JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzymatic Synthesis and Characterization of L-Methionine and 2-Hydroxy-4-(methylthio)butanoic Acid (HMB) Co-oligomers

MATHUR RAJESH,† SHUBHEN KAPILA,*,† PAUL NAM,† DANIEL FORCINITI,† STEPHEN LORBERT,‡ AND CHARLES SCHASTEEN‡

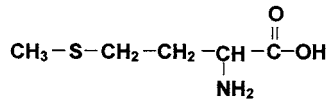
Center for Environmental Science and Technology and Department of Chemistry, University of Missouri−Rolla, 1870 Miner Circle, Rolla, Missouri 65409-0530, and Novus International, Inc., St. Charles, Missouri 63304

Oligomers of L-methionine (Met) and its hydroxy analogue, 2-hydroxy-4-(methylthio)butanoic acid (D,L-HMB) were synthesized with the proteolytic enzyme papain. The Met homooligomers and HMB−Met co-oligomers obtained through the enzymatic reactions were subjected to persulfonation and separated with reverse phase liquid chromatography (RPLC). The separated oligomers were characterized with electrospray ionization-mass spectrometry (ESI-MS). The oligomers were also characterized with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). The results showed that co-oligomers were predominantly composed of 4−8 Met residues and one HMB residue. The data also suggest that in the co-oligomers, HMB is attached at the N-terminal end of the oligopeptide chain.

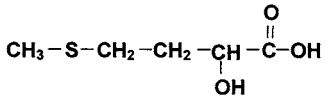
KEYWORDS: Oligopeptides; co-oligomers; methionine; methionine hydroxy analogue; MHA; HMB; papain; mass spectrometry

INTRODUCTION

Met (**Figure 1**) and L-lysine are limiting amino acids in poultry and ruminants. To enhance nutrition, these amino acids are supplemented as crystalline amino acid salts or oligopeptides in the diet (*1*, *2*). However, it has been shown that the bioavailability of orally administered amino acid salts and oligopeptides in ruminants is very limited (*1*, *2*). Oligopeptides are efficiently hydrolyzed in the rumen, and the free amino acids are readily converted to short chain fatty acids and ammonia by rumen microbes. As a result, orally administered peptides are not available to the animal (*3*). Chemical modification or protection of the oligopeptide could render such oligopeptides inaccessible to rumen microorganisms. A modified form of Met, HMB (**Figure 1**), has been used as a Met supplement in animal nutrition for over three decades. HMB is also referred to as MHA in the literature. HMB itself is not an amino acid; however, both the D and the L isomers are enzymatically converted to Met via stereospecific amination pathways (*4*, *5*). Administration of HMB has been found to enhance protein utilization, egg production, and feed conversion efficiency in the poultry (*5*). HMB has been shown to be more resistant to attack from rumen microbes than Met (*6*, *7*). In a recent study on HMB rumen bypass and subsequent gastrointestinal absorption of HMB, Koenig et al. (*8*) showed that up to 50% of HMB escaped microbial degradation and was available for intestinal absorption. However, the effectiveness of administering free HMB has been questioned in a study on nutrition in lactating dairy cows. The study showed that HMB supplementation did not alter milk production or milk composition (*9*). To take advantage of HMB resistance to rumen microbial attack and enhance Met availability, administration of HMB-capped Met oligomers has been considered. However, such oligomers are not available; therefore, synthesis of the oligomers through enzymatic reactions was evaluated.



Methionine (Met)



2-Hydroxy-4-(methylthio)butanoic Acid (HMB)

Figure 1. Structures of Met and HMB.

The use of proteolytic enzymes for the synthesis of oligopeptides has been reported in a number of articles dating back to the 1930s. It is assumed that the protease-catalyzed oligomerization of hydrophobic amino acids such as Met, leucine, tyrosine, tryptophan, and phenylalanine is driven by precipitation of the growing hydrophobic peptide chains (*10*). Protease (chymotrypsin) has been used to synthesize threonine, pheny-

* To whom correspondence should be addressed. Tel: 1(573)341-4986. Fax: 1(573)341-6605. E-mail: kapilas@umr.edu.
† University of Missouri−Rolla.
‡ Novus International, Inc..

10.1021/jf026093g CCC: $25.00
Published on Web 03/22/2003

lalanine, tyrosine, and Met oligomers by Brenner et al. (*11*, *12*). Synthesis of phenylalanine oligomers with six residues has been reported by Dannenberg and Smith (*13*). Papain, a "hardy" protease, has been widely used in the synthesis of amino acid oligomers. Peptide synthesis with this enzyme has been evaluated by Anderson and Luisi (*14*), who determined that the reaction rate is dependent on the type of initiators, the ionic strength, and the buffer composition. These parameters affect both the yield and the polydispersity of the product (*14*). A papain-catalyzed synthesis of leucine oligomers has been described by Sluyterman and Wijdenes (*15*). Synthesis of tyrosine heptamers with papain has been reported by Selvi et al. (*16*). Papain and α-chymotrypsin-catalyzed synthesis of oligo-L-glutamic acid with 5−9 residues has been reported by Aso et al. (*17*). Papain has also been used by Arai et al. (*18*) to catalyze synthesis of Met oligomers in a buffered medium at pH 9; the yield of the crude oligomers was reported to be approximately 80%. Jost and co-workers (*19*) synthesized Met oligomers with the same enzyme in a 1 M citrate buffer at pH 5.5; the yield of purified oligomers was approximately 50%. Application of papain for the synthesis of leucine, phenylalanine, and tryptophan oligomers at pH 7.5 has also been demonstrated by Lee et al. (*20*). However, enzymatic synthesis and characterization of α-hydroxy acid and α-amino acid co-oligomers in general and HMB-α-amino acids in particular have not been described in the literature. This study was undertaken to explore enzymatic synthesis and characterization of HMB and Met co-oligomers.

