

Modification of Wheat Flour with Bromelain and Baking Hypoallergenic Bread with Added Ingredients

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Based on the wheat glutenin IgE-binding epitope, Gln-Gln-Gln-Pro-Pro, a practical method is proposed for the production of hypoallergenic wheat flour. Bromelain was found effective for decomposing the epitope structure. In practice, soft flour was mixed with water dissolving bromelain and the mixture was incubated at 37°C for 4 h. The result of IgE-ELISA (enzyme-linked immunosorbent assay) suggested negative allergenicity. A mixture of bromelain-modified flour, glucose, citric acid, a surfactant and sodium hydrogen carbonate was baked to produce hypoallergenic bread, resembling English muffins.

Key words: hypoallergenic flour; hypoallergenic bread; allergy; bromelain; wheat

We have previously reported^{1,2)} that hypoallergenic wheat flour could be produced by treating soft flour with collagenase, and that pasta-like noodles and bread could be made from this hypoallergenic flour. The collagenase preparation we used, however, was of *Clostridium* origin,³⁾ and its use in food is not legally permitted. We also reported that most wheat-associated allergic patients were sensitive to gluten, and identified the major epitope structure as Gln-X-Y-Pro-Pro, where X and Y are replaceable amino acid residues.^{4,5)}

We therefore tried to screen for a food-usable enzyme with high activity to hydrolyze peptide bonds near the essential proline residues of the epitope. We propose here a practical procedure for enzymatically producing hypoallergenic wheat flour with the aim of producing bread for wheat-allergic patients.

Materials and Methods

Reagents. 2-Furanacryloyl-Leu-Pro-Ala (FALGPA) was obtained from Sigma Chemical Co. Sorbitan monostearate (commercial name: Emasol S-18F) was donated by Kao Co. Other reagents used were all of reagent grade.

Enzymes. Protease A (371 units/mg), protease B (2.12 units/mg), protease N (189 units/mg), protease S (11.5 units/mg), proleather (10.5 units/mg), and papain (W-40, 477 units/mg) were obtained from Amano Pharmaceutical Co. Bromelain (1030 units/mg) and Maxrase (53.6 units/mg) were obtained from Wako Pure Chemical Ind. and Kaken Pharma Co., respectively.

Wheat flour. Commercially available soft flour (Showa Sangyo Co.; commercial name: Cleopatra) was used as the material for producing hypoallergenic flour.

Immunoassay. Flour samples (0.5 mg each) were extracted with 1.0 ml of 0.1 M Tris-HCl (pH 8.6) containing 4 M urea (UTH buffer solution), and the allergenicity of the extract was tested by ELISA.⁶⁾ Briefly, microtiter plates were coated with 100 μ l of the extract overnight at 4°C. Unoccupied sites in the wells were saturated by incubating with 200 μ l of bovine serum albumin (2%) at 37°C for 2 h. Serum samples from patients sensitive to gluten were kindly provided by Dr. Zenro Ikezawa of Yokohama City University Hospital, informed consent being obtained from all donors. For the assay, serum samples were used at a dilution of 1:10. Serum samples from healthy adults were used as controls. Sera were added to the wells and incubated overnight at room temperature. This was

followed by the additions of biotinylated anti-human IgE (Kirkegaard & Perry Lab.), streptavidin-peroxidase conjugate (Boehringer Mannheim), and *o*-phenylenediamine (Wako Pure Chemical Ind.). The wells were washed between each addition with PBS Tween-20 (0.05%). After the reaction, the OD value was read by an ELISA reader, using a 490 nm filter. Allergenicity is expressed in arbitrary units based on the absorbance at 490 nm.

FALGPA hydrolyzing activity. Enzyme activity was determined with FALGPA as a substrate by the method of Van Wart *et al.*⁷⁾ under the following reaction conditions: substrate, 0.05 mM; solvent, 0.4 M NaCl, 10 mM CaCl₂ in 50 mM Tris-HCl (pH 7.5); enzyme concentration, 5 mg/ml of solvent; and reaction temperature, 25°C. Activity is expressed as units/mg of enzyme, where one unit is equivalent to one μ mol of FALGPA hydrolyzed/min at 25°C.

