Purification, Characterization, and cDNA Cloning of a Galactosespecific C-Type Lectin from Drosophila melanogaster*

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We purified a lectin from a pupal extract of Drosophila melanogaster. This lectin agglutinated trypsinized and glutaraldehyde-fixed bovine red blood cells in the presence of calcium or magnesium. The hapten sugar of this lectin was galactose. The molecular mass of the intact lectin was determined to be 41 kDa, and it comprised 14- and 17-kDa subunits. The 17-kDa subunit was shown to be a glycosylated form of the 14-kDa subunit. Analysis of the cDNA for this lectin revealed that the 14-kDa subunit consists of 163 amino acid residues and contains all residues conserved in various C-type lectins. It was suggested that the Drosophila lectin and Sarcophaga lectin share some properties and function similarly in defense and development, but probably they are not structural homologues.

Many humoral lectins have been purified from various invertebrate organisms including insects (1–10). The biological roles of these lectins are not well known, but it is suggested that they participate in the self-defense mechanisms of these organisms (11–15). Among these lectins, those that require calcium for their activity are called C-type lectins (16). The biological roles of these lectins, for instance in defense and development, are similar in defense and development, but probably they are not structural homologues.

We have been studying the hemolymph of Sarcophaga peregrina (flesh fly), focusing mainly on its biological function (18–20). The Sarcophaga lectin was found in the hemolymph of immunized third instar larvae of Sarcophaga during a study on antibacterial proteins of this insect. Although naive larvae do not contain Sarcophaga lectin, it is induced promptly when larvae are injected with foreign cells such as bacteria or when their body wall is simply injured. The same lectin was found to appear at the embryonic and pupal stages in naive animals (21). Subsequently, it was suggested that Sarcophaga lectin is essential for the differentiation of imaginal discs at the pupal stage (22). Thus, it is becoming more and more clear that this lectin participates in both the defense and development (ontogeny) of this insect, which indicates that a single lectin can play two independent biological roles (23, 24). To determine the biological roles of Sarcophaga lectin more precisely, it would be useful to identify the Drosophila counterpart of the Sarcophaga lectin, if any, because genetical techniques are available for Drosophila.

In this paper, we report the purification, characterization, and cDNA cloning of a Drosophila lectin. So far, no lectin activity has been reported in Drosophila. We found that intact red blood cells are not effective for the detection of Drosophila lectin activity. This seems to be the reason why lectin activity was not detected in Drosophila for a long time. Like the Sarcophaga lectin, the Drosophila lectin is a C-type lectin and specifically recognizes galactose. From the similarity of the expression of the genes for the Drosophila and Sarcophaga lectins, we propose the functional similarity of these two lectins. However, their molecular masses are different, and their amino acid sequence identity is at most 25%.

MATERIALS AND METHODS

Animals and Pupal Extract Preparation—Drosophila melanogaster CS2 pupae collected within 4–8 h after pupation were used throughout. Packed pupae (30 ml) were homogenized in 30 ml of buffered insect saline (10 mM Tris/HCl, pH 7.4, 130 mM NaCl, 5 mM KCl, and 1 mM CaCl2) and then centrifuged at 35,000 × g for 10 min, and the resulting supernatant (pupal extract) was used as the starting material for purification of the Drosophila lectin.

Assay of Hemagglutinating Activity—Bovine red blood cells (BRBC)1 trypsinized and fixed with glutaraldehyde were used as target cells. These cells were prepared according to the method of Nowak et al. (25). Briefly, a suspension of 4% (w/v) BRBC in 100 mM phosphate buffer, pH 7.4, containing 40 mM NaCl was treated with 1 mg/ml trypsin for 1 h at 37 °C. The cells were washed well with phosphate-buffered saline (PBS; 75 mM phosphate buffer, pH 7.2, containing 75 mM NaCl) and then suspended in PBS at a concentration of 20%. Glutaraldehyde was added to this suspension (final, 1% [v/v]), which was then mixed well and kept for 1 h at room temperature. The cells were successively washed well with PBS containing 0.1% glycine and PBS, respectively, and then stored as a 10% suspension in PBS (BRB suspension).

