Research of a *Bombyx* Humoral Lectin Activating Factor by FPLC

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INTRODUCTION

A lectin has the specific property reacting reversibly with a specific sugar residue. For this specificity, various animal lectins seem to play important roles in the living body ; for instance, the function as specific endocytosis, the fluid defense factor, the opsonin-like action and so on (KAWASAKI, 1993). 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).

On the other hand, invertebrate lectins play a significant role in a non-specific self defense mechanism, such as the defense substances of insects (KURATA, 1992 ; KOTANI *et. al*., 1995 ; HOMMA and NATORI, 1997). We reported that a 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 remains obscure where the lectin is produced and activated in the living body.

In the previous paper (KATO and NAKAMURA, 1997), we emphasized the possibility that the humoral lectin-protein (130K-glycoprotein) is produced and activated in the fat body of *Bombyx mori*, and that it is moved into the haemolymph, because all chromatograms obtained by means of FPLC system revealed similar patterns on the fractions obtained from the haemolymph and the fat body of each periods and the result of SDS-polyacrylamide gel electrophoresis analysis showed similar electrophoretic patterns on the fractions. This paper reports the research of a *Bombyx* humoral lectin activating factor in the fat body, by means of FPLC system. 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, haemolymph and fat body were collected daily. The haemolymph was collected by cutting pleopods and abdominal legs of larvae. The fat body was collected from the dissected larvae, 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. Haemagglutination

Sheep red blood cells (SRBC) were washed three times in phosphate-buffered saline (PBS) (75 mM NaCl, 75 mM Na_2HPO_4/KH_2PO_4, pH 7.2) and resuspended to be 2% (V/V) of the solution in PBS. Five mg of each samples was dissolved in 100 μl of insect saline (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$), and 25 μl of the solution was used for the assay on the haemagglutination activity. The haemagglutination activity was determined by serial two-fold dilution in a microtiter V-plate. Twenty five μl of SRBC suspension was mixed with 25 μl of sample solution in each well of the microtiter V-plate. The plate was shaken on a microtiter mixer for 5 min, and then incubated for 30 min at 37℃. The plate was then kept at 20℃ for 2 hr and the haemagglutination activity was recorded.

3. Superdex 200 gel filtration

Gel filtration on Superdex 200 (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 2.5 ml per minute. The effluent was collected in 5 ml fractions and measured at 280 nm.

4. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of proteins on Phast Gel Gradient 10-15 was performed by means of Pharmacia Phast System.

5. 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.

RESULTS and DISCUSSION

Figures 1 and 2 show the results of the haemagglutination assay. We took the photograph one minute after the microtiter V-plate, in which the haemagglutination assay by

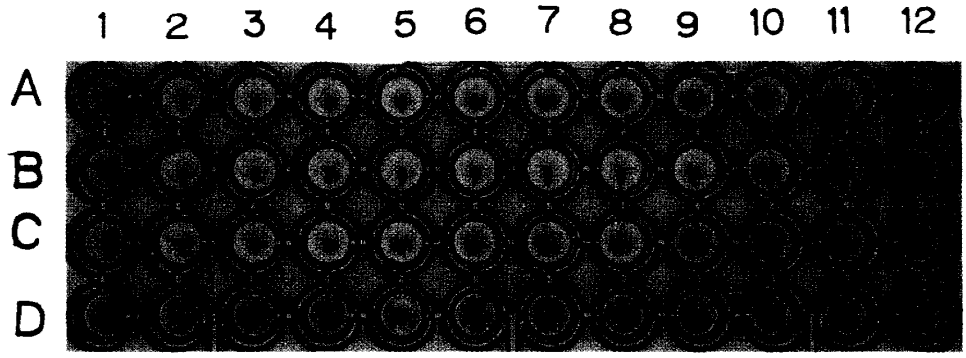

Fig. 1 Assay of haemagglutination activity
A : Inactive lectin (larval haemolymph, 3 rd day),
B : Fraction of mature larval haemolymph,
C : A+B,
D : Control.