Amylase activity. Gelatinized potato starch was dispersed in water (4.5 ml) to a final concentration of 1%, and hydrolyzed with 2.0 mg of each enzyme at 37°C for 30 min. The resulting reducing group was determined by the method of Luchsinger and Cornesky,⁸⁾ using 3,5-dinitrosalicylic acid. Amylase activity is expressed as μ mol glucose equivalents/min/mg of enzyme.

Electrophoresis. Enzyme-treated flour samples (50 mg) and intact flour (5 mg) were separately extracted in UTH buffer solution (1 ml) while shaking. Extracts were obtained by centrifuging at 1300 $\times$ g for 5 min, and 5 μ l portions were electrophoresed by the procedure of Laemmli.⁹⁾ SDS 10-20% gradient polyacrylamide gel was obtained from Daiichi Pure Chemicals. A mixture of rabbit muscle phosphorylase *b* (MW 97 k), bovine serum albumin (MW 67 k), bovine carbonic anhydrase (MW 31 k), soybean trypsin inhibitor (MW 22 k), and hen's egg lysozyme (MW 14 k) was used as the molecular-weight markers. The gel was stained with Coomassie brilliant blue R-250 (Sigma Chemical Co.).

Gel filtration. The hypoallergenic flour (50 mg) was added to water (0.5 ml) while stirring, and the suspension was centrifuged at 1300 $\times$ g for 5 min. The supernatant was loaded onto a Biogel P-2 (Bio-Rad) column (1 $\times$ 24.5 cm), and eluted with water at a flow rate of 28.5 ml/h. The void volume of the column was 15.5 ml. Bacitracin (MW 1411) and tetraglycine (MW 246) were used as molecular weight markers.

Resistography. The hypoallergenic flour (86 g on a dry matter basis) was mixed with water (74 ml) using a resistograph (Brabender) at 63 rpm and 30°C for 20 min to obtain a mixing time-resistance curve. A mixture of intact flour (100 g) and water (60 ml) was also resistographed in the same manner.

Farmography. A raw baker's yeast preparation (Oriental Yeast) was suspended in water at a concentration of 2 g/ml. The suspension (0.35 ml)

was mixed with 50 g of hypoallergenic flour batter and intact flour dough as described for the resistography experiments. The mixtures were analyzed with a farmograph (Ato, AR-1000) for carbon dioxide generation and retention at 24°C for 90 min.

Bread making. Bread was made from the hypoallergenic flour by the method described in the previous paper.²⁾ A mixture of hypoallergenic batter (1.6 kg), glucose (10 g), citric acid monohydrate (13 g), sorbitan monostearate (10 mg), and salt (10 g) was vigorously stirred with sodium hydrogen carbonate (20 g). The mixed batter (30 g each) was immediately poured into a muffin cup and baked at 180°C for 20 min. Control bread was made from soft flour instead of the hypoallergenic flour. In this case, water was added to the mixture to a final water/flour ratio of 6:10. A commercially available English muffin was used as a control in the photographs of crumb grains.

Specific loaf volume. Loaf volume was measured by the rapeseed replacement method.¹⁰⁾ Specific loaf volume was represented as loaf volume (ml)/loaf weight (g). Standard errors were less than 10% of the respective values.

Color parameters. The color parameters (L-, a-, and b-values) of the upper part of each crust were measured with a color-difference meter, Suge Test Instruments (SC-2-XCH). Standard errors were less than 10% of the respective values.

Sensory test. A trained panel of 7 members was used. Scores for the hypoallergenic bread sample or for the control bread were obtained by grading between 5 (very good) and 0 (very bad).

Statistical analysis. Student's *t*-test¹¹⁾ was adopted.

Results and Discussion

As in our previous study,⁴⁾ we isolated an allergenic peptide from a chymotryptic hydrolysate of gluten by gel filtration and reversed-phase HPLC. The primary structure of this peptide was found to have the motif Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Pro-Pro-Phe. We also reported that the epitope structure of this peptide was Gln-X-Y-Pro-Pro, where X and Y are replaceable amino acid residues.⁵⁾ This finding led us to presume that wheat flour would become hypoallergenic by hydrolyzing peptide bonds near the essential proline residues.

