# Preparation of Microcapsulated Enzymes for Lowering the Allergenic Activity of Foods

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Alcalase from *Bacillus licheniformis* was microcapsulated by the spray-dry method for degradation of ovalbumin (OVA) in the small intestine. Capsules were prepared with yield of about 65% and a mean particle diameter of 10−20 $\mu$m. The microcapsulated alcalase was very resistant to digestion in the gastric environment (pH 1.2), and the efficiency of release was about 100% within 10 min in intestinal fluids (pH 6.8). Moreover, when microcapsules were incubated with a solution of OVA, >99% of OVA was hydrolyzed within 120 min. In addition, microcapsules of protease YP-SS from *Aspergillus niger* were also prepared for degradation of OVA in the stomach. Almost all protease YP-SS activity was released from capsules within only 10 min in gastric fluids. Oral administration of the microcapsulated enzyme to Sprague-Dawley rats resulted in a significant decrease in the serum level of OVA-specific IgE.

**Keywords:** *Microcapsulated enzymes; degradation of OVA; Eudragit; spray-dry method; oral administration test*

## INTRODUCTION

Food allergies have become a serious health problem not only for children but also for adults. For allergic patients, avoidance of the intake of foods that are responsible for allergies is recommended as a conventional therapy. It is, therefore, necessary to ensure removal of the corresponding allergens from foods before they are eaten. Watanabe et al. (1990) developed a hypoallergenic rice using enzymatic degradation to generate an allergen-free food, and they characterized the product in terms of color and mechanical strength. Izumi et al. (1992) determined the amino acid sequence of the major allergenic protein of rice, and they attempted to modify the protein by genetic manipulation. However, no significant improvements have been made with regard to the properties of foods, such as their processing characteristics.

Our interest has been focused on the identification and microcapsulation of proteolytic enzymes that can degrade ovalbumin (OVA) as the major allergen in egg white. In a previous study (Matsumoto et al., 1997), we found that alcalase from *Bacillus licheniformis* and protease YP-SS from *Aspergillus niger* were useful for degrading OVA in the upper intestinal tract and the stomach, respectively, and they catalyzed the marked and rapid proteolysis of OVA to smaller peptides.

There are many reports of potential applications of microcapsules. For example, sustained-release preparations of microcapsulated drugs, which reduce the required frequency of administration, are more convenient for patients and might, thus, improve compliance (Jeong et al., 1985; Okahata et al., 1986). Microcapsules for controlled release of drug have become increasingly important in the development of methods for targeting drugs to particular body sites or organs (Kato et al., 1981; Yoshioka et al., 1982). However, despite many potential uses of microcapsules in medicine, they have not been exploited in the food industry to any major extent because of limited availability of coating agents or solvents and high cost. However, it would seem to be desirable to help allergic patients to digest allergens *in vivo* after the intake of untreated food. Therefore, we tried to prepare microcapsulated enzymes, which can degrade allergens in the small intestine and/or the stomach after intake, to prevent the intestinal absorption of intact allergens.

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## EXPERIMENTAL PROCEDURES

**Materials.** OVA (from turkey egg, grade V) was purchased from Sigma Chemical Co. (St.Louis, MO). Porous starch was kindly supplied by San-ei Sucrochemical Co., Ltd. (Aichi, Japan). Eudragit L100 and Eudragit E100, which are synthetic acrylic copolymers, were kindly supplied by Röhm Pharma GmbH (Darmstadt, Germany) and were used as coating agents. These coating agents are readily dissolved under alkaline conditions and acidic ones, respectively. Alcalase from *B. licheniformis* (2.4 L, type FG) was obtained from Novo Co. (Chiba, Japan) and protease YP-SS from *A. niger* from Yakult Medical Co. (Tokyo, Japan). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and were of analytical reagent grade.

