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Purification of Sarcotoxin II, Antibacterial Proteins of *Sarcophaga peregrina* (Flesh Fly) Larvae†

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ABSTRACT: Three antibacterial proteins with almost identical primary structures termed sarcotoxin IIA, IIB, and IIC were purified to homogeneity from the hemolymph of third instar larvae of *Sarcophaga peregrina*. The molecular masses of these proteins were about 24 000. These proteins were found to have common antigenicity, and antibody against sarcotoxin IIA cross-reacted with sarcotoxin IIB and IIC. Radioimmunoassay using this antibody showed that these proteins are induced in the hemolymph in response to injury of the larval body wall.

Humoral antibacterial substances are known to be induced in some insects on injection of dead or living bacteria (Whitcomb et al., 1974; Boman, 1981; Chadwick, 1982). These substance are thought to participate in the defense system of insects, which have no immune network. However, the molecular properties of most of these antibacterial substances have not well been characterized. The proteins studied most extensively are cecropins and attacins of *Hyalophora cecropia* (giant silk moth), which are groups of proteins induced in the hemolymph of pupae of *H. cecropia* by immunization (Steiner et al., 1981; Hultmark et al., 1982, 1983; Lee et al., 1983; Engström et al., 1984; Kockum et al., 1984; von Hofsten et al., 1984).

Previously, we reported the induction of bactericidal proteins in the hemolymph of third instar larvae of *Sarcophaga peregrina* (flesh fly) by injection of a light suspension of *Escherichia coli* (Natori, 1977). Subsequently, we found that mere pricking of the larval body wall with a hypodermic needle is sufficient for induction of antibacterial proteins. We purified one of these proteins, termed sarcotoxin I, to homogeneity (Okada & Natori, 1983) and found that it is a mixture of three proteins (sarcotoxin IA, IB, and IC) with almost identical primary structures. We showed that these proteins consisted of 39 amino acid residues and differed only in 2–3 amino acid residues (Okada & Natori, 1985a). The primary target of sarcotoxin I was shown to be the bacterial membrane (Okada & Natori, 1984). Treatment of *E. coli* with sarcotoxin I resulted in almost instantaneous inhibition of active transport and in a rapid decrease in ATP levels (Okada & Natori, 1985b).

This paper describes the purification of another group of antibacterial proteins termed sarcotoxin II. The molecular mass of sarcotoxin II is much higher than that of sarcotoxin I, being about 24 000. Sarcotoxin II was found to consist of three proteins (sarcotoxin IIA, IIB, and IIC) with almost identical primary structures. It is possible that the cooperative actions of multiple antibacterial proteins with different antibacterial spectra form a potent defense system protecting insects from bacterial infection.

Materials and Methods

Animals and Collection of Hemolymph. Third instar larvae of *Sarcophaga peregrina* were used throughout. For induction of antibacterial activity, larvae were pricked with a hypodermic needle. The injured larvae were kept in contact with water for 24–48 h at room temperature in plastic containers.

Hemolymph was collected by cutting off the anterior tip of the larvae with fine scissors and collecting the drop of hemolymph that exuded in a Petri dish on ice. The resulting hemolymph was centrifuged for 5 min at 200*g* to remove hemocytes, and the clear supernatant was stored at −20 °C (Okada & Natori, 1983).

Fractionation of Hemolymph. Previously, we found that the antibacterial activity in the hemolymph could be separated into three fractions (GI, CI, and CII). We purified and characterized a group of antibacterial proteins named sarcotoxin I from fraction CII. This paper describes purification of another group of antibacterial proteins, termed sarcotoxin II, from fraction GI. The GI fraction was obtained as described before (Okada & Natori, 1983).

Assay of Antibacterial Activity. The following method was used for determination of antibacterial activity. *E. coli* K-12 594 (streptomycin resistant) was grown in antibiotic medium (Difco). Cells in the exponential phase of growth were collected and suspended in 10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 6.0) containing 130 mM NaCl at an A_{650} of 0.3 (2.5×10^8 cells/mL) determined in a Shimazu 150-02 spectrometer. The sample (200 μL), antibiotic medium (190 μL), and *E. coli* suspension (10 μL) were mixed in a test tube and incubated at 37 °C for 140 min with shaking. Then the mixture was rapidly chilled, and the A_{650} was measured.

