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Matsumoto, Kiyoshi
Laboratory of Food Quality Control, Faculty of Agriculture, Kyushu University

Yoshimaru, Tetsuro
Laboratory of Food Quality Control, Faculty of Agriculture, Kyushu University

Matsui, Toshiro
Laboratory of Food Analysis, Faculty of Agriculture, Kyushu University

Osajima, Yutaka
Laboratory of Food Analysis, Faculty of Agriculture, Kyushu University

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Enzymatic Degradation of Ovalbumin by Various Proteases

Kiyoshi Matsumoto, Tetsuro Yoshimaru, Toshiro Matsui* and Yutaka Osajima*

Laboratory of Food Quality Control, Faculty of Agriculture,
Kyushu University, Fukuoka 812-81, Japan
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An investigation was made of the enzymatic hydrolysis of ovalbumin (OVA), a major allergen in egg white, by various acid and alkaline proteases. Protease YP-SS (acid protease) from *Aspergillus* niger and alcalase (alkaline protease) from *Bacillus licheniformis* were found to be useful for the degradation of OVA, respectively. OVA was almost totally hydrolyzed within 15 hr at 37°C by alcalase. Alcalase acted rapidly to hydrolyze OVA, with about 90% of OVA being hydrolyzed within 30 min., the reaction then proceeded more slowly. The antigenicity of the OVA hydrolysate was investigated with IgG raised in rats against OVA and it was apparent that the antigenicity had been almost completely destroyed.

INTRODUCTION

For allergenic patients, the choice of processed foods that are free from allergens is important for preventing a specific immunological response. Therefore the elimination of allergens from processed food before intake are necessary. From this viewpoint, Watanabe et ***al.*** (1990) produced a hypoallergenic rice as an allergen-free food, and they characterized the quality of the color and mechanical strength. Izumi et ***al.*** (1992) determined the amino acid sequence of the major allergenic protein of rice, and they have attempted to modify this protein at the gene level. It is, however, difficult to apply them directly as a "physiologically functional food", because of some disadvantages relating to the native physical properties (e.g. flexibility or texture).

In this study, ovalbumin (OVA) from turkey egg was hydrolyzed by using pepsin, trypsin, chymotrypsin and other 1'7 kinds of food processing proteolytic enzymes for depression of allergenic activity. Further it would seem that addition of temporary inactivation forms (e.g. microcapsulation of these enzyme) in food systems may have degradated allergens selectively in the gastrointestinal tract without damaging the nutritional and functional benefits of native foods, and we are investigating in favor of the practical use.

In this paper, the authors have examined proteolytic enzyme that would digest OVA, which is a major allergens in egg white. The depression of allergenic activity in *vivo* by the enzyme will be reported subsequently.

* Laboratory of Food Analysis, Faculty of Agriculture, Kyushu University 46-09, Fukuoka 812-81, Japan.

MATERIALS AND METHODS

Materials

Ovalbumin (OVA, from turkey eggs) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Seventeen of proteolytic enzymes were those used commercially in the food industry and three others (pepsin, chymotrypsin and trypsin) were degradative enzymes used in the laboratory. All the enzymes are listed in Table 1, together with the origin, optimal pH and specific activity of each enzyme preparation (data produced by the manufacturers). Pepsin, chymotrypsin and trypsin were obtained from Boehringer Mannhaim Yamanouchi Co. (Tokyo, Japan).

Table 1. List and Properties of Enzyme Used.

Enzyme	Optimum pH	Origin	Specific activity
Flavourzyme	5.0-7.0	*Aspergillus oryzae*	20-50 LAPU/g*
Denapsin 2P	3.0	*Aspergillus niger*	20,000 APUN/g*
Protease YF-SS	2.5-3.0	*Aspergillus niger*	100,000 u/g
Newlase F	3.0	*Rhyzopus niveus*	7,000 u/g
Protease M	3.0	*Aspergillus oryzae*	5,500 U/g
Molsin F	2.0-3.0	*Aspergillus Saitoi*	Not clarified
Orientase 20A	2.5	*Aspergillus niger*	200,000 u/g
Pepsin	2.0	Porcine gastric mucosa	2,500 U/mg
Denazyme AP	7.5	*Aspergillus oryzae*	110 U/mg
Orientase ONS	7.0	*Aspergillus oryzae*	100,000 U/g
Bioprase SP-4	7.0	Bacteria	20,000 pun/g*
Tasinase N-11-100	7.4	Not clarified	100,000 U/mg
Papain W-40	8.0	*Carica papaya*	400,000 U/mg*
Protease A	7.0	*Aspergillus oryzae*	10,000 u/g*
Actinase	9.0	*Streptomyces griseus*	250,000 U/g
Pantitase NP-2	7.0	*Aspergillus oryzae*	40,000 U/g
Aroase AI-10	7.0	*Bacillus subtilis*	100,000 u/g
Alcalase	8.3	*Bacillus licheniformis*	2.4 AU/g*
Chymotrypsin	8.0	Bovine pancreas	40,000～60,000 U/g
Trypsin	8.0	Bovine pancreas	10,000～13,000 u/g

