Evidence of Stepwise Digestion of Protein in the Digestive System of the Silkworm, *Bombyx mori* (Lepidoptera : Bombycidae)

Yasuhiro HORIE and Kijiro WATANABE

Sericultural Experiment Station, Yatabe, Ibaraki 305, Japan

Eisuke SAKAMOTO¹

*Department of Sericulture, Faculty of Agriculture, Tokyo University of
Agriculture and Technology, Fuchu, Tokyo 183, Japan*

(Received December 24, 1981)

The proteolytic activity in the digestive system of the silkworm was measured by determining of U. V. absorption and the ninhydrin reaction. When the substrate was incubated with digestive fluid at pH 11.2, O. D. at 280 nm was increased very rapidly, whereas the amount of ninhydrin positive substances reached 10% of complete hydrolysis of substrate and maintained this level. Subsequently, the product by protease of digestive fluid was incubated with midgut enzyme preparation at pH 6.85. The ninhydrin positive substances were increased markedly. Furthermore, the peptides produced by protease of the digestive fluid were separated by means of gel filtration on Sephadex column and their molecular weight was estimated to be about from 600 to 4,000.

These results indicate that there is a functional differentiation between digestive fluid and midgut tissue concerning the protein hydrolysis in the silkworm: High molecular proteins are hydrolyzed into peptides in digestive fluid at strong alkaline condition, and the peptides absorbed in the tissue were hydrolyzed into amino acids by the midgut peptidases.

INTRODUCTION

It is known that very active protease is present in the digestive fluid of the silkworm, *Bombyx mori*, and that the optimal pH of this enzyme is 11.2 (HORIE et al., 1963; HAMANO and MUKAIYAMA, 1970; IWAMOTO and EGUCHI, 1975). The several properties of this enzyme have been studied, including substrate specificity (HAMANO and MUKAIYAMA, 1970; IWAMOTO and EGUCHI, 1978), the effect of temperature on the activity (HORIE et al., 1963; EGUCHI and IWAMOTO, 1976; IWAMOTO and EGUCHI, 1978), the effect of divalent metals (HAMANO and MUKAIYAMA, 1970; IWAMOTO and EGUCHI, 1978), electrophoretic variation of this enzyme among silkworm strains (EGUCHI and YOSHITAKA, 1967), and change of activity during development (HORIE et al., 1963; EGUCHI and IWAMOTO, 1975; IWAMOTO and EGUCHI, 1975).

¹ Present address: *Isehara Agriculture Extension Office, Isehara, Kanagawa 259-11, Japan.*
Please address reprint request to Y. Horie.

Recently, it has been reported that the protease of digestive fluid consists of at least three components of enzyme, and that the properties of these enzymes were characterized (IWAMOTO and EGUCHI, 1978, 1979). On the other hand, SHINODA (1930) and HORIE et al. (1963) have suggested that there is a functional differentiation between digestive fluid and the midgut tissue as to the hydrolysis of protein in the silkworm.

This work was undertaken to clarify the mechanism of proteolysis in the digestive system of the silkworm.

MATERIALS AND METHODS

Animals and enzyme preparation. Fifth-instar larvae of the silkworm of the N 124 × C 124 strain reared on mulberry leaves, were used. The digestive fluid was collected from mid-5th instar larvae previously starved for 24 hr, by applying a weak electric shock. The larvae were sacrificed by opening ventrally, and the midgut contents were rinsed in an ice-cold 0.85% KCl solution. The digestive fluid and the midgut tissues were kept at -20°C prior to further preparation. 0.5 g of frozen midgut tissues was homogenized in a glass-teflon homogenizer in ten vol. of 0.1 M Tris-maleate buffer (pH 6.5), centrifuged at 15,000 g for 30 min, and the supernatant was obtained. The digestive fluid was used for assay after the filtration through a cotton pad.

Assay of enzyme activities. The measurement of proteolytic activity was carried out according to the previous paper (Horie et al., 1963); 0.5 ml of digestive fluid was added to 10 ml of the substrate solution which was prepared by dissolving 1% casein (Merk, Hammarsten) in 0.1 M borate buffer, adjusted to pH 11.2 with 1N NaOH solution. The reaction mixture was incubated at 30°C and the each 0.5 ml aliquot of the reaction mixture was added to 10 vol. of 10% TCA solution to stop the reaction. After keeping the tube in ice-cold water for 2 hr, the protein precipitated was removed by centrifuging at 5000 g for 10 min. The optical density of the supernatant was measured at 280 nm by means of the spectrophotometer (Hitachi Type 124).

