Immunochemical and Structural Analysis of Pepsin-Digested Egg White Ovomucoid

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Ovomucoid, an egg protein comprising ~10% egg white, was digested using the enzyme pepsin, and fragments were isolated by anion-exchange and reverse phase HPLC. Four distinct fragments were identified by analysis with SDS−PAGE, including three large fragments with molecular weights of around 24, 18, and 14 kDa. N- and C-terminal and amino acid sequencing analyses identified the fragments as $V^{134}-C^{186}$ (domain 3), $V^{21}-A^{133}$, and $A^{1}-A^{133}$ (domain 1+2). Further separation and sequencing of the fraction composed of small peptides, to determine their exact makeup and location in the protein, remained to be carried out and identified a peptide $G^{51}-Y^{73}$. All four fragments showed IgE-binding activity, as measured by ELISA, using human sera from egg-allergic individuals. Little change in the digestibility of ovomucoid by trypsin and chymotrypsin was observed following digestion with pepsin, indicating that pepsin-digested ovomucoid retains its trypsin (protease) inhibitor activities. Reduced carboxymethylated ovomucoid was prepared, and digestion with pepsin produced significantly more peptides than did the digestion of the native ovomucoid, indicating that the disulfide bonds play a significant role in the digestive resistance of ovomucoid. The reduction of ovomucoid enhanced its digestibility and lower allergenicity of the protein.

Keywords: *Egg allergy; ovomucoid; IgE epitope; pepsin; digestibility; allergenicity*

INTRODUCTION

Approximately 2−3% of adults and 5−7% of children are plagued by food allergy (Fuchs and Astwood, 1996). Not to be confused with other food sensitivities, such as metabolic disorders and allergy-like intoxications, food allergy is an immune-mediated state of hypersensitivity resulting from exposure to a food allergen and is most often mediated by immunoglobulin (Ig) E (Mekore, 1996). It is also known that food allergies are more prevalent in children, due to an immature gastrointestinal epithelial membrane barrier, allowing more proteins through the barrier and into circulation (Hefle, 1996). A number of pathways permit or facilitate the uptake of proteins by the epithelial barrier, including endocytosis and exocytosis, via specific receptors, and across the tight junctions of epithelial cells; however, these may vary depending on the specific protein and on the state of intestinal maturity. Intestinal absorption of proteins is limited by a variety of factors, including gastrointestinal secretions (e.g., proteolytic enzymes), the peristaltic activity of the intestine, and the properties of the epithelial membrane. In most species, intestinal maturation results in a progressive decrease in protein uptake, due to an increase in the protein to lipid ratio, resulting in a decrease in the fluidity of the epithelial membrane, and is referred to as "closure" (Murphy and Walker, 1997).

A number of food allergens that cause such reactions have been identified. In general, these food allergens are water-soluble proteins (Metcalfe, 1992), with acidic pH (Hefle, 1996) and molecular weight ranges between 18000 and 40000 Da. These molecules tend to be heat and acid stable and, thus, resistant to digestion (Astwood et al., 1996). They must also be small enough to gain access through the intestinal epithelium, yet large enough that bridging on the mast cell surface occurs, in order to elicit an immune response (Hefle, 1996; Taylor, 1992).

One such food allergen is ovomucoid, a major allergen derived from egg white. Egg allergy is one of the most frequent causes of immediate food-allergic reactions in children; however, sensitivity frequently disappears around age 5 or 6, with only one-third of individuals displaying sensitivity past 6 years of age, until around 10 years (Bush and Hefle, 1996). The egg white is more allergenic than the yolk and contains 23 different glycoproteins, with ovomucoid, the dominant allergen, comprising ~10% of the egg white (Kato et al., 1987; Sampson and Cook, 1997). Ovomucoid, a highly glycosylated protein containing 20−25% carbohydrate, has a molecular weight of 28 kDa and an isoelectric point of 4.1 (Kato et al., 1987). It is composed of 186 amino acids, arranged in three tandem domains, each ~60 amino acids in length. Each domain is cross-linked by three intradomain disulfide bonds; however, they lack any interdomain disulfide bonds. Domains 1 and 2 contain four carbohydrate chains, and about half of all domain 3 contains one large carbohydrate chain (the other half lacks the carbohydrate chain). It has been suggested that the carbohydrate chains, especially in the case of domain 3, may play a role as an antigenic determinant of the ovomucoid against IgE (Matsuda et al., 1985a,b). Ovomucoid has been found to be relatively

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heat (Konishi et al., 1985; Matsuda et al., 1983) and acid (Matsuda et al., 1983) stable and possesses trypsin inhibitory activity (Konishi et al., 1985). As well, its disulfide bonds and tertiary structure allow it to retain its allergenicity following proteolytic digestion (Matsuda et al., 1983), making it a potent food allergen. A further exploration of the proteolytic digestion of native and chemically modified ovomucoid, and the resultant allergenic peptides, is required to fully understand the mechanism of ovomucoid hypersensitivity and to determine methods for the management of egg sensitivity.