To measure lectin activity, 25 μl of a serially diluted sample was mixed with 25 μl of a 2.5% BRBC suspension containing 1% (w/v) bovine serum albumin in a well of a V-bottomed microtiter plate and then incubated for 1 h at 37 °C. Agglutinated BRBC formed a diffuse mat, whereas unagglutinated BRBC formed a clear dot on the bottom of a well. Lectin activity (titer) was expressed as the reciprocal of the maximum dilution of the test sample causing hemagglutination.

Purification of Drosophila Lectin—About 50 ml of the pupal extract was mixed well with an equal volume of the 10% BRBC suspension and then kept for 1 h at 4 °C. The cells were then washed well with buffered insect saline containing 1 mM CaCl2 by repeated centrifugation until the absorbance at 280 nm dropped below 0.005. Then the cells were suspended in 10 ml of buffered insect saline containing 2 mM EDTA instead of CaCl2 (EDTA-eluted lectin) or 0.2 M galactose (galactose-eluted lectin) and incubated for 1 h at 4 °C to elute the Drosophila lectin from the BRBC. Then the suspension was centrifuged to remove the cells, and the clear supernatant was collected. The galactose-eluted lectin was dialyzed against buffered insect saline for 12–16 h to remove galactose. CaCl2 was added to the EDTA-eluted lectin at a final concentration of 4 mM. Homogeneous Drosophila lectin was eluted from the BRBC with both elution procedures.

Determination of Partial Amino Acid Sequences of Drosophila Lec-

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1 The abbreviations used are: BRBC, bovine red blood cells; PBS, phosphate-buffered saline; CAPS, 3-cyclohexylaminopropanesulfonic acid; HPLC, high pressure liquid chromatography.
The DNA used as a probe was 32P-labeled and finally once for 15 min at 42°C with 0.2% SDS, twice for 10 min each time with 0.2% SDS containing 25 μg/ml of single-stranded salmon sperm DNA for 16 h at 42°C. Then the filters were washed three times with 2× SSC containing 0.1% SDS for 20 min each time at room temperature and then at 50°C. The blot was then exposed to x-ray film at −80°C.

For sequencing, the isolated cDNA clone in Agt10 was first subcloned into pGEM-3Zf(+) (+). The nucleotide sequences of both strands were determined using a Taq Dye Deoxy Cycle Sequencing kit (Applied Biosystems).

Northern and Southern Blot Hybridizations—Northern blot hybridization was performed in 50% (v/v) formamide, 5× SSC, 1× Denhardt’s solution, 50 mM phosphate buffer, pH 7.9, containing 0.5% (w/v) SDS, 50 mg/ml of each 2-mercaptoethanol, phenylmethylsulfonyl fluoride, and leupeptin, and 1 mg/ml EDTA for 18 h at 37°C.

cDNA Cloning of Drosophila Lectin—We determined the sequences of five peptides derived from the 14-kDa subunit of the purified Drosophila lectin. Then we synthesized degenerate primers corresponding to RELNSELVT and DPNSLFK, which were included in two of these peptides, and performed reverse transcription-polymerase chain reaction with these primers using pupal mRNA as a template. The amplified sequence comprising 296 base pairs was cloned and used as a probe for screening Drosophila early pupal cDNA library in Agt10. For this, recombinant plagues were plated with Escherichia coli C600 hflA1 at a density of about 20,000 plaques/plate, and the resulting plaques were blotted onto two nylon filters. Prehybridization was performed in 3× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate), 10× Denhardt’s solution (1× Denhardt’s solution = 0.02% (w/v) each of Ficoll-400, bovine serum albumin, and polyvinylpyrrolidone) containing 25 μg/ml of single-stranded salmon sperm DNA at 50°C for 5 h. Hybridization was performed in 3× SSC, 10× Denhardt’s solution containing 25 μg/ml of single-stranded salmon sperm DNA and the 32P-labeled probe at 50°C for 12 h. Then the filters were washed three times with 2× SSC containing 0.1% SDS for 20 min each time at room temperature and then at 50°C. The blot was then exposed to x-ray film at −80°C.

