Tissue Distribution of Sialic Acid Relating to Humoral Lectin Activity of *Bombyx mori*

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INTRODUCTION

A lectin–protein has the specific property reacting reversibly with a specific sugar residue. For this specificity, various animal lectins seem to play fundamental roles in the living body ; for instance, the function as specific endocytosis, the fluid defence factor, the opsonin–like action and so on (KAWASAKI, 1992). Recently, insect humoral lectins have been of interest for the defence and differentiation mechanisms of the living body (NATORI, 1991 ; KURATA, 1992, KOTANI *et al*., 1995).

We recently reported that the humoral lectin (130 K–glycoprotein) played physiological role through metamorphosis of the silikworm, *Bombyx mori*, because it seemed to possess the highest activity on spinning day at all times, and that the lectin activity was regulated by sialic acid (KATO *et al*., 1991, 1994). We also reported that the appearance and the disappearance of the lectin activity were controled with neuraminidase and galactosidase, because these enzymes seemed to be activated with the ecdyson in the mature larval haemolymph of *Bombyx mori* (NAKAMURA and KATO, 1994, 1995). However, it still remains obscure where the lectin–protein is produced and activated in the living body.

The purpose of the present work is to confirm the tissue distribution of sialic acid relating to the humoral lectin activity in various tissues of *Bombyx mori*. This investigation will provide useful information for understanding the production and activation mechanisms 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, haemocyte, fat body and midgut were collected daily. Figure 1 shows the procedure in detail.

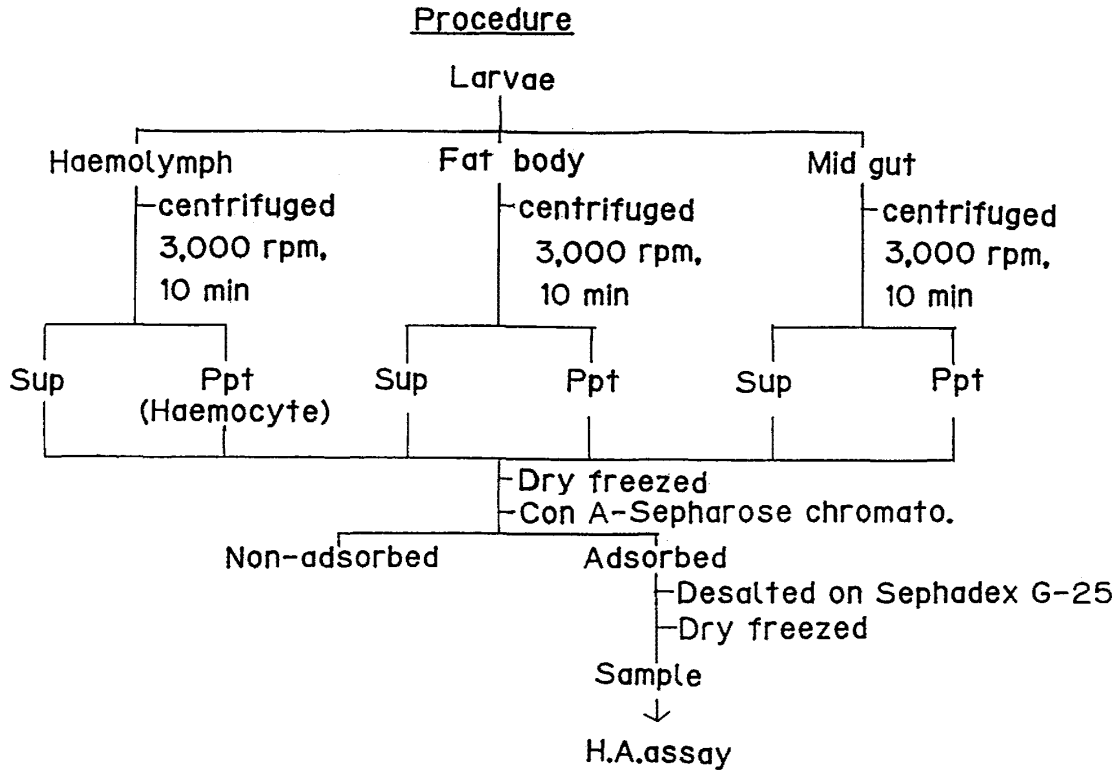


Fig. 1. Procedure for isolation.

