

Comparative Studies of Two-Dimensional Electrophoresis on Galactosidase Relating to *Bombyx* Lectin Activity.

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INTRODUCTION

Biological activities elicited by lectins include cell agglutination, mitosis, apoptosis and so on. For these activities, animal lectins seem to play an important role in a defense mechanism of the living body, such as removal of a foreign matter or a biopolymer which has lost the normal function in the living body (KAWABATA, 2000 ; FURUTA *et al.*, 2001 ; KAWASAKI, 2001 ; WAGO, 2001 ; KOYAMA *et al.*, 2002 ; HIRABAYASI, 2004). On the other hand, 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).

In a previous study, I showed that a humoral lectin (130 K-glycoprotein) of *Bombyx mori* played a physiological role on cellular communication through metamorphosis of the domesticated silkworm, *B. 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 *Bombyx* humoral 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 of *B. mori*. Accordingly, I tried the novel technology to fractionate galactosidase in the haemolymph

of *B. mori* by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (KATO, 2004), because major advancements are being made in the field of proteomics with mass spectrometric identification of proteins separated by 2-D PAGE, currently (FURUTA *et al.*, 2002 ; OH-ISHI, 2002 ; KONDO, 2004).

Here I describe comparative studies of 2-D PAGE on galactosidase relating to *Bombyx* lectin activity. 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 domesticated 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 by cutting the larval abdominal legs. After centrifuging the haemolymph at 3,500 rpm for 15 min at 4°C, each of resultant supernatant was lyophilized.

(2) The first method of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The first method of 2-D PAGE was performed using the 2-D minislab system (Atto Co.). The proteins were subjected to agar gel for isoelectric focusing (the first dimension) with a linear immobilized pH gradient (range 5–8). Electrophoresis was carried out for 210 min with a constant voltage of 300 V per gel tube. The proteins were then separated by SDS-PAGE (10%) in the second dimension, and electrophoresis was carried out for 90 min with a constant electric current of 20 mA per gel. After electrophoresis, the gel was stained with 0.1% coomassie brilliant blue R-250 in 30% methyl alcohol and 10% acetic acid. The gel was then destained in 30% methyl alcohol and 10% acetic acid.

(3) The second method of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The second method of 2-D PAGE was performed using the Mini-PROTEAN mini tube gel 2-D PAGE system (Bio-Rad Laboratories, Inc.). The proteins were subjected to isoelectric focusing (the first dimension) with a linear immobilized pH gradient (range 5–7). 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% coomassie brilliant blue R-250 in 40% methyl alcohol and 7.5% acetic acid. The gel was then destained in 10% methyl alco-

hol and 7.5% acetic acid.

(4) 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 ml/hr. The effluent was collected in 5 ml fractions and measured at 280 nm with a Shimadzu spectrophotometer type UV 1200.

(5) Assay of galactosidase activity

Galactosidase activity was assayed according to the method of LI and LI (1972), with slight modifications. *p*-nitrophenyl- α -galactopyranoside or *p*-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°C 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 *p*-nitrophenol by monitoring the absorbance at 400 nm with the spectrophotometer.

RESULTS and DISCUSSION

Figure 1 shows the result of the first method of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) on the haemolymph of the domesticated silkworm, *Bombyx mori*, on 10 th day in the fifth instar. The first method of 2-D PAGE was performed using the 2-D minislabs system (Atto Co.). The good result on various electrophoretic conditions using the haemolymph-protein was obtained when 4 mg of the haemolymph-protein was solubilized with 1 ml of the first dimension sample buffer containing 0.1 M Tris, 6 M urea, 1 M thiourea, Complete mini EDTA-free (a grain 10 ml), 1% CHAPS, 1% Triton X-100 and 1% DTT, and then subjected to agar gel (pH 5~8) for isoelectric focusing (the first dimension). Twenty five μ l of the solution was an optimum volume for loading on to the tube. An optimum first dimension (isoelectric focusing, IEF) run time was 210 min with a constant voltage of 300 V per gel. After the first dimension run was complete, the 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, 5% bromophenol blue. An optimum 2-D PAGE run time was 90 min with a constant electric current of 20 mA per gel. The horizontal way of Fig. 1 shows the first dimension (IEF) electrophoresis and the vertical way shows 2-D PAGE. The right lane of Fig. 1 shows SDS-PAGE profile of LMW (Low molecular weight) Kit-protein. The result showed the possibility that the

pH gradient (pH 5-8)

