International Scholars Journals

International Journal of Food Safety and Public Health Vol. 10 (4), pp. 001-005, April, 2023. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Anti-allergic effects on addition of hydroperoxide to dough fermentation

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Accepted 23 November, 2022

The anti-allergic effects on dough fermentation of hydroperoxides produced by lipid peroxidation through lipoxygenase induction were studied. The anti-allergic effect of hydroperoxides to dough fermentation on antigen-antibody reaction involving IgE was examined. Crude proteins extracted from the dough including hydroperoxides showed weaker antigen-antibody reactions on allergic tests such as precipitin ring test with human-specific IgE, and the IgE binding activity on ELISA. This inhibitory effect on the antigen-antibody reaction increased with increased hydroperoxide and a parallel relationship was observed between hydroperoxide level and inhibitory effect. The crude proteins extracted from the dough with hydroperoxides were applied to an affinity choromatography column of immobilized-trypsin chitin. Then proteins having affinity was recovered. The recovered proteins were separated by SDS- PAGE. Each proteins was examined for the IgE binding activity on ELISA. Therefore, ovomucoid, which is present in egg, was identified as the main allergen. Crude proteins of baked bread made from dough including hydroperoxides showed weaker IgE binding activity on ELISA than proteins of baked bread made from dough without hydroperoxides. Suppression of antigen-antibody reactions with IgE due to denaturation of allergen protein (ovomucoid in the present study) present in dough by the produced hydroperoxide may be identified as a major factor. The importance of hydroperoxides as an ingredient of baked bread was confirmed.

Key words: Hydroperoxide, IgE, allergy, dough, baked bread, ELISA.

INTRODUCTION

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

Proteins in normal bread also cause allergies for allergy sufferes. Allergens are primarily thought to be proteins and gluten of origin from wheat flour or additional ingredients such as hen eggs in the bread-making process. Most allergy sufferers require commercially prepared allergen-free bread because of the allergy risk. To overcome this issue, development of allergy- free bread has been studied from various viewpoint: the interaction between the allergens in hen eggs (mainly ovomucoid) and gluten in the bread effects on allergy. Herein, the details are described.

The aim of this study was to determine the effects of peroxides on allergic reactions by closely investigating the relationship between peroxides produced from the induction of lipid peroxidation reactions in the process of dough fermentation and proteins, also egg proteins that act as allergens.

MATERIALS AND METHODS

Materials

The ovomucoid (Type III), ovomacroglobulin (Type II), ovomucin (Type I), ovalbumin (Type VII), ovotransferrin (Type I), lysozyme, linoleic acid (more than 99% pure) and lipoxygenase (from soybean, type I, lyophilized) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kieselgels 60G and PF254 were purchased from Merck (Darmstadt, Germany). The 0.25 mm thin-layer chromatography (TLC) was done with a TLC apparatus from Yazawa

Scientfic Apparatus Manufacturing Company Limited. (Tokyo, Japan). N',N'-dimethylformamide, Chondroitin sulfate sodium salt, hematin, 2',7'-dichlorofluorescin diacetate (DCFDA) were purchased from Tokyo Kasei Kogyo Company Limited. (Tokyo, Japan). Gluten and other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Preparation of linoleic acid hydroperoxide

Linoleic acid hydroperoxide was prepared by the procedure of Matsuda et al. (1978) by enzymatic peroxidation with lipoxygenase. The standard reaction mixture containing 32 mM linoleic acid, 0.1% Tween 80, 50 mM Na_2HPO_4 /NaOH buffer (pH 9.0), and 50 units of the lipoxygenase in a total volume of 20 ml was incubated in a conical flask to facilitate flushing with pure oxygen. The reaction mixture was stirred mechanically for 40 min at 30ºC under a stream of pure oxygen. After incubation, hydroperoxide was extracted with diethyl ether. The formation of linoleic acid hydroperoxide was monitored by measurement of the increase in the absorbance at 234 nm. The hydroperoxide was purified by TLC with n-hexane/diethyl ether/ acetic acid (60:40:0.1, v/v/v) as the solvent system and was monitored under UV light.

Bread making and preparation of the model system of bread

Conditions were as follows: wheat flour (1.0 kg), fresh live yeast (40 g), NaCl (15 g), saccharose (40 g), hen egg (120 g) were added to water (350 ml), the materials were mixed using a mixer. The lipid added was linoleic acid (more than 95% pure), which was added at a 3% level. Other ingredients used to make the bread were all commercially available. Lipoxygenase was 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 15 min, the dough underwent final fermentation for 35 min and was then baked for 20 min at 180°C.

