The Relationship between Sialidase Activity and Lectin Activity in Haemolymph of *Bombyx mori*

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INTRODUCTION

A family of lectins is generally called as one of the characteristic protein group which has the specific property reacting reversibly with a specific sugar residue. For this specificity, an animal lectin binds with glycoproteins of cell membrane and it seems to be concerned in the various reaction of the living body.

Invertebrate lectins, especially soluble lectins in the haemolymph of some insects, have been of interest because they seem to play fundamental roles in the defence and differentiation mechanisms of the living bodies (KOMANO *et al*., 1980; SUZUKI and NATORI, 1983; KATO *et al*., 1988; MORI *et al*., 1989). Moreover, KURATA (1992) emphasized the importance of insect lectins for insect metamorphosis and self-non self recognition.

We reported previously (KATO *et al*., 1991, 1993) that the lectin activity of *Bombyx mori* may be influenced by the administration of terpenoid imidazole (KADONO-OKUDA *et al*., 1987; AKAI and MAUCHAMP, 1989) and juvenile hormone analogue (KAJIURA *et al*., 1987). Moreover, it has been shown that the lectin plays physiological role through metamorphosis of *Bombyx mori* because the lectin seems to possess the highest activity on spinning day at all times. However the activation mechanism of the lectin still remains obscure.

In the present investigation, we have made a comparative study of sialidase activity in the larval haemolymph of *Bombyx mori* treated with juvenile hormone analogue (JHA) to obtain further information on the appearance mechanism of the lectin activity *in vivo*.

MATERIALS AND METHODS

1. Preparation of samples

A hybrid race, Shunrei × Shogetu, of the silkworm, *Bombyx mori*, was used in this experiment. Thirty μg/larva of methoprene (JHA; Ohtsuka Pharmaceutical Co.) was applied topically to larval skin on the second day of the fifth instar. After the application of JHA, the larvae were reared on mulberry leaves, while the control larvae were reared on

mulberry leaves without application of JHA.

In preparing the samples for this research, larval haemolymph was collected daily. After centrifuging the haemolymph at 3,500 rpm for 15 min at 4°C, the supernatant that resulted was lyophilized.

2. Thiobarbituric acid assay

Thiobarbituric acid assay was performed according to the method of WARREN (1959). To a sample of 0.2 *ml* was added 0.1 ml of 0.2 M periodate solution. The tubes were shaken and allowed to stand at room temperature for 20 min. Asenite solution (10%), 1 *ml*, was added and the tubes were shaken until a yellow-brown color disappears. Thiobarbituric acid solution (0.6%), 3 *ml*, was added, the tubes shaken, capped with a glass bead, and then heated in a virgorously boiling water bath for 15 min. The tubes were then removed and placed in cold water for 5 min. After 4.3 *ml* of cyclohexanone was added, the tubes were shaken and then centrifuged for 3 min in a clinical centrifuge. The clear upper cyclohexanone phase was red. The spectrum was estimated at the scan speed of 60 nm per min and the chart speed of 120 mm per min with a Hitachi spectrophotometer type 100-50.

3. Sialic acid analysis by high performance liquid chromatography (HPLC)

Five mg of a lyophilized product was hydrolyzed with 1 ml of 0.2 N-H_2SO_4 for 30 min at 80°C to yield sialic acid. Then sialic acid was analyzed on a column of Shim-pack SCR-101H according to the method of MURAKITA (1987). Elution was performed with aqueous solution of phospholic acid (pH 1.5) and the flow rate was 1.0 *ml* per min. Column eluates were monitored at 205 nm with a ultra violet spectrophotometric detector SPD-6A.

