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# Purification of Endopeptidase in Yam *Dioscorea opposita* and Its Characterization

Yasuyuki TSUKAMASA, Masashi ANDO and Yasuo MAKINODAN

*Department of Fisheries, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan*

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**An endopeptidase in powdered yam was purified using a combination of anion-exchange and gel-filtration chromatographies. The preparation gave 9.2-fold purification with yield of 19% against the crude extract. The endopeptidase was classified as a serine endopeptidase with inhibitory spectrum, with an optimum temperature and pH of approximately 60°C and 7.1, respectively. It also had high heat stability and high salt resistance.**

Keywords: yam, endopeptidase, purification

We (Tsukamasa *et al.*, 1999) previously showed the presence of an endopeptidase in powdered yam (PY) which hydrolyzes myofibrillar proteins which are components of Kamaboko and causes Kamaboko-gel degradation. However, the crude extract from PY was used only for characterization in the earlier study. In this study, we purified and characterized the endopeptidase from PY.

## Materials and Methods

*Preparation of crude extract from powdered yam* PY (Yamaimo special A), which was prepared by lyophilization, was supplied by Senba Touka Kogyo (Tokyo). Thirty grams of PY was dissolved in 450 ml of 10 mM Tris-HCl (pH 7.5) containing 5 mM 2-mercaptoethanol (buffer A) and stirred at 4°C overnight. The solution was then centrifuged at 13,000× *g* for 40 min, and the supernatant was filtered through glass wool. The filtrate was dialyzed with 10 volumes of buffer A overnight and then the dialysate was used as a crude extract.

*Assay of endopeptidase activity* The reaction mixture was prepared with 600 $\mu$l of buffer B composed of 100 mM $KH_2PO_4$ and 50 mM $Na_2B_4O_7$, pH 6.8, 200 $\mu$l of 5% casein, and 200 $\mu$l of enzyme solution, and then incubated for 30 min at 60°C. The reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA). After filtration, TCA-soluble materials in the filtrate were measured by the method of Lowry *et al.* (1951). The difference of the absorbance at 750 nm between sample and blank, $\Delta A_{750}$, was measured and we defined 1.0 of $\Delta A_{750}$ as one unit.

*Preparation of myofibril* Myofibril was prepared from the white croaker muscle. The ordinary muscle of the white croaker was collected and homogenized with 6 volumes of 39 mM borate buffer, pH 7.0, containing 100 mM KCl and 4.6 mM EDTA and then centrifuged at 600 ×*g* for 15 min. The precipitate was suspended in the same buffer and used.

*SDS-polyacrylamide gel electrophoresis analysis* SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970), using 12.5% gel.

E-mail: tukamasa@nara.kindai.ac.jp

## Results and Discussion

*Purification of endopeptidase* All purification procedures were done at 4°C. Solid ammonium sulfate was added to the crude extract to give 60% saturation at pH 7.5. After 3 h, the precipitate was collected by centrifugation at 13,000×*g* for 40 min. Obtained precipitate was dissolved in buffer A and dialyzed with the same buffer overnight. This dialyzate was applied to a DEAE-cellulose column (3.0×50 cm), equilibrated with buffer A and washed thoroughly with the same buffer. Elution was performed with 0–0.4 M NaCl gradient in a total volume of 1000 ml. Two active peaks, fraction numbers 86–110 and 134–160, are observed in Fig. 1. Because the endopeptidase activity of the former peak fraction was greater than that of the latter, the former was used for further purification. After concentration by ultrafiltration, the concentrate was dialyzed with buffer A overnight. The dialysate was absorbed in DEAE-Sephadex A-50 equilibrated with buffer A by batch procedure. After a column (3×14 cm) was filled with the DEAE-Sephadex, elution was performed with 0–0.5 M NaCl gradient in a total volume of 300 ml of 10 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol (buffer C). An active peak, fraction number 34–54, is observed in Fig. 2, therefore, the fractions were pooled and

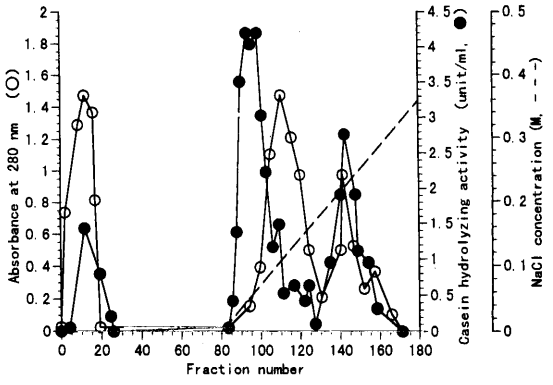


**Fig. 1.** DEAE-cellulose column chromatography of the crude extract. The column (3.0×50 cm) was equilibrated with 10 mM Tris-HCl (pH 7.5) containing 5 mM 2-mercaptoethanol.

concentrated to about 10 ml by ultrafiltration. The concentrate was applied to an Ultrogel AcA22 column (1.5×105 cm) eqilibrated with buffer C. A chromatogram of gel-filtration using the column is shown in Fig. 3. The active peak, fraction numbers 67–83, was pooled. The purification process so far noted is summarized in Table 1. The endopeptidase was purified 9.2-fold with yield of 19%. The partially purified endopeptidase migrated as a single band in SDS-PAGE (Fig. 4). The molecular weight of the endopeptidase was estimated to be about 370,000 from the calibration curve.

