



Assessment of the potential allergenicity of a Milk Basic Protein fraction

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Abstract

Background: A specific basic fraction of bovine milk, termed Milk Basic Protein (MBP), has the potential to provide nutritionally important benefits if used as a food ingredient. Although derived from milk, MBP is intended for use as an ingredient in other foods. Cows' milk is a well studied, commonly allergenic food. Although the proteins in MBP are not identified as milk allergens, food products containing MBP will be labelled as containing milk as a caution to milk allergic consumers under food labelling guidelines in the US and the European Union as MBP has not been demonstrated to be free of milk allergens. However, as part of an overall safety evaluation of MBP, the developers sought to evaluate the potential allergenicity of the primary protein components for characteristics of allergenic food proteins and to assess whether intake of these proteins at intended use levels could present a significant new allergenic risk for consumers.

Objective: To evaluate the potential allergenicity of the five identified proteins in MBP. While extensive studies have not demonstrated allergenicity of lactoferrin, the four other proteins are less studied. The four were tested here by sequence identity comparison to known allergens, and for stability of these proteins in acidic pepsin as a characteristic common to many food allergens.

Methods: Sequences of the proteins were compared to those listed in AllergenOnline.com, by methods recommended for the evaluation of proteins introduced in crops through genetic engineering. Pepsin stability was assessed by incubating the various proteins in simulated gastric fluid at pH 1.2 with porcine pepsin for up to 60 min at 37 °C, with samples withdrawn and analyzed at specific times.

Results: No significant sequence similarities were identified for the MBP proteins compared to known allergens. All but one of the protein components of MBP were digested relatively quickly by pepsin. The more stable protein will be of low abundance as consumed in contrast to most pepsin-stable food allergens.

Conclusions: Based on molecular characteristics and expected exposure, the protein components in MBP are unlikely to present any increased risk of allergy for milk allergic subjects or of cross-reactivity for other allergic subjects. However, since the proteins are derived from milk, products containing MBP will need to be labelled as containing milk proteins to warn milk allergic subjects of the potential risk of allergic reactions.

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1. Introduction

Food allergies affect an estimated 3.5–4% of the US population (Sicherer et al., 2004) with symptoms ranging from relatively mild to severe and life-threatening (Sampson, 2005). Because of the increasing awareness and the apparent increasing prevalence of food allergy, many new foods

Abbreviations: Cys, bovine cystatin C; HMG, bovine high mobility group protein 1; Kin, bovine high molecular weight kininogen I; LPO, bovine lactoperoxidase.

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and food ingredients are being subjected to pre-market allergenicity assessment (Poulsen, 2004; Hansen et al., 2004; Bindslev-Jensen et al., 2003; Crevel et al., 2002). If there is any indication that a newly introduced food ingredient might pose a risk to a sensitive group of consumers, appropriate labelling strategies are needed to protect potentially allergic individuals (Mills and Breiteneder, 2005; Mills et al., 2004; Hefle and Taylor, 2004).

The current study was designed to evaluate the potential allergenicity of a new food ingredient that is derived from cows' milk as part of the self-affirmation process to establish a food ingredient as generally recognized as safe (GRAS) under the Food and Drug Administration regulations of the US (21 CFR §170.30(b)). A specific basic protein fraction, Milk Basic Protein (MBP), of cows' milk contains several milk proteins including: lactoferrin, cystatin C, kininogen fragment 1·2, high mobility group-like protein and lactoperoxidase. In vitro and in vivo studies of these protein components from the milk whey protein source for MBP (Takada et al., 1996, 1997a,b,c) and the MBP (Toba et al., 2000; Takada et al., 2001) have shown that they contribute to bone health by both stimulating osteoblast activity and inhibiting osteoclast activity, thus promoting bone formation and suppressing bone resorption. Lactoferrin has previously been evaluated and found to be Generally Recognized as Safe (GRAS) as a food additive under the US Food and Drug Administration (FDA). The current study was performed to provide the FDA with information about the potential that the other proteins in MBP might elicit allergic reactions for a GRAS determination for MBP (FDA GRAS Notification, 2006).