RESULTS AND DISCUSSION

The larvae of *Bombyx mori* which were treated with methoprene (JHA, 30 μg/larva) on day 2 of the fifth instar showed a prolonged feeding period and the lectin obtained from the JHA-treated mature larvae was similar to the active lectin such as the one obtained from the control mature larvae. The result seems to coincide with the relationship described in the previous paper (NAKAMURA and KATO, 1992) between the JHA-treated larvae and the controls. Next, individual haemolymph was obtained from the JHA-treated larvae on day 4, on day 7, on day 10 and on day 12 of the fifth instar by means of the method described as "Materials and Methods" for assay of sialidase activity in the

haemolymph of *Bombyx mori*. Because the JHA-treated larvae began spinning on the twelfth day of the fifth instar, while the controls began spinning on the tenth day of the fifth instar in the present experiment.

N-acetylneuramin lactose from bovine colostrum was used as substrate for assay of sialidase activity in the haemolymph and the activity was estimated according to determine free sialic acid separated from N-acetylneuramin lactose treated with the haemolymph of *Bombyx mori*. Then analysis of sialic acid was performed by means of thiobarbituric acid assay and high performance liquid chromatography (HPLC).

Figure 1 shows the ultra violet absorption spectrum of the clear upperred cyclohexanone phase obtained by means of thiobarbituric acid assay. Figure 1(a) shows the spectrum from N-acetylneuraminic acid (NANA), while Fig. 1(b) shows the spectrum from sample haemolymph. Each spectrum showed a maximum of absorbance at 549 nm. Accordingly, it was confirmed that the sample haemolymph included sialic acid.

Figure 2(a) shows a chromatogram of N-acetylneuramin lactose in Tris-HCI buffer by means of HPLC. The chromatogram revealed several peaks, although it revealed no peak caused by sialic acid. Next, N-acetylneuramin lactose was treated with neuraminidase solution for 1 hr at 37°C, because neuraminidase was used as standard sialidase. Then analysis of sialic acid was performed by means of HPLC. The result is shown in Fig. 2(b). The chromatogram revealed a peak caused by NANA, which had a retention time of 5.98 min.

Figure 3(a) shows a chromatogram of the JHA-treated haemolymph on day 10 in Tris-HCI buffer. The chromatogram revealed several peaks. The peak which had the

