available in the market.

Water Resistance

Susceptibility of coatings to water was assessed with ASTM D 870: Standard Practice Aluminum panels with coatings were immersed in glass container with water at 30^0C for 3 days. The samples were then removed and dried and observed for blush and cloudiness in water. None of coatings including the EAS coating showed any blemishes or blisters on the surface in addition water remained clear in all cases clearly showing that all coatings were resistant to water.

Blind Organoleptic evaluation of beer by expert panel

The test containers with EAS and commercial epoxy coated coupons were delivered to the Anheuser Busch Corp. The test beer cans were aged at 25 ^{0}C for 15 days.

At the end of the two week period beer from the containers was subjected to a blind organoleptic test by a panel of experts assembled at the Anheuser-Busch facility in St. Louis. The panel rated the beer from each container for its taste, aroma, and other qualities. The panel ranked beer from each container on a 3-5 point system where a beer with a lower number is considered to possess higher quality. Beers with numerical values within 3.0-4.0 are considered acceptable. Beers from all containers with EAS coated coupons were rated at better than 4.5 thus falling well within the acceptable range.

1.4.2. BPA Leaching from Epoxy Resin in the Simulants. The amount of Bisphenol A leached from the EAS, EPON, and the proprietary BPA based can coating in the different simulants were determined by LCMS.

1.4.2.1. Identification and Quantification. An isocratic HPLC program was used to separate BPF and BPA. BPF eluted at 4.5 minutes where as BPA eluted at 5.3 minutes, chromatographic separation obtained for BPA and BPF is shown in Figure 1.17. APCI mass spectrum of deprotonated BPF and BPA with psuedomolecular ions at m/z 199 and 227 respectively are shown in Figure 1.18.

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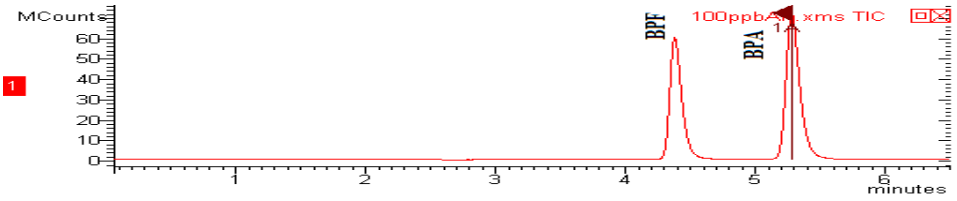
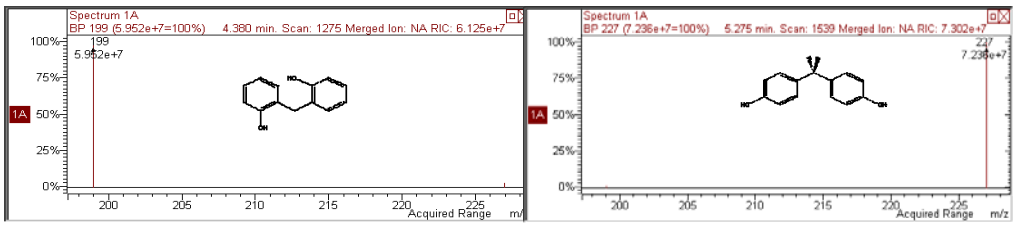


Figure 1.17: Reverse phase liquid chromometry separation of BPF and BPA obtained under isocratic elution



Bisphenol F

Bisphenol A

Figure 1.18: APCI mass spectra of BPF and BPA

Calibration standards were prepared through serial dilution of a stock solution with a 50:50 acetonitrile -water (v/v) mixture. The BPA concentrations in the calibration standards ranged from 0.1 - 100 parts per billion (ppb) all standards contained a fixed concentration of internal standard. Bisphenol F was selected as an internal standard because of its suitable retention time characteristics and stability. A calibration curve was obtained by plotting response ratio of BPA to the internal standard, Bisphenol F. The linear regression analysis of the calibration data showed that a correlation coefficient of 0.9999, the calibration curve is shown in Figure 1.19. The detection limit of BPA with the APCI source was found to be ~0.1ppb, where as the detection limit with ESI source was found to be 0.5 ppb. The detection limits were calculated as the analyte concentration that yields a signal which equal to three times the noise.

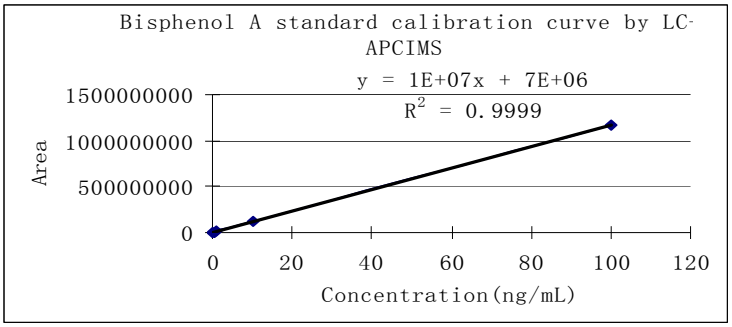


Figure 1.19: Calibration curve for BPA obtained by LC-APCI- MS

BPA in the leachate samples was monitored with the LC-APCI-MS system. The presence of BPA was identified by matching the retention time and mass spectrum. Quantification was carried out by integrating the areas for ions at m/z 199 and m/z 227 in the extracted ion chromatograms.

1.4.2.2. Recovery of BPA from the Aqueous Solution. BPA leached into the saline aqueous solution was recovered from the solution with solid phase extraction (SPE).To establish the efficiency of the SPE for BPA recovery from saline solutions, saline solutions containing 0.9% and 5.0% of sodium chloride were fortified with 100 ppb of BPA and BPF. The BPA / BPF containing solutions were passed through conditioned SPC cartridge. The SPC cartridges with bonded C18 siloxane were rinsed with 5mL Methanol followed by 5mL acetonitrile and finally with 5mL water. 5mL aliquots of the leaching solutions were loaded onto the cartridge. Cartridges were eluted with a 5mL mixture of acetonitrile and water. The eluent was analyzed with LC-ESI-MS and checked for the recovery of BPA. The results showed that the recoveries of BPA from the saline solution ranged between 100.3-108.5%.

1.4.2.3. BPA Leaching from Epoxy Coatings in Ethanol Water Mixture. To monitor the effect of ethanol content on the extent of BPA leaching from the coated panels, panels were immersed in water – ethanol mixtures with varying amounts of ethanol (0 – 95%) for periods ranging between 1-30 days. Aliquots of the water – ethanol mixtures were removed and analyzed for BPA leaching. The results of the experiments with the Epon coatings are summarized in Table 1.3. The results showed that BPA

concentrations in the mixtures increased with the immersion period and ethanol concentration. BPA concentration in water (without) ethanol was less than detectable at immersion period upto 30 days indicating little or no leaching of BPA from the coatings occurs with only water as the leaching medium. Similarly, BPA was not detected above detection limits in water- ethanol mixture containing 10% ethanol at immersion period less than 7 days. However, BPA concentration above detection limit was found after longer immersion periods, after 14 days the concentration was found to be 0.5 ppb it increased to 0.8 ppb after 30 days. Similarly, BPA was not detected water - ethanol mixture with 20% ethanol after 7 day immersion period. Concentrations well above the detection limit were found after longer immersion periods 14.1 ppb and 33.1 ppb after 14 day and 30 day immersion respectively. Immersion in mixtures with higher ethanol lead to enhanced leaching of BPA from the coatings, e.g. in mixture with 55% ethanol concentration of leached BPA was found to be 23.3 ppb after 7 days and it increased to 106 ppb after 30 day immersion. Higher concentration of BPA were found in 90% ethanol in the mixture BPA concentration was found to be 46.8 ppb after 7 days and it increased to 127 ppb after 30 days. Results showed that leaching of BPA from coatings is dependent on the ethanol content and the duration of exposure of the coatings to the leaching solution.

Table 1.3: BPA leaching from epoxy coatings obtained with EPON resin

Leaching Medium	BPA Concentration(ppb)			
% Ethanol	day 1	day 7	day 14	day 30
95%	<DL	46.8	50.9	127
55%	<DL	23.3	43.8	106

20%	<DL	<DL	14.1	33.1
10%	<DL	<DL	0.5	0.8
0%	<DL	<DL	<DL	<DL

A some what similar trend for BPA leaching was observed from the coatings obtained with the proprietary epoxy resin coating obtained from the Anheuser Busch, results obtained with this coating are summarized in Table 1.4. However, the extent of BPA leaching was considerably lower with these coatings. In both leaching test, no BPA was detected in the D.I water blank. But more BPA was detected in the aqueous solutions with higher ethanol content. It is speculated that ethanol can penetrate the coating and extract BPA from the coating more than water. There are free BPA monomers present in the BPA based epoxy coatings even after epoxy resins were cured with all types of hardener at a specified high temperature. When the panels coated with epoxy resins exposure in the different solvents with a long time, it is possible that BPA leached out from the epoxy coating into the solvents. BPA was extracted by ethanol by passing through polymeric coatings more than water. Thereby, more BPA was detected in the aqueous solution with higher ethanol content.

Table1.4: BPA leaching from epoxy coatings obtain with proprietary the resin obtained from the Anheuser Busch

Leaching Medium	BPA Concentration (ppb)			
% Ethanol	day 1	day 7	day 14	day 30
95%	5.1	13.9	18.1	22.3
55%	2.3	7.6	9.4	13.6
20%	<DL	<DL	1.1	4.9
10%	<DL	<DL	<DL	<DL
0%	<DL	<DL	<DL	<DL

1.4.2.4. BPA Leaching from Epoxy Coatings in Salt Solutions. Effect of salt concentration on BPA leaching from epoxy coatings was examined by immersing panels with epoxy coatings in solution with varying salt concentrations for periods ranging upto 30 days. Aliquots of salt solutions were collected at different time periods and analyzed for BPA. Results of experiments with the EPON coatings are summarized in Table 1.5.

Table 1.5: BPA leaching from epoxy coatings obtained with EPON

Leaching Medium	BPA Concentration(ppb)			
% Salt	day 1	day 7	day 14	day 30
10%	<DL	6.4	14.0	28.7
5%	<DL	7.1	15.9	31.6
0.90%	<DL	6.4	15.7	31
0.50%	<DL	5.9	16.0	29.8
0	<DL	<DL	<DL	DL

Concentration of BPA in leachate without salt was less than the detection limit even after 30 day immersion of coated coupon. However, BPA concentrations above the detection limit were found in leachates with salt concentration higher than 0.5%. In these leachates concentration increased with the immersion period. Leachate with salt concentration between 0.5 – 10% showed essentially the same trend and no correlation between the salt concentration and BPA concentration in the leachate could be discerned. The extend of BPA leached from the Epon coating in the salt solutions was appreciably lower than the one observed with the water ethanol mixtures.

BPA concentration in all salt containing leachates obtained with the proprietary epoxy resin coating obtained from the Anheuser Busch were below the detection limit. The coatings obtained with this resin more highly cross linked because of oligomerization and are more resistant to degradation in salt solution than the Epon coatings.

1.4.2.5. BPA Leaching from Epoxy Coatings in Presence of Acid and Base. Solutions with pH of 2, 7, and 10 were used to simulate the acid and basic foods or beverages. Acetic acid was used for lowering the pH to 2 and sodium hydroxide was used to prepare the pH 10 solution. The distilled deionized water (pH ~7.0) served neutral solution. BPA concentrations in all leachates were below the detection limit of the LC-MS system.

As expected no BPA residues were detected in leachates from EAS coatings under all immersion conditions. Thus, the EAS coatings are superior to the BPA based epoxy coatings.

1.4.3. Determination of phthalic acids in leachate from EAS, EPON, AB Coatings.

1.4.3.1. Identification and Quantification The identification and quantification of dicarboxylic acid in the leaching samples was carried out as methyl esters with a GC-MS system equipped with an electron ionization (EI) source. To obtain better detection limit and quantification the MS was operated in the selective ion monitoring (SIM) mode, details of operational parameters are given in the experimental section. MHHPA dimethyl ester (Di methyl methyl hexahydro phthalate - DMMHP) and phthalic acid dimethyl ester (Dimethyl phthalate - DMP) were readily separated with GC. The GC-MS output obtained by introducing 50ng of MHHPA dimethyl ester and phthalic acid dimethyl ester

is shown as the total ion chromatrogram in Figure 1.20. MHHPA dimethyl ester eluted at 15.73 min. where as phthalic acid dimethyl ester eluted at 16.05 min.

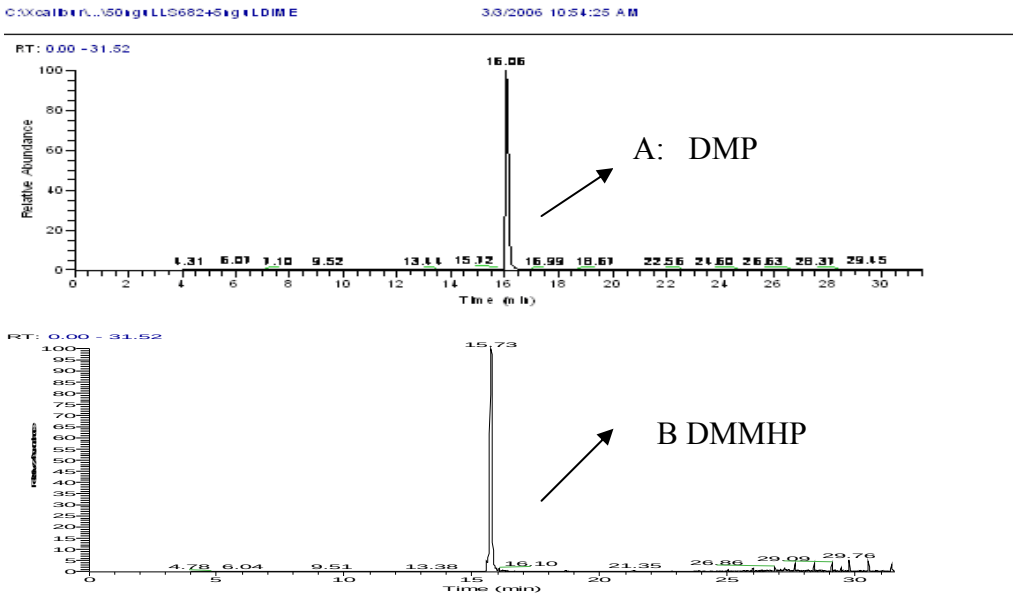


Figure 1.20: Total ion chromatogram of 50 ng DMP (A) and DMMHP (B).

Total ion chromatograms of 50ng LS-682 and 5ng DMP are shown in Figure 1.21. The DMP peak appears at 16.05 min, the dominant ion its mass spectrum appeared at m/z 163 resulting from α – cleavage leading to the formation of an acylium ion and the departure of a radical (CH3-O.). The DMMHP present in LS – 682 eluted after 15.73 min., it mass spectrum contained a characteristic ion at m/z 183; this ion also resulted from α – cleavage leading to the formation of an acylium ion and the departure of a radical (CH3-O.).

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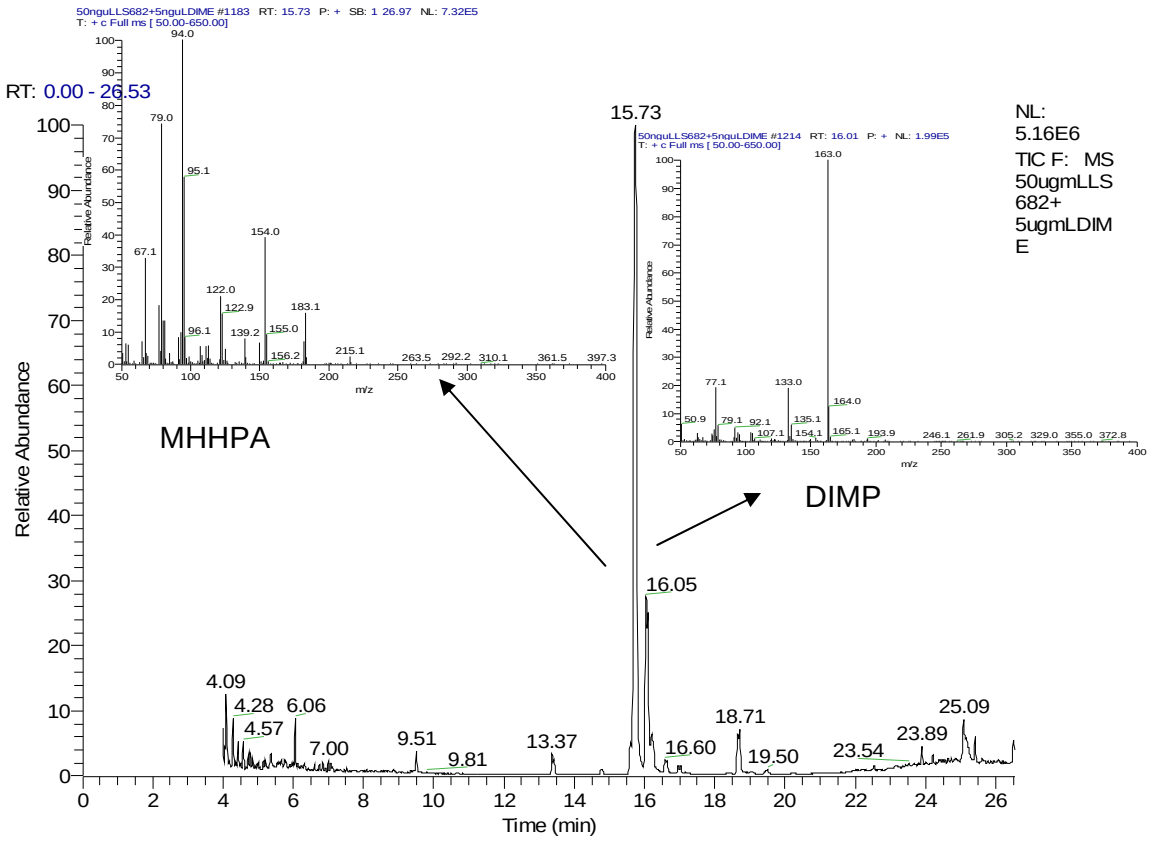

Figure 1.21: A total chromatogram and mass spectra of esterification product of LS-682 and DMP.

The esterification of LS682 was carried out with a one mL stock solution (100mg LS682 in 100mL hexane). The resulting esters solution was diluted to obtain calibration standards with nominal concentrations of 5, 10, 20, and 50ng μL^{-1}. The DMP calibration standard solutions were prepared from commercially available DMP at concentrations 0.1, 1.0, 5.0, and 20ng μL^{-1}. The calibration curve for DMP, depicting amount introduced vs mass spectrometer signal is shown Figures 1.22, the DMP response was found to be linear over the concentration range the correlation co-efficient was found to be 0.9997.

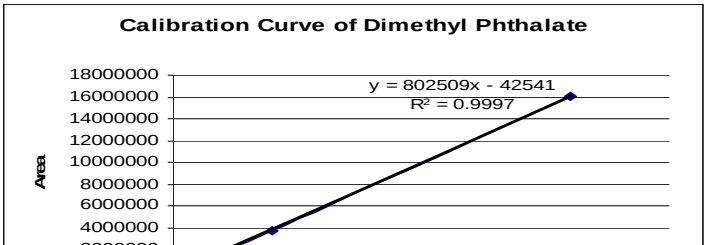

Figure 1.22: Calibration curve for DMP

The calibration curve for LS-682 (DMMHP) over the 5 – 50 ng µL-1 concentration range is shown in Figure 1.23. The response was found to be linear over the concentration range the correlation co-efficient was found to be 0.9994.

The calibration curve for LS-682 (DMMTP) over the 5 – 50 ng µL-1 concentration range is shown in Figure 1.24. The response was found to be linear over the concentration range the correlation co-efficient was found to be 0.9994.

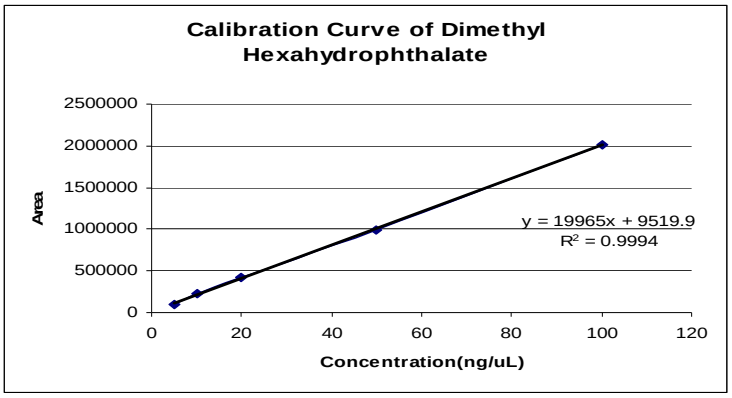

Figure 1.23: Calibration curve for DMMHP

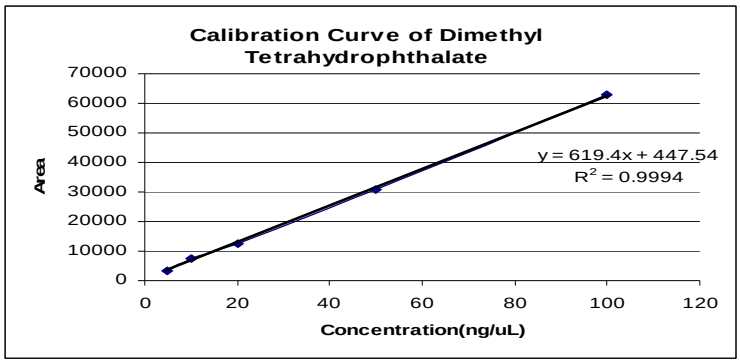

Figure 1.24: Calibration curve for DMMTP

1.4.3.2. MHHPA Leaching from Epoxy Coatings in Ethanol Water Mixture.

Concentration of DMMHP found in water ethanol mixtures obtained after immersing EAS resin coated aluminum coupons in the mixtures for varying time periods are given in Table 1.6. The EAS formulation used for coating aluminum coupons comprised of 1 part of EAS resin and 0.7 parts of LS-682. Unreacted methyl hexahydrophthalic anhydride or hyrohydized resin would lead to methyl hexahydrophthalic acid in the leaching solution and would be detected as dimthyl methyl hexahydrophthalate (DMMHP). Results showed that DMMHP concentrations increased with immersion period and ethanol content of the immersion solution. DMMHP concentrations were below the detection limit when the immersion solution did not contain ethanol even after a thirty day period. Measurable levels of DMMHP were observed when ethanol concentration was increased to 10% and coupons were immersed for periods longer than 7 days, however, concentration did not show any change beyond that observed after the 7 day period. The increase in ethanol content of the leaching solution to 20% led to detection of DMMHP in the leachate after just one day immersion but concentrations were low and remained constant at 0.1ppb upto 30days. The initial DMMHP levels in leachates with 55 and 90 % ethanol were also quite low but showed an increase with the immersion period.

