

Profile Analysis and Immunoglobulin E Reactivity of Wheat Protein Hydrolysates

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Key Words

Wheat protein hydrolysate · Immunoglobulin E · Food allergy

Abstract

Background: Wheat protein hydrolysates have been traditionally used as food additives and are now being used in cooking worldwide. There have been a few studies on the relationship between the molecular mass distribution and the immunoglobulin E (IgE) reactivity of the wheat protein hydrolysates. **Method:** We analyzed the peptide profile of commercial wheat protein hydrolysate samples from enzymatic or acid hydrolysis of wheat protein using size exclusion chromatography. We further investigated the IgE reactivity of the wheat protein hydrolysates using the inhibition ELISA method and sera of 5 patients sensitive to wheat. **Results:** The wheat protein enzymatic hydrolysate samples showed high concentrations of peptides with molecular masses greater than 1,050 Da, whereas in contrast, the wheat protein acid hydrolysates showed extremely low concentrations of peptides with molecular masses greater than 1,050 Da. Tested wheat protein acid hydrolysates hardly inhibited the patient IgE binding ability to wheat proteins

in the five patient sera. On the contrary, some tested wheat protein enzymatic hydrolysate samples inhibited the IgE binding ability to wheat proteins. **Conclusion:** These results suggested that the uptake of wheat protein enzymatic hydrolysates might still have the possibility of causing food allergic reactions in patients allergic to wheat and the processed foods containing them.

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Introduction

Protein-specific immunoglobulin E (IgE)-mediated allergic reactions are known to play a major role in food allergies [1]. Especially, IgE-mediated allergic reactions to wheat have long been an important public health problem, since wheat is consumed as a staple food in many countries. Generally, wheat proteins are comprised of salt-soluble (albumin and globulin) and salt-insoluble (glutenin and gliadins) fractions. While most of the components of the salt-soluble proteins have a physiological function, the salt-insoluble proteins, that are also known as prolamins and constitute the gluten proteins, are the storage proteins of the seed. Both fractions are considered to be responsible for the IgE-mediated allergy to wheat

Table 1. Patient serum information

	Age	Sex	CAP-FEIA scores							Allergy
			wheat	corn	soybean	tomato	egg	milk	rice	
I	–	–	4	5	4	5	–	–	–	wheat
II	–	–	4	5	4	5	1	2	–	wheat
III	–	–	3	–	2	–	3	3	–	wheat
IV	1	male	6	2	5	–	6	6	4	wheat, egg, milk
V	3	male	4	–	–	–	5	2	–	wheat

[2–4]. Therefore, we have to consider the structure characteristics of both wheat fractions in order to assess IgE binding.

The most effective means to reduce the allergenicity of food allergens has been to decrease the molecular mass of the native food allergenic proteins by enzymatic or acid hydrolysis. Some studies have attempted to develop hypoallergenic wheat using wheat hydrolysis [5–9]. However, the optimal extent of hydrolysis of wheat regarding its immunological properties is still unclear.

Wheat protein hydrolysates have been traditionally used as a seasoning in Japan and several Asian countries and are now being used in cooking worldwide [10]. In recent years, the labeling system for allergenic food materials has been enforced in some countries including Japan, the EU, Korea and USA to provide information about allergenic foods for the consumer. Nevertheless, there are presently only a few methods and few studies predicting the allergenicity of wheat protein hydrolysates.

In the present study, we analyzed the peptide profile of commercial wheat protein hydrolysate samples after the enzymatic or acid hydrolysis of wheat protein using size exclusion chromatography (SEC). Additionally, we investigated the IgE reactivity of wheat protein hydrolysates using the inhibition ELISA method and sera of patients allergic to wheat. We also discussed the association between the distribution of the molecular mass and the IgE reactivity of the wheat protein hydrolysates.

Material and Methods

Samples and Reagents

Two typical commercial enzymatic (samples A and B) and three typical acid hydrolysates (samples C–E) of wheat protein were kindly provided by the Japanese Assembly for Manufacturers of Hydrolyzed Protein. Aprotinin, angiotensin, bacitracin, secretin and gly-

gly-tyr-arg used as standard peptides for the SEC were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). All test samples were dissolved in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS/Tween).