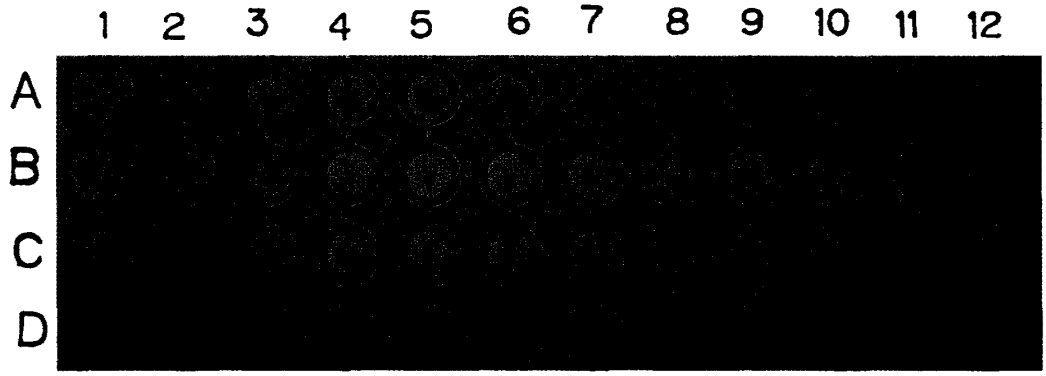

Fig. 2 Assay of haemagglutination activity
A : Inactive lectin (larval fat body, 3 rd day),
B : A+Fraction of pupal fat body,
C : Active lectin (pupal fat body),
D : Control.

serial two-fold dilution was performed, was rotated from its normal horizontal position to a vertical position. On that occasion, sheep red blood cells (SRBC), which were not agglutinated, ran from the center of a bottom in the well. On the other hand, SRBC, which were agglutinated, did not run and retained their circular shape. These observations suggested that the sample was able to agglutinate SRBC. Line A in Fig. 1 shows the haemagglutination assay of the inactive lectin-protein (130K-glycoprotein) obtained from the larval haemolymph on day 3 in the fifth instar. A weak activity was detected as shown in the photograph. Line B in Fig. 1 shows the assay of the mature larval haemolymph fraction, and a weak activity was also detected as shown in the photograph. However, the inactive lectin-protein (as line A), which was treated with the mature larval haemolymph fraction (as line B) for one hour at 37℃, shows the result of the assay that a remarkable activity was recognized as shown in line C of Fig. 1. Moreover, line D in Fig. 1 shows the control section in which the fraction was not added. On the other hand, line A in Fig. 2 shows the haemagglutination assay of the inactive lectin-protein obtained from the larval fat body on day 3 in the fifth instar. A weak activity was detected as shown in the photograph. However, the inactive lectin-protein (as line A), which was treated with the pupal fat body fraction for one hour at 37℃, shows the result of the assay that a remarkable activity was recognized as shown in line B of Fig. 2. Line C in Fig. 2 shows the assay of the active lectin-protein obtained from the pupal fat body, and a remarkable activity was detected as shown in the photograph. Moreover, line D in Fig. 2 shows the control section in which the fraction was not added.

Table 1 summarizes the haemagglutination activities of the fractions shown in Figs. 1 and 2. The mature larval haemolymph fraction and the pupal fat body fraction had the ability to activate the inactive lectin-protein as shown in the table. The fact suggests the possibility that an enzyme activating the inactive lectin-protein presents in the fat body, that the inactive one is activated with the enzyme in the fat body, and that the active one is moved into the haemolymph. In the previous paper (NAKAMURA and KATO, 1995),

Table 1 Haemagglutination activity

	Activity (titer^{-1}L)	Specific activity (titer^{-1}/mg protein/ml)
A : Inactive lectin	2^1= 2	0.04
B : Sup of mature larval haemolymph	2^0= 1	0.02
C : A+B	2^5= 32	0.64
D : A+Sup of pupal fat body	2^6= 64	1.28
E : Active lectin	2^9=512	10.2

we took notice of neuraminidase as the enzyme activating the inactive lectin–protein. Accordingly, we tried gel filtration on a Superdex 200 column for researching neuraminidase in the fat body of *Bombyx mori*.