Table 1.6: DMMHP concentration from the 70% EAS coating

Leaching medium	DMMHP ppb			
Ethanol	day 1	Day 7	day 14	day 30
0	<DL	<DL	<DL	<DL
10%	<DL	0.1	0.1	0.1
20%	0.1	0.1	0.1	0.1
55%	0.1	0.2	0.2	0.3
95%	0.1	0.3	0.4	0.5

Table 1.7 shows the concentration of DMMHP found in ethanol stimulants from the 68% EAS can coating. In the blank, 10%, and 20% ethanol simulants, no DMMHP was detected from all samples collected at different times. In the 55 % ethanol simulants, no DMMHP was detected upto 7 days. The concentration of DMMHP remained constant at 0.1 ppb upto 30 days. The initial DMMHP levels in leachates with 90 % ethanol were also quite low but showed an increase with the immersion period.

Table1.7: DMMHP concentration from 68% EAS coating

Leaching medium	DMMHP(182.5-183.5) ppb			
Ethanol	day 1	Day 7	day 14	day 30
0	<DL	<DL	<DL	<DL
10%	<DL	<DL	<DL	<DL
20%	<DL	<DL	<DL	<DL
55%	<DL	<DL	0.1	0.1
95%	<DL	0.1	0.2	0.2

A some what similar trend for MHHPA leaching in the ethanol water mixture was observed from 65% EAS coating upto 30days. In the blank, 10%, 20% and 55% ethanol simulants, DMMHP concentration was less than the detection limit upto 30 days. In 95% ethanol simulants, no DMMHP was detected for the day 1 samples. The concentration of DMMHP remained constant at 0.1 ppb upto 30 days.

Table1.8: DMMHP concentration from 65% EAS coating

65%EAS	DMMHP(182.5-183.5) ppb			
Ethanol	day 1	Day 7	day 14	day 30
10%	<DL	<DL	<DL	<DL
20%	<DL	<DL	<DL	<DL
55%	<DL	<DL	<DL	<DL
95%	<DL	0.1	0.1	0.1

In all leaching tests from EAS coating with different formulation, no DMMHP was detected in the D.I water blank. But more DMMHP was detected in the aqueous solutions with higher ethanol content and higher anhydride hardener formulation. It is speculated that MHHPA is extracted from polymeric coating by ethanol higher than by water. And when more anhydride hardener was added into the coating more than it was needed for the polymerization, more MHHPA was eluted out from coating. So it is necessary to optimize the formulation of the coating.

The leaching amount of MHHPA from the EPON can coatings found in the ethanol simulants from day 1 to day 30 was shown in Table 1.9. DMMHP concentration from EPON coating was below the detection limit in all 10% ethanol water mixture upto

30 days. The initial DMMHP levels in leachates with 90 % ethanol were also quite low but showed an increase with the immersion period.

Table 1.9: DMMHP concentration from the EPON coatings

EPON	DMMHP(182.5-183.5) ppb			
Ethanol	day 1	day 7	day 14	day 30
H_2O	<DL	<DL	<DL	<DL
10%	<DL	<DL	<DL	<DL
95%	0.2	0.2	0.3	0.3

DMMHP concentration from AB coatings was less than the detection limit upto 30 days. AB is a coating with proprietary hardener obtained from the beer company, It is possible the hardener was not based on phthalic anhydride. So no DMMHP was found in the leachate.

1.4.3.3. MTHPA Leaching from Epoxy Coatings in Ethanol Water Mixture. A some what similar trend for MTHPA leaching was observed from the EAS coatings. The results show DMMTP concentration was below the detection limit in the blank, and 10% ethanol mixture from the 68% and 65% EAS can coating upto 30 days. For the 70% EAS coating (Table 1.10), the MTHPA leaching amount was only detected in the 95% ethanol water mixture. DMMTP concentration was below the detection limit in the day 1 samples. The concentration of DMMTP remained constant at 0.1 ppb from 7 days upto 30 days.

Table1:10: DMMTP concentration of EAS coatings

70%EAS	DMMTP(180.5-181.5) ppb			
Ethanol	day 1	day 7	day 14	day 30
H_2O	<DL	<DL	<DL	<DL
10%	<DL	<DL	<DL	<DL
95%	<DL	0.1	0.1	0.1

A some what similar trend for MTHPA leaching was observed from the EPON coatings in ethanol water mixture. Table 1.11 shows the DMMTP concentration from the EPON can coatings in the ethanol water mixture upto 30 days. DMMTP concentration was only detected in the 95% ethanol water mixture and remained constant at 0.1 ppb upto 30 days.

Table 1.11: DMMTP concentration from the EPON coatings

EPON	DMMTP(180.5-181.5) ppb			
Ethanol	day 1	day 7	day 14	day 30
H_2O	<DL	<DL	<DL	<DL
10%	<DL	<DL	<DL	<DL
95%	0.1	0.1	0.1	0.1

1.5. CONCLUSIONS

A series of experiments proved that it is possible to obtain beverage can coatings from soybean oil based epoxy resin. Newly developed EAS based coatings fulfilled industry curing requirements and passed the recommended mechanical property measurement tests. Experimental results show that EAS has potential for use as a

protective coating because of its a low film weight, possession of an exceptional combination of toughness, adhesion, formability, chemical resistance, flexibility, and impact and scratch resistance properties. It also does not impart flavors to beverages.

LC-APCI-MS provides a simple and sensitive method for determination of Bisphenol-A, with the detection limit of 0.1ppb. BPA concentration in leachates depends on the ethanol content of the leaching solution and storage time. The leaching level of BPA was increased with ethanol content in the simulants and also increased with the time varying. BPA leaching is not affected by pH (2-10) of the leaching solutions. GC-MS provides a sensitive method for monitoring of phthalic acids in simulants. The use of anhydrite hardeners in epoxy coatings can lead to the presence of trace levels of phthalic acids in leaching solutions trace levels. Organic acid concentration in leachates depends on epoxy formulations and the ethanol content of the leaching solution and storage time. With more content of phthalic anhydride hardener in the coating formulation, more phthalic acids were present in the leachate. The migration level of phthalic acids was increased with the ethanol content in the simulants and also increased with the time varying. In addition to EAS coating fulfills the industry curing requirements. The coating is concluded to be as good as, if not better than, the commercial coating currently used. This study proved that it is possible to use EAS as a beverage can coating.

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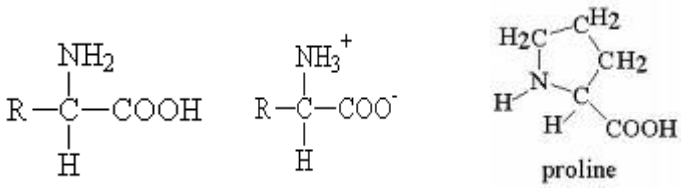
2. ENZYMATIC SYNTHESIS AND CHARACTERIZATION OF TAILORED N&C END PROTECTED OLIGOPEPTIDES

2.1. INTRODUCTION

2.1.1. Amino Acids. Proteins are made from a set of 19 α-amino acids and the imino acid proline. α-amino acids are organic molecules possessing both carboxyl and amino groups. At the center of the amino acid is an asymmetric carbon atom called α carbon. Its four different parts are an amino group, a carboxyl group, a hydrogen atom, and a variable group symbolized by R. The R group, the functional group of amino acid is called the side chain, differs with each amino acid. Figure 2.1 shows the Fisher-Romanoff projection of amino acid and L-amino acid in the ionization form and the structure of proline.



(A) (B) (C)

Figure 2.1: Fisher -Rosanoff projection of (A) amino acid, (B) amino acid in the ionized form, and (C) the structure of proline.

With four different groups connected to the tetrahedral α-carbon atom, amino acids with the exception of glycine, are chiral; the two mirror-image forms are called the L isomer and the D isomer. Only L amino acids are constituents of proteins. The side chains of these 20 building blocks vary tremendously in size, shape, and the presence of functional groups. They can be classified as follows: (1) aliphatic side chains—glycine,

alanine, valine, leucine, isoleucine, methionine, and proline; (2) aromatic side chains—phenylalanine, tyrosine, and tryptophan; (3) hydroxyl-containing aliphatic side chains—serine and threonine; (4) sulfhydryl-containing cysteine; (5) basic side chains—lysine, arginine, and histidine; 6) acidic side chains—aspartic acid and glutamic acid; and 7) arboxamide-containing side chains—asparagine and glutamine. Depending on the polarity of the side chain, amino acids vary in their hydrophilic or hydrophobic character (Berg et al., 2001; Jones, 2002; Doonan, 2002).

Most microorganisms such as E. coli can synthesize the entire basic set of 20 amino acids, whereas human beings cannot make nine of them. They are isoleucine, leucine, lysine, threonine, tryptophan, methionine, histidine, valine, and phenylalanine. The amino acids that must be supplied in the diet are called essential amino acids, whereas the others are termed nonessential amino acids (Berg et al., 2001). Both types are listed in Table 2.1.

Table 2.1: Basic set of 20 amino acids

Nonessential amino acids	Essential amino acids
Alanine	Histidine
Arginine	Isoleucine
Asparagine	Leucine
Aspartate	Lysine
Cysteine	Methionine
Glutamate	Phenylalanine
Glutamine	Threonine
Glycine	Tryptophan
Proline	Valine
Serine	
Tyrosine	

2.1.2. Peptides. Peptides are polymers of amino acids connected by amide bonds (peptide bonds) between the carboxyl group and the amino group (Sewald and Jakubke, 2002). Because both the amine and carboxylic acid groups of amino acids can react to form amide bonds, one amino acid molecule can react with another and become joined through an amide linkage. This condensation reaction yields the newly formed peptide bond and a molecule of water, as shown in Figure 2.2.

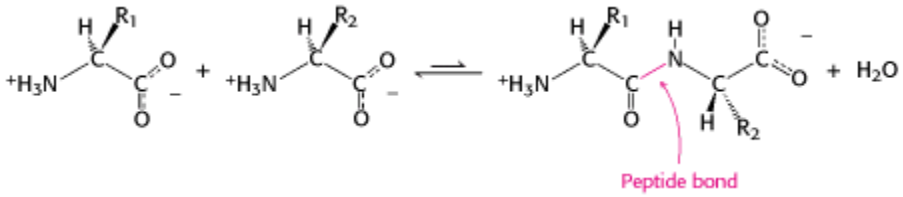


Figure 2.2: Peptide-Bond Formation, the linking of two amino acids is accompanied by the loss of a molecule of water.

The linear arrangement of amino acids in the polypeptide chain comprises the primary structure of the polypeptide/protein. The amino acids present in a polypeptide are termed as “residues. The polypeptide chain have two functional end groups present at the end of the chain, the end with the amine functionality is designated as the N-terminal end and the one with the carboxylic acid functionality is designated as the C-terminal end (Jones, 2002). A basic skeleton of a poly peptide chain is given in Figure 2.3, in which “n” designates the number of amino acid residues present in the poly peptide chain other than terminal amino acids.

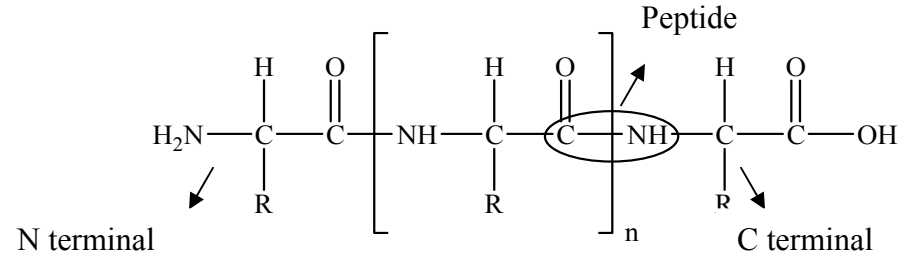


Figure 2.3: Basic skeleton of polypeptide chain showing N and C terminal functionalities

Most natural polypeptide chains contain between 50 and 2,000 amino acid residues and are commonly referred to as proteins. Peptides made of small numbers of amino acids are called oligopeptides or simply peptides. The mean molecular weight of an amino acid residue is about 110, so the molecular weights of most proteins are between 5,500 and 220,000(Berg et al., 2001).

2.1.3. Proteins. Proteins are the key molecules in the processes of life, virtually all the activities which sustain living organisms are carried out by proteins. The following are a few examples of protein and what they do: enzymes catalyze the reactions of life processes, often increasing their rates by many orders of magnitude; Regulatory proteins control functions such as the expression of genetic information and the balance of the chemical reactions that are going on in a cell at any time; Transport proteins carry other molecules from place to place in the organism. For example, the protein haemoglobin transports oxygen around the blood stream; Immunoglobulin provides a first line of defense against foreign proteins or invading pathogens; Muscle proteins carry out the work of muscular contraction(Doonan, 2002).

A polypeptide is a polymer of amino acids connected in a specific sequence. A protein consists of one or more polypeptide chains folded into a specific three-dimensional conformation. The amino acid sequence of a polypeptide determines what three-dimensional conformation the protein will take. The four superimposed levels of structure in the complex architecture of a protein are primary, secondary, tertiary, and quaternary. The primary structure of a protein is its unique sequence of amino acids. The secondary structure is the folding or coiling of the polypeptide into repeating configurations, mainly the α-helix and the β-pleated sheet that results from hydrogen bonding between the parts of the polypeptide backbone. The tertiary structure is the overall three-dimensional shape of a polypeptide and results from interactions between amino acid R groups. Proteins made of more than one polypeptide chain have a quaternary level of structure. Protein shape is ultimately determined by its primary structure, but the structure and function of a protein are sensitive to physical and chemical conditions (Campbell et al., 2004).

2.1.4. Protein as Animal Nutrients. Oral administration of proteins and peptides has received widespread attention because they supply the amino acid requirement of living beings (Kompella et al., 2001; Senel et al., 2001). Animal products have been major constituents of human diet for centuries because they provide adequate nutrients intake in forms of high biological activity. Poultry and livestock contribute major nutrition sources in the human diet. Protein requirements as a percent of the diet of an animal are at its highest during their stages of growth, pregnancy, and lactation. Protein plays an

important role in growth, maintenance and productive requirements of the animals (Pond et al., 1995; Asplund, 1994).

2.1.4.1 Amino Acid Metabolism in Animals. The process of protein digestion and amino acid metabolism in animals can be subdivided into 3 steps: catabolism, absorption and anabolism. In catabolism step, dietary proteins (or rumen microbial proteins) undergo acid and enzymatic hydrolysis in the gastro-intestinal tract (GIT) of animal which refers to the digestive tract, the system of organs within multicellular animals that takes in food, digests it to extract energy and nutrients, and expels the remaining waste from the mouth to the rectum. The digestive process refers to the mechanical, chemical, and enzymatic breakdown of macromolecular food into simpler molecules that can be absorbed into the body and used as nutrients. In absorption step, the hydrolyzed forms of L-amino acids are absorbed at the brush border membrane of the intestines through an enantio-specific transport system. In the last step, the absorbed amino acids are utilized for growth, maintenance and productive requirements during deamination and transamination. The oxidative deamination of amino acids leads to the removal of the amino group and production of the corresponding α-keto acid analog. Then, in transamination reactions, an amino group is transferred from an amino acid to an α-keto acid. The carbon skeleton of the α-keto acids can be either catabolized as a source of energy or used to synthesize the α-keto acid analogs of other amino acids. These α-keto acids can then be utilized to synthesize the respective amino acids through transamination reactions (Stryer, 1981; Lehninger, 1982).

The stomach of ruminants is divided into four compartments, reticulum, rumen, omasum and abomasums, which is different from monogastric species including humans, swine, poultry, dogs, cats, rodents etc. The rumen and reticulum are often physically indistinguishable and are referred collectively as the reticulo-rumen. The reticulum serves as the organ that facilitates redistribution of the ingested food between the rumen and the omasum (Pond, et al., 1995). The rumen serves as a fermentation vat and hosts a number of microorganisms (bacteria and protozoa). This modified foregut permits a pregastric fermentation step. During the microbial fermentation, the deamination of amino acids leads to the formation of free ammonia and organic acids. The ammonia/ammonium ion and acids may be either absorbed through the rumen wall or utilized by rumen microorganisms. The rumen microorganisms can utilize the ammonia and organic acids as an energy source or synthesize new microbial proteins (Asplund, 1994). The rumen microbes break down amino acids for production of energy rather than their utilization as nitrogen source for their growth. This microbial metabolism step leads to the alteration of the amino acid balance present in the dietary proteins. The fraction of proteins that escape rumen degradation and are available directly to the animals is referred to as “by-pass” protein (Satter, et al., 1975). So the total protein available for the animal is a sum of the dietary protein that escapes from the rumen degradation and the microbial proteins coming from the metabolism of rumen degradable form of the dietary protein

2.1.4.2. Bioavailability of Essential Amino Acid. Those amino acids that are not synthesized in an animal or not synthesized in amounts required by the animal are classified as the "essential amino acids". It is believed that the inability to synthesize amino acid is dependent on the animal's inability to synthesize the α-keto acid precursors. On the other hand, requirements for non-essential amino acids in animals can be met through transamination reactions as long as an adequate supply of amino groups is present. The protein requirement of animals is based on their essential amino acid requirement and the true nutritive value of a protein feed is dependent on the digestibility of the feed protein, absorbability of the particular essential amino acids from the hydrolyzed feed protein and the amino acid composition in the absorbed feed, which is also referred to as "bioavailability" of essential amino acids (Pond, et al., 1995; Cheeke, 1999).

The essential amino acids present in the concentrations lower than the required concentrations are termed the "limiting amino acids" (Pond, et al., 1995; Schwab, et al., 1992). For most of the animal species, methionine, lysine, and arginine are considered the primary limiting amino acids in proteins from plants, single cells, and other less conventional sources (Satter and Roffler, 1975; Alex and Marshall, 2000; Younge et al., 2001; Froidmont et al., 2002;). In lactating cows, milk production accounts for nearly 95% of these amino acids requirement (Guan et al., 2002). Abdominal (post-rumen) infusion of these amino acids for supplementation has also received widespread attention, especially in the cases of lysine, methionine, and arginine (Schwab et al., 1992; Robinson et al., 2000; Robinson et al., 1999).

2.1.5. Supplementation of Limiting Amino Acids. The amino acid supplementation in the animal diet is very important to meet the amino acid balance in the animal feed or to increase the productivity of the animals. The limiting amino acids can be directly supplemented along with animal diet or it can be supplemented in the form of high protein rich animal diets such as meat meal, fish meal, feather meal etc. But these high protein feeds are too expensive to be used and their amino acid composition may not meet the required composition (Perry et al., 1999)

The crystalline amino acids are direct supplementation of limiting amino acids which are produced by either fermentation or by chemical synthesis. The nutritive value of feed is enhanced by supplementation of crystalline amino acids or oligopeptides (Chalupa et al., 1975; Schwab et al., 1975). But a principal barrier to the utilization of crystalline of essential amino acid supplements is the pre-gastric fermentative step in ruminants. These amino acids are prone to the microbial degradation by rumen microbes into short chain free organic acids and ammonia which are not available to the animal in required quantities (Mathur, 2003； Asplund, 1994; Schwab et al., 1992; Onodera, 1993).

Protected amino acid is another form of the amino acids supplementation. The amino acids are encapsulated into a polymer matrix that is resistant to rumen degradation at rumen condition (pH 6.5 to 6.8). The polymer encapsulation dissolves, liberating the amino acids and making them bio-available to the animal when these protected amino acids reach the actual stomach (abomasums) due to the significant change in pH (Schwab, 1995). Because encapsulation process is complicated and costly, protected amino acid is not feasible for routine supplementations. To enhance bioavailability of amino acid supplementation, different strategies have been explored.

2.1.5.1. Tailored Oligopeptides. A tailored oligopeptide is a peptide with a group of amino acids in a desired sequence or ratio. The sequence of amino acids in a peptide determines the type of tailored peptide. It has frequently been found that a change in the sequence of amino acids in a peptide changes the nature and activity of tailored peptides.

In the animal food industry, the oligopeptides are usually synthesized from L-amino acids through chemical and enzymatic methods. Synthesis of homo or hetero oligopeptides of Met and Lys has been reported (Mathur, 2003; Bercovici et al., 1989; Sampath et al., 2002; Takanori et al., 1996). Studies have proven that ruminally protected forms of these amino acids help improve milk production (Alex et al., 2000; Guan et al., 2002; Polan et al., 1991; Rogers et al., 1989; Onodera, 1993).

Studies carried by Schwab and Pisulewski indicate that Lys must contribute about 7 percent and Met about 2.5 percent of the total amino acids in duodenal digesta to maximize protein in cow's milk (Schwab et al., 1992; Pisulewski et al., 1996). Studies have shown that supplementation of Lys or Met individually has little effect on milk production when compared to their combined addition to the ration in the proportion of 3:1 (Robinson et al., 2000; Nichols et al., 1998). This nutritional requirement can be handled if these two amino acids are given in the form of oligopeptides to avoid rumen degradation. Synthesis of the Lys and Met co-oligopeptides in 3:1 ratio (Lys to Met) has been reported in literature (Roy, 2003; Santhana, 2006; Bercovici et al., 1989). However, higher Lys content of such oligopeptides results in higher water solubility of leading to lower rumen by-pass and decreased the bioavailability to the animals.

Many studies have been conducted to decrease the ruminal degradability of protein in oilseed meals using a formaldehyde treatment. The decrease in protein degradation is considered to be the result of lowering the solubility of protein in the rumen (Spears et al., 1980; Nishimuta et al., 1974). It has been shown that protein degradation is inversely related to solubility in rumen fluid and as solubility decreases, degradation of dietary protein in rumen decreases (Hungate, 1966). It has also been shown that ruminal bacteriacan deaminate hydrophilic peptides at a faster rate than peptides that contain higher proportion of phenolic or aliphatic amino acids (Chen et al., 1987). Thus, the availability of supplemented amino acids and peptides can be enhanced by decreasing their water solubility. The amount of feed protein that by-passes the rumen is dependent not only on the quantity of more slowly degraded protein in the diet, but also on the rate of degradation of these fractions and the flow rats of liquid and solids through the rumen (Wohlt et al., 1976; Perry et al., 1999). Thereby, hydrophobic amino acid oligopeptides are more resistant to degradation than the hydrophilic amino acids oligopeptides.

2.1.5.2. Amino Acid Analogue. Amino acid analogues such as α-keto acids or α-hydroxy acids have been used for amino acid supplementation. These keto or hydroxy acids can be converted to L-amino acids by a transamination reaction (Dibner and Knight, 1984). The efficacy of this supplementation procedure depends on the resistance of amino acid analogues to ruminal microbial degradation and their subsequent absorption (Koenig et al, 2002).

2-Hydroxy-4-(methylthio) butanoic acid (HMTBA), the alpha hydroxy analog of methionine, has been used as a methionine supplement in animal food. It has a hydroxyl group adjacent to the carboxyl group, making it an α-hydroxy acid (AHA). Figure 2.4 shows the structure of methionine and HMTBA.

