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A Study on Galactosidase relating to a *Bombyx* Humoral Lectin Activity

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INTRODUCTION

An animal lectin plays a significant role in a defense mechanism of living body such as cell adhesion or endocytosis (FUJITA, 2000 ; NOMURA *et al*., 2000). For instance, invertebrate lectins play a significant role in a non-specific self defense mechanism (KAWABATA, 2000) and in an apoptosis mechanism (HIRABAYASHI and KASAI, 1998). Especially, humoral lectins of insects play a significant role through metamorphosis of insects and will be useful defense substances for human being in future (KOTANI *et al*., 1995 ; FUJITA *et al*., 1998 ; NATORI, 1998). 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, we reported that a humoral lectin-protein (130 K-glycoprotein) played a physiological role through metamorphosis of the silkworm, *Bombyx mori*, because it always seemed to possess the highest lectin activity on spinning stage (KATO *et al*., 1994). We also reported on the appearance and the disappearance of the lectin activity. We emphasized the possibility that the humoral lectin-protein was produced and activated in fat body of *Bombyx mori*, and that it was secreted into haemolymph (KATO *et al*., 1998). Moreover, we researched a *Bombyx* humoral lectin activating factor in fat body by means of FPLC system, and obtained neuraminidase-like enzyme from the fat body (KATO and NAKAMURA, 1999, 2000). On the other hand, we have found that the active lectin disappeared the activity, when it was treated with galactosidase (KATO and NAKAMURA, 1987).

This paper reports on galactosidase activity relating to a *Bombyx* humoral lectin activity, especially on a method of an estimation of galactosidase 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 silkworm, *Bombyx mori*, was used in this experiment. Larvae were reared with fresh mulberry leaves. In preparing the samples for this research, haemolymph and fat body were collected. Haemolymph samples were collected by cutting the larval abdominal legs or pupal abdomen. Fat body samples were collected from dissected larvae or pupae, washed with cold 0.7% NaCl solution and homogenized in a glass homogenizer with a Teflon pestle. After centrifuging them at 3,000 rpm for 10 min at 4℃, each of resultant supernatant was lyophilized.

2. 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 a Shimadzu spectrophotometer type UV 1200. The scheme of the procedure was shown in Fig. 1.

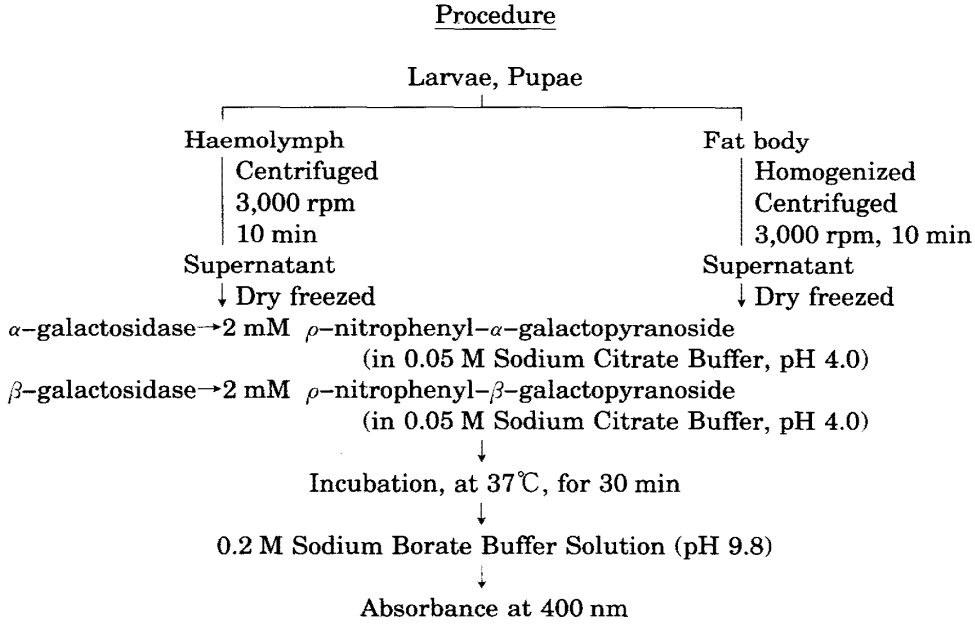

Fig. 1 Procedure of estimation

3. Gel filtration

Gel filtration was performed using a Superdex 200 (Pharmacia) column (2.6×60 cm) 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 the spectrophotometer.

