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# Solubilization of meat & bone meal protein by dilute acid hydrolysis for the production of bio-based flocculant☆

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ABSTRACT

Flocculants are substances that cause suspended particles to aggregate, therefore accelerating sedimentation to produce a clarified solution. They find use in a huge variety of applications including wastewater treatment, erosion control, and paper manufacture. Meat and bone meal (MBM) is a high protein by-product of meat processing. Protein extracted from MBM has been shown to have good flocculation activity, but this protein has poor solubility. Previous attempts to improve solubility through alkaline or enzymatic hydrolysis have produced small protein fragments with low flocculation activity. The objective of this project was to produce large, soluble MBM protein fragments by applying reaction conditions that are known to hydrolyze peptide bonds only at aspartic acid and asparagine residues. In this project, milled MBM was suspended in 0.03N hydrogen chloride solution, under vacuum, and incubated at 108 °C. Samples were removed after 1, 2, and 4 h, and the soluble and insoluble material was separated. The molar mass distribution of soluble material was examined by size exclusion chromatography (SEC), progressive solubilization of protein was measured by using a test for organic nitrogen, and flocculation activity was tested by measuring kaolin clarification effectiveness (KCE). Results showed that at all hydrolysis times, concentration of solubilized material was increased approximately 3-fold compared to the acid-free controls. Results also showed that as reaction duration increased, more MBM protein was solubilized, but average molecular weight (MW) of solute was reduced, and flocculation activity was dependent on reaction duration. The dilute acid hydrolysis (DAH) employed in this project could be a simple and inexpensive treatment that has potential to add value to MBM and provide a new source for bio-based flocculants.

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## 1. Introduction

Flocculants are substances that facilitate the removal of suspended particles or colloidal material from a liquid (Krishnan and Attia, 1988). In agriculture, they are used to control erosion in irrigation furrows and to clarify livestock waste liquids. Flocculants are also widely used in processes such as concentration during chemical operations, paper manufacture (Piazza and Garcia, 2010a), dewatering in mining operations (Svarovsky, 2000), and aiding filtration and centrifugation (Lewellyn and Avotins, 1988).

A major class of flocculants are synthetic polymers, most commonly derivatives of polyacrylamide (PAM). While synthetic polymer

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flocculants are attractive due to their high effectiveness and low cost (Brostow et al., 2009; Nasser and James, 2007), there is growing concern regarding the environmental and health impacts of these substances (Acharya et al., 2010; Harford et al., 2011; Letterman and Pero, 1990; Park et al., 2009). These concerns have driven academic and commercial interest in the development of bio-based alternatives to synthetic polymer flocculants.

Meat and bone meal (MBM) is a product of the rendering industry, typically containing 45–63% protein (Garcia et al., 2006). Piazza and Garcia (2010a) showed that water-extracted MBM protein has flocculant functionality. It was shown to be effective at flocculating fine clay particles from suspension. Although compared to anionic PAM a much greater concentration of MBM protein is required to achieve equivalent clarification, MBM protein is not as dependent on the presence of calcium ions as anionic PAM is.

The main obstacle to utilizing MBM protein as a flocculant is its poor solubility. Water or dilute salt solutions extract less than 10% of MBM protein (Garcia et al., 2006; Piazza and Garcia, 2010a). Research efforts at maximizing the non-hydrolytic solubilization of MBM protein achieved solubilization of no more than 30.1% of MBM protein, and this was only possible using conditions that are impractical for the manufacture of a moderate value product (Garcia and Phillips, 2009).

It is commonly observed that hydrolysis of insoluble protein will produce peptides with increased solubility. The downside of hydrolytic solubilization is that it also tends to diminish functional properties such as gelling or foaming. Researchers have applied a variety of hydrolysis methods, attempting to achieve a high degree of MBM protein solubilization while retaining the flocculant activity of MBM protein. Hydrolysis with alkali, specific proteases, or non-specific proteases each greatly increased the proportion of MBM protein solubilized, but the solubilized protein fragments were generally quite small and had to be used much higher doses compared to intact MBM protein to achieve equivalent flocculation. (Garcia et al., 2011; Piazza and Garcia, 2010b, 2014).