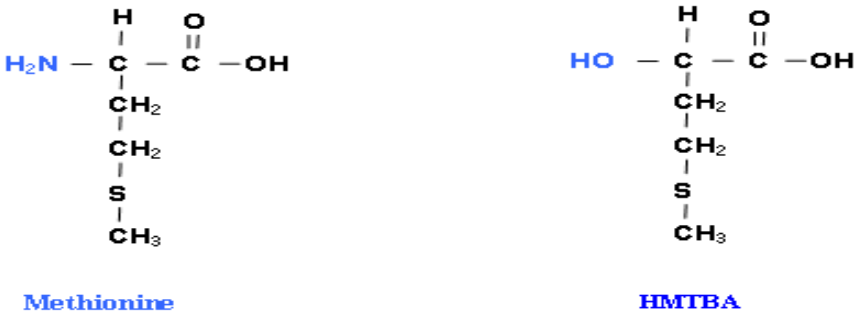


Figure 2.4: Structure of methionine and HMTBA

HMTBA was oxidized to its α-keto acid by an enzyme in the body. The α-keto acid undergoes transamination with glutamate to form the L-methionine (Dibner and Knight, 1984; Dibner, 1983; Schwab, 1995). D, L-HMTBA and D, L-methionine provides equimolar levels of methionine activity in swine, showing that D, L- HMTBA are good L-methionine substitutes in piglets (Knight et al., 1998).

HMTBA is more resistant to ruminal degradation than methionine and exits higher rumen by pass. It is readily converted to methionine via amination pathways mentioned above. Numerous studies have been carried out to evaluate the efficiency of supplementation based on the milk production in cows. Carbon-14 labeled HMTBA fed to cows showed greater persistence in the rumen fluid than methionine. HMTBA is more slowly degraded than L-methionine and increased the tissue supply of L-methionine by resisting ruminal degradation (Belasco, 1980; Patterson, 1988; Koenig et al., 1999).

Peptide structure affects peptide degradation. Most of the peptidases present in the rumen fluid are amino peptidases acting from the N termini of the peptides. Acetylation of the peptides more significantly inhibits the degradation by the rumen microorganism than do the non-acetylated peptides (Wallace, 1992, Wallace and Mckain, 1989). The presence of amino acids such as glycine and proline at the N termini lowers hydrolysis of rumen microorganisms. The N-α-acetylation of methionine and lysine dipeptide (Met-Lys) has shown improved rumen resistance (Wallace, 1992). Thus, protection of the N termini of the peptides can significantly enhance their rumen bypass of these peptides (Amos et al., 1974; Wallace et al., 1990, 1993; Broderick et al., 1988; Chen et al., 1987). To take advantage of HMTBA resistance to rumen microbial attack and enhanced amino acid bioavailability, HMTBA-capped oligopeptides of amino acids such as Met, Lys, and Arg have been developed to test their efficiency as feed supplements (Rajesh, 2003; Roy, 2003).

However, no attempt to covalently link HMTBA to Lys-Met co-oligopeptide has been reported in the literature. Since, the presence of HMTBA would increase the bioavailability in the polypeptide of the amino acids that are relatively inaccessible to ruminant microorganisms. In this study, HMTBA capping co-oligopeptides will be synthesized that would be expected possess high by-pass characteristics.

2.1.6. Peptide Synthesis. Peptide synthesis has drawn considerable attention during the recent years. The interest stems wide applicability of peptides in diverse fields, e.g. several peptides have been introduced as pharmaceuticals and several hundred peptides are undergoing clinical trials, or in advanced preclinical trials. Several approaches for

peptide synthesis have been reported these include chemical synthesis (Marder and Alberício, 2003), recombinant DNA technology (Hershfield et al., 1974), and enzymatic synthesis (Andersen et al., 1991).

2.1.6.1. Chemical Synthesis. Chemical methods are the most popular means of synthesis of peptides. Many peptides have been routinely prepared by solution and solid-phase methods (Jones, 2002; Albericio, 2004). The chemical synthesis is normally started with the main chain and side chain functionalities of the adduct-amino acid or peptide selectively protected so as not to participate in the reaction. The carboxyl component is then activated to contribute the carbonyl group and form the peptide bond by coupling with amine component via an ∝-amide linkage. The protecting groups must be removed in total if the synthesis is completed or by selective cleavage of the ∝-amine or carboxyl protection if the synthesis is to be continued. Chemical synthesis is advantageous in its suitability for large-scale production. Nevertheless, these methods have "undiminished challenge" disadvantageous mainly from the fact that the individual steps of the pathways are relatively unspecific in nature, so many side reactions also occur. Harsh conditions lead to secondary reactions such as degradation of amino acid residues. Consequently, the success of the synthesis is jeopardized by appearance of racemization and a time-consuming side chain protection/deprotection strategy, side by-products, lengthiness, cost effectiveness, and lack of specificity (Jakubke, 1994).

2.1.6.2. Recombinant DNA Technology. Recombinant DNA technology for peptide synthesis was developed in the 1970s (Jackson et al., 1972; Cohen and Chang, 1973; Morrow et al., 1974; Thomas et al., 1974). This new technology for the insertion of genes across species and genetic barriers arose as a result of many important findings in the field of genetics. The approach involves five main steps: (1) isolation of the encoding DNA fragment from the donor organism; (2) insertion of the DNA into an appropriate vector, usually a circular plasmid; (3) transfer of the vector into the host organism; (4) cultivation of the host organism (cloning), which leads to gene amplification, mRNA synthesis, and protein synthesis; and (5) isolation of the recombinant protein, expression in the host to produce the desired product. In principle, the fact that any protein or peptide can be synthesized is the major advantage of this method. However, the limitation of this method is that large-scale production is difficult, particularly for the small peptides, purification of the product from the cell lysate or the medium is not simple, and a cloned gene is not always stable over many generations. Additionally, the incorporation of unnatural amino acids is limited when recombinant DNA methods are used (Jakubke, 1994; Sewald and Jakubke et al, 2002).

2.1.6.3. Enzymatic Peptide Synthesis. Enzymatic synthesis makes use of proteases that usually act as hydrolases, catalyzing the cleavage of a peptide bond and degrading the proteins to yield amino acids. The reaction of proteolysis is irreversible because peptide bond hydrolysis is exergonic. Figure 2.5 shows the reversible equilibrium of protease action. The concept of reversibility of mass action would suggest that with proteases, synthesis of peptide bond (the reverse reaction) should be possible

under the proper conditions. Although ionic hydrolysis products are thermodynamically more stable, the fundamental suitability of proteases to catalyze the formation of peptide bond is based on the principle of microscopic reversibility (Sewald and Jakubke, 2002). All though proteases are primary protein degrading enzymes, they can be used as catalysts for peptide synthesis if the equilibrium is shifted to the right side.

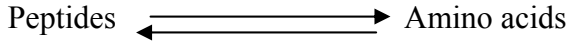


Figure 2.5: Reversible reaction of proteases on proteins and amino acids

An outline of the different steps for a rational approach to a desired enzymatic peptide synthesis has been developed (Andersen et al., 1991; Gupta, 2000). First, a suitable protease as a biocatalyst and a thermodynamically or kinetically controlled approach should be selected. The starting amino acid or peptide derivatives are then prepared. After optimizing the reaction media, the reaction kinetics, yields, and factors influencing the reaction need be studied. Protection of both the N-terminal and C-terminal ends of amino acids with suitable protecting group is needed in enzymatic peptide synthesis. The selection of the protection group should be based on the amino acid substrate and the type of the target peptide. The obtained peptide yield is dependent on the concentration and reactivity of the nucleophile, as well as the degree of hydrolysis of the peptide product.

Proteases tend to restrict their activities to one or to a very limited number of closely related substrates. As a consequence, the use of proteases as catalysts for the peptide-bond forming steps of in vitro synthesis offers ideal opportunities to suppress most of the undesired side reactions often encountered during relatively unspecific

chemical synthesis. The stereo- and regio-specificity of enzymatic synthesis has made it an attractive alternative for chemical synthesis. Racemic compounds can be used as substrates. Enzymatic reaction takes place under very mild conditions and with minimal solvent consumption. (Furutani et al., 1999; Jakubke 1994; Stryer, 1981; Lehninger, 1982.). The enzyme can be made reusable through immobilization on suitable surfaces (Gupta, 2000).

However, several disadvantages of enzymatic synthesis have prevented the application of this method in industrial production. Enzymes are highly specific, special enzymes that catalyze every kind of peptide bond are not available, so appropriate enzyme must be found for the desired reactions. Attention must be given to possible side reactions for trans-peptide formation and esterase activity of the enzymes. Enzymes are sometimes not sufficiently stable, even at ordinary temperatures (Shigeru et al., 1995; Uemura et al., 1990).

2.1.6.3.1. Equilibrium controlled. The enzyme-catalyzed synthesis of peptide bonds can be carried out under equilibrium control or kinetic control which is classified according to the type of carboxyl component used. As shown in Figure 2.6(Sewald and Jakubke et al., 2002), the carboxyl component contains a free carboxyl group in the equilibrium controlled approach, while an alkyl ester or other activated form is in the carboxyl component in the kinetically controlled approach. The energy required for the conversion of the starting components into the peptide products is fundamentally different.

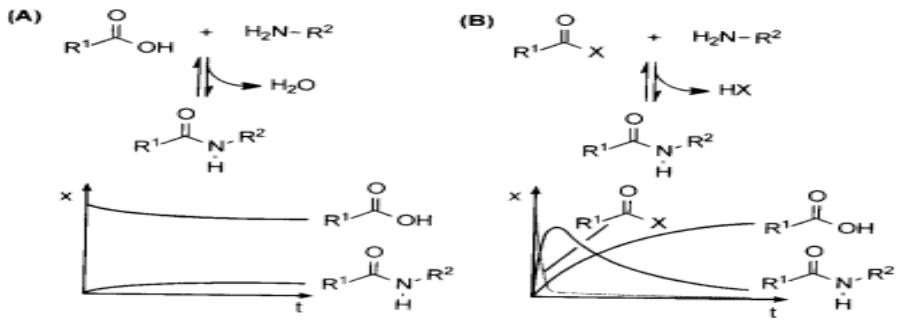


Figure 2.6: Comparison of the equilibrium (A) and the kinetically controlled approach (B) of protease-catalyzed peptide synthesis. R^1-COOH=carboxyl component; R^1-CO-X =slightly activated carboxyl component; H_2N-R^2 =amino component; HX=leaving group.

The equilibrium controlled (thermodynamic approach) represents the direct reversal of proteolysis. The large amount of enzyme required for this approach and the reaction rate is low. The ionization equilibrium must be manipulated to shift the equilibrium towards the peptide bond formation. The ionization of the α-carboxylic acid and α-amino groups can be reduced of water-miscible organic solvents is added to the aqueous reaction mixture. However, the catalytic activity of the enzyme may decrease under such conditions. Other manipulations can also be employed to reduce either the concentration of the peptide product (i.e., by precipitation or selective extraction) or increase the reaction media, for example: reaction conditions promoting product precipitation, reverse micelles, anhydrous media containing minimal water concentrations, and water mimics (Sewald and Jakubke et al., 2002; Jakubke, 1994).

2.1.6.3.2. Kinetically controlled synthesis. A kinetically controlled model of peptide synthesis through a protease-catalyzed reaction is shown in Figure 2.7. In this model an acyl transfer occurs through a two-step reaction (Sewald and Jakubke et al.,

2002). In the first reaction, an acyl donor ester (Ac-X) reacts with enzyme (a protease) to form an acyl enzyme intermediate (Ac-E). Then, a peptide or hydrolyzed product (Ac-N) is formed by the nucleophilic attack of the second substrate or water on the complex. The enzyme (EH) acts as a transferase catalyzing the transfer of the acyl moiety to the nucleophilic amino component (HN). The yield of an enzymatic synthesis is a function of the relative rates of hydrolysis and aminolysis (Chen, et al., 1992).

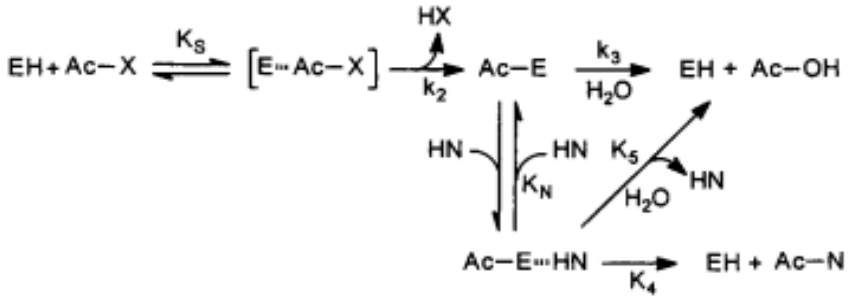


Figure 2.7: Kinetic models for protease-catalyzed acyl transfer reaction. EH = Free enzyme; Ac-X = acyl donor (carboxy component); HX = Leaving group; Ac-E = Acyl enzyme complex; Ac-OH = hydrolysis product; HN= acyl acceptor (amino component); Ac-N = aminolysis product (synthesized peptide).

In contrast to the equilibrium controlled approach, Kinetically controlled protease-catalyzed peptide synthesis requires much less enzyme, its reaction time to obtain maximum product yield is significantly shorter, and its product yield depends on both the properties of the enzyme used and its substrate specificity (Gupta, 2000). Whereas the equilibrium controlled approach ends in equilibrium, the concentration of the product formed in the kinetic approach passes a maximum before the slower hydrolysis of the product becomes important. Amidase activity of most proteases is lower

than esterase activity. The product inevitably is consumed (Sewald and Jakubke et al., 2002).

2.1.6.3.3. Proteases. Enzymes are protein molecules that play the role of biocatalysts in the organism. They catalyze the reactions of all metabolic processes. Enzymes are divided into classes, one of which is the class of hydrolases that catalyze the reaction of hydrolysis of various bonds (peptide bonds, ester bonds, etc.) with the participation of a water molecule. Proteases belong to the class of hydrolyases. Enzymes catalyze the reaction and enhance the reaction rate by one million times over the un-catalyzed reaction. Enzymes bind to the substrate molecules with their active sites and lower the activation barrier so that most of the substrate molecules convert to products (Stryer, 1981; Lehninger, 1982). Enzymes do not alter the equilibrium of a reaction, but they accelerate the rate at which the equilibrium of a reaction is achieved. The equilibrium of the reaction can be manipulated towards the proteosynthesis by shifting the ionic equilibria, by shifting the chemical equilibria, and by kinetically controlled synthesis.

Proteases occur naturally in all organisms and are involved in various metabolic processes. Acid proteases secreted into the stomach (such as pepsin) and serine proteases present in duodeum (trypsin, chrymotrypsin) enable us to digest the protein in food. Proteases present in blood serum (thrombin, plasmin, Hageman factor, etc.) play an important role in blood clotting, as well as in lysis of the clots and the correct action of the immune system. Other proteases are present in leukocytes (elastase, cathepsing) and play several different roles in metabolic control (Barrett et al., 2003; Hooper, 2002).

Most enzymes are specific on the substrates to which they exert their action. Enzymatic action can be reversibly or irreversibly ceased by inhibiting the active sites. Proteases are divided into four major groups according to the character of their active site (catalytic site) and conditions of action: serine proteinases, cysteine (thiol) proteinases, aspartic proteinases, and metalloproteinases. Attachment of a protease to a certain group depends on the structure of catalytic site and the amino acid (as one of the constituents) essential for its activity. Serine and cysteine proteases are able to form a covalent acyl-enzyme complex with an amino acid or peptide carboxyl ester as an acyl donor. However, serine and cysteine proteases are not perfect acyl transferases, so undesired reactions may take place due to their limited specificity. For example, hydrolysis of the acyl enzyme, secondary hydrolysis of the ligation product and proteolytic cleavage of other protease-labile peptide bonds present in the segments to be ligated (Sewald and Jakubke, 2002).

Papain belongs to a class of thiol proteases that have a cysteine residue at their active center. Cysteine proteases play a significant role in the immunological responses and muscular protein degradation. Among the proteases, papain is the most efficient catalyst for oligomerizing amino acids in aqueous phase (Ferjancic et al., 1991). Papain is extracted from the latex of the Carica Papaya fruit. The purified protein is present in a mixture with other enzymes such as chymopapain and papaya proteinase. Papain acts as an endopeptidase-type enzyme that randomly cuts any peptide bonds existing in the main chain. Papain has high structural stability over a wide pH range. The reaction yield is also dependent on the pH. A pH of 5 to 6 was found to be a good range for papain (Anderson and Luisi, 1979). The properties of amino acids and their binding on either side of the

groove hosting the active site and their corresponding interaction with the substrate molecule dictate the specificity of enzymes (Berger and Schechter, 1970).

Papain has proven to be a versatile protease for both thermodynamically and kinetically controlled reactions for the synthesis of a variety of peptides, due to its broad specificity for amino acid side chains (Stehle et al., 1990; Stevenson et al., 1991). Papain is reported to be 23 KD proteins and consists of 212 amino acid residues divided among two domains. The left domain (L-domain) consists of residues 12 to 112 and 208-212 and the right domain (R-domain) is comprised of residues 1-11 and 113-207, As shown in Figure 2.8 (Kamphuis et al.,1984). The L-domain is mainly α-helical and the R-domain is made of twisted antiparallel β-sheets. The active site of the enzyme is located in a cleft situated between the two domains. The amino acid residue responsible for the catalytic action is cysteine residue (Cys25 for papain). An acyl enzyme intermediate is formed through the essential "thiol" group of cysteine residue. Another amino acid playing a crucial role in the enzyme mechanism is the histidine. The hydrogen bonding of the thiol group of cysteine and the imidazole group of His159 (papain) leads to a high degree of polarization. The catalytic Cys-25 residue is provided by the L-domain, while the His-159 is provided by the R-domain. These residues are responsible for the catalytic activity of papain (Lowe and Yuthavong, 1971). The degree of polarization may be further enhanced by the ester and amide linkages present in the substrate molecule. A tetrahedral intermediate is formed between the thiol moiety of enzyme and the carbonyl moiety of the substrate. The hydrolytic step in the deacylation process is a general base catalyzed (imidazole) reaction.

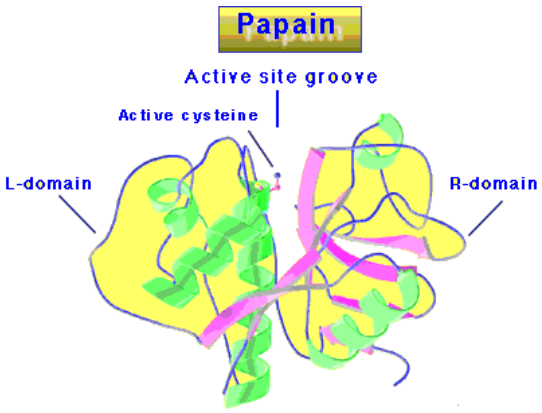


Figure2.8: Structure of papain

Peptide synthesis catalyzed by cysteine proteases requires the presence of an anti-oxidant in the reaction medium to ensure enzymatic activity. Figure 2.9 shows the structure of cysteine. It is an amino acid containing an active thiol group. Thiol groups are prone to oxidation and a reducing agent is required for them to exist in thiol form. Mercaptoethanol has been used as a reducing agent for papain catalyzed hydrolysis and synthesis (Fergancic-Biagini et al., 1993; Kimura et al, 1990). However, the high toxicity of mercaptoethanol has limited its application in the food industry as a trace residue of mercaptoethanol is left in the product. L-Cysteine was used as antioxidant for papain catalyzed synthesis of oligopeptides of Leu, Met, Phe and Tyr in citric acid buffers with yields ranging from 51 to 96% (Anderson and Luisi, 1979).

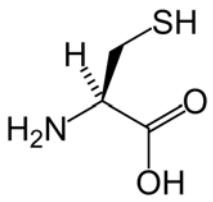


Figure 2.9: Structure of the Cysteine Molecule

2.1.6.3.4. Enzymatic Synthesis Reaction Media. Enzymatic peptide synthesis has been carried out in monophasic aqueous, bi-phasic, and tri-phasic reaction media (Mathur, 2003). For hydrophilic amino acids, thermodynamically controlled synthesis of enzymatic oligopeptides in an aqueous medium cannot be carried out because the product is soluble in aqueous media. Consequently, oligopeptides are susceptible to the hydrolysis. To overcome this problem, oligomerization of hydrophilic amino acids such as lysine is traditionally done in a biphasic medium where the product can distribute them in the water-organic inter-phase. In three-phase reaction system, sandwiching the water between two organic phases enhances this phenomenon.

Higher reaction rates have been reported in water organic monophasic systems because of the reduction in mass-transfer resistance of bi-phasic systems from the inadequate partitioning of the hydrophobic substrates through the aqueous layer are uncommon (Hideo et al., 1990; Mitin, 1988). The synthesis of both polar and non-polar amino acid oligopeptides can be achieved in aqueous organic solvent systems with proper manipulation of the water content of the reaction medium. When the reaction is carried out in such solvent systems, low water content media are highly attractive in kinetically controlled peptide synthesis because they minimize the secondary hydrolysis of the product peptide (Clapes et al., 1999; Clapes et al., 1995). The insolubility of proteases in such monophasic medium seems to sieve them from the undergoing severe inactivation and keep the enzyme locked up in its native orientation, thereby maintaining its catalytic activity (Kazandjian et al., 1986). Monophasic solvent systems increase stability of enzyme to thermo-inactivation. Covalent alterations in the primary molecular structure and the partial unfolding of the enzyme lead to its inactivation at high temperatures in

aqueous media (Tanford, 1968; Freeney, 1980; Ahern et al., 1985). In addition, Monophasic reaction media hold ease of enzyme recovery eliminating/reducing the need for immobilization and low microbial contamination (Cremonesi et al., 1974). These advantages make aqueous organic monophasic reaction media an ideal choice for synthesis of targeted/tailored peptides.

A low water content favors enzyme stability, mass transfer of hydrophobic substrates to biocatalyst phase and the thermo-dynamic equilibrium in reversed hydrolytic reaction. However, Enzymes are usually denatured and inactivated in the presence of organic solvents (Ogino et al., 2001). Selection of proper solvent for a reaction has been a critical point. Solvents have been characterized by many parameters. The best for evaluating the suitability in bioorganic syntheses so far has been the partition coefficient (P) in an octanol/water two-phase system. It has been concluded that the use of enzymes in solvents with high log P values have the stability, i.e. solvents of very hydrophobic character (Reslow et al., 1987 and 1988). Studies showed observed relation between enzyme behavior concerning activity and stability and the properties of the organic solvent used was severely influenced by the amount of water dissolved in the organic solvent. Papain exhibited high stability in aqueous acetonitrile, ethanol and 1, 4-dioxane. Decreases in the activity of the enzyme were observed at organic solvent concentrations above 60%. Tetrahydrofuran (THF) caused a dramatic reduction in activity even at low concentrations (5-10%). At 90% ethanol and 90% acetonitrile, papain exhibited an increased amount of the α –helical conformation, with little change in the tertiary structure (Szabo et al., 2006). In this study, acetonitrile was chosen to be the water/organic monophasic reaction media.

Papain-catalyzed oligomerization of Met, Tyr, Lys, and Gly in monophasic aqueous organic media has been reported comprising of varying amounts of acetonitrile/water. The optimal condition for Lys and Gly oligomerization was around 10 (%v/v) of water in acetonitrile while the oligomerization of Met and Tyr proceeded when the water content of the reaction medium was equal to or greater than 15 (%v/v) of water in acetonitrile. This unique property of monophasic solvent systems could be advantageous in the synthesis of co-oligopeptides of Lys-Met with tailored composition.