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One unit of antibacterial activity was defined as the amount that caused 50% inhibition of bacterial growth relative to the control, as described previously (Okada & Natori, 1983).

Polyacrylamide Gel Electrophoresis. Electrophoresis on SDS[1]–polyacrylamide slab gels was carried out essentially by the method of Laemmli (1970). In preparation of gels containing 4 M urea, diallyltartardiamide (39:1 acrylamide/diallyltartardiamide, by weight) was used as a cross-linker instead of *N,N'*-methylenebis(acrylamide), as described by Brock and Roberts (1980). Protein samples were dissolved in 1% (w/v) SDS solution containing 2% (v/v) β-mercaptoethanol, heated at 100 °C for 5 min, and subjected to SDS–polyacrylamide gel electrophoresis. The acrylamide content of the gel was 12.5%. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 by the method of Fairbanks et al. (1971).

Peptide Mapping. Protein samples were digested with tosylphenylalanyl chloromethyl ketone treated trypsin (Worthington) in 0.2 M NH_4CO_3 solution (pH 8.0) for 13 h at 37 °C, at a molar enzyme to protein ratio of 1:50 (Yuan et al., 1982). The resulting tryptic peptides were analyzed in a Gilson HPLC system with a column of Synchropak RP-P, C18. The solvents used were 0.05% (v/v) trifluoroacetic acid and acetonitrile containing 0.05% (v/v) trifluoroacetic acid. Peptides were monitored by measuring the absorbance at 215 nm.

Antibody against Antibacterial Protein Sarcotoxin IIA. A sample of 75 μg of purified antibacterial protein sarcotoxin IIA mixed with complete Freund's adjuvant was injected into a male albino rabbit weighing about 4 kg. The protein was injected into the footpads and into four or five sites in the back. A booster injection of 50 μg of sarcotoxin IIA in incomplete Freund's adjuvant was given 21 days later. The animal was bled 8 days after the booster injection. The reactivity of the antiserum with purified sarcotoxin IIA was determined by the double-diffusion method of Ouchterlony (1958).

Radioimmunoassay Procedure. Assays were done in 1 mL of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 1% (w/v) Triton X-100, 0.1% (w/v) gelatin, and 0.02% sodium azide. A mixture of a fixed amount of radioiodinated sarcotoxin IIA, 0–1 μg of unlabeled sarcotoxin IIA, and 20 μL of antiserum diluted 1:20 were incubated at 4 °C for 14 h. Then, 50 μL of Cowan I solution prepared by the method of Kessler (1975) was added. After 60 min, the reaction mixture was centrifuged for 10 min at 8800*g*, and a sample of 900 μL of the resulting clear supernatant was used for determination of radioactivity. Radioactivity increased linearly with increase in the amount of unlabeled sarcotoxin IIA. For determination of the amount of sarcotoxin II in the hemolymph, samples were diluted serially and added to the reaction mixture instead of unlabeled sarcotoxin IIA. The amount of sarcotoxin II was determined from a dose–response curve obtained with unlabeled sarcotoxin IIA.

Sarcotoxin IIA was radioiodinated by the modified method of Hunter and Greenwood (1962). The sample, which usually had an activity of 10^6 cpm/μg, was stored at −80 °C in the presence of 0.2% bovine serum albumin.

