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Two Dimensional Electrophoresis of Galactosidase Relating to the Disappearance of *Bombyx* Lectin Activity.

Yasuo Kato

INTRODUCTION

Lectins are a class of proteins that bind to a carbohydrate moiety of glycoconjugates. Biological activities elicited by lectins include cell agglutination, mitosis, apoptosis and so on. For these activities, animal lectins seem to play a significant role in a defense mechanism of the living body, such as removal of a foreign matter or a biopolymer which has losed the normal function in the living body (KAWABATA, 2000 ; FURUTA *et al*., 2001 ; KAWASAKI, 2001 ; WAGO, 2001 ; KOYAMA *et al*., 2002).

Insect lectins are important as part of nonspecific self defense. Especially, humoral lectins of insects play a specific physiological role on cellular communication through metamorphosis of insects and will be useful defense substances for human being in future (KOTANI *et al*., 1995 ; FUJITA *et al*., 1998 ; UJITA *et al*., 2002). On the other hand, AMANAI *et al*. (1994) suggested that a *Bombyx* lectin in haemolymph served some function in gonadal development.

In a previous study, I showed that a humoral lectin (130 K-glycoprotein) of *Bombyx mori* played a physiological role through metamorphosis of the domesticated silkworm, *Bombyx mori* (KATO *et al*., 1994). I also emphasized the possibility that the humoral lectin was produced and activated in fat body of *B. mori*, and that it was secreted into haemolymph (KATO *et al*., 1998). Moreover, I reported on neuraminidase as a *Bombyx* humoral lectin activating factor (KATO and NAKAMURA, 2000). On the other hand, I tried to make a study on galactosidase because the active lectin disappeared the activity when it was treated with galactosidase *in vitro*. The result showed that both of α-galactosidase activity and β-galactosidase activity were recognized in the haemolymph and the fat body of *B. mori* (KATO and NAKAMURA, 2001). In the previous papers, I described on the activity of galactosidase in the fractions obtained from the haemolymph and the fat body by means of gel filtration and Mono Q ion exchange chromatography. These results suggested the possibility that galactosidase presented actually in the haemolymph and the

fat body of *B. mori*, and that galactosidase related to the *Bombyx* humoral lectin activity (KATO, 2002 ; KATO, 2003). However, it was difficult to purify galactosidase from the haemolymph and the fat body.

Here I describe the novel technology to fractionate galactosidase in the haemolymph of *B. mori* by two dimensional polyacrylamide gel electrophoresis, because major advancements are being made in the filed of proteomics with mass spectrometric identification of proteins separated by two dimensional polyacrylamide gel electrophoresis, currently (FURUTA *et al*., 2002 ; OH-ISHI, 2002). This investigation will provide useful information for understanding an original role of the lectin-protein *in vivo*.

MATERIALS and METHODS

(1) Preparation of samples

A hybrid race, Shunrei × Shougetu, of the silkworm, *Bombyx mori*, was used in this experiment. Larvae were reared with fresh mulberry leaves. In preparing the samples for this research, haemolymph was collected. Haemolymph was collected by cutting the larval abdominal legs. After centrifuging the haemolymph at 3,500 rpm for 15 min at 4℃, each of resultant supernatant was lyophilized.

(2) Two dimensional polyacrylamide gel electrophoresis (2 D-PAGE)

2 D-PAGE was performed using the Mini-PROTEAN mini tube gel two dimensional polyacrylamide gel electrophoresis system (Bio-Rad Laboratories, Inc.). The proteins were solubilized with 9.5 M urea, 2.0% Triton X–100, 5.0% 2–mercaptoethanol, 1.6% Bio-lite 5/7 and 0.4% Biolite 3/10, and then subjected to isoelectric focusing (the first-dimension). Electrophoresis was carried out for 5 hr with a constant voltage of 500 V per gel capillary tube. The proteins were then separated by SDS-PAGE (10%) in the second dimension, and electrophoresis was carried out for 45 min with a constant voltage of 200 V per gel. After electrophoresis, the gel was stained with 0.1% coomasie briliant blue R–250 in 40% methyl alcohol and 7.5% acetic acid. The gel was then destained in 10% methyl alcohol and 7.5% acetic acid.