**Preparation of Microcapsules.** With the goal of preparing microcapsulated enzymes that can degrade OVA in the small intestine, we attempted the microcapsulation of alcalase from *B. licheniformis* by the spray-dry method because of its convenience and high degree of reproducibility. The procedure for preparation of the microcapsules was as follows. Fifteen grams of porous starch with a number of holes in each particle of a few micrometers in diameter was added to 45 mL of alcalase (2.4 Anson units), and the mixture was stirred for 2 h. The solution was then sonicated for 5 min to integrate the alcalase into the porous starch, which was then freeze-dried and coated with Eudragit L100. The freeze-dried material was combined with 600 mL of a 5% solution (w/v) of Eudragit L100 in ethanol, as an enteric-coating copolymer that is soluble at pH 6.0 and above (Morishita et al., 1992). The microcapsulated alcalase was prepared using a CL-8 spray-dryer (Ohgawara-Kakouki Co., Tokyo, Japan) equipped with a rotary atomizer nozzle, a nozzle speed of 10 000 rpm, and inlet and outlet air temperatures of 105 and 62−79 °C, respectively.

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Similarly, for degradation of OVA in the stomach, protease YP-SS from *A. niger* was microcapsulated according to the same procedure but with Eudragit E100 as the polymer that would dissolve in gastric juices.

**Assay of Enzymatic Activity.** Enzymatic activities were determined by a spectrophotometric method (Anson, 1938), by reference to a calibration curve for standard tyrosine solutions (20−80 μg/2 mL of 0.2 M HCl). For each assay, 1 mL of enzyme solution (suitably diluted so that the absorbance is revealed in the range of a calibration curve) was incubated with 5 mL of a 0.6% (w/v) solution of Hammersten casein (Wako Pure Chemicals Ltd., Osaka, Japan), which dissolved in a 0.75% solution of lactic acid (pH 3.0) or in a 0.05 M solution of $Na_2HPO_4$ (pH 7.5) in the case of assay for protease YP-SS activity or for alcalase activity, respectively, as substrate at 37 °C for 10 min. The reaction was then stopped by addition of 5 mL of a 0.11 M solution of trichloroacetic acid solution. After incubation at 37 °C for 30 min, the solution was filtered with filter paper (No. 5B, Advantec Toyo Co., Tokyo, Japan). Five milliliters of a 0.55 M solution of $Na_2CO_3$ and 1 mL of Folin−Ciocalteu reagent (Nacalai Tesque Co.) were then added to 2 mL of the filtrate, and the absorbance was measured at 660 nm with a spectrophotometer (UV-1200, Shimadzu Co., Kyoto, Japan). One unit was defined as the enzymatic activity that, in 1 min under the present assay conditions, liberated Folin-positive amino acids and peptides equivalent to 1 μmol of tyrosine.

**Efficiency of Encapsulation of Each Enzyme into Microcapsules.** Before measurements, 50 mg of microcapsulated enzyme was ground by a mortar and a pestle in 1 mL of 0.1 M phosphate buffer (pH 7.5) to dissolve the enteric-coating agent Eudragit L100 or 1 mL of 0.1 M citrate buffer (pH 3.0) to dissolve Eudragit E100. A suitable dilution of resultant solution was assayed for enzymatic activity as described above. The encapsulation efficiency was calculated by expressing the amount of enzyme encapsulated as a percentage of the initial amount of enzyme used for preparing a microcapsulated enzyme.

**Stability and Release of Microcapsulated Enzyme.** The stability of microcapsulated alcalase in gastric fluids was investigated. A 100-mg aliquot of the microcapsulated alcalase was incubated at 37 °C with reciprocal shaking (100 strokes/min) in 15 mL of the first fluid (a solution of HCl containing 0.2% NaCl; pH 1.2) as described in the Japanese Pharmacopoeia (JP XII). The microcapsules were then collected by filtration and washed with distilled water. The collected microcapsules were dissolved completely in phosphate buffer (pH 7.5) and assayed for alcalase activity that was retained after the treatment with acidic solution. The resistance of alcalase was expressed as the amount of alcalase activity retained as a percentage of the initial alcalase activity.

