Studies on the β-1,3-glucan recognition protein of the prophenoloxidase activating system in insect hemolymph

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Contents

Abbreviations

AMC, 7-amino-4-methylcoumarin;

BAEE, N-α-benozoyl-L-arginine ethylester;

BAEEase, an esterase hydrolyzing BAEE;

bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol;

Boc, t-butoxycarbonyl;

BSA, bovine serum albumin;

BTP, Bis-Tris propane;

CPB, beads of curdlan type polysaccharide 13140, β-1,3-glucan produced by Ascaligenes faecalis var. myxogenes IFO 13140;

D.D.W. distilled deionized water;

FPLC, fast protein liquid chromatography;

GARC-10, goat anti-rabbit IgG linked to colloidal gold particle with a diameter of 10 nm;

IEF, isoelectric focusing;

K-P, potassium phosphate;

MCA, methylcoumarin;

PBS, phosphate buffered saline;

plasma-CPB, silkworm plasma passed over CPB column;

plasma-PG, silkworm plasma passed over peptidoglycan-Sepharose 4B column;

PMSF, phenylmethanesulphonyl fluoride;

pNPGB, p-nitrophenyl-p'-guanidino-benzoate;

PO, phenoloxidase(o-diphenol:O_2 oxidoreductase, (EC 1.10.3.1));

proPO, prophenoloxidase;

PPAE,	prophenoloxidase activating enzyme;
SDS,	sodium dodecyl sulfate;
SDS-PAGE,	SDS polyacrylamide gel electrophoresis;
Suc,	succinyl;
TBS,	Tris-HCl buffered saline;
TBS-BSA,	TBS containing 0.1 %(w/v) bovine serum albumin;
TBS-Tween,	TBS containing 0.1 %(v/v) Tween 20;
T-M,	Tris-maleate;

Introduction

β-Glucans containing β-1,3-glycosidic linkages (β-1,3-glucan) are cell wall component of fungi or algae. They displays a remarkably diverse array of activities including potentiation of immune system of mammal, activation of the blood coagulation system of horse-shoe crab, Limulus polyphemus, triggering of the prophenoloxidase activating system of insects and crustaceans, induction of phytoalexin synthesis in plants and so forth, which has well been documented in literature (Lotzova and Gutterman, 1979; Hamuro et al., 1978; Ashida, 1981; Söderhäll, 1981; Boegwald et al., 1982; Morikawa et al., 1985; Hihara et al., 1970; Reynolds et al., 1980; Müller-Eberhard and Schreiber, 1980; Morita et al., 1981; Sharp et al., 1984). It is reasonable to assume that the molecules having affinity with β-1,3-glucan exist in these biological systems. However, the primary site(s) of action for β-1,3-glucan has poorly been understood except for that in the activation of the alternative complement pathway by β-1,3-glucan (Horstmann et al., 1985). Apart from C3b of the alternative complement pathway, which has a very broad specificity and, in a strict sense, could not be considered a specific binding protein for β-1,3-glucan, and none of the specific binding protein for β-1,3-glucan has been isolated.

Insects live in almost every habitat on the earth and the number of their species has been estimated to be more than 3 millions, which are over three-quarters of animal species known to man. Their prosperity might have been impossible without developing effective defense mechanisms against

attacks from various invaders such as parasites, parasitoids and microorganisms. The front line for the defense against the attacks is organized by integument and digestive tract as the physical barriers against the invaders as they are tissues surrounding insect body and contacting directly to environment. Although the tissues are effective as the barriers, however, invaders such as fungi often break through them. In that case the humoral and cellular defense mechanisms in the hemolymph of insects are known to start acting upon them.

The defense mechanisms of insects are different from those of vertebrates. A most conspicuous difference known at present is that insects are lacking in the system having "specific immune responses" and "immunological memory", which corresponds to B cell- and T cell-mediated immune system in vertebrates. Unfortunately, however, our knowledge on the defense mechanisms of insects is poor and is not enough to answer the following questions: Whether or not insects have molecules which are responsible to recognize specific component(s) of microorganisms; whether or not the specific interaction between such molecule(s) and the microbial component(s) is the cause for insects to evoke its defense reactions known as phagocytosis, encapsulation, immune protein synthesis, activation of prophenoloxidase cascade and so forth.

From the early studies on invertebrate pathology, melanization reaction in insect hemocoel has been known to be evoked by parasites, bacteria and fungi and numerous reports have described this phenomenon (Salt, 1970; Chadwick, 1975; Nappi, 1973; Lipke, 1975). Phenoloxidase (PO) oxidizes

phenolic compounds to produce their quinone derivatives and eventually to form melanin. The quinones can react spontaneously with the thiol and amino groups of proteins and other compounds and have been claimed to be bactericidal. PO is present in hemolymph of all the insects and crustaceans so far examined and is speculated to play an important role in the defense reactions of the animals (Salt, 1970; Nappi, 1973; Ratcliffe and Gagen, 1976,1977;). In insects, the activity of the enzyme was detected not only in the cellular defense reaction such as encapsulation and nodule formation but also humoral encapsulation (Götz and Vey, 1974).

PO in insects hemolymph usually exists as an inactive precursor, prophenoloxidase (proPO) (Ohnishi,1953). The procedures for obtaining homogeneous proPO preparations have been developed in a few species of insects (Heyneman, 1965; Ashida, 1971; Aso, 1985). ProPO of the silkworm, Bombyx mori migrated to the position corresponding to 80 kDa polypeptide in sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE) and contains two cupric copper atoms per 80,000 g protein (Ashida, 1971). ProPO activating enzyme (PPAE) is present in the hemolymph (Ashida et al., 1983) and cuticle of silkworm larvae (Ashida and Ohnishi, 1967), and the cuticular PPAE is a serine protease which converts proPO to PO through a limited proteolysis, releasing a peptide (Dohke, 1973a, 1973b; Ashida et al, 1974; Ashida and Dohke, 1980). Hemolymph PPAE has not yet been purified.

With respect to the regulation mechanism of proPO activation in vivo the investigation on the subject had been hampered until the beginning of 1980's, because of spontaneous activation of proPO during the collection of

hemolymph from the hemocoel of insects.

A method for obtaining hemolymph without initiating proPO activation was developed by Ashida (1981), and the method make it possible for us to study the mechanism of proPO activation *in vitro*. From the early studies on the mechanism of proPO activation the following facts have been demonstrated: the mechanism for proPO activation (proPO activating system) is present in plasma fraction; The system is a cascade triggered by microbial products such as β-1,3-glucan of fungal cell wall or peptidoglycan of bacterial cell wall (Ashida, 1981; Ashida *et al*, 1983; Yoshida and Ashida, 1986); When the microbial products are added to silkworm plasma, at least two zymogens of serine enzymes (proBAEEase and proPPAE) are activated (Yoshida and Ashida, 1986); limited proteolysis is involved during the activation of proPO by hemolymph PPAE (Ashida and Yoshida, 1988); the proPO activating system requires Ca^{++} to be triggered by β-1,3-glucan or peptidoglycan (Ashida *et al*, 1983; Leonard *et al*, 1985; Dularay and Lackie, 1985).

The proPO activating system has been reported to be present in several species of insects and crustaceans (Pye, 1974; Ashida *et al*., 1983; Söderhäll and Häll, 1984; Smith and Söderhäll, 1983) and it is suggested that the proPO activating system functions to recognize foreigness, generate opsonin and hemokinetic factors, and activate fat body to synthesize immune proteins (Ashida *et al*., 1982; Söderhäll, 1982; Ratcliffe *et al*., 1984; Huxham and Lackie, 1988). However our knowledge on the activation mechanism of the system is incomplete to evaluate correctly its role in insect defense mechanism and much remains to be studied. The

present author has chosen the proPO activating system of the silkworm, Bombyx mori to study the action mechanisms of β-1,3-glucan in biological systems and discovered a new protein (β-1,3-glucan recognition protein) which has specific affinity to β-1,3-glucan and triggers proPO activating system cascade upon binding with β-1,3-glucan. In this thesis, the results of author's study are reported in three parts. Part 1, part 2 and part 3 describe the demonstration of the presence of β-1,3-glucan recognition protein in the proPO activating system, purification and preliminary characterizations of β-1,3-glucan recognition protein, the results of the immuno-chemical localization of the recognition protein, respectively.

1. Preparations of Silkworm plasmas deprived of β-1,3-glucan recognition protein or peptidoglycan recognition protein

Introduction

As described in introduction, the prophenoloxidase(proPO) activating system of silkworm hemolymph is a cascade and is triggered in the presence of Ca^{++} by elicitors such as β-1,3-glucan or peptidoglycan (Ashida, 1981; Ashida et al., 1983; Yoshida and Ashida, 1986). When silkworm plasma is incubated with the elicitor, at least two serine enzymes, which are BAEEase and proPO activating enzyme(PPAE), are activated. These serine enzymes are insensitive to diisopropylfluorophosphate before activation but become sensitive upon activation, suggesting that they may be present as zymogens in plasma (Yoshida and Ashida, 1986). Thus, the proPO activating system has some characteristics similar to those of the complement system of vertebrates (Müller-Eberhard and Schreiber, 1980) and the blood coagulation system of horse-shoe crab (Morita et al., 1981).

To investigate whether two separate molecules with affinity to β-1,3-glucan and to peptidoglycan in the proPO activating system were the same one or not, it was tried to obtain two plasma preparations from each of which one of the molecules has been specifically removed by affinity columns. Silkworm plasma was passed over a CPB column [CPB, curdlan type polysaccharide(β-1,3-glucan) beads] or a peptidoglycan-Sepharose 4B column in the absence of divalent cation, in order to remove the molecules with affinity to each ligand. The plasma passed over each column showed that there are two

entry sites for triggering the proPO activating system, at which two separate molecules, β-1,3-glucan recognition protein and peptidoglycan recognition protein are located.

Materials and Methods

Experimental animals

Silkworms (Bombyx mori) were reared on an artificial diet from Takeda Pharmaceutical Industry Co. Ltd., Osaka at 25°C in a 12 hr photoperiod. Fifth instar larvae at about the beginning of spinning were used for obtaining plasma.

Preparation of plasma

The plasma fraction of silkworm hemolymph was collected according to Ashida (1981). Plasma was dialyzed against several changes of Tris-maleate buffer (T-M buffer; 10 mM Tris, 10 mM maleate, 150 mM NaCl, pH 6.5) at 4°C for 72 hr to remove divalent cation. The dialyzed plasma was added, drop by drop, into liquid nitrogen and frozen plasma stored at -70°C. The frozen plasma was thawed and centrifuged at 48,800 g for 60 min. The supernatant was used as silkworm plasma.

Zymosan solution

Ten ml of 5 mg/ml zymosan suspension in distilled deionized water (D.D.W.) was sonicated with Bransonic Sonicator (Model 220) for 3 min. Large particles were removed by centrifugation at 3000 g for 10 min. The supernatant containing 1.0 mg zymosan/ml was used as the zymosan solution.

Peptidoglycan

Cell wall was prepared from Micrococcus luteus ATCC4698,

cultured in 10 l of culture medium, according to a method of Araki et al. (1972) except that crude cell wall fraction was suspended after RNase treatment into 50 ml of Tris-HCl buffer (pH 7.5) containing 50 mM Tris, 20 mM $MgCl_2$, 1 mM $CaCl_2$ and 7 mg of DNase I, and incubated for 3 hr at 37°C. 762 mg of freeze-dried cell wall was obtained. Peptidoglycan was purified from the cell wall after Araki et al. (1972) and the peptidoglycan suspension (1.0 mg/ml of D.D.W.) was used as an elicitor.

CPB column

CPB (average particle size 80 μm) was a gift from Dr. Nakao. CPB column (1.3 x 2.3 cm) was equilibrated with T-M buffer at 4°C.

Peptidoglycan-Sepharose 4B (Sepharose 4B coupled with peptidoglycan) column

Peptidoglycan was purified from 153 mg of M. luteus cell wall according to Sasaki et al. (1983). Purified peptidoglycan was suspended in 153 ml of 80 mM ammonium acetate and digested with egg white lysozyme (1.5 mg) at 45°C for the first 4 min and then at 37°C for 2 hr with stirring. The incubation mixture was filtered through 4 sheets of Millipore filter (HAWPO 4700). After lyophilizing the filtrate it was dissolved in 6 ml of D.D.W. and 5.5 ml of the solution was applied to Sephadex G-50 SF column (2.5 x 90 cm) equilibrated with 50 mM ammonium bicaronate and eluted with the same solution at a flow rate of 15 ml/hr. 3 ml-fractions were collected and assayed for reducing sugars according to Park and Johnson (1949). Reducing sugar(s) were detectable between fraction number 60 and 150. To discard large fragments of peptidoglycan fractions (Nos. 82 to 149) were

pooled. The pooled fractions were lyophilized and dissolved in 9.3 ml of 0.1 M sodium bicarbonate buffer, pH 10.0. Peptidoglycan fragments in the buffer were coupled with 2.7 g of CNBr-activated Sepharose 4B according to manufacturer's instruc-tion manual. Peptidoglycan-Sepharose 4B thus prepared was packed into a column (0.6 x 1.7 cm) and equilibrated with T-M buffer containing 1 mM EDTA.