2. Con A–Sepharose affinity chromatography

Each sample was applied to a column of Con A–Sepharose 4 B. After washing thoroughly with a 0.02 M Tris–HCl buffer solution (pH 7.4, 0.5 M NaCl), 0.3 M α–methyl–D–glucoside was added. The humoral lectin–protein (130 K–glycoprotein) was thus obtained as the adsorbed fraction to Con A–Sepharose, and the product was lyophilized after desalting with a column of Sephadex G–25.

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 system (MURAKITA, 1987). Col-

umns of Shim-pack SCR-101 H was used. 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.

5. 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 sample 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 microtiter V-plates. Twenty five μl of SRBC suspension was mixed with 25 μl of sample solution in each well of microtiter V-plates. 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.

RESULTS AND DISCUSSION

Sialic acid analysis was carried out for extracts of some tissues of the silkworm, *Bombyx mori*, including haemolymph, haemocyte, fat body and midgut. Figure 2 shows the ultra violet absorption spectrum of the clear upper cyclohexanone phase obtained by means of thiobarbituric acid assay. Figure 2-1 shows the spectrum from N-acetylneuraminic acid (NANA), used as standard sialic acid. Figure 2-2, 2-3, 2-4 and 2-6 show the spectrums from haemolymph, haemocyte, fat body (supernatant, sup) and midgut (supernatant, sup), respectivelly. Each spectrum showed a maximum of absorbance at approximately 550 nm. Accordingly, it was confirmed that the each sample included sialic acid. On the other hand, Fig. 2-5 and 2-7 show the spectrums from fat body (precipitation, ppt) and midgut (precipitation, ppt), respectivelly. Each spectrum did not show a maximum of absorbance at approximately 550 nm. Therefore, it was confirmed that the each sample did not include sialic acid.

Figure 3 shows the results of the analysis of sialic acid by means of high performance liquid chromatography (HPLC). Figure 3-A shows the chromatogram of the haemolymph on day 3 in the fifth instar. The chromatogram revealed several peaks. The peak which had the retention time of 5.7 min was caused by NANA, used as standard sialic acid. The other peaks are unknown. As shown in the chromatogram, the haemolymph on day 3 included free sialic acid. On the other hand, Fig. 3-B shows the chromatogram of the fat body on day 3. The small peak which had the retention time of 5.72 min was caused by NANA. As shown in the chromatogram, the fat body on day 3 included free sialic acid slightly. The result indicated that the amount of free sialic acid was large in larval haemolymph on day 3 and small in larval fat body on day 3. On the other hand, the amount of free sialic acid was not detectable in the mature larval haemolymph and the mature larval fat body on day 9.