RESULTS and DISCUSSION

We studied on the method of the estimation of galactosidase activity in detail, according to the procedure shown in Fig. 1. Figure 2 shows a standard curve of *ρ*-nitrophenol. The dilute solution of *ρ*-nitrophenol in 0.05 M sodium citrate buffer was colored through the addition of 0.2 M borate buffer, and then the absorbance was measured at 400 nm. It was confirmed that the optical density was directly proportional to the density of *ρ*-nitrophenol as shown in Fig. 2. Figure 3 shows the result of the study on a substrate of galactosidase. As shown in Fig. 3, it was confirmed that the color reaction looked no color in cases of only 0.2 M sodium borate buffer, *ρ*-nitrophenyl-*β*-galactopyranoside in 0.05 M sodium citrate buffer with 0.2 M sodium borate buffer and 5 mg of dried matter of haemolymph in 0.05 M sodium citrate buffer with 0.2 M sodium borate buffer. On the other hand, it was confirmed that the color reaction looked yellow in cases

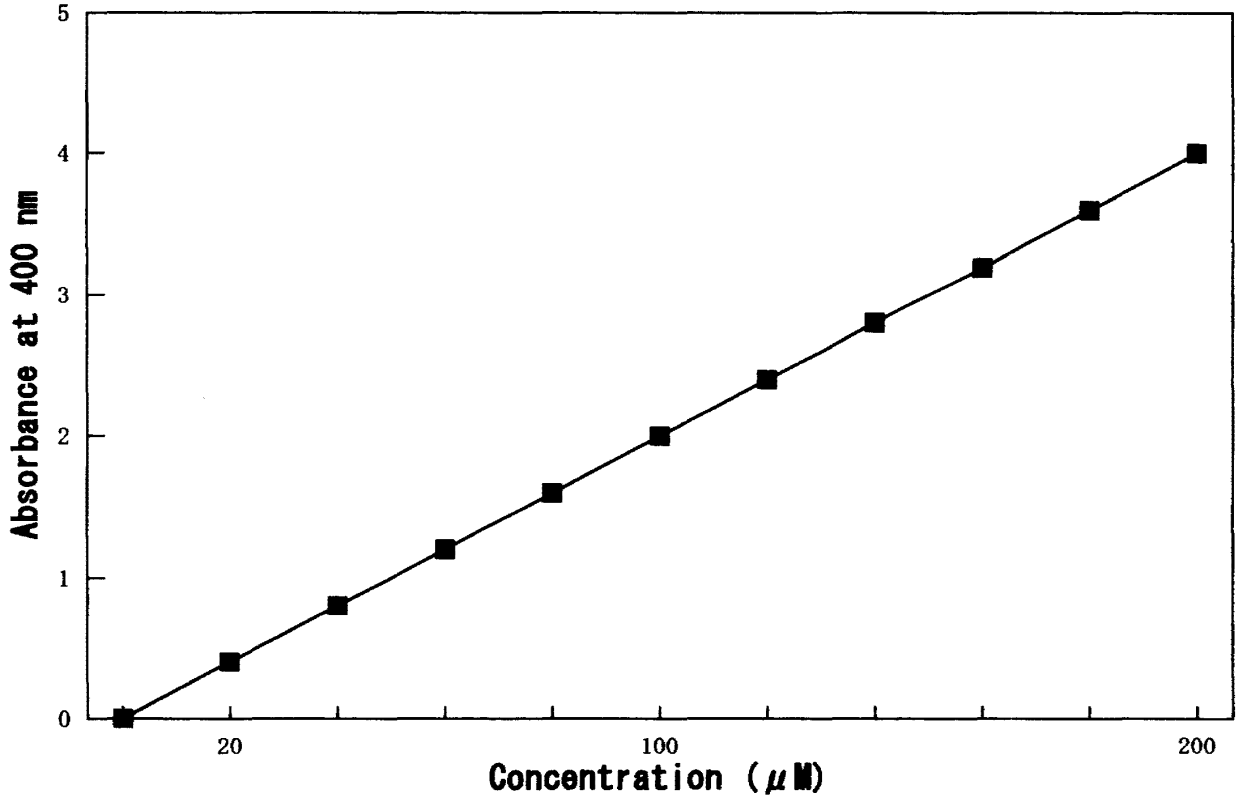

Fig. 2 Standard curve of *ρ*-nitrophenol

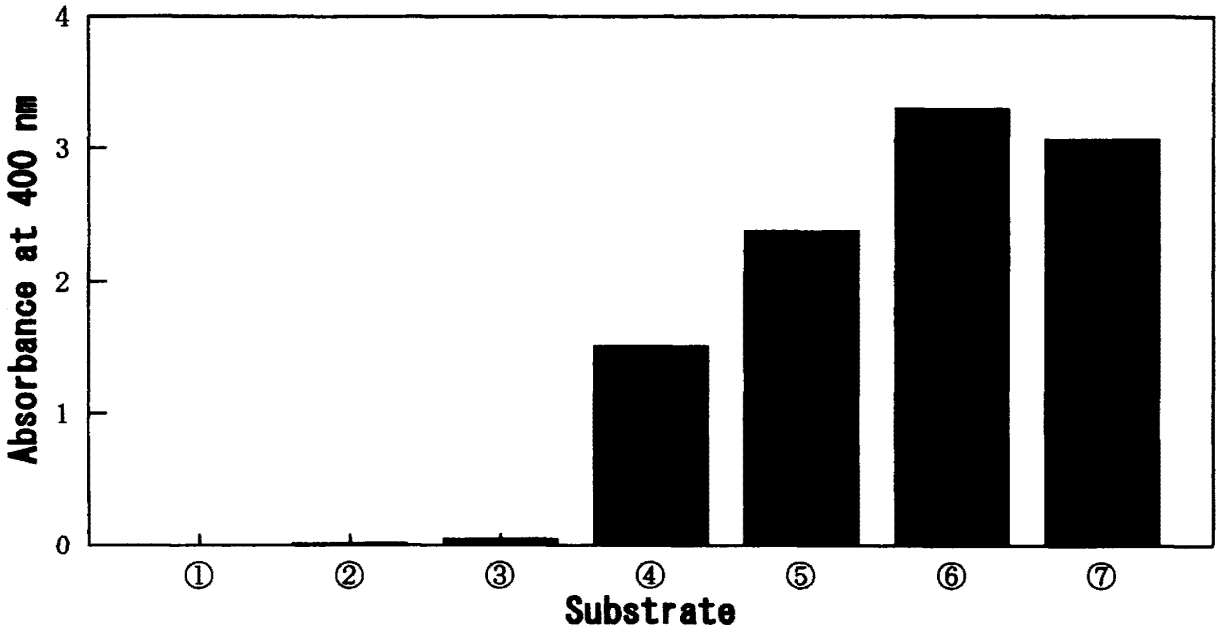

Fig. 3 Activity of galactosidase against substrate
① : 0.2 M sodium borate buffer solution
② : ρ-nitrophenyl-β-galactopyranoside
③ : 5 mg of dried matter of haemolymph
④ : 5 mg of dried matter of haemolymph + ρ-nitrophenyl-β-galactopyranoside
⑤ : 10 mg of dried matter of haemolymph + ρ-nitrophenyl-β-galactopyranoside
⑥ : 20 mg of dried matter of haemolymph + ρ-nitrophenyl-β-galactopyranoside
⑦ : β-galactosidase + ρ-nitrophenyl-β-galactopyranoside