Strong acids can hydrolyze peptide bonds between any pair of amino acids. Acid hydrolysis is typically non-specific and produces diverse populations of small peptides resulting from pseudo-random lysis of peptide bonds. Protein structural analysis literature of the 1960's contains references to reaction conditions under which acid hydrolysis is highly specific (Tsung and Fraenkel-Conrat, 1965; Schultz, 1967). When dissolved in 0.03N HCl, placed under vacuum, and incubated at 105 °C, peptide bonds on either end of aspartyl or asparaginyl residues were cleaved and free aspartic acid was liberated. It was found that under these conditions aspartic acid peptide bonds are preferentially cleaved at a rate at least 100 times greater than other bonds and that non-specific cleavages at other sites were minimal. As more modern techniques rendered this specific hydrolysis reaction obsolete for the study of protein structure, it seems to have fallen out of use. The scientific literature does not seem to contain references to the use of these reaction conditions for hydrolyzing insoluble protein or for the purposes of preparing a functional hydrolysate for practical application. The present study examines whether dilute acid hydrolysis (DAH) has potential for increasing the utility of MBM protein.

## 2. Materials and methods

### 2.1. Sample preparation

Meat and bone meal (MBM) obtained from Darling Ingredients (Irving, TX) was defatted in standard Soxhlet extractors using hexane for 4 h. Defatted MBM was reduced to a fine powder using a cryogenic mill (model 6800, Spec Certiprep, Metuchen, NJ). Defatted and milled MBM was then suspended separately in 0.03N HCl and in pure water at 2 mg/mL. Hydrolysis experiments were conducted using 15 mL aliquots in borosilicate glass vials capped with air-tight Teflon valves. The filled hydrolysis vials were placed under vacuum using a PicoTag work station (Waters Corporation, Milford, MA) according to the manufacturer's directions. The vials were then put into a 108 °C oven and incubated for varying durations. After cooling down to room temperature, samples were centrifuged at $2000 \times g$, at 10 °C for 30 min. The supernatant was saved for analysis of the solubilized protein.

### 2.2. Protein solubilization

The original protein content of the defatted, milled MBM was obtained by carrying out Kjeldahl nitrogen determination according to an AOAC official method (AOAC, 2002). Analyses were performed in quadruplicate using a system comprised of a Tecator Digestor Auto (Foss, Eden Prairie, MN) and a Kjeltec 8100 Distillation Unit (Foss). A nitrogen-to-protein conversion factor of 5.37 for MBM was applied throughout the present research, as recommended by Sriperm et al. (2011).

To determine the concentration of solubilized protein, the liquid portion of completed reactions was analyzed using a TNT 880 kit (Hach, Loveland, CO) following manufacturer's direction. This kit is designed to produce values equivalent to standard total Kjeldahl nitrogen (TKN) analysis of water. To ensure the test results are all located within the test kit measurement range, between 0.23 and 13.5 mg/L, all samples using HCl were diluted 1:20 while the samples using pure water were diluted 1:10 prior to analysis. Analyses were performed in triplicate.

### 2.3. Molar mass distribution

Hydrolysates were analyzed for their molar mass distribution using size exclusion chromatography (SEC) according to methods previously used by Piazza and Garcia (2014). Analysis was carried out on an HPLC (Waters Alliance 2695 Separations Module, Waters Corporation, Milford, MA) using a peptide column (Superdex Peptide 10/300 GL, 10 × 300–310 mm, GE Healthcare Bio-Sciences, Pittsburgh, PA) with an evaporative light scattering detector (Varian 380-LC, Varian Inc., Santa Clara, CA). The eluent was 50 mM ammonium hydrogen carbonate. Molecular weight (MW) standards were analyzed to establish the correlation between retention time and MW. Standards included thyroglobulin (#T9145, thyroglobulin from bovine thyroid, Sigma-Aldrich Co. LLC., St. Louis, MO), a kit for MW 6500-68000 (comprising aprotinin, cytochrome C, carbonic anhydrase, albumin, and bradykinin, Sigma), ovalbumin (#A5503, albumin from chicken egg white, Sigma), peptide 6A (Bachem Americas, Inc., Torrance, CA), phenylalanine (#P1150000, Sigma), glutamic acid (#1294976, Sigma), and alanine (#A7469, Sigma). Results were obtained by using the average value of 3 replications for each treatment.

### 2.4. Kaolin clarification effectiveness (KCE)

Kaolin clarification effectiveness (KCE) is a method developed in the authors' lab (Garcia et al., 2014) for testing the flocculation activity of a substance. Suspension with a final concentration of 1 g/L kaolin clay (Polygloss 90, KaMin performance minerals, Macon, GA) was prepared in 25 mM Malic-MES-Tris buffer (MMT) at pH 5.5. The MMT buffer was prepared with MES hydrate (#M5287), L-Malic acid (#M1000), and Trizma base (#T1503) which were all obtained from Sigma. Tests were conducted by measuring the turbidity of a glass vial containing 24 mL of the kaolin suspension using a ratio turbidimeter which employs an infrared light source (2100AN IS Laboratory Turbidimeter ISO, HACH, Loveland, CO). After mea-

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