The major cows' milk allergens have been identified as α -casein, β -casein, κ -casein and two whey proteins, β -lactoglobulin and α -lactalbumin (Wal, 2004; Jarvinen et al., 2001; Adams et al., 1991; Gjesing et al., 1986). The protein components of MBP are not known to cause allergies in consumers. However, several studies report some IgE binding to bovine lactoferrin using serum from cow-milk allergic subjects (Natale et al., 2004; Wal et al., 1995; Wal, 1998), although the specificity was not confirmed and there was no demonstration of biological activity. Because there is no certain test to absolutely demonstrate a lack of allergenicity, food products containing MBP would clearly be labelled as containing an ingredient from milk. Food labelling laws and regulations in the US, European Union and other countries stipulate that ingredients derived from commonly allergenic foods, including milk should reveal the source, namely milk. Thus, MBP is not intended for consumption by milk allergic consumers and will be clearly labelled as from milk even though MBP does not contain any of the major milk allergens.

The focus of the allergenicity assessment provided herein is an evaluation of the possibility that exposure to the milk protein components of MBP might lead to increased allergies due to potential cross-reactivity due to similarities of sequence/structure of the MBP with known allergenic proteins from other sources. Consequently, a

bioinformatics study was performed to compare the sequences of the proteins to those of known allergens to evaluate the potential for allergic cross-reactions (Goodman et al., 2005). Sequence searches are performed to evaluate the sequential and structural similarities between the query sequences and known allergens to evaluate the possibility that one or more of the query sequences may share one or more common IgE binding epitopes with an allergen. If true, there is a possibility the query sequence might elicit an allergic response in those with existing allergies. Epitopes that are bound by IgE may be short sequential segments of amino acids, or may be conformational, made up of amino acids that are separated in linear sequence, but due to conformational folding are very close in three dimensional space. Since the conformation of a protein is dictated to a great extent by the primary sequence, proteins sharing high percent identities by local alignment programs such as FASTA or BLASTP are highly likely to share sequential and conformational epitopes. Matches of high identity between any of the four milk proteins in the study with a known allergen would suggest a need for further evaluation of potential allergenicity through specific serum IgE tests using samples from subjects allergic to the source of sequence similar allergens (Goodman et al., 2005).

The proteins were also tested for stability in acidic pepsin. Many major food allergens are stable to pepsin, and this stability is assumed to be a risk factor for allergic sensitization through the gastrointestinal tract (Astwood et al., 1996; Bannon et al., 2002; Thomas et al., 2004). Lactoferrin has previously been tested and is susceptible to pepsin hydrolysis, but leaving at least one relatively stable short peptide that has antimicrobial activity (Tomita et al., 1991). Since the stability of four MBP proteins in pepsin was not previously known, tests were performed in conjunction with an evaluation of the expected exposure based on intended use of MBP as a further consideration of potential risk of allergic reactions. The results of those studies are reported here.

2. Materials and methods

2.1. Protein sequences

Sequences of the four bovine proteins evaluated for this product, MBP, are publicly available. Bovine cystatin C (Cys) (GI:27806675, Olsson et al., 1997) is a 118 amino acid inhibitor of cysteine proteases. High molecular weight kininogen I (Kin) (GI:125505, Kitamura et al., 1983) is a 621 amino acid protein that is processed in vivo by kallikrein (Han et al., 1976) to produced a mature glycoprotein of 110 amino acids, also known as fragment 1·2, representing amino acids 389–498 of the full length protein, which is secreted in milk (Wilson et al., 1989). As noted by Hayashi et al. (1985), bovine kininogen fragment 1·2 migrates as an apparent 24 kDa protein in SDS-PAGE, probably due to the glycan content. High mobility group protein 1 (HMG) (GI:123367, Kaplan and Duncan, 1988) is produced as a 215 amino acid translation product. While the HMG is known to be processed as a secreted protein and is found in milk as a smaller molecular weight form than expected, the C-terminus has not been determined (Walker et al., 1980; Yamamura et al., 1999), and therefore the complete sequence was used for high mobility group-like

protein to search for matches to allergens. Lactoperoxidase (LPO) is a 712 amino acid protein that is processed to a mature form representing amino acids 101–712 of the translation product (GI:129823, Dull et al., 1990).