Conditions of model system of bread were as follows. Wheat flour (1.0 kg), Fresh live yeast (40 g), NaCl (15 g), saccharose (40 g), ovomucoid (1.0 g), ovomuchin (1.0 g), ovalbumin (1.0 g), ovotransferrin (1.0 g), ovomacroglobulin (1.0 g), and lysozyme (1.0 g) were added to water (350 ml), the materials were mixed using a mixer. The various concentration of hydroperoxide was added, which was added at a 100 mM level, which served as the test sample. A sample system was created. This sample underwent primary fermentation in an incubator at 37°C with 75 - 80% humidity. After fermentation, gas was released; after a bench time of 15 min, the dough underwent final fermentation for 35 min and was then baked for 20 min at 180°C. The dough and baked bread was used for each experiment.

Preparation of crude protein extracts from dough and baked bread

Extract of crude proteins from the dough and baked bread were as following by Chung and Champagne(2001). Dough (30 g) and baked bread (50 g) were stirred in 300 ml of 0.1 M Phosphate buffer pH 6.5, and 10 mM EGTA for 30 min at 4ºC, followed by centrifugation at 85,000 x g for 10 min. The supernatants (extracts crude proteins) were filtered at 0.22 mm MILLEX-GX (Millipore Co., USA). The filtrate, crude proteins, was used for was added into experiment.Concentr ation of crude proteins in the extracts was determined with the Folin phenol reagent bassed on Lowry et al. (1951)

Measurement of linoleic acid hydroperoxides

Hydroperoxide was estimated by the method of Cathcart et al. (1984). Extracted linoleic acid (30 µl) was then measured for hydroperoxide. First, 1.0 ml of a 1.0 mM solution of DCFDA in ethanol and 2.0 ml of 0.01 M NaOH were mixed and stirred for 30 min before being neutralized with 10 ml of 25 mM phosphate buffer (pH 7.4). Then, 2.0 ml neutralized DCFDA solution were added to a solution of hematin in 25 mM phosphate buffer (pH 7.4; 0.01 mg 2',7'-dichlorofuluorescein (DCF)/ml) and 2.9 ml hematin-DCFDA solution and 10 µl supernatant containing linoleic acid were mixed and left at 50ºC for 60 min before fluorometry treatment (excitation, 400 nm; emission, 470 nm) to measure DCF. This method measures hydroperoxide with more sensitivity than the iron rhodanide method usually used. The formation of linoleic hydroperoxides and other products was checked by TLC with n-hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as the solvent system. The TLC plate was sprayed with 50% sulfuric acid, heated at 110ºC for 15 min, and checked under ultraviolet light.

Determination of antibody-antigen reaction

Human blood was collected from ten females (20 years old) with their permission. The human serum was separated in accordance with method of Mirella et al. (1995). Human serum containing K-EDTA (1.6 mg/ml) was separated with a centrifugal separator (2000 x g, 8 min) and objective serum fractions were obtained. Single-radial immunodiffusion was performed for measurement of antigen-antibody reaction of crude proteins obtained from the dough including 100 mM hydroperoxide and the dough without hydroperoxide against human-specific IgE. Human serum was added into 1% agar solution, 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 on overnight at 37ºC, the diameter of the precipitin ring obtained was measured. Enzyme-linked immunosorbent assay (ELISA) was conducted according to a method of Engvall and Perlmann (1972).

Separation of proteins by affinity chromatography and SDS−polyacrylamide gel electrophoresis (PAGE)

Protein fractions extracted from the dough with hydroperoxide and dough without hydroperoxide were separated by immobilized-trypsin affinity column chromatography (Mine and 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 eluted in the flow through void volume solution, whilst the bound fractions were eluted from the column by the buffer linear gradient elution. Latter fraction was further purified by SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed in accordance with a method of Laemmli (1970) using Ready Gel J 18 mA/gel (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. The HAST System automated electrophoresis device Cell was used as well. Amounts of protein in each fraction obtained by affinity column chromatography were measured by the Lowry et al. (1951) method.

Statistical analysis

The data were expressed as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance , and differences between means were tasted using Duncan's multiple range tests. P-values of <0.05 were considered to be significant.