Characterization of Met oligomers and HMB−Met co-oligomers was carried out with RPLC and LC-ESI-MS. Oligomers and co-oligomers were also analyzed with MALDI-TOF-MS. These techniques are well-suited for peptide characterization, and their application in the area is well-documented in review articles and books (*21*−*23*).

MATERIALS AND METHODS

Materials. Met ethyl ester hydrochloride was purchased from Fluka Chemical Corp., Milwaukee, WI. L-Cysteine, sodium citrate, TFA, and papain (EC 3.4.22.2, twice crystallized suspension, 25 units activity/mg, 28 mg protein/mL at pH 4.5) were purchased from Sigma Chemical Co., St. Louis, MO. Formic acid, hydrogen peroxide, hydrobromic acid, EDTA, methanol, and acetonitrile were obtained from Fisher Scientific, St. Louis, MO. Phenol was obtained from Chem Service, Inc., West Chester, PA. DMSO was obtained from Aldrich Chemical Co., Milwaukee, WI. Racemic HMB was procured from Novus International, Inc., St. Louis, MO. The 250 mm × 4.6 mm i.d., 5 μm C-18 and C-8 reverse phase columns used in the RPLC separation were obtained from P. J. Cobert Associates, Inc., St. Louis, MO.

Liquid Chromatography with DAD and MS Detection. Liquid chromatographic separations were carried out with a model L-7000 HPLC system (Hitachi Instruments, Inc., San Jose, CA.). The system consisted of a piston pump (L-7100), equipped with a column oven (L-7300) and an autosampler (L-7200) with a 50 μL injection loop. The analytes were separated on reverse phase columns and then introduced into a DAD, model L-7450 (Hitachi Instruments). The data were recorded with proprietary HPLC software supplied by Hitachi Instruments. The LC-ESI-MS analysis was carried out by introducing the column effluent into a model M-8000 ion trap MS (Hitachi Instruments) through an ESI.

Preparation of D,L-HMB Ethyl Ester. Twenty grams of HMB was added to 200 mL of anhydrous ethanol in a round bottom flask. The solution was heated to 80 °C, acidified with hydrogen chloride gas generated by adding 20 mL of sulfuric acid dropwise to 20 g of magnesium chloride, and refluxed for 8 h, and HMB ethyl ester was recovered by evaporating the solvent with a rotary evaporator.

Synthesis of Met and HMB−Met Oligomers. Papain-catalyzed synthesis of Met oligomers and HMB−Met co-oligomers was carried out with two procedures. In the first procedure (*18*), 10 g of methionine ethyl ester or 5 g each of HMB ethyl ester and methionine ethyl ester was dissolved in 50 mL of Nanopure water containing 0.05 mol sodium bicarbonate buffer and 2 mmol L-cysteine. The volume of the mixture was increased to 100 mL by adding Nanopure water. The pH of the mixture was adjusted to 9.0, and then, 1 mL of the papain suspension was added. After the the enzyme was added, the mixture was incubated at 37 °C for time periods ranging from 10 min to 24 h. At the end of the incubation period, the reaction was terminated by elevating the mixture temperature to 80 °C for 10 min. In the second procedure (*19*), 3 g of methionine ethyl ester or 1.5 g each of HMB ethyl ester and methionine ethyl ester were added to 10 mL of Nanopure water containing 1 mmol L-cysteine, 0.1 mmol EDTA, and 0.01 mol sodium citrate. The pH of the mixture was adjusted to 5.5 prior to the addition of 0.5 mL of the papain suspension. The reaction mixture was incubated at 37 °C for periods ranging between 10 min to 24 h. At the end of the incubation period, the reaction was terminated by thermal denaturation of enzymes at 80 °C.

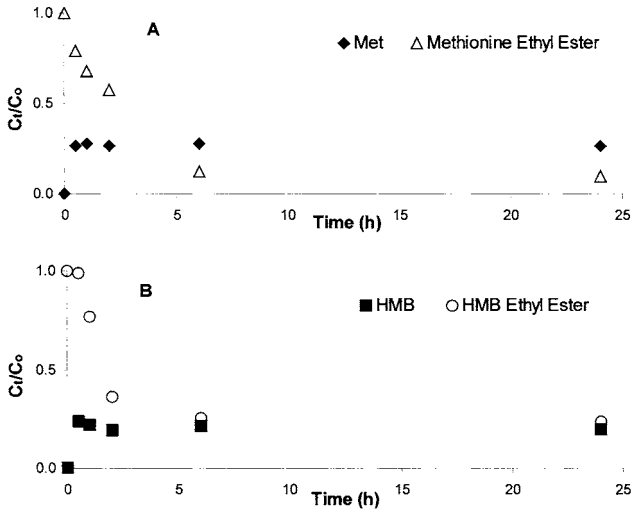


Figure 2. Change in concentrations of monomers during the oligomerization reactions with papain in a 1 M sodium citrate buffer at pH 5.5 and 37 °C. (**A**) Free methionine ethyl ester and Met after different incubation periods. (**B**) Free HMB ethyl ester and HMB after different incubation periods. C_t = concentration of Met, methionine ethyl ester, HMB, and HMB ethyl ester at time t; C_0 = initial concentration of free Met, methionine ethyl ester, HMB, and HMB ethyl ester.

Purification of Oligomers. Oligomers obtained as precipitates were separated from the residual monomers in the supernatant through centrifugation. The precipitate free supernatant was then filtered and analyzed for residual esters, free Met, and HMB. The precipitate was washed six times with 100 mL of water to remove adsorbed monomers and salts. The precipitate was then dissolved twice in DMSO and reprecipitated with water to remove residual HMB. The precipitate was finally freeze-dried to obtain "purified" oligomers.