Preparation of Acid and Enzymatic Wheat Hydrolysates

Preparations of the acid and enzymatic wheat hydrolysates are briefly described as follows. For the acid hydrolysate, the raw material, gluten, prepared from flour, was hydrolyzed by diluted hydrochloric acid under heated conditions, typically ranging from 100 to 110°C. The hydrolysate slurry was neutralized by sodium hydroxide solution. The solution of the hydrolyzed protein was separated from the residue using filtration methods. The clear solution was then purified or decolorized by an adsorption and filtration process. The solution was dried and a powder obtained. For the enzymatic wheat hydrolysate, the raw material, gluten, prepared from flour, was suspended in water and heated for the protein hydrating and denaturing. The treated material was hydrolyzed by some proteolytic enzymes, and then the enzymes were deactivated by heating. The hydrolysate slurry was clarified and sometimes decolorized using filtration methods and an adsorption process. The solution was continually dried and the powdered products obtained.

Patient Sera

The sera from 3 severe wheat-allergic patients (sera I–III) were obtained from Crystal Chem, Inc., Ill., USA. Two additional sera (sera IV and V) were obtained from Japanese patients who had a food allergy to wheat. The specific IgE titers of the patient sera were estimated by CAP-FEIA (CAP-System-FEIA, Pharmacia Diagnostic, Uppsala, Sweden) and the specific IgE titers were expressed as CAP-FEIA scores ranging from 0 to 6, according to the manufacturer's instructions. Serum information of the patients is shown in table 1. The CAP-FEIA scores of the three commercial patient sera (I–III) were 4 and those of the two Japanese patient sera (IV and V) were 6.

Size Exclusion Chromatography

The SEC system consisted of DP-8020 pumps (Tosoh, Tokyo, Japan) and a Tosoh UV-8016 detector. The mobile phase was PBS with a flow rate of 0.5 ml/min that was degassed by an ERC-3215 degasser (ERC Inc., Tokyo, Japan). The Superdex™ Peptide HR 10/30 (10 mm ID × 310 mm; Amersham Pharmacia Biotech AB, Uppsala, Sweden) was connected to a size exclusion separation column. The column was maintained at room temperature. All test

sample solutions were prepared in PBS/Tween at 50 µg/ml for the SEC analysis and inhibition ELISA analysis. The peptides of the test sample solutions were detected at a wavelength of 254 nm during SEC. The injection volume was 100 µl. The resolution range of this column was between 100 and 7,000 Da, and the molecular weight cut-off was 20,000 Da. To determine the molecular mass distribution of the wheat protein, the wheat hydrolysates were analyzed by SEC (fig. 1). According to the general theory, the retention time of the peptides increases as the peptide size decreases during SEC. Figure 1 shows the relationship between retention time and standard protein molecular weight size. The retention time of each standard protein is well correlated with each molecular weight size.

