

Proteolysis of meat and bone meal to increase utilisation

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Abstract. Meat and bone meal (MBM), an important by-product of the meat industry, is the ground, rendered remainder of farm animals after removal of the hide and meat. Most protein in MBM is insoluble, which limits its usefulness. Defatted, milled porcine MBM was subjected to saturating amounts of trypsin, a selective protease, and subtilisin, a protease with broad selectivity. Samples were withdrawn over a 48-h time course of hydrolysis and filtered to remove insoluble material. The rate at which the MBM protein was converted to a soluble form was equivalent for both proteases. Over the time course, trypsin generated fewer free amino groups than did subtilisin, and at a specified time, the molecular weight (MW) of the soluble trypsin hydrolysate was higher than that of the subtilisin hydrolysate. Assay of amino group formation showed that the proteases were still active even after soluble protein generation had ceased. The hydrolysates are useful for a variety of food and non-food uses. The hydrolysates were tested for flocculation activity in an ongoing effort to find sources for renewable flocculant. Kaolin flocculant activity was observed with the soluble fraction obtained before hydrolysis of MBM and also observed with the relatively high MW hydrolysates from short treatment with trypsin and subtilisin. Low MW fractions obtained from by subtilisin treatment at 30–48 h also showed kaolin-settling ability, probably through a coagulation or charge neutralisation process.

Additional keywords: bridging, kaolin, patch.

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Introduction

Approximately 10% of the gross income of the meat industry comes from by-products (Jayathilakan *et al.* 2012). One such by-product is meat and bone meal (MBM). MBM is the ground, rendered remainder of farm animals after removal of the hide and meat for leather and food use. MBM demand has been limited by governmental restrictions due to disease concerns (Arvanitoyannis and Ladas 2008) and by an increase in available protein. MBM is currently used as an animal feed ingredient in some countries and as a solid fuel. MBM has poor solubility, due to the nature of its protein component plus the high heat used in its preparation (Garcia *et al.* 2006).

Recently there has been an influx into the market place of protein-rich materials, which are by-products of biofuel production. There has also been a surge of interest in the use of proteins in non-food applications due to a need for renewable materials. A partial list of such applications is wood adhesives (Park *et al.* 2000; Liu and Li 2007), media ingredients for industrial fermentation (Garcia *et al.* 2011; Liang *et al.* 2011; Solaiman *et al.* 2011), commodity chemicals from protein-derived amino acids (Lammens *et al.* 2012), bio-based plastics (Swain *et al.* 2004), and renewable flocculant (Piazza and Garcia 2010b).

It has been known for some time that protein hydrolysis of meat industry by-products gives soluble extracts with high protein content (Webster *et al.* 1982). This research area is still under active investigation (Bhaskar *et al.* 2007) and has been recently expanded to include the production of bioactive peptides by hydrolysis of edible meat by-products (Toldrá *et al.* 2012). Nevertheless, a review of the literature shows few studies of MBM hydrolysis. Acid hydrolysis was applied for the determination of amino acid content of MBM (Rayner 1985). Basic and enzymatic hydrolysis was applied to MBM to obtain soluble hydrolysates (Garcia *et al.* 2011). Basic hydrolysis gave a product with degraded amino acids and high ash content. Enzymatic hydrolysis employed non-specific proteases, which gave soluble hydrolysates with number average molar mass of only 500 Da, and the percent organic matter solubilised was only 40%.

An aqueous extract of (unhydrolysed) MBM was reported to have kaolin (clay) flocculant activity (Piazza and Garcia 2010a). This extract contained only a fraction of the MBM protein, since most MBM protein is insoluble. Base-catalysed hydrolysis of MBM gave hydrolysates, which showed intriguing differences in their flocculation ability depending upon the time they were taken from the hydrolysis mixture (Piazza and Garcia 2010b). The

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only data concerning flocculation by protease-derived MBM hydrolysates in the literature is a test of a single Versazyme hydrolysate. Thus a more rigorous investigation of flocculation by enzyme-produced MBM hydrolysates is warranted.