Plasma-CPB(plasma passed over CPB column)

Five ml of plasma was added to 20 ml of T-M buffer. The diluted plasma was passed over a CPB column at a flow rate of 50ml/hr at 4°C. Effluent, containing proteins (about 25 ml), was collected into 225 ml of saturated ammonium sulfate (pH 6.5), which was being stirred. Stirring was continued overnight and precipitate was collected by centrifugation at 16,000 g for 20 min. To the precipitate, 4 ml of T-M buffer was added and the resultant protein solution dialyzed against three changes of the same buffer (500 ml) overnight. The dialyzed solution was centrifuged as above and the supernatant used as plasma-CPB after its volume was adjusted to 5 ml with T-M buffer.

Plasma-PG (plasma passed through peptidoglycan-Sepharose 4B column)

One-hundredth volume of 100 mM EDTA (pH 6.5) was added to the plasma and 15 ml of the mixture applied to peptidoglycan-Sepharose 4B column at a flow rate of 6 ml/hr. Twelve ml of effluent from the beginning of the plasma application was pooled and used as plasma-PG.

Reaction mixture for triggering proPO activating system in plasma by elicitor

Each plasma preparation (10 volume) was added to the

mixture of zymosan or peptidoglycan (one volume) and 80 mM $CaCl_2$ (one volume). The reaction mixture was incubated at 25°C and used as an enzyme solution of PO and BAEEase.

Assay of PO activity

PO activity was assayed after Pye (1974). A 10 µl aliquot of enzyme solution was added to 1 ml of substrate solution (0.1 M potassium phosphate buffer, pH 6.0 containing 4 mM 4-methylcathechol and 8 mM 4-hydroxyproline ethylester). After incubation at 30°C for 10 min, the increase of absorbancy at 520 nm was measured with a Shimazu spectrophotometer (Model 150).

Assay of BAEEase

An esterase hydrolyzing BAEE was assayed according to Trautschold et al (1974) with some modifications as given by Yoshida and Ashida (1986). The unit of the enzyme activity has been defined after Yoshida and Ashida (1986).

Chemicals

Chemicals were obtained from the following sources: CPB was a gift from Dr. Y. Nakao of Applied Microbiology Labs., Takeda Chemical Ind. Ltd.(Osaka); zymosan, NAD and alcohol dehydrogenase (from yeast) were from Sigma; N-α-benzoyl-L-arginine ethyl ester were from Peptide Institute Inc. (Minoshi, Osaka); Sephadex G-50 SF and CNBr-activated Sepharose 4B were from Pharmacia LKB Biotechnology Inc.; chicken egg white lysozyme (crystallized 6 times) were from Seikagaku Kogyo Co., Ltd., (Tokyo). Other chemicals used were the highest grade commercially available.

Results and Discussion

As is evident from the results presented in Fig. 1. 1. and Table 1. 1, the proPO activating system in plasma was not triggered during chromatography on CPB or peptidoglycan-Sepharose 4B. This result may look strange, since CPB and peptidoglycan-Sepharose 4B have β-1,3-glucan and peptidoglycan, respectively. However, it is understandable if we take into account of the following facts: the column chromatography was carried out in the absence of Ca^{++} ion which is required to trigger the system by elicitor; the peptidoglycan coupled to CNBr-Sepharose 4B were small fragments, which been shown to have no activity as elicitor; the flow rate of plasma though CPB column was maintained very high (50 ml/hr) in order to shorten the period of time for plasma to stay with β-1,3-glucan (CPB); the plasma was applied to CPB column after it had been diluted to one-fifth, rendering the plasma less reactive to β-1,3-glucan.

Plasma-CPB and plasma-PG were incubated separately with zymosan (β-1,3-glucan) and peptidoglycan, respectively, and activities of PO and BAEEase activity of the incubation mixtures were assayed. The proPO activating system in both of plasma-CPB and plasma-PG was not triggered without the elicitor during the incubation (Fig. 1. 1. and Table 1. 1.). However, the system in plasma-CPB could be triggered by peptidoglycan but not by zymosan (β-1,3-glucan) and the activities of PO and BAEEase appeared in the manner similar to that observed in plasma incubated with each of the elicitors.

The above results could be understandable if one assumes that the β-1,3-glucan recognition protein and peptidoglycan

recognition protein, which have affinity in the absence of Ca^{++} ion to β-1,3-glucan and peptidoglycan, respectively, were removed by the affinity columns and that they are necessary for the proPO activating system to be triggered with the elicitors. The scheme based on the above results and our previous findings of the silkworm proPO activating system is presented in Fig. 1. 2.

In the next part of this thesis, the putative β-1,3-glucan recognition protein will be shown to exist in the silkworm plasma and the procedure to purify the protein to homogeneity is described.

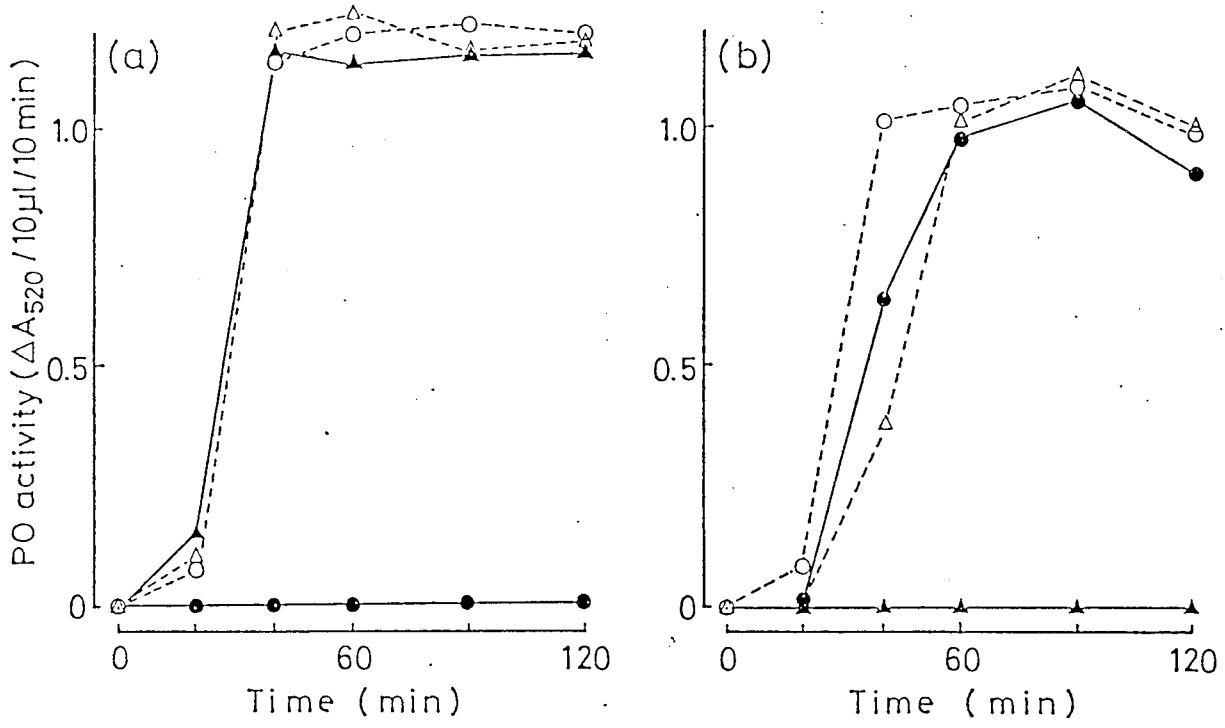

Fig. 1. 1.

a) Activation of proPO in plasma-CPB with β-1,3-glucan or peptidoglycan.

Plasma preparation were incubated with elicitor for triggering the proPO activating system and an aliquot was assayed for PO activity at intervals as described under Materials and Methods. ○-○, plasma and zymosan; △-△, plasma and peptidoglycan; ●-●, plasma-CPB and zymosan; ▲-▲, plasma-CPB and peptidoglycan.

b) Activation of proPO in plasma-PG with β-1,3-glucan or peptidoglycan.

Experimental procedures were the same as in a) except that plasma-PG was employed instead of plasma-CPB. ○-○, plasma and zymosan; △-△, plasma and peptidoglycan; ●-●, plasma-PG and zymosan; ▲-▲, plasma-PG and peptidoglycan.

Table 1. 1.

Activation of BAEEase in plasma, plasma-CPB and plasma-PG by elicitors

plasma preparation	added elicitor	BAEEase activity* (units/ml of plasma)
plasma	zymosan	93.7
	peptidoglycan	91.0
plasma-CPB	zymosan	0.0
	peptidoglycan	89.3
plasma-PG	zymosan	84.2
	peptidoglycan	0.0

* Plasma preparation and elicitor were incubated as described under Materials and Methods. BAEEase activity was assayed after 20 min incubation at 25°C.

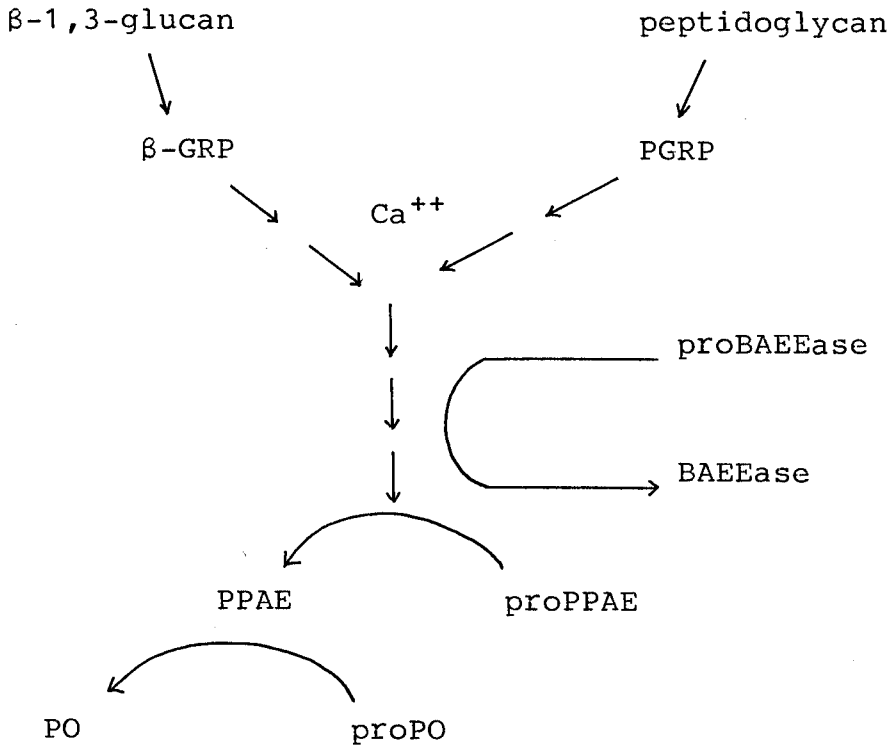

Fig. 1. 2. Scheme of the proPO activating system in silkworm plasma.

β-GRP ; β-1,3-glucan recognition protein

PGRP ; peptidoglycan recognition protein

BAEEase ; an esterase hydrolyzing BAEE

proBAEEase; proenzyme of BAEEase

PPAE ; prophenoloxidase activating enzyme

proPPAE ; proenzyme of PPAE

PO ; phenoloxidase

proPO ; proenzyme of PO

2. Purification of a β-1,3-glucan recognition protein

Introduction

In part 1, it was suggested that β-1,3-glucan and peptidoglycan recognition protein occur as separate entities and that the interaction of the recognition proteins with the respective elicitors causes to triggers the prophenoloxidase (proPO) activating system in silkworm plasma. It was also described a method to specifically remove the β-1,3-glucan recognition protein from the proPO activating system using beads composed of β-1,3-glucan. Plasma, deprived of the recognition protein (referred to as plasma-CPB), may then be used to assay for the β-1,3-glucan recognition protein.

The present part describes an assay method for the β-1,3-glucan recognition protein of the proPO activating system in silkworm plasma and a procedure for obtaining a homogeneous and functionally active β-1,3-glucan recognition protein preparation, together with a preliminary characterization of the molecule.

Materials and Methods.

Experimental animals.

Silkworm (Bombyx mori) larvae were reared on an artificial diet as described in part 1.

Preparation of silkworm plasma (plasma-CPB) for assaying β-1,3-glucan recognition protein.