Inhibition ELISA

Inhibition ELISA was performed according to the previously described method, with some modifications [11, 12, 13]. Fifty milligrams of commercially available wheat flour (Nippon Flour Mills Co., Ltd., Tokyo, Japan) was extracted with 20 ml of 0.1 M Tris-HCl buffer (pH 8.6, 1.0 ml) containing 4 M urea, and 100 µl of the extract solution was coated as an antigen on a microplate (Nunc-Immuno plate, F96 Maxisorp, Nunc A/S, Roskilde, Denmark) overnight at 4°C. The extract solutions were discarded, and each well was washed four times with 200 µl of PBS/Tween. To minimize the nonspecific binding of the serum proteins, 200 µl of 0.1% human serum albumin (HSA) in PBS was added, and the plates were incubated for 2 h at 37°C and then washed with PBS/Tween. Before the wheat protein hydrolysate samples and patients' sera were added to the well, they were mixed in tubes. As a positive control, a pancreatic hydrolysate of wheat gluten was used, as previously described [12, 13]. Sixty microliters of the test peptide sample solutions (pancreatic hydrolysate of wheat gluten or wheat hydrolysates, each serial dilution) prepared in PBS/Tween was preincubated with 60 µl of patient sera in tubes at 25°C for 1 h. Therefore, the final concentrations of the patient sera in the tubes were estimated to be a 20-fold dilution for patient sera I–III and a 100-fold dilution for patient sera IV and V with PBS/Tween. Preincubated test peptide sample solution, 100 µl each, was added to the wells, and the preincubated serum solutions (final concentration: 20-fold dilution for patient sera I–III or 100-fold dilution for patient sera IV and V), which were only preincubated with PBS/Tween without the addition of the inhibitory sample, were used as the control. Fifty microliters of rabbit anti-mouse IgE (Fc) (10^{-3} dilution in PBS/Tween containing 0.1% HSA; Yamasa Shoyu Co., Ltd., Chiba, Japan) was added to each well, and the plates were incubated for 1 h at room temperature; then, each well was washed as before. Fifty microliters of goat anti-rabbit IgG β-galactosidase conjugate (10^{-3} dilution in PBS/Tween containing 0.1% HSA; Amersham, UK) was added to each well, and the plates were incubated for 1 h at room temperature and the wells washed as before. The wells were incubated for 1 h at 37°C with 100 µl PBS containing 0.1 mM 4-methylumbelliferone β-galactoside (Sigma). Finally, 25 µl of 1 M anhydrous sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was monitored by a Titertek Fluoroskan reader (Flow Laboratories, Inc., Costa Mesa, Calif., USA). The relative ELISA value was calculated as A/B, where A is the ELISA absorbance value of the patient serum preincubated with the peptide sample and B is that of the control. The binding of a test peptide sample and a patient's IgE antibody was judged by the 50% inhibition (IC_{50}) value [11].

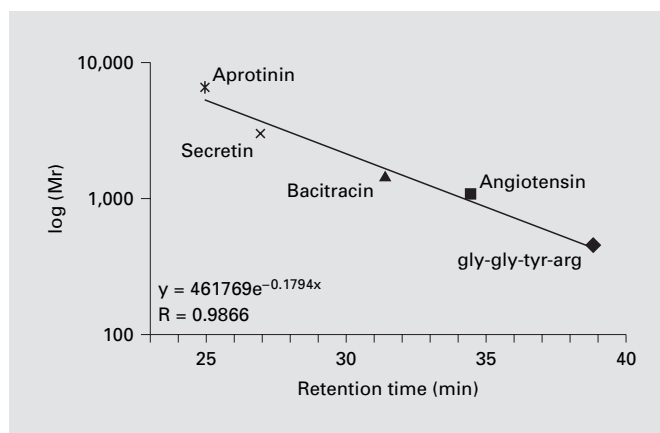


Fig. 1. Relationship between the retention time and molecular mass of standard proteins in the supercritical fluid chromatography analysis. Standard proteins (molecular mass): gly-gly-tyr-arg (451 Da), angiotensin (1,050 Da), bacitracin (1,450 Da), secretin (3,038 Da) and aprotinin (6,500 Da).

Results

Peptide Profile of Wheat Protein Hydrolysates

Figure 2 shows the typical chromatograms of five wheat protein hydrolysates and angiotensin from SEC analysis. As shown, samples A and B, the wheat protein enzymatic hydrolysates, showed high concentrations of peptides having a molecular mass greater than 1,050 Da, corresponding to the molecular mass of angiotensin. However, samples C–E of the wheat protein acid hydrolysates showed extremely low concentrations of peptides with a molecular mass greater than 1,050 Da. The relative molecular mass of the main peak of the fragment appeared to be approximately 500–1,050 Da according to the standard curve. These results suggest that the acid hydrolysates could contain a number of peptides with a molecular mass of less than 1,050 Da, while the enzymatic hydrolysates could still contain some peptides with a molecular mass greater than 1,050 Da, and the absorbance reduction in the 1,050 Da peaks of the wheat protein acid hydrolysates appears to be attributed to the reduction in the molecular mass of the wheat proteins.