(3) Gel filtration

Gel filtration was performed using a Superdex 200 column (2.6 × 60 cm, Amershan Pharmacia Biotech Ltd.) equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, at a constant flow rate of 150 m*l*/hr. The effluent was collected in 5 m*l* fractions and measured at 280 nm with a Shimadzu spectrophotometer type UV 1200.

(4) Assay of galactosidase activity

Galactosidase activity was assayed according to the method of Li and Li (1972), with slight modifications. ρ-nitrophenyl-α-galactopyranoside or ρ-nitrophenyl-β-galactopyranoside was used as a substrate. One ml of 2 mM substrate in 0.05 M sodium citrate buffer (pH 4.0) was added in enzyme solution or sample solution. The reaction mixture was incubated at 37℃ for 30 min. The reaction was stopped through 3 ml of 0.2 M borate buffer (pH 9.8). The mixture was measured for liberated ρ-nitrophenol by monitoring the absorbance at 400 nm with the spectrophotometer.

RESULTS and DISCUSSION

Figure 1 depicts the result of two dimensional polyacrylamide gel electrophoresis (2 D-PAGE) analysis on the domesticated haemolymph of *Bombyx mori* on 10th day in the fifth instar. 2 D-PAGE was performed using the Mini-PROTEAN mini tube gel two dimensional polyacrylamide gel electrophoresis system (Bio-Rad Laboratories, Inc.), as described in "MATERIALS and METHODS". The good result on various electrophoretical conditions using the haemolymph-protein was obtained when 4 mg of the dried haemolymph was solubilized with 1 ml of the first-dimension sample buffer containing 9.5 M urea, 2.0% Triton X–100, 5% 2–mercaptoethanol, 1.6% Biolite 5/7 and 0.4% Biolite 3/10. Twenty five μl of the sample solution was an optimum volume for loading on to the capillary tube. An optimum first-dimension (isoelectric focusing, IEF) run time was 5 hr with a constant voltage of 500 V per gel. After the first-dimension run was complete, the capillary tube gel was loaded on the slab gel for 2 D-PAGE with SDS-PAGE buffer containing 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, 0.5% bromophenol blue. An optimum

Fig. 1 Two dimensional polyacrylamide gel electrophoresis (2 D-PAGE) profiles of the haemolymph on 10 th day in the fifth instar of *Bombyx mori*.

2 D-PAGE run time was 45 min with a constant voltage of 200 V per gel. The horizontal way of Fig 1 shows the first-dimension (IEF) electrophoresis and the vertical way shows the 2 D-PAGE. The result showed the possibility that the haemolymph-protein was separated actually by means of this method, as shown in Fig. 1.

Figure 2 shows an elution pattern of gel filtration of the haemolymph of *B. mori*. A column of Superdex 200 was equilibrated with 0.1 M Tris-HCl buffer containing 0.1 M NaCl (pH 8.0) and eluted with the same buffer. Three significant fractions, Fraction I, Fraction II and Fraction III were obtained by gel filtration as shown in Fig. 2.

Figure 3 shows the result of the research of the activities of α-galactosidase and β-galactosidase in the fractions obtained by gel filtration of the haemolymph of *B. mori* showed in Fig. 2. Galactosidase activity was assayed according to the method of LI and LI (1972), with slight modifications, as described in "MATERIALS and METHODS". Both of α-galactosidase activity and β-galactosidase activity were recognized in the fractions, as shown in Fig. 3. Especially, Fraction II was rich in both of α-galactosidase and β-galactosidase. In the previous paper (KATO, 2002), I described that the result of FPLC Mono Q ion exchange chromatography on Fraction II obtained from the haemolymph of *B. mori* by gel filtration showed that the chromatogram revealed a single peak at the salt concentration of 0.25 M. However, the result of the research of galactosidase activities that both of α-galactosidase activity and β-galactosidase activity were recognized actually on the fraction at the salt concentration of 0.25 M by Mono Q ion exchange chromatography. Namely, the significant fraction obtained by Mono Q ion exchange chromatography of Fraction II from the *Bombyx* haemolymph seemed to contain both of α-