In the research results presented here, MBM was hydrolysed with two proteases, trypsin and subtilisin. Trypsin is a selective protease that preferentially cleaves the peptide bond on the carboxyl side of arginine and lysine (Yin *et al.* 2008). Subtilisin (protease from *Bacillus licheniformis*) is a protease with broad reactivity (Hill and Schmidt 1962; Guérard *et al.* 2001). The soluble hydrolysates were tested for their flocculant activity. Finding sources of renewable flocculants is important because currently used flocculants are prepared from materials manufactured from natural gas and petroleum (Salehizadeh and Shojaosadati 2001). Also renewable flocculants may be safer than synthetic flocculants, which are known to be toxic or to degrade to toxic products (Bolto and Gregory 2007).

Materials and methods

Materials

A fine kaolin (clay) with the trade name 'Polygloss 90' from Huber Engineered Materials was a gift from the M. F. Cachat Co. (Lakewood, OH, USA). Polygloss 90 had a surface area of 22.0 m²/g. Porcine MBM was a gift from Hormel (Austin, MN, USA). DL-dithiothreitol, MES [2-(morpholino)ethanesulfonic acid], *o*-phthalaldehyde, sodium tetraborate decahydrate, protease from *Bacillus licheniformis* (subtilisin) [Type VIII, lyophilised powder, 7–15 units/mg (1 unit = 1.0 μmol trypsin/min), CAS number 9014–01–1, EC number 3.4.21.14], and trypsin from bovine pancreas [powder, ≥7500 BAEE units/mg solid, CAS number 9002–07–7, EC number 3.4.21.4] were obtained from Sigma-Aldrich (St Louis, MO, USA). Ammonium hydrogen carbonate 98% was from Alfa Aesar (Lancaster, UK). Titan 2, 30-mm Filter Red 0.45 μm, PVDF membrane filters were from Sun Sri (Rockwood, TN, USA). Water was purified to a resistance of 18 MΩ-cm using a Barnstead E-pure system from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation of MBM fractions

Fat removal from MBM was conducted by mixing 3 mL hexane per 1 g MBM for 1 h at 22°C and centrifugation of the mixture at 7000g for 45 min at 20°C. The MBM was air-dried overnight. In 100-g portions, part of the MBM was processed in a cryogenic mill (6800 Freezer Mill, SPEX CertiPrep, Metuchen, NJ, USA) to reduce the particle size. Small portions of the defatted, milled MBM were vacuum dried to constant weight, which showed that MBM contained 2.8 ± 0.1% (s.e., *n* = 6) water, and relevant calculations were corrected for this water. The defatted, milled MBM contained 48.1 ± 1.1% protein and had an ash content of 34.4 ± 0.1%.

Extraction of MBM protein without protease

Milled, defatted MBM (625 mg; dry weight 606 mg) was extracted with 24 mL aqueous NH₃HCO₃ (50 mM, pH 9.0) at 30°C in a rotary shaker. At measured times, a portions of the mixture was filtered through 0.45-μm PVDF filters (17 mm,

Titan, Rockwood, TN, USA). Duplicate 1-mL portions of the filtrates were transferred to preweighed vials. The buffer and water were removed by placing the vials in a nitrogen stream at 55°C for 12 h (Nitrogen Evaporator, Organomation Associates, Berlin, MA, USA), followed by placement in a vacuum oven at 55°C until constant weight was obtained.

Proteolysis and extraction of MBM protein

Milled, defatted MBM was subjected to hydrolysis by proteases. In a typical procedure 10 g (dry weight 9.7 g) of MBM was suspended in 400 mL aqueous NH₃HCO₃ (50 mM, pH 9.0), and the reaction vessel was placed in a rotary shaker at 30°C. After 20 min, a 10-mL sample was removed for the 'zero' point. The indicated amount of protease was added, and samples were subsequently withdrawn at the indicated times. The pH of the samples was lowered to 3.0 to terminate the reaction. The acidified samples were filtered through 0.45-μm PVDF filters, and dry weight of the filtrates was determined as described above.

Protein measurement of MBM from nitrogen analyses

Nitrogen content was determined using an automated analyser (model FP-2000, Leco Corp., St Joseph, MI, USA). A nitrogen to protein conversion factor of 5.37 is based on complete amino acid analysis of MBM (Sriperm *et al.* 2011).