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The probe was labeled with [α-32P]dCTP using a multiprime labeling kit.

Other Methods—DNA manipulations, including restriction enzyme digestion, DNA ligations, plasmid isolation, and E. coli transformation, were carried out by standard methods. Chromosomal localization of the Drosophila lectin gene was performed according to the Drosophila laboratory manual (26). RNA was extracted by the guanidine-thiocyanate method (27). Protein was determined by the method of Lowry et al. (28) using bovine serum albumin as a standard.

RESULTS

Detection of Drosophila Lectin in the Pupal Extract—Sarcophaga lectin is known to be synthesized by the fat body at the pupal stage and to be secreted into the hemolymph (20), so a pupal extract of Drosophila was thought to be an appropriate source for the purification of Drosophila lectin, if any. We examined the hemagglutinating activity of the pupal extract using various red blood cells. No intact or trypsinized and glutaraldehyde-fixed red blood cells of bovine, horse, sheep, guinea pig, and chicken were agglutinated in the presence of the pupal extract, but significant agglutination was detected only when trypsinized and glutaraldehyde-fixed BRBC were used. It is known that the susceptibility of red blood cells to agglutinin generally increases when they are treated with trypsin (29).

We examined the effects of various sugars and divalent cations on the hemagglutinating activity of the pupal extract. As shown in Table I, galactose and lactose caused 50% inhibition of the hemagglutinating activity at concentrations of 0.1 and 1 mM, respectively. Other sugars so far tested had almost no effect, except o-glucose, which inhibited the agglutinating activity at a much higher concentration. We found that the hemagglutinating activity required divalent cations. Essentially no hemagglutinating activity was detected in the absence of a divalent cation, but a saturation level of activity was detected in the presence of 1 mM CaCl2, MgCl2 was also effective, but its effect was a little weaker than that of CaCl2 (Table I). These results suggested that the hemagglutinating activity in the pupal extract of Drosophila is due to a galactose-binding C-type lectin like the Sarcophaga lectin.

The hemagglutinating activity in the pupal extract was found to significantly decrease when it was kept at room temperature for several hours, suggesting that Drosophila lectin in the extract is unstable.

Purification of Drosophila Lectin—Taking advantage of the galactose specificity and calcium dependence, we were able to purify the Drosophila lectin in the pupal extract to homogeneity in a single step using the affinity to trypsinized and glutaraldehyde-fixed BRBC. The Drosophila lectin bound to BRBC was eluted with either a galactose or EDTA solution. The eluted Drosophila lectin gave 14- and 17-kDa protein bands on SDS-polyacrylamide gel electrophoresis, but the intensity of the 17-kDa band was much fainter than that of the 14-kDa one. As summarized in Table III, the galactose solution was better than the EDTA one for elution of Drosophila lectin from BRBC in terms of its specific activity and recovery. Therefore, we routinely used the former solution.

To determine whether or not the 14- and 17-kDa proteins are both subunits of Drosophila lectin, we further subjected the purified lectin to HPLC on a molecular sieve column (Superose 12). As shown in Fig. 1A, the Drosophila lectin was eluted as a single peak, and its molecular mass was estimated to be 41 kDa. Fractions corresponding to this peak were subjected to SDS-polyacrylamide gel electrophoresis. Again one major 14-kDa and one minor 17-kDa band were detected, as shown in