Superdex 200 gel filtration was tried on standard proteins shown in Fig. 3. 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. As shown in Fig. 3, five fractions, blue dextrane and four standard proteins (Ferritin ; MW 440,000, Aldolase from rabbit muscle ; MW 158,000, Albumin from hen egg ; MW 43,000, Ribonuclease A ; MW 13,700), were obtained by gel filtration. Next, gel filtration of standard neuraminidase, type V from Clostridium perfringens by Sigma Co., was performed at the same condition. The result shows that the chromatogram revealed two peaks, No. 51 and No. 56, as shown in Fig. 4. Figure 5 shows the elution pattern of the pupal fat body fraction on a column of Superdex 200. A fraction of No. 50 nearby in Fig. 5 corresponded to the fraction containing neuraminidase in Fig. 4. Therefore, the fraction seemed to crude neuraminidase fraction of the pupal fat body.

Figure 6 depicts the result of SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis on the fractions obtained by gel filtration. Figure 6–1 shows the fractions ob-

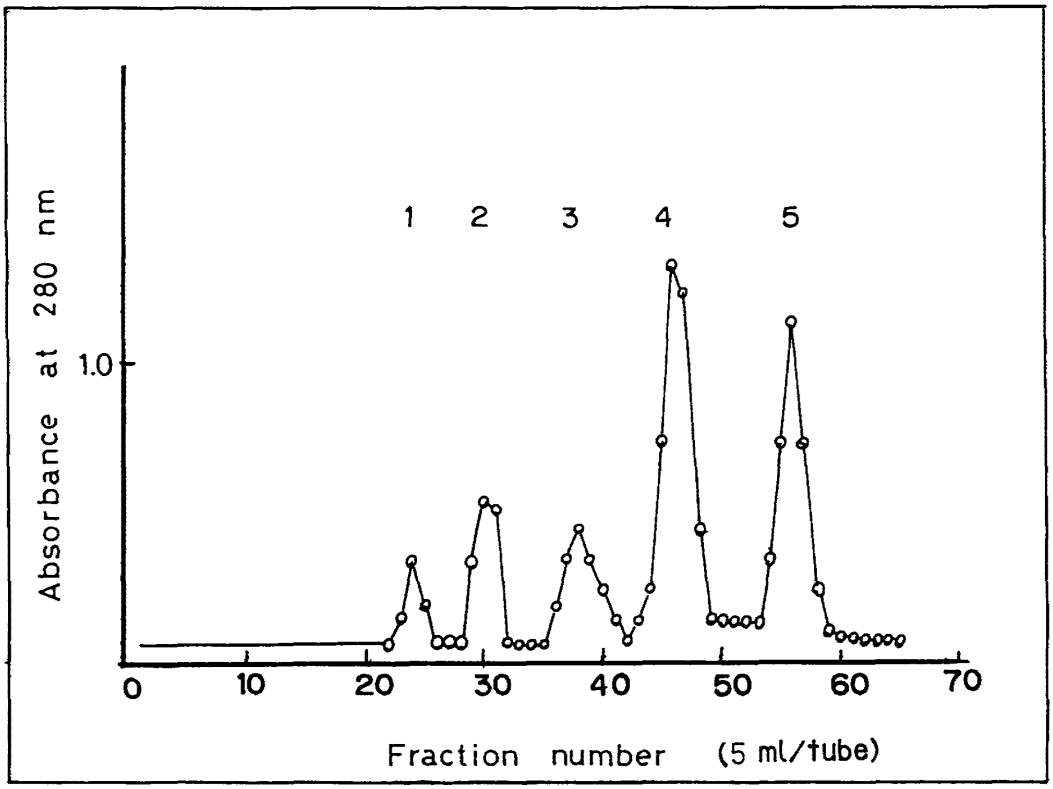

Fig. 3 Elution pattern of gel filtration on a Superdex 200 column (Standard proteins)
1 : Blue dextrane, 2 : Ferritin, 3 : Aldolase from rabbit muscle,
4 : Albumin from hen egg, 5 : Ribonuclease.

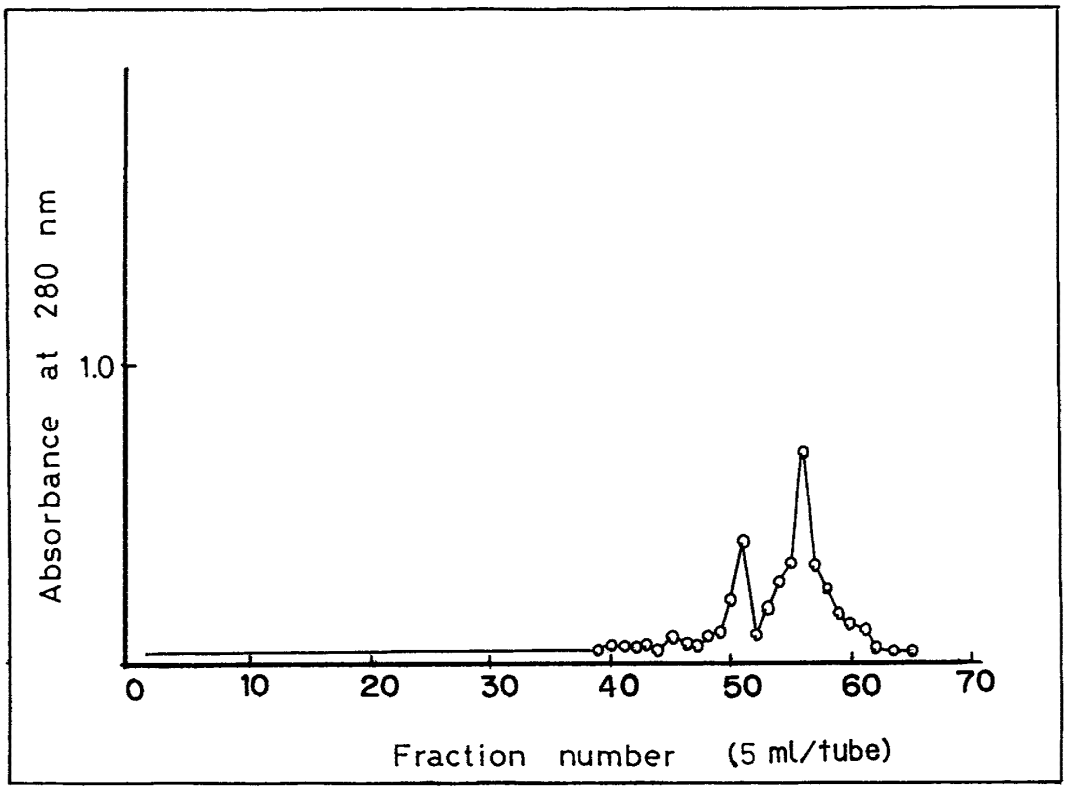

Fig. 4 Elution pattern of gel filtration on a Superdex 200 column (Standard neuraminidase)

