



Effects of hydroperoxide in breads on IgE antibody responses

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ABSTRACT

The effect of hydroperoxide in breads on antigen–antibody reaction against IgE was examined. We compared the antigen–antibody reaction against IgE between dough including hydroperoxide and that not including hydroperoxide. Crude proteins extracted from the dough including hydroperoxide showed weak antigen–antibody reaction on allergic tests such as ring precipitin test with human-specific IgE, and the IgE binding activity on ELISA. In contrast, the dough without hydroperoxide showed antigen–antibody reaction. The crude proteins extracted from the dough with hydroperoxide or without hydroperoxide were applied by affinity chromatography of trypsin-immobilized chitin. Then proteins having affinity were recovered. The recovered proteins were separated by SDS-PAGE. Each protein was examined for the IgE binding activity on ELISA. The IgE binding activity of baked bread was studied as well. Crude proteins of baked bread made from dough including hydroperoxide showed weaker IgE binding activity on ELISA than proteins of baked bread made from dough without hydroperoxide. Those results suggested that addition of hydroperoxide into dough affects on antigen activation.

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1. Introduction

Over the last several years, numerous researchers have studied food allergies from a variety of angles and clarified various facts (Fuchs & Astwood, 1996; Sampson, 1999; Buck & Hefle, 1995; Sampson & McCaskill, 1985; Crespo, Pascual, Burks, Helem, & Esteban, 1995). Various foods show food allergies, and it is not easy to find safe foods for allergy patients. In addition, most of the allergens in food allergies are proteins, and various proteins are known as allergens, but detection of specific allergens in foods is difficult.

The oxidation of lipids in foods is responsible for the formation of off-flavors and chemical compounds which may be detrimental to health, and it is well known to give a demerit food chemistry or biochemistry fields (Ames, Shigena, & Hagen, 1994; Gardnaer, 1996; Grosch, 1987; Pokorny, 1999; Shewfelt & Del Rosario, 2000; Mercier & Gelinas, 2001; Toyosaki & Sakane, 2002; Toyosaki & Koketsu, 2004; Toyosaki, 2007; Rabia, Suhur, & Nazlin, 2008; Wong & Chye, 2009; Wei-Dan et al., 2010). In the current climate, various lipid peroxides produced by such lipid peroxidation are treated only as a detriment. However, among longstanding traditional foods there are foods with fine flavors brought out by inducing lipid peroxidation like fine, thin noodles and dried foods, so there are instances when lipid peroxide produced by lipid peroxidation can also be an advantage in the field of food products. The properties of foods can be rendered an advantage through better use of the properties of lipid peroxides.

By the way, proteins in normal bread also cause allergies for allergy patients. Allergens are primarily thought to be proteins of origin from flour or additional ingredients such as eggs in the making bread process. Most allergy patients could not consume commercially prepared breads because it is risky. We have studied to overcome this issue from various viewpoints for development of anti-allergy bread. The object of the current study was to investigate the anti-allergic action of hydroperoxide in bread dough and its mechanism.

2. Materials and methods

2.1. Materials and samples

Spring wheat flour (Super King; 13.8% protein, 0.42% ash, and 14% water) was obtained from Nisshin Flour Milling Inc. (Chiyoda, Tokyo, Japan). Dried yeast (*Saccharomyces cerevisias*) was purchased from S.I. Lesaffre (Marcq-en-Baroeul, France). Lipoxygenase (from soybean, Type I-B, lyophilized) and linoleic acid (99% pure) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Gluten (more than 90% pure), 2',7'-dichlorofluorescein (DCF), hematin (more than pure 90%), and Tween 80 were purchased from Nakarai Tesque, Inc. (Kyoto, Japan). Diethylaminoethyl (DEAE) cellulose (DE52) was purchased from Whatman, Ltd (Maidstone, UK). Macro-Prep 25Q Strong Anion Exchange support (Macro-Prep 25Q, 200 mL), Bio-Scale Mini Bio-Gel P-6 Desalting Cartridges (5 × 10 mL), and a Bio-Scale Empty Column (MT20, 15 mm × 113 mm, 19.4–21.9 mL), 10x Tris/glycine/SDS (25 mM Tris, pH 8.3, 192 mM glycine, and 0.1% SDS), Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, and 0.01% Bromophenol Blue), PROTEAN II Ready Gel (10% acrylamide, 15-well),