| Sugars             | Concentration for 50% inhibition (mM) |
|--------------------|---------------------------------------|
| Galactose          | 0.1                                   |
| Lactose            | 1                                     |
| o-Glucose          | 10                                    |
| o-Mannoose         | 100                                   |
| Fructose           | 100                                   |
| o-Xylose           | 100                                   |
| Raffinose          | >100                                  |
| Sucrose            | >100                                  |
| Trehalose          | >100                                  |
| Turanose           | >100                                  |
| N-Acetyl glucosamine | >100                              |
| N-Acetyl galactosamine | >100                             |
to the 14-kDa protein on treatment with glycosylated form of the 14-kDa protein and that it is converted into a cosylated 17-kDa protein comigrated with the 14-kDa protein. The disappearance of the 17-kDa band, indicating that the deglycosylation reaction product (296 base pairs) was cloned. Using this clone as a probe, we isolated two hybridization positive clones from a cDNA library of Drosophila pupa. The complete nucleotide sequences of both clones were determined and found to be identical. This cDNA encoded a protein consisting of 183 amino acid residues, and the sequences of all of peptides 1–5 derived from the 14-kDa protein and the amino-terminal 20 residues were included in this sequence, indicating that this is Drosophila lectin cDNA. The complete nucleotide sequence together with the putative amino acid sequence encoded by this cDNA are shown in Fig. 3A.

The amino-terminal amino acid residue was assigned as Arg at position 21. Therefore, the 14-kDa protein consists of 163 amino acid residues, and the 20 residues from the first Met are thought to be a leader peptide containing a signal sequence, which was supported by the hydropathy profile of this protein shown in Fig. 3B. On comparison of the sequence of Drosophila lectin with that of Sarcophaga lectin, the molecular size of Drosophila lectin was found to be about 2/3 that of Sarcophaga lectin, and Drosophila lectin lacked a large part corresponding to the carboxyl-terminal region of Sarcophaga lectin. The sequence of about 30 residues at the amino-terminal region was unique to Drosophila lectin, but most of the other sequence corresponded to that of carbohydrate recognition domain. Drosophila lectin contained 9 amino acid residues conserved in the carbohydrate recognition domains of known C-type lectins, as shown in Fig. 4. Moreover, Glu, Asp, and Asn residues, which were recently pointed out to be responsible for sugar and Ca²⁺ binding (30), were also conserved in Drosophila lectin. Thus, this is clearly a C-type lectin. The Sarcophaga lectin was the lectin with the highest percentage of identity with the Drosophila lectin in the Protein Information Resource, SWISS-PROT, and Protein Research Foundation protein data bases. The overall sequence identity between the Sarcophaga and Drosophila lectins was at most 25%, but the sequence identity between their carbohydrate recognition domains was much higher.

The sequence Asn-Xaa-Ser or Thr is known to be the consensus sequence for the N-glycosylation site (31). There was one
such sequence, Asn-Gly-Ser, in this lectin. Possibly, this Asn residue in the 17-kDa subunit is glycosylated.

Expression of the Drosophila Lectin Gene—To determine the functional similarities of the Sarcothopa and Drosophila lectins, we investigated the expression of the Drosophila lectin gene by Northern blot hybridization. It is known that the Sarcothopa lectin gene is activated when the body wall of third instar larvae is injured (21). In Drosophila, the situation was essentially the same as in Sarcothopa. The Drosophila lectin gene was activated 4–6 h after the larval body wall had been pricked with a thin needle, as shown in Fig. 5A. This was especially clear in second instar larvae, because no appreciable basal level expression of the Drosophila lectin gene was detected in naive larvae. In third instar larvae, significant basal level expression was detected, which was different from in Sarcothopa larvae, but this expression was clearly enhanced on their body injury.

The Sarcothopa lectin gene is known to be expressed transiently at the embryonic and early pupal stages during the normal development of Sarcothopa (21). We examined the expression of the Drosophila lectin gene at various developmental stages of Drosophila. As shown in Fig. 5B, no significant expression of the Drosophila lectin gene was detected in embryos or first or second instar larvae. However, its expression commenced in third instar larvae and was enhanced in early pupae but then decreased significantly at the late pupal stage. Significant expression of the Drosophila lectin gene was also detected in adults, but a clear difference existed between males and females. The expression in males was much more than that in females, suggesting that this lectin participates in a male-specific function in adults.