The release of alcalase from the microcapsules was evaluated as follows. A 100-mg aliquot of the microcapsulated alcalase was incubated at 37 °C with reciprocal shaking (100 strokes/min) in 15 mL of the second fluid (phosphate buffer; pH 6.8) described in JP XII. At appropriate times, the solution was filtered and alcalase activity in the filtrate was assayed.

Similarly, the release of protease YP-SS from microcapsules was examined with the first fluid of JP XII as solvent, and proteolytic activity in the filtrate was assayed.

**Hydrolysis of OVA by the Enzyme Released from Microcapsules.** For evaluation of the hydrolysis of OVA by protease released from the microcapsules, we added 50 mg of microcapsulated enzyme to 0.5 mL of a solution of OVA (10 mg/mL) and incubated the mixture for various periods of time (10, 30, 60, and 120 min) at 37 °C. The reaction was stopped by heating at 95 °C for 10 min or by addition of 0.5 mL of 0.1 M HCl in the case of hydrolysis by protease YP-SS or by alcalase, respectively. The hydrolysate was then subjected to gel permeation chromatography analysis on an Asahipak GS-320 column (7.6 mm i.d. × 500 mm; Asahikasei Co., Tokyo, Japan) to evaluate the extent of degradation of OVA. Elution was performed with 50 mM ammonium acetate (pH 6.7) at a flow rate of 0.5 mL/min, with monitoring of absorbance at 280 nm.

**Oral Administration Protocols and ELISA.** The oral administration test was performed using male 4-week-old Sprague-Dawley (SD) rats. Rats were housed in five groups of four animals each as follows: groups fed OVA or casein; and groups fed OVA and microcapsulated protease YP-SS, OVA and microcapsulated alcalase, and OVA and both microcapsulated protease YP-SS and microcapsulated alcalase. Each rat was given a diet that was based on the American Institute of Nutrition Rodent Diets (Reeves et al., 1993) and water *ad libitum*. Thus, OVA [20% (w/w) of the diet] was used as protein in the diet instead of casein. Furthermore, the diet was supplemented with 11 mg of 145 900 units/g of microcapsulated protease YP-SS or 45 mg of 25 800 units/g of microcapsulated alcalase per 1.0 g of OVA, respectively. Blood samples were collected from tail veins at weekly intervals and, finally, rats were anesthetized with ether and blood was collected from the aorta. Serum was prepared by centrifuging the blood at 3000*g* for 15 min. Serum was tested in a direct ELISA according to the method of Yamada et al. (1994). That is, to measure OVA-specific IgE, 150 μL of OVA solution (50 μg/mL) dissolved in the 50 mM carbonate buffer (pH 9.8) was added to each well of a 96-well ELISA plate (Maxisorp; Nunc Co., Roskilde, Denmark) and treated for 1 h at 37 °C. After blocking with 300 μL of the blocking solution [0.1% fish gelatin dissolved in TPBS (0.05% Tween 20 dissolved in a phosphate buffered saline, pH 7.4)] for 1 h at 37 °C, each well was reacted with 100 μL of rat serum diluted with the blocking solution for 1 h at 37 °C. Then, bound IgE was detected by reaction with biotin-conjugated mouse anti-rat IgE (1000× diluted) and then with POD-conjugated avidin (5000× diluted). Wells were rinsed three times with TPBS between each step. After incubation at 37 °C for 15 min with 100 μL of substrate solution, the reaction was stopped by adding 100 μL of 1.5% oxalic acid, and results were expressed as absorbance at 415 nm for each serum sample. The substrate solution 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 of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, diammonium salt).

**Statistical analysis** was performed using one-way analysis of variance ($p < 0.05$ or $p < 0.1$), followed by Student's *t*-test for examination of the significance of differences.

## RESULTS AND DISCUSSION

**Efficiency of Encapsulation and Morphology of Microcapsules.** With the goal of preparing microcapsulated enzyme that can degrade OVA selectively in the small intestine and/or the stomach, we used porous starch as the core material in this study for convenience and for simplicity of microcapsulation. The porous starch was produced by enzymatic degradation of corn starch, and Suzuki (1995) reported that microcapsulation using porous starch allows preparation of microcapsules with sustained release and stabilization of docosahexaenoic acid.

