Characterization of a *Bombyx* Humoral Lectin Activating Enzyme

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INTRODUCTION

A lectin is a functional protein which has the specific property reacting reversibly with a specific sugar residue on the cellular membrane. For this specificity, various animal lectins seem to play fundamental roles in the living body. However, biological phenomena based on the specific recognition of sugar chains have attracted much less attention in comparison with those of nucleic acids and proteins. Accordingly, the biological significance of glycoconjugates is not yet fully understood, and too many questions remain unanswered (KASAI and HIRABAYASHI, 1996).

Recently, it has been reported that invertebrate lectins play a significant role in a non-specific self defense mechanism (HATAKEYAMA, 1997). Especially, humoral lectins of insects have been concerned by various investigators, because they play a significant role through metamorphosis of insects and will be useful defense substances for human being in future (KURATA, 1992 ; KOTANI *et. al*., 1995 ; HOMMMA and NATORI, 1997).

We reported that the humoral lectin (130K-glycoprotein) played a physiological role through metamorphosis of the silkworm, *Bombyx mori* (KATO *et al*., 1991, 1994). We also reported on the appearance and the disappearance of the lectin activity (NAKAMURA and KATO, 1995, 1996). However, it still remained obscure where the lectin was produced and activated in the living body. Accordingly, we emphasized the possibility that the humoral lectin-protein (130K-glycoprotein) was produced and activated in the fat body of *Bombyx mori*, and that it was moved into haemolymph (KATO and NAKAMURA, 1997 ; KATO *et al*., 1998). Moreover, we researched a *Bombyx* humoral lectin activating factor in the fat body by means of FPLC system, and obtained a *Bombyx* humoral lectin activating enzyme from the fat body in the previous paper (KATO and NAKAMURA, 1998).

This paper reports the chracterization of a *Bombyx* humoral lectin activating enzyme in the fat body, by means of HPLC. This investigation will provide useful information for understanding the activation mechanism 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. The larvae were reared on mulberry leaves.

In preparing the samples for this research, fat body was collected daily. The fat body was collected from the dissected larvae and pupae, washed with cold 0.7% NaCl solution and homogenized in a glass homogenizer with a Teflon pestle. After centrifuging them at 3,500 rpm for 15 min at 4℃, each resultant supernatant was lyophilized.

2. Superdex 200 gel filtration

Gel filtration on a Superdex 200 column (Pharmacia) was performed using 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl as the eluting buffer. The column (2.6× 60 cm) was eluted with the buffer solution and a flow rate was kept at 150 ml per hour. The effluent was collected in 5 ml fractions and measured at 280 nm with a Shimadzu spectrophotometer type UV 1200.

3. Thiobarbituric acid assay

Thiobarbituric acid assay was carried out 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. Arsenite 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 vigorously 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 with a Shimadzu spectrophotometer type UV 1200.

4. High performance liquid chromatography (HPLC)

The sialic acid analysis was performed with a Shimadzu HPLC LC-5 A system (MURAKITA, 1987). A column of Shim-pack SCR-101 H (7.9 mm×30 cm) was used and the column temperature was kept at 55℃. Elution was performed with aqueous solution of phospholic acid (pH 1.5) and the flow rate was 1.0 ml per minute. Column eluates were monitored at 205 nm with a ultra violet spectrophotometric detector SPD-6 A. Absorp-

tion intensity was determined with a chromatopack C-R 1 B automatically.

RESULTS and DISCUSSION

Figure 1 shows comparative elution patterns of gel filtration on the fat body of the