Analysis of the Drosophila Lectin Gene—To determine the copy number of the Drosophila lectin gene, we performed Southern blot hybridization with Drosophila DNA digested...
with various restriction enzymes. The results are shown in Fig. 6. A single band was detected irrespective of the restriction enzyme used, indicating that the Drosophila lectin gene is a single copy gene like the Sarcophaga lectin one (32). On chromosome mapping, the Drosophila lectin gene was found to be located at position 37B on the left arm of the second chromosome, as shown in Fig. 7.

**DISCUSSION**

We isolated and characterized a galactose-binding C-type lectin from a pupal extract of Drosophila. We succeeded in detecting lectin activity using trypsORIZED and glutaraldehyde-fixed bovine red blood cells. The carbohydrate specificity and divalent cation requirement of the Drosophila lectin were almost the same as those of the Sarcophaga lectin (18). However, analysis of Drosophila lectin cDNA revealed that the sequence identity between the Drosophila and Sarcophaga lectins was at most 25%. There are many proteins with much higher sequence identity in Sarcophaga and Drosophila. For instance, the sequence of Drosophila cecropin A was found to be identical with that of Sarcophaga sarcotoxin IA (33). Considering these facts, the 25% sequence identity of the two lectins is too low to assume that they are structurally related proteins and that the genes for these lectins were derived from a common ancestral gene. Moreover, the molecular masses of these two lectins are very different.

Sarcophaga lectin was originally isolated from the hemolymph of injured larvae, indicating that it is a hemolymph lectin (18). Although Drosophila lectin was soluble in the pupal extract, we cannot exclude the possibility that it is a membrane-bound lectin, because several hydrophobic regions were found in the molecule on hydropathy analysis, whereas no appreciable hydrophobic region was detected in Sarcophaga lectin (32). Therefore, it is difficult to conclude at this stage that the Drosophila lectin is a true structural homologue of the Sarcophaga lectin, if any, in Drosophila. However, as the pupal extract does not seem to contain other lectins, it would be worthwhile determining whether or not this Drosophila lectin is a functional homologue of the Sarcophaga lectin.

Drosophila lectin was suggested to be a trimer of the 14-kDa subunit, and one of these subunits was sometimes glycosylated, resulting in a 17-kDa subunit. However, the molar ratio of the 14- and 17-kDa subunits was estimated to be more than 2:1, judging from the intensities of the electrophoretic bands of these two subunits of Drosophila lectin. Therefore, all the lectin molecules do not necessarily contain a glycosylated subunit, and the biological relevance of glycosylation of the Drosophila lectin is not clear. This situation was essentially the same as in the case of the Sarcophaga lectin.

We found that expression of the Drosophila lectin gene was enhanced significantly by pricking of the larval body wall with a thin needle, suggesting that it is a defense molecule like the Sarcophaga lectin. Expression of the Drosophila lectin gene was also enhanced at an early pupal stage when imaginal discs differentiate. These expression patterns of the Drosophila lectin gene are very similar to those of the Sarcophaga lectin gene (21). As Sarcophaga lectin was shown to be a defense molecule as well as an autocrine regulator of imaginal disc differentiation (24), Drosophila lectin is assumed to have similar functions in the defense and development of Drosophila.

Although the two lectins are assumed to have common roles, they also seem to have their own roles. The Sarcophaga lectin gene was expressed at an embryonic stage (21), whereas no significant expression of the Drosophila lectin gene was detected in embryos. The Drosophila lectin gene was expressed in third instar larvae, whereas no significant expression of the Sarcophaga lectin gene was detected in third instar larvae (21). These differences in gene expression may be due to a functional difference between the two lectins. In adults, expression of the Drosophila lectin gene in males is significantly higher than that in females, irrespective of their age, which is also different from the expression of the Sarcophaga lectin gene.

We think that the Sarcophaga and Drosophila lectins share some properties and function similarly in the defense and development of these insects. Because the Drosophila lectin gene was assigned on the second chromosome, analyses of mutant and/or transgenic flies will provide more information on the function of this lectin.

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