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Novel Method for Producing Hypoallergenic Wheat Flour by Enzymatic Fragmentation of the Constituent Allergens and Its Application to Food Processing

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A novel method is proposed to produce hypoallergenic wheat flour suitable for patients allergic to wheat. Wheat flour was mixed with a cellulase solution, and the mixture was incubated at 50° C for 1 h to hydrolyze the carbohydrate allergens. The hydrolysate was further incubated with actinase at 40° C for 1 h while gently stirring to decompose the proteinaceous allergens. The product was evaluated for its allergenicity by an enzyme-linked immunosorbent assay, the results of which suggested negative allergenicity in most cases. The product changed to a batter state that was difficult to process by the usual methods. Gelatinization of the starch in the product and the addition of a surfactant were beneficial for food processing.

Key words: hypoallergenic flour; wheat allergy; hypoallergenic food

The number of allergic patients has been increasing in recent years, particularly in developed countries.[1] An allergy is physiologically defined as a state of hypersensitivity in an immune response, sometimes with fatal results.[2] Food allergy is the hypersensitivity caused by consuming particular foods or, more precisely, their constituent allergens. In most cases, these allergens are proteins. It is well known that allergens in eggs, milk, soybeans and wheat often cause allergies when they are continuously eaten.

Wheat allergy describes the allergic reaction taking place in those who continuously consume wheat products including bread, noodles, cookies, cakes and even alcoholic beverages. The main symptom is atopic dermatitis that develops shortly after a wheat-based product has been eaten, usually resulting in skin eruption and itching. Wheat allergy is thus a serious problem in many countries where the custom of consuming wheat products has been traditionally established.

Although statistical data showing a rapid increase in wheat allergy have not been officially presented, the major opinion is that the number of clinical data on wheat allergy is increasing.

Based on such opinion, we have previously reported that a major allergen in wheat flour was the low-molecular-weight glutenin in gluten,[3] and that it had a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding epitope.[4] We thus presented a method for decomposing gluten with bromelain.[5] However, the process was not completely satisfactory, since some glycoproteins[6] belonging the α-amylase inhibitor families and an polysaccharide[7] were also found to be allergens.

The first objective of this present work is to propose a two-step method using a carbohydrate-decomposing enzyme and a protease to produce hypoallergenic flour that would be tolerated by most patients allergic to wheat. The second is to make wheat products from this hypoallegenic flour that has lost its original dough-forming property.

Materials and Methods

Wheat flour. Commercially available soft flour (Showa Sangyo Co.; commercial name, Cleopatra) was used as the starting material.

Enzymes. Actinase AS (250 Tyr units/mg of solid, Kaken Pharma Co.), bromelain (1030 units/mg of solid, Wako Pure Chemical Ind.), collagenase (from *Clostridium histilyticum*, 150 units/mg of solid, Wako Pure Chemical Ind.), transglutaminase (from *Streptoverticillum* sp., 3 units/mg of solid, Ajinomoto Co.), cellulase (from *Trichoderma viride*, 86.8 units/mg of solid, Amano Pharmaceutical Co.), cel-

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Abbreviations: CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; UTH, 0.1 M Tris-HCl (pH 8.6) containing 4 M urea

lobiase (105 units/mg of solid, Amano Pharmaceutical Co.) and hemicellulase (98.6 units/mg of solid, Amano Pharmaceutical Co.) were used.

Ingredients. Hypoallergenic margarine from palm oil, tapioca starch and sugar stearate ester (SE S-1670) were generously presented by Asahi Denka Kogyo, Showa Sangyo Co., and Kao Co., respectively. All reagents used were of normal reagent grade, and the other ingredients were purchased from market sources.

Enzymatic treatment of the flour. In a one-step process, a given amount of each carbohydrate-decomposing enzyme or protease in water (60 ml) was mixed with the flour (100 g), the mixture then being incubated at 50°C for the former enzyme, or at 40°C for the latter. In a two-step process, a similar procedure was carried out, except that 55 ml of water was used, and then a second step was performed with another enzyme in water (5 ml). The product formed by using cellulase (enzyme/flour ratio of 0.5%) in the first step and actinase (the same ratio) in the second step is referred to as hypoallergenic batter.