With this idea, we screened for an enzyme with high ability to hydrolyze peptide bonds near proline residues in attempt to produce hypoallergenic flour. The enzyme chosen should be characterized by its low amylase activity,

which is expected to minimize the development of sweetness, deterioration of starch functionality, and volume loss of the product. In addition, an amylase inhibitor cannot be used for the production of hypoallergenic flour because of its allergenic potency.¹²⁾ The ability to hydrolyze peptide bonds near proline residues was determined using FALGPA. Table I shows the FALGPA hydrolyzing and amylase activities of the enzymes tested. Bromelain showed the highest FALGPA hydrolyzing activity of the enzymes tested and also had low amylase activity. We, therefore, selected bromelain for producing the hypoallergenic flour.

We have previously reported²⁾ that a water/flour ratio of 0.6 was favorable for hypoallergenic bread making. Considering this result, we treated a 6:10 mixture (1.6 kg) of water and flour with bromelain (10 g) at 37°C for 8 h. Trace allergenicities were found with the 1–4 h reaction products, but no allergenicity was detected with the 8 h products (Table II). However, under conditions of 37°C for 8 h, the product became spoiled by microorganisms. To shorten the reaction time, we first treated a 5:10 mixture (1.5 kg) of water and flour with bromelain (10 g) at 37°C for 2 h. Bromelain (10 g) in water (100 ml) was then added again, and the incubation was continued at 37°C for another 2 h. This two step procedure shortened the reaction time

Table II. Allergenicities of Bromelain-treated Flour under Different Conditions

Condition		Allergenicity (Relative ELISA value)
Water/flour ratio	Incubation time (h)	
0.6	0 ^a	1
	1	0.05
	2	0.03
	4	0.03
	8	nd ^b
0.5→0.6 ^c	2+2 ^c	nd

^a Intact flour.

^b Not detected (<0.02).

^c Reaction was done by the two-step method.

Flour (1 kg) was first treated with bromelain (10 g) in water (500 ml) at 37°C for 2 h. Bromelain (10 g) in water (100 ml) was then added again, and the incubation was continued at 37°C for an additional 2 h.

Table I. FALGPA Hydrolyzing and Amylolytic Activities of Enzymes for Food Use

Enzyme	Activity	
	FALGPA hydrolyzing (unit/mg)	Amylolytic (μmol glucose equivalent/min/mg)
Bromelain	1.52	0.072
Protease B	0.78	0.703
Papain	0.21	0.040
Protease A	0.20	0.759
Protease N	nd ^a	— ^b
Protease S	nd	—
Proleather	nd	—
Maxrase	nd	—

^a Not detected.

^b Not determined because no FALGPA hydrolytic activity was found.

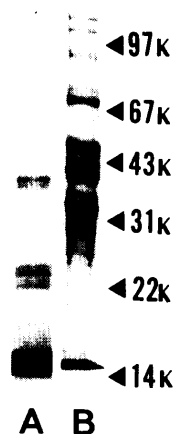


Fig. 1. SDS-PAGE of Bromelain-treated Flour (A) and Intact Wheat Flour (B).

from 8 h to 4 h, and the resulting product showed no allergenicity without spoiling. As shown in Fig. 1, the electrophoretic patterns of the bromelain-treated products indicate that most wheat proteins were converted to low-molecular-weight peptides. The hypoallergenic product contained proteinaceous components of nearly 20 kDa and 30–40 kDa, which were considered to be non-allergenic. From these results, we propose the two-step method for the practical production of hypoallergenic flour from soft flour.

The molecular weight distribution of the hypoallergenic flour was evaluated by gel filtration on Biogel P-2 (Fig. 2). The elution curve indicates that some low-molecular-weight peptides and free amino acids were produced during the enzymatic reaction. According to the resistographic data (Fig. 3A), soft flour changed to the batter state upon the proteolysis of gluten, but the farmogram shown in Fig. 4 indicated that the carbon dioxide generated was well-retained in the batter. From the farmogram, we judged that

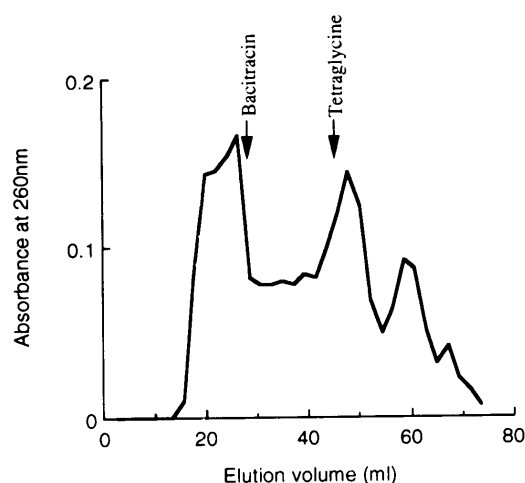


Fig. 2. Biogel P-2 Gel Filtration Pattern of Bromelain-treated Flour.