The plasma fraction of hemolymph and plasma-CPB were prepared in the same way as part 1. Plasma-CPB was used for assaying the β-1,3-glucan recognition protein. The proPO activating system in plasma-CPB is triggered with peptidoglycan but not with β-1,3-glucan.

Assay of β-1,3-glucan recognition protein activity.

The sample solution to be assayed for β-1,3-glucan recognition protein was serially diluted and 10 µl of each diluted solution added to a mixture of 78 µl of plasma-CPB, 10 µl of zymosan solution (100 µg zymosan/ml of double distilled water, prepared after Yoshida and Ashida (1986)) and 2 µl of 250 mM $CaCl_2$, followed by incubation at 25°C for 120 min. After the incubation, phenoloxidase(PO) activity of the reaction mixtures was assayed spectrophotometrically. To eliminate the possibility that the observed activation of proPO was independent of β-1,3-glucan action, PO activity of the reaction mixture devoid of zymosan was always checked after incubation.

The most dilute solution resulting in more than 18 units of PO activity in the reaction mixture was determined and the reciprocal of the dilution factor was used to express the amount of β-1,3-glucan recognition protein activity/ml of sample solution. One unit is defined as the amount of β-1,3-glucan recognition protein contained in 1 ml of β-1,3-glucan recognition protein solution for which the reciprocal of the dilution factor is 1.

β-1,3-Glucan recognition protein activity determined as above provides a relative, rather than quantitative estimation, since different activity values were obtained for a given β-1,3-glucan recognition protein solution when

different lots of plasma-CPB preparations were used. Thus, a single preparation of plasma-CPB was used throughout the purification of β-1,3-glucan recognition protein described in the present study.

Purification of β-1,3-glucan recognition protein.

Fifth instar larvae of silkworm (Bombyx mori) at the 5th or 6th day were bled by cutting abdominal legs with scissors. Hemolymph was immediately mixed with saturated ammonium sulfate (pH 6.5) under vigorous stirring. Three hundred ml of hemolymph from about 650 larvae was collected into 650 ml of saturated ammonium sulfate and stored at 4°C until use.

All subsequent procedures were performed at 0 - 4°C and centrifugation carried out at 12,300 g for 30 min, unless otherwise specified.

The hemolymph preparation was centrifuged and the precipitated materials dissolved into 20% saturated ammonium sulfate containing K-P buffer (10 mM potassium phosphate buffer, pH 6.5), 1 mM phenylmethylsulfonyl fluoride(PMSF), 0.1 mM p-nitrophenyl-p'-guanidinobenzoate, 5 mM phenylthiourea and 5 mM EDTA to make the total volume 300 ml. After centrifugation of the solution, the supernatant was brought to 35% saturation by addition of saturated ammonium sulfate solution(pH 6.5) and left overnight. The resulting precipitate was collected by centrifugation, dissolved in 50 ml of K-P buffer containing 1 mM PMSF and 5 mM phenylthiourea and dialyzed against 2 l of the same buffer for 48 hr with 6 changes of the buffer. The dialyzed solution was used as ammonium sulfate fraction after centrifugation.

The ammonium sulfate fraction was applied to a DEAE-Toyopearl column (2.5 x 55 cm) pre-equilibrated with K-P buffer.

After washing the column with 400 ml of the same buffer, adsorbed proteins were eluted by applying a linear salt gradient (0 to 0.3 M KCl in K-P buffer) in a total volume of 1300 ml. The flow rate was maintained at 80 ml/hr and 9 ml-fractions were collected. An elution profile is shown in Fig. 2. 2. Fractions with β-1,3-glucan recognition protein activity as indicated with a horizontal bar in the figure were pooled and dialyzed against 1000 ml of K-P buffer overnight, followed by centrifugation to remove flocculent materials.

The supernatant was applied to an Affigel-heparin column (1.4 x 22 cm), previously equilibrated with K-P buffer. After washing the column with 150 ml of the buffer, bound materials were eluted at a flow rate of 30ml/hr using a 600 ml linear salt gradient, 0 to 0.25 M KCl, established in K-P buffer. Five ml-fractions were collected. The active fractions as indicated with a horizontal bar in Fig. 2. 3, were pooled and dialyzed against 1000 ml of BTP buffer (20 mM bis-Tris propane buffer, pH 6.5), followed by centrifugation at 30,000 g for 10 min.

The following FPLC chromatography was performed at room temperature. The supernatant obtained in a preceding step was applied to a Mono Q column (HR 5/5), equilibrated to BTP buffer and adsorbed proteins were eluted with a linear salt gradient established in the same buffer (Fig. 2. 4). The flow rate was maintained at 1 ml/min and 0.5 ml-fractions were collected. Fractions (fr. nos. 15 to 16) were pooled.

A two hundred µl portion of the pooled fractions was applied to a Superose 12 (HR 10/30) column equilibrated with K-P buffer containing 150 mM NaCl and eluted at a flow rate

of 250 µl/min with the same buffer; 0.8 ml-fractions were collected. This chromatography was carried out repeatedly to purify all the sample obtained in the preceding purification step. Fractions with β-1,3-glucan recognition protein activity were combined and used as a purified β-1,3-glucan recognition protein preparation.

Sedimentation equilibrium ultracentrifugation.

The molecular weight of native β-1,3-glucan recognition protein in K-P buffer containing 150 mM NaCl (0.5 mg protein/ml) was determined by the method of Yphantis (1964) using a Hitachi analytical ultracentrifuge (Model 282) equipped with Hitachi ultracentrifuge processor (Model DA-7).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE).

SDS-PAGE was carried out in a 1 mm thick slab gel according to Laemmli (1970). Samples were incubated in the presence of 82 mM Tris-HCl buffer, pH 8.8 containing 1% SDS, 1% β-mercaptoethanol, 30% glycerol and 0.01% bromophenol blue (SDS-PAGE solubilizing buffer) in boiling water for 5 min.

IEF-PAGE was performed according to the method of Wrigley (1971).

Gels were stained for proteins with Coomassie brilliant blue R-250.

Procedures for examining ability of plasma proteins or purified β-1,3-glucan recognition protein to bind to insoluble glucans.

Hemolymph of 4 silkworm larvae (5th instar 5-day) was collected into 4 ml of T-M buffer (10 mM Tris-maleate buffer, pH 6.5 containing 150 mM NaCl) containing 0.1 mM p-

nitrophenyl-p'-guanidinobenzoate. Hemocytes were removed by centrifugation at 800 g for 30 min at 2°C. The remaining supernatant was used as plasma.

Twenty mg (dry weight) of beads of curdlan-type polysaccharide (β-1,3-glucan), and a mixture of Sephadex G-100 (20 mg) and cellulose (20 mg) suspended in double distilled water (D.D.W.) were packed into columns (i.d. 8 mm) and equilibrated to T-M buffer. Plasma (2 ml) was applied to each column. After washing the columns with 10 ml portions of T-M buffer or consecutively with 10 ml portions of the buffer and 8 M urea, the washing solution was drained from glucans as thoroughly as possible. Protein(s) adsorbed on beads of β-1,3-glucan and a mixture of Sephadex G-100 and cellulose was extracted with 25 µl and 50 µl, respectively, of SDS-PAGE solubilizing buffer in boiling water for 5 min. In the case of purified β-1,3-glucan recognition protein, 10 µg protein in 50 µl of T-M buffer was applied to glucan columns and extracted as above. Two µg of β-1,3-glucan recognition protein, 10 µl of the extracts from beads of β-1,3-glucan or 20 µl of extracts from the mixtures of the glucans was subjected to SDS-PAGE to examine if any of the extracts contained protein with the same electrophoretic mobility as that of β-1,3-glucan recognition protein.

Assay of amidase activity of β-1,3-glucan recognition protein incubated with zymosan.

Amidase activity of β-1,3-glucan recognition protein or the molecule bound to zymosan was assayed using fluorogenic substrates, peptidyl-7-amino-4-methyl-coumarins (referred to as peptidyl-MCAs).

Pre-incubation mixtures, comprising 5 volumes of β-1,3-

glucan recognition protein solution (0.3 mg protein/ml of K-P buffer containing 150 mM NaCl) and 1 volume of zymosan solution (0.1 mg/ml of D.D.W.) or 5 volumes of the β-1,3-glucan recognition protein solution and 1 volume of D.D.W., were incubated at 25°C. After 10 min incubation, 10 µl aliquots of the pre-incubation mixtures were assayed for amidase activity. The reaction mixture for the assay consisted of 480 µl of T-M buffer containing 5 mM $CaCl_2$, 10 µl of 5 mM fluorogenic substrate and 10 µl of the above pre-incubation mixture. After incubation of the mixtures at 30°C for 120 min, 500 µl of 50% (v/v) acetic acid was added to terminate enzyme reaction. The amount of liberated 7-amino-4-methylcoumarin (referred to as AMC) was determined after Kojima et al. (1979) from fluorescence intensity read at 460 nm with excitation at 380 nm, using a Hitachi 204-A fluorescence spectrophotometer. For controls the same pre-incubation mixtures except for the β-1,3-glucan recognition protein was prepared and their amidase activity was assayed as above. Peptidyl-MCAs used were as follows: Arg-MCA, Bz-Arg-MCA, Boc-Glu-Lys-Lys-MCA, Boc-Glu(OBzl)-Gly-Arg-MCA, Boc-Gln-Arg-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, Boc-Leu-Gly-Arg-MCA, Boc-Leu-Ser-Thr-Arg-MCA, Boc-Leu-Thr-Arg-MCA, Boc-Phe-Ser-Arg-MCA, Boc-Val-Leu-Lys-MCA, Boc-Val-Pro-Arg-MCA, Glt-Gly-Arg-MCA, Gly-Pro-MCA, Leu-MCA, Lys-Ala-MCA, Pro-Phe-Arg-MCA, Pyr-MCA, Suc-Ala-Pro-Ala-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, Suc-gly-Pro-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Z-Arg-Arg-MCA and Z-Phe-Arg-MCA. These substrates were dissolved in either D.D.W., dimethylsulfoxide or dimethylformamide according to manufacturer's instruction.

Analyses of amino acid composition.

Purified β-1,3-glucan recognition protein was dialyzed against D.D.W. and lyophilized. The lyophilized powder (about 0.5 mg) was hydrolyzed with constant boiling point HCl in sealed ampoules at 110°C for 24 hr. Amino acids in the hydrolyzate were analyzed on a Hitachi 835 amino acid analyzer. Tryptophan content was determined separately after hydrolysis of sample with 4 N methane sulfonic acid at 115°C for 24 hr (Simpson et al. 1976).

Determination of protein.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Chemicals.

Chemicals were obtained from the following sources: beads of curdlan-type polysaccharide 13140 (β-1,3-glucan produced by Ascaligenes faecalis var. myxogenes IFO 13140)(Harada et al., 1968) was a gift from Dr. Y. Nakao of Applied Microbiology Labs. Takeda Chemical Ind. Ltd. (Osaka); zymosan and phenyl-methanesulfonyl fluoride from Sigma; molecular weight standard and Affigel-heparin from Bio-Rad Labs.; Ampholine for pH range 3 to 6 from LKB; DEAE-Toyopearl and cellulose powder from Advantec Toyo Co. Ltd. (Kanda, Tokyo); standard proteins for isoelectric point calibration, Sephadex G-100 and pre-packed columns (Mono Q column, HR 5/5 and Superose 12 column, HR 10/30) from Pharmacia Fine Chemicals; p-nitrophenyl-p'-guanidinobenzoate from Vega Chemicals (Tuscon, Arizona); peptidyl-7-amino-4-methyl-coumarins and 7-amino-4-methyl-coumarin from Peptide Institute Inc. (Mino-shi, Osaka). Other chemicals used were the highest grade commer-cially available.

Results and Discussion

Assay of β-1,3-glucan recognition protein.

The procedure for assay of β-1,3-glucan recognition protein in the proPO activating system was developed as described in Materials and Methods. As shown in Fig. 2. 1, a linear relation was observed between the amount of β-1,3-glucan recognition protein and the activity, although the linear curve is displaced from the origin. A method for the assay of β-1,3-glucan recognition protein relies the linear relationship and was used successfully in the course of the purification of the recognition protein from larval hemolymph of the silkworm, Bombyx mori.