Immunoassay. Each enzymatically treated sample (25 mg) was extracted with a UTH buffer solution (1 ml). The resulting extract (0.1 ml) was tested by ELISA.[8] Biotinylated anti-human IgE (Kirkegaad & Perry Lab.), streptoavidin-peroxidase conjugate (Roche Diagnostics Co.) and *o*-phenylenediamine (Wako Pure Chemical Ind.) were used for the assay. Eight out-patients allergic to wheat at National Sagamihara Hospital were asked to donate blood for the assay, their sera being pooled for use in the assay unless otherwise noted. Intact flour (control, 2.5 mg) and enzymes (2.5 mg) were also tested in the same manner. Allergenicity is expressed as the ELISA value relative to the control.

Starch hydrolyzing activity. Potato starch (1 g) was gelatinized in water (100 ml) at 100°C for 20 min and then cooled to 37°C. An aliquot (1 ml) of the starch solution was hydrolyzed with each enzyme (1 mg) at 37°C for 30 min. Reducing sugars in the reaction mixture were determined by the method of Luchsinger and Cornesky[9] with 3,5-dinitrosalicylic acid. Starch hydrolyzing activity is expressed as glucose equivalent μmole/min/mg of enzyme.

Viability of the bacterial cells. Flour (1 g) was suspended in water (5 ml), and the resulting suspension was incubated at 50°C for 1 h. CFUs were assayed on a peptone-tryptone medium (Pseudomonas agar F, Difco Lab.) containing 1% glycerol by incubating overnight at 37°C.

Protein solubility. The hypoallergenic batter (1 g) was extracted with water or with a UTH buffer (100 ml each), and the protein content of the extract was measured with a protein assay kit (Bio-Rad Lab.). Protein solubility is expressed as the ratio of water-soluble to UTH buffer soluble proteins. An aliquot of the water extract was submitted to gel filtration.

Gel filtration. The water extract (10 ml) from the cellulase-actinase-treated flour was, after concentration, loaded into a polyhydroxymethacrylate column (Japan Spectroscopic Co., Biofine PO-4, 7.5×300 mm) and eluted with water at a flow rate of 1 ml/min. The eluate was measured for its absorbance at 220 nm. Bacitracin A (MW 1422), fluorescein isothiocyanate-glutaminyl glutaminyl glutaminyl prolyl proline (MW 967), glutathione (MW 307) and leucyl glycine (MW 188) were used as molecular weight markers.

Gelatinization of the starch. The hypoallergenic batter (100 g) was heated in a water bath at 65°C for 30 min (partially gelatinized batter) or steamed for 20 min (exhaustively gelatinized batter). The heat-treated batter samples were submitted to measurement of the viscosity, degree of gelatinization and textural parameters. The samples were also processed into food items.

Viscosity. The hypoallergenic batter (30 g) was poured into the sample cup of a rapid-visco analyzer (Newport Scientific), and the change in viscosity of the sample was measured according to the method of Deffenbaugh *et al.*[10] The sample temperature was maintained at 65°C.

Processed food items. The ingredients in the processed food items are shown in Table 4. For noodle making, the exhaustively gelatinized batter was mixed with salt in a temperature range of 65-70°C, and the mixture was passed through an extruder (Yasukawa Electric Mgf.). For cup cakes, the mixture (20 g each) was poured into alumina cups (6 cm in base diameter) and then steamed for 7 min. For pizza, the mixture (130 g each) was molded on a plate (18 cm in diameter) and baked at 170°C for 10 min. For cookies, the mixture was shaped into discs 3 cm in diameter and 5 mm in thickness and then baked at 160°C for 7 min. For wafers, the batter (10 g) was suspended in water (50 ml) and then heated to 90°C to induce gelatinization before the other ingredients were added. The wafers were baked at 130°C with a test baking unit (Hebenstreit Co., model 965) equipped with two plates (each 156×110 mm) until steam was no longer emitted. For puffed items, the noodles prepared as already described were used as the starting material, being cut into grits and dried. The dry grits were humidified until their moisture content had reached 15%. The moist grits (10 g each)

were puffed with a rice cake machine (We-Pop Co., model AP-01) equipped with a mold (10 cm in diameter and 1 cm in depth).