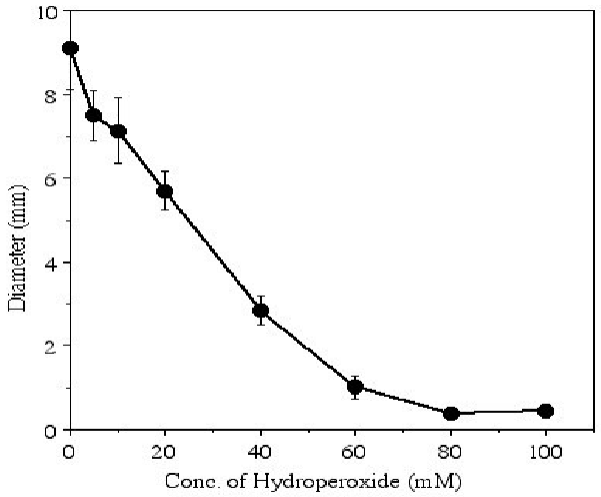

Figure 1. Evaluation of binding activity of human-specific IgE with dough added various concentrations of hydroperoxide by single-radial immunodiffusion. Values are the means of three replicates ± standard deviation.

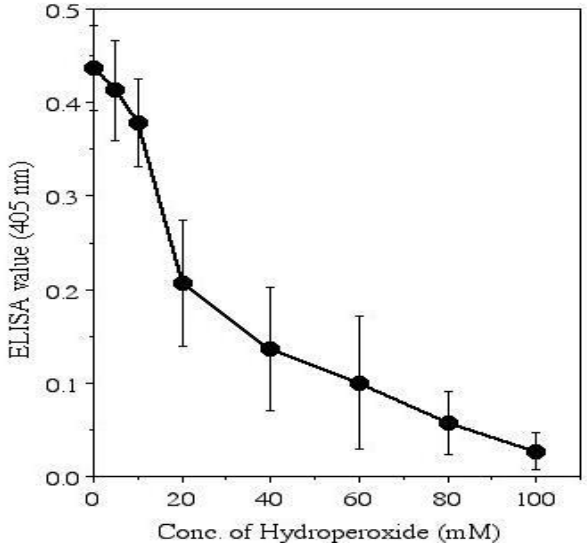

Figure 2. Evaluation by ELISA of human-specific IgE binding to dough with various concentrations of hydroperoxide. Values are the means of three replicates ± standard deviation.

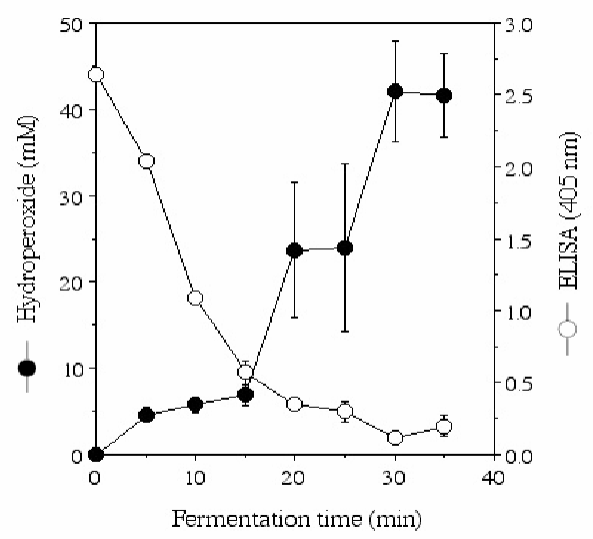

Figure 3. Relationship between the concentration of hydroperoxide and time-course of the binding activity of human-specific IgE with dough during fermentation process. Values are the means of three replicates ± standard deviation.

RESULTS AND DISCUSSION

Effects of hydroperoxide on IgE antibody

Figure 1 shows the measurement results obtained using a single radial immunodiffusion method for IgE antibody in a solution from which crude proteins in dough were extracted using 50 mM phosphate buffer (pH 7.4) . Crude protein solution extracted from dough without hydroperoxide showed a concentration dependent antigen-antibody reaction (diameter of precipitin ring: 12 mm) against IgE in the single-radial immunodiffusion method. In contrast, crude protein solution extracted from dough added hydroperoxide decreased precipitin ring. The diameter of the precipitin ring of crude protein extracted from dough having 60 mM hydroperoxide was 2.5 mm. The diameter of the precipitin ring of dough having a higher concentration of hydroperoxide decreased drastically (Figure 1).

Figure 2 shows the results of confirmation using an enzyme-linked immunosorbent assay (ELISA). The reaction of dough extracts of a higher concentration of hydroperoxide against IgE got weaker. Crude protein extracted from dough having more than 20 mM hydroperoxide decreased antigen-antibody reaction against IgE drastically. It was concentration-dependent (Figure 2) and these results consisted with the results in Figure 1.

These facts strongly suggested that the addition of hydroperoxide affected antigen-antibody reaction of dough crude protein.