2.2. OBJECTIVES.

The overall objective of this research was develop and evaluate efficacy of papain catalyzed synthesis approach for obtaining C and N terminal end protected high rumen by-pass oligopeptides comprised of Lys and Met, the two principle limiting amino acids in bovines. The overall objective was met through a series of experiments carried out to meet specific sub-objective:

1. Papain catalyzed synthesis of Lys – Met oligopeptides in monophasic systems comprised of water and acetonitrile.

2. Monitor the effect of substrate ratio and substrate introduction period on the oligopeptide composition.

3. Optimization of reaction parameters that yield oligopeptides with a protected C terminal (an intact ester) end.

4. Development and evaluation of chymotrypsin catalyzed addition of HMTBA to the N terminal end of oligopeptides obtained through papain catalyzed synthesis.

5. Assess solubility of C & N terminal protected tailored Lys-Met oligopeptides.

6. Assess rumen by-pass potential of the tailored peptides.

2.3. EXPERIMENTAL

2.3.1. Materials. L-lysine (Lys), L-lysine ethyl ester (LysEE) dihydrochloride, L-methionine (Met), L-methionine ethyl ester (MetEE) hydrochloride, L-arginine (Arg), L-arginine ethyl ester (ArgEE) dihydrochloride, N,N diisopropylethylamine (DIPEA), L-cysteine, and trifluoroacetic acid (TFA), and papain (EC 3.4.22.2, twice crystallized suspension, 25 units activity/mg, 28mg protein/ml at pH 4.5) were purchased from Sigma Chemical Co.(St. Louis, MO.). Sodium salt of hexane sulfonic acid, Absolute ethanol, methanol (anhydrous and HPLC grade), and acetonitrile (HPLC grade), and 12N Hydrochloric acid were obtained from Fisher Scientific, (St. Louis, MO)2-hydroxy-4-(methythio) butanoic acid (HMTBA) was obtained from Novus International, Inc. of St. Louis, Missouri. Chymotrypsin (C4129) was obtained from Sigma-Aldrich (St. Louis, MO). The nanopure water used in the experiments was obtained from a Synergy 185 filtration unit manufactured by the Millipore Corporation, (Billerica, MA.).

2.3.2. Synthesis and Quantitation of Ethyl Ester of Amino Acids and Hydroxy Acid.

2.3.2.1. Synthesis of Amino Acids and Hydroxy Acid Ethyl Esters. Approximately 50 grams of L-Met, L-Lys, L-Arg, and HMTBA were separately weighed out and added to a 1 L three neck round bottomed flask. To the same flask 500 mL of anhydrous ethanol was added. A reflux condenser was connected to the middle opening of the flask. Anhydrous hydrous hydrogen chloride gas was introduced into the flasks through one of the side openings. And bubbled through the reaction mixture for

30minutes. The reaction mixture was stirred by a magnetic bar, heated to ~85 ºC with heating mantles. The content of the flasks were refluxed, aliquot of the reaction mixture was taken out at 2, 4, 6, and 8 hours. After proper dilution with methanol, the sample was injected into HPLC. Ethyl ester formed through the reaction was recovered by removing the excess ethanol via rotary evaporation. The residue ester and residual amino acid from rotary evaporation was then poured into a flat plate. The residual ethanol in the product was allowed to evaporate in a fume hood overnight. Aliquots of the products were dissolved in methanol, solutions were centrifuged and then filtered through 0.22µm membrane filters. A 10µL portion of the filtered sample was injected into a reverse phase C-18 column installed in HPLC-UV system. HPLC separations were carried out with a bench top HPLC system, model L-7000 from Hitachi Instruments Inc. (San Jose, CA) The HPLC system consisted of a reciprocating piston pump (L-7100), a column oven (L-7300), an auto sampler (L-7200) with a 50µL injection loop, and a diode array UV-Vis absorbance detector (L-7420). A proprietary HPLC software supplied by Hitachi Instruments Inc. was used for control of the system and data collection. Separations of free amino acids, free HMTBA and their respective ethyl eaters were achieved with 250mm X 4.6mm ID column packed with a stationary phase comprised of octadecyl silane covalently bonded on 5µm silica gel particles which were purchased from P.J. Cobert Associates, Inc. (St. Louis, MO).

2.3.2.2. Quantitative Determination of Lys, Arg, and Their Ester. Quantitative determination of hydrophilic amino acids (lysine and arginine) and their esters was carried out with ion pair RPLC on the C-18 column. The separations were achieved with

a gradient program in which mobile phase composition was changed from 100% mobile phase A (water with 0.5mM 1-hexanesulphonic acid and 0.1% orthophosphoric acid) to 75% mobile phase A and 25% mobile phase B (50:50 acetonitrile to water mixture with 0.5mM 1-hexanesulphonic acid and 0.1% orthophosphoric acid) in 30min. The mobile phase flow rate was maintained at 1mg/mL.

2.3.2.3. Quantitative Determination of Met, HMTBA and Their Ester. The separations of Met, HMTBA and their esters were achieved with RPLC on the C-18 column. Separations were facilitated through gradient elution in which mobile phase composition was changed from 100% A (Water + 0.1% TFA) to 60 % A and to 40% B (Acetonitrile) in 15min. The mobile phase flow rate was maintained at 1mL/min. The absorption wavelength was set at 210 nm.

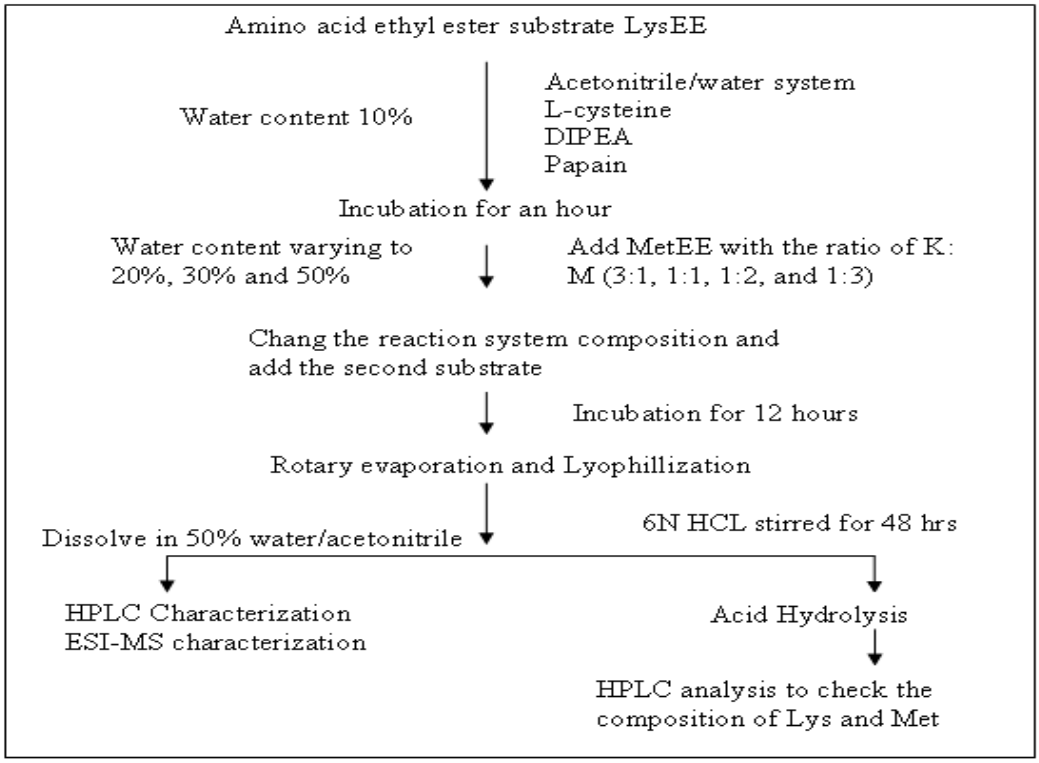
2.3.3. Evaluation of Papain Stability in Monophasic Reaction Media. Since the stability of the enzyme in the reaction system is of concern, the activity of papain in acetonitrile/water system was first studied. The free papain was dissolved in 5mL of the acetonitrile/water mixture with the water content varying from 5%, 10%, 15%, 20%, 30%, and 50% (v/v). The enzyme was recovered from solution after period varying between 1 – 24 hours by removing the solvent mixture with rotary evaporator. The activities of the recovered enzymes were evaluated through Lys and Met oligomerization in appropriate reaction systems.

2.3.4. Synthesis of Oligopeptides, Co-Oligopeptides and HMTBA Attached Co-Oligopeptides. Homo-oligomers of Lys and Met were first synthesized with papain in the water/ acetonitrile monophasic reaction media to check the papain stability and the feasibility of monophasic reaction media. Then Lys-Met co-oligomers were synthesized in the same system with sequential addition approach. Finally, HMTBA was attached to the Lys-Met co-oligomers with chymotrypsin in the buffer.

2.3.4.1. Papain Catalyzed Synthesis of Homo-oligopeptides from Met and Lys in Monophasic Reaction Media. 0.5mM L-Lys EE dihydrochloride (123.6mg) and 0.5mM L-MetEE hydrochlorie (106.9mg) were separately added to a reaction vial containing 5mL of the acetonitrile water mixture with 5%,10%, 15%, 20%, 30%, and 50% (v/v) water in acetonitrile. 20mg of L-cysteine 100µL of DIPEA and 30mg of papain were added to this vials. The vials were placed in an incubator for 24 hours, incubator temperature was maintained at 37 ºC. To end the reaction vials were taken from the incubator and placed in a water bath at 80^{o} C for 10 minutes. The vials were transferred to a centrifuge and spun at 3,500 rpm for 10 minutes, the supernatant in vials was separated from the precipitate and transferred to 25 mL round bottom flasks and brought dryness with a rotary evaporator. The precipitate was lyophilized. Dry residues from the supernatant and the precipitate were reconstituted in 50% ethanol water mixture. The solutions were centrifuged to remove suspended matter, filtered through a 0.22 µm filter, and 10 µL aliquots were introduced into HPLC system. The solutions were also analyzed with an ESI-MS system.

2.3.4.2. Papain Catalyzed Synthesis of Lys-Met Co-oligopeptides in Monophasic Reaction Media. A sequential addition strategy was adopted for the synthesis of Lys-Met co-oligopeptides in an acetonitrile-water monophasic reaction media. Table 2.2 is the schematic of the strategy adapted for enzymatic synthesis of tailored peptides in a monophasic reaction media.

Table 2.2: A simple schematic of the strategy adapted for enzymatic synthesis of tailored peptides in monophasic reaction media.



The substrate concentrations were varied from a relative molar ratio 3:1, 1:1, 1:2, and 1:3 of LysEE to MetEE. Lys ester was added first to a 7mL amber reaction vial. The reaction was started with water content favoring the oligomerization of Lys with a 10:90 of water/acetonitrile system. To each vial, 0.5mL water and 4.5mL ACETONITRILE was

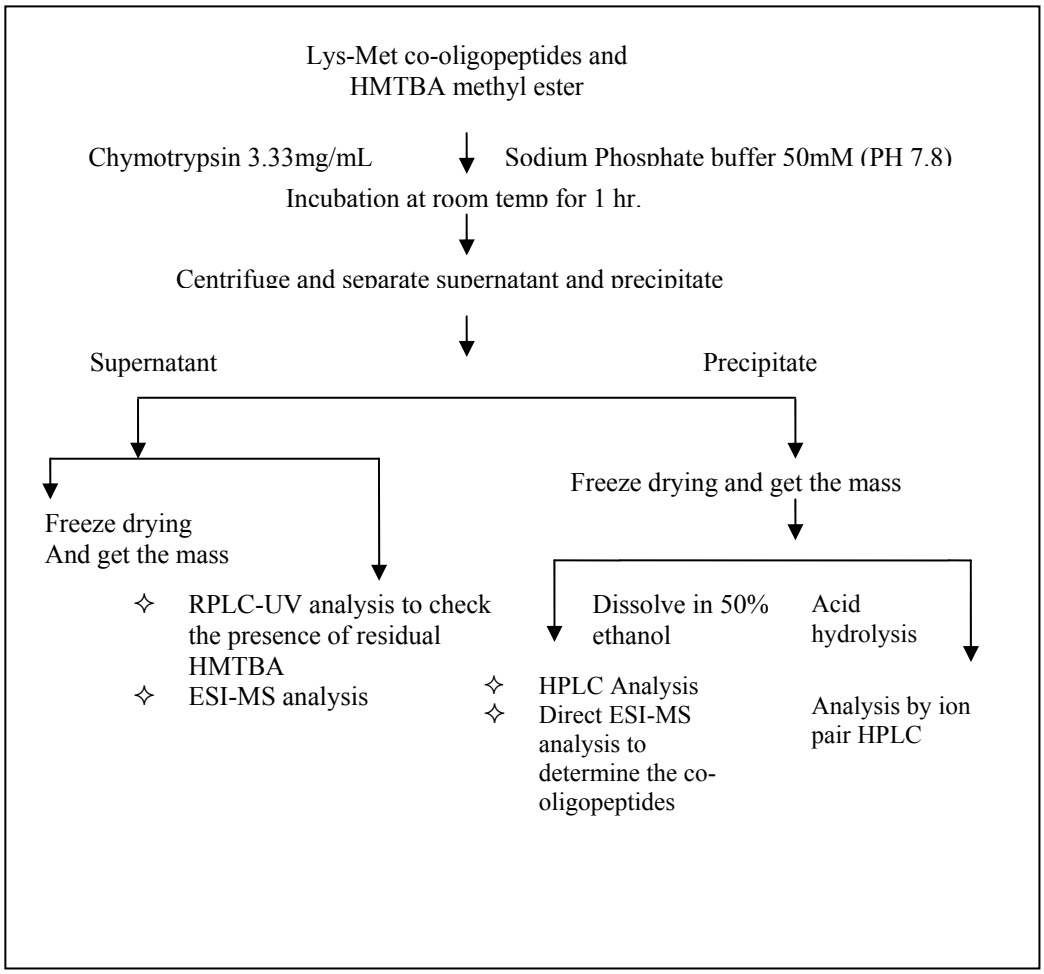
added. Then 20.0mg of L-cystein and 100uL of DIPEA was also added to the mixture. Finally, 30.0mg of papain was added to the reaction mixture to catalyze the oligomerization reaction. The vial was closed with a PTFE lined screw cap and placed in an incubator. After an hour minute period, MetEE was added as the second substrate and the additional water was added.

The reaction system water/acetonitrile content ratio was varied from 10:90 to 20:80, 30:70 and 50:50. The vial was closed and placed in the incubator for 12 hours. After the set incubation period, the reaction was terminated by heating the reaction mixture 80 ºC for 10 minutes. The reaction mixture was brought to near dryness with a rotary evaporator. The product was brought to complete dryness with a freeze drier. A 50 mg aliquot of product was transferred to a 7 mL vial containing a 5mL water-ethanol mixture (50:50). Contents of the vial were shaken for 5 minutes to dissolve the reaction product. A 1mL aliquot of the solution was transferred to another 7 mL vial, then, 4mL of water-Aetonitrile (50:50) was added to the vial. The contents of the vial were shaken and mixed with a vortex mixer. The solution was then centrifuged. The supernatant was passed through a 0.2 μm filter and collected in a 7 mL vial.

2.3.4.3. Chymotrypsin Catalyzed Synthesis of HMTBA Capped Co-oligopeptides of Lys-Met. 50mg of purified Lys-Met oligomers and 50mg of HMTBA-ME was weighed out and taken to a an amber reaction vial containing 5mL of 50mM sodium phosphate (dibasic) buffer solution at a certain pH. To this vial, 250μL (50 enzymatic units) of chymotrypsin solution (3.33mg/mL of sodium phosphate-dibasic) was also added. This reaction mixture was incubated in a shaker for 15, 30, 45, and 60min at room temperature. Table 2.3 shows the Schematic of enzymatic synthesis of

HMTBA capped co-oligopeptides. A 250µL aliquot was taken out at each incubation period and diluted proportionately, centrifuged, and filtered. Then, 5µL aliquot of the filtered product was injected directly into ESI (+) -MS to determine the product profile. The same procedure was repeated with HMTBAEE as the substrate.

Table 2.3: Schematic of enzymatic synthesis, enzymatic hydrolysis of HMTBA capped co-oligopeptides



2.3.4.4. Purification of Lys-Met Co-oligopeptides and HMTBA Capped Lys-Met Co-oligopeptides. The co-oligopeptides present in the precipitate of the reaction mixture were separated from the supernatant containing the residual co-oligopeptides and monomers. The organic solvents were removed by rotary evaporation and then the sample was subjected to dryness using freeze dryer. The freeze-dried oligopeptides were then washed three times with 10mL of water/ethanol to remove any residual monomers and smaller cooligopeptides of Ly-Met. This washed precipitate was then lyophilized to obtain pure co-oligopeptides.

2.3.5. Characterization of Homo-oligopeptides, Co-oligopeptides and HMTBA Attached Co-oligopeptides. HPLC technology was used to determine the oligomerization yield of monomers. To character the oligopeptides, direct ESI-MS was used to analysis the residues of the oligopeptides. HPLC was used to obtain the composition of each monomer joined the oligopeptides chain.

2.3.5.1. HPLC Analysis of Homo- and Co-oligopeptides. The separation of Lys homo-oligopeptides, Lys-Met and Arg-Met co-oligopeptides and residual monomers was achieved in a reverse phase C-18 column (250mm x 4.6mm i.d, 5μm), then detected with a fixed wavelength UV-Vis detector (Hitachi Instruments). 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. The separated analytes from the column were monitored at 210nm. In all cases, the mobile phase flow rate was maintained at 1mL/ min. 10μL of the sample after filtration with a

0.22µ membrane filter was injected into the column. The percentage yield of the reaction was monitored by estimating the residual amount of monomer of Lys and Met ethyl ester left un-reacted. Met homo-oligopeptides were dissolved in 70% acetonitrile. The separation was achieved with a gradient program in which mobile phase A (Water + 0.1% TFA) was changed to 80% of B (acetonitrile + 0.1% TFA) in 50min. The mobile phase flow rate was maintained at 1mL/min. The eluent coming from the column was introduced into UV detector) set at 210nm.

2.3.5.2. ESI-Mass Spectrometry Characterization of Oligopeptides. A Hitachi M-8000 ion trap and a Varian 1200 triple quadruple mass spectrometry system with electrospray ionization interface were used to characterize synthesized Lys-Met co-oligopeptides. A 0.5mg/mL Lys-Met co-oligopeptides solution was obtained by dissolving the synthesized co-oligopeptides in 50% ethanol in distilled water. The supernatant and precipitate of synthesized HMTBA capped Lys-Met oligopeptides was diluted with 50% ethanol to form a 0.5mg/mL solution. The sample with a make-up solution comprised of 50% acetonitrile in water with 0.1% acetic acid was infused at a flow rate of 0.2mL/min into the MS system using a syringe pump (Harvard Apparatus). The operating parameters of the 3D Q-Ion Trap were as follows: +3.5KV electrospray capillary voltage; 400V detector voltage; 200^{o} C assistant gas heater temperature; and 150 ^{o} C the aperture-1 temperature respectively. The 3D Q- Ion Trap mass analyzer was scanned from 100–1500amu.

2.3.5.3. Analysis to Determine the Composition of Co-oligopeptides and HMTBA Attached Co-oligopeptides. To estimate the composition of each amino acid and/or hydroxyl acid present in the oligopeptides, the oligopeptides were hydrolyzed to free amino acid by strong acid. Approximate 100mg of oligopeptides was placed into 20mL amber vial containing 5mL of 6N hydrochloric acid. The contents were stirred and digested at 110ºC on a sand bath for 120 hours. A 1mL aliquot of the digest solution was transferred into a 25mL round bottomed flask. The solution was dried with a rotary-evaporator and reconstituted with 5mL 50% acetonitrile-water. This sample was diluted further and filtered through 0.22µm membrane filters. A 10µL portion of the sample was used for HPLC analysis of the amounts of each amino acid present in the oligopeptides. The separation was carried out using a gradient program in which mobile phase A (water + 10mM hexane sulfonic acid + 0.1% o- phosphoric acid) was changed from 100% to 25% of mobile phase B (50% water + 50% acetonitrile + 10mM hexane sulfonic acid + 0.1% o- phosphoric acid) in 30min. A 10µL portion of the sample was introduced into C-18 column (250mm x 4.6mm i.d., 5µm). The mobile phase flow rate was maintained at 1mL/min. The eluent from the column was introduced into a fixed wavelength UV detector monitored at 210nm.

2.4. RESULTS AND DISCUSSIONS

2.4.1. Synthesis of Ethyl Ester of Met, Lys, Arg, and HMTBA. Amino acid esters were used as substrates in the enzymatic synthesis reactions. The amino acid esters have a stronger acylating power than the corresponding amino acids. They also have higher potential energy then the corresponding amino acids, so they are more likely to

overcome the activation energy hurdle. The first step was to synthesize amino acid esters. The ethyl esters of amino acids and HMTBA were carried out with amino acid and anhydrous ethanol in presence of an acid catalyst (hydrogen chloride gas).

The acids and esters products were monitored and separated well by RPLC. Because additional amine group in the R group makes Lys a hydrophilic amino acid that is not usually retained on a regular reverser phase C18 column. Hexane sulfonic acid was added as a counter ion for the protonated amino group to increase the retention time of Lys. The LC chromatograms of acid substrates and the esterification reaction product of MetEE, ArgEE, and LysEE are shown in Figure 2.10. The reaction yield of ethyl esters was determined based on the residual free acid at the end of the reaction, calculating by the following equation:

Percent ester yield = Peak area of the ester/ (peak area of the ester + Peak area of the amino acid) x 100.

The results show that the concentration of free amino acid in the reaction mixture decreased rapidly in the presence of hydrogen chloride gas. Less than 10% of the initial amino acid added to the mixture was found after a 3 hours incubation period. Figure2.11 showed that the esterification yield varied from 2 to 8 hours. After a 4 hour incubation period, the residual free acid concentration was less than 5% of the initial acid concentrations in the reaction product mixture. Thus, the ester yield was larger than 95%. The data clearly shows that amino acid esters can be readily obtained in high yields through acid catalyzed esterification reaction.