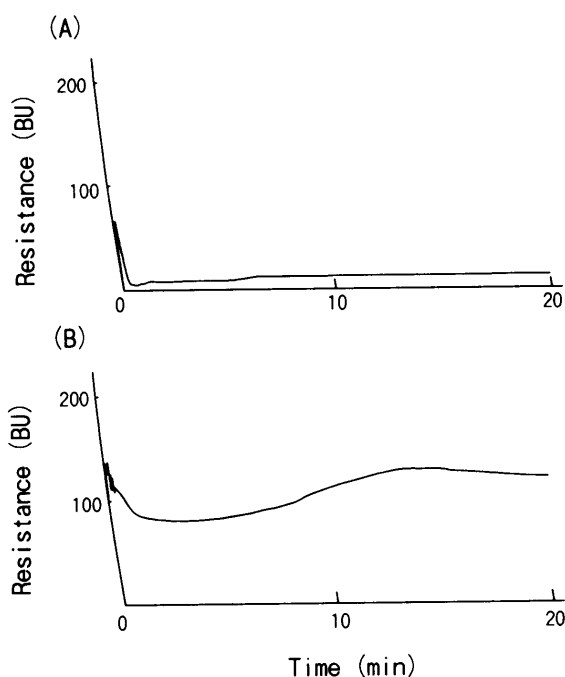


Fig. 3. Resistograms of Bromelain-treated (A) and Intact Flour (B).

it would be possible to make bread if the batter was baked in a cup to stabilize the shape. The glutenin-type protein in baker's yeast shows high structural similarity to the allergenic peptide of gluten.⁴⁾ We, therefore, used sodium hydrogen carbonate and citric acid as carbon dioxide generators. The hypoallergenic batter (1.6 kg) was mixed with citric acid (13 g), glucose (10 g), salt (10 g), and surfactant (10 mg), and then mixed with sodium hydrogen carbonate (20 g) to generate carbon dioxide. Portions of

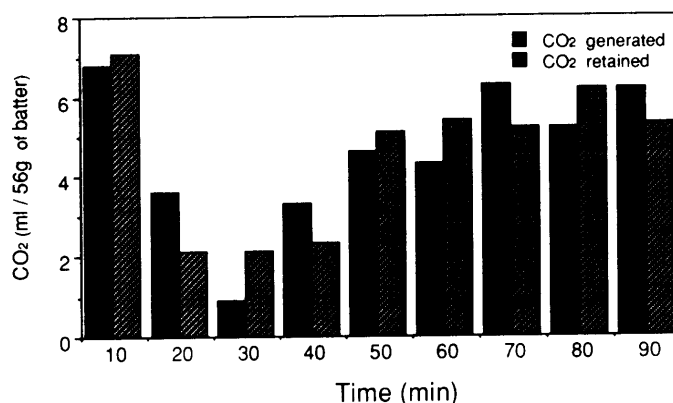


Fig. 4. Farmogram of Bromelain-treated Flour.