Degree of peptide bond hydrolysis

The time course of MBM hydrolysis was followed by peptide bond cleavage using a spectrophotometric assay for amino group formation by derivation with *o*-phthalaldehyde (Nielsen *et al.* 2001) with the addition of dimethylsulfoxide to stabilise the chromophore, as recommended (Church *et al.* 1985). The '*o*-phthalaldehyde reagent' contained 90 mg *o*-phthalaldehyde, 127 mg sodium dodecyl sulfate, 100 mg dithiothreitol. To this mixture, the following liquids were added sequentially with stirring: 2 mL ethanol, 80 mL sodium tetraborate decahydrate (50 mM), and 18 mL dimethyl sulfoxide. To assay amino groups, 1 mL of *o*-phthalaldehyde reagent was added to a 0.2-mL sample with mixing. The absorbance at 340 nm was measured after 3 min. Calibration curves were prepared using serine as an amino group standard. Total amino groups in completely hydrolysed MBM were obtained using the hydrolysis method described in Gehrke *et al.* (1985).

Flocculation testing

A published method was used for measuring the flocculation ability of the hydrolysates (Piazza and Garcia 2010a). Hydrolysate (volume adjusted to give 8 mg dry weight) was dispersed in aqueous buffer [2-(N-Morpholino)ethanesulfonic acid], and the pH was adjusted to 5.5. Settling of kaolin (clay) was determined at 1 and 5 h. All measurements were repeated 5 times, and the mean values ± s.e. were calculated.

Gel filtration chromatography

Molecular weight (MW) standards: dipeptide (Ser-Gly) and pentapeptide (Peptide 6A) were from Bachem Americas (Torrance, CA, USA). Aprotinin, cytochrome C, carbonic anhydrase, albumin, and bradykinin were from a kit for MW

6500–68 000 for gel filtration chromatography, Sigma-Aldrich. Thyroglobulin, gamma globulin, ovalbumin were components of a gel filtration standard from Biorad (Richmond, CA, USA). Alanine, glutamic acid, and phenylalanine were from Sigma.

HPLC gel filtration chromatography was conducted under isocratic conditions on a Waters 2695 Separation Module with Varian 380-LC evaporative light scattering detector using a Superdex Peptide column 10/300 GL (300 × 10 mm, GE Healthcare, Piscataway, NJ, USA). Each chromatography run was for 60 min. The eluting solvent was 50 mM ammonium hydrogen carbonate.

Results and discussion

Extraction of MBM without protease

Milled, defatted MBM was extracted with aqueous ammonium hydrogen carbonate buffer (pH 9.0) to measure its water-soluble content. The time course of extraction of soluble mass (15 min to 24 h) was followed, and the percent mass was calculated. After 15 min of extraction, the mass percent of extracted material was $28.3 \pm 1.5\%$. The mass percent of extracted material at 24 h was identical within experimental error to that obtained after 15 min. Thus, the majority of MBM consists of insoluble material under these extraction conditions, and increasing extraction time does not increase MBM solubility.

Enzymatic extraction of MBM mass

Milled, defatted MBM was hydrolysed with the proteases trypsin and subtilisin. The amount of protease used was determined empirically to give the maximal rate of hydrolysis. The time course of hydrolysis was followed by measuring the dried mass of the water-soluble material and calculating percent mass. Fig. 1 shows composite data from three experiments for each protease.

The percent of soluble mass increased at a rapid rate during the first 7 h of hydrolysis with trypsin (Fig. 1a). At times greater than 7 h, the percent of soluble mass increased only modestly. The curved line in Fig. 1a is the fit of the data by non-linear regression analysis to the following function: $f(t) = [(M_p t) / (K + t)] + M_{int}$ where M_p is the percent of soluble mass attainable at infinite time by protease addition; K is a constant; t is the hydrolysis time; M_{int} is the initial percent of soluble mass obtained by mixing MBM and aqueous buffer before protease was added. For the data in Fig. 1a the values of M_p , K , and M_{int} are $49.7 \pm 1.2\%$, 1.6 ± 0.2 , and $28.2 \pm 1.4\%$. The sum of M_{int} and M_p is the maximum percent of soluble MBM mass (M_{max}) and is $77.9 \pm 1.9\%$.

The data shown in Fig. 1b shows the percent dried mass of the water-soluble material obtained by treatment of MBM with the protease subtilisin. From non-linear regression analysis, the values of M_p , K , and M_{int} are $48.7 \pm 1.1\%$, 1.6 ± 0.2 , and $29.1 \pm 1.9\%$, giving a value for M_{max} as $77.8 \pm 1.9\%$. Thus, the estimates of M_p , K , M_{int} , and M_{max} obtained from trypsin or subtilisin hydrolysis were remarkably similar.