Results

Purification of Sarcotoxin II. About 10 mL of fraction GI obtained from about 12000 larvae was heated at 100 °C for 5 min to remove heat-precipitable proteins completely. The preparation was then centrifuged for 10 min at 40000*g*, and the resulting supernatant was diluted 10-fold with 2.5 mM Tris-HCl buffer (pH 7.6) and applied to a column of DEAE-cellulose (3 × 5 cm, Whatman DE52). Antibacterial activity was recovered in the flow-through fraction. This step was useful for removing the bulk of higher molecular proteins contaminating the GI fraction. The resulting flow-through fraction was applied to a column of hydroxylapatite (1 × 4 cm) that had been equilibrated with buffer A. The column was washed with buffer A, and then adsorbed material was eluted with 40 mL of a linear gradient of 10–200 mM phosphate buffer (pH 6.0). Antibacterial activity was eluted as a single peak coinciding with a peak of protein, as shown in Figure 1. The active fraction gave a single protein band with a molecular mass of about 24000 on SDS–polyacrylamide gel electrophoresis but gave multiple, closely adjacent bands when subjected to SDS–polyacrylamide gel electrophoresis in the presence of 4 M urea. Therefore, we tried to separate these proteins by HPLC. The active fraction from hydroxyapatite was applied to a column of Synchropak RP-P, C 18 (250 × 4.1 mm), connected to a Gilson HPLC system, and protein was eluted with a linear gradient of 30–45% acetonitrile in

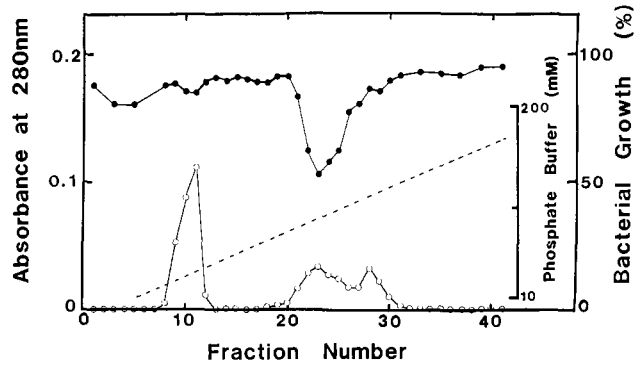

FIGURE 1: Chromatography of antibacterial activity on a column of hydroxylapatite. The flow-through fraction from DEAE-cellulose was applied to a column (1 × 4 cm) of hydroxylapatite. The column was washed with 10 mM phosphate buffer, pH 6.0, and then antibacterial activity was eluted with 40 mL of a linear gradient of 10–200 mM phosphate buffer, pH 6.0, at a flow rate of 0.25 mL/min. Fractions of 6 mL were collected, and their antibacterial activity was assayed with 20 μL of each fraction. (○) A_{280}; (●) bacterial growth; (– –) concentration of phosphate buffer.

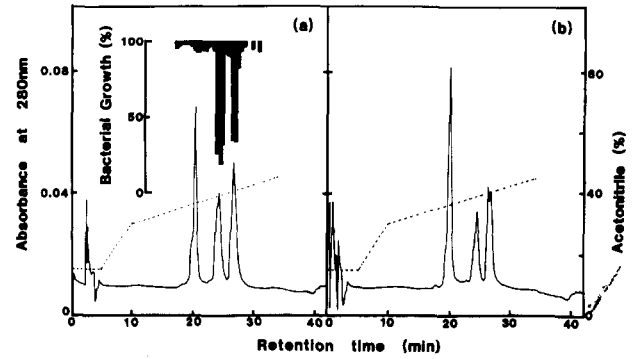

FIGURE 2: Reverse-phase HPLC analysis of sample from hydroxylapatite. The active fraction from hydroxyapatite was analyzed in a Gilson HPLC system. Chromatographic conditions were as follows: column, Synchropak RP-P, C18 (250 × 4.1 mm); solution A, 0.05% trifluoroacetic acid in H_2O; solution B, 0.05% trifluoroacetic acid in acetonitrile; gradient, first 15–30% solution B and then 30–45% solution B; flow rate, 0.5 mL/min. The absorbance at 280 nm was monitored. (a) Peak fractions were lyophilized and dissolved in 100 μL of 10 mM phosphate buffer, pH 6.0, containing 130 mM NaCl, and 20 μL of each fraction was assayed for antibacterial activity (histograms). (b) HPLC pattern of a different preparation.