This paper describes the proteolytic digestion of native and chemically modified ovomucoid, using pepsin, trypsin, and chymotrypsin, and the isolation and characterization of the resulting ovomucoid peptides, in an effort to determine the peptides responsible for ovomucoid sensitivity and to achieve a better understanding of the mechanisms of ovomucoid-induced allergy.

MATERIALS AND METHODS

Isolation and Purification of Ovomucoid. Separation of ovomucoid was carried out according to the modified method of Fredericq and Deutsch (1949). Further purification of the crude ovomucoid was carried out by HPLC (Bio-Rad Laboratories, Hercules, CA) using a Bio-Scale Q5 column (Bio-Rad Laboratories). Sample was loaded using 20 mM Tris-HCl, pH 7.0, and the protein was eluted with 20 mM Tris-HCl containing 1 M NaCl, pH 7.0, at a flow rate of 1 mL/min. Purified protein was then lyophilized for further use.

Peptic Digestion of Ovomucoid. Twenty milligrams of ovomucoid was dissolved in 2 mL of distilled water, adjusted to pH 2.0 with 0.1 N HCl. One milliliter of porcine pepsin (Sigma Chemical Co., St. Louis, MO) solution (1 mg/mL in 10 mM HCl) was added to the ovomucoid solution, for an enzyme to substrate ratio of 1:20 or 1:200. The solution was incubated at 37 °C, and 200 μL aliquots were removed after 10, 20, 30, 60, and 120 min and 6 h and placed into 200 μL of 20 mM Tris-HCl, pH 8.0.

Tryptic and Chymotryptic Digestion of Ovomucoid. Ten milligrams of ovomucoid was first digested with pepsin, using an enzyme to substrate ratio of 1:200, as described above. After 30 min, the pH of the solution was adjusted to between 7 and 8, to inactivate the pepsin. To individual 500 μL aliquots of the digested ovomucoid were added trypsin and chymotrypsin (Sigma Chemical Co.), at an enzyme to substrate ratio of 1:500. The digestions were carried out at 37 °C, for 10, 20, 30 60, 120, and 180 min, followed by heating at 95 °C for 10 min.

Chemical Modification of Ovomucoid. Reduced carboxymethylated ovomucoid (RCM) was prepared by first dissolving 10 mg of ovomucoid in 2 mL of 6 M guanidium hydrochloride (Fisher Scientific, Fair Lawn, NJ) prepared in 0.6 M Tris-HCl, pH 8.6. Two hundred microliters of 0.4 M dithiothreitol (DTT) (Sigma Chemical Co.) was added, and the solution was incubated under nitrogen gas for 4 h, at 40 °C. Two hundred microliters of 500 mM iodoacetate (Sigma Chemical Co.) solution was added dropwise, with shaking, and the solution was then incubated in the dark, at 37 °C, for 30 min, with shaking (Zhang and Mine, 1998). The RCM ovomucoid was dialyzed overnight at 4 °C against water adjusted to pH 7−8 and then concentrated by ultrafiltration using YM10 Centricon concentrators (Amicon Inc., Beverly, MA), with a molecular weight cutoff of 10 kDa.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). Fifteen percent acrylamide gels were prepared, and SDS−PAGE was carried out according to the method of Laemmli (1970). Gels were stained using Coomassie Brilliant Blue R-250 (Sigma Chemical Co.) and destained using a 40% methanol, 7% acetic acid solution. Molecular weights were estimated using a wide range molecular weight marker (Sigma Chemical Co.).