First, we investigated the efficiency of encapsulation of alcalase into the microcapsules. We found that microcapsulated alcalase was generated with a yield of about 65%. From the efficiency (about 72%) of integration of alcalase into porous starch, we assumed that coating with Eudragit L100 by the spray-dry method was very efficient, without loss of alcalase activity. Furthermore, the activity of integrated alcalase was hardly affected by the ethanol used as the solvent for Eudragit L100. In the case of microcapsulation of protease YP-SS, we obtained a similar result (yield of about 70%). Thus, the microcapsulation method (spray-dry method) used in this study seemed to be effective and applicable to the food industry because of both its convenience and safety.

Typical scanning electron micrographs (SEM) of porous starch and microcapsulated alcalase are shown in Figure 1. The prepared capsule had a mean particle

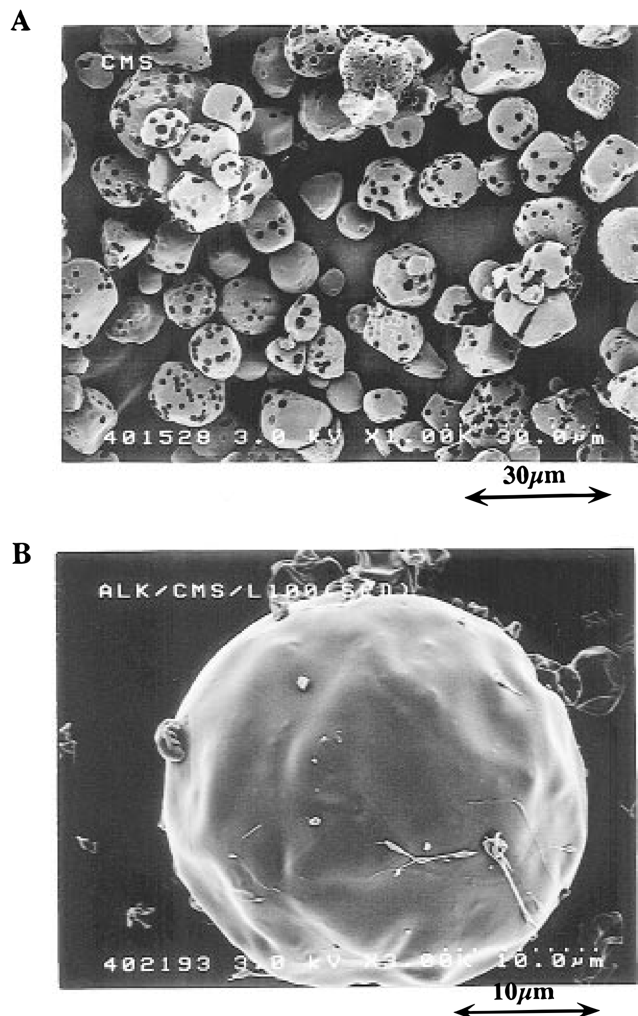


**Figure 1.** SEM of porous starch (A) and microcapsulated alcalase prepared by the spray-dry method (B).

diameter of 10−20 μm; the microcapsules were spherical, and their surfaces were almost smooth. The microcapsules containing protease YP-SS had a structure similar to that shown in Figure 1B (data not shown). These results suggest that the microcapsules prepared by the spray-dry method were fine particles and homogeneous and that the quality (in particular the texture) of original foods would not be influenced by the addition of such microcapsulated enzymes.

**Evaluation of a Microcapsulated Enzyme for Degradation of OVA in the Intestinal Tract or Stomach.** To investigate the resistance of microcapsulated alcalase to degradation in the gastric environment, microcapsulated alcalase was treated with a gastric solution (pH 1.2, HCl solution containing 0.2% NaCl). As shown in Figure 2, we observed the high stability of microcapsulated alcalase in the gastric solution. That is, even though the alcalase activity retained in the microcapsules was slightly reduced during a 2-h incubation, the activity was barely affected during a 1-h treatment. This result indicates that the enzyme should not be susceptible to any damage in the gastric environment and that it should pass through the stomach without loss of activity.