*manufacturer's method

Hydrolysis of OVA by various enzymes

OVA was hydrolyzed by seventeen enzymes used in the food industry and by three, namely pepsin, chymotrypsin and trypsin, that are used in the laboratory. Each enzyme was added at 1% (w/w) with each substrate because each preparation of enzyme had different specific activity. For evaluation of hydrolysis of OVA by various enzymes, 50 μl of each solution of enzyme (1 mg/ml) was added to 0.5 ml of an OVA solution (10 mg/ml),

dissolved in 0.1 M citrate buffer (pH 3.0) or in 0.1 M phosphate buffer (pH 7.5) for hydrolysis by acid or alkaline proteases, respectively. Each mixture was incubated at 37 °C for 15 hr. After hydrolysis of OVA, the hydrolysis reaction was stopped by heating at 95°C for 10min or by addition of 0.5 ml of 0.1 M HCl in case of hydrolysis by acid or alkaline proteases, respectively.

The molecular sizes of fragments in hydrolysates of OVA

The molecular sizes of fragments in hydrolysates of OVA were estimated by gel-permeation chromatography (GPC, model LC-9A; Shimadzu Co., Kyoto, Japan) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; mini-PROTEIN II; Japan Bio-Rad Co., Tokyo, Japan). For the estimation of the degradation efficiency of OVA by GPC, the hydrolysates were applied to an Asahipak GS-320 column (7.6 mm i.d. x **50** cm; Asahikasei Co., Tokyo, Japan). The elution was performed with 50 mM ammonium acetate (pH 6.7) at a flow rate of 0.5 ml/min, with monitoring the absorbance at 280 nm. The molecular masses of the fragments in hydrolysates were estimated by referece to retention times of myoglobin (17 kDa), bradykinin (1 kDa) and Gly-L-Tyr (0.3 kDa) as standards. SDS-PAGE of OVA and of hydrolysate of OVA was performed on 16% polyacrylamide gels by the method of Laemmli (1970) and the bands were stained with amide black 10 B.

Allergenic activity of the hydrolysates of OVA

The allergenic activity of OVA and of an OVA hydrolysate produced by enzyme were tested with a direct enzyme-linked immunosorbent assay (ELISA) and the results were calculated from the absorbance at 405 nm, by reference to a calibration curve for a standard antigen-antibody reaction: purified rat IgG and goat anti-rat IgG $F(ab')_2$ (CAPPEL, West Chester, PA, U.S.A.). OVA-specific rat IgG was kindly donated by Dr. K. Yamada of Kyushu University (Yamada et *al.*, 1994). In brief, male Brown Norway (BN)/Sea [SPF] rats (4 weeks old) were intraperitoneally immunized 3 or 4 times with OVA dissolved in **3%** $Al(OH)_3$-0.15 M NaCl at intervals of one week. After 4 weeks, rats were anesthetized with ether and blood was collected from aorta. Then, serum was prepared by centrifuging the boold at 3000 x g for 15min. Peroxidase-conjugated affinity-purified $F(ab')_2$ fragments of goat antibodies raised against rat IgG (CAPPEL) were used as the labelled antibody. The substrate solution for the ELISA was a 10:9:1 mixture (v/v) of 0.006% H_2O_2 dissolved in 0.2 M citrate buffer (pH 4.0); H_2O; and a solution of 6 mg/ml 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid, diammonium salt) (ABTS; Wako Pure Chemicals Ltd., Osaka, Japan).