Acid Hydrolysis of Oligomers. A 25 mg aliquot of purified oligomers was transferred to an 8 mL vial containing 2 mL of 6 N HCl. The contents of the vial were heated to 110 °C and maintained at this temperature for periods ranging from 24 to 168 h. To determine the relative concentrations of Met and HMB residues in the precipitate, aliquots of the hydrolysate were removed at different time intervals and analyzed with the LC-DAD system.

Liquid Chromatographic Analysis of Acid Hydrolysate. An aliquot of the acid hydrolysate was neutralized with 6 M sodium hydroxide solution, and solvents were removed with a rotary evaporator. The residue was dissolved in 5 mL of ethanol and filtered through a 0.2 μm membrane filter. A 10 μL portion of the filtered solution was injected into a C-8 reverse phase column installed in the LC-DAD system. The separation was achieved with a linear gradient program. In the gradient, the mobile phase composition was changed from 100% water with 0.1% TFA (eluent A) to 55% eluent A and 45% eluent B (0.1% TFA in acetonitrile) in 30 min. The mobile phase flow rate was

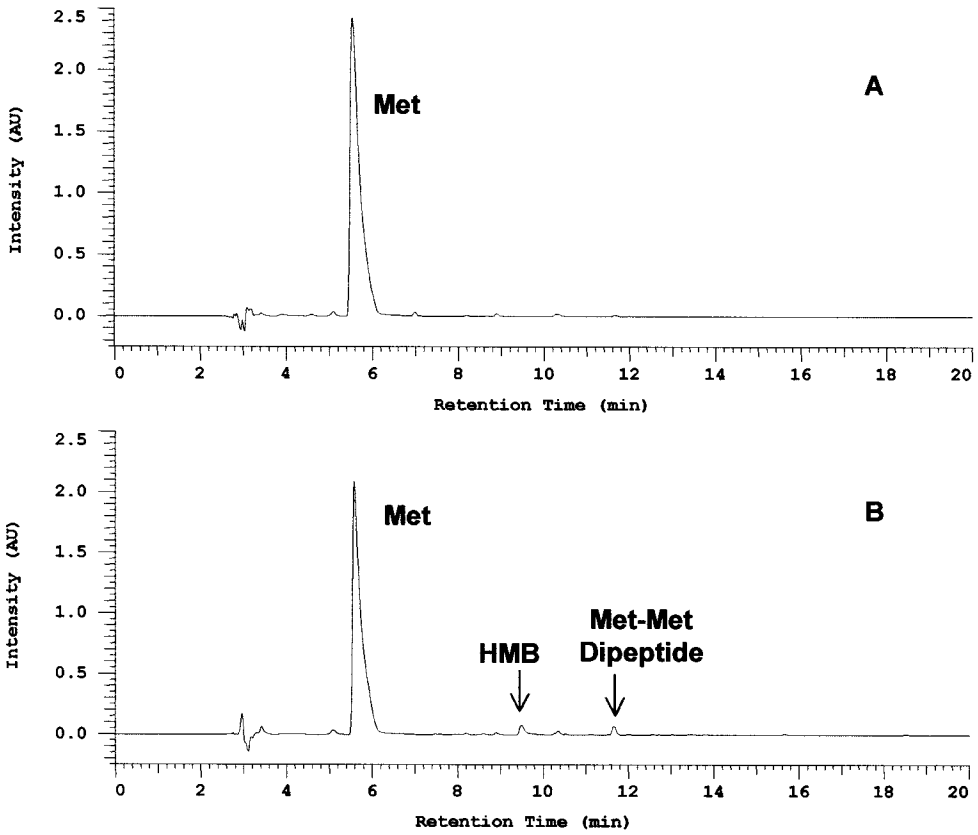


Figure 3. Chromatogram of free Met, HMB, and Met–Met dipeptide detected in acid hydrolysate of Met and HMB–Met oligomers. Oligomers were synthesized with papain in a 1 M sodium citrate buffer at pH 5.5 and 37 °C. (**A**) Chromatogram of Met oligomer acid hydrolysate. (**B**) Chromatogram of HMB–Met co-oligomer acid hydrolysate.

maintained at 1 mL/min. The eluent was introduced into the DAD and monitored over the wavelength range of 210−350 nm.

MALDI-TOF-MS Analysis. Oligomer aliquots dissolved in DMSO were analyzed with MALDI-TOF-MS using thioglycerol as the matrix. The operating parameters of the MALDI-TOF-MS system were as follows: acceleration potential, +20 KV; grid voltage, 80%; low mass gate, 191.0; flight tube pressure, $\sim 2 \times 10^{-7}$ Torr.

Persulfonation of Oligomers. Purified oligomers and co-oligomers were persulfonated to facilitate liquid chromatographic separations. Persulfonation was carried out in a manner similar to the one described by Spindler et al. (*24*). The persulfonation involved oxidation of all sulfide moieties to sulfones with performic acid. The performic acid was prepared by oxidation of formic acid with hydrogen peroxide. A 0.5 mL solution of 30% hydrogen peroxide was mixed with 4.5 mL of 88% formic acid and 25 mg of phenol. The mixture was allowed to stand for 30 min at room temperature. The mixture was then cooled to 0 °C for 15 min in an ice bath. Fifty milligrams of finely divided oligomer powder was added to the mixture and stirred for 15 min in the ice bath. The mixture was stored in a refrigerator overnight at 4 °C. The mixture was removed from the refrigerator, and the excess performic acid in the mixture was reduced by adding 0.7 mL of 48% hydrobromic acid. After the mixture was stirred for 30 min, the residual bromine and formic acid were removed with a rotary evaporator at 50−60 °C.