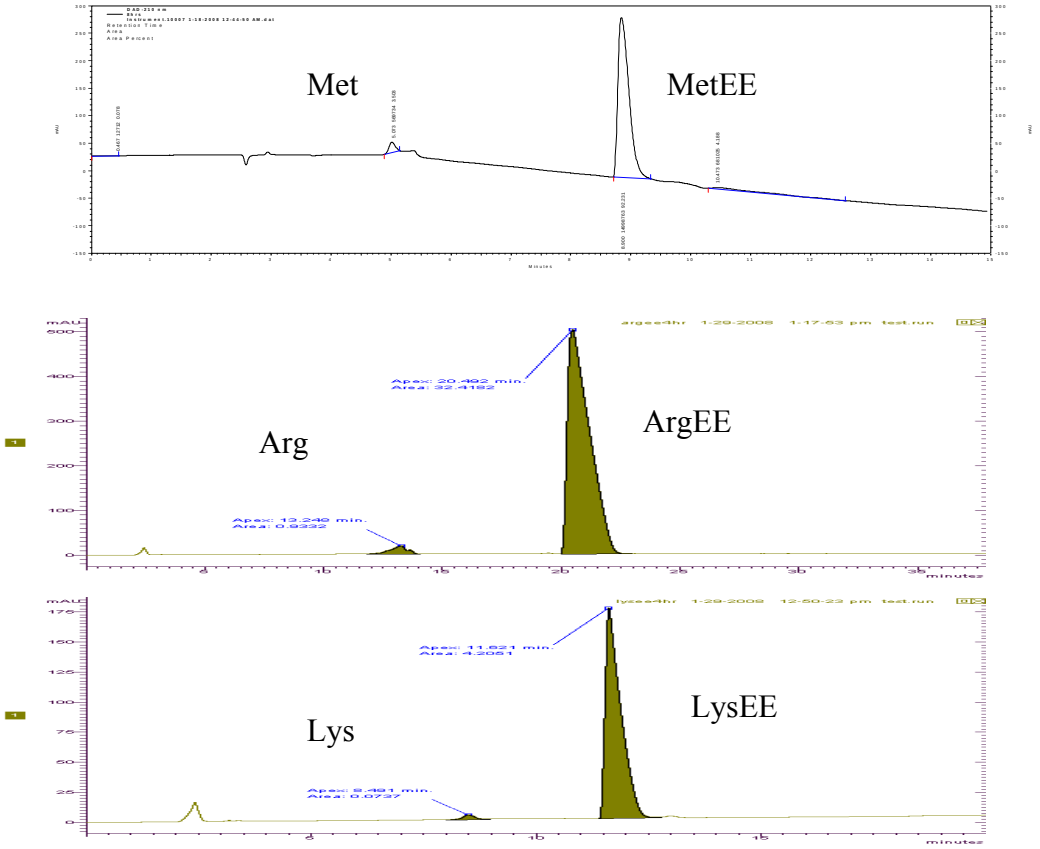


Figure 2.10: RPLC separation amino acid and ester after esterification

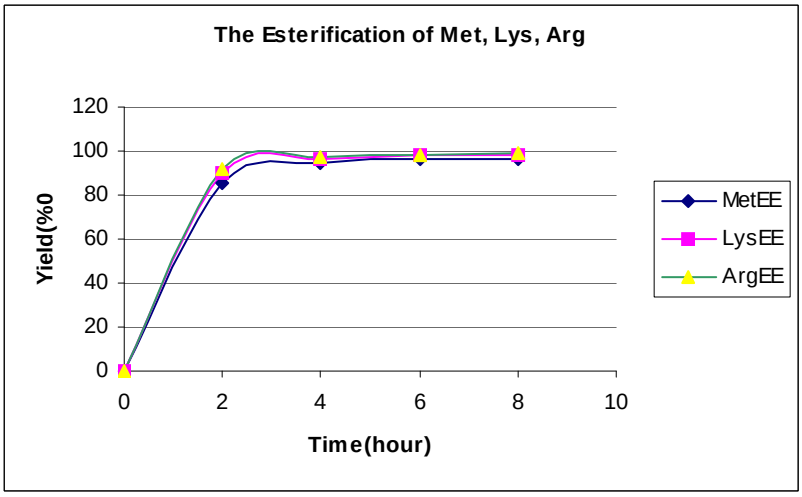


Figure 2.11: The esterification yield of Met, Lys, and Arg with the time varying

2.4.2. Evaluation of Papain Stability in Monophasic Reaction Media. It has been shown in several cases that activity and stability of the enzymes are better in organic solvents than in water. Since enzymes are insoluble in most organic solvents, the primary concern about papain in a acetonitrile-water system is their relative instability. The stability of papain in 5%, 10%, 15%, 20%, 30%, and 50% water-acetonitrile systems was studied. Papain was incubated in these water/acetonitrile mixtures for 24hrs. The HPLC chromatogram shown in Figure 2.12 A) and B) corresponds to Lys oligopeptides synthesized in water/acetonitrile system using papain recovered from acetonitrile/water solvent mixture at 24hrs and from fresh papain. The chromatogram shows no marked deactivation of papain in this system. The chromatogram contains a series of peaks eluting after the retention time of LysEE that correspond to residues of oligomers of Lys.

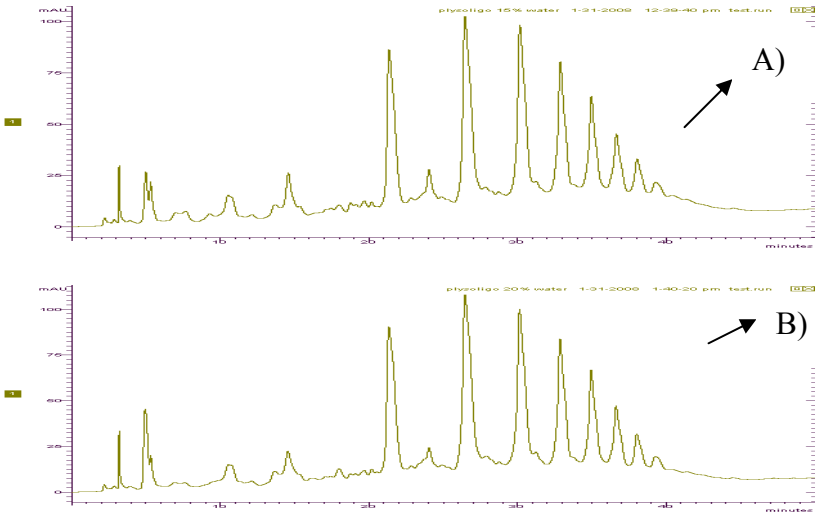


Figure 2.12: HPLC chromatogram of Lys homo-oligopeptides synthesized in 10% water/acetonitrile system A) with recovered papain from 10% water/ acetonitrile system. B) with fresh papain.

The ESI-MS spectrum of Lys homo-oligopeptides synthesized in a 10% water-acetonitrile system was shown in Figure 2.13. The m/z 303, 431, 559, 687, 815, 943, 1071, 1199, and 1327 are corresponding to oligo-Lys residues with intact ester at the C-terminal from $(Lys)_2COOEtH^+$ to $(Lys)_{10}COOEtH^+$. The m/z 403, 531, 659, 787, 915, 1043, 1171, 1299 are corresponding to oligo-Lys residues with free acid end at the C-terminal from $(Lys)_3H^+$ to $(Lys)_{10}H^+$. The ions were 128amu apart representing a repeating Lys moiety. Trimer, tetramer, pentamer with free acid and ester at C-terminal end are the dominant ions in the spectra. Since most of the peptide synthesis described in this research was carried out over 24hrs, the decrease in enzyme activity was not monitored beyond this time to obtain any half-life information on proteolytic activity as has been reported in many cases.

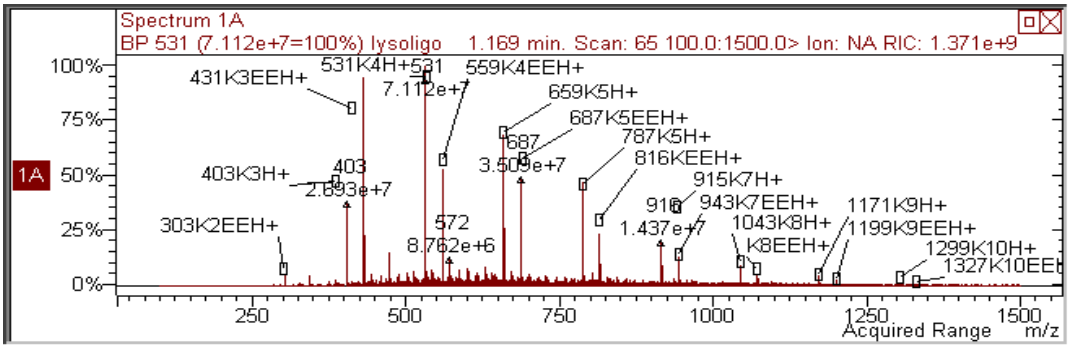


Figure 2.13: The ESI-MS spectrum of Lys homo-oligopeptides synthesized in 10% water-acetonitrile monophasic reaction media.

2.4.3. Lys, Met Homo-oligopeptides Synthesized with Papain in Monophasic Reaction Media. The enzymatic synthesis strategies were undertaken over the years to pull the equilibrium of enzymatic hydrolysis towards the reverse side by removing a component involved in the equilibrium from the system concerned. According to the laws

of mass action the equilibrium can be shifted either side. In the case of hydrophobic amino acids in aqueous media, peptides are precipitated out because they are more hydrophobic than the reactant monomers. Therefore, if the product is precipitated out, then the equilibrium can be pulled towards the product side. In case of Hydrophilic amino acids, the products are soluble in the aqueous media and the yield decreases sharply in aqueous medium. The use of an organic solvent could pull the equilibrium to the peptide side. In the case of co-oligopeptides of Lys and Met, the strategy was undertaken to synthesize Lys hydrophilic amino acid oligopeptides in acetonitrile with low water content and synthesize of Met hydrophobic amino acid oligopeptides with high water content.

Before synthesis of Lys-Met co-oligopeptides, the suitable water/acetonitrile reaction system was optimized for individual oligomerization of Lys and Met homo-oligomers. 5%, 10%, 15%, 20%, 30%, and 50% (v/v) water/acetonitrile monophasic reaction media was tested in this study. Dried Lys oligopeptides and Met oligopeptides from both the supernatant and the precipitate were then separately reconstituted in 50% (v/v) ethanol/water and 70% (v/v) acetonitrile/water and analyzed by HPLC. Orthogonal information was obtained by analyzing the resultant products in ESI-MS. Figure 2.14 shows the HPLC chromatograph of Lys homo-oligopeptides precipitate synthesis in a) 5%, b) 10%, c) 15%, d) 20%, e) 30%, and f) 50% water-acetonitrile systems.

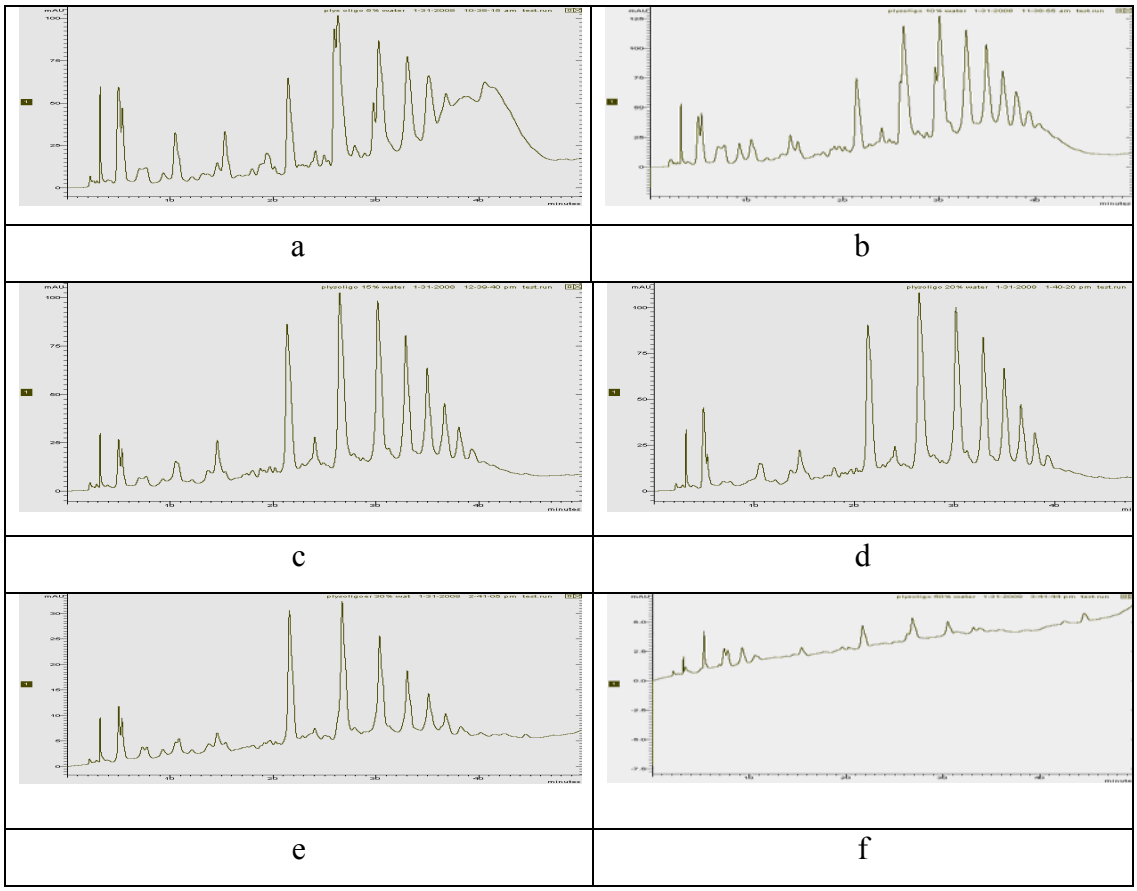


Figure 2.14: HPLC chromatograph of Lys homo-oligomers precipitate synthesized in a) 5%, b) 10%, c) 15%, d) 20%, e) 30%, and f) 50% water/acetonitrile system

The chromatogram contains a series of peaks eluting after the retention time of LysEE that correspond to residues of oligomers of Lys. The oligomerization yield of Lys was higher in b)10%, c) 15%, d) 20% water/acetonitrile system than that in the a) 5%, e) 30%, and f) 50%. It is speculated that papain denatured in 5% water/acetonitrile systems contribute the lower yield of oligomerization. For 30% and 50% water/acetonitrile system, the system tended to behave in a manner similar to a 100% aqueous system, resulting in an increased hydrolysis of preformed Lys oligopeptides that lowered the net oligomerization of Lys. Oligomerization of Lys decreased as the water content was

increased in the acetonitrile system. The data proved that oligomerizaiton of Lys prefers in the lower water content system.

Figure 2.15 shows the ESIMS spectra of Lys oligomers synthesized in a) 5%, b) 10%, and c) 20% water/acetonitrile reaction media. Lys oligopeptides of Lys3- Lys10 are formed in the reaction media. In the 5% and 10% water/acetonitrile system, as long as the oligopeptides with free acid ends are shown, the corresponding oligopeptides with ester at C terminal end are also shown up. The oligopeptides with free acid end are more dominant than the oligopeptides with the ester end. However, when the water content is larger than 15% in the reaction media, most oligopeptides are produced with free acid end.

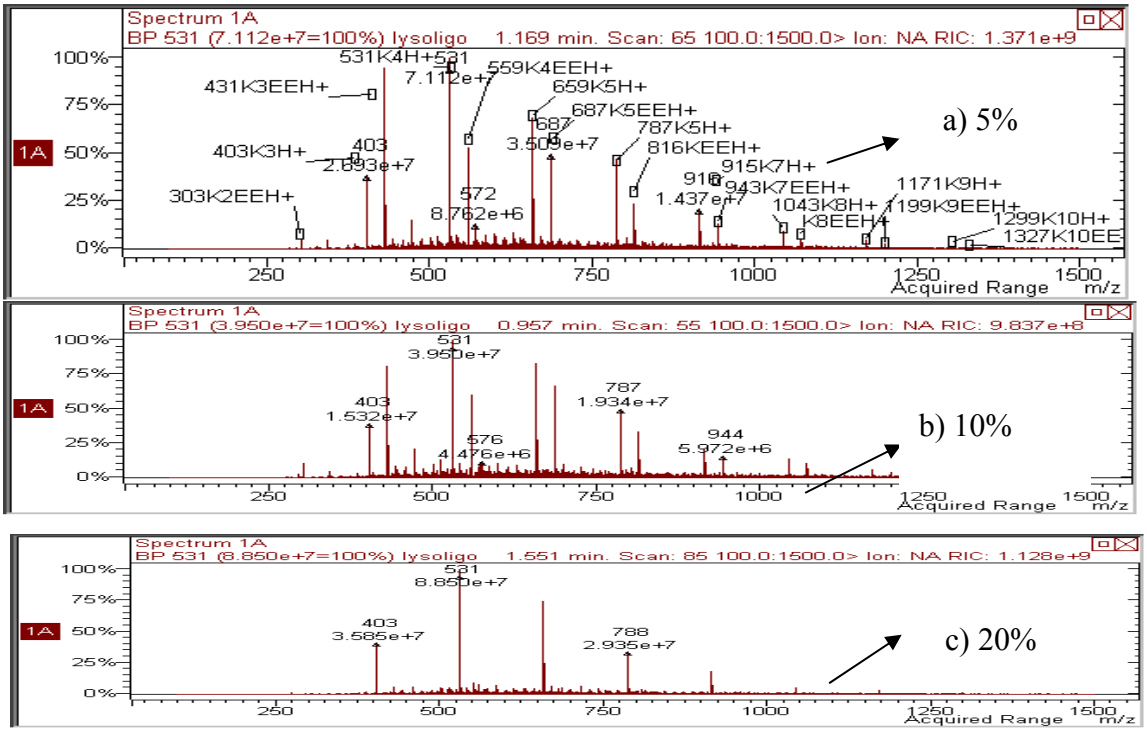


Figure 2.15: ESI-MS spectrum of Lys oligopeptides precipitate synthesized in a) 5%, b)10%, and c) 20% water/acetonitrile system.

Figure 2.16 shows the ESIMS spectra of Met oligopeptides synthesized in a) 10% and b) 20% water/acetonitrile system. Met oligopeptides of Met_3-Met_8 are shown in all the spectra. And in the 5%, 10%, and 15% water/acetonitrile reaction media, as long as the oligopeptides with free acid end are shown, the corresponding oligopeptides with ester ends are also shown up. Oligopeptides with an ester end are more dominant than the oligopeptides with free acid end. When the water content is lager than 20% in the media, most oligopeptides are produced with free acid at C terminal end. It is supposed that the oligopeptides with esters at C terminal end are hydrolyzed to oligopeptides with free acid at C terminal end in the system with higher water content.

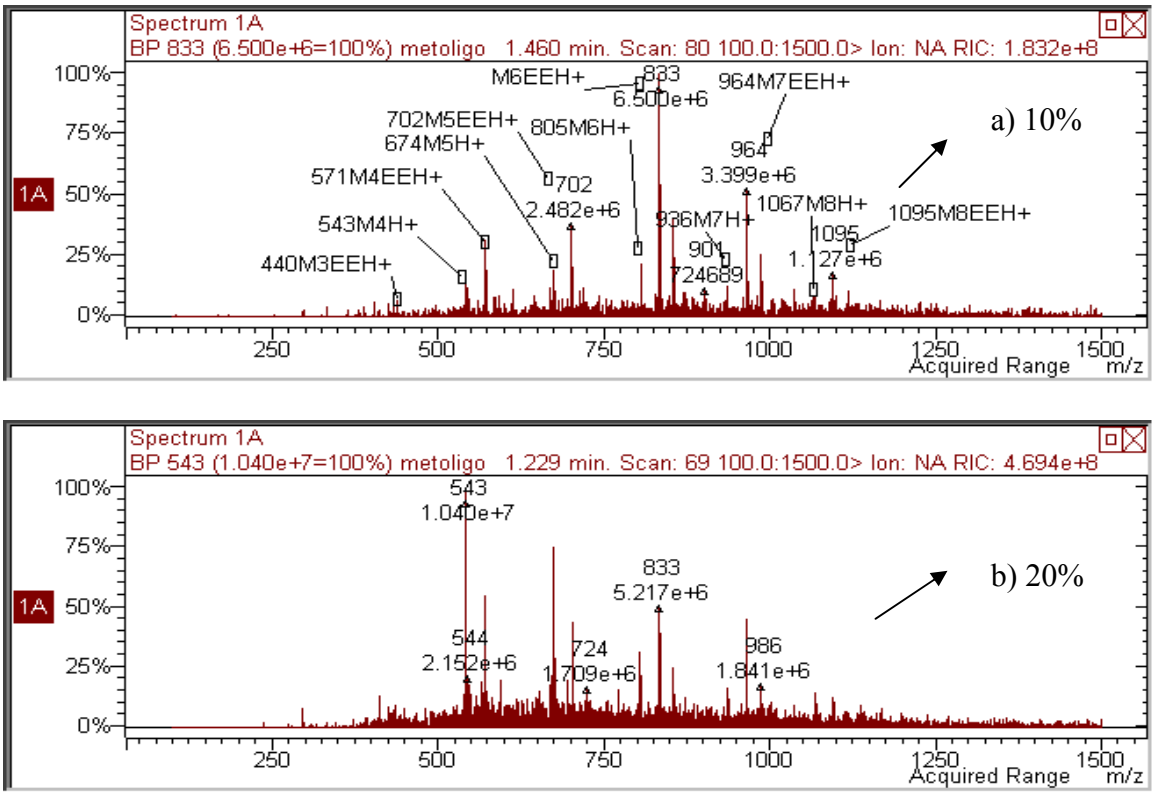


Figure 2.16: ESI-MS spectrum of Met oligopeptides precipitate synthesized in a) 10%, and b) 20% water/acetonitrile system.

Figure 2.17 shows the effect of water content in the reaction media on the percent oligomerization yield of LysEE and MetEE. Two different curves are shown in the plot. Lys oligomerization in water/acetonitrile reaction medium increased with decreasing water content. The lower water composition in the reaction media resulted in the higher oligomerization yield of LysEE. Conversely, Met oligomerization in acetonitrile-water system increased with increasing water content. When the water composition was increased beyond 20%, the oligomerization of Lys dropped noticeably while the oligomerization of Met was considerably stable. As the water composition increased beyond 20% (v/v) of water in acetonitrile, the system tended to behave in a manner similar to a 100% aqueous system, resulting in an increased hydrolysis of preformed Lys oligopeptides that lowered the net oligomerization of Lys but favoring the formation of Met oligopeptides. The results also show that oligomerization of Lys was essentially completed in a 2h incubation period. After this time period, the concentrations of free Lys and LysEE remained essentially the same.