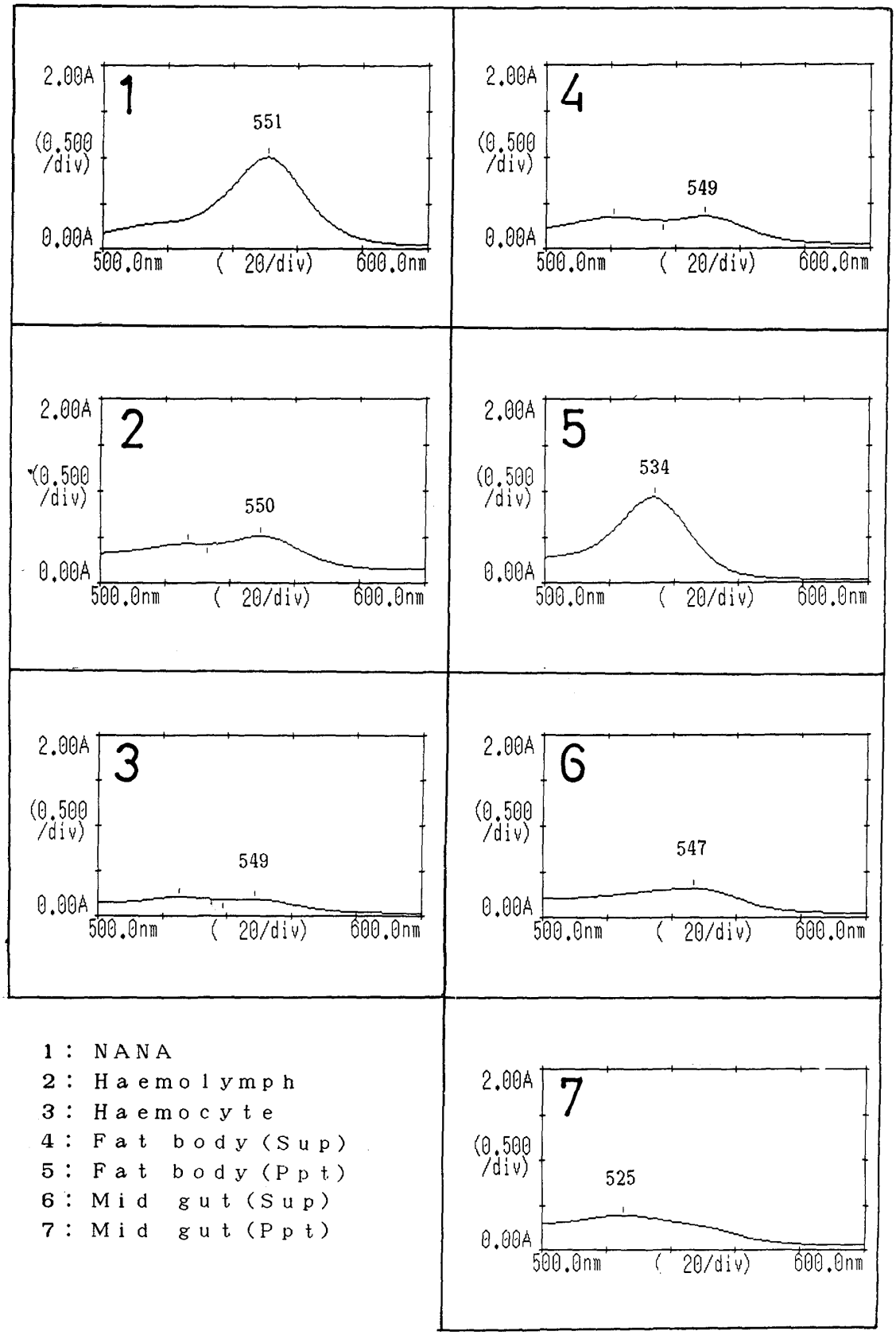
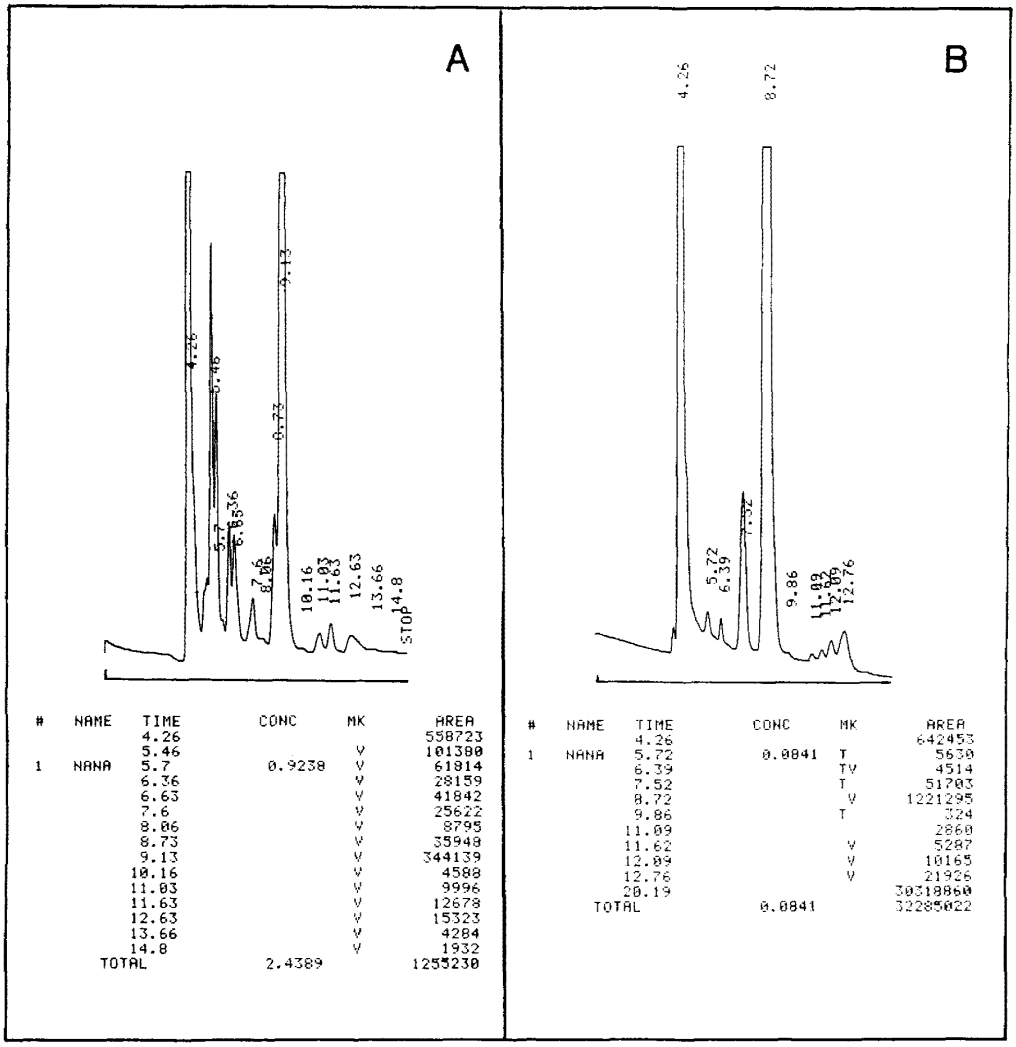


