Note

Inhibition of Specific Degradation of 57-kDa Protein in Royal Jelly during Storage by Ethylenediaminetetraacetic Acid

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We have previously shown that 57-kDa protein in royal jelly (RJ) was specifically degraded in proportion to both storage temperature and storage period, and we suggested that it could be useful as a marker of freshness of RJ (Kamakura, M., Fukuda, T., Fukushima, M. and Yonekura, M., Biosci. Biotechnol. Biochem., 65, 277-284 (2001)). Here, we investigated the effects of various proteinase inhibitors on proteinase activity in RJ and on the specific degradation of 57-kDa protein during storage. Ethylenediaminetetraacetic acid (EDTA), but not other inhibitors, inhibited the proteinase activity in RJ, and dose-dependently suppressed storage-dependent degradation of 57-kDa protein. These results suggest that EDTA inhibits a specific proteinase activity in RJ, thereby suppressing the degradation of 57-kDa protein during storage at high temperature.

Key words: royal jelly; 57-kDa protein; metalloproteinase; ethylenediaminetetraacetic acid; storage-dependent degradation

Royal jelly (RJ) is the exclusive food of the queen honey bee (Apis mellifera) larva, and is secreted from the hypopharyngeal and mandibular glands of the worker honey bees.1) RJ possesses several pharmacological activities2) and consists mainly of proteins, sugars, lipids, vitamins and free amino acids,3) together with a large number of such bioactive substances as 10-hydroxy-2-decenonic acid,4) antibacterial protein,5) and a stimulating factor for the development of genital organs in male mice.6) Therefore, RJ has been widely used in commercial medical products, health foods and cosmetics in many countries.

The physical properties and chemical composition of RJ are dependent on the storage conditions, and undergo various changes such as acceleration of the Maillard reaction, and increases of viscosity, acidity, and protein degradation during storage of RJ at high temperature.7) We previously investigated the compositional changes of RJ during storage under various conditions (from 4°C to 50°C for up to 7 days), seeking a factor that could be used as a marker to evaluate freshness of RJ. We found that 57-kDa protein in RJ was specifically degraded in proportion to both storage temperature and storage period, whereas the contents of other constituents in RJ did not change during storage under the conditions examined.8) Therefore, we suggested that it is reasonable to evaluate the freshness and quality of RJ in terms of the change of 57-kDa protein.9) RJ has been reported to contain a proteinase,9) and this proteinase may be involved in the specific degradation of 57-kDa protein during storage. For quality control of RJ, it is desirable to identify the factor responsible for the degradation.

In this study, we investigated the effects of several proteinase inhibitors on proteinase activity in RJ and on the specific degradation of 57-kDa protein during storage.

Boc-Glu (Obzl)-Ala-Arg-MCA was purchased from Peptide Institute Inc. (Osaka, Japan). RJ was purchased from Hangzhou Green Forever Apiculture Company (Hangzhou, China). Phenylmethylsulfonl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), N-ethylmaleimide (NEM), diisopropyl fluorophosphate (DFP), benzamidine, pepstatin A, o-phenanthroline and iodoacetic acid (IAA) were from Wako Pure Chemicals (Osaka, Japan). N-α-Tosyl-l-lysine chloromethyl ketone (TLCK), ovooinhibitor, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64), diazoacetyl-norleucine methyl ester (DAN) and phosphoramidon were from Sigma (St. Louis MO, USA). All other reagents were of analytical grade.

The proteinase activity in RJ was measured by the method of Funakoshi et al.9) with some modifications. RJ (1.0 g) was dissolved in 10 ml of 50 mM Tris-HCl (pH 8.0) buffer, and the suspension was centrifuged at 5000 x g for 10 min. The supernatant was taken as the enzyme solution. Enzyme solution

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Abbreviations: MCA, 7-amino-4-methylcoumarinamide; RJ, royal jelly
(20 µl) was mixed with 120 µl of 0.5 mM Boc-Glu (Obzl)-Ala-Arg-MCA in 50 mM Tris-HCl (pH 8.0) buffer containing 100 mM NaCl and 10 mM CaCl2, and incubated for 30 min at 37°C. After the incubation, the reaction was stopped by the addition of 4 ml of 10% acetic acid. Then, the fluorescence of 7-amino-4-methylcoumarin (MCA) produced in the reaction mixture was measured with excitation at 380 nm and emission at 460 nm. One unit of activity was defined as the amount of enzyme that liberated 1 µmol of MCA per min. Protein concentration was determined by the method of Lowry et al., using bovine serum albumin as the standard. The residual proteinase activity after incubation with or without various proteinase inhibitors at room temperature (25°C) for 60 min was measured.

As summarized in Table 1, the proteinase activity in RJ was inhibited by such metal chelators as EDTA and EGTA. However, serine-proteinase inhibitors (PMSF, ovoinhibitor, benzamidine, DFP and TLCK), cysteine-proteinase inhibitors (NEM, IAA and E-64) and aspartic-proteinase (DAN and pepstatin A) inhibitors showed no inhibitory effect. Metalloproteinase inhibitors such as phosphoramidon and o-phenanthroline also did not inhibit the proteinase activity in RJ. Overall, the results suggest that the inhibition of proteinase activity in RJ by EDTA may be attributable to the elimination of calcium ion, which is implicated in stabilization of the proteinase.