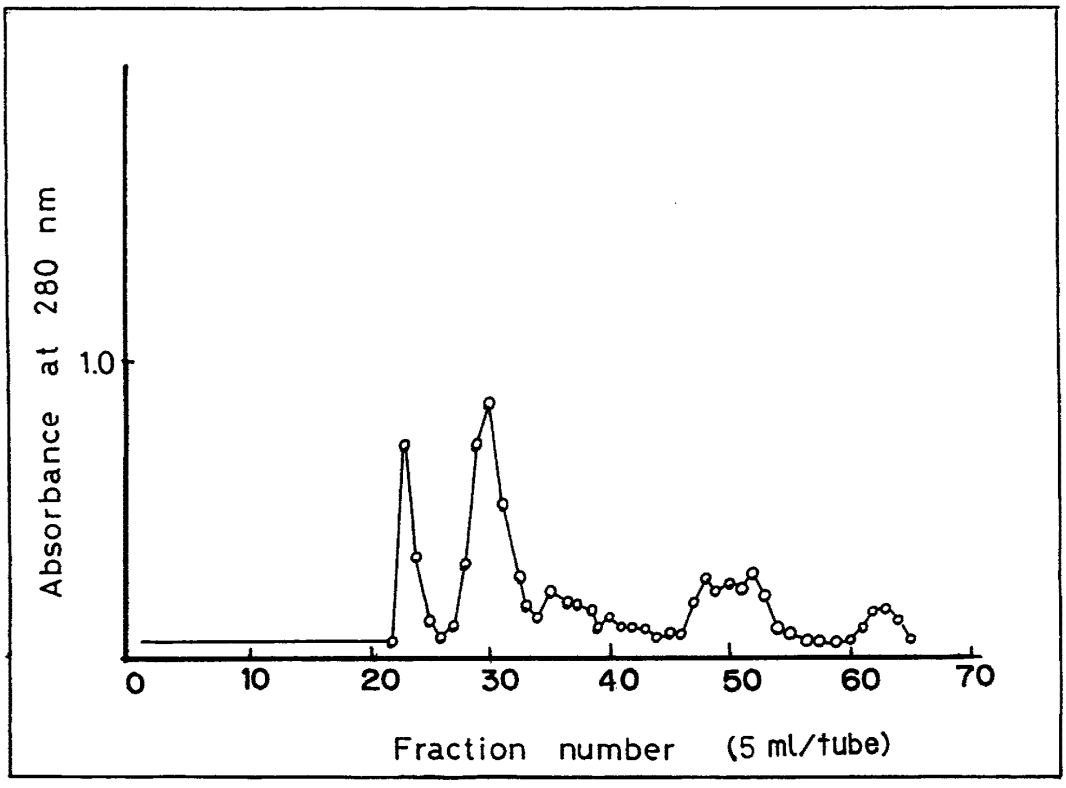

Fig. 5 Elution pattern of gel filtration on a Superdex 200 column (Pupal fat body)