of 5 mg of dried matter of haemolymph in 0.05 M sodium citrate buffer containing ρ-nitrophenyl-β-galactopyranoside with 0.2 M sodium borate buffer and β-galactosidase in 0.05 M sodium citrate buffer containing ρ-nitrophenyl-β-galactopyranoside with 0.2 M sodium borate buffer. The optical density increased as well as the increase of the density of the haemolymph, as shown in Fig. 3. Therefore, next experiment was performed using 5 mg of the dried matter of the haemolymph. It was also confirmed that each of ρ-nitrophenyl-α-galactopyranoside and ρ-nitrophenyl-β-galactopyranoside was a substrate of α-galactosidase and β-galactosidase, respectively.

Table 1 shows the result of the research of α-galactosidase activity and β-galactosidase activity in haemolymph during the fifth instar to pupal stage of *Bombyx mori*. Both of the activities were recognized in haemolymph as shown in Table 1. It showed that β-galactosidase activity was higer than α-galactosidase activity in all days. The activity was the highest on spinning stage. Besides, it was guessed that the activity became high on the young day of the fifth instar, because galactosidase for the ecdysis of the fourth instrar remained high. Table 2 shows the result of the research of α-galactosidase activity and β-galactosidase activity in fat body during the fifth instar to pupal stage of *Bombyx mori*. Both of the activities were recognized in fat body as shown in Table 2. Also, it showed that β-galactosidase activity was higer than α-galactosidase activity in all days.