Protein measurement of extracts

Duplicate portions of the dried trypsin and subtilisin-treated soluble extracts obtained during the time course were analysed for nitrogen, and percent protein was calculated (Sriperem *et al.*

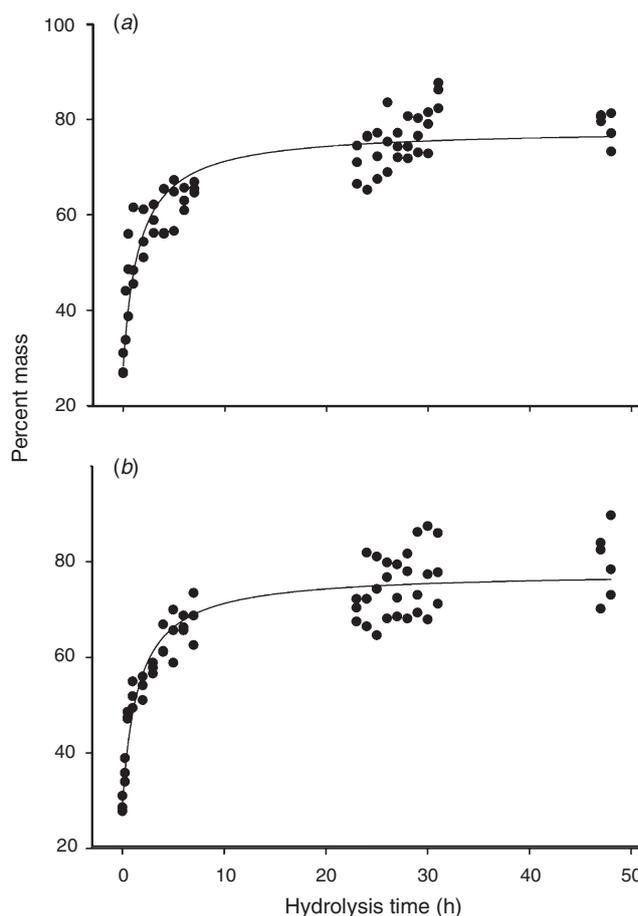


Fig. 1. Effect of hydrolysis time on the percent of soluble mass released from defatted meat and bone meal (MBM). For each time point, two 1-mL filtered portions from the hydrolysis assay were added to weighed vials. The portions were dried to constant weight, and the average weight was calculated. Percent mass was calculated from the dried weight of water-soluble material divided by the dried weight of MBM before hydrolysis. Results from three hydrolysis trials for each protease were combined. Each data point is the average of two measurements. (a) Hydrolysis with 500 mg trypsin; (b) hydrolysis with 40 mg subtilisin. The curves were generated by non-linear regression as described in the text.

2011). The mean percent protein in the soluble extracts was $63.9 \pm 1.2\%$ after correction for the protein in trypsin or subtilisin. Since average M_{max} is $77.85 \pm 1.90\%$ for trypsin and subtilisin-treated MBM, the percent of protein extracted from MBM is $49.7 \pm 1.5\%$ (0.7785×63.9). This product is, within experimental error, equal to the percent protein in dried MBM: $48.1 \pm 1.1\%$. The equivalence of these values shows that all the protein contained in MBM was converted to a soluble form by proteolysis with trypsin or subtilisin.

A reviewer questioned whether the collagen present in MBM is hydrolysed by proteases. While it is true that the triple helix structure of collagen usually prevents or dramatically slows proteolysis, the collagen structure is disrupted by heat which allows protease digestion (Bruckner and Prockop 1981). Specific recent examples of proteolysis of collagen by trypsin are given in Yuanlong *et al.* (2012) and Zhang *et al.* (2006). An example of proteolysis of collagen by subtilisin is given in Vasileva-Tonkova

et al. (2007). Additionally, it has been claimed that finely divided collagen powders are also digested by trypsin (Thomas and Seymour-Jones 1923), although the heat generated in the collagen sample by the grinding process may have been sufficient to denature the collagen. Normal procedures for cooking and drying MBM use temperatures in the range of 130–150°C, which are sufficiently high to denature the collagen in MBM and make it susceptible to proteolysis.