Degree of gelatinization. The β-amylase-pullulanase method[11] was applied to measure the degree of gelatinization. The partially and exhaustively gelatinized batter samples (80 mg each) were suspended in water (8 ml). The suspension before and after gelatinization with 1 N NaOH was hydrolyzed with the enzymes, and reducing sugars in the hydrolysate were measured by the method of Luchsinger and Cornesky.[9] The degree of gelatinization is expressed as the ratio of the values before and after alkaline gelatinization.

Textural parameters. The hypoallergenic batter was poured into an alumina cup (38 mm in diameter and 13 mm in height) and then steamed for 10 min to form a gel. This gel was cut back to a height of 13 mm. The hardness, cohesiveness and stickiness of the gel were measured with a texturometer (General Foods, GTX-2) equipped with an alumina plunger (13 mm in diameter). The clearance, sample temperature and bite speed were set at 2 mm, 65°C and 6 times/min, respectively. The pizza was measured under the same conditions, and the noodles were measured as described in the previous paper.[12] The cup cakes were cut into cubes (20×20×20 mm), each cube being measured for hardness and cohesiveness in a similar manner, but with a nickel plunger (50 mm in diameter) at a sample temperature of 25°C and a clearance of 4 mm.

Rupture stress. Rupture stress of the cookies, wafers and puffed items was measured by a texture measuring system (Yamaden Co., Rheoner RE 3305) with a polyacetal plunger (5 mm in diameter) at a compression speed of 1 mm/sec.

Specific volume. The volume of each sample was measured by the rape seed replacement method,[13] specific volume being expressed as the volume (ml)/weight (g).

Results and Discussion

We reported in our previous papers,[5,14] that actinase, bromelain, collagenase and transglutaminase effectively decreased the allergenicity of gluten, a major allergen in wheat flour, and that bromelain was the first choice because of its high ability to decompose the allergen and its low cost. Bromelain itself, however, cross-reacted with the pooled sera from wheat allergic patients (Table 1). We thus modified the process for producing hypoallergenic flour from that reported in the previous paper.[14] Based on its high hypoallergenizing ability,[14] negligible cross-reactivity with serum, low starch- hydrolyzing ability (Table 1) and cost, we instead selected actinase for decomposing the proteinaceous allergens. Table 2 shows that actinase-treated flour retained a small degree of allergenicity against the pooled sera, indicating the contribution of carbohydrates, glycolipids and/or glycoproteins. We selected an enzyme with a low starch-hydrolyzing activity and the ability to reduce the remaining allergeniciy. Cellulase was the best enzyme tested for this purpose (Tables 1 and 2), and was used at the first step in the enzymatic process to avoid its inactivation by actinase. According to the previous report,[14] flour (100 g) was mixed with water (55 ml) containing a given amount of cellulase. The reaction temperature was set at 50°C, at which no gelatinization of the starch occurred, and CFUs decreased from 2,000/g of flour (before incubation) to less than 10/g of flour after 60 min. A reaction time of 1 h and an enzyme/flour ratio of 0.5% were enough to decompose the carbohydrate allergens (Table 2). The reaction product, after being cooled to 40°C, was then subjected to the subsequent actinase treatment.

Actinase was used for the second step of the process to decompose the proteinaceous allergens.

Table 1. Allergenicity and Starch Hydrolyzing Activities of the Enzymes

Enzyme	Relative ELISA value (%)	Starch hydrolyzing activity* (μmol/min/mg of enzyme)
Actinase	nd**	0.1
Bromelain	60.0	nd**
Collagenase	nd**	nd**
Transglutaminase	5.1	0.1
Cellulase	nd**	0.2
Cellobiase	nd**	3.7
Hemicellulase	nd**	3.7

* Glucose equivalents.
** Not detectable.