*Properties of purified endopeptidase* As shown in Table 2, the casein-hydrolyzing activity of this purified endopeptidase was inhibited by serine protease inhibitors, SBTI and antipain, but little by cysteine protease inhibitors, monoiodoacetic acid or E-64, a metallo-protease inhibitor, 1,10-phenanthroline, or an aspartic protease inhibitor, pepstatin A. Therefore, the endopeptidase was suggested to be a serine endopeptidase. The effect of temperature on the casein-hydrolyzing activity was determined at from 40°C to 80°C (Fig. 5). Highest activity was observed at 60°C and linearity of the 60°C-reaction was retained for up to 30 min. The endopeptidase was inactivated at 80°C. The effect of pH on the casein-hydrolyzing activity was determined (Fig. 6), and optimum pH was around 7.1. Heat stability was determined

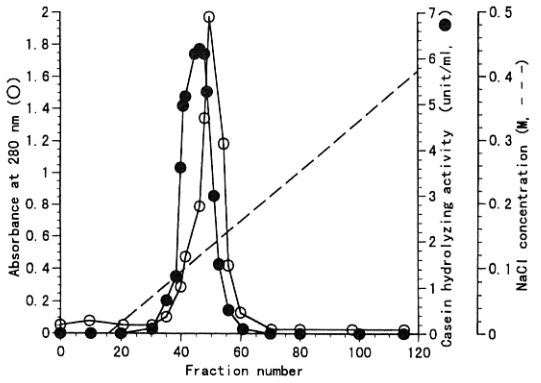


**Fig. 2.** DEAE-Sephadex A-50 column chromatography of the fractions obtained from DEAE-cellulose column. The column (3.0×14 cm) was equilibrated with 10 mM Tris-HCl (pH 7.5) containing 5 mM 2-mercaptoethanol.

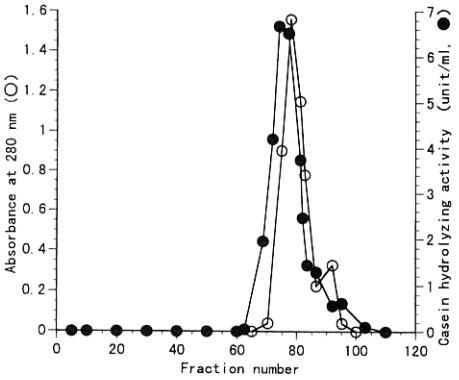


**Fig. 3.** Ultrogel AcA22 column chromatography of the fractions obtaied from DEAE-Sephadex column. The column (1.5×105 cm) was equilibrated with 10 mM Tris-HCl (pH 8.0) containig 5 mM 2-mercaptoethanol.

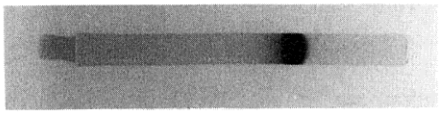

**Fig. 4.** SDS-PAGE analysis of the purified endopeptidase.

**Table 2.** Effect of various protease inhibitors on the casein hydrolyzing activity of purified endopeptidase.

| Inhibitor | Final concentration | Relative activity (%) |
|---|---|---|
| None | — | 100 |
| E-64 | 1 mg/ml | 89 |
| Antipain | 1 mg/ml | 54 |
| Monoiodoacetatic acid | 5 mM | 94 |
| Pepstatin A | 5 μg/ml | 103 |
| Soybean trypsin inhibitor | 500 μg/ml | 62 |
| 1,10-Phenanthroline | 5mM | 101 |

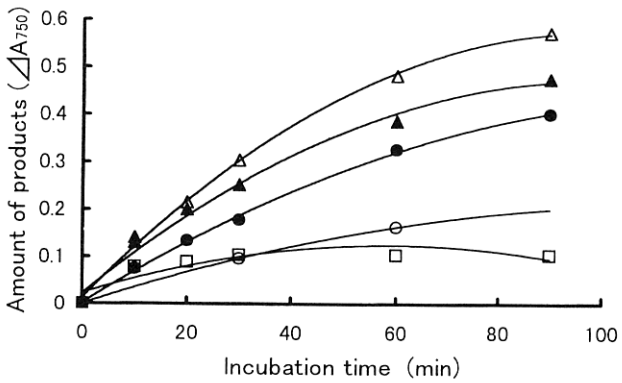


**Fig. 5.** Effect of temperature on the casein hydrolyzing activity of the purified endopeptidase. ○, 40°C; ●, 50°C; △, 60°C; ▲, 70°C; □, 80°C.