Tricine SDS—PAGE. Tris-Tricine ready gels, 16.5% acrylamide (Bio-Rad Laboratories), based on the formulation by Schagger and von Jagow (1987), were used to resolve the peptides and small proteins produced during proteolytic digestion of ovomucoid. Samples were diluted in sample buffer containing 20% 1.0 M Tris-HCl, pH 6.8, 40% glycerol, 2% SDS, 0.04% Coomassie Blue R-250 (Sigma Chemical Co.), and 2% 2-mercaptoethanol (Fisher Scientific). Electrophoresis was carried out at 100 V, for ~110 min, at 4 °C, in running buffer composed of 0.1 M Tris-HCl, 0.1 M Tricine, and 0.1% SDS. Gels were fixed in a 40% methanol, 10% acetic acid solution, followed by staining with 0.025% Coomassie Blue R-250 (Sigma Chemical Co.) in 10% acetic acid and destaining in 10% acetic acid.

HPLC Fractionation of Peptic Digests. Ovomucoid fragments, produced by the peptic digestion of native ovomucoid, were separated by HPLC, using a Bio-Scale Q2 column (Bio-Rad Laboratories). The column was equilibrated with 20 mM Tris-HCl, pH 7.0, and protein fragments were eluted with 20 mM Tris-HCl containing 1 M NaCl, pH 7.0. Collected fractions were concentrated by ultrafiltration, using YM3 Centricon concentrators (Amicon, Inc.), with a molecular weight cutoff of 3000 Da. RCM ovomucoid digests and tryptic/chymotryptic digests were analyzed by reverse phase HPLC, using a Source 15RPC ST 4.6/100 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated using 0.1% trifluoroacetic acid (TFA) (Aldrich Chemical Co. Inc., Milwaukee, WI), and samples were eluted using 0.1% TFA in 70% acetonitrile.

Enzyme-Linked Immunosorbent Assay (ELISA). Allergenicity of the digested ovomucoid fragments was evaluated using a half-sandwich ELISA format, to measure IgE binding. A 96-well microwell plate (Corning Costar Corp., Cambridge, MA) was coated with 100 μL/well of 5 μg/mL ovomucoid and ovomucoid fragments, in 0.1 M sodium carbonate buffer, pH 9.6, and incubated at 4 °C, overnight. The plate was washed four times with Tris-buffered saline containing 0.05% Tween 20 (TBST), then blocked with 150 μL/well of 0.1 M sodium carbonate buffer, pH 9.6, containing 2% bovine serum albumin (BSA) (Sigma Chemical Co.), and incubated at 37 °C, for 2 h, with shaking. The plate was then washed with TBST. Human sera from patients with egg-white allergy (Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan, and Faculty of Medicine, Gunma University, Gunma, Japan), with previously determined high IgE titers, were diluted 1:25 in phosphate-buffered saline (PBS), pH 7.2, containing 1% BSA, and 100 μL/well was applied to the plate and incubated overnight, at room temperature, with shaking. The plate was washed with TBST and incubated with 100 μL/well of monoclonal anti-human IgE alkaline phosphatase conjugate (Sigma Chemical Co.), diluted 1:2000 in PBS containing 1% BSA, overnight, at room temperature, with shaking. The plate was washed and developed using 100 μL/well of *p*-nitrophenol phosphate (Sigma Chemical Co.) in 1 M diethanolamine buffer, pH 9.8, for 2 h at room temperature, with shaking. The reaction was stopped using 25 μL/well of 3 N NaOH. Absorbance at 405 nm was read using a Bio-Rad model 550 plate reader (Bio-Rad Laboratories). All ELISA values were expressed after subtraction of blank absorbance (<0.10).

Amino Acid Sequence Analysis. Sequence analysis of the ovomucoid fragments produced by peptic digestion was carried out with a PE Applied Biosystems 491 Protein Sequencer (Foster City, CA). Each protein sample containing ~10 pmol was blotted on a PVDF membrane (ProSorb, PE Applied Biosystems), followed by analysis of N-terminal sequences.

C-Terminal Sequence Analysis. C-Terminal analysis of peptides was carried out acording to the modified method of Klemm (1984). Twenty nanomoles of each peptide was dissolved in 200 μL of digestion buffer (0.1 M pyridine acetate, pH 5.6, containing 0.1 mM norleucine and 1% SDS) and mixed with 2 nmol of carboxypeptidase Y (Sigma Chemical Co.) in 10 μL of digestion buffer. The mixture was incubated at room temperature, and 25 μL aliquots were removed at 1, 5, 10, 20, and 30 min. Five microliters of acetic acid was added to stop