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and Precision Plus Protein Dual Color Standards, were purchased from Bio-Rad Laboratories (Tokyo, Japan). Silicagel70 F254 (20×20 cm) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Other reagents were of special grade and were obtained from Nacalai Tesque.

2.2. Preparation of linoleic acid hydroperoxide

Linoleic acid hydroperoxide was prepared according to the produce of Matsuda, Yasumoto, and Iwami (1978) by enzymic peroxidation with lipoxygenase. The standard reaction mixture, containing 32 mM linoleic acid (99% pure), 0.1% Tween 80, 0.2 M borate buffer (pH 9.0), and 50 units of lipoxygenase in a total volume 20 mL, was incubated in a conical flask to facilitate its being flushed with pure oxygen. The reaction mixture was stirred mechanically for 40 min at 30 °C under a stream of pure oxygen in a separating funnel. Then hydroperoxide was extracted with diethyl ether and concentrated by removal of the solvent. The formation of linoleic acid hydroperoxide was monitored by measurement of the increase in the absorbance at 234 nm. Absorbance at 234 nm was used as a direct index of the amount of the conjugated diene and as an indirect index of the amount of hydroperoxide. The hydroperoxide was checked by TLC for purity with a mixture of *n*-hexane diethyl ether, and acetic acid (60:40:1,v/v/v) as the solvent system and was monitored under UV light.

2.3. Formula for dough and baking bread

The wheat flour (strong flour) that was used to make adjustments in the bread dough was the type that is readily commercially available. The lipid added was linoleic acid (more than 95% pure), which was added at the 3% level. Other ingredients used to make the bread were all commercially available. Various concentrations of hydroperoxide (0–500 µM), and lipoxygenase (35,000 units) were added at the end of bread dough adjustment and underwent primary fermentation in an incubator at 37 °C with 75–80% humidity. After fermentation, gas was released; after a bench time of 10 min, the dough underwent final fermentation for 90 min and was then baked for 12 min at 200 °C (Toyosaki, 2007).

2.4. Measurement of hydroperoxide

Hydroperoxide concentration was calculated in terms of 2',7'-dichlorofluorescein (DCF). To de-emulsify, the 5.0 mL reaction product with DCF and linoleic acid was centrifuged (10,000×g, 30 min). The linoleic acid of the supernatant was then measured to determine the hydroperoxide level using the method of Cathcart, Schwieters, and Ames (1984). First, 1.0 mL of a 1.0 mM solution of DCF in ethanol and 2.0 mL of 0.01 N NaOH were mixed and stirred for 30 min before being neutralized with 10 mL of 25 mM phosphate buffer (pH 7.2). Then 2.0 mL of the neutralized DCF solution was added to a solution of hematin (10 mM) in 25 mM phosphate buffer (pH 7.2; 0.01 mg DCF/mL); subsequently, 2.0 mL of this hematin-DCF solution and 10 mL of the linoleic acid sample were mixed and left at 50 °C for 50 min, before fluorometry treatment (excitation. 400 nm; emission. 470 nm) to measure DCF.

2.5. Separation of the human serum fraction

Human blood was collected from a best description of the ten females with their permission. There was no allergic case history, and it collected blood from ten healthy females. It was confirmed not to show an abnormal allergic sensitivity to the actual experiment system. These bloods were supplied to the experiment.

The human serum was separated in accordance with the method of Mirella, Massiomo, Gianni, Vincenzo, Maurizio, and Cristima (1995). Human serum containing K-EDTA (1.6 mg/mL) was centrifuged

(2000×g, 8 min) and the serum fraction necessary for the experiment was obtained.