We next examined the release of alcalase from the microcapsules in intestinal fluid (pH 6.8, phosphate buffer). Almost all encapsulated alcalase was released within only 10 min in intestinal fluid (Figure 3). Thus, alcalase released from microcapsules seemed to have the

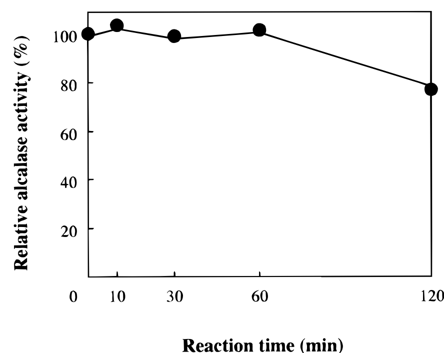


**Figure 2.** Stability of microcapsulated alcalase in artificial gastric juice (pH 1.2) at 37 °C.

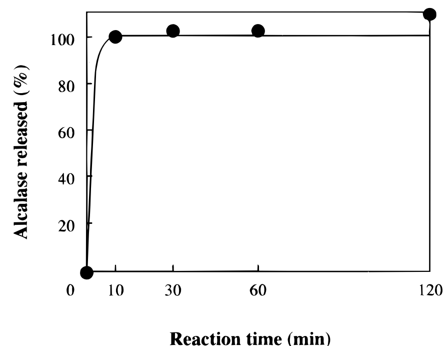


**Figure 3.** Release of alcalase from microcapsules under conditions that mimic the intestinal tract (37 °C and pH 6.8).

potential to digest OVA within its residence time in the intestinal tract (a few hours) (Muto, 1990). Furthermore, it has already been reported that alcalase can hydrolyze rigid proteins such as β-lactoglobulin (Matsumoto et al., 1997) (a major allergen in milk) and actin (Sugiyama et al., 1991), and Matsui et al. (1993) reported that a hydrolysate generated by alcalase from sardine muscle had little taste, as well as high solubility in water. It appeared, therefore, that the degradation of OVA could be done in the intestinal tract by use of the microcapsulated alcalase.

In addition, for degradation of OVA in the stomach, we also prepared microcapsulated protease YP-SS. The capsules had excellent release efficiency, with approximately 100% within 10 min in gastric fluids; this behavior was the same as that of microcapsulated alcalase. This result was probably due to the ready solubility of Eudragit L100 and Eudragit E100, which were used as coating agents. Eudragit L100 and Eudragit E100 are synthetic acrylic copolymers that have recently been used as additives to medicines and have been employed as coating agents for controlled-release capsules (Kawata et al., 1986). Our results suggest that the degradation of OVA might be achieved in the stomach, as well as in the small intestine, by simultaneous usage of microcapsulated protease YP-SS and microcapsulated alcalase.

We investigated the long-term stability of the microcapsulated enzymes in 0.1 M phosphate buffer (pH 5.5), without agitation, to mimic normal food storage conditions. Little degradation of protease YP-SS or of alcalase was observed for up to 6 weeks. We have also examined the heat resistance of these microcapsulated enzymes at 100 °C. As a result, little inactivation of protease YP-SS and of alcalase capsulated was observed for up to 60 and 90 min, respectively. This is an important consideration for food systems and, thus,

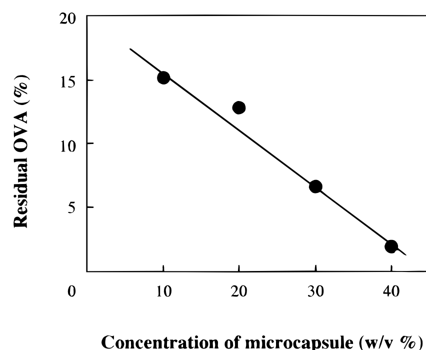


**Figure 4.** Effects of the concentration of microcapsulated alcalase on the degradation OVA. OVA was hydrolyzed by alcalase released from microcapsules for 30 min at 37 °C and pH 6.8.