The amount of ninhydrin-positive substances was measured by the method of YEMM and COCKING (1954) using the supernatant after deproteinizing with TCA, and the results were expressed in terms of the optical density at 570 nm. The substrate was hydrolyzed with 6 N HCl solution at 110°C for 12 hr under reducing pressure, and then the amount of ninhydrin-positive substances was determined.

RESULTS AND DISCUSSION

The hydrolysis of casein was followed in the process of incubation by measuring absorbance at 280 nm and the amount of ninhydrin-positive substances. As shown in Fig. 1, the increase of optical density at 280 nm was very rapid and approximately 80% of casein added as a substrate was hydrolyzed after 2 hr by the addition of the digestive fluid, and the optical density eventually reached the plateau within 2 hr. On the other hand, the increase of ninhydrin-positive substances in the deproteinized supernatant was not parallel to that of the density at 280 nm; it increased very slowly reaching the plateau level of about only 10% of the total value of ninhydrin-positive substances which was obtained by HCl hydrolysis.

Fig. 1. Time-course of protein hydrolysis by the digestive fluid. The reaction mixture is shown in the text. The activity is expressed in terms of optical density at 280 nm or 570 nm per μ l of digestive fluid. The theoretical maximum was obtained by complete hydrolysis in HCl solution as shown in the text. ●—●; Optical density at 280 nm. ○—○; Optical density at 570 nm. (ninhydrin reaction).

Fig. 2. Time-course of protein hydrolysis by the digestive fluid and enzyme preparation from midgut. The reaction mixture and condition are shown in the text. The activity is expressed in terms of hydrolysis percentage to the complete hydrolysis. The time of addition of midgut enzyme to the reaction mixture is indicated by an arrow. ●—●; Optical density at 280 nm when the reaction mixture with digestive fluid was incubated at pH 11.2. ◐—◐; Optical density at 570 nm (ninhydrin reaction) After 3 hours, pH was adjusted to 6.85 and midgut enzyme was added. ○—○; Optical density at 570 nm Midgut enzyme was added but pH was kept 11.2.

These results suggest that the proteolysis in the digestive fluid of the silkworm does not proceed to formation of amino acids, but it remains at intermediary peptides level. Subsequently, the effect of the addition of enzyme solution prepared

Fig. 3. Elution patterns of substrate protein and digested product by the digestive fluid on Sephadex G-100. —; substrate protein (before reaction), ----; digested product (after 24 hr-incubation), A ; Bovine albumin (MW. 67,000), B ; Ovalbumin (MW. 45,000), C ; Myoglobin (MW. 17,800).

from the midgut tissue on further hydrolysis of the peptides was tested.

The casein solution was at first incubated with the digestive fluid for 3 hr at pH 11.2 as shown above, and then the half of the reaction mixture was taken out and boiled for 15 min to stop the reaction. The pH value of the reaction mixture boiled was adjusted with 10% acetic acid solution to 6.85 which has been known to be optimal pH for dipeptidase of the midgut tissue (HORIE et al., 1963). The final volume of the neutralized reaction mixture was made up to twice volumes with distilled water.

Two ml of midgut enzyme was added to 5 ml of the reaction mixture and incubated at 30°C. The reaction was followed by measuring the amount of ninhydrin-positive substances in 0.5 ml aliquot of the reaction mixture. The result is shown in Fig. 2, in which the activity is expressed in terms of hydrolysis percentage of the substrate. The ninhydrin-positive substances increased very rapidly in the first 30 min after addition of the midgut enzyme, and then hydrolysis proceeded almost linearly. As a result, the percentage of hydrolysis reached approximately 85% after 6 hr-incubation. On the contrary, the amount of ninhydrin-positive substances was kept at a constant level when the reaction mixture was incubated at pH 11.2.

It is proved from these results that the proteolytic process in the digestive system of the silkworm consists of at least two steps; the enzymes of digestive fluid appear to belong to the group of endopeptidases which hydrolyze protein into peptides and the enzymes of the midgut tissue seem to be exopeptidases which split the peptides to amino acids.

Although weak activity of protease has been observed in the midgut tissue (HORIE et al., 1963; IWAMOTO and EGUCHI, 1975; EGUCHI and IWAMOTO, 1976), it does not seem to play an important role in protein digestion in this tissue, because the optimal pH for midgut protease is in accord with that for digestive fluid (HORIE et al., 1963; EGUCHI and IWAMOTO, 1976) and midgut proteases mainly consist of membrane-bound type (EGUCHI and IWAMOTO, 1976).