Liquid Chromatographic Analysis of Persulfonated Oligomers. Aliquots of persulfonated oligomers were dissolved in 5 mL of a acetonitrile/water mixture (40:60) and filtered through a 0.2 μm membrane filter. A 10 μL portion of the filtered solution was injected into a C-18 reverse phase column installed in the liquid chromatographic system. The separation was achieved with linear gradient program. In the gradient, the mobile phase composition was changed from 100% 0.022 M phosphate buffer, pH 6.5 (eluent A), to 60% eluent A and 40% eluent B in 20 min. The eluent B consisted of 80% 0.022 M phosphate buffer plus 20% acetonitrile. The mobile phase flow rate was maintained at 1 mL/min. The eluent was introduced into the DAD and monitored over the wavelength range of 210−350 nm.

Liquid Chromatography Mass Spectrometric Analysis of Persulfonated Oligomers. To prevent the plugging problems encountered with the use of buffered eluents in the LC-ESI-MS interfaces, the LC-ESI-MS separations were carried out with mobile phases consisting of water with 0.1% acetic acid (eluent A) and acetonitrile with 0.1% acetic acid (eluent B). A linear gradient program was used to optimize separation on a C-18 column. In the gradient, the mobile phase composition was changed from 100% eluent A to 40% eluent A and 60% eluent B over 40 min. The mobile phase flow rate was maintained at 1 mL/min. The LC effluent was split 4/1, and 800 μL was introduced into a fixed wavelength UV detector set at 210 nm. The remaining 200 μL was introduced into the ESI-MS. The MS was operated in both positive and negative ion monitoring modes over the 100−2000 amu mass range.

RESULTS AND DISCUSSION

Synthesis of Met and HMB−Met Oligomers. The amount of oligomer formed was determined by weighing the dried precipitate. The extent of monomer consumption was assessed by determining residual methionine ethyl ester, HMB ethyl ester, Met, and HMB in the supernatant after different incubation periods. The results of measurements for oligomer synthesized with the procedure outlined by Jost et al. (*19*) are shown in **Figure 2**. The results show that the concentration of methionine ethyl ester in the reaction mixture decreased rapidly in the presence of the enzyme. Less than 10% of the initial methionine ethyl ester added to the mixture was found after a 6 h incubation period (**Figure 2A**). The concentration of HMB ethyl ester also decreased but to a lesser degree, and nearly 30% of the HMB ethyl ester remained intact even after 24 h of incubation (**Figure**

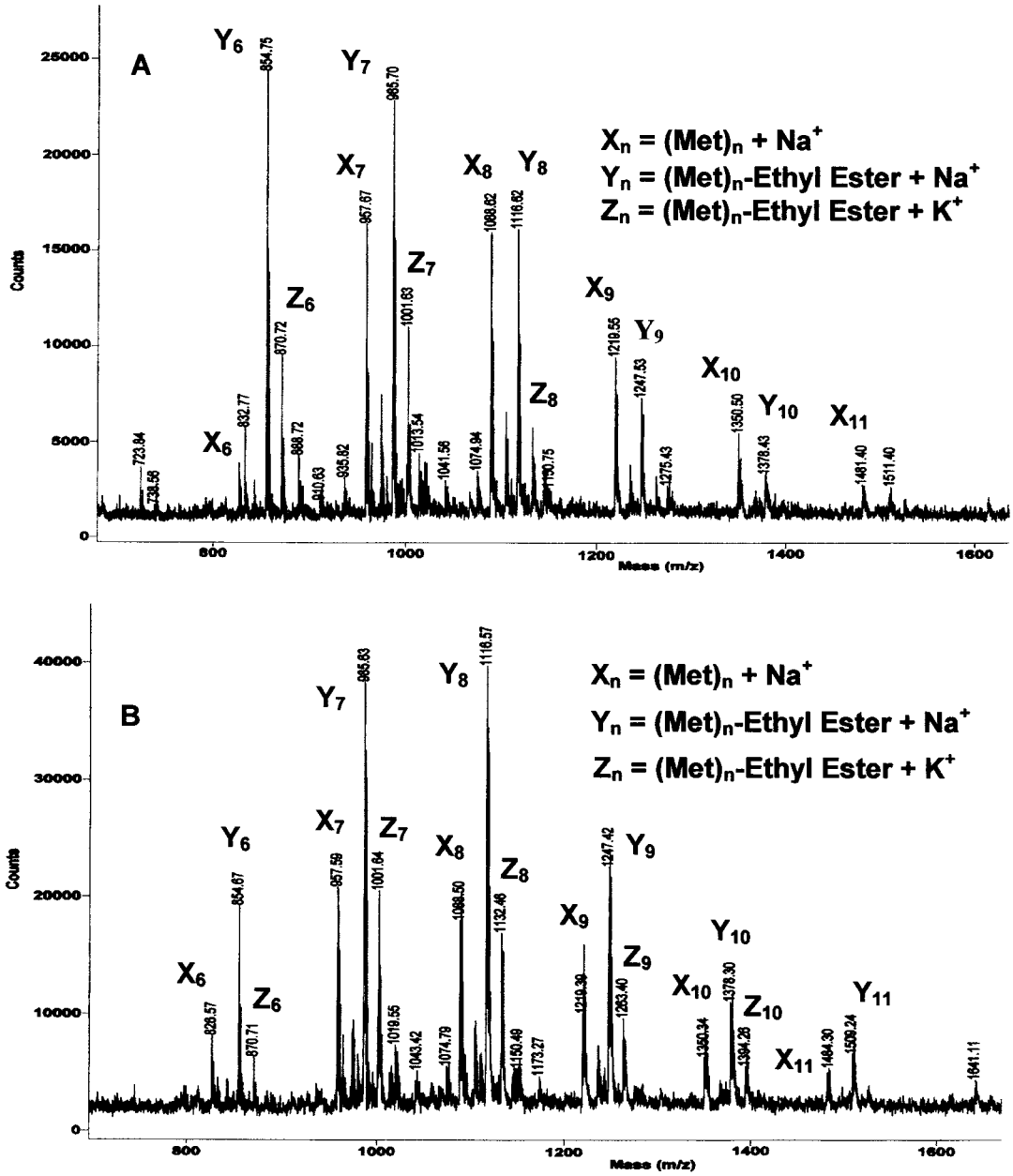


Figure 4. MALDI-TOF-MS of Met oligomers and HMB–Met co-oligomers synthesized with papain in 1 M sodium citrate buffer at pH 5.5. The incubation period was 24 h at 37 °C. (**A**) Mass spectra of Met oligomers. (**B**) Mass spectra of HMB–Met co-oligomers