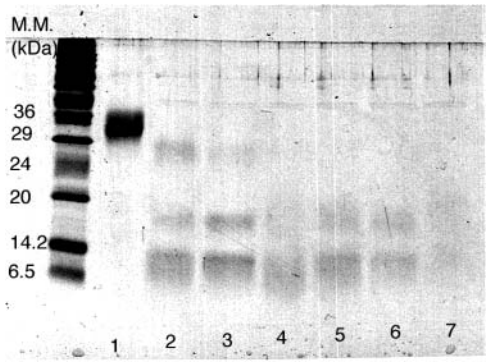

Figure 1. SDS−PAGE gel of native ovomucoid (1) and ovomucoid digested with pepsin, at an enzyme to substrate ratio of 1:20, in deionized water, pH 2.0, for 10 (2), 20 (3), 30 (4), 60 (5), and 120 (6) min and for 6 h (7), at 37 °C.

the reaction. The sample was then lyophilized, followed by amino acid analysis as described above.

RESULTS AND DISCUSSION

Peptic Digestion of Ovomucoid. The digestion of ovomucoid by pepsin was assessed using SDS−PAGE (Figure 1). The sample digested for 10 min showed three broad bands: one around 24 kDa, only slightly smaller than the native ovomucoid, and two lower molecular weight bands, around 18 and 14 kDa. Only the two lower molecular weight bands were visible after 20 min of digestion (the larger one was likely further digested) and diminished as digestion time increased. By 120 min, the bands were very faint, and even after 6 h retained. The possibility of acid hydrolysis can be rejected, as Matsuda et al. (1983) observed that ovomucoid is highly resistant to extreme pH values. Therefore, under normal digestive conditions, large ovomucoid fragments remain and may act as allergens.

Isolation of Digested Ovomucoid Fragments. Digestion using both enzyme to substrate ratios (E:S) of 1:20 and 1:200 (which would more closely represent the pepsin concentration in the human stomach) produced HPLC elution profiles with four peaks, as can be seen in Figure 2. The first three peaks, which eluted before the salt gradient, indicating an overall neutral or positive charge, were common to all digests. A fourth peak was evident in both the 1:20 and 1:200 digests; however, the fourth peak produced at an E:S of 1:200 was present early on in the salt gradient, whereas the fourth peak produced at an E:S of 1:20 eluted late in the gradient and is likely a product of the further digestion of the fourth peak, occurring at higher enzyme concentrations. These fractions were subsequently labeled, in order of increasing elution time, fractions 1−5.

From the SDS−PAGE of the five fractions (Figure 3), the approximate molecular weight of fraction 1 was determined to be 18 kDa, fraction 2 was determined to be 14 kDa, and fraction 5 was determined to be ∼24 kDa. Fraction 3 did not produce a visible band (likely due to smaller peptides), and fraction 4 appeared to be a combination of fractions 1, 2, and 5, supporting the idea that this fragment becomes digested to produce fraction 5 at higher enzyme concentrations. These results correspond to the findings of Matsuda et al. (1985a), who isolated ovomucoid fragments of 25, 18, 13, and <2 kDa by digestion with pepsin. Taking this into consideration, it is possible that fraction 3 may have been of low molecular weight or composed of low molecular weight fragments held together by disulfide bonds and ran out of the gel during SDS−PAGE under reduced conditions. Thus, fraction 3 was further purified by a reverse phase HPLC using a Source 15RPC ST 4.6/100 column as described under Material and Methods (data not shown).

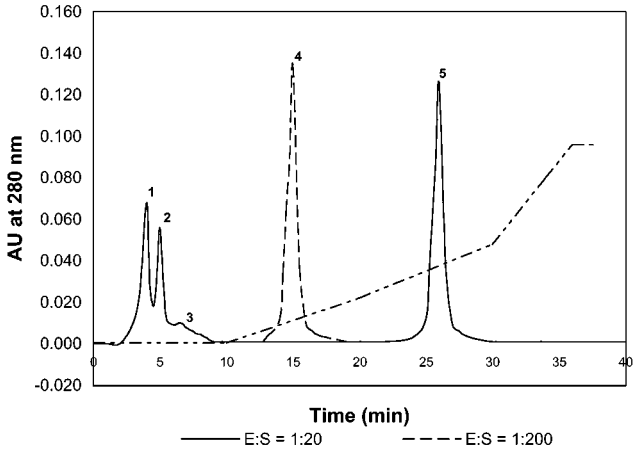

Figure 2. HPLC elution profile of pepsin-digested ovomucoid. Equilibration of the Bio-Scale Q2 column and loading of the sample onto the column were carried out using 20 mM Tris-HCl, pH 7.0, and elution of the bound fragments was carried out with a linear gradient of 20 mM Tris-HCl, pH 7.0, containing 1 M NaCl, at a flow rate of 1 mL/min. Peaks 1−3 were common to all ovomucoid digests. Peak 4 was visible only at an enzyme to substrate ratio of 1:200, and peak 5 was visible at an enzyme to substrate ratio of 1:20.