Evaluation of IgE Reactivity of Wheat Protein Hydrolysates

To assess the IgE reactivity of the wheat protein hydrolysates, we investigated whether the five wheat protein hydrolysates retained their IgE binding ability by inhibition ELISA using the sera of patients sensitive to the

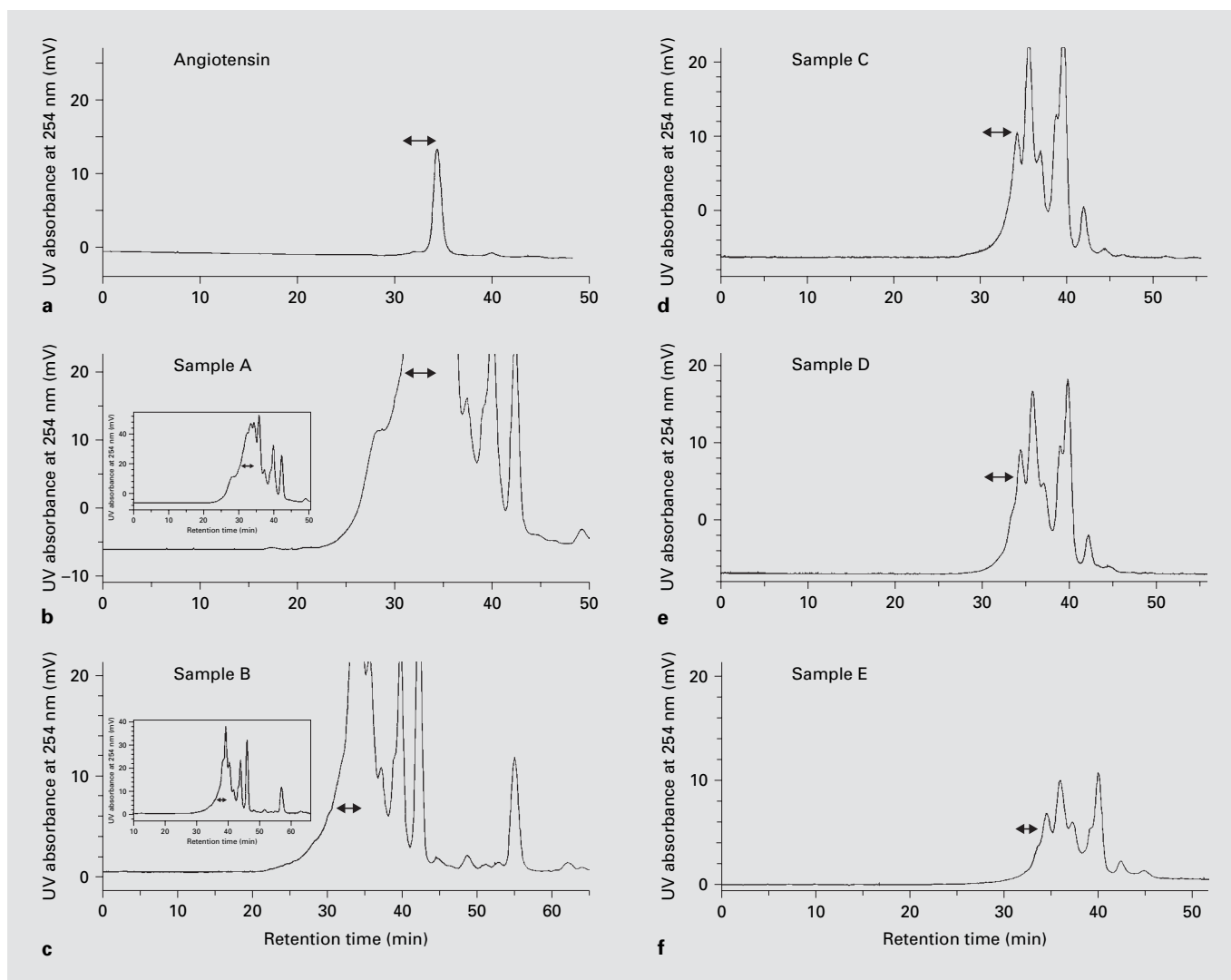
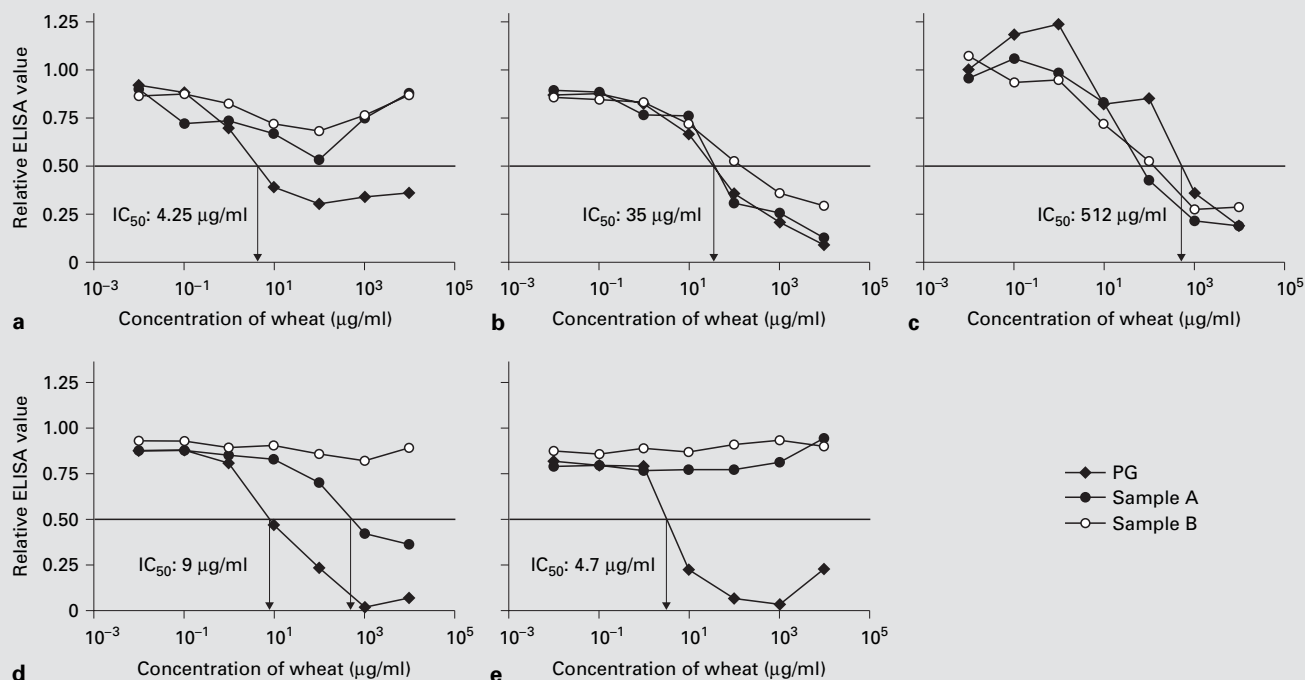