**Table 1.** Purification chart of endopeptidase from powdered yam.

| Fraction | Volume (ml) | Protein (mg) | Total activity[a] | Specific activity[b] | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Crude extract | 420 | 1205 | 604.1 | 0.500 | 1 | 100 |
| Ammonium sulfate | 180 | 527.3 | 518.9 | 0.980 | 2.0 | 86 |
| DEAE-Cellulose | 158 | 75.8 | 291.4 | 3.84 | 7.7 | 48 |
| DEAE-Sephadex A-50 | 57 | 35.6 | 161.6 | 4.53 | 9.1 | 27 |
| Ultrogel AcA22 | 35 | 25.1 | 82 | 4.57 | 9.2 | 19 |

[a]unit
[b]unit/mg

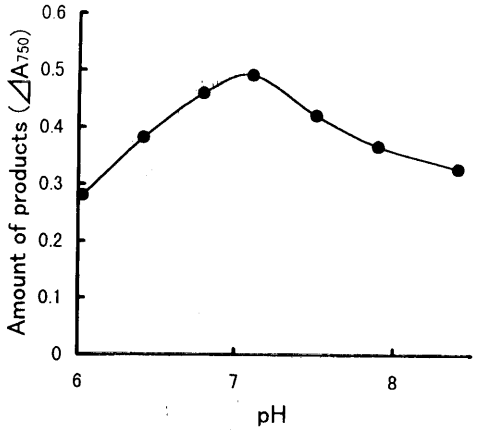


**Fig. 6.** Effect of pH on the casein hydrolyzing activity of the purified endopeptidase. Casein hydrolyzing activity was measured at 60°C for 30 min.

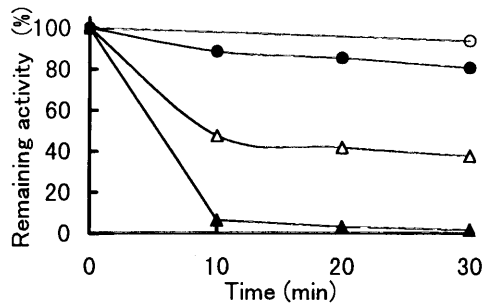


**Fig. 7.** Heat-stability of the casein hydrolyzing activity of the purified endopeptidase. Casein hydrolyzing activity was measured at 60°C for 30 min after heating at various temperature up to 30 min. ○, 50°C; ●, 60°C; △, 70°C; ▲, 80°C.

at 50, 60, 70 and 80°C for 30 min (Fig. 7). The remaining activity after 30 min-heating at each temperature was 100, 80, 40 and 0%, respectively. In the effect of NaCl on the casein-hydrolyzing activity, the higher the NaCl concentration was, the

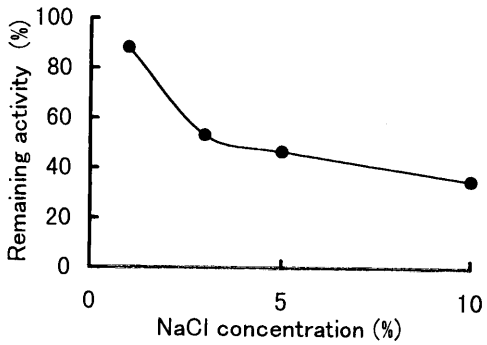


**Fig. 8.** Effect of NaCl on the casein hydrolyzing activity of the purified endopeptidase. Casein hydrolyzing activity was measured at pH 7.1 at 60°C for 30 min.

lower was the endopeptidase activity (Fig. 8). However, about 30% of proteolytic activity remained even at 10% of NaCl.

As mentioned above, we found a serine endopeptidase from yam which has high heat stability and high salt resistance. Therefore, if we add yam to food composed mainly of protein, we must be attentive to the serine endopeptidase. For example, hanpen, a surimi-based product, and ganmodoki, a tofu-based product, containing yam. Protein and NaCl concentration of the former are 9.9% and 2.0% and of the latter are 15.3% and 0.5%, respectively. It is possible that the endopeptidase in yam hydrolyzes their content before and during the heating process. Therefore, hanpen and ganmodoki should be prepared at low temperature and quickly heated up to more than 80°C in the center point.

## References

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