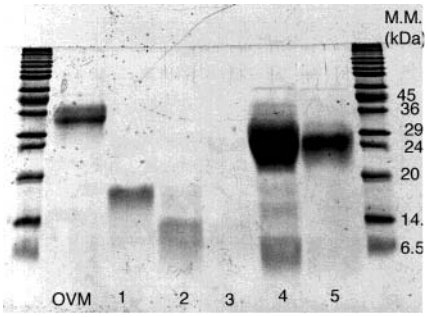

Figure 3. SDS−PAGE gel of the five ovomucoid fragments, produced by peptic digestion at enzyme to substrate ratios of 1:20 (peaks 1−3 and 5) and 1:200 (peaks 1−4) for 60 min, and separated by anion-exchange HPLC.

Table 1. N- and C-Terminal Amino Acid Sequences of Pepsin-Digested Ovomucoid Fractions 1−3 and 5

sample	N-terminal amino acids	C-terminal amino acids
fraction 1	VSVDCSE	KC
fraction 2	AEVDCSR	LAA
fraction 3	GTNISKE	Y
fraction 5	VCNKDLRPICG	LAA

Sequence Analysis of Ovomucoid Fragments. N- and C-terminal amino acids were identified for fractions 1−3 and 5 (Table 1). Fraction 1 was determined to have an N-terminal sequence containing the amino acids VSVDCSE, which corresponds to cleavage of the A^{133}–V^{134} peptide bond, between domains 2 and 3. Furthermore, its migration pattern was identical to that of domain 3 derived from Spase V8 protease as described before (Zhang and Mine, 1998). Therefore, fraction 1 must be made up of ovomucoid domain 3. The large molecular weight of fraction 1, as determined by SDS−PAGE, is likely due to the presence of a large carbohydrate chain, which adds considerable mass, and, as

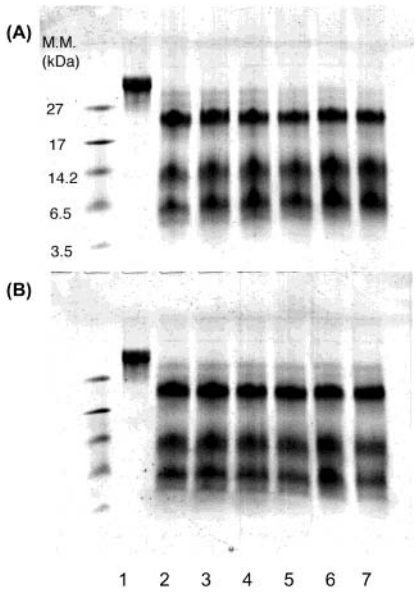

Figure 4. Tricine SDS−PAGE gel of chymotryptic (A) and tryptic (B) ovomucoid digests. Ovomucoid (1) was first digested with pepsin, at an enzyme to substrate ratio of 1:200, followed by digestion with trypsin or chymotrypsin, at enzyme to substrate ratios of 1:500, for 10 (2), 20 (3), 30 (4), 60 (5), 120 (6), and 180 (7) min.

shown by Zhang and Mine (1998), results in a significant difference in molecular weight between the glycosylated and nonglycosylated forms, thereby rendering it difficult to accurately estimate the molecular weight of domain 3.

Fraction 2 was determined to have an N-terminal sequence of AEVDCSR and C terminus of LAA, corresponding to $A^{1}-A^{133}$, and the N- and C-terminal sequences of fraction 5 were found to be VCNKDLRPICG, starting from V^{21} and ending at A^{133}. Both of these sequences are found in domain 1 and are therefore fragments containing some, or all, of domain 1, overlapping each other.