Peptide bond hydrolysis

Peptide bond hydrolysis was monitored over the hydrolysis time course by measuring the appearance of free amino groups. After an initial rapid increase in free amino groups, the rate of amino formation continued from 5 to 48 h at a reduced rate. At times after 30 min, the free amino groups generated by subtilisin were greater than those generated by trypsin. By 48 h, the free amino groups given by subtilisin were 1.15 ± 0.10 mmol/g MBM, while those generated by trypsin were 0.66 ± 0.04 mmol/g MBM. It is notable, that there was a significant increase in amino groups going from the second day of collection (23–31 h) to the third day of collection (47–48 h) for both subtilisin- and trypsin-promoted hydrolysis. This shows that subtilisin and trypsin were still active during the 23–48-h time period. The flattening of the soluble mass curves (Fig. 1) during this time period was not due to insufficient proteolytic activity, but was instead due to depletion of insoluble protein from MBM.

Fig. 2 shows the concentration of amino groups ($\mu\text{mol/mL}$). The amino group concentration data show that amino groups increased throughout the time course. Subtilisin generated a higher concentration of free amino groups than did trypsin. Fig. 2 also shows the ratio of amino groups to the soluble mass ($\mu\text{mol/mg}$). In contrast to the concentration data, the ratio of amino to soluble mass for trypsin was approximately constant up to 30 h. After 30 h, there was almost no additional soluble

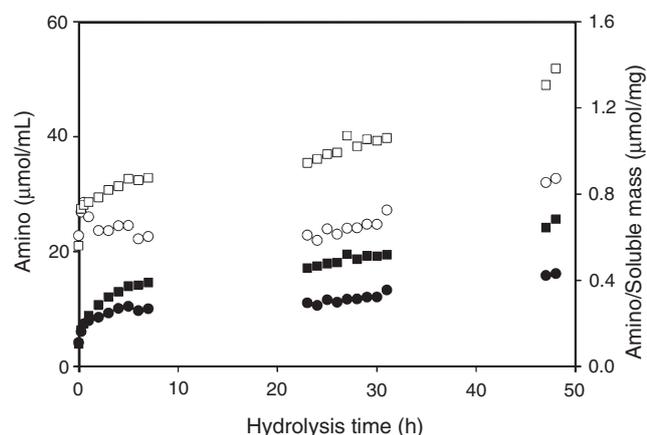


Fig. 2. Effect of hydrolysis time on amino group released from defatted meat and bone meal. From a single hydrolysis trial, five amino group assays were conducted. Results are the mean of 10 assays. Open symbols: amino/soluble mass ($\mu\text{mol/mg}$); closed symbols: free amino ($\mu\text{mol/mL}$). Trypsin: (○) amino/soluble mass, (●) free amino. Subtilisin: (□) amino/soluble mass, (■) free amino.

mass released from MBM, but amino groups continued increasing, and the ratio of amino to soluble mass increased. In contrast to the trypsin results, the ratio of amino to soluble mass for subtilisin increased throughout the time course. According to Figs 1a and b, subtilisin released soluble mass at nearly the same rate as trypsin. However, with subtilisin, the degree of proteolysis was in excess of what was needed to release soluble mass. Thus the data show that while proteolysis is important for mass extraction, there is not a correlation between the degree of proteolysis and the formation of soluble mass.

The equivalence in the rates of soluble mass generation by subtilisin and trypsin suggests that in addition to amide bond hydrolysis rate, there is a factor which is important in controlling the rate of soluble mass generation. One possibility is that after the enzymes hydrolyse the protein in MBM, the process of solubilising the resulting protein fragments is not instantaneous, and the rate of this solubilising process limits the overall rate of formation of soluble mass. Also, the proteases may not be able to contact all of the MBM protein at once and may instead only hydrolyse that protein localised at or near the surface of the MBM particle. The proteases are able to increase their penetration into the MBM particle as the surface proteins are hydrolysed and solubilised. This overall process, penetration and hydrolysis, occurs at a rate which is equal for each protease.

Molecular weight distribution

The influence of time on the MW distribution of material in the soluble fraction of the trypsin hydrolysates is shown in Fig. 3. In the first 15 min (0.25 h) large decreases in >30 and 10–30 kDa materials were observed, with a corresponding increase in material with MW <10 kDa. With times greater than 15 min, there was a relatively gradual change in MW distribution with the lower MW materials gradually increasing. At 47–48 h, the material of greatest abundance was 1–5 kDa, and there was also some increase in material with MW <0.2 kDa (free amino acids).