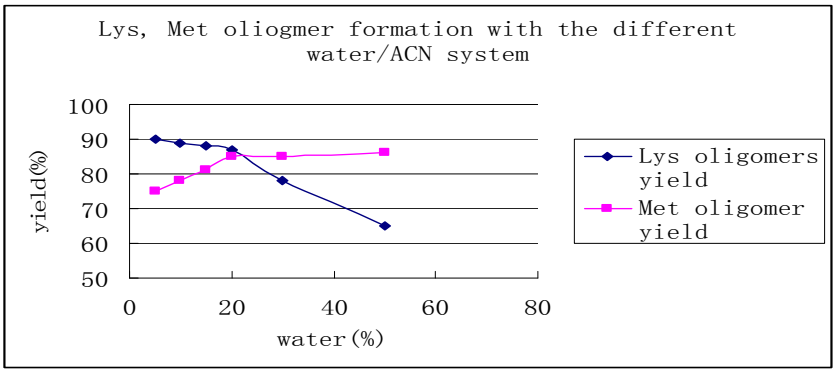


Figure 2.17: Effect of water content in the reaction media on the percent oligomerization yield of LysEE and MetEE

2.4.4. Tailored Lys-Met Oligopeptides Synthesized with Papain in Monophasic Reaction Media. The procedure of synthesizing of Lys-Met tailored peptides was carried out in the acetonitrile-water system shown in Table 2.2. It is well known fact that papain prefers hydrophobic amino acids to hydrophilic amino acids, and Lys oligomerization prefers lower water composition, and Met oligomerization prefers higher water composition of the reaction medium. Thus, a sequential addition approach was adopted to facilitate the synthesis of the co-oligopeptides of Lys–Met. When such an approach was adopted, the reaction medium began with the water content 10% in water/acetonitrile system for favoring the oligomerization of Lys. The second substrate MetEE was added once the first substrate has been oligomerized considerably, but has not reached completion. In the mean time, the water composition of the reaction medium was altered to favor its incorporation in the oligopeptide chain. The additive L-cystein, which was added as antioxidant to reduce the oxidation of the cysteine moiety in the active site of the enzyme, also helped stabilize of the enzyme. DIPEA was used as chelated reagent to neutralize the acid components and free metal ions from the vicinity of enzyme active site of papain. The effect of various reaction parameters such as the final water composition of the reaction media, substrate concentration ratio, incubation period and temperature, and time of addition of second substrate were studied in an effort to synthesize a Lys-Met peptide with a composition of Lys_m: Met_n (n>m) which should have a lower water solubility.

2.4.4.1. Effect of Starting Substrate Ratio. Concentrations of Lys and Met esters added to the reaction media were varied to monitor their effect on the oligopeptide yield and amino acid composition. Because of the hydrophilic nature of Lys and the hydrophobic nature of Met, the higher Met ester substrate ratio was supposed to synthesize lower water solubility of Lys-Met peptides. Thus, the effect of a substrate concentration of 3:1, 1:1, 1:2, and 1:3 LysEE to MetEE was studied to obtain final hetero-oligopeptides with low water solubility. It is obvious that with more hydrophobic amino acid substrate should generate hydrophobic oligopeptides. With higher content of Met in the oligopeptides should have the lower water solubility and consequently with a rumen bypass. So MetEE substrate content in the reaction media was largely increased. In this study, the substrates LysEE were first added to the 10:90 water/acetonitrile monophasic reaction medium. After an incubation period of 1h, the second substrate MetEE was added and the final reaction medium was adjusted to water/acetonitrile at 20:80 in the mean time. The reaction was stopped after 12h incubation period.

The HPLC chromatogram of each co-oligomer is given in the Figure 2.18. The chromatographic output clearly shows a series of peaks eluting after the retention time of LysEE and MetEE. These peaks correspond to the oligopeptides or co-oligopeptides of Lys and Met. As the oligomerization process proceeds, the chain length increases, rendering the hetero-oligopeptides or homo-oligopeptides more hydrophobic and causing them to be precipitated out. The peaks were co-eluting in some cases and individual peak characterization was not feasible due to the use of ion-pairing agent hexane-sulfonic acid used in the separation. The percent yield of oligomerization was estimated by analyzing

the supernatant for the residual amounts of un-reacted monomers left according to the following equation:

Percent oligomerization yield = (Initial amt of amino acid ester- left amt of amino acid ester) / Initial amt of amino acid ester

The results showed that percent oligomerization yield of LysEE and MetEE reached around 75% in this monophasic reaction media when the Lys ethyl ester and Met ethyl ester were added in 1:1, 1:2, and 1:3 mass ratios. The oligomerization yield of MetEE is a little lower when the ratio of LysEE to MetEE is 1:3. It is speculated that because excess of Met EE was added into the reaction system.

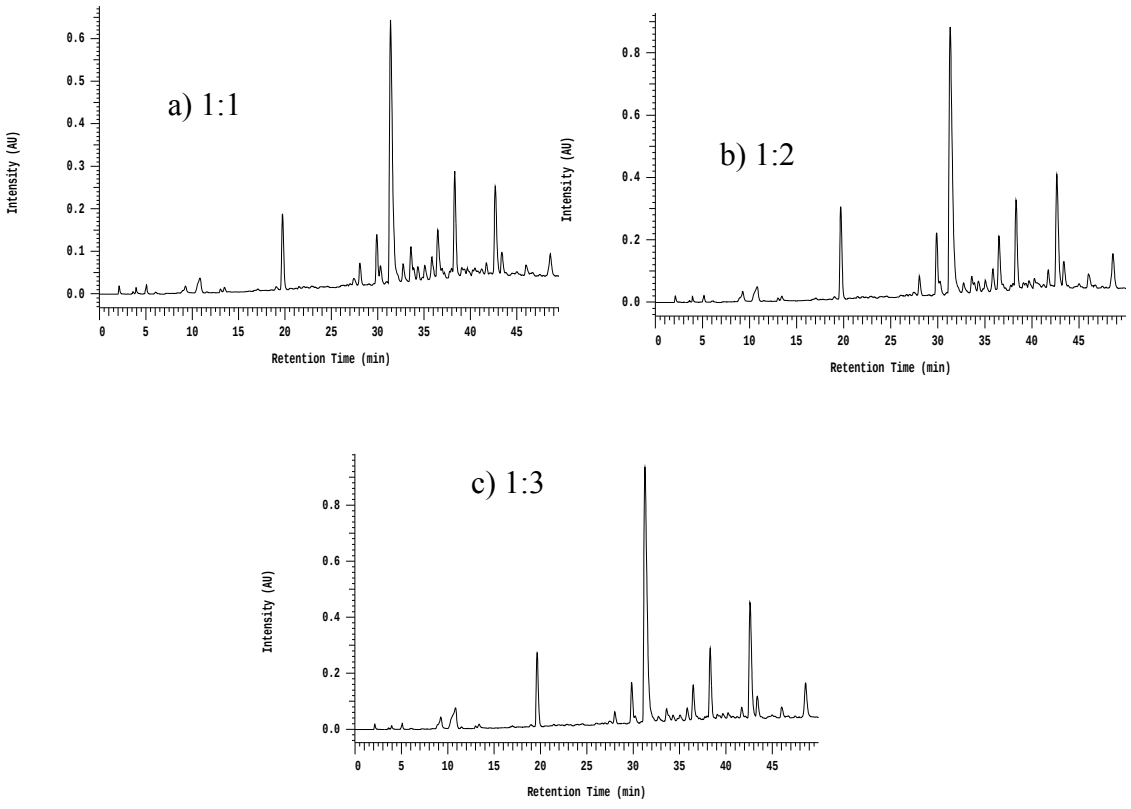
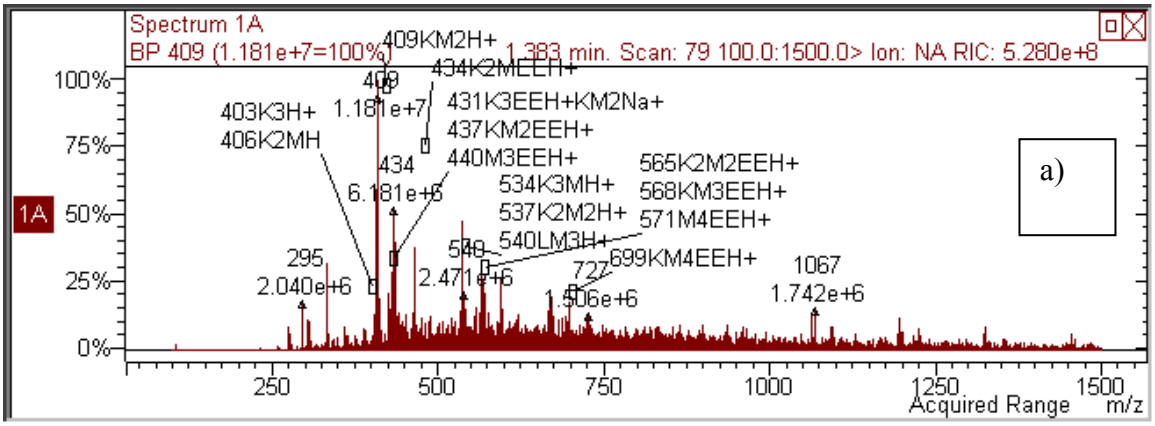


Figure 2.18: HPLC separation of un reacted monomers and Lys-Met Co-oligopeptides synthesized for 12h incubation period with a starting substrate ratio of Lys: Met a) (1:1), b) (1:2), and c) (1:3).

The ESI-MS spectrum of the synthesized oligopeptides in the final reaction medium of 20:80 (v/v) water/acetonitrile is shown in Figure 2.19. The product profile also showed a series of ions corresponding to Lys-Met co-oligopeptides and homo-oligopeptides of Lys or Met. The results also indicated that an increase in Met ethyl ester concentration led to the formation of Met homo-oligopeptides with a corresponding decrease in the hetero-oligopeptides and Lys homo-oligopeptides. These results were not surprising because since Met with its non-polar side chain is a preferred substrate for papain. Figure 2.19 (a) shows the spectrum of oligopeptides of Lys and Met synthesized when the ratio of substrate Lys to Met concentration was 3:1. Homo-oligopeptides of Lys or Met present in the spectrum were less dominant than the hetero-oligopeptides. Around 95% of the oligopeptides were with the free carboxylic acid end and only 5% of the oligopeptides were with the ester end. Figure 2.19 (b), (c), and (d) show the spectrum of oligopeptides of Lys-Met synthesized when the ratio of the substrate of LysEE to MetEE was respectively 1:1, 1:2, and 1:3. The oligomerization process tended to favor the formation of hetero oligopeptides of Lys-Met. Figure 2.19 (b) shows the ESIMS spectrum with the substrate ratio of LysEE to MetEE 1:1. $Lys_{(m)}$-$Met_{(n)}$ co-oligomers and homo-oligomers with ester end at C terminal are dominant in the mass spectrum. The most dominant ions are $K_1M_{(n)}EEH^+$ with C terminal end protected with ethyl ester such as $437KM_{(2)}EEH^+$, $568KM_{(3)}EEH^+$, $699KM_{(4)}EEH^+$, $306KM_{(1)}EEH^+$, $830KM_{(5)}EEH^+$, and $961KM_{(6)}EEH^+$. LysMet co-oligomers with free acid C terminal end are also appeared such as $406K_2MH^+$, $409KM_2H^+$, $534K_3MH^+$, $537K_2M_2H^+$, $540KM_3H^+$, $662K_4MH^+$, $668K_2M_3H^+$, $671KM_4H^+$, $799K_2M_4H^+$, and $802KM_5H^+$. Lys homo-oligopeptides and Met homo-oligopeptides are dominant in a small amounts in the

spectrum, such as $403K_3H^+$, $412M_3H^+$, $531K_4H^+$, $543M_4H^+$, $659K_5H^+$, $674M_5H^+$, $805M_6H^+$, and $936M_7H^+$.

When the substrate ratio of LysEE to MetEE was changed to 1:2, no Lys oligopeptides were present in the spectrum but more dimmer, trimer, tetramer, pentamer, and hexamer of Met oligopeptides with ester ends were precipitated out including: $309M_2EEH^+$, $440M_3EEH^+$, $571M_4EEH^+$, and $702M_5EEH^+$. Lys-Met co-oligopeptides such as, $KMEEH^+$, K_2MEEH^+, KM_2EEH^+, K_3MEEH^+, $K_2M_2EEH^+$ and KM_4EEH^+ appeared in the spectrum . Among the oligopeptides, the Lys-Met dimmers with ethyl ester C terminal ends are most dominant in the spectrum. The second dominant is Met-Met dimmer with ethyl ester end.

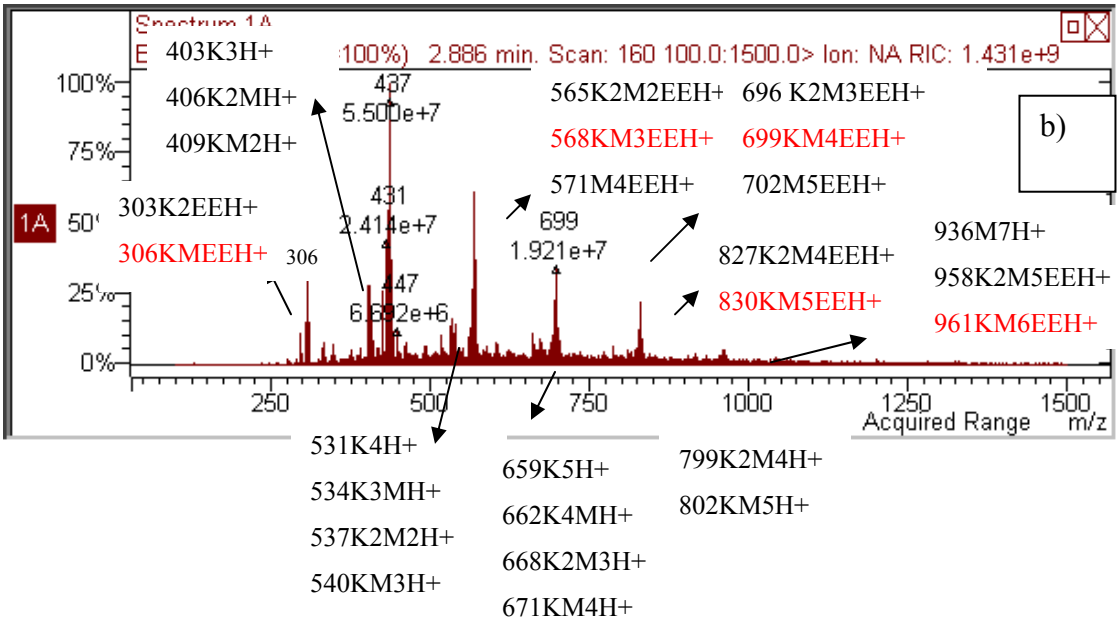


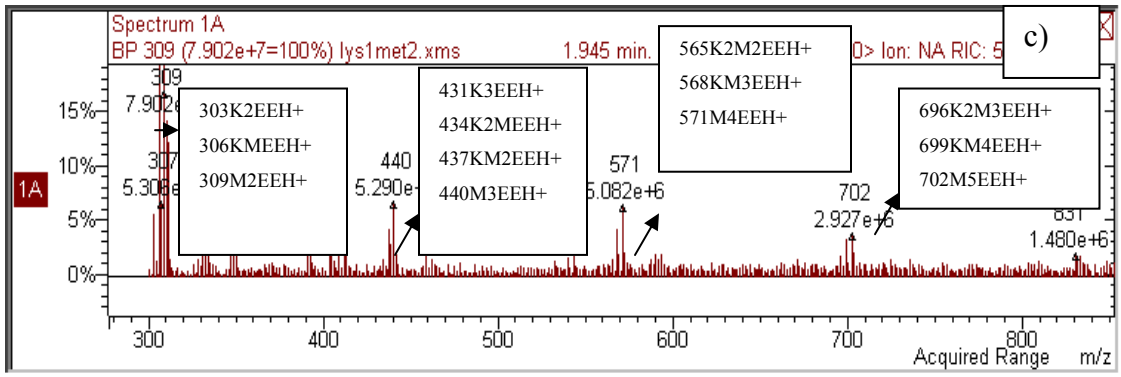
431K3EEH+KM2Na+

434K2MEEH+

437KM2EEH+

Figure 2.19: Direct Injection ESI (+)-MS spectrum of Lys-Met Co-oligopeptides for a starting substrate ratio of Lys: Met (a) (3:1), (b) (1:1), (c) (1:2), and (d) (1:3).





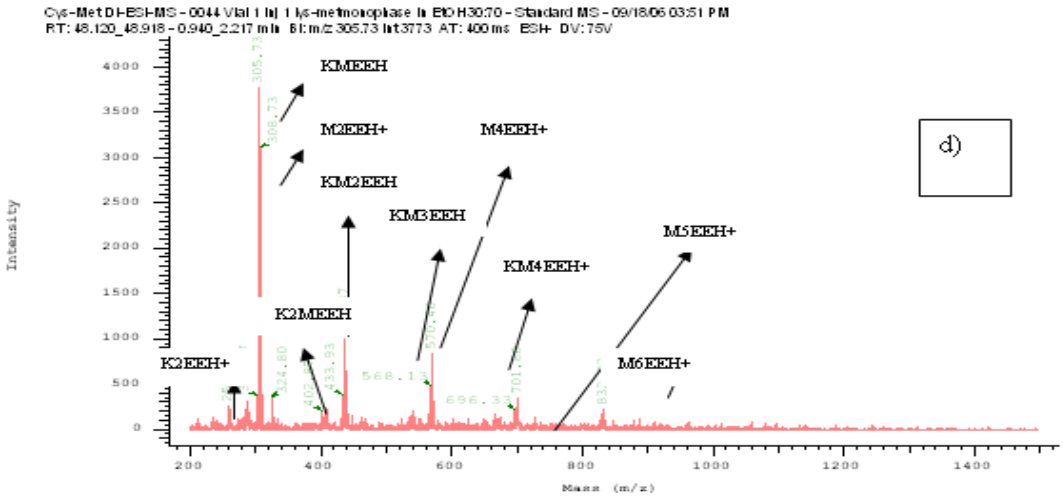


Figure 2.19 Cont: Direct Injection ESI (+)-MS spectrum of Lys-Met Co-oligopeptides for a starting substrate ratio of Lys: Met (c) (1:2), and (d) (1:3).

2.4.4.2. Effect of Incubation Time. For the incubation time effect study, Lys-Met cooligopeptides are synthesized in a water/acetonitrile system with the substrate of LysEE to MetEE 1:1 at an incubation temperature of 37^{o} C. Lys oligomerization is known to complete in 2 hours. After one hour incubation of LysEE, the second substrate MetEE was added to the system. To monitor the Met oligomerization during the incubation time, the reaction was stopped at 2, 4, 8, 12, to 24 hours. HPLC and ESIMS were carried out to characterize oligopeptides synthesized with different incubation times. HPLC chromatograms show that the residue MetEE have more than 50% left at 2 hour incubation period. The oligomerization yield of MetEE increases along with increasing the incubation time. The Figure 2.20 shows that the oligomerization yield of MetEE reaches near 85% at 12 hours and stayed the similar yield until 24 hours. ESI-MS results show few ions are characterized as Lys-Met co-oligopeptides for the product for 2 hours. The co-oligopeptides and homo-oligopeptides of Met are shown up in the mass spectra starting from 4 hour incubation time.

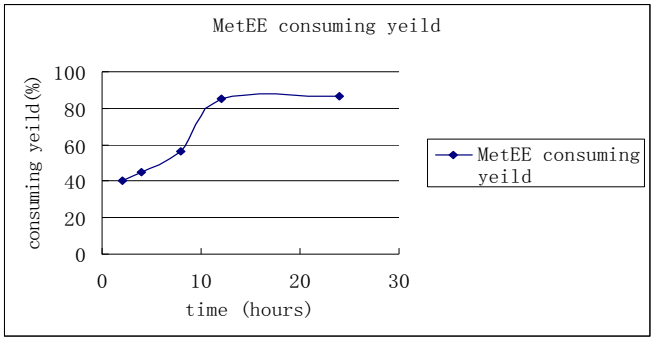


Figure 2.20: Percent conversion of Met into oligopeptides and cooligopeptides synthesized in water/acetonitrile system with different incubation time

2.4.4.3. Effect of Temperature. Incubation temperature is an important parameter governing enzymatic activity. The synthesis of Lys-Met co-oligopeptides was studied under elevated temperature conditions with 0.5mM commercial LysEE and MetEE (1:1) standard in the same reaction media. The synthesis was carried out at 25° C, 37° C, 45° C and 60° C. The results show that at an incubation temperature of 25° C and 37° C, the concentration of substrates in the reaction media decreased rapidly in the presence of enzymes. The yield of the oligomerization reached the highest and more than 80% of the initial LysEE, MetEE substrate was incorporated into the oligopeptide chain. When the incubation temperature was increased to 45° C and 60° C, a measurable drop in the product yield occurred. The oligomerization yields of LysEE and MetEE were lower than 60%. ESIMS results are compatible with the HPLC results. Figure 2.21 shows the ESIMS spectra of Lys-Met oligomerization present in the precipitate of the reaction mixture obtained at varying reaction temperatures. ESI-MS spectra of both 25° C and 37° C show the similar results that the spectrum consists of a series of peaks corresponding to hetero-oligopeptides of Lys-Met and homo-oligopeptides of Lys, Met. The hetero-oligopeptides, through present in the spectrum, were more dominant than the homo-oligopeptides. Only the most dominant ion present at the spectrum of 25 ° C is $568KM_3EEH^+$, not $437KM_2EEH^+$ at the spectrum of 37° C. At both 45° C and 60° C, not much homo- or hetero- oligopeptide ions are present in the spectra, which match the lower oligomerization yield from HPLC analysis. It is hypothesized that at higher temperatures enzyme denaturation causes a drop in product conversion or the drop could be caused by some degradation of co-oligopeptides occurring at higher temperature. In this study, the temperature was selected at 25° C and 37° .

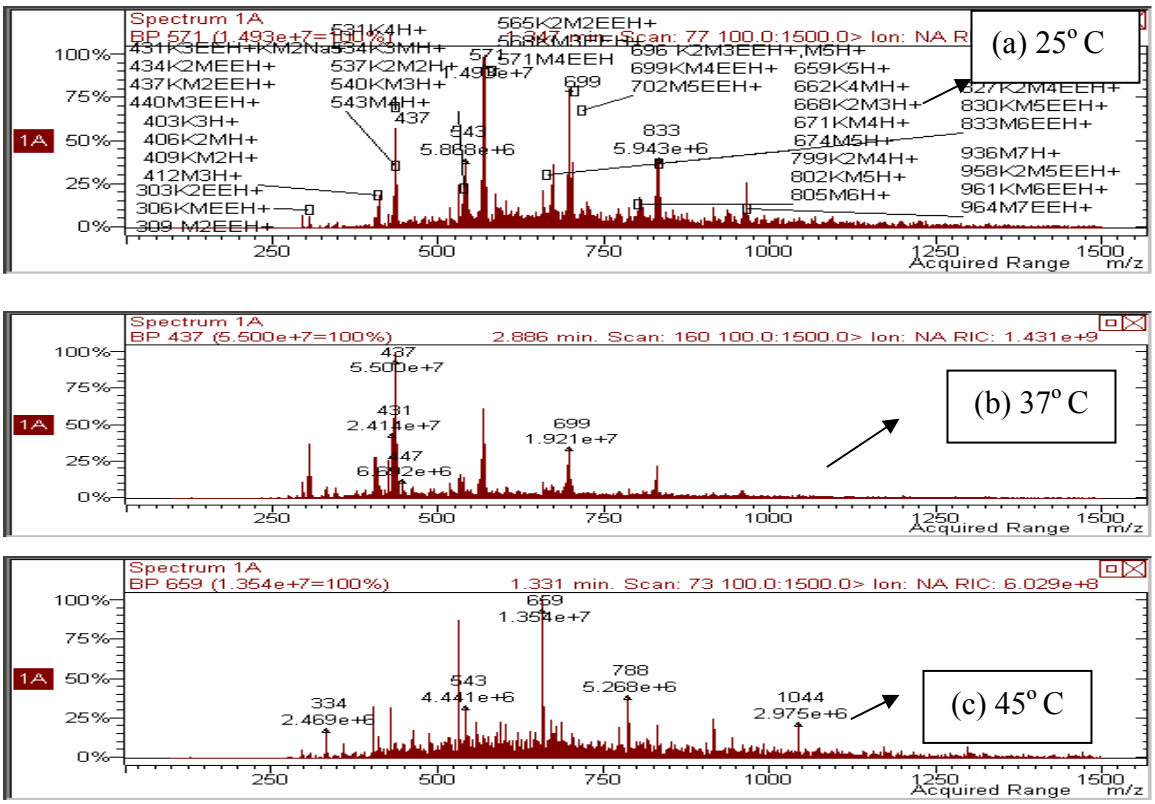


Figure 2.21: The ESIMS spectra of Lys-Met oligomerization present in the precipitate of the reaction mixture obtained at (a) 25° C, (b) 37° C, and (c) 45° C.