Table 1 Activities of α-galactosidase and β-galactosidase in haemolymph of *Bombyx mori*

	Activity of α-galactosidase	Activity of β-galactosidase
	Absorbance	Absorbance
3 rd day	0.226	3.010
6 th day	0.148	1.637
8 th day	0.157	1.724
9 th day	0.189	1.755
10 th day	0.187	1.744
Pupae	0.263	1.614

Table 2 Activities of α-galactosidase and β-galactosidase in fat body of *Bombyx mori*

	Activities of α-galactosidase	Activities of β-galactosidase
	Absorbance	Absorbance
3 rd day	0.125	0.360
6 th day	0.107	0.298
8 th day	0.122	0.350
9 th day	0.366	0.506
10 th day	0.117	0.298
Pupae	0.273	1.458

The activity was the highest on spinning stage, more distinctly than the occasion of haemolymph. Moreover, the time of the highest activity in fat body seemed to be differ from one in haemolymph. Namely, the time of the highest activity in fat body seemed to be earlier than one in haemolymph. We reported previously on the relationship between galactosidase and haemagglutination activity of the *Bombyx* lectin *in vitro* (1987). Namely, the active lectin disappeared the activity, when it was treated with galactosidase. On that occasion, β-galactosidase was more effective than α-galactosidase against disappearance of the lectin activity. The fact seemed to coincide with the result that β-galactosidase activity was higer than α-galactosidase activity in all days as described above.

Figure 4 shows an elution pattern of gel filtration on haemolymph of *Bombyx mori* on day 10 of the fifth instar. A column of Superdex 200 pg 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. 4. Figure 5 shows the results of the research of α-galactosidase activity and β-galactosidase activity in the three fractions obtained from haemolymph by gel filtration. As shown in Fig. 5, it was suggested that both of them were present in Fraction II. We reported previously that the active lectin-protein contained galactose as neutral sugar in its sugar-chain, and that galactosidase seemed to be present in haemo-