2.6. Extract of crude protein from dough

0.1 M Phosphate buffer (pH 6.5, 30 mL) was added into dough (50 g) and crude proteins in it were extracted. The mixture was filtered. The filtrate, crude proteins, was used for each experimental.

2.7. Measurement of the amount of protein

The amount of protein was measured by the Lowry, Rosebrough, Farr, and Randall (1951).

2.8. Measurement of antigen–antibody reaction by single-radial immunodiffusion

Single-radial immunodiffusion was performed for measurement of human-specific IgE antigen–antibody reaction of crude proteins obtained from the dough. Human serum (2.0 mL) was added into 30 mL of 1% agar solution, and the agar solution was coated to a thickness of 4 mm on a slide glass surface. Holes (2.5 mm diameter) were formed in the agar. The proteins obtained from the dough were added into the hole. After the glass was maintained overnight at 37 °C, the size of the ring precipitin obtained was measured.

2.9. Measurement by enzyme-linked immunosorbent assay (ELISA)

For measurement of the IgE binding activity of protein, ELISA (direct ELISA) was conducted according to a method of Engvall and Perlmann (1972). The direct ELISA uses the method of directly labeling the antibody itself.

2.10. Separation of proteins by affinity chromatography

Protein fractions extracted from the dough were separated by trypsin-immobilized affinity column chromatography (Mine & Zhang, 2001). The adsorbed substances were eluted by the linear gradient of pH 6.5 to 7.5 in 1.0 M phosphate buffer. The non-affinity fraction came into the flow through solution, while the affinity fraction was eluted from the column by the buffer linear gradient elution. The latter fraction was further purified by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The amounts of protein in each fraction obtained by affinity column chromatography were measured by the Lowry et al. (1951).

2.11. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed in accordance with a method of Laemmli (1970). That is, electrophoresis was performed with Ready Gel J 18 mA/gel (concentration gradient gel) using a Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Inc, Tokyo, Japan). After electrophoresis, the gel was stained using Coomassie brilliant blue R250.

2.12. Statistical analysis

Data were reported as mean±standard deviation. Analysis of variance and least significant difference tests were conducted to identify differences among means. Statistical significance was declared at $p < 0.05$.

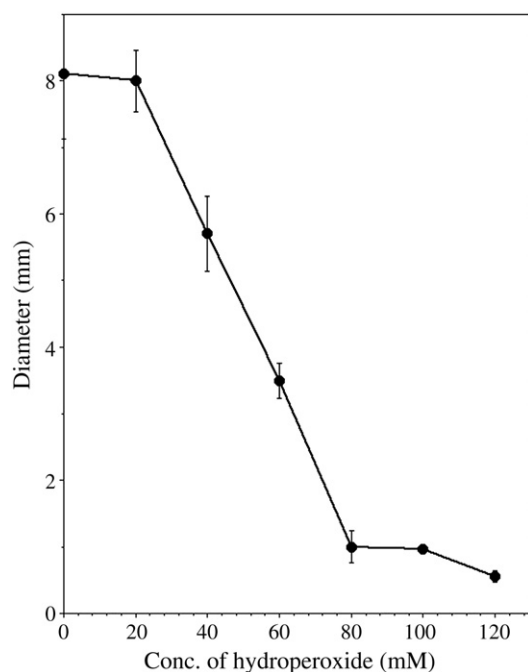


Fig. 1. Evaluation of the binding activity of human-specific IgE with dough added various concentrations of hydroperoxide by single-radial immunodiffusion. Each value represents the mean $\pm$ standard deviation in triplicate.