Fraction 3 contained four peptides, with N-terminal sequences beginning at V^{136}, A^{1}, V^{21}, and G^{51}. Two of these N-terminal sequences overlap with the sequences determined for other fractions (fractions 2 and 5) and, therefore, may be smaller peptides produced by further digestion of fractions 2 and 5 or simply by the digestion of ovomucoid at alternate sites on domain 1. Further separation by reverse phase chromatography under reduced condition was carried out to separate a smaller peptide and identified its N-terminal sequence beginning at G^{51} and ending Y^{73}. Gu et al. (1988) isolated a glycopeptide between domains 1 and 2, produced by peptic digestion, by reducing the fragment of interest prior to fractionation. Fraction 3 was identical to that which they isolated.

Tryptic and Chymotryptic Digests of Ovomucoid. Tryptic and chymotryptic digestion of ovomucoid was carried out following digestion with pepsin, to observe any change in the susceptibility of ovomucoid to trypsin or chymotrypsin attack. In its native state, ovomucoid possesses serine protease inhibitor activity (Kato et al., 1987; Matsuda et al., 1985a); however, this activity has been observed to diminish with increasing peptic digestion (Konishi et al., 1985).

Tricine SDS−PAGE gels of tryptic and chymotryptic ovomucoid digests, carried out following digestion with pepsin, can be seen in Figure 4. Little to no change in

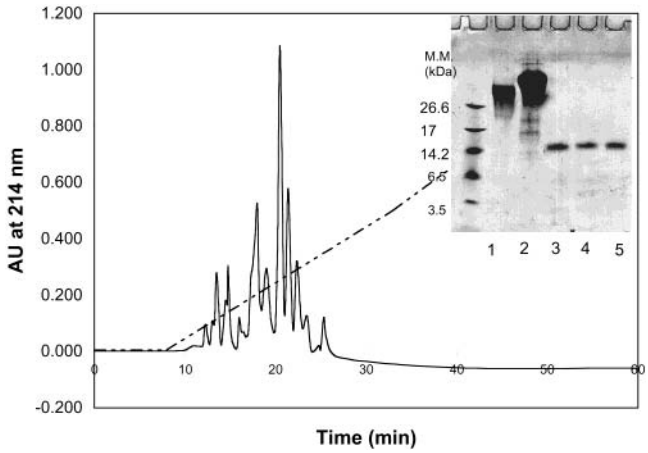

Figure 5. Tricine SDS−PAGE gel and HPLC elution profile of pepsin-digested RCM ovomucoid. Ovomucoid (1) was reduced and carboxymethylated (2) and digested with pepsin, at an enzyme to substrate ratior of 1:200, for 10 (3), 20 (4), and 30 (5) min. HPLC was carried out with a linear gradient of 0.1% TFA in 70% acetonitrile, at a flow rate of 1 mL/min.

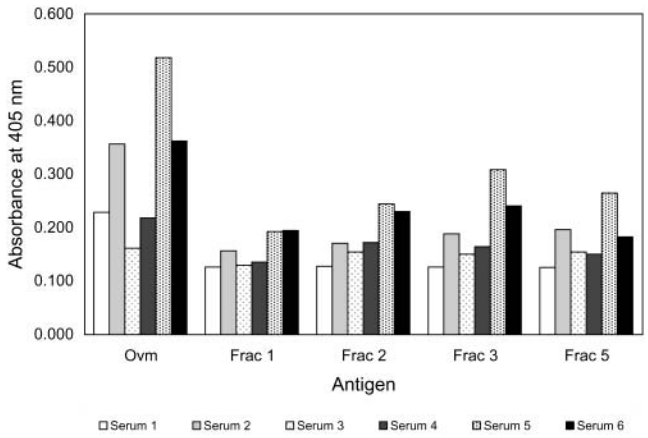

Figure 6. IgE-binding activity of the four fragments of ovomucoid, produced by pepsin digestion, at enzyme to substrate ratios of 1:20.

the banding pattern of ovomucoid digested with pepsin and ovomucoid digested with pepsin and then trypsin or chymotrypsin, over a period of 2 h, could be observed. The two highest molecular weight bands appeared to diminish slightly over the 2 h chymotrypsin digestion period, but not in the case of trypsin digestion.