Fig. 2. Chromatograms from SEC of samples A–E. Angiotensin (50 $\mu\text{g/ml}$) (a), enzymatic hydrolysates (b, c), acidic hydrolysates (d–f). Two-sided arrows represent the range between the retention time of angiotensin (1,050 Da) and the retention time of bacitracin (1,450 Da). SEC was performed using the SuperdexTM Peptide HR 10/30 (10 mm ID $\times$ 310 mm; Amersham Pharmacia Biotech AB). The mobile phase was PBS with a flow rate of 0.5 ml/min. Angiotensin was eluted at a retention time of 35 min.

wheat protein (fig. 3, 4). With 0.1 mg/ml of a pancreatic hydrolysate of wheat gluten as the positive control [12, 13], the ELISA inhibition of the pancreatic hydrolysate of the wheat gluten reached about 60–70% with 1,000 $\mu\text{g/ml}$ using the three patient sera (fig. 3). The concentrations of the pancreatic hydrolysates of the wheat gluten for the IC_{50} were 4.25 $\mu\text{g/ml}$ (serum I), 35 $\mu\text{g/ml}$ (serum II), 512 $\mu\text{g/ml}$ (serum III), 7.8 $\mu\text{g/ml}$ (serum IV) and 2.4 $\mu\text{g/ml}$ (serum V), respectively. These results suggest that the five patient sera should be judged to be ap-

propriate for the IgE antibody to wheat in this assay. As shown in figure 3, the wheat protein enzymatic hydrolysates clearly inhibited the binding ability between the IgE antibodies of the sera II–IV and wheat proteins, though they did not clearly inhibit the binding ability of those in sera I and V. On the contrary, as shown in figure 4, only sample D of the wheat protein acid hydrolysates appeared to inhibit the binding ability between the IgE antibodies in the patient sera and wheat proteins, when 10,000 $\mu\text{g/ml}$ of wheat gluten acid hydrolysates were incubated with

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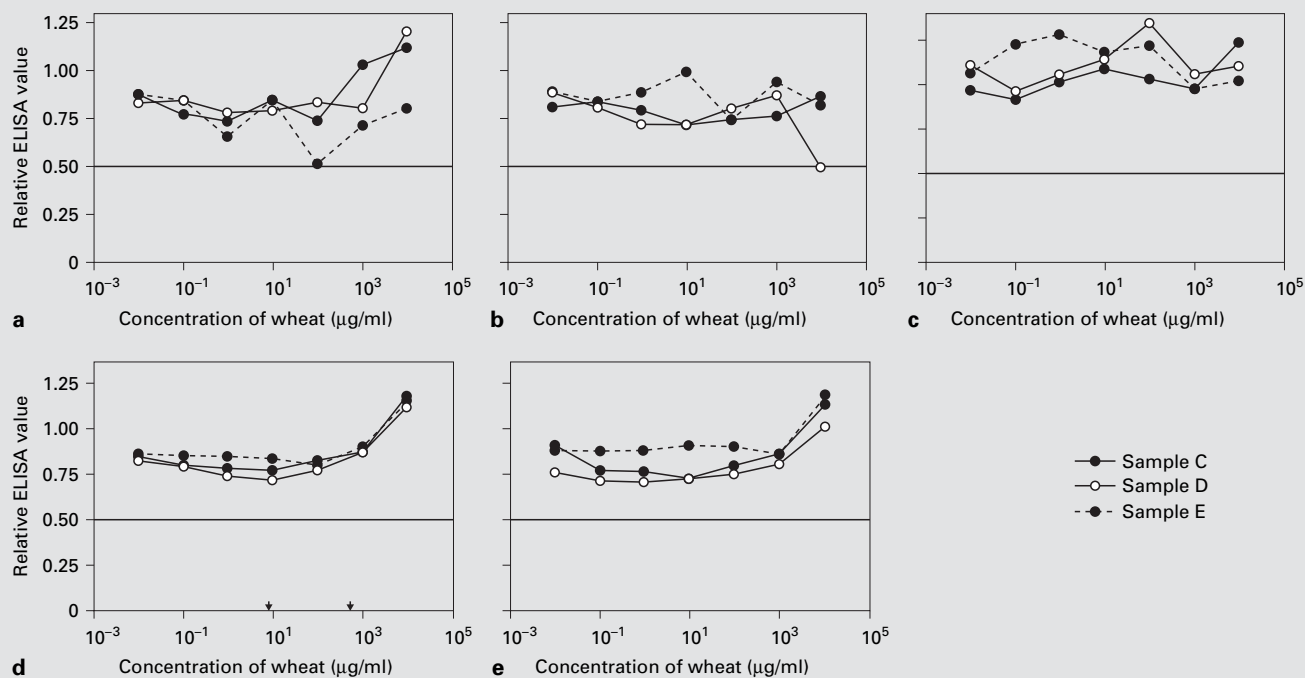


Fig. 3. Inhibition ELISA of wheat protein acidic hydrolysate using five patient sera: serum I (a), serum II (b), serum III (c), serum IV (d) and serum V (e). The relative ELISA value was calculated as A/B, where A is the ELISA absorbance value of the preincubated test peptide sample solution and B is that of the control. PG = Pancreatic hydrolysate of wheat gluten.