[1] Abbreviations: SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

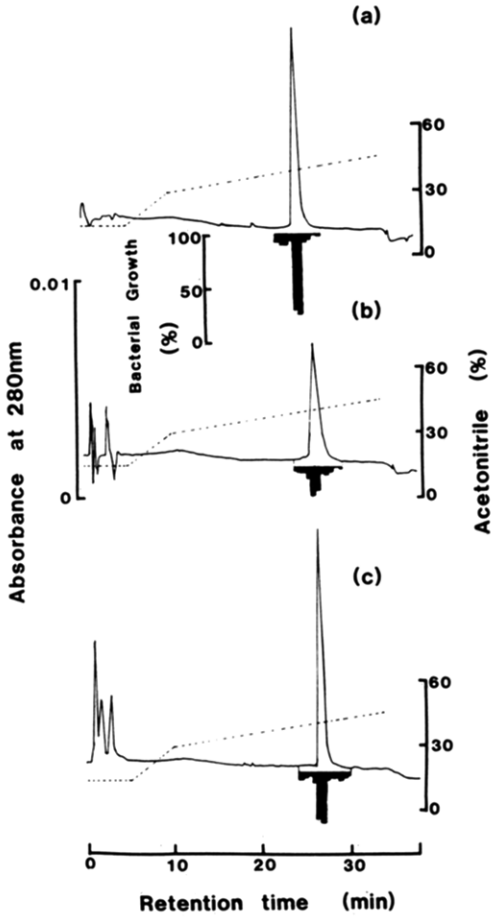

FIGURE 3: Reverse-phase HPLC analysis of purified sarcotoxin II. Conditions were as for Figure 2. (a) Sarcotoxin IIA; (b) sarcotoxin IIB; (c) sarcotoxin IIC.

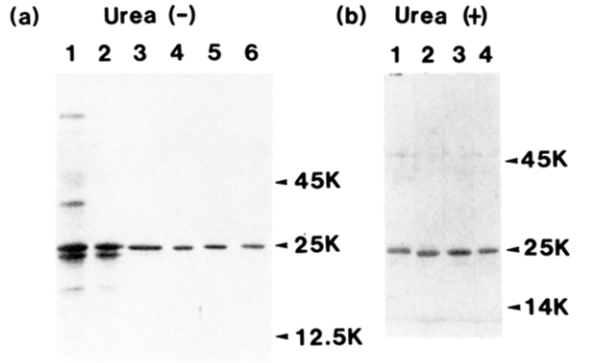

FIGURE 4: Electrophoretic profiles of sarcotoxin II. (a) SDS–polyacrylamide gel electrophoresis (12.5%) of antibacterial protein fractions obtained at each step of purification: (lane 1) GI fraction (about 5 μg); (lane 2) DEAE-cellulose flow-through fraction (about 4 μg); (lane 3) active fraction from hydroxylapatite (about 4 μg); (lane 4) sarcotoxin IIA; (lane 5) sarcotoxin IIB; (lane 6) sarcotoxin IIC (each 2 μg). (b) SDS–polyacrylamide gel electrophoresis in the presence of 4 M urea: (lane 1) active fraction from hydroxylapatite (about 2 μg); (lane 2) sarcotoxin IIA; (lane 3) sarcotoxin IIB; (lane 4) sarcotoxin IIC (2 μg each). Arrows indicate the positions of the marker proteins ovalbumin (45 000), α-chymotrypsinogen (25 000), lysozyme (14 000), and cytochrome *c* (12 500).

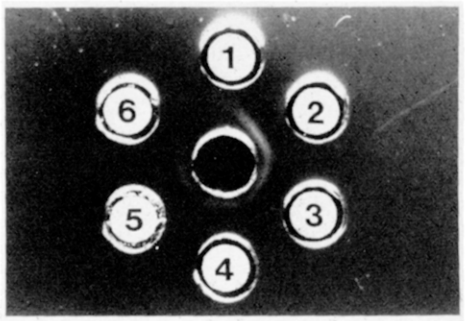

FIGURE 5: Immunodiffusion pattern of antibody to sarcotoxin IIA. Immunodiffusion was carried out in 1% agarose gel containing phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Antiserum was placed in the center well. (Well 1) Phosphate-buffered saline; (wells 2–4) 1 μg each of purified sarcotoxin IIA, IIB, and IIC, respectively; (wells 5 and 6) 10 μL each of hemolymph from injured larvae and normal larvae, respectively.