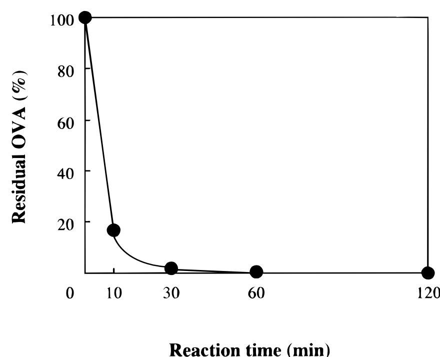


**Figure 5.** Time course of the hydrolysis of OVA in solution (0.5 mL, 10 mg/mL) with 40% (w/v) microcapsulated alcalase under conditions that mimicked the intestinal tract at 37 °C.

these microcapsules seem likely to be useful and acceptable to some extent in a variety of food systems.

**Rates of Hydrolysis of OVA by Enzyme Released from Microcapsules.** The microcapsulated alcalase and microcapsulated protease YP-SS were rapidly released in the intestinal tract and stomach, respectively. Each microcapsulated enzyme must be released to degrade the allergen in a particular environment. In the case of microcapsulated alcalase, from our knowledge of the residence time of alcalase in the intestinal tract, it seems to be desirable that OVA be rapidly digested by released alcalase. With the goal of developing the use of microcapsules in foods, we investigated the effect of the concentration of microcapsulated alcalase on the hydrolysis of OVA in a 30-min period. Microcapsulated alcalase was added from 10% (w/v; i.e. 50 mg) to 40% (w/v; i.e., 200 mg) to a solution of OVA (1 mg/mL, 0.5 mL), and each hydrolysate was applied to an Asahipak GS-320 column. As shown in Figure 4, the amount of OVA decreased linearly with increasing concentrations of microcapsulated alcalase. With 40% (w/v) microcapsulated alcalase, about 98% of OVA was degraded within 30 min. Thus, the concentration of microcapsules for complete degradation of OVA within the residence time was assumed to be 40% (w/v). Figure 5 shows the time course of the hydrolysis of OVA for addition of 40% (w/v) microcapsulated alcalase. OVA was degraded completely during a 1-h incubation.

We also examined the degradation of OVA by microcapsulated protease YP-SS. Microcapsulated protease YP-SS was added at 10% (w/v; i.e. 50 mg) to a solution of OVA (1 mg/mL, 0.5 mL) because an addition of 10% microcapsulated protease YP-SS (159 000 units/g) was almost the same as that of 40% microcapsulated alcalase (25 800 units/g). Figure 6 shows the time courses of the

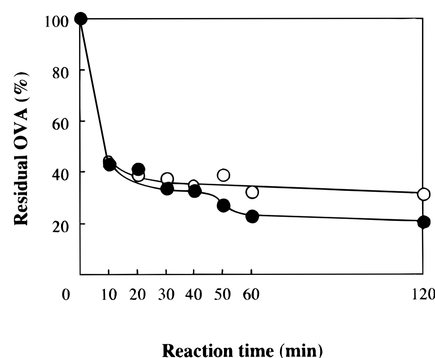


**Figure 6.** Time course of the hydrolysis of OVA in solution (0.5 mL, 10 mg/mL) with 10% (w/v) microcapsulated protease YP-SS in artificial gastric fluid at pH 2.0 (●) and pH 3.0 (○).

**Table 1. Food Intake Efficiency of SD Rats Fed a Diet That Contained Microcapsulated Enyzme(s)**

| group | food intake[a] (g/day) | wt gain[a] (g/day) | food eff[b] |
|---|---|---|---|
| casein | 22.7 ± 1.5 | 6.5 ± 1.7 | 0.29 |
| OVA | 17.8 ± 3.1 | 4.1 ± 1.6 | 0.23[c] |
| microcapd protease YP-SS + OVA | 20.0 ± 3.0 | 5.6 ± 1.7 | 0.28 |
| microcapd alcalase + OVA | 20.0 ± 2.3 | 5.4 ± 1.8 | 0.27 |
| microcapd protease YP-SS and microcapd alcalase + OVA | 18.6 ± 3.3 | 5.2 ± 1.6 | 0.28 |