2B). The results also show that the reaction was completed within the first 6 h; after this time period, the concentrations of free HMB and free Met remained relatively constant. These results were in agreement with the gravimetric measurements, which revealed that the mass of oligomer precipitates did not increase significantly after the 6 h incubation. The results further show that nearly 80% of Met introduced as methionine ethyl ester is incorporated into the oligomers. The results are in general agreement with the results obtained for Met oligomers by Jost et al. (*19*) and Anderson and Luisi (*14*).

Acid Hydrolysis of Oligomers. Chromatograms of purified Met oligomers and HMB−Met co-oligomers acid hydrolysate obtained after 96 h of incubation are shown in **Figure 3**. Chromatograms depict the output of the DAD at 210 nm. The upper trace (**A**) depicts the chromatography of Met homooligomers hydrolysis products, while the lower trace (**B**) depicts the chromatography of HMB−Met co-oligomer hydrolysis products. The chromatogram of Met oligomer hydrolysate contained only one peak, and the retention time of this peak was identical to that of Met. The co-oligomer hydrolysate chromatogram contained two peaks. The retention times for the peaks matched those of Met and HMB. The relative concentration of Met and HMB in the hydrolysate was determined to be 16:1.

MALDI-TOF-MS Analysis. The positive ion MALDI-TOF spectrum of Met oligomers is shown in **Figure 4A**. The spectrum contains ion clusters, which are 131 amu apart. This mass difference corresponds to the repeating Met moiety (C_5H_9NOS). Because the masses of the N- and C-terminal Met residues ($C_5H_{10}NOS$ and $C_5H_{10}NO_2S$) are 132 and 148, respectively, a Met hexamer, $^{N}Met-(Met)_4-Met^{C} + H^+$ should appear at *m*/*z* 805 while a heptamer, $^{N}Met-(Met)_5-Met^{C} + H^+$, should appear at *m*/*z* 936. The *m*/*z* values of the dominant ions in the spectra did not coincide with these ions or other ion in this series; instead, the dominant ions appeared at *m*/*z* 827, 958, 1089, 1220, 1351, and 1482. These ions most likely