2.4.4.4. Effect of Addition Time of the Second Substrate. A sequential addition approach was used to synthesis Lys-Met co-oligopeptides in a water- acetonitrile reaction medium to avoid the homo-oligopeptides formation. Thus, it became necessary to optimize the addition time of the second substrate. The second substrate should be added when the oligomerization of the first substrate has occurred considerably, but has not reached completion. If the second substrate is added after the completion of oligomerization of the first substrate, it leads to the formation of a mixture of homo-oligopeptides of both the substrates with few co-oligopeptides. From the time course study of Lys oligomerization, it became clear that for the success of the sequential

addition strategy, MetEE should be added before 2h. To test this strategy, MetEE was added to the reaction media at three different times respectively: addition at the same time with LysEE, addition after 0.5 hour incubation and addition after 1 hour incubation. Figure 2.22 shows ESIMS spectra of the oligopeptides precipitate obtained after total 12 hours incubation with a different addition time for MetEE.

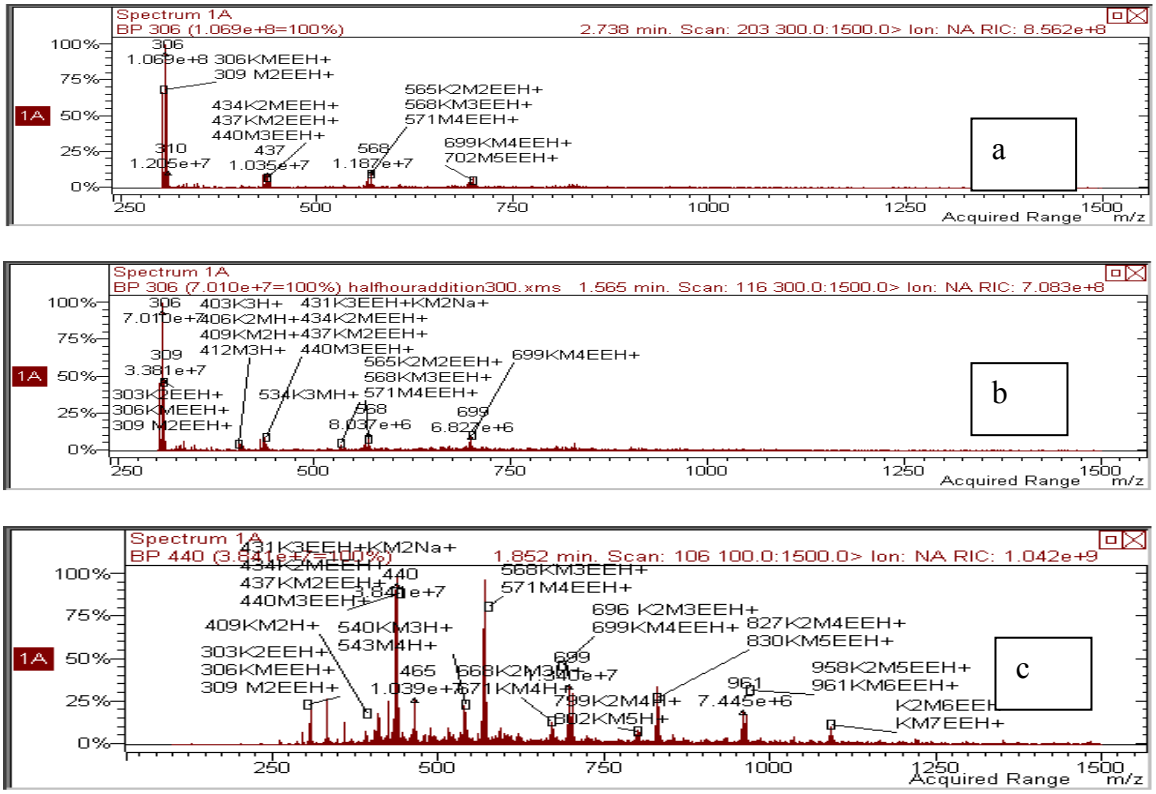


Figure 2.22: The ESIMS spectra of Lys-Met oligomerization present in the precipitate of the reaction mixture obtained with different addition time for the second substrate: (a) at the same time, (b) after 0.5 hour, (c) after 1 hour.

When LysEE and MetEE were added to the system at the same time, Met homo-oligopeptides and Lys-Met hetero-oligopeptides were more dominant than the Lys-oligopeptides which prove that papain prefers hydrophobic amino acids to hydrophilic amino acids. Few differences were observed in the spectrum for the addition of MetEE

after 0.5 hour addition. The dominant ions are Lys-Met co-oligopeptides 306KMEEH+, 437KM2EEH+, 568KM3EEH+, and 699KM4EEH+, 434 K2MEEH+, and 565K2M2EEH+ and met homo-oligopeptides 309M2EEH+, 440M3EEH+, 571M4EEH+, and 702M5EEH+. A few homo-oligopeptides of Lys also appeared in the spectrum after 0.5 hour addition in the second substrate such as 303K2H+, 403K3H+, and 431K3EEH+. However, in the spectrum of addition of the second substrate after the 1 hour, big difference was observed. more ions corresponding to the hetero-oligopeptides of Lys-Met appeared, especially those oligopeptides with more Met composition such as KM2EEH+, KM3EEH+, KM4EEH+, and KM5EEH+ . They are also the most dominant ions in the spectrum. Those oligopeptides are what is desired in this study because they are supposed to have higher bioavailability.

2.4.4.5. Effect of Final Reaction Media. Because of the hydrophobicity difference of Lys and Met, the water content in the reaction media for oligomerization of Lys-Met oligopeptides is very important. It is well established that papain prefers hydrophobic amino acids to hydrophilic amino acids, and Lys oligomerization prefers lower water composition, and Met oligomerization prefers higher water composition of the reaction medium. So the effect water content in the final reaction medium on co-oligomerization of Lys-Met was studied to maximize the distribution of tailored co-oligopeptides in the final product. The water/acetonitrile reaction media started with 10% water to favor the oligomerization of Lys. And when MetEE was added to the system, the water content was changed from 10:90 to 20:80, 30:70 and 50:50. The procedure described in section 2.3.4.2 was adapted. Figure 2.23 shows the HPLC

chromatograms of unreacted monomers and Lys-Met Co-oligopeptides synthesized in the water/acetonitrile system after 12 hour incubation period with a starting substrate ratio of 1:2 LysEE to MetEE.

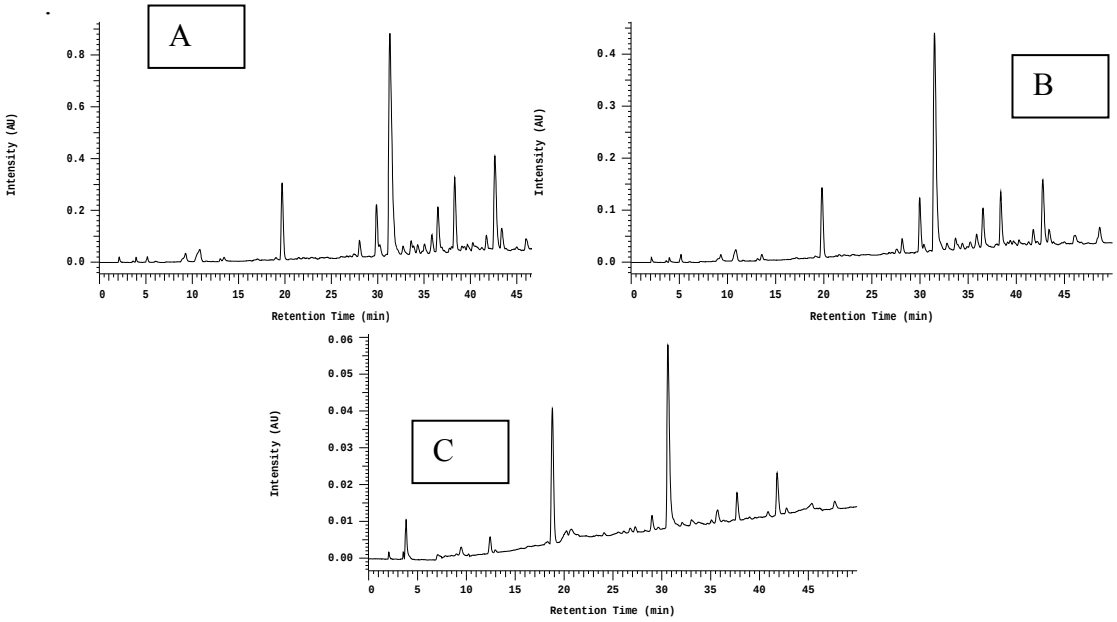


Figure 2.23: HPLC separation of un reacted monomers and Lys-Met oligopeptides synthesized in the final system with (A) 20:80, (B)30:70, and (C) 50:50(%v/v) of water/acetonitrile.

An assessment of residual amounts of Lys and Met monomer present in the product revealed that the incorporation of Lys and Met in the oligopeptide chain did not change considerably with the change in water content from 20% to 30%. Only more LysEE converted to oligopeptides in the final system with 20:80(v/v) water/acetonitrile than in the final system with 30:70(v/v) water/acetonitrile. And more MetEE converted to oligopeptides in the final system with 30:70(v/v) water/acetonitrile than in the final system with 20:80(v/v) water/acetonitrile. The oligomerization yield of LysEE was the

lowest in the final system of 50:50. As the water composition increases beyond 20% (v/v) water in acetonitrile the system tends to behave in a manner similar to 100% aqueous systems, causing an increased hydrolysis of preformed Lys oligopeptides to drop the net conversion of Lys while encouraging the formation of Met oligopeptides.

Figure 2.24 shows the ESI (+)-MS spectra of the oligopeptide product synthesized with the starting substrate ratio at 1:2 (Lys to Met) with the final water content was changed to 20%, 30% and 50% in the water/acetonitrile reaction media. A large Lys-Met co-oligopeptides are formed and accompanied by a little amount of homo-oligopeptides of Lys and Met when the final reaction medium were 20% and 30% water content. But when the water content of the reaction medium was changed to 50%, the relatively large amounts of ions (68.8%) are corresponding to homo-oligopeptides of Lys or Met. Only a small amount of co-oligopeptides presents. This result signifies an important advantage of monophasic system: by manipulating the reaction medium composition to a small degree the required product profile could be achieved without compensating the final molar concentration of the amino acids in the oligopeptide product.

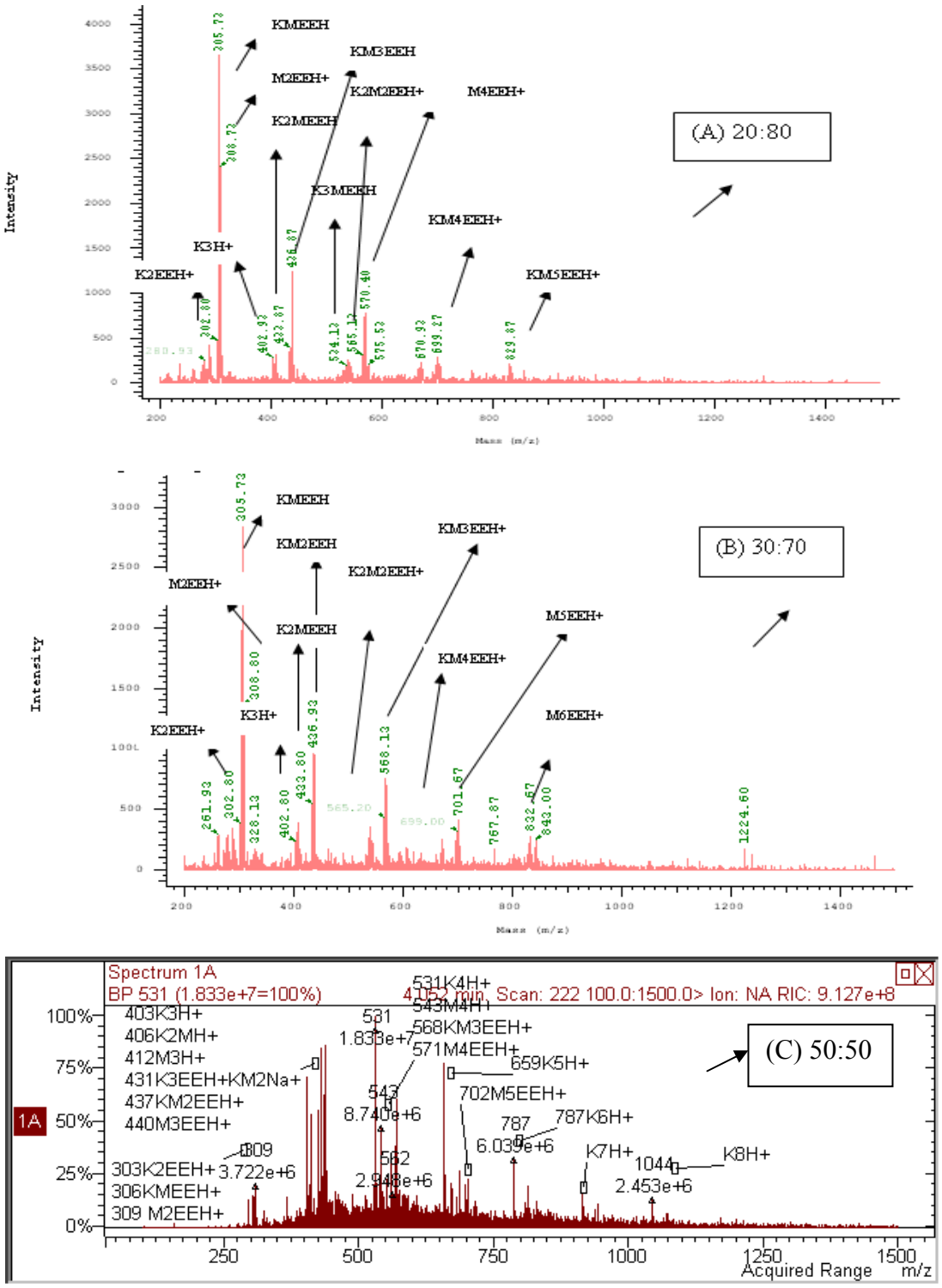


Figure 2.24: ESI (+)-MS spectra of Lys-Met oligopeptides synthesized in the final reaction media with (a) 20:80, (b) 30:70 and (c) 50:50 (%v/v) of water/acetonitrile.

To sum up previous studies, Table 2.4, 2.5, and 2.6 show the oligomerization yield and mass composition of Lys-Met co-oligomerization gained by HPLC and Direct injection ESI(+)MS analysis. Those data included the oligopeptides synthesized with various of substrate concentration ratio of (3:1, 1:1, 1:2, and 1:3)(Lys to Met) in the water/acetonitrile monophasic reaction media changed from stating (10:90)(%v/v) to final 20:80, 30:70, and 50:50(%v/v) at 25^{o}C for an incubation period of 24 hours. And the sequential addition method was adopted and the second substrate MetEE was added at 1 hour.

Table2.4: Oligomerization yield and mass composition of Lys-Met synthesized in the final reaction media at 20:80 (v/v) water/acetonitrile system gained by HPLC and direct injection ESI (+) MS analysis.

Composition of Substrate Lys:Met(20:80)	Homo oligopeptides	Co-oligopeptides	Free acid end	Ester end	KEE Yield	MEE Yield
3:1	K3H+ K4H+ K5H+ K6H+ 45%	K1M1EE+ K2M1H+ K3M1H+ K2M2H+ K4M1H+ K3M2H+ K5M1H+ K4M2H+ 55%	All others 95%	K1M1EEH+ 5%	66.32	65.45
1:1	K2EE+ M2EE+ K3H+ M3EE+ M4EE+ 32%	KMEE+ K2MEE+ KM2EE+ K3MEE+ KM4EE+ K4M5EE+ 68%	K3H+ 4%	All others 96%	75.28	72.70
1:2	K2EE+ M2EE+ K3H+ M4EE+ 36%	KMEE+ KM2EE+ K2MEE+ K3MEE+ K2M2EE+ KM4EE+ KM5EE+ 64%	K3H+ 2.5%	All others 97.5%	73.72	78.35
1:3	K2EE+ M2EE+ K3H+ M5EE+ M4EE+ M6EE+ 54%	KMEE+ KM2EE+ K2MEE+ KM3EE+ 48%	K3H+ 2%	All others 98%	80.27	79.74

Table2.5: Oligomerization yield and mass composition of Lys-Met synthesized in the final reaction media at 30:70 (v/v) water/acetonitrile system gained by HPLC and direct injection ESI (+) MS analysis.

Composition of Substrate Lys:Met(30:70)	Homo oligopeptides	Co-oligopeptides	Free acid end	Ester end	KEE Yield	MEE Yield
3:1	M2H+ M3H+ M4H+ M5H+ M6H+ M6EE+ M7H+ 84%	K1M2H+ K1M3H+ K1M6H+ 16%	All others 95%	M6EEEE+ 5%	69.15	68.45
1:1	K2EE+ M2EE+ M5EE+ 25%	KMEE+ KM2H+ KM2EE+ K2M2EE+ KM4EE+ KM3EE+ 75%	KM2H+ 4%	All others 96%	72.76	79.07
1:2	K2EE+ M2EE+ K3H+ M5EE+ M6EE= 29%	KMEE+ KM2EE+ K2MEE+ K2M2EE+ KM4EE+ KM3EE+ 71%	K3H+ 3%	All others 97%	71.26	80.39
1:3	K2EE+ M4EE+ M5EE+ 35%	KMEE+ KM2EE+ K2MEE+ KM3EEH+ K2M3EE+ K5M3EE+ KM5EE+ K4M4EE+ 48%	KM5H+ K5M3H+ 10%	All others 90%	77.04	79.36

Table 2.6: Oligomerization yield and mass composition of Lys-Met synthesized in the final reaction media at 50:50 (v/v) water/acetonitrile system gained by HPLC and direct injection ESI (+) MS analysis.

Composition of Substrate Lys:Met(50:50)	Homo oligopeptides	Co-oligopeptides	Free acid end	Ester end	Lys Yield	Met Yield
1:1	K2EE+ M2EE+ 67%	KMEE+ KM2H+ KM2EE+ 33%	KM2H+ 3.5%	All others 96.5%	70.63	72.87
1:2	K2EE+ M2EE+ K3H+ K3H+ 68.8%	KMEE+ KM2EE+ KM3EE+ 31.2%	K2H+ K3H+ 9.4%	All others 90.6%	64.71	69.91
1:3	K2EE+ M2EE+ M3EE+ 41.3%	KMEE+ 58.7%		All	66.25	68.62

2.4.4.6. Analysis of Composition of Co-oligopeptides. The amount of Lys and Met incorporated into the oligopeptide chains was estimated by subjecting the co-oligopeptides to acid hydrolysis. The hydrolysis was carried out using 6N HCl for a period of 120hours. Complete hydrolysis was observed after 48hours of hydrolysis. The Co-oligopeptides and oligopeptides were turned into the respective monomers Lys and Met. Because it is very hydrophilic, the Lys is not retained in a regular reverse phase column. Hence, the separation was done using hexane sulfonic acid as an ion pairing agent. The chromatographic outputs for washed and un-washed substrates after 48hours hydrolysis are given in Figure 2.25.

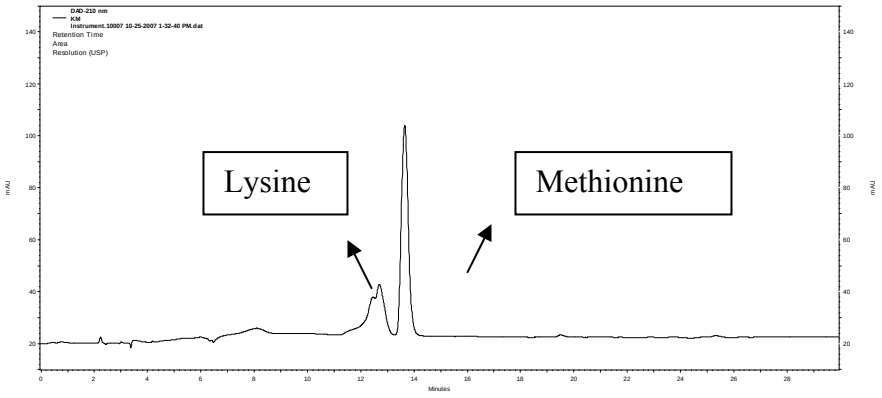


Figure 2.25: HPLC chromatograms for acid hydrolyzates of (Lys)m-(Met)n

The relative concentrations of Lys and Met were found to be 1:1.78. In conclusion, the previous research of papain catalyzed oligomerization of Lys-Met co-oligopeptides was successfully carried out in the acetonitrile/water reaction media. The reaction parameters were successfully optimized as follows: starting substrate concentration ratio 1:1 (Lys: Met); time of addition of second substrate 1h; beginning reaction medium composition 10(%v/v) of water in acetonitrile; final reaction medium composition 20 (%v/v) of water in acetonitrile, temperature of incubation 25^{o} C; and time of incubation 24 hour. Manipulating the solvent composition of the system could alter the co-oligopeptide distribution. The oligomerization yield of Lys and Met were the largest and most hetero-oligopeptides of Lys and Met were formed with 97% oligopeptides with ester end C terminal end in this system.

2.4.4.7. Synthesis of Arg-Met Co-oligopeptides with Papain in Monophasic Reaction Media. Arg is also a hydrophilic amino acid. To make Arg-Met co-oligopeptides, the same strategies were undertaken to papain catalyzed synthesize Arg hydrophilic oligopeptide in low water content monophasic media at 25° C. Then the second substrate MetEE was added and water content was increased to 20(%v/v) after 1 hour incubation, which facilitated the formation of hydrophobic amino acid oligopeptides. The reaction was incubated for 24 hours and stopped by heating the reaction mixture to 80° C for 10 minutes. The supernatant was separated and rotary evaporated to dryness. The precipitate was lyophilized. Dried oligopeptides were then reconstituted in 50% ethanol in water, then centrifuged, filtered and analyzed in HPLC and ESIMS. From the ESIMS, Arg-Met co-oligopeptides and homo-oligopeptides appeared in the mass spectrum. The dominant ions are recognized as Arg-Met$_{(m)}$EEH$^+$ with C terminal end protected by ethyl ester. Arg homo-oligopeptides and Met homo-oligopeptides are present with small amounts. Arg-Met co-oligopeptides with free acid C terminal end are also present with small amounts.