A feature of the assay method is that the lower limit of the detectable concentration of β-1,3-glucan recognition protein depends on the availability of plasma-CPB. For example, when separate preparations of plasma-CPB were used, the concentration was found to be 2.5 μg and 1 μg recognition protein/ml of reaction mixture as shown in Fig. 2. 1 and Fig. 2. 7, respectively. The method is sufficiently sensitive to allow detection of 0.1 to 0.25 μg of the recognition protein, although it is influenced by unknown factors as shown by the fact that the sensitivity of the method depends on the quality of plasma-CPB. Therefore, in using the method to quantitate β-1,3-glucan recognition protein, it is important to recognize the limit. In a series of purifications of β-1,3-glucan recognition protein described below, a single preparation of plasma-CPB was employed for the assay of

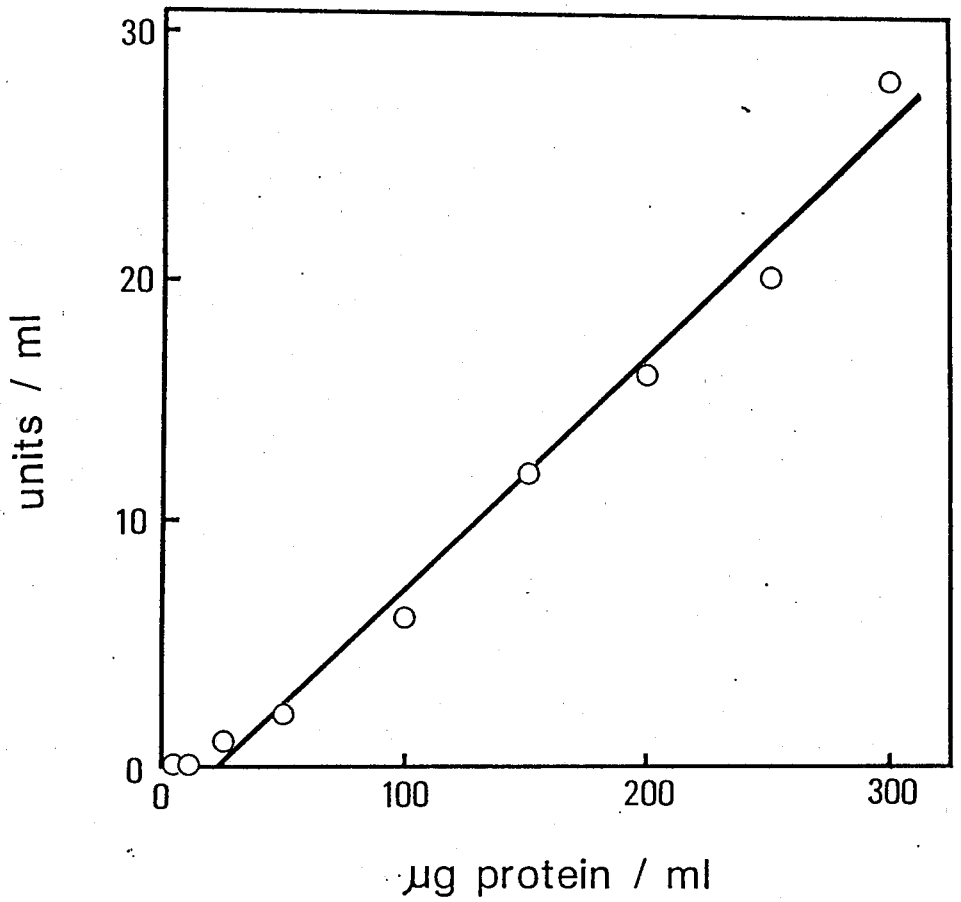

Fig. 2. 1. Relation between the amount of β-1,3-glucan recognition protein and its activity in an assay for β-1,3-glucan recognition protein activity.

Purified β-1,3-glucan recognition protein (Superose 12 fraction) was used and its activity assayed as described under Materials and Methods. Abscissa and ordinate shows the protein concentration of β-1,3-glucan recognition protein solution subjected to activity assay and the β-1,3-glucan recognition protein activity of the solution, respectively.

recognition protein activity.

Another feature of the assay method is that there is a limit in the application of the method to quantitate β-1,3-glucan recognition protein in crude preparation. The limitation became evident because silkworm hemolymph and ammonium sulfate fraction of the hemolymph were shown to trigger the proPO activating system of plasma-CPB in the absence of elicitor. As proPO in insect hemolymph is often activated spontaneously when the hemolymph is collected through cut given to body wall (integument), unidentified substance(s) other than β-1,3-glucan and peptidoglycan has been speculated to activate proPO in insect hemolymph through its direct action on proPO or through triggering the proPO activating system. It is highly probable that the assay method for β-1,3-glucan recognition protein was inapplicable to silkworm hemolymph and ammonium sulfate fraction in Table 2. 1. due to the presence of such substance(s) in them and that the substance(s) was removed from DEAE-Toyopearl fraction during the chromatography on DEAE-Toyopearl.

Purification of β-1,3-glucan recognition protein.

We tried to elute native β-1,3-glucan recognition protein from curdlan-type polysaccharide beads to achieve a one step purification of the molecule. But solutions, which could elute β-1,3-glucan recognition protein from the beads, always caused the inactivation of the molecule as is described in the next section. Therefore, for the purification of β-1,3-glucan recognition protein, larval silkworm hemolymph was fractionated by the conventional purification methods. The purification procedures consisted of ammonium sulfate fractionation and column chromatography on DEAE-Toyopearl,

Affigel-heparin, Mono Q and Superose 12 in the fast protein liquid chromatography system of Pharmacia LKB Biotechnology Inc. Elution profiles of proteins and β-1,3-glucan recognition protein are presented in Figs. 2. 2 to 2. 5. Major and minor peaks of β-1,3-glucan recognition protein activity were detected in the chromatography on Affigel-heparin and Mono Q, although it is not clear whether the β-1,3-glucan recognition protein in the minor peaks is artifact generated during purification or if more than one kind of β-1,3-glucan recognition protein occurs in vivo. In the present purification, the recognition protein in the minor peaks was discarded and only the recognition protein in the major peak was purified. In Superose 12 column chromatography β-1,3-glucan recognition protein activity was eluted at the position corresponding to a major protein peak (Fig. 2. 5). The major active fraction was used as purified β-1,3-glucan recognition protein. Typical data on the purification process of β-1,3-glucan recognition protein are summarized in Table 2. 1. About 2.03 mg of β-1,3-glucan recognition protein was obtained from 300 ml of hemolymph. The final product of the purification was a homogeneous preparation of protein (Fig. 2. 6).

Homogeneity of purified β-1,3-glucan recognition protein and preliminary characterization of the protein.

Purified β-1,3-glucan recognition protein migrated as a single band to the position corresponding to that of 62 K Da polypeptide in SDS-PAGE under reduced conditions (Fig. 2. 6a). In IEF-PAGE the recognition protein preparation gave a single band, the position of which corresponded to isoelectric point, pH 4.3 (Fig. 2. 6b). The amino acid

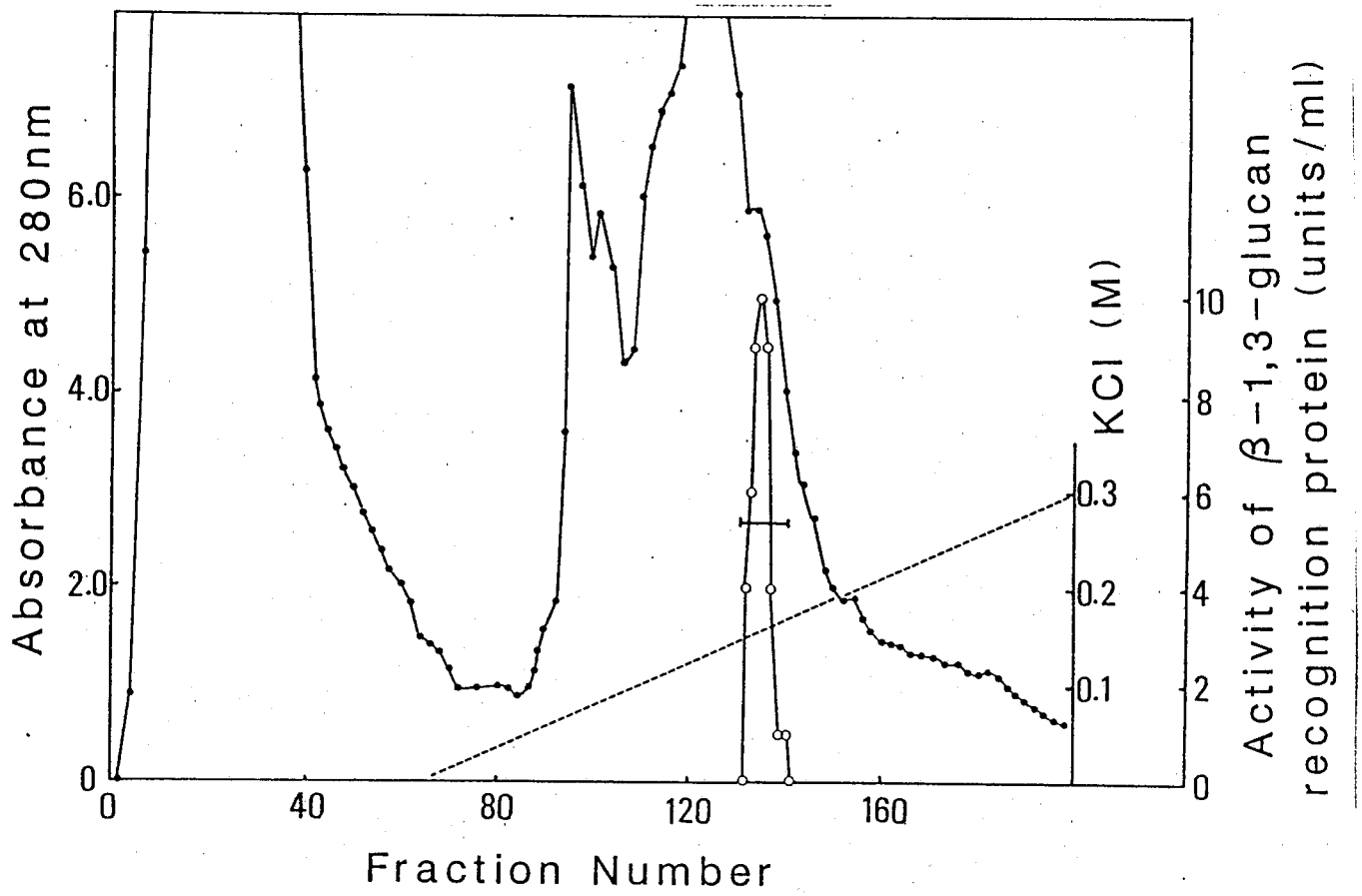

Fig. 2. 2. DEAE-Toyopearl column chromatography of β-1,3-glucan recognition protein.

Conditions are described under Materials and Methods. ● — ●, absorbancy at 280 nm; o — o, activity of β-1,3-glucan recognition protein. Broken line shows a gradient of KCl concentration. The horizontal bar indicates fractions which were pooled and subjected to next step purification.

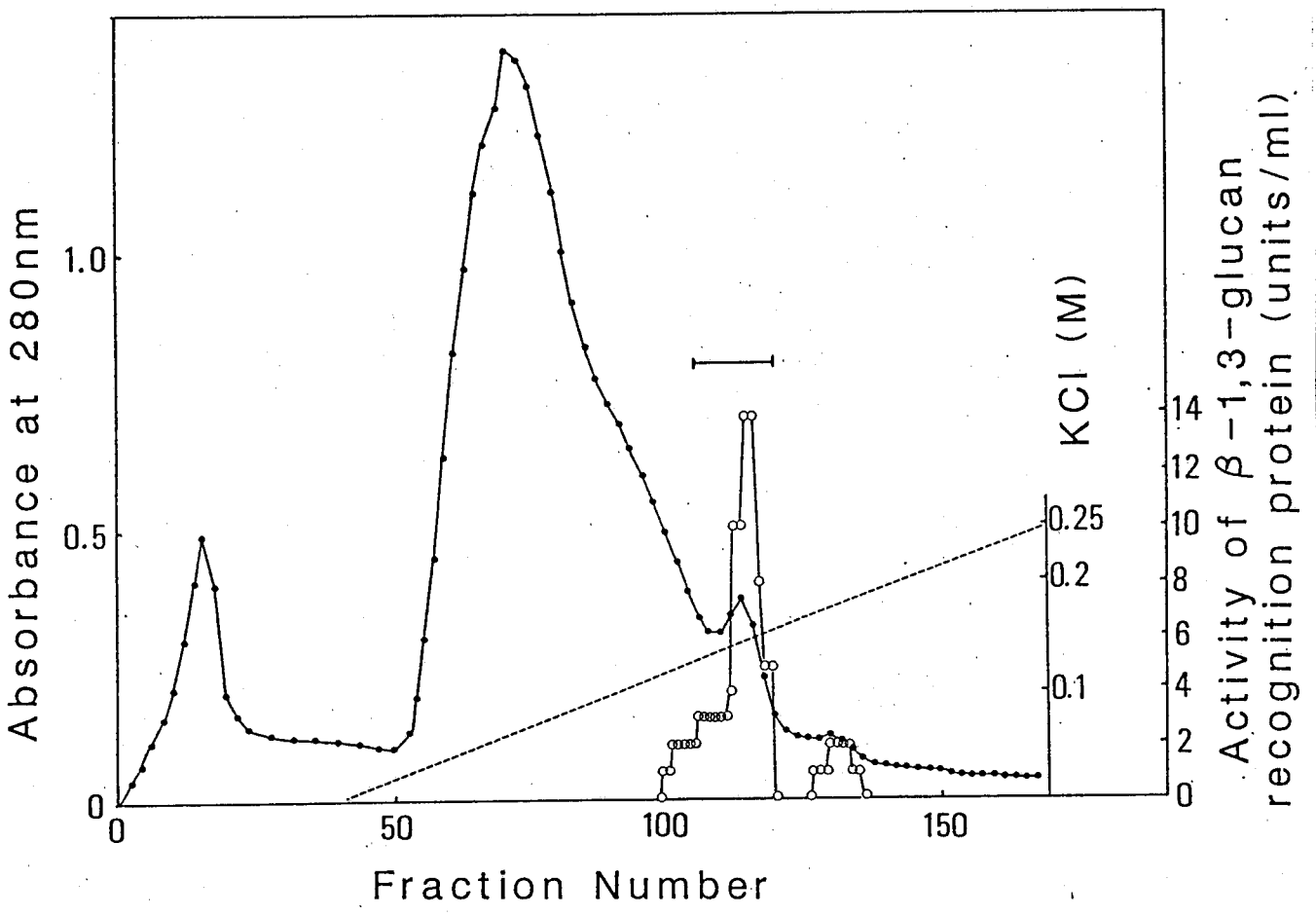

Fig. 2. 3. Affigel-heparin column chromatography of β-1,3-glucan recognition protein.