2.2. Allergen sequences for bioinformatics

2.2.1. The AllergenOnline version 5.0 (updated 1 October, 2004) database

This was used for the primary comparisons to allergens. The database is available at <http://www.allergenonline.com/>. The database contains 1191 known and/or putative allergens associated with food, airway, contact or injected (biting or stinging insects) allergenic sources. This curated database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. All database entries are linked to sequences in the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH).

2.2.2. NCBI entrez protein database

Protein entries in the Entrez search and retrieval system, maintained by the NCBI of the National Institutes of Health (USA.), were compiled from the following sources: SwissProt, PIR, PRF, PDB and translations from annotated coding regions in GenBank and RefSeq. The database is potentially updated or modified daily, and therefore the date of sequence searches by BLASTP is relevant to the dataset used in the BLASTP searches. For this study, all BLASTP searches were run on 20 January 2005, using entries posted on GenBank on 18 January, 2005, 8:02 AM. A total of 2,309,749 sequences were searched. The searches with BLASTP were performed using sequences from all organisms, with a filter of “allergen” in the Entrez query.

2.3. Sequence database methods

2.3.1. FASTA3 overall search of AllergenOnline

The potential sequential and inferred structural similarity of each of these four proteins in MBP were evaluated relative to all allergens in AllergenOnline version 5.0, by performing a FASTA3 search using the default search and scoring criteria of Pearson (2000). The default scoring matrix is BLOSUM 50 (Henikoff and Henikoff, 1992, 1996). The penalty for each gap inserted into query or searched sequences to obtain optimal alignments is calculated as $(-q + -r \cdot k)$, where $q(10)$ is an initial penalty for each independent gap, $r(2)$ is a penalty for each amino acid position within the gap and k is the number of amino acid positions within the gap (Reese and Pearson, 2002). The default word size (ktup) is two (Pearson, 2000). The FASTA3 version used in these searches was 3.4t25b1, dated November 12, 2004 (<ftp://ftp.virginia.edu/pub/fasta/>). Statistical values are calculated for each search and compared to expected values, as illustrated in the histogram of the computer output. Alignment of regions containing low sequence complexity may lead to irrelevant alignments and are expected to show skewed distributions and should be reanalyzed after removing the low complexity regions (Pearson, 2000). Very small expectation values (E values) indicate probable evolutionary homology, and structural similarity. While the E value default for FASTA3 is set to 10, a value that does not indicate significant similarity, distantly related sequences will generally have E values less than 0.01, and highly similar sequences that probably represent close homology are more likely to have E values less than $1e-7$. If the statistical parameters calculated for any alignment appears to indicate significant similarity, the percent identity over the length of the intact proteins should be evaluated. As discussed by Aalberse (2000), a protein sharing greater than 70% identity over its length, relative to an allergen is likely to be cross-reactive, or share IgE binding. Those that have less than 50% identity are not very likely to be cross-reactive. Such matches would require serum testing and probably clinical testing to verify actual cross-reactivity.

2.3.2. FASTA3 of AllergenOnline by 80 aa segments

The identification of relatively short regions of high identity shared by a query sequence and an allergen may indicate similarities that could also share IgE binding, or cross-reactivity. Based on the recommendation of

Codex (2003), the FASTA3 algorithm was used to compare all possible contiguous amino acid segments of each of these four proteins in MBP with all sequences listed in AllergenOnline. Every possible contiguous 80-amino acid sequence of each query protein was searched, beginning with amino acids 1–80, and continuing until the last 80 amino acid segment of each protein was compared with the database on AllergenOnline, using the FASTA3 algorithm. In this case, the percent identities over the individually aligned 80 amino acid segments [(# identical residues/80 or more amino acids $\times 100\%$)] were evaluated to consider potential cross-reactivity.