3. Results and discussion

3.1. Measurement of antigen–antibody reaction of crude proteins extracted from dough of hydroperoxide, and lipoxygenase using single-radial immunodiffusion and ELISA

As soon as mixing of whole materials was done, crude proteins were extracted using phosphate buffer. The crude protein solution extracted from dough without hydroperoxide showed antigen–antibody reaction (size of diameter of ring: 8.1 mm) against IgE

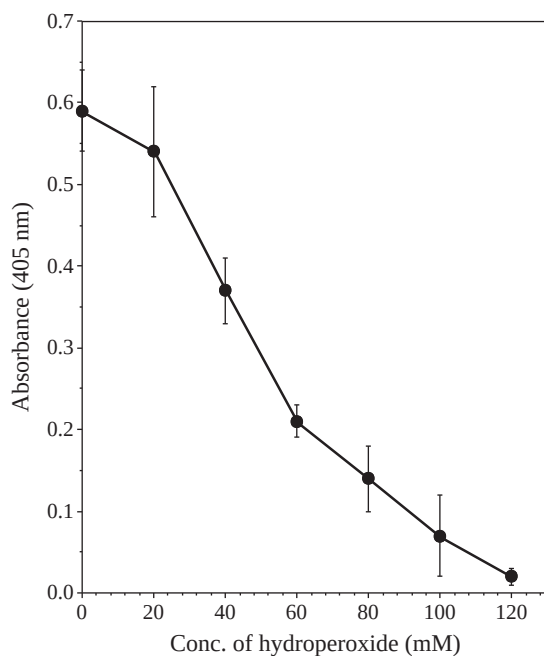


Fig. 2. Evaluation of the binding activity of human-specific IgE with dough added various concentrations of hydroperoxide by ELISA. Each absorbance represents the mean $\pm$ standard deviation in triplicate.

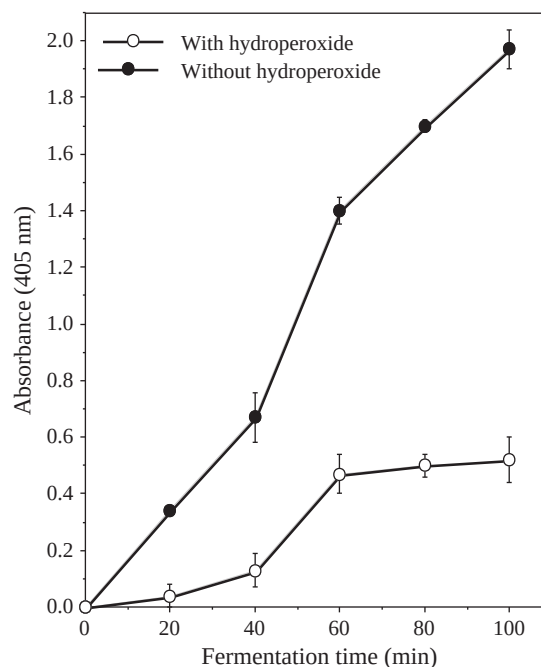


Fig. 3. Effect of hydroperoxide to changes in the binding activity of human-specific IgE with the fermentation of dough. Each absorbance represents the mean $\pm$ standard deviation in triplicate.

concentration-dependently in the single-radial immunodiffusion method. In contrast, the crude protein solution extracted from dough including hydroperoxide decreased the size of the diameter of ring precipitin concentration-dependently. The size of the diameter of the ring precipitin of crude protein extracted from dough including 20 mM hydroperoxide was 8.1 mm. The size of the diameter of the ring precipitin of dough including a higher concentration of hydroperoxide decreased drastically (Fig. 1).