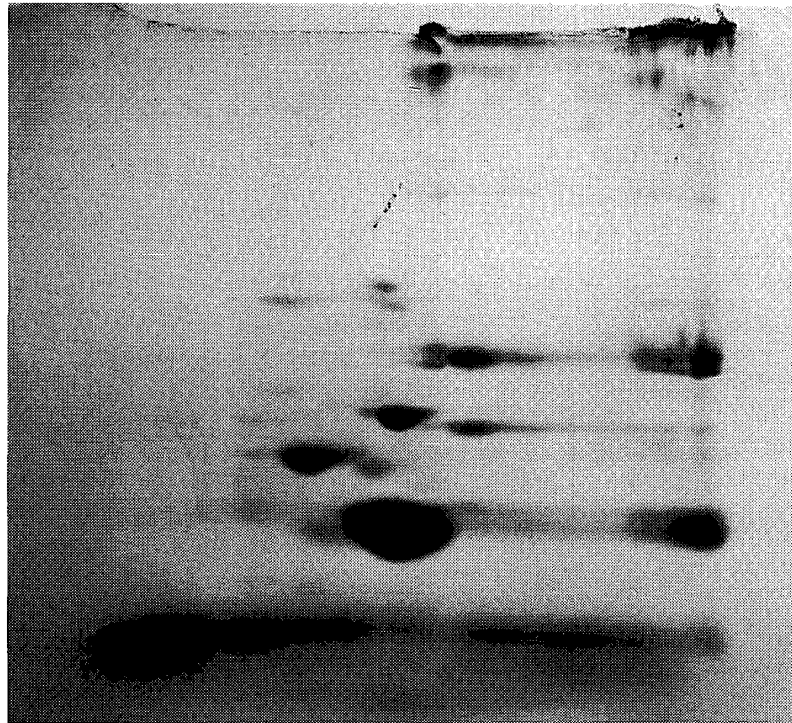


Fig. 1 The first method's profile of 2-D PAGE on the haemolymph of *Bombyx mori*

haemolymph-protein was separated clearly by means of this method, as shown in Fig. 1. Moreover, the result showed the possibility that molecular weight of the haemolymph-protein might be determined with isoelectric point, by means of SDS-PAGE on LMW Kit-protein.

Figure 2 shows the result of the second method of 2-D PAGE on the haemolymph of *Bombyx mori* on 10 th day in the fifth instar. The second method of 2-D PAGE was performed using the Mini-PROTEAN mini tube gel 2-D PAGE system (Bio-Rad Laboratories, Inc.). 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 μ l 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 2-D PAGE run time was 45 min with a constant voltage of 200 V per gel. The horizontal way of Fig. 2 shows the first dimension (IEF) electrophoresis and the vertical way shows 2-D PAGE. The result showed that the haemolymph-protein was separated faintly by means of this method, as shown

pH gradient (pH 5-7)

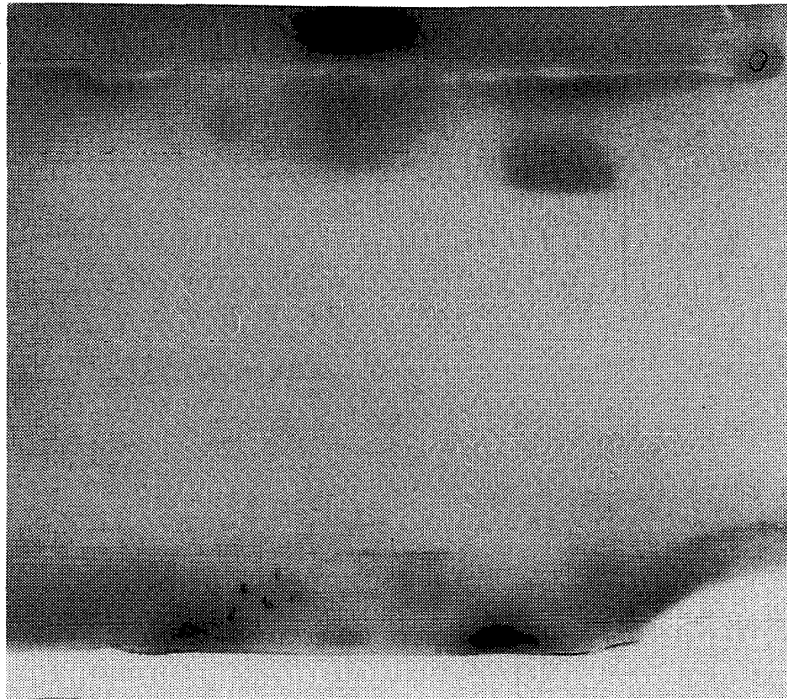


Fig. 2 The second method's profile of 2-D PAGE on the haemolymph of *Bombyx mori*