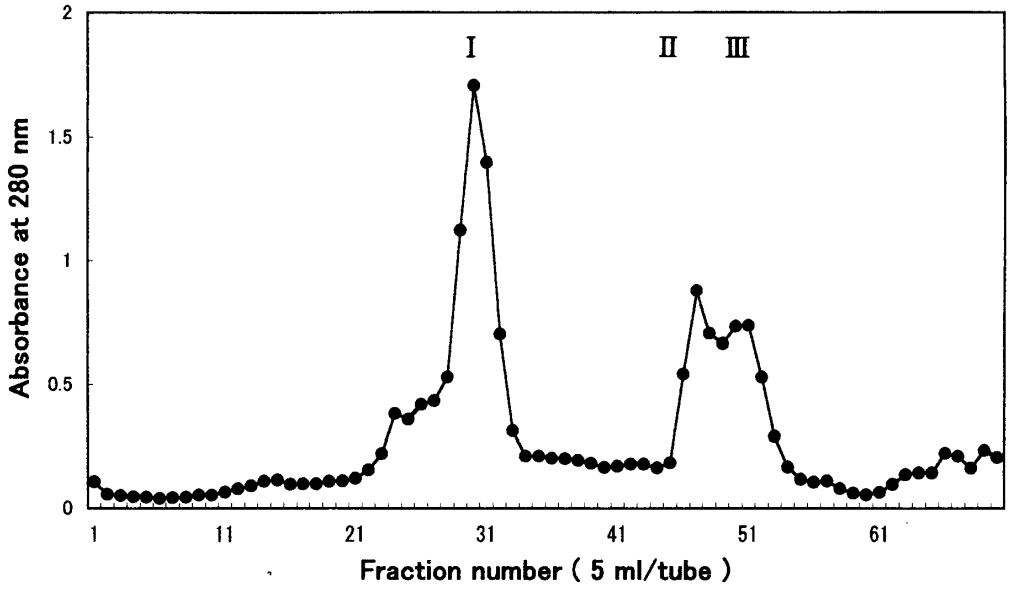

Fig. 2 Gel filtration of the haemolymph on a Superdex 200 column

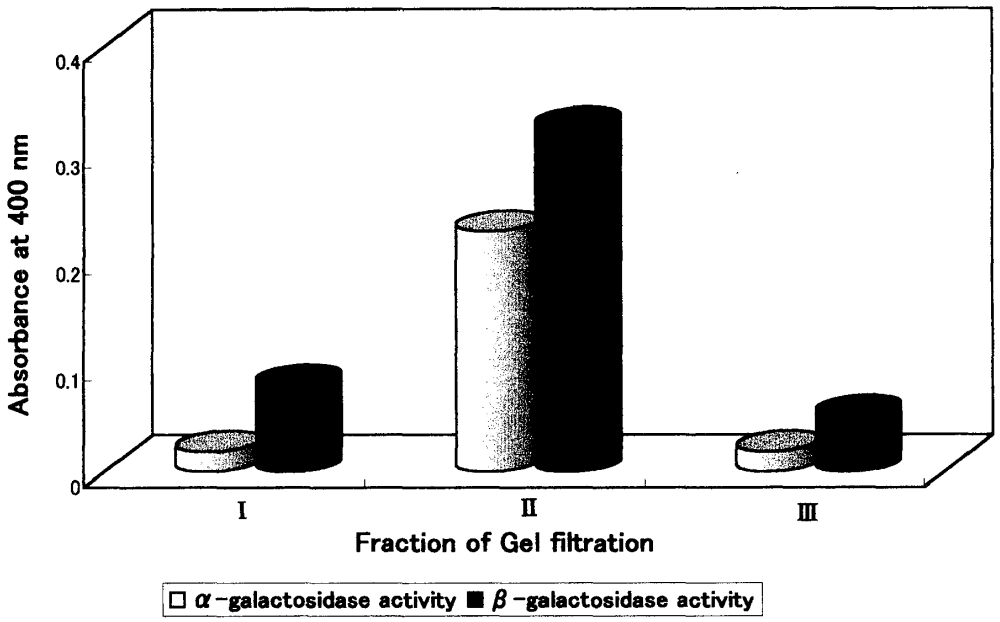

Fig. 3 Activities of α-galactosidase and β-galactosidase in the fractions obtained from the haemolymph by gel filtration on a Superdex 200 colum.