Table 2. Allergenicity of the Enzymatically-treated Wheat Flour

Enzyme		E/F ratio* (%)		Reaction time (h)		Relative ELISA value (%)
1st step	2nd step	1st step	2nd step	1st step	2nd step	
Actinase		1		1		5
Actinase		1		2		5
Cellulase		1		1		90
Cellulase	Actinase	0.25	0.5	1	1	4
Cellulase	Actinase	0.25	0.5	2	1	4
Cellulase	Actinase	0.5	0.5	1	1	<1**
Cellulase	Actinase	0.5	0.25	1	1	2
Cellulase	Actinase	0.5	0.25	1	2	2

* Enzyme/flour ratio.
** A relative ELISA value of less than 1% was obtained, when flour was reacted with either cellobiase or hemicellulase at an E/F ratio of 0.5% for 1 h instead of cellulase.

Table 3. Allergenicity of the Cellulase-actinase-treated Product against Sera* from Patients Allergic to Wheat

Serum sample	Relative ELISA value (%)
A	<1
B	<1
C	<1
D	<1
E	<1
F	<1
G	3
H	4

* Individual serum samples were used.

Table 4. Optimal Composition of Ingredients for the Processed Food Items

Ingredient	Composition (g)				
	Cup cakes	Noodles	Pizza	Cookies	Wafers
Batter*	100	100	100	100	100
Salt	0.3	0.6	1		0.2
Sugar	9			30	
Surfactant**	6×10^{-6}		6×10^{-6}		6×10^{-6}
Baking powder	3		0.25	3.7	
Liquid jelly	26				
Margarine***	3				
Vanilla essence	sv****			sv	
Tapioca starch			30		
Water			19		50
Olive oil			9		
Wheat starch				62	
Palm oil				55	
Ammonium carbonate					0.7
Sodium hydrogen carbonate					0.2

* Completely gelatinized batter was used for noodle making. The other items were made from partially gelatinized batter.
** Stearate sugar ester.
*** Hypoallergenic margarine.
**** Small volume.

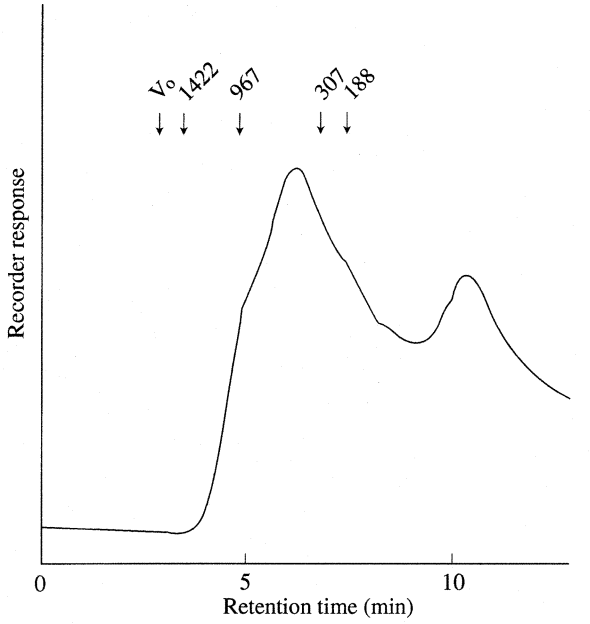

Fig. 1. Gel Filtration Pattern of the Water-soluble Fraction of Cellulase-actinase Treated Flour.

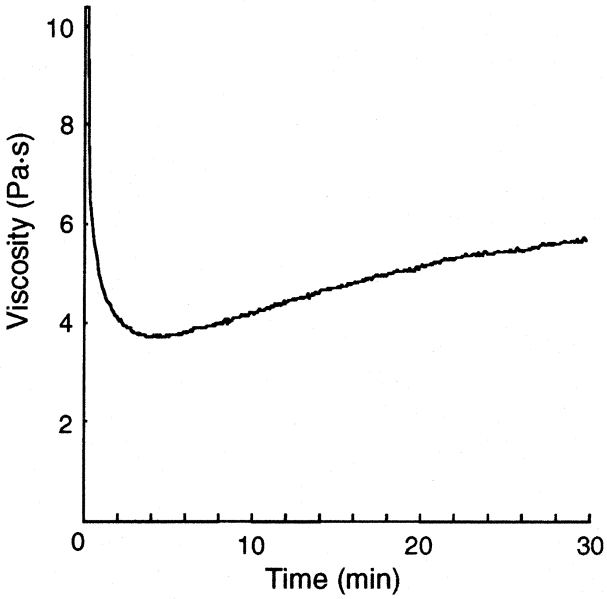

Fig. 2. Change in Viscosity of the Hypoallergenic Batter during Heating at 65°C.