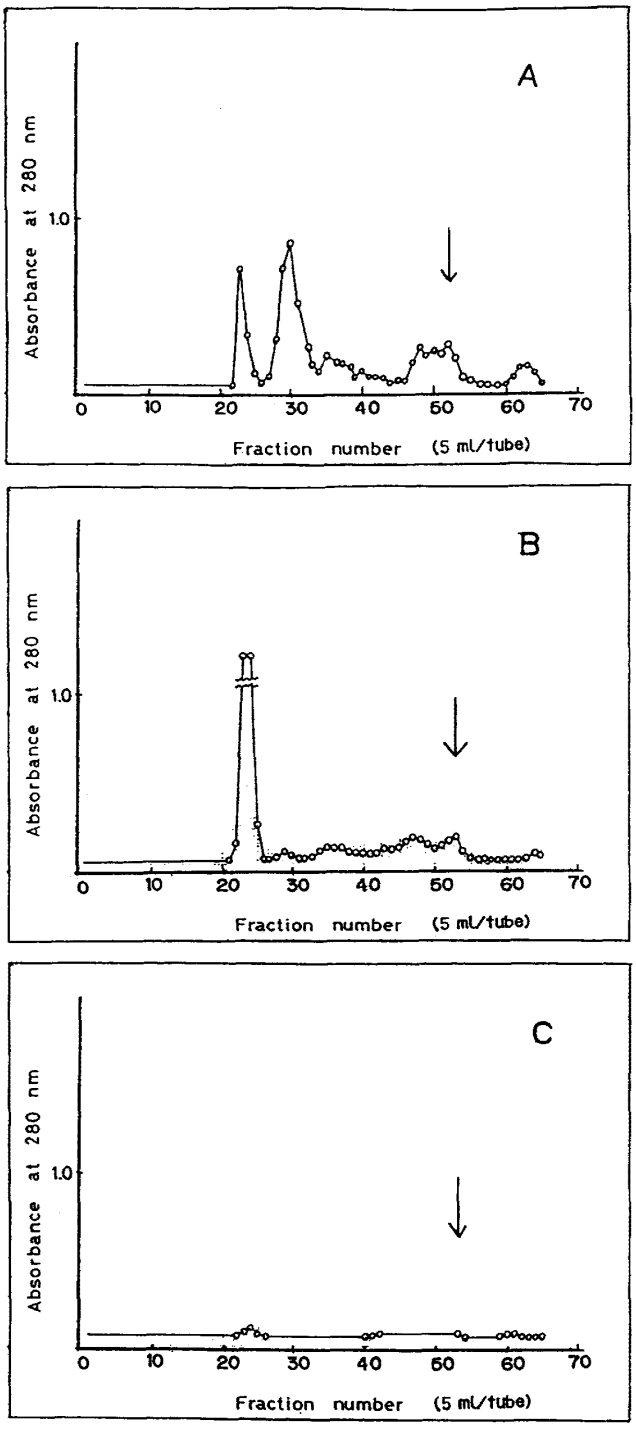

Fig. 1 Elution pattern of gel filtration on a Superdex 200 column
A : Pupal fat body, B : Mature larval fat body, C : Young larval fat body.

silkworm, *Bombyx mori*, in cource of time. 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. Figure 1-A shows the elution pattern of the pupal fat body on a column of Superdex 200. As shown in Fig. 1-A, five fractions were obtained by gel filtration. A fraction of No. 50 nearby showed as an arrow in Fig. 1-A corresponded to the fraction containing neuraminidase, because the result showed that the chromatogram revealed a peak of No. 50 nearby, when gel filtration of standard neuraminidase, type V from Clostridium perfringens by Sigma Co., was performed at the same condition as described in the previous paper (KATO and NAKAMURA, 1998). Therefore, the fraction seemed to neuraminidase fraction of the pupal fat body. Figures 1-B and 1-C show the elution patterns of the mature larval fat body and the young larval fat body on day 3 in the 5th instar, respectively. The result showed that the enzyme fraction of No. 50 nearby showed as arrows in Fig. 1 was detected only from the mature larval fat body, but not from the young larval fat body.

The color reaction was perfomed by means of the thiobarbituric acid assay (WARREN, 1959), to research neuraminidase in the enzyme fraction. N-acetylneuraminic acid (NANA) was used as standard sialic acid. Then, N-acetylneuramin lactose was used as a substrate against neuraminidase. Analysis of sialic acid, separated from N-acetylneuramin lactose with the neuraminidase solution for one hour at 37℃, was performed by