Alignments of less than 80 amino acids in length were recalculated to normalize the identity to an 80 amino acid score, by increasing the denominator to 80, without altering the numerator. Therefore an alignment with 38 identical amino acids over a length of 40 (=95%), would be recalculated to 47.5%. The reason for the adjustment is that alignments less than 80 amino acids long may have very high identities, and would therefore be more likely to act as a cross-reactive allergen if the matched region represented an IgE epitope, than longer alignments of markedly lower identity scores. When the FASTA3 program inserts gaps in the query sequence to provide optimal alignment, the length of the alignment will exceed 80 amino acids. Rather than “correcting” the alignment identity scores, the same criterion of 35% identity is maintained as per the recommendation of Codex (2003). The rationale by Codex (2003) for recommending that alignments of $>35\%$ identity over segments as short as 80 amino acids is that proteins sometimes contain structural motifs that are comprised of sequences much shorter than the intact protein, and that these structural motifs may include a conformational IgE binding epitope. This criterion is more conservative than empirical data would suggest is common for cross-reactive proteins (Aalberse, 2000).

2.3.3. Exactmer (8aa) search of AllergenOnline

The identification of exact matches of any sequence eight amino acids long, between a query protein and an allergen, has been suggested as a useful indicator of possible allergic cross-reactivity (Metcalfe et al., 1996). We used a string-search algorithm to search for any possible 8-contiguous amino acid segment (word) of the query protein against the entire AllergenOnline version 5.0 database, by creating a “word” from each possible 8 aa segment of the query protein. The list of “words” was compared to the set of 1191 sequences in the AllergenOnline database. Each exact matching sequence and sequence position was compiled for each query protein.

2.3.4. BLASTP of NCBI Entrez “allergen”

The BLASTP is available on the NCBI Entrez website (<http://www.ncbi.nlm.nih.gov/BLAST/>). A BLAST search was used comparing each complete query sequence against the entire Entrez Protein database, with a limit option selected to query entries for “*allergen*”, to align only with proteins identified as allergens. The purpose of this BLAST search is to ensure that a significant match with a newly discovered allergenic sequence that has not yet been entered into AllergenOnline, is not overlooked. The default conditions for Expectation value (10), word size (3), scoring matrix (BLOSUM 62) and gap penalties (–11 existence, plus extension of –1) were used. The “low complexity” filter was turned off, meaning that irrelevant low complexity alignment matches may be identified as significant, so each identified match should be further evaluated to understand the significance. Evaluation of the E value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment. Significant scores are those with E values less than $1e-7$ and identities greater than 50%.

2.4. Pepsin digestion

Purified pepsin with 4150 units of activity per mg of protein was purchased from Sigma/Aldrich (product #P-6887, from porcine stomach). LPO from bovine milk was purchased from Tatua (Morrinsville, New Zealand). Cys was purified from bovine milk according to the method of Matsuoka et al. (2002). Kininogen fragment 1·2 was purified from bovine plasma and prepared by enzymatic cleavage in our laboratory according

to the method of Shimada et al. (1985). HMG was purchased from Wako Pure Chemicals (Tokyo, Japan; Cat. #080-07041, from bovine thymus). Polyacrylamide Tricine gels, 10–20%, 15 well, 1 mm thick, were purchased from Invitrogen (product #EC66255). Protein molecular weight marker was purchased from Tefco (product #03-064). Coomassie brilliant blue G-250 staining solution was purchased from Bio-Rad (product #161-0786).