0.05% trifluoroacetic acid. A typical elution profile is shown in Figure 2a. Three distinct peaks were eluted, and the last two peaks were found to have antibacterial activity when assayed after lyophilization of peak fractions. The middle peak contained a single protein, but the last peak was found to contain two proteins giving closely adjacent bands on SDS–polyacrylamide gel electrophoresis in the presence of 4 M urea. This last peak was separated into two peaks depending upon the preparation, as shown in Figure 2b. By collecting fractions of both sides of this peak separately and repeating HPLC, it was possible to purify two proteins in this peak. Thus, three proteins with antibacterial activity were purified from the GI fraction to near homogeneity and were named sarcotoxin IIA, IIB, and IIC in order of their elution from HPLC. The elution profiles of these proteins from HPLC with their antibacterial activities are shown in Figure 3. Although the positions of elution of sarcotoxin IIB and IIC from HPLC were very similar, they were clearly different.

The electrophoretic profiles of protein at each purification step of sarcotoxin II and those of purified sarcotoxin IIA, IIB, and IIC are shown in Figure 4. As is evident from lane 1 in Figure 4a, the major component of the GI fraction was sarcotoxin II. The purified proteins each gave a single band on SDS–polyacrylamide gel electrophoresis irrespective of the presence of 4 M urea. The molecular masses of sarcotoxin IIA, IIB, and IIC were all about 24 000, but they seemed to be slightly different and to increase in the order sarcotoxin IIA, IIB, and IIC, as shown in lanes 4–6 in Figure 4a.

Results of a typical purification are summarized in Table I. Usually, the specific activity increased 3–4-fold on purification of the GI fraction to the first HPLC step, which separated sarcotoxin IIA from IIB and IIC, and the overall increase in specific activity from the hemolymph was about 300–500-fold.

Table I: Summary of Purification of Sarcotoxin II from 260 mL of Hemolymph

	total act. (units)	total protein (mg)[a]	sp act. (units/mg)	yield (%)
GI fraction	1120	27	41.5	100
DEAE-cellulose	747	15	49.8	67
hydroxylapatite	179	1.7	105.3	16
sarcotoxin IIA	84	0.45	183	7.5
sarcotoxin IIB and IIC	68	0.49	138	6.1

[a] Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Relation of Sarcotoxin IIA, IIB, and IIC. Since sarcotoxin IIA, IIB, and IIC behave in almost the same way during purification and since the molecular masses of these proteins are also similar, it was expected that these proteins would have almost identical primary structures, as in the case of sarcotoxin IA, IB, and IC (Okada & Natori, 1985a). We carried out two experiments to examine this possibility. First, we raised