Ovomucoid may therefore retain some degree of trypsin inhibitory activity and, to a lesser extent, its chymotrypsin inhibitory activity, upon digestion with pepsin. At the same time, however, the major trypsin active site was identified by Kato et al. (1987) as being the $R^{89}-A^{90}$ peptide bond, in domain 2. Digestion at this site (under nonreducing conditions), held together by its disulfide bonds, would likely not result in the formation of any new (i.e., free) peptides, regardless of trypsin inhibition, due to the retention of secondary structure. It is probable that this situation would also occur in the case of chymotrypsin digestion; however, the point of attack of chymotrypsin is not as well documented. As well, in general, chymotrypsin hydrolyzes sites similar to those of pepsin and therefore may not produce any new peptides.

Tricine SDS—PAGE and HPLC Fractionation of RCM Ovomucoid Digests. Four distinct bands were visible for all three peptic digests of RCM ovomucoid,

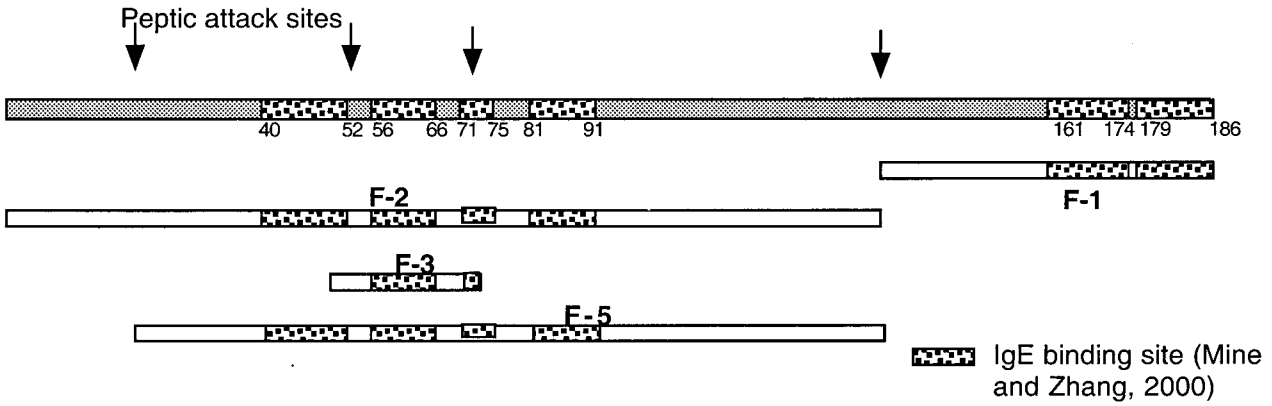

Figure 7. Localization of IgE-binding sites and peptic-digested fragments in ovomucoid.

with observed molecular weights lower than that of the native ovomucoid digestion (Figure 5). One intense band was visible around 14.2 kDa, with three lighter bands around 6, 4, and 1 kDa. Any peptides with a molecular weight of <1 kDa would have run off of the gel and therefore cannot be visualized.

Considerably more peptides were visible by HPLC analysis. Separation of the peptides by reverse phase HPLC revealed the presence of ~12 peptides, as can be seen in Figure 5, compared to only 4 peaks for the native ovomucoid digests, indicating that significant digestion of the RCM ovomucoid had occurred in the absence of the disulfide bonds. The loss of secondary and tertiary structure not only should have exposed more active sites, at which peptic digestion could occur, but would also release those peptides which in the protein's native state remain bound by disulfide bonds, thereby resulting in an increased number of peptides.

Allergenecity of the Pepsin-Digested Ovomucoid. All ovomucoid fragments displayed reduced IgE binding activity, to varying degrees depending on patient serum, compared to the native ovomucoid. However, all four fragments retained some allergenicity, with fractions 2 and 3 showing consistently high allergenicity with all six patient sera (Figure 6). This agrees with the findings of Matsuda et al. (1985c) that IgE-binding activity, and therefore allergenicity, of ovomucoid was reduced, but not eliminated, upon peptic digestion and therefore can elicit an immune response once digested and absorbed.