Relationship between hydroperoxide and antigenic activity

Investigation of changes in peroxides produced by lipid peroxidation reactions in the process of dough fermentat-ion, in addition to associated changes in antigenic activity, revealed decreases in antigenic activity with progress of fermentation (Figure 3).This finding suggests that hydroperoxides produced during fermentation were highly likely to have caused denaturation of allergen proteins, which led to inhibition or loss of antigenic activity.

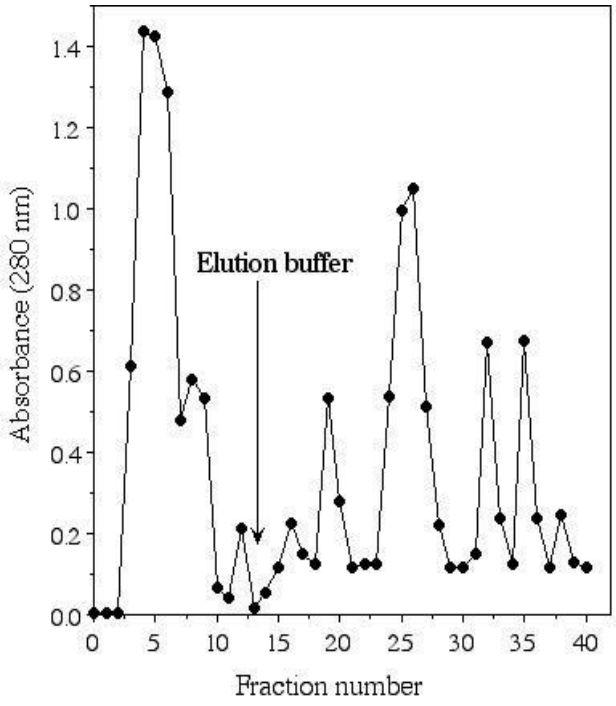

Figure 4. Elution profiles of proteins extracted from dough by affinity chromatography using immobilized-trypsin chitin.

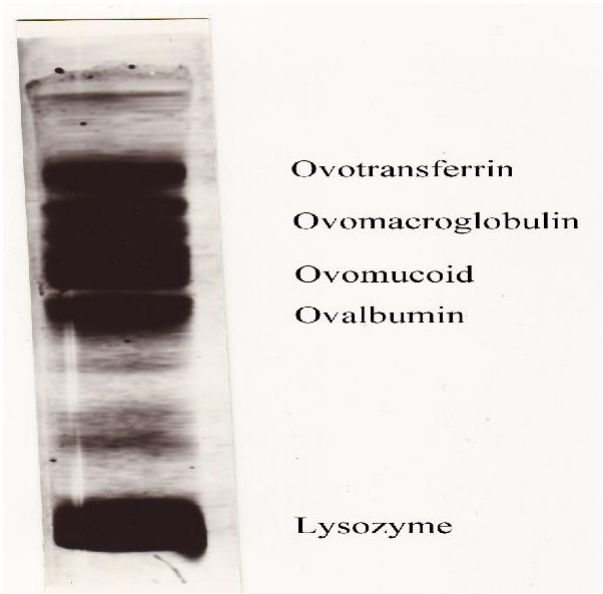

Figure 5. SDS-PAGE of 14-40 fractions numbers from the affinity chromatography studies.

Isolation and purification of allergen proteins using affinity chromatography

As shown in Figure 4, after the extraction of crude proteins from dough, proteins with antigenic activity were isolated and purified using affinity chromatography.

Figure 5 shows the results of sodium dodecyl sulfate polyacrylamide gel electrophoresis for fractions (fraction numbers 14 - 40) collected using elution buffer. Eluted proteins primarily included ovomucoid, ovalbumin, ovotransferrin, ovomacroglobulin, and lysozyme. Measurements of antigenic activity for these protein fractions confirmed a particularly high antigenic activity for ovomucoid (Figure 6).