The MW distribution of soluble material in the subtilisin hydrolysates is shown in Fig. 4. A gradual decrease in the >30 kDa proteinaceous material was observed to 31 h. The 10–30 and 5–10 kDa materials also decreased to 23 h, and thereafter the amounts of these materials remain approximately constant until 47–48 h where there was a decrease. At 47–48 h, the >30 kDa materials were absent, and the 10–30, 5–10, and 1–5 kDa materials were at their lowest levels. At 47–48 h there was a gradual increase in the <0.2 kDa material up to 31 h, and at 47–48 h, there was a large increase in the <0.2 kDa material.

The MW distribution data in Figs 3 and 4 are consistent with the amino group formation data in Fig. 2, which show that more amide bond hydrolysis is catalysed by subtilisin than by trypsin. Trypsin is a highly selective proteinase, and this selectivity promotes the formation of higher MW fragments, although some lower MW fragments form also. In contrast, subtilisin has relatively broad selectivity, and its action gives a higher amide bond hydrolysis rate resulting in greater amounts of lower MW material.

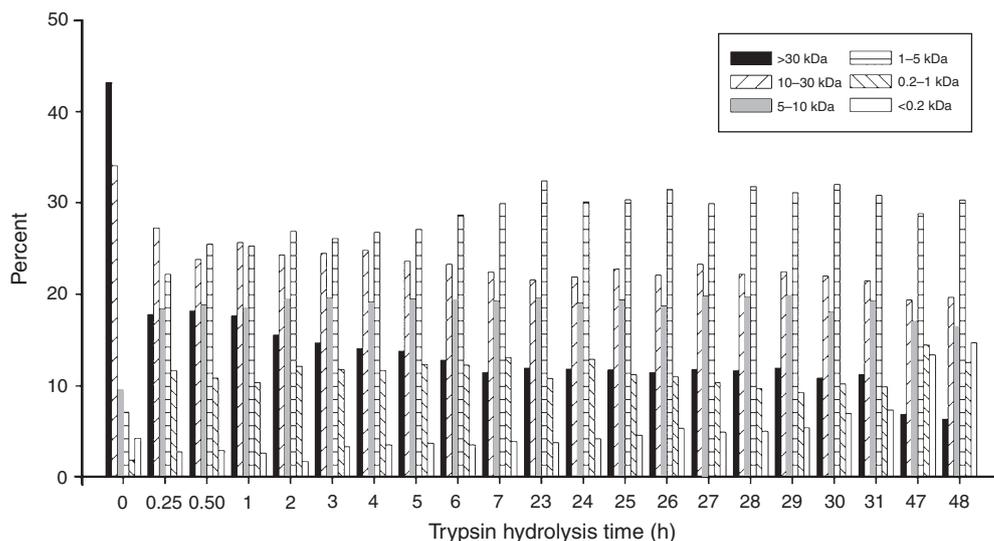


Fig. 3. Effect of trypsin hydrolysis time on the molecular weight distribution of the soluble meat and bone meal hydrolysate. The bar height represents the percent of hydrolysate within this molecular weight range. The results are the mean of two hydrolysis trials. The molecular weight range represented by each bar is shown in the legend on the upper left side of the figure.