Pepsin digestion was performed according to the method of Thomas et al. (2004). In brief, a ratio of 10 units of pepsin activity/mg of test protein was used throughout the study. The pepsin/protein ratio in the digestion reaction was approximately 2.2:1 (w:w). For each protein, a single tube containing simulated gastric fluid (SGF; 0.084 N HCl, 35 mM NaCl, pH 1.2 and 4,000 U of pepsin) was preheated to 37 °C prior to the addition of test protein solution (5 mg/ml). The tube contents were mixed and placed in a 37 °C water bath. The samples were removed at 0.5, 2, 5, 10, 20, 30, and 60 min after initiation of the incubation and the reaction quenched by addition of alkali solution (200 mM NaHCO₃, pH 11) and 5x Laemmli sample buffer (40% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.33 M Tris–HCl, 0.05% bromophenol blue, pH 6.8). Quenched samples were immediately heated to >75 °C for 10 min. Equal volume samples from each time point and control reactions were separated in SDS–PAGE under reducing conditions using 10–20% Tris–Tricine gels. The gels were fixed with 40% methanol, 10% acetic acid for 30 min, then stained with Coomassie brilliant blue (CBB) G-250 for 1 h, destained and photographed.

3. Results

3.1. Bioinformatics

Comparison of the amino acid sequences of all four of the proteins in MBP with those of known and putative allergens listed in AllergenOnline.com version 5.0 did not reveal any significant matches. The best predictor of cross-reactivity would be a match of greater than 50% identity over the full-length of the query protein and an allergen. None of the four proteins had a match of even 35% identity over the full length.

The closest match to any allergen by Cys was to cat cystatin, also known as Fel d 3 (GI:17939981) with an *E* score of 2.9 e-4, and 29% identity over an alignment of 58 amino acids. While these are likely to be evolutionary homologues, the low degree of identity indicates a very low probability of any shared IgE binding epitopes. There were no matches of Cys to any allergen using a sliding window of 80 amino acids, or 8 amino acids. Using BLAST with public database sequences listed on Entrez revealed the same low identity match with cat cystatin.

The highest scoring match of Kin with an allergen was with an *E* value of 3e-2, with 36% identity over 50 amino acids to an uncommonly known allergen, polyprotein Hgg-14 of a soybean cyst nematode (GI:18677166). There were no matches using the 80 or 8 amino acid sliding windows. The closest match using BLAST with Entrez sequences was the same Hgg-14 polyprotein.

The highest scoring match for LPO was to a parasitic scabies (mite) antigen of *Sarcoptes scabiei*, with an *E* score of 3.2 and 26% identity over 109 amino acids. There were no matches of LPO to any allergen with 80 or 8 amino acid sliding windows. There was also no match with an *E* score less than 10 to any allergen listed in Entrez.

The HMG did have some matches that appeared to be over the precautionary limits of 35% identity (GI:24711753 from *Brugia malayi* and GI:3687326 from *Necator americanus*) or matches of 8 identical amino acids (eecedee) from Ara h 1 of peanut (GI:1168390). However, the matched area was due to similarities with the carboxy-terminus of HMG that is a low complexity acidic segment of glutamic and aspartic acid residues which is apparently cleaved or not present in the protein as it is expressed in milk (Yamamura et al., 1999) (see Fig. 1).

3.2. Protein stability in pepsin

The four purified proteins digested at somewhat different rates under the standard conditions used in this assay. The full-length LPO (Fig. 2a) was no longer visible by 0.5 min, showing very rapid degradation. A very small amount of residual 4,000 MW stainable protein was seen at 0.5 min, but after that only a faint, diffuse band was visible, and this staining disappeared through 60 min. The primary band of Cys (Fig. 2b) was nearly undetectable by 2 min, although apparently low concentrations of small molecular weight partially resistant peptides (approximately 4000 and 5500 MW) remained visible up to 60 min after initiation of digestion. Kininogen fragment 1·2 was clearly visible up to 5 min after initiation of digestion, but markedly reduced by 10 min and the band disappeared by 60 min. However, two semi-stable protein bands began to accumulate at 0.5 min, and continued to increase

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1  mgkgdppkpr gkmssyaffv qtcreehkkk hpdasvnfse fskkcserwk tmsakekgkf
61  edmakadkar yeremktyip pkgetkkkfk dnpaprppps afflfcseyr pkikgehpql
121 sigdvakklg emwnntaadd kqpyekkaak lkeyekdia ayrakgkpa akkgvvkaek
181 skkkkeeeed eedeedeede eedeedeede dddde //

