Note

Degradation of Soybean Protein by an Acid Proteinase from *Monascus anka*

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Degradation of soybean protein by *Monascus*-proteinase was investigated in order to reveal the role of the enzyme in the process of tofuyo ripening. The ratio of trichloroacetic acid-insoluble nitrogen of soybean protein to the total nitrogen in the reaction mixture decreased with increasing enzymatic reaction time. It was found that the digestion of soybean protein by this enzyme progressed as follows: initially, α' -, α -, and β -subunits in β -conglycinin, and then, the acidic subunit in glycinin were degraded. However, the basic subunit of glycinin still remained, and some polypeptide bands (around 10 kDa) were formed during the enzyme reaction. The degradation rate of soybean protein by this enzyme was affected by the ethyl alcohol concentration in the reaction mixtures.

Keywords: soybean protein, degradation, acid proteinase, *Monascus* fungus, red koji, tofuyo

The mold *Monascus* has been used for tofuyo and food coloring in Okinawa Prefecture, Japan (Yasuda, 1983). Tofuyo is a vegetable protein food made from tofu by the action of microorganisms. This food is a creamy cheese-type product with a mild flavor, fine texture, and good taste. It is unique and characteristic that the ripening was carried out in the presence of the ethyl alcohol of awamori (Okinawan traditional distilled liquor) (Yasuda, 1990). Changes in soybean protein and nitrogen compounds (Yasuda *et al.*, 1993, 1994) and physical changes (Yasuda *et al.*, 1996) of tofuyo during the ripening period were examined in order to reveal the ripening mechanism. An acid proteinase from *Monascus anka* (*Monascus*-proteinase) is considered to serve as a key enzyme for the ripening of the product. Although fungal proteinases have been extensively investigated (Fukumoto *et al.*, 1967; Ichishima, 1970; Tsujita & Endo, 1977), information on the enzyme produced by *Monascus* fungus was very limited. The authors reported the purification and some properties of acid proteinase obtained from red koji of *Monascus* fungus which was used for making tofuyo (Yasuda *et al.*, 1984).

In this paper, the effects of ethyl alcohol on the activities of the purified *Monascus*-proteinase and the mode of action toward soybean protein are described.

Materials and Methods

Microorganism, cultivation and purification of the enzyme The microorganism used throughout this study was *Monascus anka* No. 3403 which was isolated in our laboratory from the red koji obtained in Hong Kong. The culture of the microorganism and purification of the enzyme used in this work were carried out as previously reported (Yasuda *et al.*, 1984). The purified enzyme was confirmed to be homogeneous by disc-gel electrophoresis and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Assay method Proteinase activity was assayed as in

our previous paper (Yasuda *et al.*, 1984), except soybean protein isolate (SPI). The amounts of trichloroacetic acid (TCA)-soluble nitrogen which formed by the enzyme reaction were determined by the following methods: The reaction mixture, containing 5.0 ml of 2% SPI solution, 5.0 ml of 0.4 M lactate buffer (pH 3.0), 10 ml of enzyme solution (15.6 μ g/ml), and toluene, was incubated at 37°C for an appropriate reaction time with shaking. After the enzyme reaction, 1 ml of 0.4 M TCA solution was added to 1.5 ml of the reaction mixture, and the reaction was stopped. Thereafter, the reaction mixture was filtrated with Toyo-filter paper No. 5B. The amounts of nitrogen in the filtrate (TCA-soluble nitrogen) or in the residue (TCA-insoluble nitrogen) were determined by a micro kjeldahl method ("Nippon Shokuhin Kogyo Gakkai," 1982; Yasuda *et al.*, 1993).

Slab SDS-polyacrylamide gel electrophoresis The samples for gel electrophoresis were prepared as follows: The enzyme reaction was carried out as previously described. After the enzyme reaction, 187.5 μ l of SDS-buffer (β -mercaptoethanol: 0.5 M Tris-HCl buffer (pH 6.8): 10% sodium dodecyl sulfate solution; 50:125:100, v/v/v) was added to 1 ml of the reaction mixture, followed by boiling for 2 min. Each sample was then lyophilized.

Slab SDS-gel electrophoresis in 15% polyacrylamide gel (10 $\times$ 14 $\times$ 0.2 cm) was performed according to the method of Laemmli with a pH 8.3 buffer system (Laemmli, 1970). After the run, the protein was stained with 0.25% Coomassie brilliant blue in a methanol-acetic acid solution (methanol: acetic acid: water; 50: 10: 43, v/v/v) at room temperature.

Results and Discussion

Effect of ethyl alcohol on the enzyme activity The effect of ethyl alcohol on the activity of the enzyme used in this work is shown in Fig. 1. The enzyme activity decreased as the ethyl alcohol concentration increased. The relative activities of 10, 20, and 30% ethyl alcohol in the reaction

Fig. 1. Effect of ethyl alcohol on the activity of *Monascus*-proteinase.

mixture, were 63, 25, and 3%, of the nonalcohol treatment, respectively. It is known that the maturation of tofuyo progresses in the presence of 15–20% ethyl alcohol. Thus, it is considered that the protein of tofu is degraded slowly, being affected by reduced proteinase activities (Yasuda *et al.*, 1993).