Fig. 2. Ultraviolet absorption spectrum.



#	NAME	TIME	CONC	MK	AREA
		4.26			558723
		5.46		V	101380
1	NANA	5.7	0.9238	V	61814
		6.36		V	28159
		6.63		V	41842
		7.6		V	25622
		8.06		V	8795
		8.73		V	35948
		9.13		V	344139
		10.16		V	4588
		11.03		V	9996
		11.63		V	12678
		12.63		V	15323
		13.66		V	4284
		14.8		V	1932
	TOTAL		2.4389		1255230

#	NAME	TIME	CONC	MK	AREA
		4.26			642453
1	NANA	5.72	0.0841	T	5630
		6.39		TV	4514
		7.52		T	51703
		8.72		V	1221295
		9.86		T	324
		11.09			2860
		11.62		V	5287
		12.09		V	10165
		12.76		V	21926
		20.19			30318860
	TOTAL		0.0841		32285022

Fig. 3. Chromatograms of sialic acid in the haemolymph (A) and the fat body (B) by using the SCR 101 H column.

Moreover, it was not detectable in the pupal haemolymph and the pupal fat body on day 15. These facts suggested that the environment contributed to the activation of the lectin-protein of *Bombyx mori*.

Figure 4 shows the results of Con A-Sepharose affinity chromatography. Figure 4-A shows the chromatogram of the haemolymph on day 9. Fifty five mg of the Con A-adsorbed fraction obtained from 500 mg of the whole dried matter. Figure 4-B shows the chromatogram of the fat body on day 9. The yield of the Con A-adsorbed fraction was 37 mg. Figure 4-C shows the chromatogram of the midgut on day 9. The yield of the Con A-adsorbed fraction was 29 mg. Then the analysis of sialic acid of the Con A-adsorbed fraction was performed by means of HPLC. Five mg of each lyophilized product was hydrolyzed with 1 ml of 0.2 N-H_2SO_4 for 30 min at 80℃ to yield sialic acid. Sialic acid was analyzed on a column of Shim-pack 101 H. The result showed that the amount of sialic acid was not detectable in the Con A-adsorbed fraction from the mature larval haemolymph on day 9, the fraction from the mature larval fat body and the fraction from the mature larval midgut.