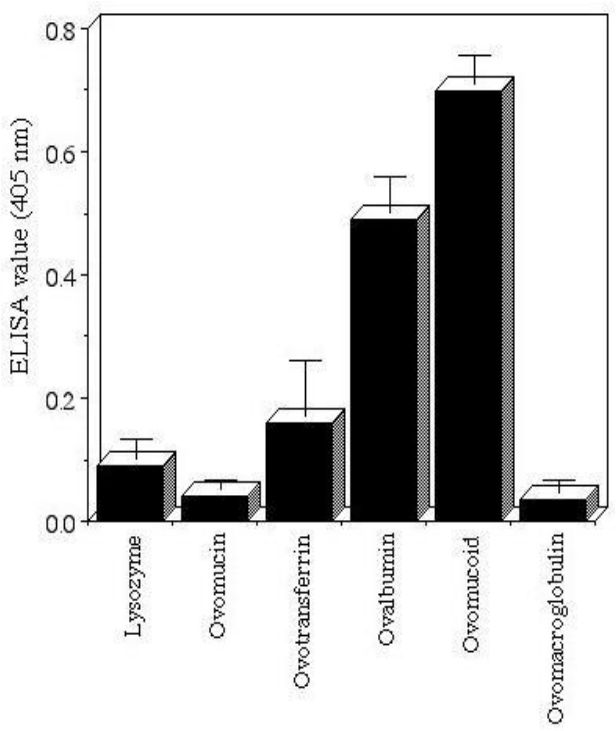

Figure 6. Comparison of the binding activity of human-specific IgE to proteins from dough with hydroperoxide. Values are the means of three replicates ± standard deviation.

Allergens are primarily thought to be proteins and gluten of origin from wheat flour or additional ingredients such as hen eggs in the bread-making process. It has already been reported that ovomucoid in the hen egg or in gluten of origin from wheat flour causes the Allergy (Mine and Zhang, 2001; Buck and Helfe, 1995; Fuchs and Astwood, 1996). Therefore, ovomucoid, which is present in hen eggs or wheat flour was may be identified as the main allergen. Peroxides produced during dough fermentation were thought to cause denaturation of the conformation of ovomucoid and thereby prevent the induction of antigen-antibody reactions with IgE.

Antigen-antibody reactions of baked bread

Bread using the dough with 100 mM hydroperoxide or without hydroperoxide was actually made. Antigen-antibody reactions of crude proteins extracted from both bread samples were studied. Bread in which lipid peroxidation reactions were induced had a clearly lower antigenic activity than bread in which these reactions were

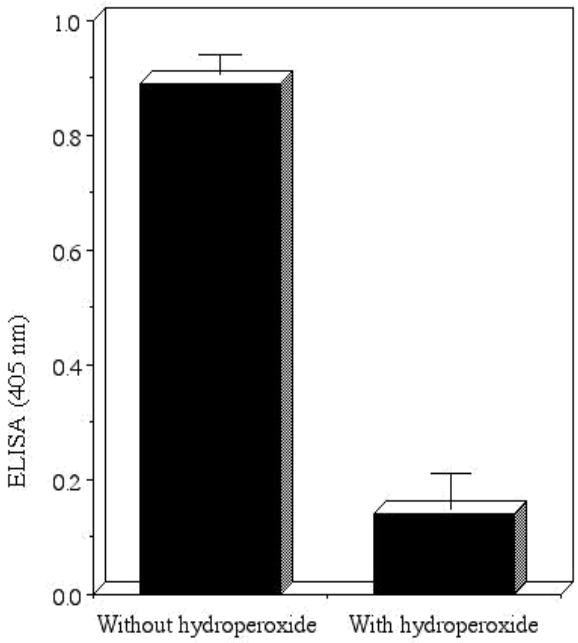

Figure 7. Comparison of the binding activity of human-specific IgE to crude proteins from baked bread made from dough with 100 mM hydroperoxide and baked bread made from dough without hydroperoxide. Values are the means of three replicates ± standard deviation.

were not induced (Figure 7).

These findings indicate that induction of lipid peroxidation reactions during dough fermentation results in production of hydroperoxides that cause changes in the conformation of allergen proteins (ovomucoid in the present study) and consequently prevent induction of antigen-antibody reactions.

The mechanism by which hydroperoxides acts promote fermentation

The various experimental results that were obtained were comprehensively analyzed to determine the mechanism of action by which hydroperoxide acts to promote fermentation. During gluten formation or additional ingredients such as hen eggs, gluten is formed when gliagin and glutenin, and ovomucoid form a network structure. When is allergen protein denaturation in the presence of hydroperoxide, the molecules themselves from macromolecules. As a result, antigen-antibody reaction involving IgE was become unresponsive.

Conclusions

The current research demonstrated that the effects of lipid peroxides produced during dough fermentation on allergic reactions. The mechanism of inhibition on allergic reaction of crude protein (ovomucoid in the present study) by hydroperoxide induction is discussed in detail. Crude protein (ovomucoid in the study) was denatured by hydroperoxides, the structure of ovomucoid was destroyed which consequently prevent induction of antigen-antibody reactions.

This phenomenon is advantageous when baking bread and can be used to enhance the quality of baked bread. Based on the results of these tests of chemical properties, further detailed study is needed of the effect of lipid peroxides on the flavor of baked bread.

ACKNOWLEDGMENTS

This work was supported by a The Elizabeth Arnold Fuji Foundation to which we are grateful.

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