Conditions are described under Materials and Methods. Symbol assignments are the same as in Fig. 2. 2.

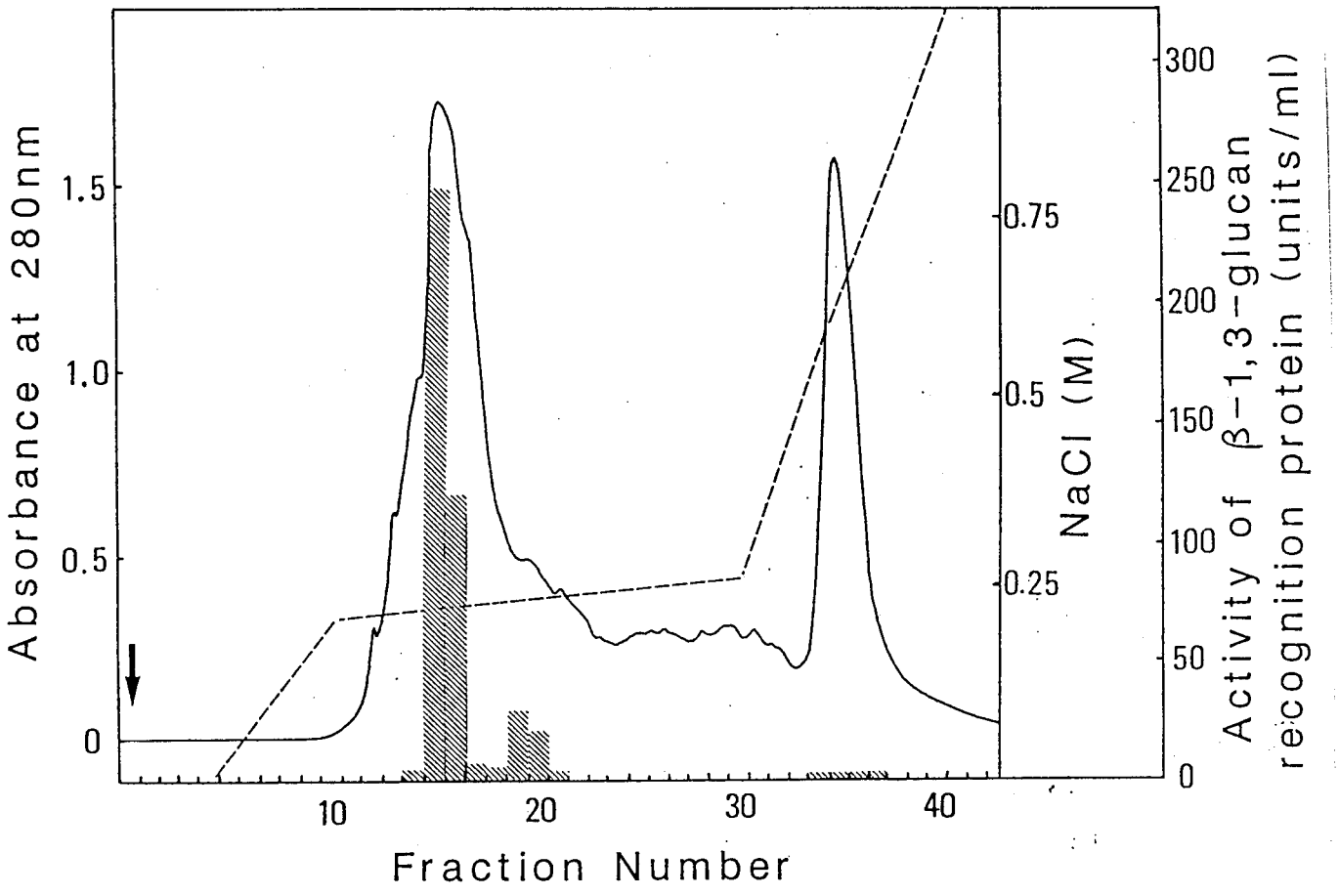

Fig. 2. 4. Mono Q column chromatography of β-1,3-glucan recognition protein.

Conditions are described under Materials and Methods. Solid line, broken line and vertical bars show absorbancy at 280 nm, NaCl concentration and β-1,3-glucan recognition protein activity, respectively.

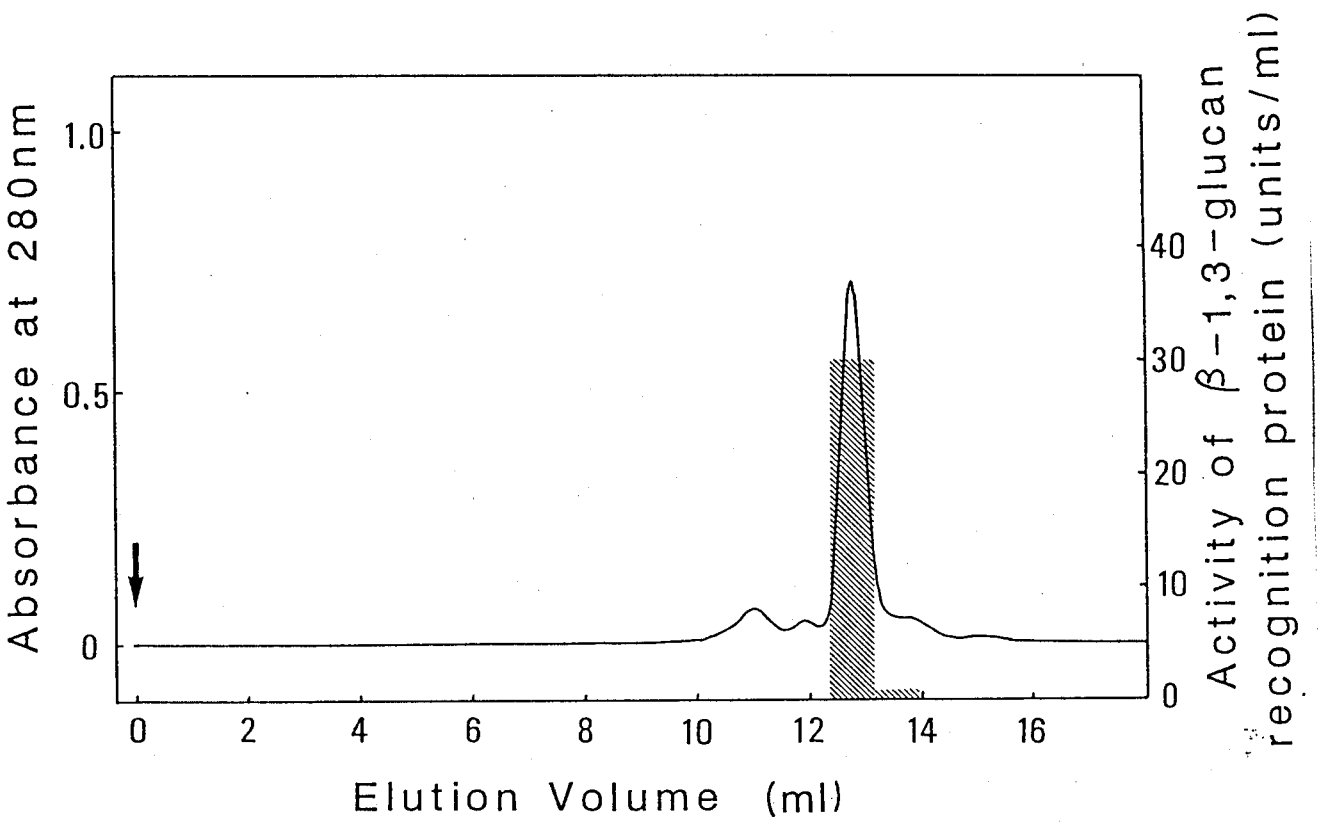

Fig. 2. 5. Superose 12 column chromatography of β-1,3-glucan recognition protein.

Sample was injected at time 0 as indicated with an arrow. For other details see Materials and Methods. Symbol assignments are the same as in Fig. 2. 4.

Table 2. 1. Summary of purification of β-1,3-glucan recognition protein from larval hemolymph of the silkworm, Bombyx mori.

	total volume (ml)	protein (mg/ml)	activity (units/ml)	specific activity (units/mg)	purification[c]	recovery[c] (%)
hemolymph[a]	300	85.0	--[b]	---	---	---
ammonium sulfate precipitate	155	60.0	--[b]	---	---	---
DEAE-Toyopearl	91.0	4.85	4.55	0.94	1	100
Affigel-Heparin	71.0	2.71	6.18	2.28	2.43	106
Mono Q	1.00	2.70	185	68.5	73.0	44.7
Superose 12	5.34	0.38	29.8	78.5	83.7	38.6

a, The volume of hemolymph collected from about 650 silkworm larvae.

b, As hemolymph and ammonium sulfate precipitate contained un-identified factor(s) which causes activation of prophenoloxidase in plasma-CPB without β-1,3-glucan and inhibitors for protease and phenoloxidase, respectively, β-1,3-glucan recognition protein activity in these fractions could not be quantitated.

c, Purification fold and recovery was calculated based on the specific activity and total activity, respectively, of DEAE-Toyopearl fraction.

composition of β-1,3-glucan recognition protein is presented in Table 2. 2, from which partial specific volume was calculated to be 0.736 ml/g (Cohn and Edsall, 1943). No amino sugars were detected in the amino acid analysis of the recognition protein, suggesting that the molecule is not a glycoprotein.

Molecules of native β-1,3-glucan recognition protein were sedimented to equilibrium at 10,000 rpm. A plot of $\ln(A_{280})$ versus $(\text{radius})^2$ gave a straight line, the slope of which together with a partial specific volume, 0.736 ml/g, gave a molecular weight of 61,000. Thus, it is obvious that the native molecule is composed of single polypeptide of about 62 K Da.

Purified β-1,3-glucan recognition protein could restore the activity of the proPO activating system in plasma-CPB with respect to β-1,3-glucan as shown in Fig. 2. 7. Under the experimental conditions, the concentration of β-1,3-glucan recognition protein in plasma-CPB influences the lag period of proPO activation, whereas maximum velocity of proPO activation was constant irrespective of the concentration. Almost no difference of the lag period was observed in a range of concentrations higher than 5 μg β-1,3-glucan recognition protein/ml of plasma-CPB. The proPO activating system in plasma-CPB could not be triggered at concentrations of β-1,3-glucan recognition protein lower than 1 μg/ml of plasma-CPB. Although these phenomena appear to relate to the mode of action of β-1,3-glucan recognition protein in triggering the system, they remain to be studied in the future.

β-1,3-Glucan recognition protein showed specific and

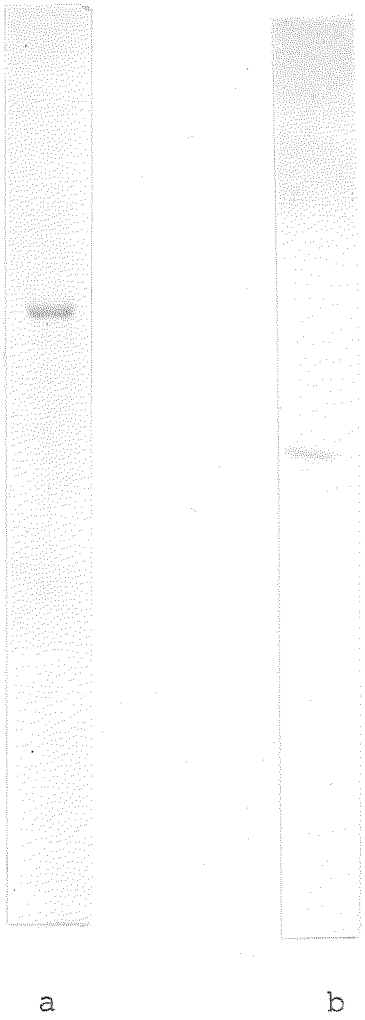

Fig. 2. 6. SDS-PAGE and IEF-PAGE of purified β-1,3-glucan recognition protein.