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Fig. 1. HMG sequence. The full length sequence of HMG represents the full-length protein found inside cells (GI:123367). However, the purified protein as it exists in bovine milk has been demonstrated to migrate at just over 10,000 to 12,000 MW based on SDS–PAGE reducing gel analysis (Yamamura et al., 1999). Since Yamamura et al. (1999) identified the N-terminus of the protein by N-terminal sequencing the secreted milk protein should begin at amino acid one or two and end at approximately amino acid 110 (underlined). Low significance matches were identified for HMG (between 35% and 40% identity over 80 amino acids with a protein from *Brugia malayi* and one from *Necator americanus* as well as an eight amino acid identity match to allergen Ara h 1 of peanuts). However, those matches were due to regions of much higher identities in the final carboxy-terminal 35 amino acids (enclosed in the box), which is apparently not part of the protein as it is secreted in milk.

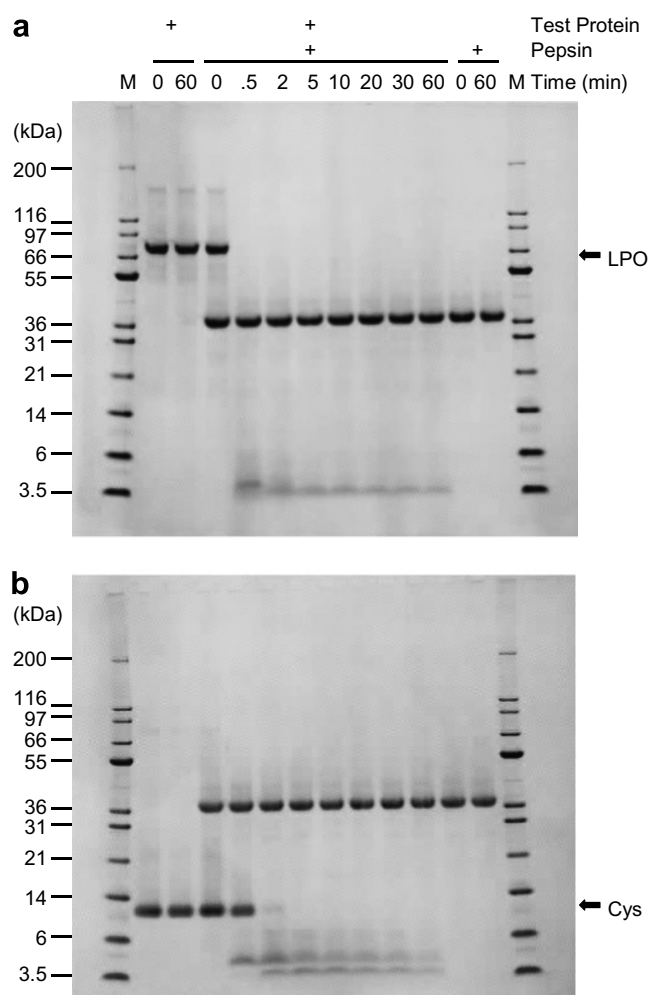


Fig. 2. Test protein digested at different rates in a standardized in vitro pepsin digestion assay. A pattern of digestion results of LPO (panel a) and Cys (panel b) were observed in this study. The LPO was rapidly digested, but produced resistant fragments. The Cys was rapidly digested, but produced resistant fragments. The gels were loaded in the same order with same amount of digestion reaction mix or markers. Molecular weights (kDa) are indicated for lanes labelled "M." All other lanes contained 15 μ l of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF without pepsin (simulated gastric fluid; 0, 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0, 60 min). These 10–20% polyacrylamide Tricine gels were fixed and stained with CBB-G250. The position of the LPO and the Cys were indicated by arrows.

in concentration through 20 min (13,000 MW band) or through 60 min (8000 MW band) as apparent degradation products from the intact protein (Fig. 3a). A somewhat similar pattern was seen for the digestion of concanavalin A from jackbeans and lysozyme from hen's egg (Thomas et al., 2004). The stability does indicate some potential increased risk of food allergy although the low abundance of the protein in milk and MBP should mitigate this factor (see Section 4). The digestion pattern of HMG (Fig. 3b) was quite similar to LPO except that a diffuse, semi-stable digestion product band of approximately 5000 MW was clearly still visible at 60 min of digestion. The results dem-