RESULTS AND DISCUSSION

Hydrolysis of OVA by vaious enzymes

The degradation of OVA by 20 proteolytic enzymes was examined. As shown in Fig. 1, OVA was barely hydrolyzed by gastrointestinal proteases (pepsin, chymotrypsin and trypsin). In general, allergens have a very tight tertiary structure and, thus, they are resistant to hydrolysis by gastrointestinal proteases. These results confirm that OVA has

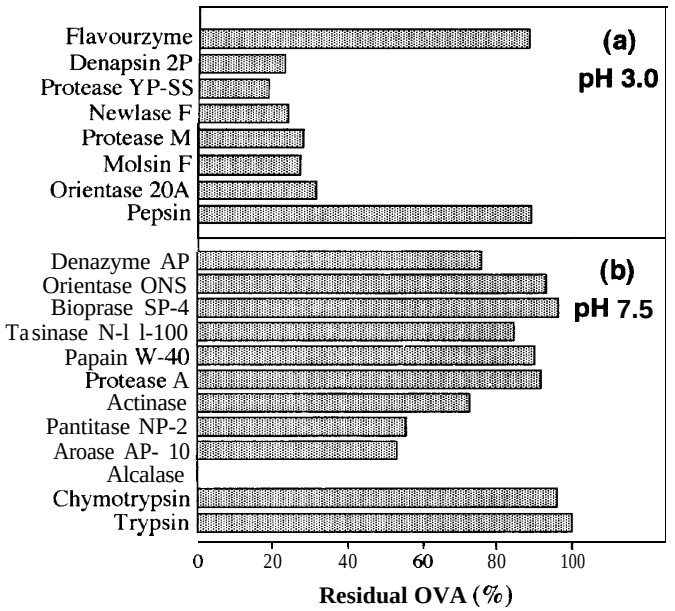

Fig. 1. Effectiveness of various proteases in the hydrolysis of ovalbumin (OVA).

a tightly folded tertiary structure.

To facilitate degradation of OVA in the gastric tract, we investigate the effects of eight acid proteases. Figure l(a) shows the residual OVA after hydrolysis for 15hr. As shown in Fig. 1 (a), 81% of OVA was hydrolyzed by protease YP-SS (from *Aspergillus niger*). This relatively strong hydrolytic activity of protease YP-SS suggested that it might be useful for the degradation of the allergen (OVA) to a certain extent only in the gastric tract. However, it is inappropriate to refer only to results of a 15-hr hydrolysis because the residence time of proteins in the gastric tract has been estimated to be 3 to 6hr(Muto, 1990). Therefore, it is necessary to investigate the rate of degradation and the allergenic activity of residual OVA, taking into account the residence time in the gastric tract.

For degradation of OVA in the upper-intestinal tract, we investigated 12 digestive enzymes at pH 7.5. As shown in Fig. 1 (b), more than 99% of OVA was hydrolyzed within 15 hr by alcalase (from *Bacillus licheniformis*, 2.4 L, Type FG, Novo Co., Tokyo, Japan). The apparent distribution of molecular masses in the hydrolysate was estimated by GPC (Fig. 2). As shown in Fig. 2, 95% of the hydrolysate of OVA eluted in the range of molecular masses below 10 kDa. This result suggests that marked hydrolysis of OVA to small peptides had occurred. Moreover, fragments of about 41% of the hydrolysate proceeded to peptides less than 1 kDa by the action of alcalase. Thus, OVA had been degraded via multiple degradation steps during digestion by alcalase, which has broad

substrate-specificity and acts as an endo-type enzyme. Almost the same result was also observed after SDS-PAGE (Fig. 3). Accordingly, we concluded that the alcalase used in this study had the ability to degrade one hundred times its weight of OVA under the conditions described.

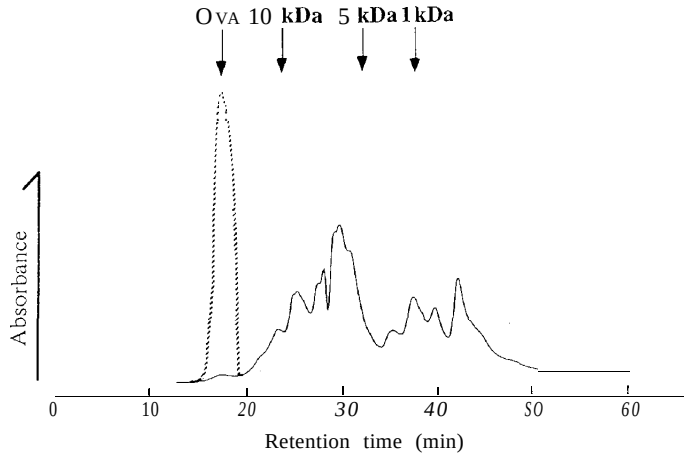

Fig. 2. Gel-permeation chromatogram of a hydrolysate of OVA generated by treatment with alcalase for 15 hr. Column, Ashahipak GS-320 (7.6 mm i.d. X 50 cm); eluent, 50mM ammonium acetate (pH 6.7); flow rate, 0.5 ml/min; monitoring, 280 nm.