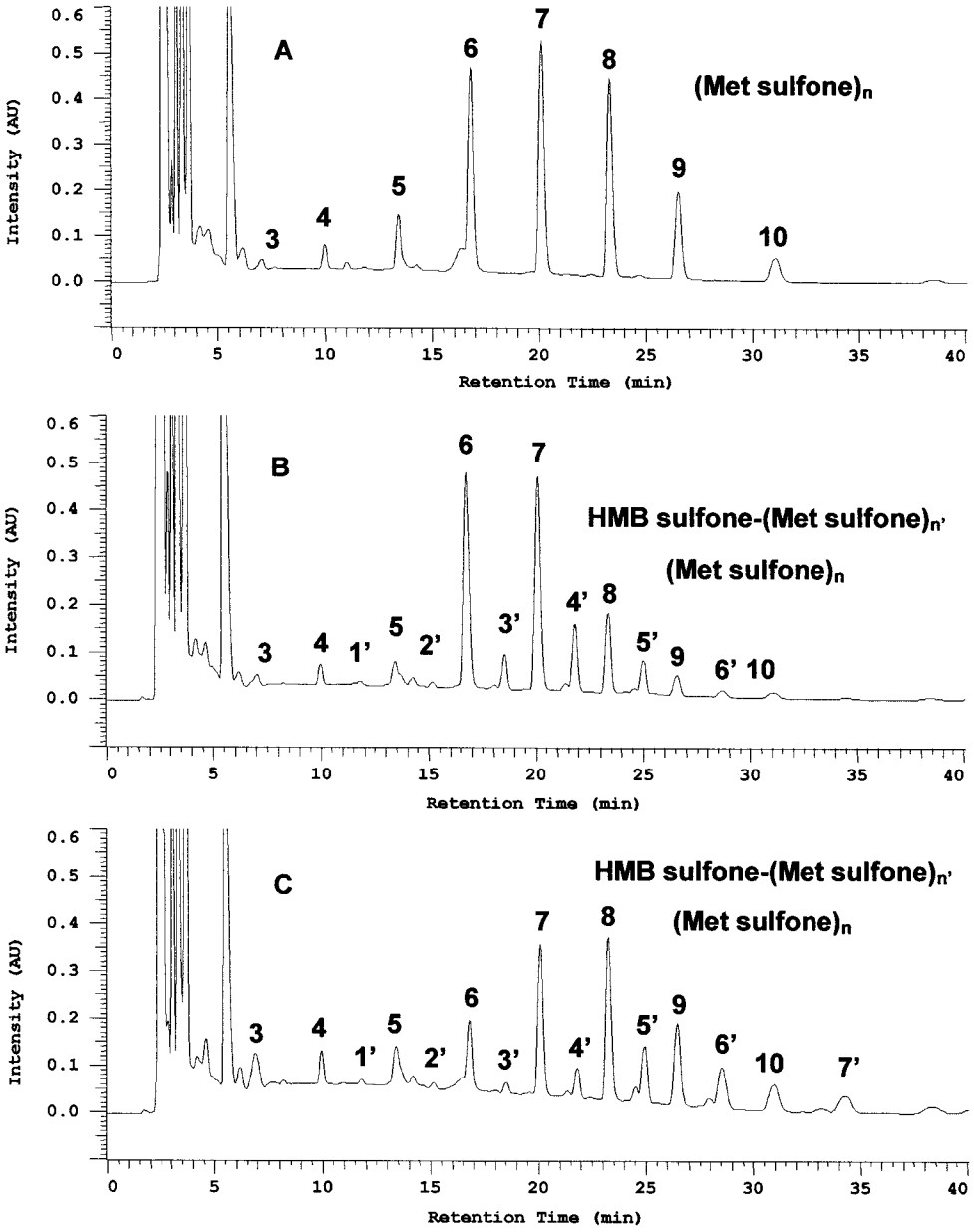


Figure 5. Chromatography of oligomer sulfones synthesized with papain in 1 M sodium citrate buffer at pH 5.5 and 37 °C. (**A**) Met oligomer sulfones obtained after 24 h of incubation. (**B**) HMB–Met co-oligomer sulfones obtained after 10 min incubation. (**C**) HMB–Met co-co-oligomer sulfones obtained after 24 h of incubation.

correspond to sodiated species of the type $^{N}Met-(Met)_n-Met^{C} + Na^+$ where $n = 4-9$. A second set of ions appeared at *m*/*z* 724, 855, 986, 1117, 1248, and 1379. These ions are most likely the sodiated Met oligomer ions with an intact ethyl moiety at the C-terminal end, $^{N}Met-(Met)_n-Met-O-C_2H_5 + Na^+$. A third set of ions appeared at *m*/*z* 740, 871, 1002, and 1135, and these ions correspond to series $^{N}Met-(Met)_n-Met-O-C_2H_5 + K^+$. A fourth set of ions appeared at 843, 974, 1105, 1236, and 1377, and these correspond to the series $^{N}Met-(Met)_n-Met^{C} + K^+$. In all cases, the number of Met residues in the oligomers was found to lie between 5 and 11.

A spectrum of HMB−Met co-oligomers is shown in **Figure 4B**. In this spectrum, ions corresponding to the series $^{N}Met-(Met)_n-Met^{C} + Na^+$, $^{N}Met-(Met)_n-Met-O-C_2H_5 + Na^+$, $^{N}Met-(Met)_n-Met^{C} + K^+$, and $^{N}Met-(Met)_n-Met-O-C_2H_5 + K^+$ were readily observed. However, none of the observed ions indicated the presence of HMB residue. The apparent absence of such ions can be attributed to the lack of a good protonation site in HMB oligomers and/or the low concentrations of co-oligomers of the type $HMB-(Met)_n$ in the mixture.

HPLC Separations of Met Oligomer Sulfones and HMB−(Met)$_n$ Co-oligomer Sulfones. Chromatographic separations of Met homooligomer sulfones are shown in **Figure 5A**. The chromatogram depicts the output of the DAD at 216 nm. A series of peaks indicating the presence of different oligomers were observed. The chromatographic profile of the oligomers is similar to the profile obtained by Kasai et al. (*25*) for Met oligomer sulfones. The chromatographic separation of the mixture of persulfonated Met oligomers and HMB−Met co-oligomers is shown in **Figure 5B**. This chromatogram contained a series of peaks, which were not present in the chromatogram of the Met oligomer sulfones (**Figure 5A**). The extra peaks indicate the formation of HMB oligomers or the incorporation of HMB in the Met oligomers at the N- or C-terminal end. HMB sulfone with a hydroxy group would be less polar than the corresponding Met oligomer sulfone with an amine moiety.