Actinase (500 mg) was dissolved in water (5 ml), and the solution was mixed with the cellulase-reaction mixture. The subsequent reaction was carried out at 40°C for 1 h (Table 2), yielding a product that was hypoallergenic in most cases (Table 3). Most (96.4%) of the constituent proteins were water-soluble. The gel filtration pattern of the water-soluble fraction is shown in Fig. 1 and indicates that the hypoallegenic flour contained starch, oligopeptides and probably oligosaccharides.

The product was in a batter state, and this characteristic limits its use as a material for food processing. To solve this problem, we gelatinized the starch present in the hypoallergenic batter before processing. The viscosity of the batter was increased by heating at 65°C for 30 min (Fig. 2), and the result was more easily handled. The partially gelatinized batter was suitable for making cup cakes, pizza and cookies. The exhaustively gelatinized batter produced by steaming formed a gel, the hardness, cohesiveness and stickiness of this gel being 10.9 g/mm^2, 0.67 and 5.5 g/mm^2, respectively. The degrees of gelatinization of the partially and exhaustively gelatinized batter samples were 3.2% and 87.3%, respectively.

The food items selected as products were cup cakes, pasta, pizza, cookies, wafers and a puffed item. The optimal ingredient compositions of these items are shown in Table 4. We have described in a previous paper[12] that the hypoallergenic batter

Table 5. Representative Textural Parameters[a] of Food Items[b] Made from the Hypoallergenic Batter

Food item	Textural parameter	Flour source		
		Gelatinized batter	Non-heated batter	Original flour[c]
Cup cakes	Specific volume	3.8	2.4	2.5
	Hardness	0.6	0.8	2.1
	Cohesiveness	0.56	0.55	0.61
Noodles	Hardness	9.0	—[d]	5.1
	Cohesiveness	0.83[f]	—[d]	0.65
Pizza	Specific volume	2.2	2.3	2.7
	Hardness[e]	4.6	4.6	12.3
	Cohesiveness[e]	0.51	0.51	0.56
Cookies	Specific volume	4.1	—[d]	2.2
	Rupture stress	7.1×10^4	—[d]	3.7×10^5
	Base area[f]	172	—[d]	102
Wafers	Specific volume	6.4	—[d]	5.9
	Rupture stress	3.2×10^5	—[d]	5.6×10^5
Puffed item	Specific volume	8.7	—[d]	8.7
	Rupture stress	5.9×10^5	—[d]	6.1×10^5

[a] Units of the parameters: specific volume, ml/g; hardness, g/mm^2; cohesiveness, no unit; and rupture stress, N/m^2. Each value is expressed as the average of the measured values with standard errors of less than 10 %.
[b] Compositions are shown in Table 4.
[c] Flour with a 0.6-fold weight of water.
[d] The item could not be produced.
[e] A mixture (20 g each) of the ingredients was steamed for 10 min, and the textural properties of the inner part were measured. The sample temperature was 65°C.
[f] % of the area to that before baking.

retained carbon dioxide well and that muffin-like bread with a specific volume of about 3.5 could be made. It is expected that more-expanded cup cakes could be obtained if the viscosity of the batter was made sufficiently high to restrict gas escape. When the partially gelatinized batter was mixed with the other ingredients, the cakes possessed a higher specific volume of 3.8 (Table 5). The cup cakes could be stored frozen, and frozen cup cakes have been used in clinical tests at Kansai Medical University Hospital. A previous paper[12] also describes that pasta-like noodles could be obtained by extrusion cooking. When the exhaustively gelatinized batter was extruded without heating, almost the same noodle quality was obtained (Table 5). Pizza, cookies, wafers and a puffed item were also prepared, and their textural profiles are summarized in Table 5. The addition of a surfactant was preferable for the cup cakes, pizza and wafers.

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