PAGE. Other experimental details are described under Materials and Methods. a, SDS-PAGE; b, IEF-PAGE.

In SDS-PAGE, gel was calibrated with the following protein molecular weight markers and their weights in kilodaltons (K) are indicated at the left of (a): phosphorylase b (92,500); bovine serum albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); lysozyme (14,400). In IEF-PAGE, the gel was calibrated with the following isoelectric point markers and their isoelectric points are indicated at the right of (b): amyloglucosidase (pH 3.50); glucose oxidase (pH 4.15); soybean trypsin inhibitor (pH 4.55); β-lactoglobulin A (pH 5.20); bovine carbonic anhydrase B (pH 5.85).

Table 2. 2. Amino acid composition of β-1,3-glucan recognition protein.

amino acid	recovered amino acid[a] (mol./1,000 mol.)
Asp	110
Thr	44
Ser	61
Glu	111
Gly	90
Ala	65
Cys/2	5
Val	57
Met	10
Ile	59
Leu	74
Tyr	42
Phe	53
Lys	67
His	14
Trp	19
Arg	41
Pro	78

a, Values except for tryptophan are means of the results of three determinations in which separate samples were analyzed. Content of tryptophan was determined two times after hydrolyzes with methanesulfonic acid of separate samples as described under Materials and Methods and mean of the values is presented here.

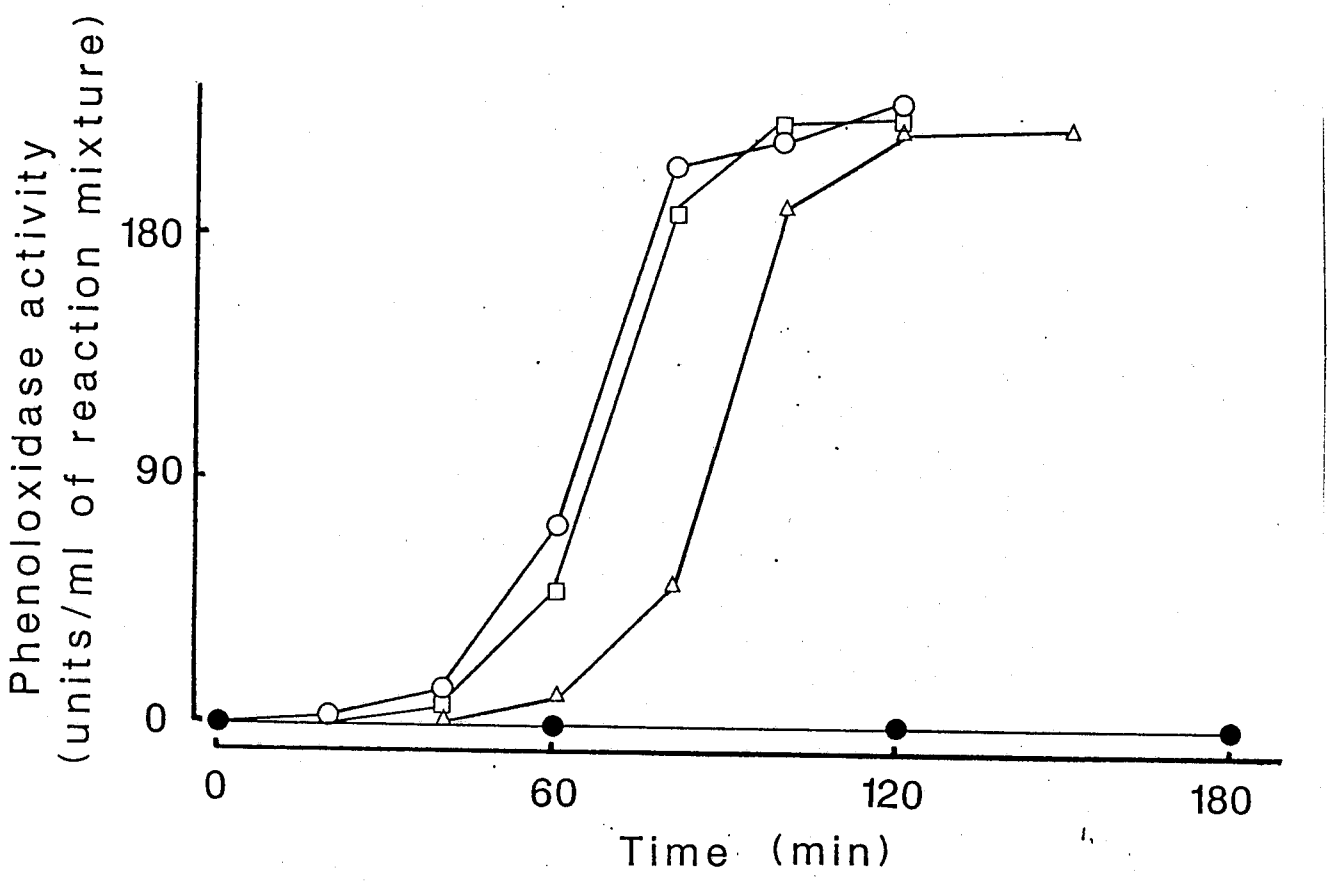

Fig. 2. 7. Activation of prophenoloxidase activating system by β-1,3-glucan in plasma-CPB supplemented with purified β-1,3-glucan recognition protein.

Each reaction mixture consisted of 25 µl of β-1,3-glucan recognition protein solution, 200 µl of plasma-CPB containing 5 mM $CaCl_2$ and 25 µl of zymosan solution (100 µg/ml). Reaction mixtures were incubated at 25°C and at intervals an aliquot was assayed for PO activity to monitor the activation of proPO activating system.

Concentrations of β-1,3-glucan recognition protein in reaction mixtures(µg protein/ml of reaction mixture): o — o, 10 µg; □ — □, 5 µg; △ — △, 1 µg, ● — ●, 0.1 µg.

strong affinity to β-1,3-glucan (Fig. 2. 8). In the light of the previous observation (Ashida et al., 1983) that only glucans with β-1,3-glycosidic linkages could trigger the proPO activating system in silkworm plasma, the demonstrated specificity of binding of the purified β-1,3-glucan recognition protein indicates that binding of the recognition protein to β-1,3-glucan is a necessary condition for the molecule to display its potential activity for triggering the proPO activating system.

The purified β-1,3-glucan recognition protein bounds to β-1,3-glucan did not dissociate appreciably at neutral pH even in a high salt concentration (3.0 M NaCl, data not shown) or 8 M urea (Fig. 2. 8 lane 3), but dissociated in the presence of 1 % SDS, 1 % β-mercaptoethanol at 100°C (Fig. 2. 8). The dissociated β-1,3-glucan recognition protein did not differ in terms of its polypeptide molecular weight from that of the native molecule.

The strong and specific affinity of β-1,3-glucan recognition protein to β-1,3-glucan enabled the detection of the β-1,3-glucan recognition protein without assaying its activity. As shown in lane 6 of Fig. 2. 8, polypeptide with relative molecular mass of 62 K Da was practically the only protein with the same degree of affinity as that of purified β-1,3-glucan recognition protein to β-1,3-glucan among those present in the silkworm plasma under the experimental conditions. β-1,3-Glucan recognition protein did not bind to Sephadex G-100 or cellulose, which are composed of α(1 → 6) [with minor part of α(1 → 2), α(1 → 3) and α(1 → 4)] or β(1 → 4) glycosidic linkages, respectively, suggesting that the recognition protein has a specific affinity to β(1 → 3)

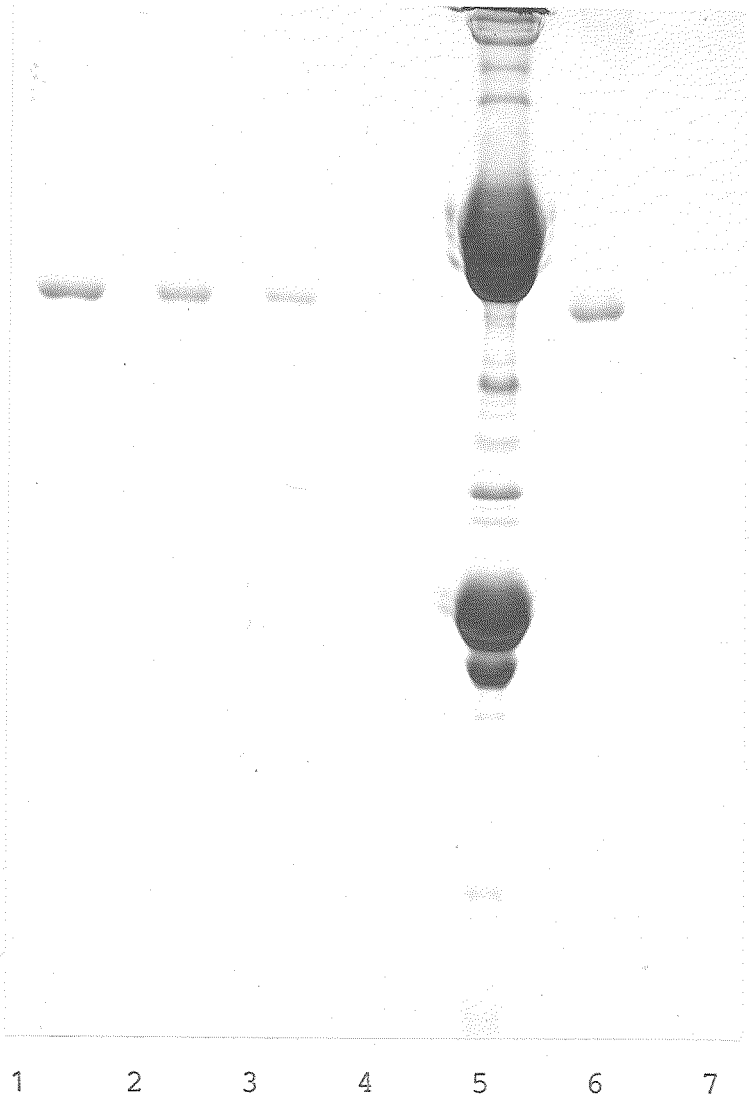

Fig. 2. 8. Examination of binding of β-1,3-glucan recognition protein to insoluble glucans.

Procedures for binding of plasma proteins and purified β-1,3-glucan recognition protein onto beads of curdlan-type polysaccharide(β-1,3-glucan) and a mixture of Sephadex G-100 and cellulose, and for elution of the adsorbed proteins from the glucans are described under Materials and Methods.

Samples subjected to SDS-PAGE: 1, purified β-1,3-glucan recognition protein; 2, protein eluted from beads of curdlan-type polysaccharide previously treated with purified β-1,3-glucan recognition protein and washed with T-M buffer; 3, same as in 2 except that beads of curdlan-type polysaccharide was washed sequentially with T-M buffer and 8 M urea; 4, same as in 3 except that a mixture of Sephadex G-100 and cellulose was used instead of beads of curdlan-type polysaccharide; 5, plasma (2 µl); 6, protein eluted from beads of curdlan-type polysaccharide previously treated with plasma (2 ml) and washed as in 3; 7, same as in 5 except that a mixture of Sephadex G-100 and cellulose was employed.

glycosidic linkages (Fig. 2. 8).

In the horse-shoe crab, the well known blood coagulation system is triggered by β-1,3-glucan or lipopolysaccharide. A protein (factor C) has been shown to be activated through interaction with lipopolysaccharide and the activated factor C to have amidase activity (Nakamura et al., 1986). The possibility of a similar mechanism operating in the activation of the proPO activating system by β-1,3-glucan was investigated. Amidase activity of β-1,3-glucan recognition protein bound to zymosan was examined using 26 commercially available peptidyl-MCAs as listed under Materials and Methods. None of the substrates was hydrolyzed appreciably by bound β-1,3-glucan recognition protein under the experimental conditions, suggesting that β-1,3-glucan recognition protein is not an inactive form of protease.

A feature of the proPO activating system that contrasts with the alternative complement pathway, a cascade present in mammalian blood, is that every component of the system is not inactivated by 1 mM (Yoshida and Ashida, 1986) or 10 mM (unpublished observation) diisopropylfluorophosphate before being triggered by elicitor. This indicates that all the serine enzymes in the proPO activating system (BAEEase, proPO activating enzyme and, may be yet unknown serine enzymes) are activated from zymogens as a consequence of binding of β-1,3-glucan recognition protein to β-1,3-glucan. It is probable that β-1,3-glucan recognition protein bound to β-1,3-glucan offers an environment in which one of the above zymogens is activated.