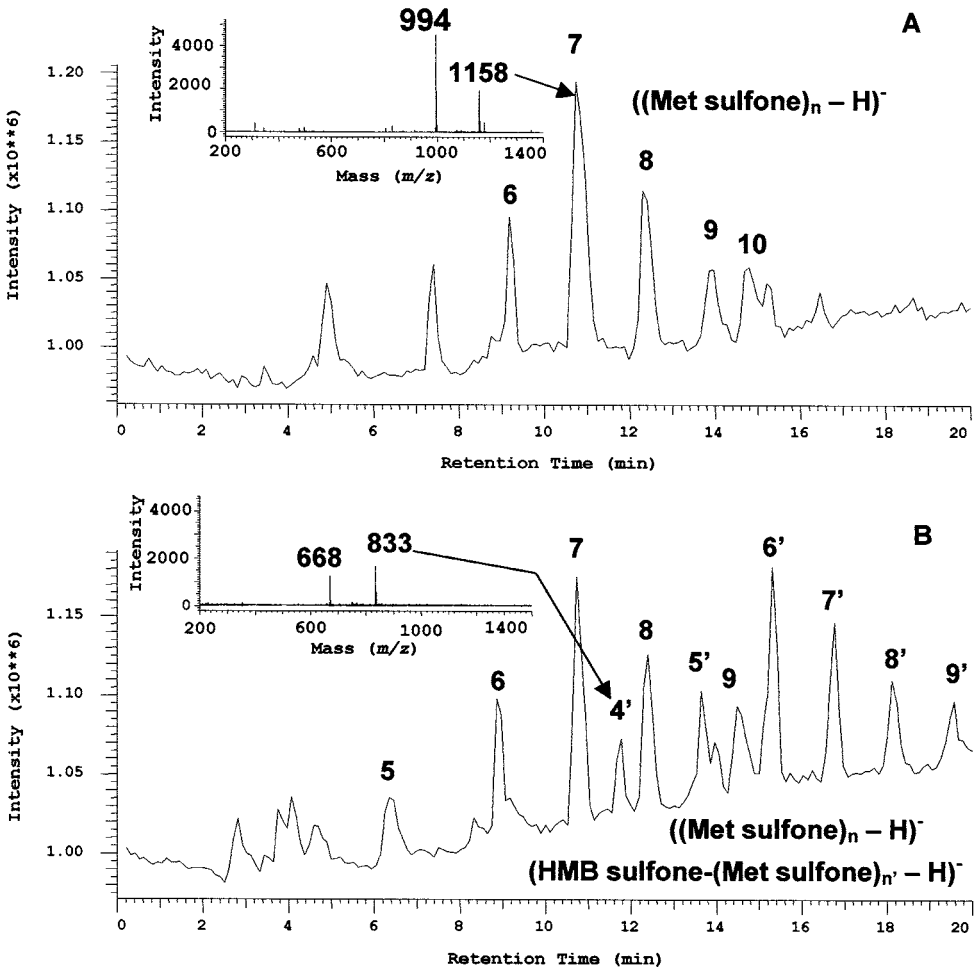


Figure 6. Negative total ion chromatogram of oligomers synthesized with papain in 1 M sodium citrate buffer at pH 5.5 and 37 °C. (**A**) Met oligomer sulfones; insert shows the mass spectrum for peak number 7, a methionine heptamer sulfone. (**B**) HMB–Met co-oligomer sulfones; insert shows the mass spectrum for peak number 4′, a HMB sulfone–$(Met sulfone)_4$.

Table 1. LC Elution Times for Met Oligomer Sulfones and HMB–Met Co-oligomer Sulfones[a]

oligomer sulfones	elution time (min) present study	elution time (min) Kasai et al. (*25*)
$(Met)_4$	10.0	N/A
HMB–(Met)	11.8	N/A
$(Met)_5$	13.4	14.0
HMB–$(Met)_2$	15.1	N/A
$(Met)_6$	16.8	17.8
HMB–$(Met)_3$	18.5	N/A
$(Met)_7$	20.1	21.0
HMB–$(Met)_4$	21.8	N/A
$(Met)_8$	23.3	24.0
HMB–$(Met)_5$	24.9	N/A
$(Met)_9$	26.5	26.9
HMB–$(Met)_6$	28.6	N/A
$(Met)_{10}$	31.1	29.5
HMB–$(Met)_7$	34.2	N/A

[a] N/A, not available.

Therefore, the HMB−Met co-oligomer should elute later than the corresponding Met oligomer. The relative intensity of the Met oligomer and possible HMB−Met peaks was found to be 3:1, respectively. Similar chromatographic results were obtained for the HMB−Met co-oligomers synthesized at pH 9.

Chromatographic separations of persulfonated Met and HMB−Met oligomers obtained after different incubation periods were also performed. The results of these separations indicated that the degree of polymerization is dependent on the incubation period. The intensities of longer chain oligomers were found to increase with the incubation period. Larger oligomers were observed in the chromatogram of oligomers obtained after 24 h of incubation as compared to the oligomers obtained after a 10 min incubation period (**Figure 5B**,**C**). Chromatographic data also indicated that the presence of HMB might affect the relative distribution of Met oligomers.

LC-ESI-MS Analysis of Met Oligomer Sulfones and HMB−$(Met)_n$ Co-oligomer Sulfones. Oligomer sulfones separated with the RPLC were characterized with ESI-MS. The ESI-MS was operated in both the positive ion and the negative ion mode. The HMB-containing oligomer sulfones were observed only in the negative ion mode. The negative ion mode TICs of the oligomers are shown in **Figure 6A**,**B**. The Met oligomer sulfone peaks in the two sets of chromatograms could be readily matched; the chromatogram of HMB−Met co-oligomer sulfones, however, contained extra peaks. The mass spectrum of the peak with a retention time of 10.7 min in the chromatogram of Met oligomer sulfones is shown in the **Figure 6A** insert. The base ion in spectrum appeared at *m*/*z* 994, while the highest mass ion was at *m*/*z* 1158. The difference of 164 would correspond to the loss of Met sulfone residue from the N-terminal end of the oligomers chain. The spectra indicate that the peak is most likely that of a Met heptamer sulfone, $(Met sulfone)_7$. The spectrum for one of the "extra" peaks (retention time of 11.8 min) in the HMB−Met co-oligomer sulfones

chromatogram is shown in the **Figure 6B** insert. The base ion in this spectrum appeared at 668, while the highest mass ion, the likely pseudo-molecular ion, appeared at *m*/*z* 833. The molecular ion represents persulfonated tetramethionine with one sulfonated HMB residue, HMB sulfone−(Met sulfone)$_4$. The base ion at *m*/*z* 668 results from the loss of mass 165 from the pseudo-molecular ion. This corresponds to the loss of HMB sulfone residue from the N-terminal end of the Met oligomer sulfones chain. The retention time of the HMB sulfone−(Met sulfone)$_4$ co-oligomer is higher than the Met pentamer sulfone, (Met sulfone)$_5$. This is in agreement with an expected increase in retention resulting from the substitution of an amino group with a hydroxyl group. A tentative identification of all Met oligomer sulfones and HMB−Met co-oligomer sulfones was made from the mass spectrometric data. These data were used to make a tentative identification of the Met oligomer peaks and the HMB−Met co-oligomer extra peaks in the LC-DAD data (**Table 1**).

The results indicate that the proteolytic enzyme papain can catalyze formation of Met homooligomers and HMB−Met co-oligomers. The total number of Met residues in the oligomers was found to vary between 4 and 11, while only one HMB residue was detected in each co-oligomer chain. The data also suggest that HMB is attached at the N-terminal end of the Met oligomer chain.

ABBREVIATIONS USED

Met, L-methionine; HMB, 2-hydroxy-4-(methylthio)butanoic acid; RPLC, reverse phase liquid chromatography; ESI-MS, electrospray ionization-mass spectrometer; MALDI-TOF-MS, matrix-assisted laser desorption time of flight mass spectrometry; MHA, methionine hydroxy analogue; LC-ESI-MS, liquid chromatography-electrospray ionization mass spectrometry; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; DAD, diode array detector; ESI, electrospray interface; LC-DAD, liquid chromatography-diode array detection; MS, mass spectrometer.