It has been reported that the combination of all three domains might be essential for retaining a full IgE-binding activity, suggesting some epitopes consisting of conformational epitopes on ovomucoid (Zhang and Mine, 1998). Therefore, some of the ovomucoid IgE epitopes (mainly conformational type) were destroyed by peptic digestion; however, ~70% of the binding sites remain intact in most molecules after digestion (Matsuda et al., 1983). Recently, several groups have reported sequential IgE epitope mapping of ovomucoid. With enzymatic cleavage of ovomucoid, two distinct IgE-binding regions (90−121 and 134−186) were identified (Besler et al., 1999). Five IgE-binding epitopes (1−20, 49−56, 85−96, 115−122, and 175−186) were indentified using synthetic peptides (Sampson and Cook, 1997). Furthermore, the author's group determined seven IgE epitopes (40−52, 56−66, 71−75, 81−91, 161−174, and 179−186) (Mine and Zhang, 2000) (Figure 7). Taking all of this into consideration, the present results demonstrate that pepsin does not destroy allergenic epitopes except for the 71−75 region of ovomucoid. This is why the peptic-digested ovomucoid retained its binding capacity with human IgE antibody. Intestinal absorption of macromolecules is limited by a variety of factors, such as gastrointestinal secretions, the propulsive activity of the gut, and the properties of the "mucosal barrier" (Knippels et al., 1998). However, the intestinal barrier is incompletely developed in premature infants, which may contribute to the high incidence of necrotizing enterocolitis. In infancy, increased quantities of foreign antigens penetrate the relatively immature barrier. It also has been proven that antigen absorption apparently increase in patients with various intestinal disorders (Walker-Smith, 1982). Thus, peptic-digested fragments could pass through the epithelial membrane, interact with T cells, and stimulate B cells to produce the corresponding IgE. Therefore, it is possible that peptic-digested ovomucoid will cause allergic reactions in a patient already sensitized to ovomucoid. It will be of interest to further investigate the transport across human intestinal epithelium of peptic digests of ovomucoid.

Astwood et al. (1996) and Kimber et al. (1999) have reported that important food allergens were stable to digestion in the gastric model (simulated gastric fluid) in vitro. Food allergens must exhibit sufficient gastric stability to reach the intestinal mucosa where absorption and sensitization can occur. Thus, increasing the digestivity of a food allergen by altering its protein structure can decrease its allergenicity. Nondigestive RCM ovomucoid showed significantly lower IgE-binding activity because of destruction of the conformational structure of ovomucoid. It has been reported that ovomucoid might have some conformational IgE epitopes, but the major contribution to allergic epitopes is sequential form, and hydrophobic amino acids are critical for IgE binding (Zhang and Mine, 1998, 1999). RCM ovomucoid, digested with pepsin for 10, 20, and 30 min, showed little to no allergenicity (Figure 8). This could be due to significant digestion and destruction of IgE epitopes, which could indicate that the reduction of the disulfide bonds in ovomucoid, prior to ingestion, could significantly reduce or eliminate the allergenicity of ovomucoid.

Ovomucoid is comprised of three tandem domains, and each domain is cross-linked by three intradomain disulfide bonds (Kato et al., 1987). This is why ovomucoid is so resistant to proteolytic digestion and heat denaturation (Matsuda et al., 1982). Bovine milk β-lactoglobulin has two intramolecular disulfide bonds that may be responsible for its allergic effects. β-Lactoglobulin was reduced specifically by the thioredoxin, and it became strikingly sensitive to pepsin and lost aller-

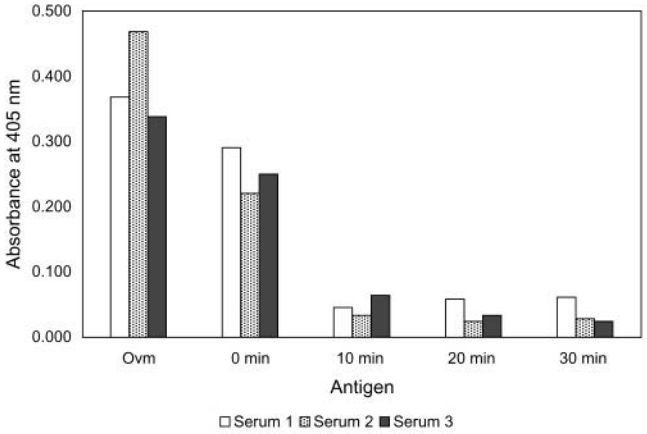

Figure 8. IgE binding activity of RCM ovomucoid digested with pepsin for 10, 20, and 30 min, at an enzyme to substrate ratio of 1:200: native ovomucoid (Ovm), as a control; RCM ovomucoid for 0, 10, 20, and 30 min of digestion.

genicity as determined by the skin test (Gregorio et al., 1999). Our result indicates that reducing the disulfide bonds in ovomucoid enhances its digestibility and can lower allergenicity in vitro. This would enable a new approach to reduce the food hypersensitivity reaction of ovomucoid using genetic engineering.

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