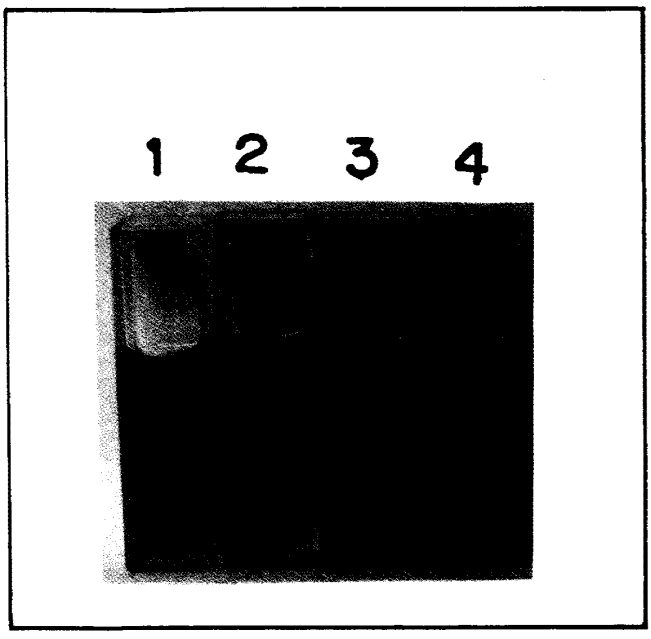

Fig. 2 Color reaction
1 : N-acetylneuraminic acid,
2 : N-acetylneuramin lactose+Neuraminidase,
3 : N-acetylneuramin lactose+Neuraminidase fraction,
4 : N-acetylneuramin lactose+Enzyme fraction from pupal fat body.

the thiobarbituric acid assay. Figure 2 shows the color of the clear upper cyclohexanone phase obtained by means of the thiobarbituric acid assay. Lane 1 in Fig. 2 shows the color of NANA, used as standard sialic acid. Lane 2, lane 3 and lane 4 show the color of free sialic acid obtained from N-acetylneuramin lactose treated with neuraminidase, the color from N-acetylneuramin lactose treated with the neuraminidase fraction obtained by gel filtration and the color from N-acetylneuramin lactose treated with the enzyme fraction of the pupal fat body, respectively. Each color showed a maximum of absorbance at approximately 550 nm. Therefore, it was confirmed that the each sample included sialic acid.

The analysis of sialic acid was performed by means of high performance liquid chromatography (HPLC). Figure 3-A shows a chromatogram of NANA obtained by using a SCR 101 H column. NANA was used as standard sialic acid. The chromatogram revealed a peak caused by NANA, which had a retention time of 5.95 minutes. Figure 3-B shows a chromatogram of standard neuraminidase, type V from Clostridium perfringens by Sigma Co., in Tris-HCl buffer by means of HPLC. The chromatogram revealed no peak caused by sialic acid. Figure 4-A shows a chromatogram of N-acetylneuramin lactose in