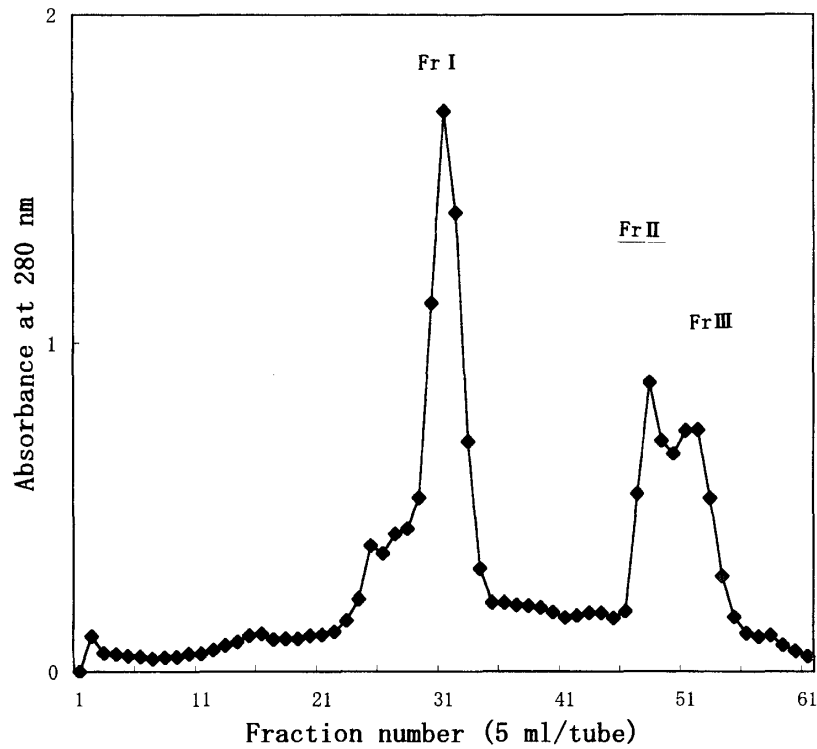


Fig. 3 Gel filtration of the haemolymph on a Superdex 200 column

in Fig. 2. As shown in Fig. 1 and Fig. 2, more distinct 2-D PAGE profile of the haemolymph-protein was obtained by means of the first method of 2-D PAGE than the second method of 2-D PAGE.

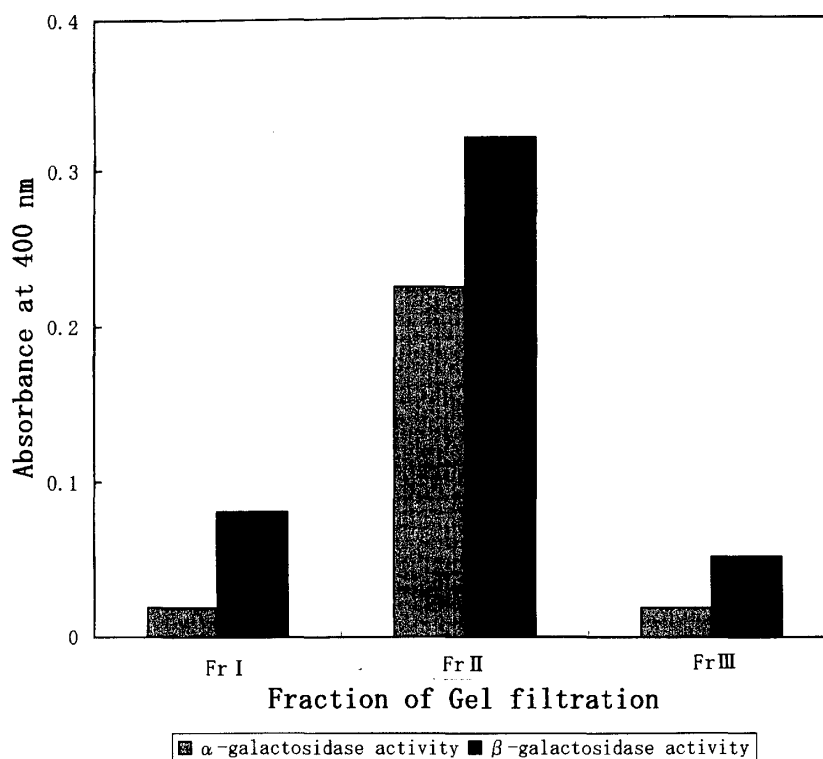


Fig. 4 Activity of galactosidase in the fractions obtained from the haemolymph by gel filtration

Figure 3 depicts 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. 3.