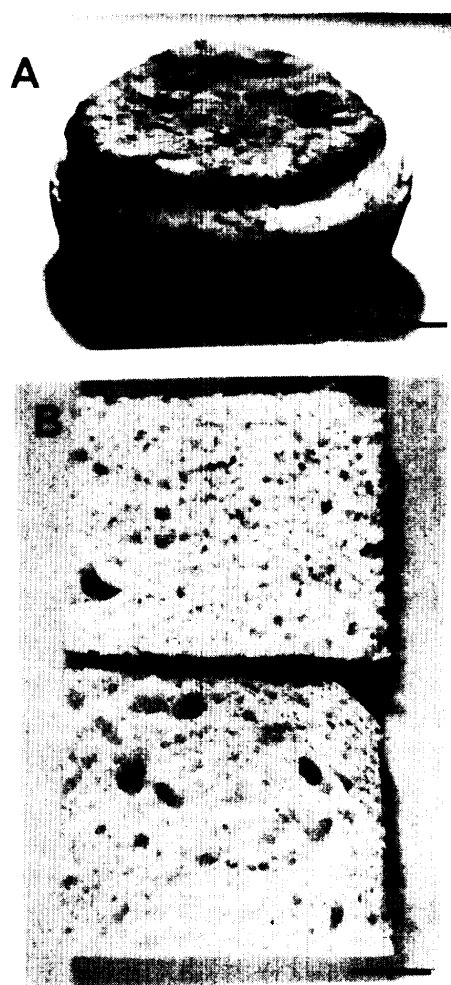


Fig. 5. Photographs Showing the Appearance of the Hypoallergenic Bread (A), and the Crumb Grains (B) of the Hypoallergenic Bread (Upper) and English Muffins (Lower, Control).

the mixture (30 g each) were poured into muffin cups and baked at 180°C for 20 min as reported in the previous paper.²⁾

The hypoallergenic bread resembled English muffins in terms of the crumb grains (Fig. 5). The specific loaf volume of the hypoallergenic bread was estimated to be 3.2, which was nearly equal to that of English muffins.²⁾ On the other hand, the color parameters were different between the hypoallergenic bread and English muffins; L-, a-, and b-values of the hypoallergenic bread were 43.3, 10.1, and 12.4, respectively, whereas, as previously reported,²⁾ those of English muffins were about 65, 5, and 22, respectively. The data indicated that a non-enzymatic browning reaction occurred between the added glucose and the amino compounds generated in the hypoallergenic bread during the baking process. As shown in Table III, the hypoallergenic bread was superior to the control bread in all sensory parameters tested, particularly in respect of color and odor.

Table III. Sensory Scores^a for the Hypoallergenic Bread

Item	Hypoallergenic bread	Control
Color	4.6 ± 0.2**	0.6 ± 0.4
Taste	2.4 ± 0.4	2.1 ± 0.3
Odor	3.5 ± 0.3	2.2 ± 0.5
Total	3.0 ± 0.4*	1.4 ± 0.3

^a Data are given as means ± standard errors.

** Significantly different from the control at 0.1% level.

* Significantly different from the control at 1% level.

Since it is clinically important to supply organoleptically acceptable samples to patients, this hypoallergenic bread will have practical application. Clinical tests are being carried out at Yokohama City University Hospital to confirm the effectiveness of the hypoallergenic bread.

References

- 1) M. Watanabe, T. Suzuki, Z. Ikezawa, and S. Arai, *Biosci. Biotech. Biochem.*, **58**, 388–390 (1994).
- 2) M. Watanabe, Z. Ikezawa, and S. Arai, *Biosci. Biotech. Biochem.*, **58**, 2061–2065 (1994).
- 3) E. Yoshida and H. Noda, *Biochem. Biophys. Acta*, **105**, 562–574 (1965).
- 4) M. Watanabe, S. Tanabe, T. Suzuki, Z. Ikezawa, and S. Arai, *Biosci. Biotech. Biochem.*, **59**, 1596–1597 (1995).
- 5) S. Tanabe, S. Arai, Y. Yanagihara, H. Mita, K. Takahashi, and M. Watanabe, *Biochem. Biophys. Res. Commun.*, **219**, 290–293 (1996).
- 6) B. J. Walsh and M. E. Howden, *J. Immunol. Methods*, **121**, 275–280 (1989).
- 7) H. E. Van Wart and D. R. Steinbrink, *Anal. Biochem.*, **113**, 356–365 (1981).
- 8) W. W. Luchsinger and R. A. Cornesky, *Anal. Biochem.*, **4**, 346–347 (1962).
- 9) U. K. Laemmli, *Nature*, **227**, 680–685 (1970).
- 10) American Association of Cereal Chemists, in "Approved Methods of the AACC," 8th Ed., The Association, St. Paul, 1983, Method 10–10B.
- 11) R. A. Fisher, in "Statistical Methods for Research Workers," 13th Ed., Hafner Publishing Company Inc., New York, 1958, pp. 122–128.
- 12) L. Gómez, E. Martín, D. Hernández, R. Sánchez-Monge, D. Barber, V. del Pozo, B. de Andrés, A. Armentia, C. Lahoz, G. Salcedo, and P. Palomino, *FEBS Lett.*, **261**, 85–88 (1990).