[a] Values are means ± SE ($n$ = 4). [b] Food efficiency = weight gain/food intake. [c] Significantly different from the casein-intake group at $p < 0.05$.

hydrolysis of OVA at pH 2.0 and 3.0 with 10% (w/v) microcapsulated protease YP-SS. In a 2-h incubation, the extent of degradation of OVA at pH 2.0 (79%) was higher than that at pH 3.0 (66%), although the optimum pH of protease YP-SS is pH 3.0. The lower rate of degradation of OVA at pH 3.0 is presumed to have been due to self-degradation of OVA under the more acidic conditions. Moreover, about 95% of OVA was degraded by simultaneous usage of protease YP-SS (2-h incubation) and alcalase (0.5-h incubation; data not shown). Consequently, we concluded that microcapsulated alcalase and microcapsulated protease YP-SS might be useful and acceptable for degradation of allergens after intake of foods that contain these microcapsules.

**Depression of the Allergenic Activity of OVA by Oral Administration of Microcapsulated Enzyme.** To assess the safety and allergen-degrading activity of the microcapsulated enzymes *in vivo*, we performed an oral administration test with SD rats. As shown in Table 1, in the case of OVA-fed rats, food intake and weight gain were markedly lower than in the casein-fed group (control). However, although food intake and weight gain by the groups fed the diets that contained microcapsulated enzyme were slightly inferior to those of the casein-fed group, food efficiencies (= weight gain/food intake) of the former were almost the same as that of the latter. These results indicated that inhibition of growth of SD rats by OVA was markedly diminished by coadministration of microcapsulated enzyme, and there were no significant differences between the diet that contained microcapsulated enzyme and the casein diet, as a control. Accordingly, it appeared that the microcapsulated enzyme had efficiently degraded OVA in the gastrointestinal tract and had no ill influence on the growth of rats.

Serum levels of OVA-specific IgE of SD rats before and after the intake of the diets that contained microcapsulated enzyme were measured by ELISA. Figure

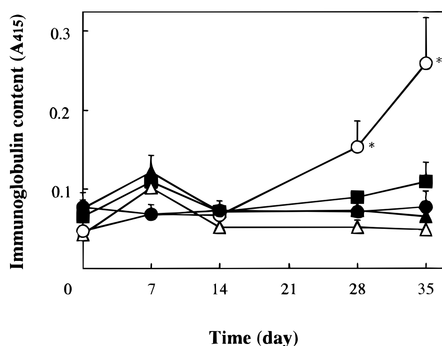


**Figure 7.** Quantitation of OVA-specific IgE in Sprague-Dawley rats fed a diet that contained microcapulated enzyme(s). Means ± SE of results from four rats are shown for each group (*$p < 0.05$ vs casein intake). Experimental groups: casein intake (●); OVA intake (○); diet containing OVA and microcapsulated protease YP-SS (▲); diet containing OVA and microcapsulated alcalase (△); diet containing OVA, microcapsulated proteaseYP-SS, and microcapsulated alcalase (■).

7 shows the changes in levels of OVA-specific IgE in serum. In the OVA-fed group, a significant increase in OVA-specific IgE was observed on day 35, as compared with the casein-fed group ($p < 0.05$, 5.5-fold increase). By contrast, the level of OVA-specific IgE did not increase in the groups fed OVA and microcapsulated enzymes or in the casein-fed group. These findings suggest that the synthesis of OVA-specific IgE might be reduced when the microcapsulated enzymes are administered orally.

In summary, we have researched the possibility of lowering allergenic activities as a model system. As a result, the microcapsulation method (spray-dry method) established in this study allows us to prepare target-specific microcapsules by selection of appropriate coating agents. In addition, the microcapsulated enzymes prepared in this study might be effective as a model system to assist in prophylactic treatment of food allergens without any negative effect on quality of life.

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