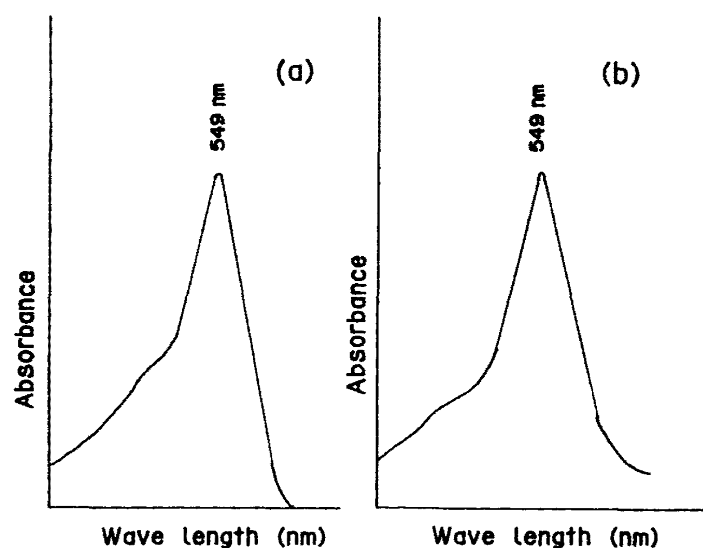


Fig. 1 Ultraviolet absorption spectrum

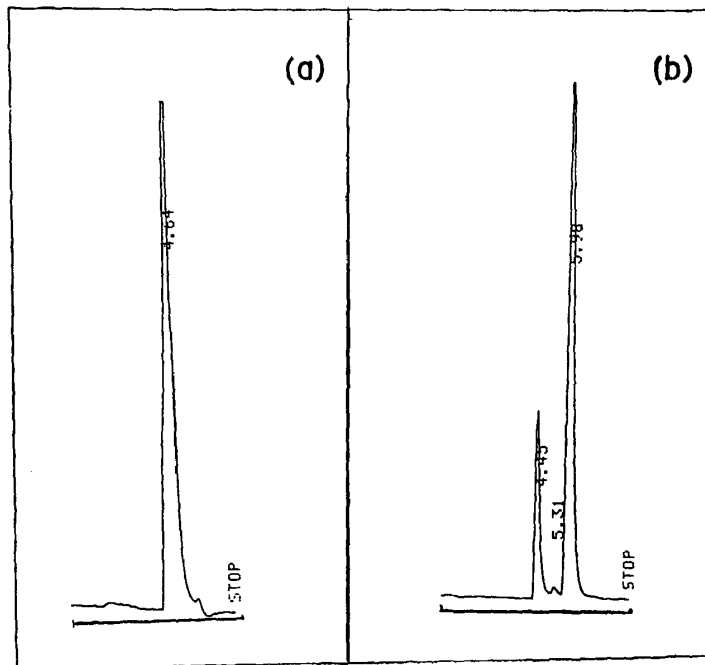
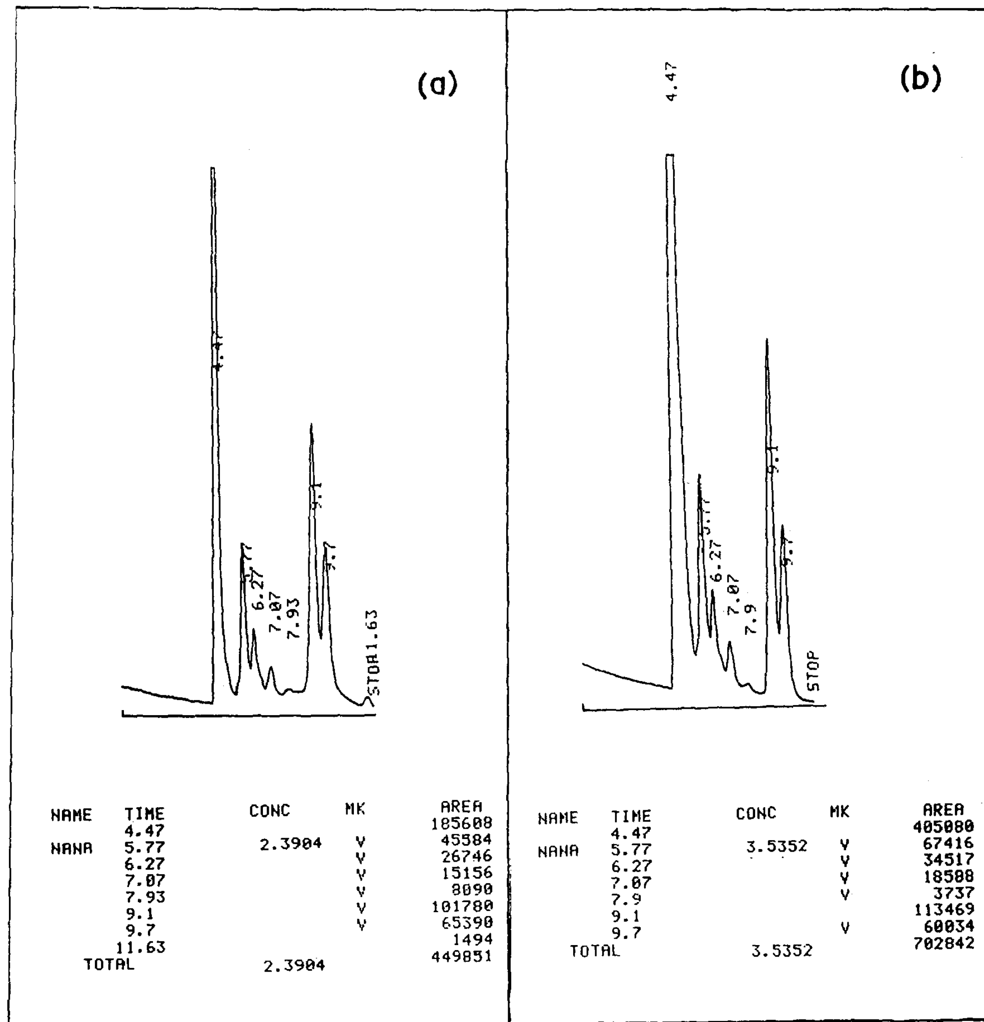


Fig. 2 Chromatogram of N-acetylneuramin lactose treated with neuraminidase by HPLC.