Figure 4 depicts 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. 3. 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. 4. Especially, Fraction II was rich in both of α -galactosidase and β -galactosidase. Accordingly, I tried to study the possibility about further separation of Fraction II using the 2-D PAGE method as described above.

Figure 5 shows the result of the first method of 2-D PAGE on Fraction II obtained by gel filtration from the haemolymph of *B. mori* as shown in Fig. 4. The first method of 2-D PAGE was performed using the 2-D minislabs system. The result showed the possibility that Fraction II was separated clearly by means of this method, as shown in Fig. 5.

Figure 6 shows the result of the second method of 2-D PAGE on Fraction II obtained by gel filtration from the haemolymph of *B. mori*. The second method of 2-D PAGE was performed using the Mini-PROTEAN mini tube gel 2-D PAGE system. The result showed

pH gradient (pH 5-8)

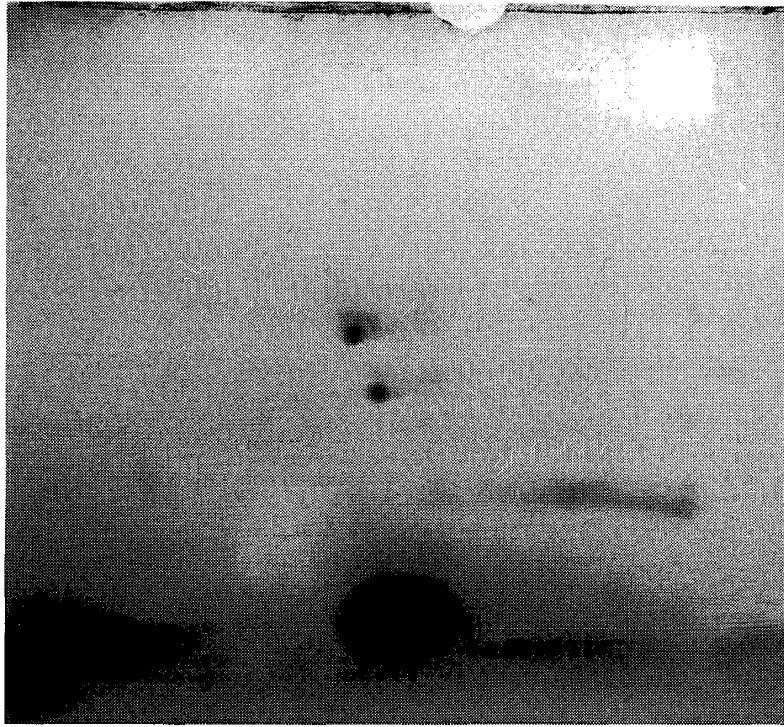


Fig. 5 The first method's profile of 2-D PAGE on the Fraction II obtained from the haemolymph by gel filtration

pH gradient (pH 5-7)