Fig. 4. Inhibition ELISA of wheat protein enzymatic hydrolysate using five patient sera: serum I (a), serum II (b), serum III (c), serum IV (d) and serum V (e). The relative ELISA value was calculated as A/B, where A is the ELISA absorbance value of the preincubated test peptide sample solution and B is that of the control.

patient serum II. These results suggested that wheat protein enzymatic hydrolysates still retain a binding ability to the patient IgE antibody and could cause food allergies.

Discussion

Considering the profile analyses and the assessment of allergenicity of the wheat protein hydrolysates in this study, we presumed that the wheat protein enzymatic hydrolysates containing a high concentration of peptides with a mass greater than 1,050 Da could cause some food allergic reactions after their consumption because of the IgE binding ability. However, we also considered that the wheat protein acid hydrolysates might cause some food allergic reactions because of the presence of the binding ability to the patient IgE with very high concentrations of these hydrolysates in terms of the immediate type allergy, though the possibility of this occurrence appears to be extremely low.

A number of studies have suggested that peptide fragmentation generated from proteolytic digestion or hydrolysis of food allergens can elicit IgE responses in already sensitized individuals [14–16]. Maynard et al. [14] showed that 8 out of 19 exhibited a specific IgE response to different tryptic peptides among the sera from patients with cow's milk allergy. Peptide fragments resulting from the proteolysis of ovomucoid and ovalbumin were also reported to react with IgE from people allergic to eggs [15, 16]. However, it has been reported that there seems to be a size limitation on the ability of peptides to react with IgE of already sensitized individuals. Van Beresteijn et al. [17] tested the immunological properties of the hydrolysates of a whey protein concentrate and found that the minimal molecular mass to elicit immunogenicity and allergenicity appeared to be between 3,000 and 5,000 Da. The minimum size for a peptide to bridge two IgE molecules on the surface of mast cells to initiate degranulation was thought to be at least 3,000 Da [18]. Considering the binding properties of one IgE antibody and peptide, these studies appear to be consistent with our present findings.

To prevent allergenic reactions, allergic individuals should strictly avoid the consumption of allergenic foods. Therefore, the Joint FAO/WHO Codex Alimentary Commission Session recommended the labeling of eight kinds of foods including wheat. In Japan, the labeling system for allergenic food materials has already been enforced since April 2002. In the Japanese labeling system, the la-

beling for five food materials including wheat has been mandatory, and for twenty other food materials, it has been recommended. Foods containing wheat protein hydrolysates should also be labeled. However, some patients have been confused by the labeling of some processed foods, because they had been able to eat such foods previously. The present study suggests that if the wheat protein acid hydrolysates are degraded into fragments less than approximately 1,050 Da, they are less likely to elicit food allergic reactions related to the IgE mediated activation of the mast cells.

In conclusion, we demonstrated that the uptake of wheat protein enzymatic hydrolysates might still possibly cause food allergic reactions in patients allergic to wheat, and the processed foods containing them should be labeled. However, we have to consider whether the processed foods containing wheat protein acid hydrolysates should be labeled.

Acknowledgments

This study was partly supported by a grant from the Ministry of Health, Labour and Welfare of Japan. We thank Dr. Tomoji Igarashi, Japan Food Research Laboratories, for giving useful suggestions in terms of size exclusion chromatography, and Mr. Tsutomu Honjoh for obtaining the patient sera. We thank the Japanese Assembly for Manufactures of Hydrolyzed Protein (Banshuu Choumiryou Co., Ltd., Cosmo Foods Co., Ltd., Kyowa Hakko Food Specialties Co., Ltd., Maruha Group, Inc., Maruhachi Muramatsu, Inc., Nitta Gelatin, Inc., Rikken Vitamin Co., Ltd., Sanyo Shouji Co., Ltd., and Takeda-Kirin Foods Corporation) for providing the samples of the wheat protein hydrolysates and their helpful information.

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