3. Immuno-cytochemical localization of β-1,3-glucan recognition protein

Introduction

The β-1,3-glucan recognition protein, which is a component of the prophenoloxidase (proPO) activating system, seems to play an important role in recognizing fungi as non-self in insect hemocoel. To advance our understanding on the function and the action mechanism of the protein, much remains to be studied. One of them is localization of the protein in hemolymph.

In this chapter the localization of the β-1,3-glucan recognition protein was studied by indirect immunogold staining technique using polyclonal rabbit anti-β-1,3-glucan recognition protein as the first antibody and goat anti-rabbit IgG linked to colloidal gold particle as the second antibody. The results indicate that plasma fraction of hemolymph and granules of granulocytes and spherules of spherulocytes contain the protein in silkworm.

Material and methods

Experimental animal

The silkworm, Bombyx mori larvae were raised on artificial diet as described in chapter 1.

Preparation of β-1,3-glucan recognition protein

β-1,3-glucan recognition protein was prepared from

silkworm hemolymph as described by Ochiai and Ashida (1988). The preparation was frozen in liquid nitrogen and stored at -70°C. After thawing the protein solution, flocculent materials were removed by centrifugation at 30,000 g for 10 min and the supernatant was used as the β-1,3-glucan recognition protein solution. The protein in the solution was demonstrated to be homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of polyclonal antibody against β-1,3-glucan recognition protein

About 50μg of β-1,3-glucan recognition protein in 0.5 ml of phosphate buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.4 containing 110 mM NaCl and 20 mM KCl) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. At 10 days intervals, two additional injections were administered. Blood was collected after 10 days following the last injection. After preparation of the serum from the blood, the IgG in the serum was purified by ammonium sulfate fractionation and Affigel-protein A column chromatography. The chromatography was performed according to the manufacture's instruction manual. Thus obtained IgG fraction was dialyzed immediately against PBS and the dialyzed IgG fraction was used as polyclonal antibody against β-1,3-glucan recognition protein. Pre-immune serum was obtained from a non-immunized rabbit and non-immunized rabbit IgG was prepared from the serum as above.

Preparation of plasma, supernatants of homogenates of hemocytes, fat body and integument for SDS-PAGE

For the preparation of plasma and homogenate of hemocytes hemolymph was collected at 4°C from 20 silkworm larvae of the 5th instar 5th day, according to the method of Mead et al. (1986) with some modifications. Hemolymph escaping from cuts on each abdominanl leg was added immediately to 40 ml of EDTA-citrate buffer (30 mM citric acid-trisodium citrate buffer, pH 4.5 containing 20 mM KCl, 110 mM NaCl and 10 mM EDTA). The diluted hemolymph (50 ml) was centrifuged at 800 g for 5 min.

The supernatant was added to an equal volume of SDS-PAGE solubilizing buffer (82 mM Tris-HCl buffer, pH 8.8 containing 1% SDS, 1% β-mercaptoethanol, 30% glycerol and 0.01% bromophenol blue) and incubated in boiling water for 5 min. The resultant solution was used as a sample of plasma.

The sedimented hemocytes were gently suspended in 10 ml of EDTA-citrate buffer and again centrifuged as above. To the sedimented hemocytes were added 500 µl of SDS-PAGE solubilizing buffer and homogenized with a glass piston homogenizer. After the homogenate was incubated in boiling water for 5 min, it was centrifuged at 4,4000 g for 10 min and the supernatant was dialyzed against 100 ml of SDS-PAGE solubilizing buffer and the dialysed solution was used as sample of homogenate of hemocytes.

For the preparation of fat body and integument silkworm larvae were dissected in EDTA-citrate buffer. Fat body (40-50 mg) was taken from the 5th abdominal segment. Two pieces of integument (5 x 5 mm) freed of accompanying fat body, muscles and tracheae was cut from the second abdominal segment. The fat body and the integument were rinsed three times each in 10 ml of EDTA-citrate buffer, and homogenized

separately in 500 µl of SDS-PAGE solubilizing buffer. The homogenates were incubated in boiling water for 5 min followed by centrifugation. The resultant supernatants were used as the homogenates of fat body and integument after they had been dialyzed against 100 ml of SDS-PAGE solubilizing buffer at room temperature.

SDS-PAGE

The above samples (twenty µl each) were subjected to SDS-PAGE after Laemmli (1970) using 4 % stacking gel and 9 % separating gel with a thickness of 1 mm.

Immunoblotting of β-1,3-glucan recognition protein on nitrocellulose membrane

After SDS-PAGE, proteins of the gel were transferred electrically to a nitrocellulose membrane (6 x 15 cm) by a Semi-dry electroblotter (Sartrius) according to manufacturer's manual. Excess sites for protein binding on the membrane were blocked by incubation with Tris-HCl buffered saline containing Tween 20 (TBS-Tween; 10 mM Tris-HCl buffer pH 7.4 containing 0.9 % NaCl and 0.1 % Tween 20) for 3 hr (Ochiai, 1982). The membrane was then incubated with 5 ml of rabbit anti-β-1,3-glucan recognition protein antibody solution 5 µg protein/ml of TBS-Tween for 6 hr at room temperature. After washing the membrane with TBS-Tween, it was incubated with 5 ml of goat anti-rabbit IgG conjugated to horseradish peroxidase 1 µg protein/ml of TBS-Tween for 6 hr at room temperature. The membrane was washed and then immunoblots were visualized by incubating the membrane with hydrogen peroxide and HRP color development reagent (Bio-Rad) according to the manufacturer's manual.

Preparation of hemocytes for electron microscopy

By giving a cut to each abdominal legs of 20 silkworm larvae (5th instar, 5th day), five ml of hemolymph was collected into 80 ml of PBS-EDTA (10 mM sodium phosphate buffer, pH 7.1 containing 140 mM NaCl, 2.7 mM KCl and 10 mM Na_2-EDTA) containing 3 % glutaraldehyde. The collected hemolymph was kept on ice for 15 min and then centrifuged at 800 g for 5 min. The sedimented hemocytes were fixed further with 20 ml of PBS-EDTA containing 3 % glutaraldehyde at 4°C for 90 min. After the cells were washed with PBS-EDTA four cycles of sedimentation-and-suspension, they were dehyderated with an ethanol series. Then the fixed hemocytes were embedded in Lowicryl K4M resin according to the manufacture's manual. The thin sections (0.08-0.09 μm in thickness) of the hemocytes were cut with a glass knife. The sections were mounted on copper grids.

Staining of thin section of hemocytes with anti-β-1,3-glucan recognition protein antibody

The ultrathin sections of the hemocytes were incubated for 5 min with Tris buffered saline (TBS; 10 mM Tris-HCl buffer, pH 7.4 containing 0.9 % NaCl) containing 1 % bovine serum albumin (BSA), and subsequently for 60 min with a mixture of one volume of TBS containing 0.1 % BSA (TBS-BSA) and 1/30 volume of normal goat serum, to block non-specific protein binding sites. Next, the sections were floated for 60 min on TBS-BSA containing 20 μg rabbit anti-β-1,3-glucan recognition protein IgG/ml. Unbound IgGs were removed by incubating the grid on TBS-BSA with four changes of the buffer, after which the sections were incubated for 60 min with goat anti-rabbit IgG linked to colloidal gold particles with a diameter of 10 nm (GARG-10) diluted 50 folds in TBS-BSA. Finally,

immunogold labeled sections were washed extensively with 4 changes of TBS-BSA, followed by 4 changes of D.D.W. and then stained with 3 % uranyl acetate and 3 % lead citrate (Reynolds, 1963).

For the control, thin sections were treated as above except that non-immunized rabbit IgG was used instead of rabbit anti-β-1,3-glucan recognition protein IgG.

All reagents except GARG-10 were passed through Millipore filters (Millex-GV, Type SLGV 025LS) before use.

Quantitation of labeling density of hemocytes sections

Electron micrographs were taken at a magnification of x 6000 or x 10,000 and enlarged to a final magnification of x 12,000 or x 20,000. The number of gold particles per square (0.25 μm^2) on section of hemocytes was counted for each hemocyte type. On micrographs of granulocytes and spherulocytes, gold particles were counted for nuclei, cytoplasms except granules or spherules, granules and spherules.

Hemocyte types were identified according to Akai and Sato (1973). Gold particles on the borderlines between cytoplasm and granules or spherules were omitted from the counts.

Chemicals

Goat anti-rabbit IgG (affinity purified) linked to colloidal gold particle with a diameter of 10 nm (GARG-10) and normal goat serum from Janssen Life Science Products Division (Beerse, Belgium); bovine serum albumin (fraction V) from Sigma; glutaraldehyde for electron microscopy grade from Wako Pure Chemical Industries (Osaka, Japan); Lowicryl K4M resin from Polaron Equipment Ltd; molecular weight standard from Bio-Rad Labs. Other chemicals used were the highest

grade commercially available.

Results and discussion

Both of hemocyte homogenate and plasma fraction of hemolymph were shown to contain a polypeptide cross reactive to rabbit anti-β-1,3-glucan recognition protein IgG. The mobility of the polypeptide in SDS-PAGE corresponded to that of the purified β-1,3-glucan recognition protein (Fig. 3. 1), indicating that the anti-β-1,3-glucan recognition protein IgG was monospecific to β-1,3-glucan recognition protein and that the protein is present not only in plasma fraction but also in hemocytes.

Electron micrographs of hemocytes sections, which were stained sequentially with rabbit anti-β-1,3-glucan recognition protein IgG and GARG-10, are presented in Fig. 3. 2-6. Numerous colloidal gold particles were detected in granules of granulocytes and spherules of spherulocytes. Although granules of granulocytes were dyed in various shades with uranyl acetate and lead citrate, gold colloid particles were clearly observed within these granules regardless of their various electron densities. The spherules of spherulocytes were labeled uniformly with gold colloid particles and the labeling density was the very high. And very few gold particles were detected in nuclei and cytoplasms of granulocytes and spherulocytes. On the sections of prohemocytes, plasmatocytes and oenocytoids, their labeling densities of gold particles were similar to those observed on control sections stained with rabbit IgG

purified from non-immunized serum instead of rabbit anti-β-1,3-glucan recognition protein IgG (Fig. 3. 4-6).

The above results indicate that β-1,3-glucan recognition protein is present at the highest concentration in the spherules of the spherulocyte (Table 3. 1). And 80 % and 98 % of the numbers of the gold colloid particles detected in granulocytes and spherulocytes concentrated to the granules and the spherules, respectively. The average of labeling densities in the spherules of the spherulocytes is calculated from the data in Table 3. 1. to be higher than that in the granules of the granulocyte by a factor of 5.2.

The granules characterizing the granulocytes are packed with fibrous materials consisted of subunits, which are claimed to be mucopolysacchride (Nittono, 1960), and the contents of mature granules are released into plasma in the silkworm hemolymph (Akai and Sato, 1973). Some section of granulocytes in the present investigation gave figures showing that they were in the process of degranulation and golds colloid particles were detected in the materials discharged from the granules of the granulocytes, suggesting that β-1,3-glucan recognition protein were released together with the mucopolysaccharide (Fig. 3. 7).

with respect to the first stage of the cellular defense reactions of insects such as nodule formation and encapsulation, it has been suggested that granule-containing cells degranulate in random contact with foreign objects such as fungi and bacteria and that a sticky substance(s) discharged from granules forms coagula where the foreign objects and reactive hemocytes are entrapped (Ratcliffe and Rowley, 1979). In Bombyx larvae the granulocytes encapsulate

a larger foreign bodies and the sheaths formed round them is composed of two layers. The outer layer consists of only plasmatocytes and the inner one of only granulocytes, which release their secretory materials to the space among the cells. Melanization also occurs in the inner sheath (Sato et al, 1976). Although the function of β-1,3-glucan recognition protein on the cellular reaction is unclear, it is obvious that the protein discharged from granulocytes enhances the activation of the proPO activating system in the case that the fungi invading into hemocoel are encapsulated.

The function of spherulocytes of silkworm is unclear. However, it has been known for long that the spherules of the cells give strong positive reactions for reagents for staining neutral mucopolysaccharides and mucoproteins (Nittono, 1960). Recently, the preliminary characterization of the cell type of two lepidopteran species was carried out by Cook et al. (1985), showing that spherules contain a sulphated glycosaminoglycan like substance, which may possess the anticoagulant activity like heparin. β-1,3-Glucan recognition protein in spherules of spherulocytes of silkworm seems to be associated with such mucopolysaccharide, since the protein was shown to be adsorbed by an Affigel heparin column in the course of its purification. Akai and Sato (1973) claimed that mature spherulocytes release the materials of spherules into plasma by exocytosis under normal physiological conditions. On the examination under the electron microscopy, spherulocytes discharging materials from their spherules were observed, supporting the inference of Akai and Sato. The empty spherules were negative to the immunogold staining of β-1,3-glucan recognition protein (Fig.