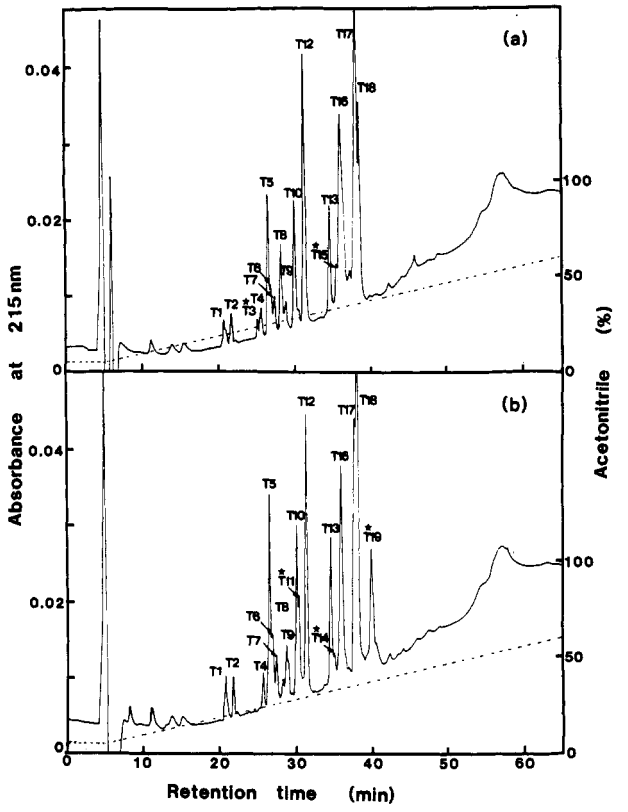

FIGURE 6: Comparison of tryptic peptides of sarcotoxin IIA and IIB. About 10 μg each of sarcotoxin IIA and IIB were digested with trypsin and analyzed by HPLC. The conditions for HPLC analysis were as for Figure 2, except that a gradient of 10–60% solution B was used. The flow rate was 1 mL/min. (a) Sarcotoxin IIA; (b) sarcotoxin IIB. Peaks were numbered from T1 to T19 in order of elution. Trypsin alone did not give a significant peak. The peaks indicated by asterisks are different for sarcotoxin IIA and IIB.

antibody against sarcotoxin IIA and tested whether the antibody cross-reacted with IIB and IIC. As is evident from Figure 5, antibody against sarcotoxin IIA cross-reacted with sarcotoxin IIB and IIC, and a fused precipitin line was obtained with sarcotoxin IIA, IIB, and IIC and hemolymph from injured larvae, but not with hemolymph from normal larvae. This finding suggests that these proteins are induced on injury of the body wall of larvae and that they have common antigenicity.

Second, we compared the tryptic peptide maps of sarcotoxin IIA and IIB. For this, purified protein was digested with trypsin, and the resulting peptides were analyzed by HPLC. The elution profiles of tryptic peptides of sarcotoxin IIA and IIB are shown in Figure 6. Clearly, the two profiles are quite similar except for a few peaks. Altogether, 19 peaks were identified in both peptide maps, of which 14 peaks were common, whereas peaks T3 and T15 were detected only in sarcotoxin IIA and peaks T11, T14, and T19 were detected only in sarcotoxin IIB. These results strongly suggest that the primary structures of sarcotoxin IIA and IIB are similar. (It was difficult to get a sufficient amount of pure IIC for tryptic analysis.)

Since the amount of sarcotoxin IIA was more than the amounts of the other two proteins, its amino acid composition could be analyzed. As shown in Table II, the contents of Gly and Ala were much higher than those of other amino acid residues, and the contents of Trp, Tyr, and Met were low. Moreover, no Cys residue was found in this protein. In these respects, sarcotoxin IIA may be similar to attacins, a group of antibacterial proteins of *H. cecropia* that have about the same molecular mass as sarcotoxin II (Hultmark et al., 1983; Engstrom et al., 1984).

Table II: Amino Acid Composition of Sarcotoxin IIA

amino acid	mol %	residues[a]
Asp + Asn	13.1	31
Thr	4.5	11
Ser	10.0	24
Glu + Gln	6.2	15
Pro	3.1	7
Gly	14.3	34
Ala	10.1	24
Cys	0	0
Val	6.4	15
Met	0.4	1
Ile	3.4	8
Leu	9.1	21
Tyr	1.2	3
Phe	4.6	11
His	2.7	6
Lys	5.5	13
Arg	4.8	11
Trp	0.7	2
total	100	237

[a] Calculated on the basis of the molecular weight of the major protein on SDS–polyacrylamide gel electrophoresis.

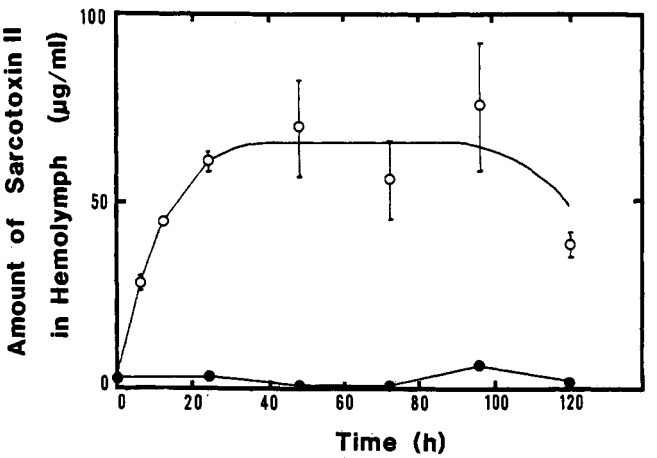

FIGURE 7: Change in amount of sarcotoxin II in hemolymph with time after injury of body wall. Hemolymph was collected with time after injury of the body wall, and its content of sarcotoxin II was measured by radioimmunoassay. Points are averages for duplicate measurements. (○) hemolymph from injured larvae; (●) hemolymph from normal larvae.