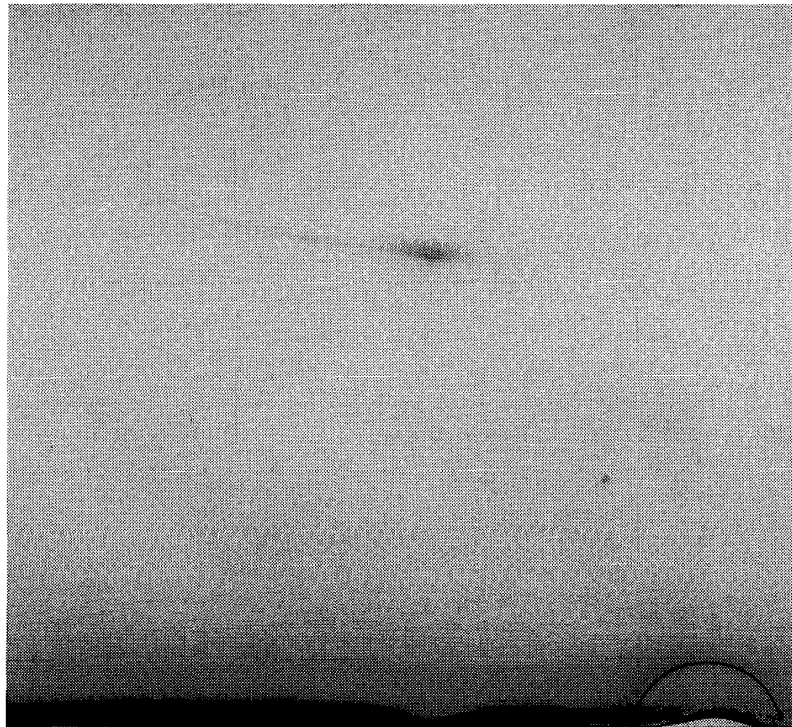


Fig. 6 The second method's profile of 2-D PAGE on the Fraction II obtained from the haemolymph by gel filtration

pH gradient (pH 5-8)

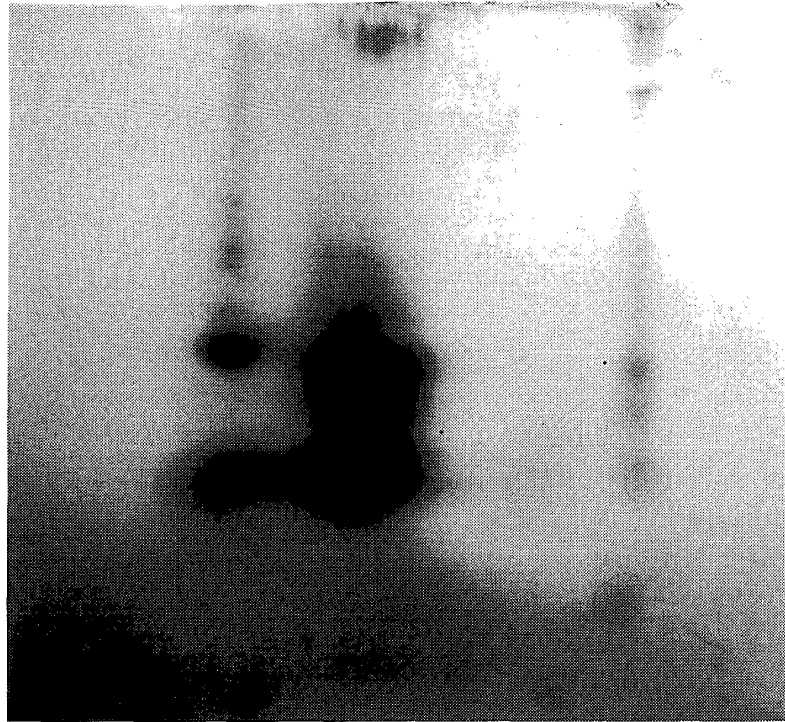


Fig. 7 The first method's profile of 2-D PAGE on standard β -galactosidase, grade III from bovine liver by Sigma Co.

that Fraction II was separated faintly by means of this method, as shown in Fig. 6. As shown in Fig. 5 and Fig. 6, more distinct 2-D PAGE profile of Fraction II was obtained by means of the first method of 2-D PAGE than the second method of 2-D PAGE, as well as 2-D PAGE profile of the haemolymph-protein.

Figure 7 shows the result of the first method of 2-D PAGE on standard β -galactosidase, grade III from bovine liver by Sigma Co. The first method of 2-D PAGE was performed using the 2-D minislabs system as described above. The result showed the possibility that the standard β -galactosidase was separated clearly by means of this method, as shown in Fig. 7.

In conclusion, I emphasize that haemolymph-protein, the significant fraction, Fraction II, obtained by gel filtration from the *Bombyx* haemolymph and standard β -galactosidase seemed to be separated clearly by the first method of 2-D PAGE, 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

Comparative two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) analysis on the haemolymph of the domesticated silkworm, *Bombyx mori* and Fraction II obtained by gel filtration from the haemolymph of *B. mori* was performed using the 2-D mini-slab system (Atto Co.) (the first method of 2-D PAGE) and the Mini-PROTEAN mini tube gel 2-D PAGE system (Bio-Rad Laboratories, Inc.) (the second method). Moreover, two-dimensional electrophoresis analysis on standard β -galactosidase, grade III from bovine liver by Sigma Co., was performed using the 2-D minislabs system (the first method). The result on various electrophoretic conditions showed the possibility that all of them seemed to be separated clearly by the first method of 2-D PAGE.

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