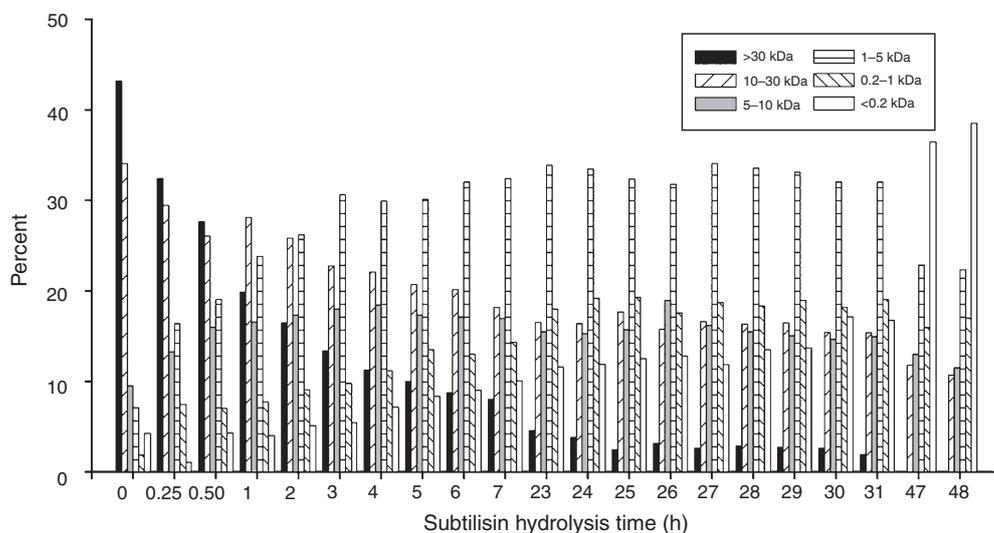


Fig. 4. Effect of subtilisin hydrolysis time on the molecular weight distribution of the soluble meat and bone meal hydrolysate.

Flocculation activity

An equal dry weight portion of the soluble filtrate from each sampled time was tested for its ability to flocculate kaolin. Flocculation testing was conducted at pH 5.5 because at this pH, the kaolin particles do not spontaneously flocculate, but proteins, peptides, and amino acids, which compose the MBM hydrolysates, have net positive charge that acts to neutralise the negative charge on the surface of the kaolin particles. The pooled results of the flocculation tests from trypsin hydrolysates are shown in Fig. 5. Kaolin settling activity was noted from the soluble portion of untreated MBM, which contained 28% of the

starting MBM mass (zero time in figure). Kaolin settling activity from the soluble hydrolysates was retained to 7 h of hydrolysis, which rendered soluble 68% of the MBM. In interpreting the results, it is important to note that Fig. 5 shows the results from a fixed quantity (0.8 g dry weight) of the soluble material. The total kaolin settling activity of the 7-h hydrolysate is actually greater than indicated in the Figure because the 7-h hydrolysate contained 40% more soluble material than prehydrolysis MBM. Nevertheless, the kaolin settling activity in the soluble hydrolysates was no longer evident in those samples taken after 7 h, and this shows that larger MW polypeptides are necessary for

flocculant activity. The need for larger MW polypeptides is consistent with the idea that the flocculant is operating through a bridging mechanism, such that the flocculant ‘bridges’ many individual particles to build a floc (Bratby 2006). The lack of kaolin settling activity from the longer time hydrolysates also shows that even a selective protease such as trypsin cannot release all the protein from MBM and maintain large enough protein fragments to give an active flocculant material.

The soluble filtrates taken from the subtilisin hydrolysis time course trials were also tested for flocculation activity (Fig. 6). Subtilisin hydrolysates gave kaolin settling only from hydrolysates obtained from 1 h or less. Since the size data (Fig. 4)

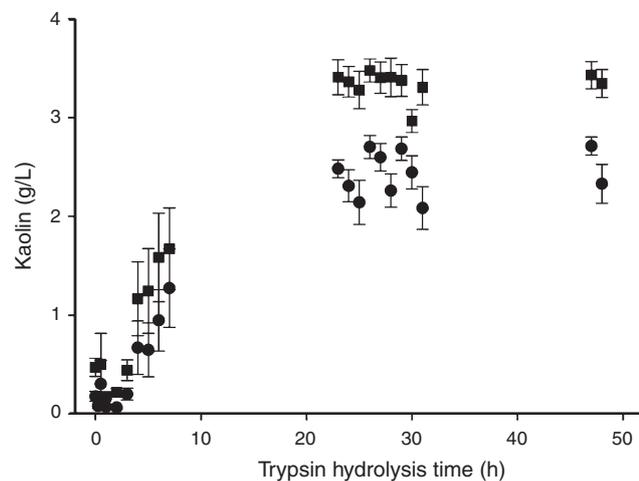


Fig. 5. Concentration of suspended kaolin after the addition of trypsin hydrolysates. Each assay contained 0.8 g (dry weight) hydrolysate/L and 5 g kaolin/L. From a single hydrolysis trial, five flocculation assays were conducted. Results are the mean of 10 assays from two hydrolysis trials. Suspended kaolin after 1 h (■) and 5 h (●). Control values were 1 h: 3.34 ± 0.14 g/L and 5 h: 2.60 ± 0.12 g/L.