(a)

NAME	TIME	CONC	MK	AREA
	4.47			185608
NANA	5.77	2.3904	V	45584
	6.27		V	26746
	7.07		V	15156
	7.93		V	8090
	9.1		V	101780
	9.7		V	65390
	11.63			1494
TOTAL		2.3904		449851

(b)

NAME	TIME	CONC	MK	AREA
	4.47			405080
NANA	5.77	3.5352	V	67416
	6.27		V	34517
	7.07		V	18508
	7.9		V	3737
	9.1			113469
	9.7		V	60034
TOTAL		3.5352		702842

Fig. 3 Chromatogram of N-acetylneuramin lactose treated with the haemolymph on day 10 by HPLC.

retention time of 5.77 min was caused by sialic acid. The other peaks are unknown. As shown in the chromatogram, the haemolymph on day 10 included free sialic acid of 2.390 μg/mg dried matter. On the other hand, Fig. 3(b) shows a chromatogram of N-acetylneuramin lactose treated with the haemolymph on day 10 for 1 hr at 37°C. The peak which had the retention time of 5.77 min was caused by sialic acid. As shown in the chromatogram, the haemolymph on day 10 seems to include free sialic acid of 3.535 μg/mg dried matter. Therefore, it seems that sialic acid of 1.145 μg was separated from N-acetylneuramin lactose by sialidase in the haemolymph, judging from these two chromatograms. The sialidase activity in the haemolymph on day 4, on day 7 and on day 12 was estimated in the same manner. The results are summarized in Table 1.

Table 1 Sialidase activity in the haemolymph.

	Sialidase activity (Unit)
Haemolymph on day 4	3.0×10^{2}
Haemolymph on day 7	3.9×10^{2}
Haemolymph on day 10	12.4×10^{2}
Haemolymph on day 12	4.6×10^{2}

The sialidase activity in the larval haemolymph of *Bombyx mori* changed through the larval maturation as shown in Table 1. The sialidase activity kept low level on day 4 and on day 7, when the lectin of *Bombyx mori* was inactive in the haemolymph. The activity increased on day 10 but decreased on day 12. In fact, the sialidase activity increased at the larval maturation but decreased on spinning day. Especially, it was evident that the activity decreased remarkably just after maturation. On the other hand, the result of sialic acid analysis in the previous paper (NAKAMURA and KATO, 1993) showed that the content of sialic acid in the larval haemolymph of *Bombyx mori* increased at the larval maturation but decreased just after spinning. Especially, it was evident that the content of binding sialic acid to the haemolymph protein decreased remarkably just after spinning. Moreover, the lectin activity became highest just after spinning. The facts suggest that the lectin activity in the haemolymph became highest after the terminal sialic acid in the sugar chains of the lectin-protein was removed with sialidase *in vivo*.

SUMMARY

A comparative study of the sialidase activity was performed by means of thiobarbituric acid assay and high performance liquid chromatography, using the JHA-treated larval haemolymph of *Bombyx mori*. The result showed that the sialidase activity increased at

the larval maturation but decreased remarkably on spinning day. Accordingly, it was emphasized that the lectin activity in the haemolymph became highest after the terminal sialic acid in the sugar chains of the lectin-protein was removed with sialidase *in vivo*, because the content of binding sialic acid to the haemolymph protein decreased just after spinning and the lectin activity became highest just after spinning.

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