LITERATURE CITED

(1) Chalupa, W. Rumen bypass and protection of proteins and amino acids. *J. Dairy Sci.* **1975**, *58*, 1198−1218.

(2) Schwab, G. C.; Satter, L. D.; Clay, A. B. Response of lactating dairy cows to abomasal infusion of amino acids. *J. Dairy Sci.* **1976**, *59*, 1254−1269.

(3) Schwab, G. C.; Bozak, C. K.; Whitehouse, N. L.; Mesbah, M. M. A. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. *J. Dairy Sci.* **1992**, *75*, 3486−3502.

(4) Dibner J. J.; Knight, C. D. Conversion of 2-hydroxy-4-(methylthio)butanoic acid to L-methionine in the chick: A stereospecific pathway. *J. Nutr.* **1984**, *114*, 1716−1723.

(5) Dibner, J. J. Utilization of supplementation methionine sources by primary cultures of chick hepatocytes. *J. Nutr.* **1983**, *113*, 2116−2123.

(6) Patterson, J. A.; Kung, L., Jr. Metabolism of DL-methionine and methionine analogues by rumen microorganisms. *J. Dairy Sci.* **1988**, *71*, 3292−3301.

(7) Belasco, I. J. Fate of carbond-14 labeled methionine hydroxy analogue and methionine in the lactating dairy cows. *J. Dairy Sci.* **1980**, *63*, 775−784.

(8) Koenig, K. M.; Rode, L. M.; Knight C. D.; McCullough, P. R. Ruminal escape, gastrointestinal absorption, and response of serum methionine to supplementation of liquid methionine hydroxy analogue in dairy cows. *J. Dairy Sci.* **1999**, *82*, 355−361.

(9) Pas Bernard, J. K. Supplemental animal-marine protein blend and a rumen protected methionine hydroxy analogue for lactating dairy cows. *Prof. Anim. Sci.* **1997**, *13*, 149−154.

(10) Ferjancic, A.; Puigserver, A.; Gaertner, H. Papain-catalyzed polymerization of amino acids in low water organic solvents. *Biotechnol. Lett.* **1991**, *13*, 161−166.

(11) Brenner, M.; Muller, H. R.; Pfister, R. W. A new enzymic peptide synthesis. I. *Helv. Chim. Acta* **1950**, *33*, 568−591.

(12) Brenner, M.; Pfister, R. W. Enzymic peptide synthesis. II. Isolation of enzymatically manufactured L-methionyl-L-methionine and L-methionyl-L-methionyl-L-methionine; A comparison with synthetic products. *Helv. Chim. Acta* **1951**, *34*, 2085−2096.

(13) Dannenberg, A. M.; Smith, E. L. Action of proteinase I of bovine lung. Hydrolysis of the oxidized B chain of insulin; Polymer formation from amino acid esters. *J. Biol. Chem.* **1955**, *215*, 55−67.

(14) Anderson, G.; Luisi, P. L. Papain-induced oligomerization of α-amino acid esters. *Helv. Chim. Acta* **1979**, *62*, 488−494.

(15) Sluyterman, L. Æ.; Wijdenes, J. Sigmoidal progress curves in the polymerization of leucine methyl ester catalyzed by papain. *Biochim. Biophys. Acta* **1972**, *289*, 194−202.

(16) Selvi, C.; Baboulene, M.; Speziale, V.; Lattes, A. Synthesis by enzymic catalysis II. Amino acid polymerization. *J. Chem. Technol. Biotechnol.* **1985**, *35B*, 282−290.

(17) Aso, K.; Uemura, T.; Shiokawa, Y. Protease-catalyzed synthesis of oligo-L-glutamic acid from L-glutamic acid diethyl ester. *Agric. Biol. Chem.* **1988**, *52*, 2443−2449.

(18) Arai, S.; Yamashita, M.; Fujimaki, M. A novel one-step process for enzymatic incorporation of amino acids into proteins: Papain-catalyzed polymerization of L-methionine ethyl ester and its regulation by adding a protein substrate. *Agric. Biol. Chem.* **1979**, *43*, 1069−1079.

(19) Jost, R.; Brambilla, E.; Monti, J. C.; Luisi, P. L. Papain catalyzed oligomerization of α-amino acids. Synthesis and characterization of water-insoluble oligomers of L-methionine. *Helv. Chim. Acta* **1980**, *63*, 375−384.

(20) Lee, H.; Fukushi, H.; Uchino, M.; Fukuzawa, Y. Papain catalyzed polymerization of L-α-amino acid methyl esters with hydrophobic side chains. *Chem. Express* **1990**, *5*, 741−744.

(21) Murgasova, R.; Hercules, D. M. Polymer characterization by combining liquid chromatography with MALDI and ESI mass spectrometry. *Anal. Bioanal. Chem.* **2002**, *373*, 481−489.

(22) Loo, J. A.; Loo, R. R. O. Electrospray Ionization Mass Spectrometry of Peptides and Proteins. In *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation, and Applications*; Cole, R. B., Ed.; John Wiley and Sons: New York, 1997; pp 385−419.

(23) Dass, C. *Principles and Practice of Biological Mass Spectrometry*; John Wiley and Sons: New York, 2000.

(24) Spindler, M.; Stadler, R.; Tanner, H. Amino acid analysis of feedstuffs: Determination of methionine and cystine after oxidation with performic acid and hydrolysis. *J. Agric. Food Chem.* **1984**, *32*, 1366−1371.

(25) Kasai, T.; Tanaka, T.; Kiriyama, S. Correlation between molecular weight distribution of oligo-L-methionine prepared by papain-catalyzed polymerization and its supplementary effect in a low protein diet. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1884−1885.

Received for review November 4, 2002. Revised manuscript received January 31, 2003. Accepted February 6, 2003.

JF026093G