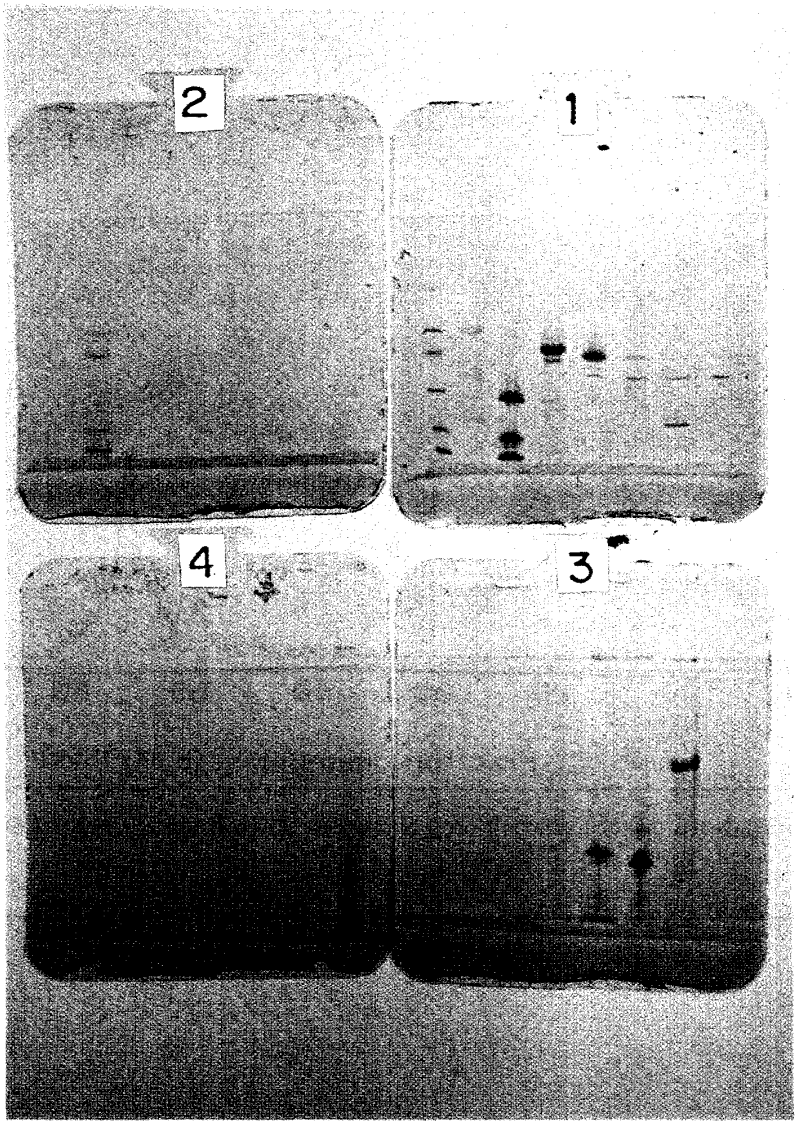

Fig. 6 Analysis of SDS-PAGE

tained from the standard proteins in Fig. 3. Figure 6-2 shows the fractions obtained from the standard neuraminidase in Fig. 4. Figure 6-3 shows the fractions obtained from the fat body fraction in Fig. 5. Moreover, Fig. 6-4 shows the fractions obtained from the mature larval fat body and the fractions from the younger larval fat body in the 5th instar. The results of SDS-PAGE analysis suggest the band corresponding neuraminidase presents in the crude enzyme fraction obtained from the fat body fraction by gel filtration. Accordingly, the colour reaction was perfomed by means of the thiobarbituric acid assay (WARREN, L., 1959), to research neuraminidase in the crude enzyme fraction. N-acetyl neuraminic acid (NANA) was used as standard sialic acid. Then, N-acetyl neuramin lactose was used as a substrate against neuraminidase. Analysis of sialic acid, which was separated from N-acetyl neuramin lactose treated with the neuraminidase solution for one hour at 37℃, was performed by the thiobarbituric acid assay.