Induction of Sarcotoxin II. We determined the amount of sarcotoxin II in the hemolymph and the time course of its induction by radioimmunoassay. As is evident from Figure 7, hemolymph from normal larvae did not contain any appreciable sarcotoxin II, but after injury of the body wall its content increased with time reaching a maximum after about 30 h. Therefore, the synthesis of sarcotoxin II is triggered by injury of the body wall. The maximum level of sarcotoxin II was usually 50–100 μg/mL of hemolymph. This level was comparable with that of *Sarcophaga* lectin, which is also induced on injury of the body wall (Komano et al., 1983). Sarcotoxin II consists of a group of proteins probably participating in the defence system of *Sarcophaga*, like sarcotoxin I, and there may be a critical mechanism controlling the syntheses of these proteins when the chance of infection increases by wounding of the body wall.

DISCUSSION

This paper describes the purification of an antibacterial protein termed sarcotoxin II from the hemolymph of third

instar larvae of *Sarcophaga peregrina* after injury of their body wall. As reported before, the antibacterial activity in the hemolymph can be separated into three fractions (GI, CI, and CII), which behave differently on polyacrylamide gel electrophoresis. A group of antibacterial protein termed sarcotoxin I has been purified from fraction CI (Okada & Natori, 1983). Therefore, sarcotoxin II, purified from fraction GI, is the second group of antibacterial proteins obtained.

Sarcotoxins I and II are completely different proteins with different molecular masses. Sarcotoxin I contains only 39 amino acid residues, whereas the molecular mass of sarcotoxin II is about 24 000. However, sarcotoxins I and II have the following two points in common. First, both proteins are heat-stable and are not inactivated by heating at 100 °C for 5–10 min. Thus, heat treatment of the hemolymph was very useful for eliminating most hemolymph proteins without inactivating the antibacterial activity (Okada & Natori, 1983). It is understandable that sarcotoxin I is heat-stable, since it is a small peptide. But the molecular mass of sarcotoxin II is 24 000, so it presumably has a stable secondary structure. Its high contents of Ala and Gly may be important for its stable structure. Second, both sarcotoxins I and II have three isoforms (Okada & Natori, 1985a). Sarcotoxin IA, IB, and IC were found to have almost identical primary structures but differed in a few amino acid residues, which suggested that they are products of three independent genes. Probably, the same is true of the three sarcotoxins II. Sarcotoxin IIA, IIB, and IIC may be almost identical proteins with small differences in their primary structures. This is clearly different from the case of *Sarcophaga* lectin, which is induced in the hemolymph simultaneously with sarcotoxins I and II. We identified only one gene for *Sarcophaga* lectin, and its product was a single protein (Takahashi et al., 1985). The biological significance of the presence of isoforms of sarcotoxin I and II is not clear, but the presence of isomers seems to be a common characteristic of insect antibacterial proteins, since cecropins and attacins of *H. cecropia* are also known to have various isoforms (Steiner et al., 1981; Hultmark et al., 1983). Possibly each isoform has a slightly different antibacterial spectrum, and the presence of multiple isoforms is effective in preventing infection by various bacteria.

As reported before, several proteins were shown to be synthesized de novo in the fat body of *Sarcophaga* larvae in response to injury of the body wall (Takahashi et al., 1984). It is not known why genes for these proteins including those of sarcotoxin I and II are selectively switched on under these conditions. A certain signal produced by injury of the body wall is presumably transmitted to nuclei of fat body cells in some way resulting in the activation of genes for specific proteins. Thus, these genes may have a common regulatory region essential for their activation in response to the stimulus of injury of the body wall.

Registry No. Sarcotoxin IIA, 105430-42-0; sarcotoxin IIB, 105430-43-1; sarcotoxin IIC, 105430-44-2.

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