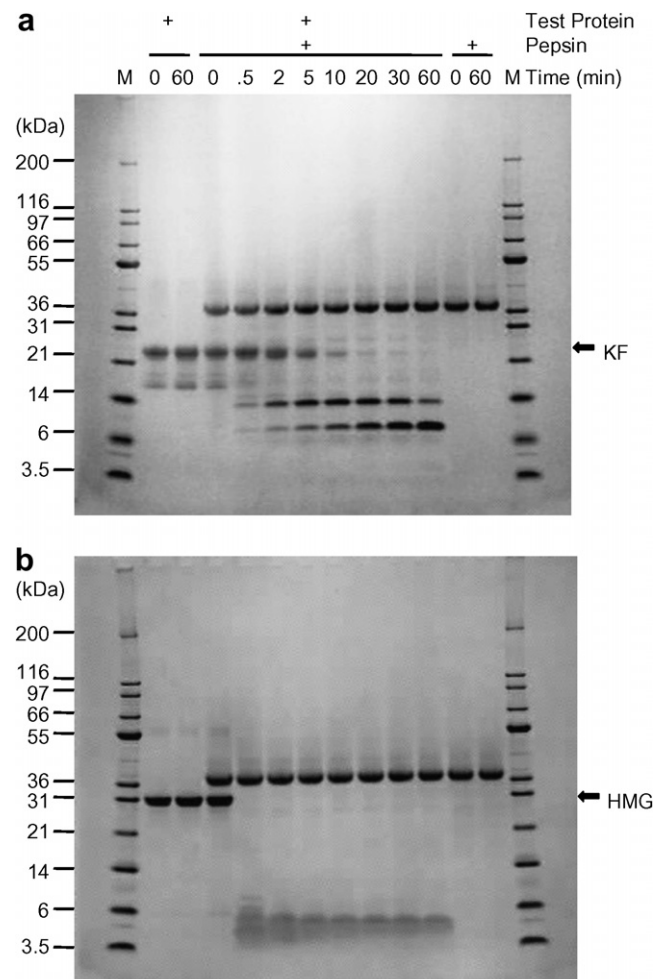


Fig. 3. Test protein digested different rates in a standardized in vitro pepsin digestion assay. A pattern of digestion results of kininogen fragment 1·2 (panel a) and HMG (panel b) were observed in this study. The kininogen fragment 1·2 was relatively resistant and also produced resistant fragments. The HMG was rapidly digested, but produced resistant fragments. The gels were loaded in the same order with same amount of digestion reaction mix or markers. Molecular weights (kDa) are indicated for lanes labeled "M." All other lanes contained 15 μ l of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF without pepsin (simulated gastric fluid; 0, 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0, 60 min). The 10–20% polyacrylamide, Tricine gels were fixed and stained with CBB-G250. The position of the kininogen fragment 1·2 (KF) and HMG were indicated by arrows.

onstrate that the four proteins in this study are digested by pepsin relatively rapidly although kininogen fragment 1·2 forms intermediately stable peptides.

4. Discussion

MBP and its principal protein components appear to carry a minimal risk of allergic sensitization. While milk contains a number of known food allergens (alpha-lactalbumin, beta-lactoglobulin and caseins), MBP is purified from bovine milk and should contain only traces of these

known milk allergens. Protein in whole milk is comprised of approximately 85% caseins and 15% whey proteins (Hermansen et al., 1999). Whey protein is made up primarily five proteins: beta-lactoglobulin (46.6%), alpha-lactalbumin (17.5%), serum albumin (5.8%), IgG (11.7%), lactoferrin (2.9%) and lactoperoxidase (0.4%) as well as approximately 14% undefined peptides called proteose-peptone (de Wit, 1998). MBP contains basic whey proteins and approximately 56% of the protein is lactoferrin and 42% is lactoperoxidase based on analysis of multiple production batches described in the GRAS notification documentation (unpublished by Environ, 2006). The remaining 2% is composed of Cys, Kin, HMG and other unidentified milk proteins. Thus most of the major allergenic proteins of milk have been removed from MBP. The goal of this study was to evaluate the characteristics of Cys, HMG, Kin and LPO to evaluate the possibility that these proteins in MBP might cause cross-reactions or lead to new allergic sensitization.