The antigen–antibody reaction of crude proteins using ELISA was confirmed. The reaction of dough of a higher concentration of

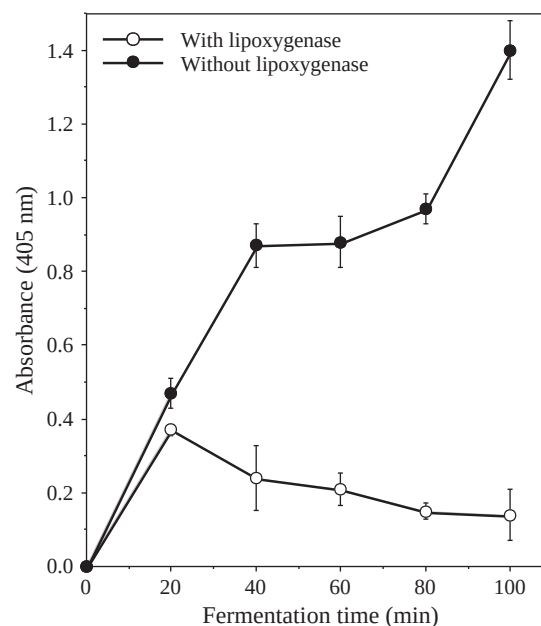


Fig. 4. Effect of lipoxygenase to changes in the binding activity of human-specific IgE with the fermentation of dough. Each absorbance represents the mean $\pm$ standard deviation in triplicate.

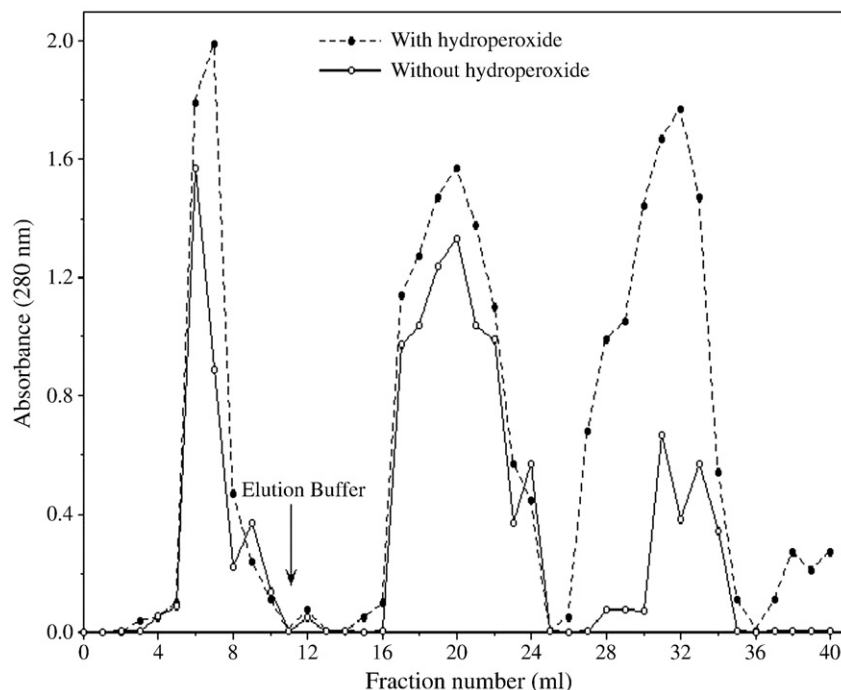


Fig. 5. Elution profile proteins extracted from doughs on affinity chromatography packed with trypsin-immobilized chitin.

hydroperoxide against IgE got weaker. Crude protein extracted from dough including more than 20 mM hydroperoxide decreased antigen–antibody reaction against IgE drastically (Fig. 2). The results are consistent with the results in Fig. 1. These facts strongly suggested that the addition of hydroperoxide affected antigen–antibody reaction of dough crude protein.

Next, the generation of crude proteins showing antigen–antibody reaction during the fermentation process of dough with or without hydroperoxide, and lipoxygenase was studied. In the case of dough including hydroperoxide, and lipoxygenase the dough has almost no reaction with IgE during a fermentation time for 20 min. However, the dough without lipoxygenase tended to increase drastically the reaction against IgE during the fermentation time (Figs. 3 and 4). The addition of hydroperoxide or lipoxygenase inhibited the production of proteins having the reaction against IgE in dough during the fermentation stage.