Subsequently, the peptides formed by the digestive fluid were characterized on

Fig. 4. Estimation of molecular weight of the digested product by the digestive fluid. A, B and C are the same as shown in Fig. 3. (1) and (2) show the elution volume of the products.

their molecular sizes. Aliquots of the reaction mixture containing digestive fluid as enzyme, were taken out after 24 hr-incubation at pH 11.2 and boiled for 15 min to stop the reaction. Then each 6.5 ml of the supernatant was fractionated by gel filtration on Sephadex G-100 (2.6×105 cm). As a control, boiled digestive fluid was added to the substrate solution and the mixture was fractionated in a manner similar to incubated one. The substrate or the reaction product was eluted with 20 mM Tris-HCl buffer (pH 7.5) with marker proteins. The flow rate was 12.6 ml per hour and 3.0 ml were collected in each tube. The elution patterns are illustrated in Fig. 3. The sample without incubation consisted of two main protein peaks which were eluted in the tubes of Nos. 56–60 and Nos. 74–84. These peaks of proteins with large molecular size were almost disappeared after 24 hr-incubation and two peaks with small molecular size were appeared in the tubes of Nos. 165–183 and Nos. 191–199, which were peptides formed by protease in the digestive fluid. These peaks of peptides were rather wide, suggesting that the peptides with various molecular size were involved in these fractions.

As shown in Fig. 4, a linear relationship was obtained between elution volume and the logarithm of molecular weight for bovine albumin, ovalbumin and myoglobin. The peaks of two groups of peptides shown in Fig. 3 were located distantly from those of the standard proteins. The average molecular weights of two groups of peptide were estimated to be approximately 4,000 and 1,500, respectively, although these groups contained peptides with various molecular sizes.

Furthermore, the same sample taken out after 24 hr-incubation was fractionated by gel filtration on Sephadex G-75 (1.7×90 cm). The sample was eluted with 20 mM Tris-HCl buffer (pH 7.5) with marker proteins (myoglobin and bacitracin). The flow rate was 12.4 ml per hour and 2.0 ml were collected in each tube. Two peaks of peptide were obtained in this case similar to that on Sephadex G-100 column. The molecular weights of two groups of peptide were estimated in the manner as shown in Fig. 4, however these average molecular weights were approximately 1,800 and 600. The reason of the difference in molecular weight between two meth-

ods using Sephadex G-100 and G-75 can still not be elucidated.

Since the increase of ninhydrin-positive substances was limited at only a 10% level, it is roughly presumed that peptide consists of on the average ten amino acids, and that the molecular weight is calculated as approximately 1,300 when the molecular weight of leucine is applied.

Although the molecular weight of peptides formed by protease in digestive fluid could not be decided exactly in this experiment, it is evident that protein is at first digested to peptide level by proteases of digestive fluid, then peptides are hydrolyzed in the midgut tissue.

ACKNOWLEDGEMENT

The authors wish to thank to Dr. T. Ito for reading the manuscript.

REFERENCES

- EGUCHI, M. and A. IWAMOTO (1975) Changes in protease, esterase, and phosphatases in the alimentary canal of the silkworm during metamorphosis. *Insect Biochem.* **5** : 495-507.
- EGUCHI, M. and A. IWAMOTO (1976) Alkaline proteases in the midgut tissue and digestive fluid of the silkworm, *Bombyx mori*. *Insect Biochem.* **6** : 491-496.
- EGUCHI, M. and N. YOSHITAKE (1967) Electrophoretic variation of proteinase in the digestive juice of the silkworm, *Bombyx mori* L. *Nature* **214** : 843-844.
- HAMANO, K. and F. MUKAIYAMA (1970) Some properties of the digestive fluid proteinase in the silkworm, *Bombyx mori*, with references to the relation between dissociation degree and nutritive value of some proteins. *J. Sericult. Sci. Japan* **39** : 371-376.
- HORIE, Y., M. TANAKA and T. ITO (1963) Proteolytic enzymes of digestive juice and midgut of the silkworm, *Bombyx mori*. *J. Sericult. Sci. Japan* **32** : 8-15.
- IWAMOTO, A. and M. EGUCHI (1975) Proteases in the midgut tissues and digestive fluid of silkworm larvae: Properties and separation. *J. Sericult. Sci. Japan* **44** : 190-194.
- IWAMOTO, A. and M. EGUCHI (1978) Enzymatic properties of three proteases in the digestive juice of the silkworm. *J. Sericult. Sci. Japan* **47** : 285-291.
- IWAMOTO, A. and M. EGUCHI (1979) Comparison of three proteases in the digestive juice of the silkworm: Substrate specificity and the effect of inhibitors. *J. Sericult. Sci. Japan* **48** : 31-36.
- SHINODA, O. (1930) Contribution of the knowledge of the intestinal secretion of insects III. On the digestive enzymes of the silkworm. *J. Biochem. Tokyo* **11** : 345-367.
- YEMM, E. W. and E. C. COCKING (1954) Estimation of amino acids by ninhydrin. *Biochem. J.* **58** : XII.