Effect of ethyl alcohol on the degradation of soybean protein by the enzyme To learn the extent of the hydrolysis of soybean protein by the enzyme isolated in this work, ethyl alcohol was added to the reaction mixture. Changes in the ratio of the TCA-soluble nitrogen content to the total nitrogen in the reaction product are shown in Fig. 2. It was found that these values rapidly increased with initial reaction time (until 1 h), after that, they increased slowly, and the ratio was affected by the concentration of the ethyl alcohol in the reaction mixture. Soybean protein was converted to low molecular weight compounds such as peptides and amino acids, being affected by these proteinase activities. It is thought that the enzyme contributes to the formation of tasty amino acids such as glutamic acid and aspartic acid together with carboxypeptidase, amino peptidase and glutaminase *etc.*, and these amino acids are closely related to the tastes of tofuyo (Yasuda *et al.*, 1993, 1994).

Mode of action toward soybean protein by the enzyme Digestion of soybean protein by the enzyme was examined by the method of slab SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of β -mercaptoethanol. The SDS-PAGE pattern of the soybean protein at various enzyme reaction stages in the presence of 20% ethyl alcohol is shown in Fig. 3. Each polypeptide band corresponding with α' -, α -, and β -subunits in β -conglycinin, and the acidic and basic subunits in glycinin were observed after 0 min incubation. After 30-min incubation, some polypeptide bands (10–12 kDa) increased. The bands of α' -, α -, and β -subunits in β -conglycinin disappeared after a 4-h incubation, but the acidic subunit in glycinin was thin, and that of the basic subunit and other relatively low molecular weight polypeptide bands (around 10 kDa) were detected after 24-h incubation.

When ethyl alcohol was absent, the SDS-PAGE pattern of soybean protein at various enzyme reaction stages was examined (the data are not shown). Some polypeptide bands appeared in molecular mass (10–15 kDa) after 5-min incubation, and the bands of α' -, α -, and β -subunits in β -congly-

Fig. 2. Changes in the ratio of TCA-soluble nitrogen content to the total nitrogen of soybean protein during the enzyme reaction. ○ 0% ethyl alcohol, △ 10% ethyl alcohol, □ 20% ethyl alcohol.

Fig. 3. Slab SDS-polyacrylamide gel electrophoretic pattern of soybean protein at various enzyme reaction stages. Preparation of the samples was described in the text. The enzyme reaction was carried out in the presence of 20% ethyl alcohol. Molecular weights were estimated by the mobilities of cytochrome c from horse heart and crossed-linked cytochrome c (cytochrome c monomer, dimer, trimer, tetramer and hexamer). A: Standard proteins described above, B: 0 min, C: 5 min, D: 10 min, E: 15 min, F: 20 min, G: 30 min, H: 1 h, I: 2 h, J: 4 h, K: 8 h, L: 24 h. Numerical values in lanes A and L showed each molecular mass (kDa). Symbols of α' , α and β in lane B showed α' -, α - and β -subunits in β -conglycinin, and A and B in lane L showed Acidic and basic subunits in glycinin.

cinin, and the acidic subunit in glycinin disappeared after 10-min incubation, but that of the basic subunit in glycinin still remained after 24-h incubation. When 10% ethyl alcohol was present, the band of the acidic subunit disappeared after 2-h incubation.

From these results, it was found that the degradation of soybean protein by this enzyme progressed as follows: initially, each subunit in β -conglycinin, and then, the acidic subunit in glycinin were degraded. However, its basic subunit still remained, and some polypeptide bands (around 10 kDa) were formed during the enzyme reaction. It is thought that the difficulty of the degradation of the basic subunit originates in the substrate specificity of the enzyme. The degradation rate of soybean protein by this enzyme was affected by the ethyl alcohol concentration in the reaction mixture. These phenom-

ena were in good agreement with the case of tofuyo during the ripening period (Yasuda *et al.*, 1993). Namely, *Monascus*-proteinase gradually degraded the protein of tofu (especially, each subunit in β -conglycinin and the acidic subunit in glycinin) in the presence of the ethyl alcohol of awamori in moromi and formed polypeptides (around 10 kDa), peptides, and amino acids during fermentation. However, it could not degrade the basic subunit in glycinin (Yasuda *et al.*, 1993, 1994, 1995). These formed polypeptides and the remaining glycinin which has strong gel-forming ability are closely related with the body of tofuyo (Yasuda *et al.*, 1993, 1995), and it is considered that the glycinin greatly contributes to preserving the good textures of tofuyo (Yasuda *et al.*, 1996). Thus, we concluded that *Monascus*-proteinase is a key enzyme for tofuyo ripening.

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