3.2. Collection and identification of proteins by affinity chromatography

The crude proteins extracted from dough with hydroperoxide or without hydroperoxide were separated using affinity chromatography

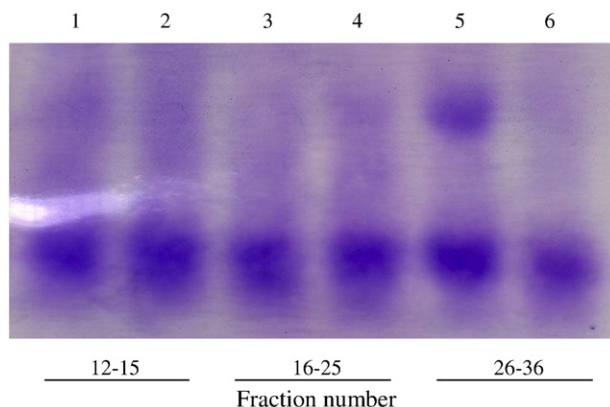


Fig. 6. SDS-page 12–36 fraction number of affinity chromatography. Nos. 1, 3, and 5: With hydroperoxide; Nos. 2, 4, and 6: Without hydroperoxide.

of a Trypsin-immobilized chitin. The crude proteins were applied on the affinity chromatography. The non-affinity fraction came into the flow through solution, while the affinity fraction of both proteins, which were dough with or without hydroperoxide, was eluted from the column by the buffer linear gradient elution and recovered fractions 12–36 between pH 6.5 and 7.5 (Fig. 5). Both elution profiles showed a different band. Proteins of both affinity fractions (fraction number 26–36) were identified by SDS-PAGE (Fig. 6). The band that the other was not able to confirm by No.5 (with hydroperoxide) was confirmed. The element of

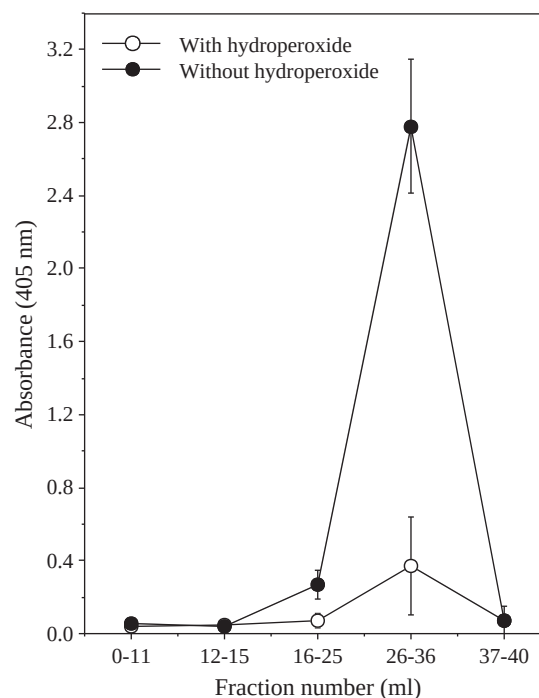


Fig. 7. Comparison of the binding activity of human-specific IgE with crude proteins extracted from dough with hydroperoxide and extracted from dough without hydroperoxide. Each absorbance represents the mean $\pm$ standard deviation in triplicate.

this band was not able to be confirmed this time. Perhaps, the possibility of the ovomucoid is high. The true colors of this band are scheduled to be confirmed in the future.

3.3. Antigen–antibody reaction of baked bread

Breads using the dough with or without hydroperoxide were actually made. Antigen–antibody reaction of crude protein extracted from both breads was studied by single-sandwich immunodiffusion and ELISA. Crude protein extracted from breads using dough including hydroperoxide obviously showed weaker antigen–antibody reaction of against IgE than crude proteins extracted from dough without hydroperoxide (Fig. 7).

Thus, the addition of hydroperoxide into dough is obviously weakened antigen–antibody reaction of dough itself and the baked breads as compared with hydroperoxide-free dough. We could confirm here the significance of addition of hydroperoxide for baked bread.

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