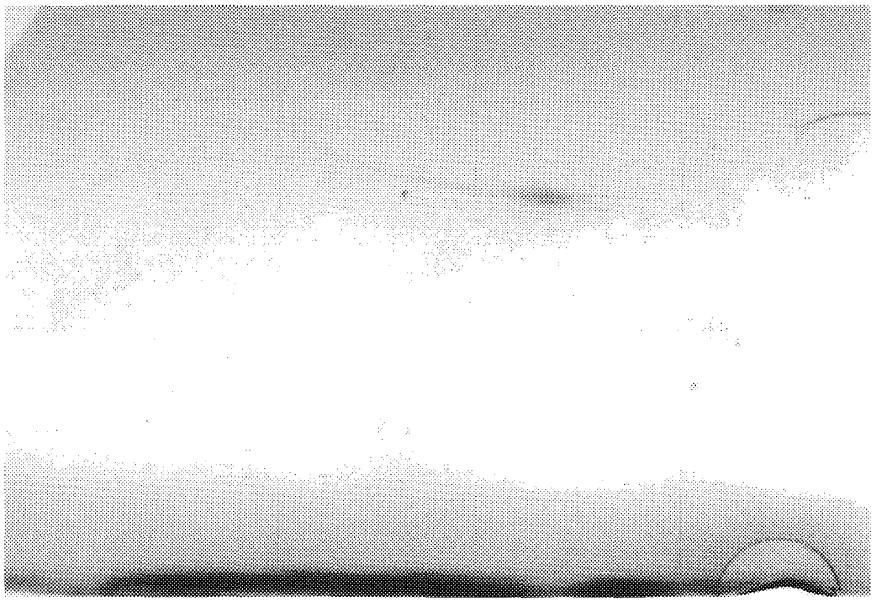

Fig. 4 Two dimensional polyacrylamide gel electrophoresis (2 D-PAGE) profiles of Fraction II obtained from the haemolymph by gel filtration on a Superdex 200 column

galactosidase and β-galactosidase, by means of FPLC. Accordingly, I tried to study the possibility about further separation of Fraction II using the 2 D-PAGE method as described above.

Figure 4 depicts the result of two dimensional polyacrylamide gel electrophoresis (2 D-PAGE) analysis on Fraction II obtained by gel filtration from the haemolymph of *B. mori*. Four mg of the dried Fraction II was solubilized with 1 m*l* of the first-dimension

sample buffer and 25 μl of the sample solution was loaded on the capillary tube gel. Electrophoresis was carried out for 5 hr with a constant voltage of 500 V per gel capillary tube. After the first-dimension (IEF) run was complete, the capillary tube gel was loaded on the slab gel for 2 D-PAGE with the SDS-PAGE buffer. Electrophoresis was carried out for 45 min with a constant voltage of 200 V per gel. The horizontal way of Fig. 4 shows the first-dimension (IEF) electrophoresis and the vertical way shows 2 D-PAGE. The result showed the possibility that the Fraction II was separated actually by means of this method, as shown in Fig. 4.

In conclusion, I emphasize that the significant fraction, Fraction II, obtained by gel filtration from the *Bombyx* haemolymph seemed to be separated by two dimensional polyacrylamide gel electrophoresis, and that it will be an useful method for studying galactosidase relating to the *Bombyx* humoral lectin activity. It needs further study from various points of view about the *Bombyx* humoral lectin and the enzymes relating to the lectin activity, such as neuraminidase and galactosidase, in view of their significance for the living body.

SUMMARY

Two dimensional polyacrylamide gel electrophoresis (2 D-PAGE) analysis on the haemolymph of *Bombyx mori* was performed using the Mini-PROTEAN mini tube gel two dimensional polyacrylamide gel electrophoresis system (Bio-Rad Laboratories, Inc.). The result on various electrophoretical conditions using the haemolymph-protein showed the possibility that the haemolymph-protein was separated actually by means of this method. Moreover, the result of 2 D-PAGE analysis on Fraction II obtained by gel filtration from the haemolymph of the domesticated silkworm of *Bombyx mori* showed that the fraction seemed to be separated by two dimensional polyacrylamide gel electrophoresis.

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