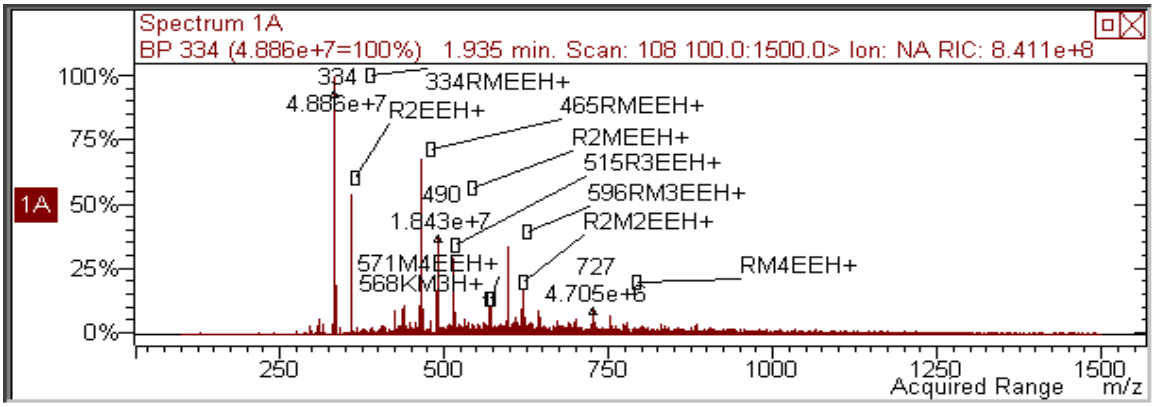


Figure 2.26: ESI(+)-MS spectra of the Arg-Met oligomerization present in the precipitate of the reaction mixture synthesized in water/acetonitrile monophase.

2.4.4.8. Solubility of Co-oligopeptides. Solubility test of co-oligopeptides were performed in Animal Science Center in University of Missouri-Columbia. Dacron bags technique (Crawford, 1978) was used to determine the extent of ruminal degradation of oligopeptides and free amino acids and to relate ruminal oligopeptides degradation to oligopeptides solubility measured in various solvents. Dacron bags containing a 0.5 gram sample of one of oligopeptides or a free amino acid were placed in the rumen of a fistulated steer. Disappearance of nitrogen compounds was determined at intervals for 6 hours to determine the relative rates of ruminal degradation. The nitrogen disappearance after 2 hours rumen exposure was correlated with the quantities of nitrogen solubilized by 1 hour extractions of the same feeds using 10% Wise Burroughs Mineral Buffer (WB), 0.15molar sodium chloride (NaCl) or autoclaved rumen fluid (ARF). The overall correlation coefficients for the 2hour degradations of all feeds with WB, NaCl and ARF were 0.66, 0.47 and 0.45, respectively. A series of oligopeptides synthesized in water/acetonitrile monophase with different starting substrate ratio were carried out the solubility test. Only the oligopeptide with Lys-Met and Arg-Met with substrate ratio 1:3(Lys to Met, Arg to Met) had satisfying results. Table 2.7 shows that Lys, Met, and Arg free amino acids are around 95% solubility. While co-oligopeptides of Lys-Met and Arg-Met with substrate ratio 1:3(Lys to Met, Arg to Met) around 68% solubility. 40% insolubility should extrapolate to 60% rumen escape, which extrapolate 60% rumen undegradable oligopeptides.

Table 2.7: Solubility of oligopeptides and free amino acids

Samples	Total N (%)	Soluble N (%)	N Solubility (%)	Average of N Solubility (%)
Lys-Met (1:3)	0.549	0.383	69.76	68.30
Lys-Met (1:3)	0.426	0.284	66.67	
Lys-Met (1:3)	0.479	0.328	68.48	
Arg-Met (1:3)	0.438	0.322	73.52	67.64
Arg-Met (1:3)	0.539	0.344	63.82	
Arg-Met (1:3)	0.497	0.326	65.59	
L-Lysine	0.393	0.393	100.00	98.73
L-Lysine	0.551	0.548	99.46	
L-Lysine	0.614	0.594	96.74	
DL-Methionine	0.304	0.27	88.82	94.24
DL-Methionine	0.282	0.27	95.74	
DL-Methionine	0.271	0.266	98.15	
Arginine-HCl	0.812	0.774	95.32	95.93
Arginine-HCl	1.059	1.014	95.75	
Arginine-HCl	0.972	0.94	96.71	

2.4.5. HMTBA Attached Oligopeptides Synthesized with Chymotrypsin.

2.4.5.1. Dimer of HMTBA Attached LysEE Synthesized with Chymotrypsin.

HMTBA methyl ester and LysEE were used as substrates for the synthesis of HMTBA-LysEE with chymotrypsin as a catalyst in a sodium phosphate dibasic buffer at pH 7.8. The ESI (+)-MS spectra of the product incubated for 10, 30, 45, and 60 minutes are shown in Figure 2.27. The spectra shows high peak at m/z 307 that corresponds to a dimer HMTBA-LysEE for 10min incubation. After 20mins, the spectrum also shows the presence of HMTBA-Lys-EENa+ with the m/z 329 along with m/z at 307. The striking

feature of the product distribution is the exclusive formation of a HMTBA-LysEE dimer with the ester protected at C terminal end. It also implies that HMTBA forms the acyl-complex with chymotrypsin while LysEE and Lys oligopeptides act as the nucleophile when HMTBA ester and LysEE were used as substrates. This result encouraged to use chymotrypsin as catalyst for capping HMTBA to co-oligopeptides of Lys-Met.

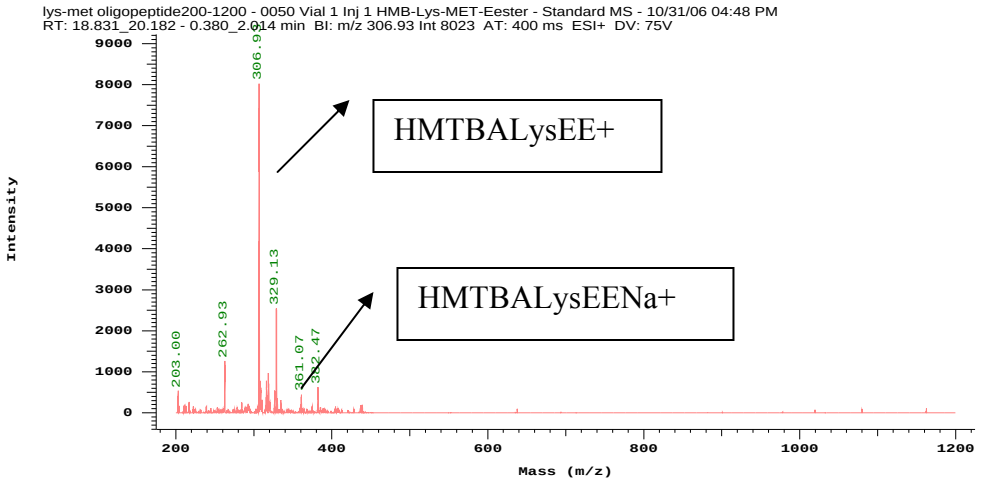


Figure 2.27: ESI(+)-MS spectra of the HMTBA attached Lys co-oliomers with chymotrypsin as catalyst in PH 7.8 buffer for 30minutes incubation.

2.4.5.2. HMTBA Attached Co-oligopeptides of Lys-Met Synthesized with Chymotrypsin. The terminal hydroxyl group in HMTBA is more hydrophobic than Met and also makes HMTBA as a higher bypass Met feed supplement for animals. It has been reported that more HMTBA than Met escapes the rumen largely intact due to the inability of the microorganisms to recognize the terminal OH group. HMTBA is known to convert to Met during the biochemical body metabolism by a two-step reaction comprised of oxidation of the hydroxyl group and subsequent trans-amination. Thus, the attachment of HMTBA to Lys-Met co-oligopeptides was supposed to increase the rumen bypass and

decrease the water solubility of the oligopeptides and finally increase the bioavailability of oligopeptides of Lys-Met. HMTBA capped Lys-Met was carried out in the sodium phosphate dibasic buffer at pH 7.8 using chymotrypsin as the catalyst. The positive ion ESI-MS spectra of an aliquot taken from the reaction mixture incubated at intervals for 15 minutes is shown in Figure 2.28 a. The spectrum consists of a series of ions corresponding to HMTBA- Lys(m)- Met (n)-oligopeptides residues. The spectrum had a series of peaks appearing at m/z 435, 535, 663, 791, and 919 corresponding to HMTBA capped poly Lys residues ranging from 2 to 6. The ion appearing at m/z 461 corresponds to HMTBA capped co-oligopeptides of Lys and Met with intact ester at the C-terminal end (NHMTBA-Lys-Met-COOEt + Na+). The ion appearing at m/z 541 corresponds to HMTBA capped co-oligopeptides of Lys and Met with free acid at the C-terminal end (NHMTBA-Lys-Met_2 + H+). Several peaks appeared at an m/z value that is 4amu higher than poly Lys residues corresponding to the addition of one HMTBA moiety to the recurring oligopeptide of Lys residues. For example, an HMTBA residue added to a pentamer of Lys (NHMTBA – (Lys)5-COOH + H+) it will appear at m/z 791, which is 4amu higher than the pentamer of Lys that will appear at m/z 787. The spectrum also shows a small peak corresponding to an unreacted dimer of Met. The ESI (+)-MS of the sample incubated for a period of 30min with HMTBAME substrate revealed the absence of those Met oligopeptide residues indicating that the process of capping was complete in 30min with HMTBAME substrate. The only peaks appearing in the spectrum were that of HMTBA capped HMTBA- (Lys)m-(Met)n residues. The positive ion ESI-MS spectra of an aliquot taken from the reaction mixture incubated at intervals for 60 minutes is shown in Figure 2.28 b.

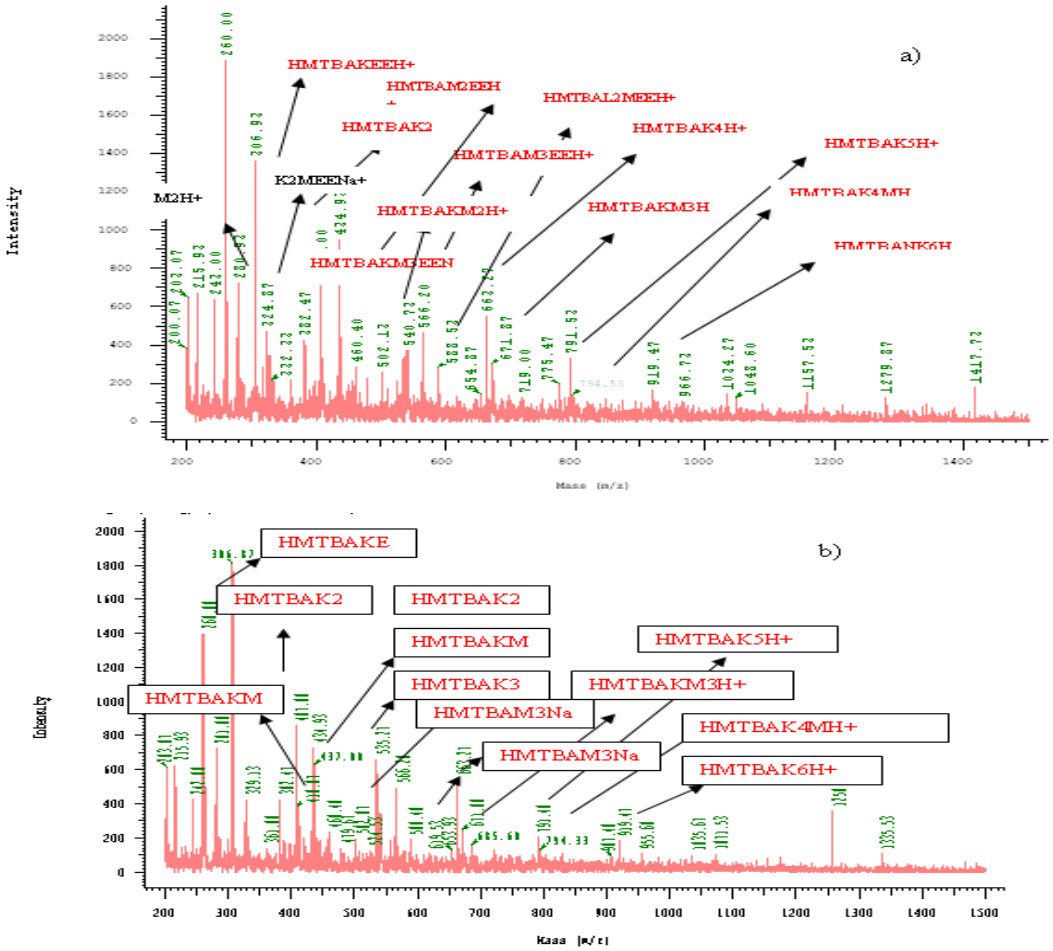


Figure 2.28: ESI (+)-MS spectra of the HMTBA attached Lys-Met co-oligopeptides with chymotrypsin as catalyst in PH 7.8 buffer for a)15, and b) 60 minutes incubation.

When the reaction period was increased beyond 30min, a significant hydrolysis of higher residues to HMTBA capped dimer and trimer of Lys, Met or Lys-Met occurred. Once capping was complete, no more Lys, Met or Lys-Met substrate was available. Hence, chymotrypsin acted on the formed higher residues of HMTBA-$(Lys)_m$-$(Met)_n$, converting it to lower HMTBA capped Lys-Met oligopeptides and performing its original function of hydrolysis. Similar results were observed when HMTBAEE was used as the

substrate. However, in this case, the reaction went to completion after an incubation period of only 60 minutes.

2.4.5.3. Analysis of Composition of HMTBA Attached Lys-Met Co-oligopeptides. The amount of HMTBA incorporated into the co-oligopeptide chains was estimated by subjecting the co-oligopeptides synthesized in the buffer system to acid hydrolysis to turn the oligopeptides and co-oligopeptides to the respective monomers HMTBA, Lys, and Met. Before hydrolyzed HMTBA attached co-oligopeptides, the product was washed with absolute ethanol to remove excess HMTBA and HMTBA-ME left unreacted. This washing step was repeated four times to remove traces of HMTBA associated with the chains. The washed ethanol from each batch was analyzed with HPLC to check for residual HMTBA or HMTBA-ME. It was observed that the concentration of HMTBA or HMTBA-ME went beyond detection limits after third wash. The hydrolysis was carried out using 6N HCl for a period of 120 hours. Ion Pair HPLC was performed to analysis the hydrolyate because Lys was not retained in regular reverse phase column. The chromatographic outputs for washed substrates after 120 hours hydrolysis are given in Figure 2.29. The relative concentrations of HMTBA, Lysine and Methionine were found to be 1: 5:10 and hence the percentage incorporation of HMTBA into oligomer chain was found to be approximately 6.2%.

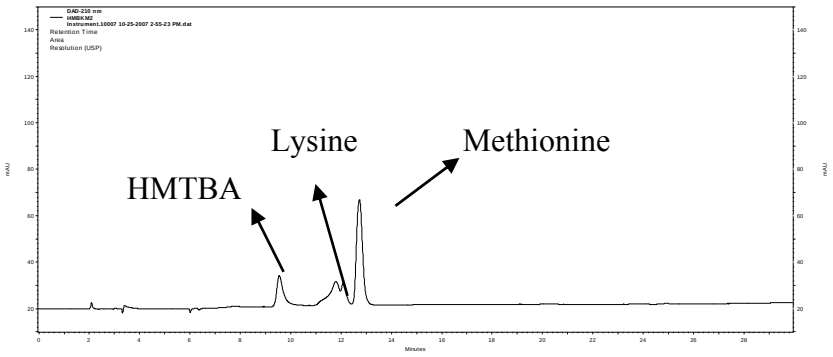


Figure 2.29: HPLC chromatograms for acid hydrolyzates of HMTBA-$(Lys)_m$-$(Met)_n$

2.5. CONCLUSIONS.

This study was undertaken to develop and evaluate efficacy of a papain catalyzed synthesis approach for obtaining C and N protected high rumen by-pass oligopeptides comprised of Lys and Met, the two principle limiting amino acids in bovines. The results of the studies show that the protease, papain with its wide specificity can efficiently catalyze the synthesis of Lys – Met cooligomers with desired composition in a water/acetonitrile monophasic reaction media with esters as the starting substrates. The composition and yields of oligopeptides can be manipulated through changes in reaction media composition and by varying the introduction time of the substrates. Oligopeptides with an ester moiety at the C-terminal end could be readily synthesized in a monophasic reaction medium with higher water content. Highest oligopeptides yields were obtained in reaction media with 20% water and 80% acetonitrile, when the substrate (Lys-EE and Met-EE) ratio was 1: 2. However, water solubility of oligopeptides was lower (~60%) when oligopeptides were synthesized with substrate (Lys-EE and Met-EE) ratio was 1: 3. Lys-Met oligopeptides with still lower water solubility and higher rumen by-pass were synthesized by incorporating HMTBA at the N terminal end of oligopeptides. Such

capping with 100% efficiency was achieved through chymotrypsin catalyzed peptide bond synthesis. The chymotrypsin catalyzed is very rapid better than 95% efficiency is readily achieved in 30 min.

2.6. APPENDICES

A list of possible m/z of ions for Met homo-oligopeptides and HMTBA attached Met oligopeptides are given in Table 2.8

Table 2.8: List of possible mass ions for Met oligopeptides

M	M	MH+	MNa+	MEEH+	MEENa+	HMTBAMH+	HMTBAMNa+	HMTBAMEEH+	HMTBAMEENa+
1	149	150	172	178	200	282	304	310	332
2	280	281	303	309	331	413	435	441	463
3	411	412	434	440	462	544	566	572	594
4	542	543	565	571	593	675	697	703	725
5	673	674	696	702	724	806	828	834	856
6	804	805	827	833	855	937	959	965	987
7	935	936	958	964	986	1068	1090	1096	1118
8	1066	1067	1089	1095	1117	1199	1221	1227	1249
9	1197	1198	1220	1226	1248	1330	1352	1358	1380
10	1328	1329	1351	1357	1379	1461	1483	1489	1511
11	1459	1460	1482	1488	1510	1592	1614	1620	1642
12	1590	1591	1613	1619	1641	1723	1745	1751	1773
13	1721	1722	1744	1750	1772	1854	1876	1882	1904
14	1852	1853	1875	1881	1903	1985	2007	2013	2035
15	1983	1984	2006	2012	2034	2116	2138	2144	2166

A list of possible m/z of ions for Lys homo-oligopeptides and HMTBA attached Lys oligopeptides are given in Table 2.9

Table 2.9: List of possible mass ions for Lys oligopeptides.

M	M	MH+	MNa+	MEEH+	MEENa+	HMTBAMH+	HMTBAMNa+	HMTBAMEEH+	HMTBAMEENa+
1	149	150	172	178	200	282	304	310	332
2	280	281	303	309	331	413	435	441	463
3	411	412	434	440	462	544	566	572	594
4	542	543	565	571	593	675	697	703	725
5	673	674	696	702	724	806	828	834	856
6	804	805	827	833	855	937	959	965	987
7	935	936	958	964	986	1068	1090	1096	1118
8	1066	1067	1089	1095	1117	1199	1221	1227	1249
9	1197	1198	1220	1226	1248	1330	1352	1358	1380
10	1328	1329	1351	1357	1379	1461	1483	1489	1511
11	1459	1460	1482	1488	1510	1592	1614	1620	1642
12	1590	1591	1613	1619	1641	1723	1745	1751	1773
13	1721	1722	1744	1750	1772	1854	1876	1882	1904
14	1852	1853	1875	1881	1903	1985	2007	2013	2035
15	1983	1984	2006	2012	2034	2116	2138	2144	2166

A list of possible m/z of ions for Met- Lys co-oligopeptides and HMTBA attached Met-Lys co-oligopeptides are given in Table 2.10.

Table 2.10: List of possible mass ions for Lys-Met oligopeptides and HMTBA attached Lys-Met oligopeptides.

K	(M)1	(M)1+H+	(M)1EEH+	(M)1EENa+	HMTBAM1H+	HMTBAM1Na+	HMTBAM1EEH+	HMTBAM1EENa+
1	277	278	306	328	410	432	438	460
2	405	406	434	456	538	560	566	588
3	533	534	562	584	666	688	694	716
4	661	662	690	712	794	816	822	844
5	789	790	818	840	922	944	950	972
6	917	918	946	968	1050	1072	1078	1100
7	1045	1046	1074	1096	1178	1200	1206	1228
8	1173	1174	1202	1224	1306	1328	1334	1356
9	1301	1302	1330	1352	1434	1456	1462	1484
10	1429	1430	1458	1480	1562	1584	1590	1612
11	1557	1558	1586	1608	1690	1712	1718	1740
12	1685	1686	1714	1736	1818	1840	1846	1868
13	1813	1814	1842	1864	1946	1968	1974	1996
14	1941	1942	1970	1992	2074	2096	2102	2124
15	2069	2070	2098	2120	2202	2224	2230	2252

K	(M)2	(M)2+H+	(M)2EEH+	(M)2EENa+	HMTBAM2H+	HMTBAM2Na+	HMTBAM2EEH+	HMTBAM2EENa+
1	408	409	437	459	540	563	569	591
2	536	537	565	587	668	691	697	719
3	664	665	693	715	796	819	825	847
4	792	793	821	843	924	947	953	975
5	920	921	949	971	1052	1075	1081	1103
6	1048	1049	1077	1099	1180	1203	1209	1231
7	1176	1177	1205	1227	1308	1331	1337	1359
8	1304	1305	1333	1355	1436	1459	1465	1487
9	1432	1433	1461	1483	1564	1587	1593	1615
10	1560	1561	1589	1611	1692	1715	1721	1743
11	1688	1689	1717	1739	1820	1843	1849	1871
12	1816	1817	1845	1867	1948	1971	1977	1999
13	1944	1945	1973	1995	2076	2099	2105	2127
14	2072	2073	2101	2123	2204	2227	2233	2255
15	2200	2201	2229	2251	2332	2355	2361	2383

K	(M)3	(M)3+H+	(M)3EEH+	(M)3EENa+	HMTBAM3H+	HMTBAM3Na+	HMTBAM3EEH+	HMTBAM3EENa+
1	539	540	568	590	672	694	700	722
2	667	668	696	718	800	822	828	850
3	795	796	824	846	928	950	956	978
4	923	924	952	974	1056	1078	1084	1106
5	1051	1052	1080	1102	1184	1206	1212	1234
6	1179	1180	1208	1230	1312	1334	1340	1362
7	1307	1308	1336	1358	1440	1462	1468	1490
8	1435	1436	1464	1486	1568	1590	1596	1618
9	1563	1564	1592	1614	1696	1718	1724	1746
10	1691	1692	1720	1742	1824	1846	1852	1874
11	1819	1820	1848	1870	1952	1974	1980	2002
12	1947	1948	1976	1998	2080	2102	2108	2130
13	2075	2076	2104	2126	2208	2230	2236	2258
14	2203	2204	2232	2254	2336	2358	2364	2386
15	2331	2332	2360	2382	2464	2486	2492	2514

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