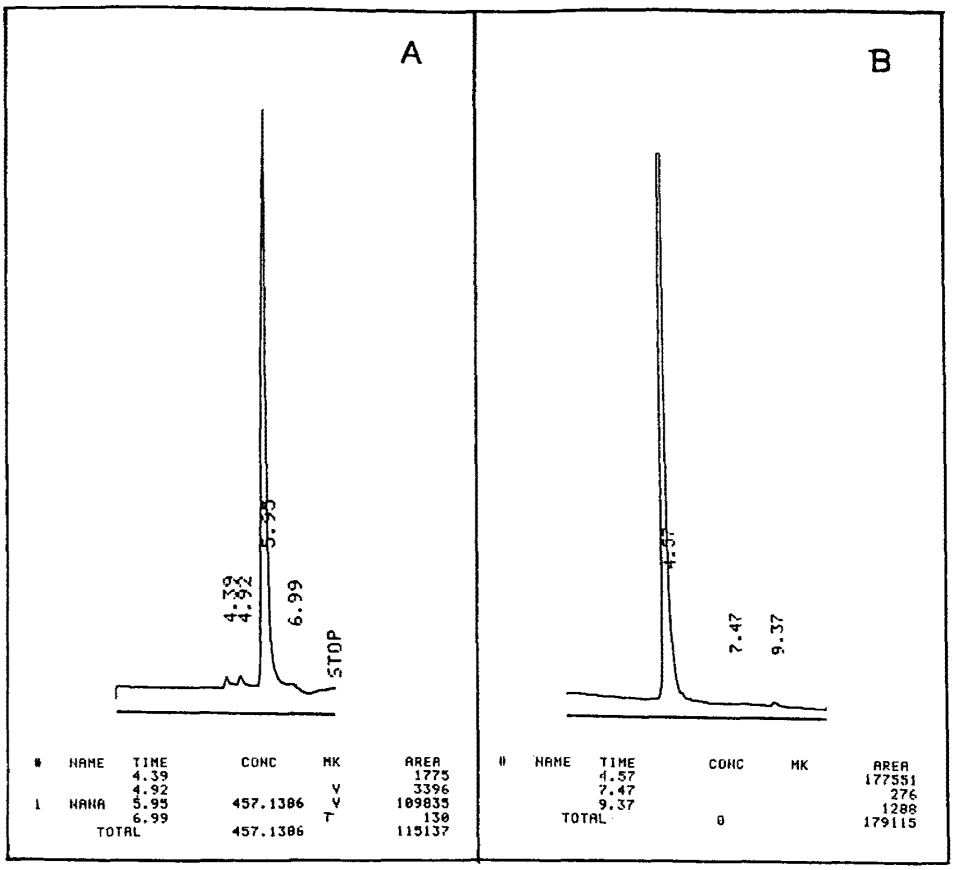

Fig. 3 Chromatograms of N-acetylneuraminic acid and neuraminidase in 0.1 M Tris-HCl buffer by HPLC
A : N-acetylneuraminic acid, B : Neuraminidase

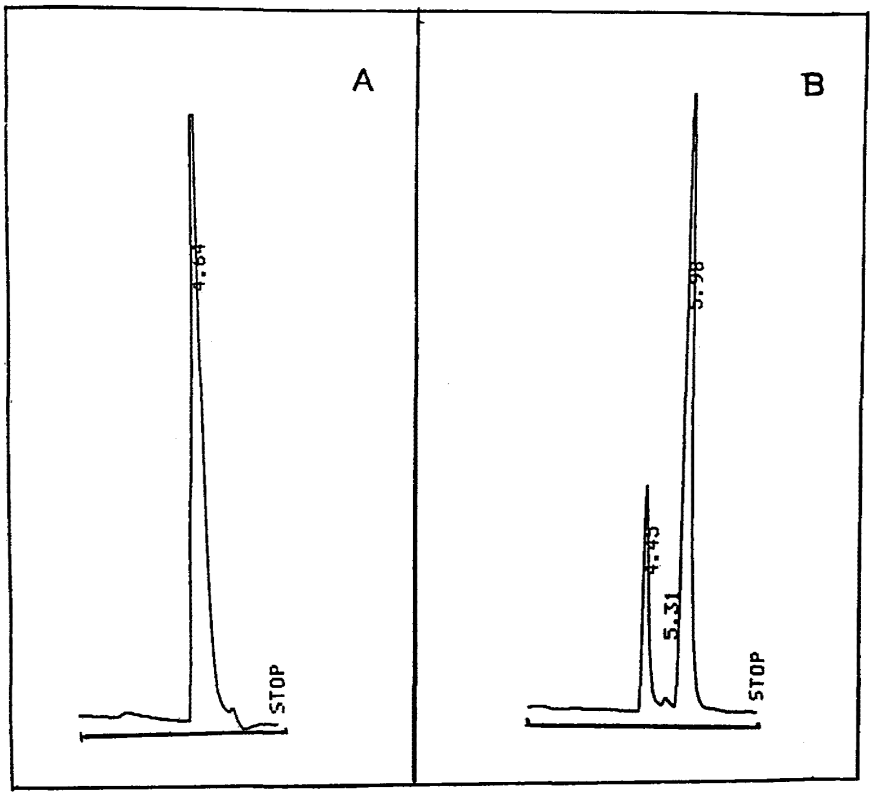

Fig. 4 Chromatograms of N-acetylneuramin lactose and one treated with neuraminidase in the buffer by HPLC
A: N-acetylneuramin lactose,
B: N-acetylneuramin lactose treated with neuraminidase.

Tris-HCl buffer by means of HPLC. The chromatogram revealed a peak, although it revealed no peak caused by sialic acid. Next, N-acetylneuramin lactose was treated with neuraminidase solution for 1 hr at 37℃, because neuraminidase was used as standard sialidase. Then analysis of sialic acid was performed by means of HPLC. The result is shown in Fig. 4-B. The chromatogram revealed a peak caused by NANA, which had a retention time of 5.98 min. Moreover, HPLC was performed on the enzyme fraction of the pupal fat body obtained by gel filtration. Figure 5-A shows a chromatogram of N-acetylneuramin lactose in Tris-HCl buffer. The chromatogram revealed several peaks, although it revealed no peak caused by sialic acid. Figure 5-B shows a chromatogram of the enzyme fraction of the pupal fat body obtained by gel filtration in Tris-HCl buffer. The chromatogram revealed several peaks, although it revealed a small peak caused by NANA, which had a retention time of 7.43 min. Next, N-acetylneuramin lactose was treated with the enzyme fraction for 1 hr at 37℃. Then analysis of sialic acid was performed by means of HPLC. The result is shown in Fig. 5-C. The chromatogram revealed a large peak caused by NANA, which had a retention time of 7.41 min. The different contents of sialic acid in Fig. 5-B and Fig. 5-C seemed to cause contents of sialic acid separated from N-acetylneuramin lactose with neuraminidase in the enzyme fraction.