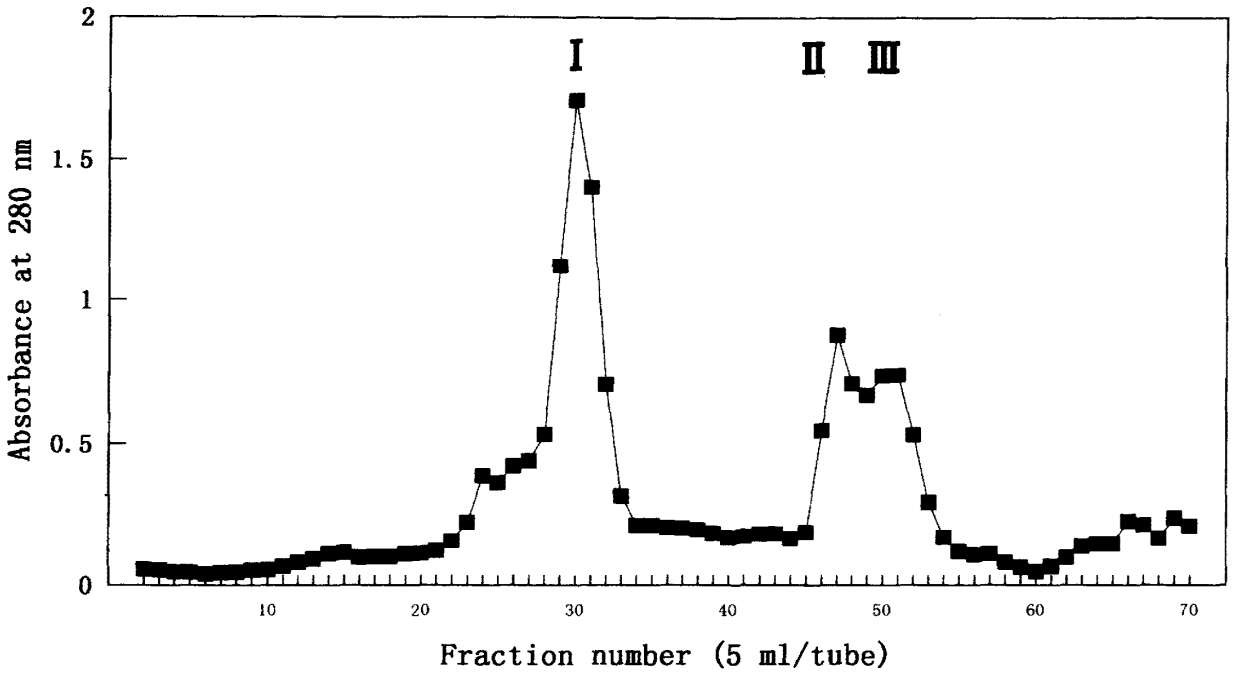

Fig. 4 Elution pattern of gel filtration on a Superdex 200 column

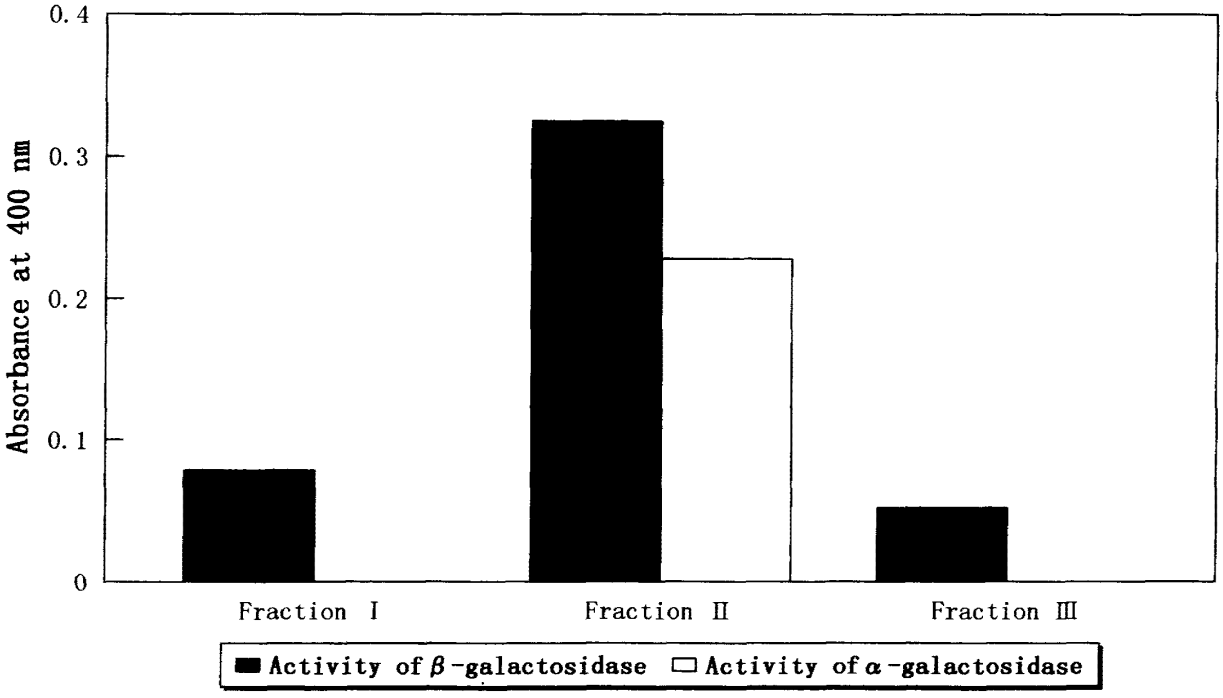

Fig. 5 Activities of α-galactosidase and β-galactosidase in the fractions obtained by gel filtration on a Superdex 200 column

lymph. However, they were reasoning on experimental results *in vitro*. Accordingly, it seems to be significance that the presence and activity of galacotosidase in haemolymph were recognized actually on experimental results *in vivo* as described above.

In conclusion, we emphasize that galactosidase might be presents actually in the living body of *Bombyx mori*. The *Bombyx* humoral lectin was activated with neuraminidase

on spinning stage as described in the previous paper (KATO *et al*., 1998). At that time, the lectin activity disappeared rapidly because galactosidase activity became high. We will try to elucidate reason why in future.

SUMMARY

We studied on the method of the estimation of galactosidase activity in detail. Both of α-galactosidase activity and β-galactosidase activity were recognized in haemolymph and fat body during the fifth instar to pupae stage of *Bombyx mori*. It showed that the β-galactosidase activity was higer than α-galactosidase activity in all days. The activity was the highest on spinning stage. Moreover, we researched on galactosidase activity in fractions obtained from haemolymph by means of gel filtration. These results suggested the possibility that galactosidase might be present actually in the living body of *Bombyx mori*, and that galactosidase related to the *Bombyx* humoral lectin activity.

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