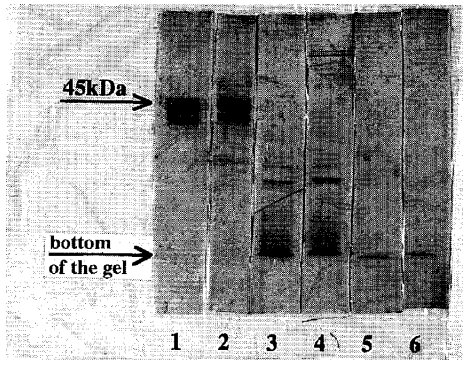

Fig. 3. Results of SDS-PAGE of OVA, a hydrolysate of OVA generated by treatment with alcalase for 15 hr, and alcalase. Lane 1,2: OVA; lane 3,4: OVA hydrolysate by alcalase; lane 5,6: alcalase.

Rate of hydrolysis of OVA by protease YP-SS and alcalase

As mentioned above, it became clear that protease YP-SS and alcalase were the most effective enzyme for the hydrolysis of OVA in acid and slightly alkaline region, respectively. By considering the residence time in the gastric and/or intestinal tract (a few hours), the rates of degradation of OVA by protease YP-SS and alcalase were investigated. Figure 4 shows the time courses of the hydrolysis of OVA by protease YP-SS and alcalase. As shown in Fig. 4, protease YP-SS hydrolyzed'about 75% of OVA in 2 hr, while alcalase hydrolyzed about 90% of OVA in 30 min, and then the reaction proceeded only slowly. Thus, alcalase seems to have the potential to digest OVA within its residence time in the intestinal tract. Furthermore, β-lactoglobulin (β-LG) was hydrolyzed completely within 30min by alcalase, in spite of its generally high resistance to digestion that is due to intramolecular cross-links (data not shown). In addition alcalase degrades relatively rigid proteins such as actin (Sugiyama et al., 1991), and Matsui et al. (1993) reported that the hydrolysate by alcalase had little unpleasant taste as well as high solubility in water. Thus alcalase seems to be useful and acceptable for the degradation of allergens, such as OVA, β-LG and so on.

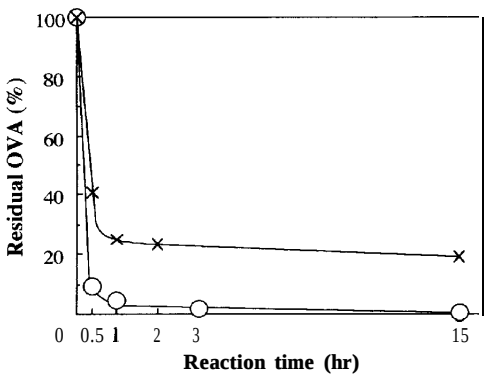

Fig. 4. Time course of the hydrolysis of OVA (0.5 ml, 10 mg/ml) by alcalase (0.05 ml, 1 mg/ml) and protease YP-SS (0.05 ml, 1 mg/ml) at 37°C. Alcalase **(0);** protease YP-ss (×).

Antigenicity of a hydrolysate of OVA by alcalase

The antigenicity of hydrolysate of OVA by alcalase was estimated from binding to IgG from rats immunized with OVA. The amount of IgG that bound OVA (50 μg/ml, 150 μl) was 4.65~ 10^4 ng/ml. A significant decrease (more than 10^3-fold) was observed for the amount of the same antibody that bound to the hydrolysate of the same amount of OVA (25.6 ng/ml) or to the alcalase used to digest OVA (19.1 ng/ml), suggesting that the antigenicity had been eliminated almost completely. Hence, the degradation of OVA by alcalase might eliminate the cause of an allergic reaction.

We are now attempting to prepare microcapsulated alcalase for attaining the degradation of allergen *in vivo* with respect to the practical use as physiologically functional materials.

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