We applied the approaches that have been identified as useful for evaluating the sensitizing potential of proteins newly expressed in genetically modified crops to evaluate the protein components in MBP. None of these protein components had any significant sequence homology with known allergens by any of several comparative strategies and therefore no allergic cross-reactions could be predicted and no at-risk population of allergic subjects could be identified to evaluate potential IgE binding, other than milk allergic subjects. Thus, ingestion of MBP and these protein components is unlikely to provoke allergic reactions in individuals who are already sensitized to any of these 1191 known allergens. Since products containing MBP will be labelled as containing milk under US and European laws, there is no need to evaluate further whether any of the four proteins bind IgE in milk allergic subjects as they should avoid the product based on labels. Furthermore, the protein components of MBP other than Kin are rather rapidly hydrolyzed by pepsin under acidic conditions. This attribute indicates that these proteins would be rapidly digested in the stomach and thus comparatively unlikely to sensitize individuals who ingest MBP. Due to the apparent stability of Kin, it is important to consider the abundance of Kin and the other proteins in foods that would be fortified with MBP.

The primary components of MBP, namely lactoferrin, lactoperoxidase, and cystatin C, are present in milk and milk-derived products and consequently have long been regularly consumed by humans. The mean intake of MBP as an ingredient in a variety of food products is likely to be in the range of 40–50 mg per day by users of these fortified foods. Ingestion of this amount of MBP provides approximately as much lactoferrin as naturally is present in one cup of milk, approximately two to three times the amount of lactoperoxidase in a cup of milk, and as much cystatin C as is found in approximately 1/4 cup of milk. Americans consume approximately one cup of fluid milk

per day (Cook and Friday, 2004). Potential intakes of MBP as an ingredient in food would therefore provide no more than approximately two to three times the quantity of any of the primary protein constituents in MBP when compared to intake from fluid milk, which is unlikely to produce an increased risk of allergic sensitization.

Kininogen fragment 1·2 and HMG-like protein also are present naturally in conventional bovine milk products, and exposure to these proteins occurs through routine consumption of dairy products. The concentration of these proteins in milk are unknown, but based on the known concentrations of the major milk proteins, Kin and HMG represent minor components. The developers of MBP have identified Kin and HMG qualitatively in MBP, but have not been able to quantify them (unpublished, Environ, 2006). However, the concentrations of lactoferrin, lactoperoxidase, and cystatin C can be used as markers for predicting the relative concentrations of various protein components in MBP relative to milk. Since these three markers together account for 98% of the protein content of MBP, exposure to Kin and HMG is expected to be less than 0.8–1.0 mg/day based on the assumption that the 2% residual MBP protein is from those two proteins. This represents a minor exposure to dietary proteins and unlike those of major food allergens. These marker proteins suggest that ingestion of MBP will not result in an exaggerated exposure to kininogen fragment 1·2 and HMG-like protein relative to background ingestion from dairy products. Importantly, MBP-containing products will be labelled as containing milk (FDA GRAS Notification, 2006).

The fact that none of these proteins have been identified as cows' milk allergens adds support to the conclusion that MBP and its protein constituents are unlikely to elicit allergic reactions in sensitized individuals and because of the relatively rapid digestion in pepsin for all MBP proteins except kininogen, and due to the very low exposure, MBP is unlikely to cause new food allergies. While the ingestion of MBP as a nutritionally beneficial food ingredient may slightly increase the exposure of consumers to these milk proteins, the likelihood that any of these proteins will elicit allergic reactions or sensitize susceptible individuals is quite low based upon the results presented here.

Potential conflict of interest

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