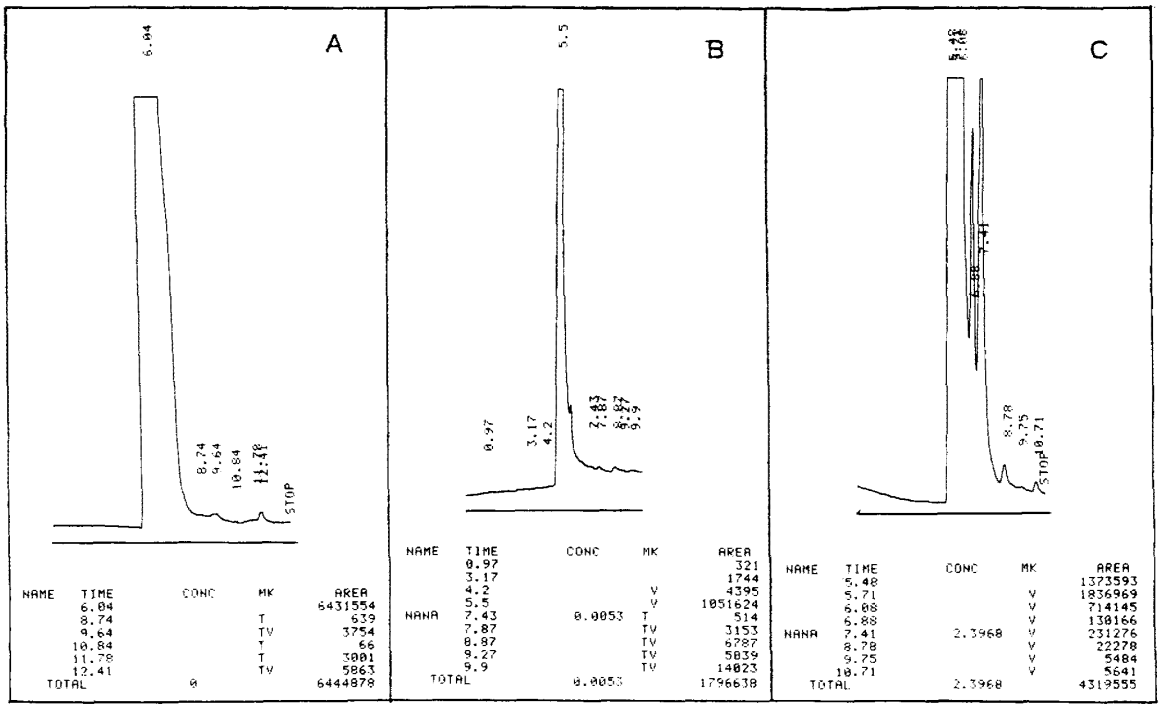

Fig. 5 Chromatograms of N-acetylneuramin lactose, enzyme fraction from pupal fat body and N-acetylneuramin lactose treated with the enzyme fraction in the buffer by HPLC
A : N-acetylneuramin lactose,
B : Enzyme fraction from pupal fat body,
C : N-acetylneuramin lactose treated with the enzyme fraction.

Namely, it was recognized that the enzyme fraction affected N-acetylneuramin lactose which was a substrate against neuraminidase.

In conclusion, the result of the analysis by means of HPLC suggested the possibility that neuraminidase exsisted in the enzyme fraction obtained from the pupal fat body, and that neuraminidase-like enzyme was isolated from the fat body by the methods described in this paper.

SUMMARY

The result of gel filtration showed that the enzyme fraction was obtained from the pupal fat body. Both the result of the color reaction by means of the thiobarbituric acid assay and the result of HPLC analysis showed that the enzyme fraction affected N-acetylneuramin lactose which was a substrate against neuraminidase. These results suggested the possibility that neuraminidase exsisted in the enzyme fraction obtained from the pupal fat body by means of gel filtration, and that neuraminidase-like enzyme was isolated from the fat body by the methods described in this paper.

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