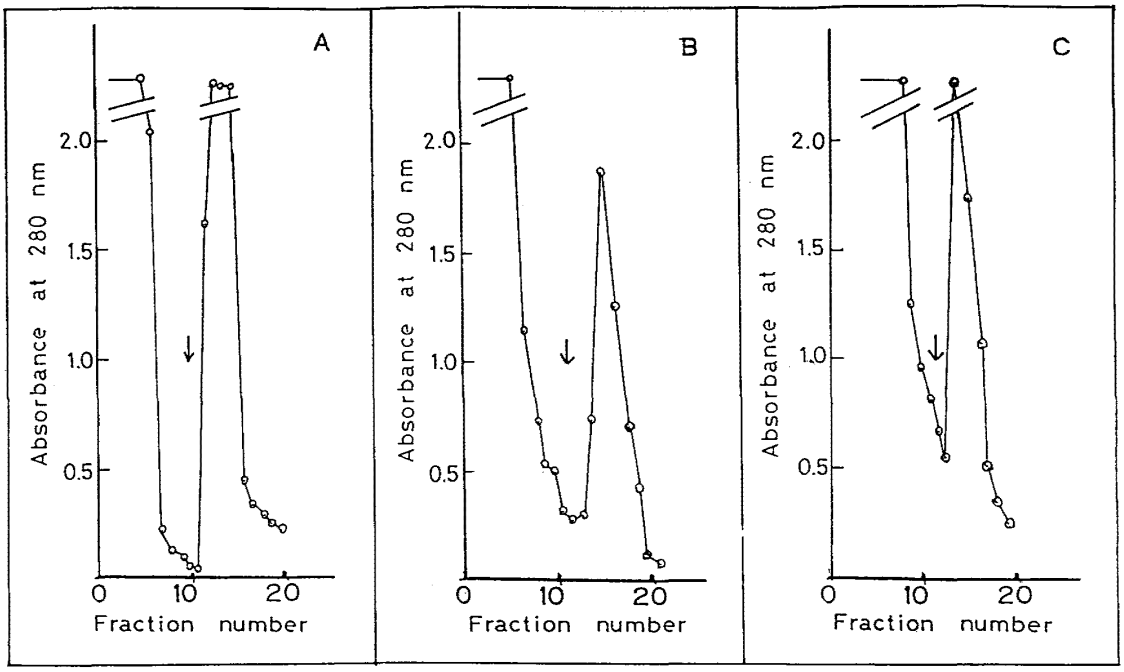


Fig. 4. Con A–Sepharose affinity chromatography
A : Haemolymph, B : Fat body, C : Mid gut

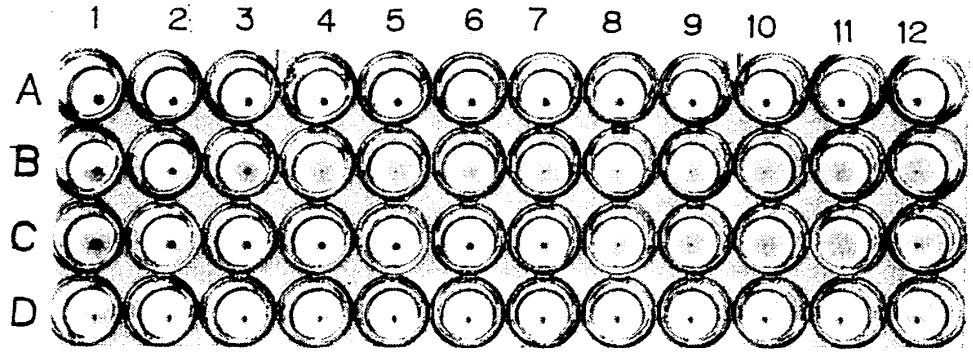


Fig. 5. Assay of haemagglutination activity of Con A–adsorbed fraction.
A : Larval haemolymph (9 th day),
B : Larval fat body (9 th day),
C : Larval mid gut (9 th day),
D : Control.

Figure 5 shows the haemagglutination activity of the ConA–adsorbed fraction. We took the photograph one minute after the microtiter V–plate, in which the haemagglutination assay by serial two–fold dilution was performed, was rotated from its normal horizontal position to a vertical position. On that occasion, 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. 5 shows the haemagglutination assay of the fraction obtained from mature larval haemolymph on day 9. A remarkable activity was detected as shown in the photograph. Line B and line C show the results of the haemagglutination assay of the fat body and the midgut, respectivelly. Remarkable activities

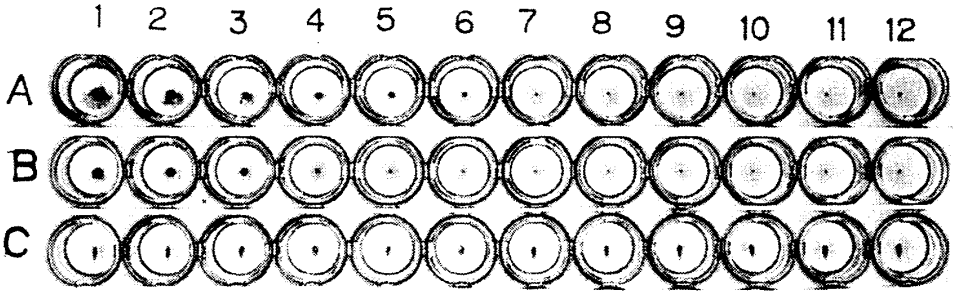


Fig. 6. Assay of haemagglutination activity of fat body.
A : Mature larval fat body,
B : Pupal fat body,
C : Control.

were recognized. Moreover, line D shows the control section in which the fraction was not added. The results of the haemagglutination assay showed that both the fractions obtained from the mature larval fat body and the mature larval midgut on day 9 were at almost the same activities as the fraction obtained from mature larval haemolymph on day 9. Namely, the facts suggested that the amount of the active 130 K-glycoprotein was at comparable levels in the mature laraval haemolymph, the fat body and the midgut.