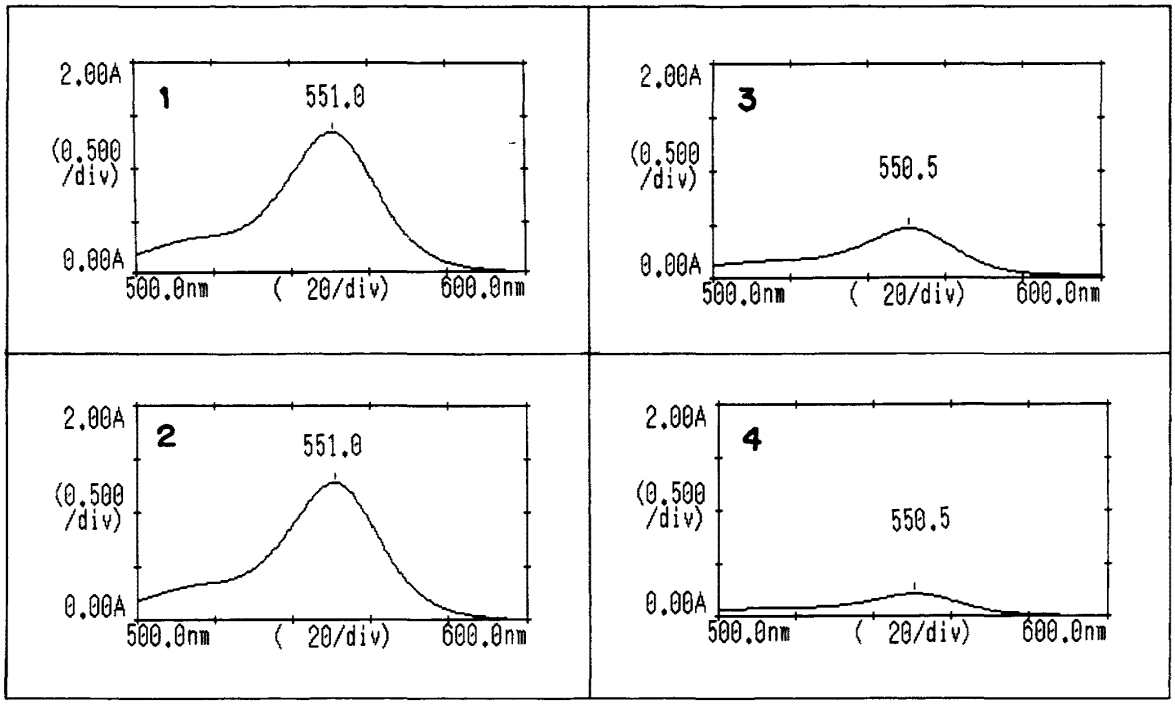

Fig. 7 Absorption spectrum
1 : N-acetylneuraminic acid (NANA),
2 : N-acetylneuramin lactose+Neuraminidase,
3 : N-acetylneuramin lactose+Neuraminidase fraction,
4 : N-acetylneuramin lactose+Crude enzyme fraction from pupal fat body.

Figure 7 shows the absorption spectrum of the clear upper cyclohexanone phase obtained by means of the thiobarbituric acid assay. Figure 7-1 shows the spectrum of NANA, used as standard sialic acid. Figures 7-2, 7-3 and 7-4 show the spectra of free sialic acid obtained from N-acetyl neuramin lactose treated with neuraminidase, one from N-acetyl neuramin lactose treated with the neuraminidase fraction by gel filtration and one from N-acetyl neuramin lactose treated with the crude enzyme fraction of the pupal fat body fraction, respectivelly. Each spectrum showed a maximum of absorbance at approximately 550 nm. Therefore, it was confirmed that the each sample included sialic acid.

In conclusion, the result of the colour reaction by means of the thiobarbituric acid assay suggests the possibility that neuraminidase as the enzyme activating the inactive lectin-protein presents in the crude enzyme fraction obtained from the pupal fat body fraction by gel filtration. In future, we try to isolate neuraminidase from the crude enzyme fraction, to provide a clue to its activation mechanism *in vivo*.

SUMMARY

The result of the haemagglutination assay showed that the mature larval haemo-

lymph fraction and the pupal fat body fraction had the ability to activate the inactive lectin–protein (130K–glycoprotein). The fact suggests the possibility that an enzyme activating the inactive lectin–protein presents in the fat body, that the inactive one is activated with the enzyme in the fat body, and that the active one is moved into the haemolymph. Accordingly, we tried gel filtration on a Superdex 200 column for researching neuraminidase in the fat body of *Bombyx mori*. The result of gel filtration showed that the crude enzyme fraction was obtained from the pupal fat body. Moreover, the result of the colour reaction by means of the thiobarbituric acid assay suggested the possibility that neuraminidase as the enzyme activating the inactive lectin–protein presented in the crude enzyme fraction obtained from the pupal fat body fraction by gel filtration.

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