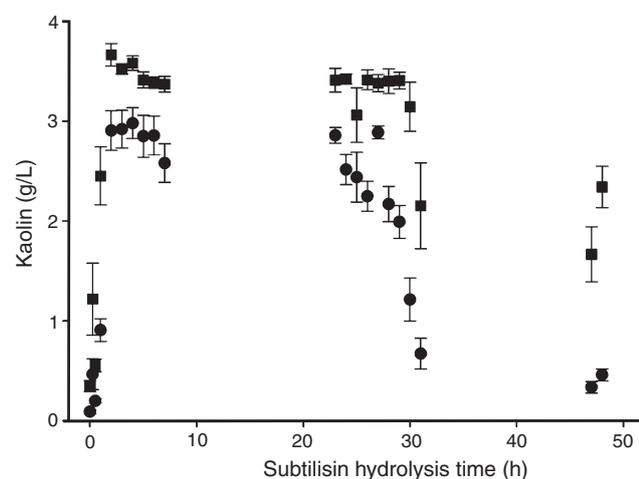


Fig. 6. Concentration of suspended kaolin after the addition of subtilisin hydrolysates. Each assay contained 0.8 g (dry weight) hydrolysate/L and 5 g kaolin/L. The data was obtained as described in Fig. 5. Suspended kaolin after 1 h (■) and 5 h (●). Control values were 1 h: 3.44 ± 0.08 g/L and 5 h: 2.78 ± 0.16 g/L.

clearly shows that subtilisin gives small polypeptide fragments, the loss of kaolin settling activity after only 1 h is consistent with the idea that flocculant that functions by the bridging mechanism which requires larger MW polypeptides. The most interesting aspect of Fig. 6 was the unexpected kaolin settling activity exhibited by the 30–48-h soluble hydrolysates. Fig. 4 shows that fractions 30–48 h contained mostly lower MW material.

The predominant mechanism of kaolin settling activity by the low MW hydrolysates is likely to be the patch or neutralisation mechanism; the positively charged amino groups in the MBM hydrolysate bind to the surface of the kaolin particles and neutralise the negative surface charge of the kaolin (Bratby 2006). In order to effectively neutralise the negative charge of the kaolin particles, the amino groups must be closely spaced on the surface of the kaolin particles. To achieve such close spacing, the size of the protein remnants needs to be relatively small. Lower MW synthetic or inorganic materials that cause sedimentation are usually referred to as coagulants (Bratby 2006).

Flocculation testing of the hydrolysates of trypsin and subtilisin revealed that there are two kinds of kaolin settling activity formed by proteolysis, a high MW and a low MW material. With respect to the high MW hydrolysate, there was $10.1 \mu\text{mol/mL}$ amino group formation at 7 h of trypsin hydrolysis and $8.9 \mu\text{mol/mL}$ amino group formation with 1 h subtilisin hydrolysis. Thus, when amino group formation is less than or equal to $\sim 9\text{--}10 \mu\text{mol/mL}$, the hydrolysates exhibited kaolin flocculant activity.

For the low MW material from subtilisin hydrolysis that gave kaolin coagulant activity, amino group formation is greater than or equal to $19.2 \mu\text{mol/mL}$ at 30–48 h (Fig. 2). This is higher than the amino group concentration measured in any of the trypsin hydrolysates. The need for a high degree of proteolysis explains why no low MW kaolin coagulant activity was observed in the trypsin hydrolysates.

Surprisingly, there is not a great deal of difference in the mass of flocculant or coagulant materials that may be generated by treatment with either enzyme. With 7 h treatment with trypsin, 17 mg/mL of high MW flocculant was prepared. With 30–48 h treatment with subtilisin, 18 mg/mL of low MW coagulant was prepared.

Conclusions

As indicated by free amine formation, the rate of proteolysis with subtilisin was greater than that given by trypsin, but the rate of soluble mass generation was the same for both proteases, suggesting that structural characteristics of MBM limit the rate at which its mass can be converted to soluble hydrolysate. Nitrogen analysis of the soluble fraction compared with the nitrogen in the original MBM indicated that all of the protein was converted to a soluble form. The soluble MBM hydrolysates gave both a relatively high MW flocculant and relatively low MW coagulant. This is the first report of two different MW kaolin settling materials being prepared from a single starting material. In addition to its use as a flocculant and coagulant, the MBM-soluble hydrolysate may also be used for animal or human nutrition in some countries (Alvarez *et al.* 2012) and as a high quality media ingredient for industrial fermentation.

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