Next, Con A-Sepharose affinity chromatography of the haemolymph on day 15 (pupa) was carried out. Forty two mg of the Con A-adsorbed fraction obtained from 500 mg of the whole dried matter. On the other hand, the yield of the Con A-adsorbed fraction from the fat body on day 15 (pupa) was 42 mg. Then the analysis of sialic acid of the Con A-adsorbed fraction was performed by means of HPLC. The result showed that the amount of sialic acid was not detectable in the Con A-adsorbed fraction from the pupal haemolymph on day 15 and the fraction from the pupal fat body on day 15. Figure 6 shows the haemagglutination activity of the fat body. Line A in Fig. 6 shows the result of the assay of the Con A-adsorbed fraction obtained from the mature larval fat body on day 9. Line B shows the result of the Con A-adsorbed fraction obtained from the pupal fat body on day 15. Moreover, line C shows the control section in which the fraction was not added. The results of the haemagglutination assay showed that the fraction obtained from the mature larval fat body was at almost the same activity as the fraction obtained from the pupal fat body.

The present study has shown the the possibility that the humoral lectin-protein (130 K-glycoprotein) is produced and activated in the fat body of *Bombyx mori*. In future, we try comparative analysis of the humoral lectin-proteins obtained from the haemolymph and the fat body in detail, to provide a clue to their physiological functions.

SUMMARY

The amout of sialic acid was determined in extracts of some tissues of *Bombyx mori* including haemolymph, haemocyte, fat body and midgut. The sialic acid was detectable in all the tissues by

thiobarbituric acid assay. The amount of free sialic acid was large in larval haemolymph on day 3 and small in larval fat body on day 3 by HPLC. The haemagglutination activity of the Con A–adsorbed fraction was at comparable levels in fat body, midgut and haemolymph of mature larvae by the haemagglutination assay. Then, haemocyte and midgut were not detectable in pupal body. Moreover, the haemagglutination activity of the Con A–adsorbed fraction obtaied from fat body was at comparable levels in mature larvae and pupa by haemagglutination assay. These results strongly suggest that the humoral lectin–protein is produced and activated in fat body of *Bombyx mori*.

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REFERENCES

KATO, Y., NAKAMURA, T. and TAKEUCHI, T. (1991): Haemagglutinating activity of haemolymph of *Bombyx mori* treated with terpenoid imidazole, J. Seric. Sci. Jpn., **60**, 208–213.

KATO, Y., NAKAMURA, T. and TAKEUCHI, T. (1994): Haemagglutination activity of haemolymph of *Bombyx mori* treated with a juvenile hormone analogue, J. Seric. Sci. Jpn., **63**, 221–228.

KAWASAKI, T. (1993): Structure and function of a macrophage lectin specific for galactose, Nippon Nogeikagaku Kaishi, **67**, 1746–1749.

KOTANI, E., YAMAKAWA M., IWAMOTO, S., TASHIRO M., MORI, H., SUMIDA, M., MATSUBARA, F., TANIAI K., KADONO–OKUDA, K., KATO, Y. and MORI, H., (1995): Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor, BBA, **1260,** 245–258.

KURATA, S., (1992): Mechanism of the dissociation of the fat body at the metamorphosis of *Sarcophaga peregrina*–Insect metamorphosis and self–non self recognition–, PNE, **37**, 101–108.

MURAKITA, H. (1987): Determination of sialic acid in serum using high performance liquid chromatography, Shimadzu Review, **44**, 225–228.

NAKAMURA, T. and KATO, Y. (1994): The relationship between sialidase activity and lectin activity in haemolymph of *Bombyx mori*, J. of Tezukayama College, **31**, 198–203.

NAKAMURA, T. and KATO, Y. (1995): Contribution of glycosidases to lectin activity in haemolymph of *Bombyx mori*, J. Tezukayama College, **32**, 257–263.

NATORI, S., (1991): Metamorphosis and self–non self recognition, Seitai Bougyo, **8**, 125–130.

WARREN, L. (1959): The thiobarbituric acid assay of sialic acids, J. Biol. Chem., **234**, 1971–1975.