Next, we examined the effects of proteinase inhibitors on the specific degradation of 57-kDa protein in RJ during storage. RJ (1 g) was dissolved in 10 ml of sterile 20 mM Tris-HCl (pH 7.0) buffer with or without various proteinase inhibitors, and the suspension was stored at 40°C for 3 days. After storage for 3 days, the suspension was centrifuged at 5000 × g for 10 min, and the supernatant was taken for RJ proteins analysis. Native PAGE was run with a 5–20% gradient polyacrylamide gel by the method of Davis. The protein in the gel was stained with Coomassie Brilliant Blue R-250. For a quantitative analysis of 57-kDa protein in RJ, the results of native PAGE of RJ proteins were analyzed by NIH image Ver. 1.62 (NIH, U.S.A.). Data were analyzed by using one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparison test. The level of P < 0.05 was used as the criterion of statistical significance.

When RJ was stored at 40°C, the results of native PAGE showed that 57-kDa protein was specifically degraded proportionally to the length of the storage period (Fig. 1). However, EDTA inhibited the specific degradation of 57-kDa protein during storage at 40°C, whereas PMSF, DAN or NEM did not (Fig. 1). The minimum concentration of EDTA suppressing the degradation of 57-kDa protein in RJ during storage at 40°C for 3 days was 30 mM. EDTA also suppressed the decrease of 57-kDa protein concentration in RJ during storage at 40°C for 3 days, while the other proteinase inhibitors did not (Fig. 2). RJ stored with 30 mM EDTA retained more than 70% of the initial content of 57-kDa protein in RJ during storage at 40°C for 3 days (Fig. 2). Furthermore, when RJ was stored with 50 mM, 100 mM or 200 mM EDTA at 40°C for 7 days, the remaining concentrations of 57-kDa protein in RJ were 56.5%, 72.9%, or 83.5% of the initial concentration, respectively. Thus, the decrease of 57-kDa protein concentration in RJ during storage at 40°C for 7 days was inhibited in proportion to the EDTA concentration. These findings suggest that EDTA may suppress the storage-dependent degradation of 57-kDa protein through the inhibition of a proteinase in RJ.

Previously, we found that 57-kDa protein stimulates hepatocyte DNA synthesis and prolongs the proliferation of primary cultured rat hepatocytes through anti-apoptotic action, as well as increasing albumin production. RJ stored at 40°C for 7 days contained little 57-kDa protein, and did not exhibit the stimulatory effect on hepatocyte DNA synthesis. Thus, the content of 57-kDa protein in RJ is considered to be associated with not only freshness, but also the physiological activity and quality of RJ. Therefore, EDTA, which inhibited storage-dependent degradation of 57-kDa protein, could be useful as a preservative agent to maintain freshness and quality of RJ.

When proteinase activity in RJ was measured by the method using casein as a substrate, proteinase activity was not detected (data not shown). However,
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Fig. 1. Change in Electrophoretic Profile of RJ Proteins during Storage.

Proteins in RJ stored at 40°C for 3 days were analyzed by native PAGE. RJ (1 g) was dissolved in 10 ml of sterile 20 mM Tris-HCl (pH 7.0) buffer with no additive (A), or with 5 mM PMSF (B), 5 mM NEM (C), 5 mM DAN (D) or 30 mM EDTA (E), and the suspension was stored at 40°C for 3 days. After storage for 3 days, the suspension was dissolved in 50 mM Tris-HCl (pH 7.0) buffer containing 20% glycerin, and the suspensions were centrifuged at 5000 × g for 10 min. The supernatants were subjected to native PAGE. Lane 1, RJ stored at 4°C for 0 days; lane 2, RJ stored at 40°C for 1 day; lane 3, RJ stored at 40°C for 2 days; lane 4, RJ stored at 40°C for 3 days. One representative experiment out of three is shown. The position of 57-kDa protein is indicated by an arrow.

the activity was detected using the synthetic substrate Boc-Glu (Obzl)-Ala-Arg-MCA. Therefore, the proteinase in RJ seemed to have a restricted substrate specificity, which is different from those of other proteinases. Further study is needed to purify and characterize the proteinase in RJ.

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Fig. 2. Changes in the contents of 57-kDa protein during storage at 40°C for 3 days.

The content of 57-kDa protein in RJ during storage at 40°C for 3 days was calculated from the result of native PAGE of RJ proteins. RJ (1 g) was dissolved in 10 ml of sterile 20 mM Tris-HCl (pH 7.0) buffer with no additive (○), or with 5 mM PMSF (△), 5 mM NEM (●), 5 mM DAN (▲) or 30 mM EDTA (▼), and the suspension was stored at 40°C for 3 days. The content of 57-kDa protein in RJ during storage at 40°C was expressed as a percentage of that of 57-kDa protein before storage. Each value is the mean ± SEM for triplicate independent experiments. Values significantly different from no additive (none) are indicated by ***p<0.001.

References