3. 8). Therefore, spherulocytes seem to supply plasma with β-1,3-glucan recognition protein.

Previously proPO was reported to be synthesized in oenocytoid of the silkworm, Bombyx mori and is released into culture medium (Iwama and Ashida, 1986). And recently, a more detailed study on the localization of the proenzyme among hemocytes of silkworm was carried out and revealed that only plasmatocytes and oenocytoids have the protein both in cytoplasm and nucleus (Ashida et al., 1988). Considering the localization of proPO and β-1,3-glucan recognition protein, it is evident that the activation of proPO induced by β-1,3-glucan take place only in plasma.

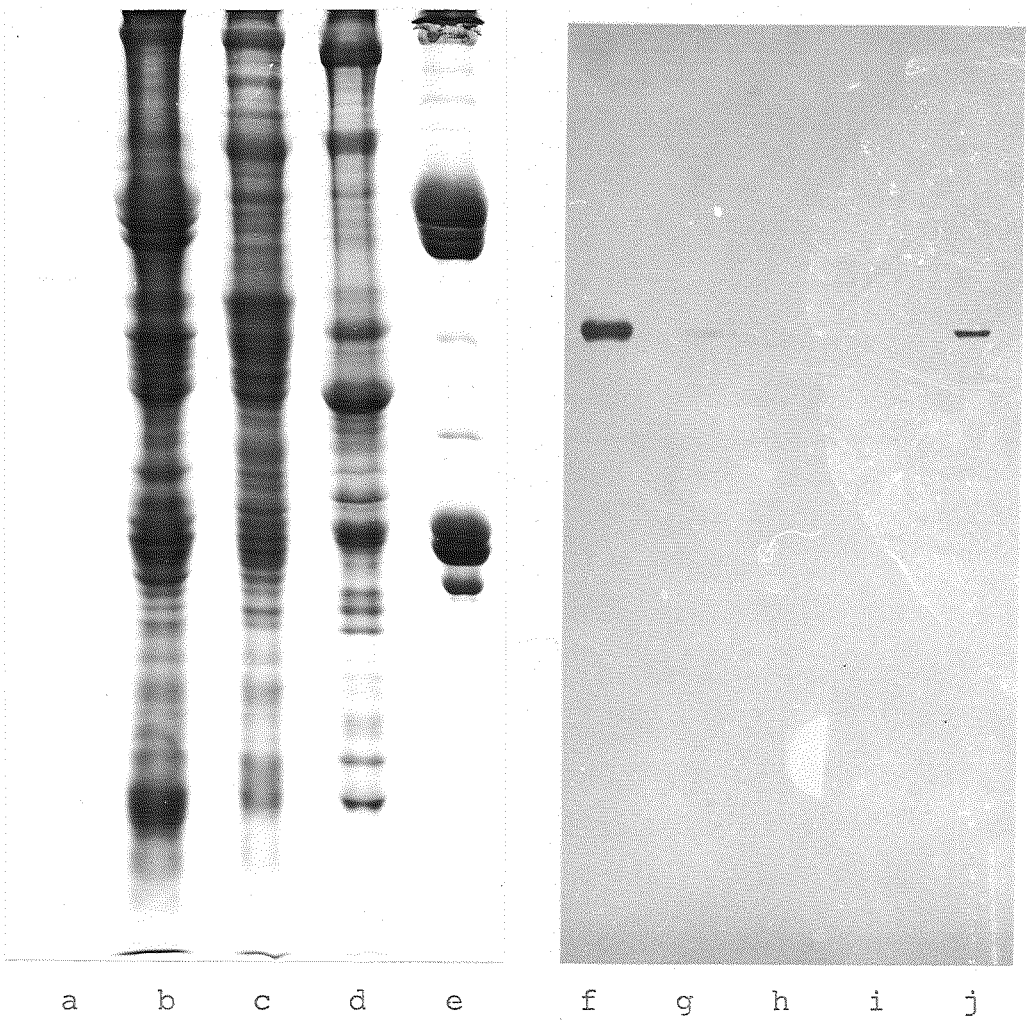

Fig. 3. 1

Specificity of β-1,3-glucan recognition protein IgG for β-1,3-glucan recognition protein.

The preparation of samples for SDS-PAGE, SDS-PAGE and immunoblotting were carried out as described under Materials and Methods. The left and the right panels are SDS-PAGE stained for proteins and immunoblotting, respectively. a and f, purified β-1,3-glucan recognition protein; b and g, homogenate supernatant of hemocytes; c and h, integument; d and i, fat body; e and j, plasma.

Fig. 3. 2 -6.

Photographs of hemocyte sections stained sequentially with anti-β-1,3-glucan recognition protein IgG and GARG-10. In the figures, nucleus, endoplasmic reticulum, mitochondrion, granule, cytoplasmic inclusion and spherule are indicated with N, ER, M, G, CI and V, respectively.

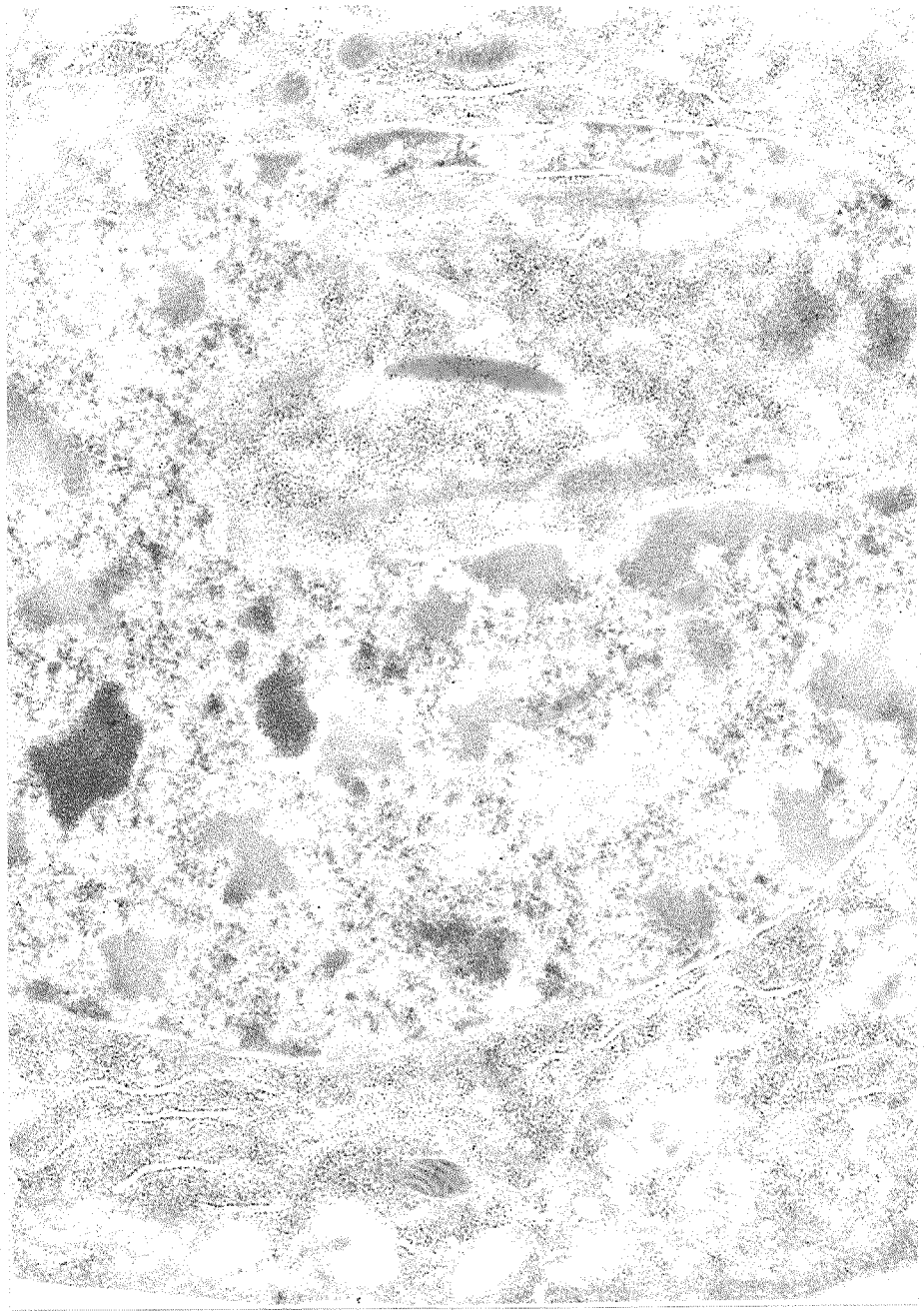

Fig. 3. 2.

Part of prohemocyte. x 20,000.

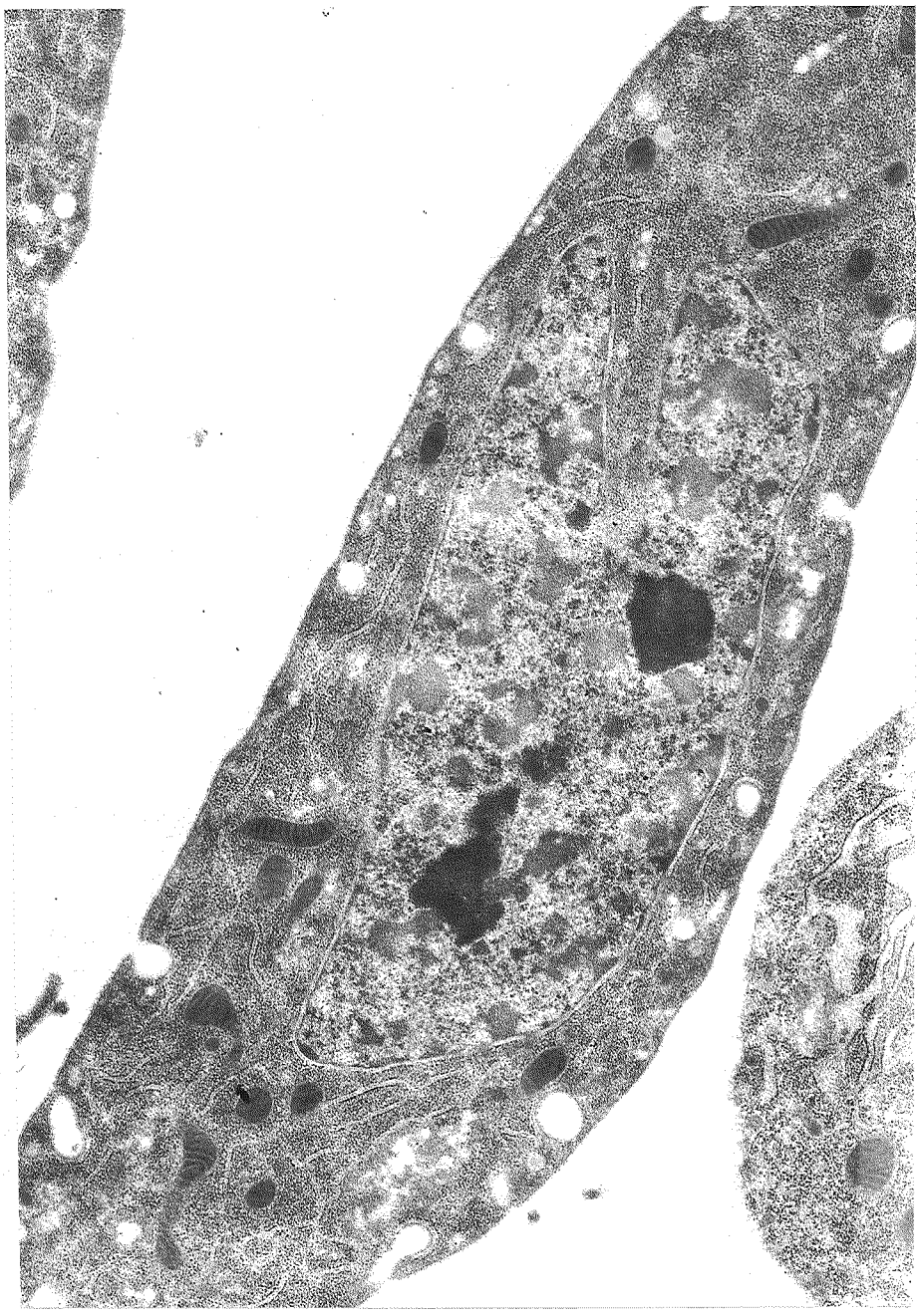

Fig. 3. 3.

Part of plasmatocyte and unidentifical hemocytes. x 12,000.

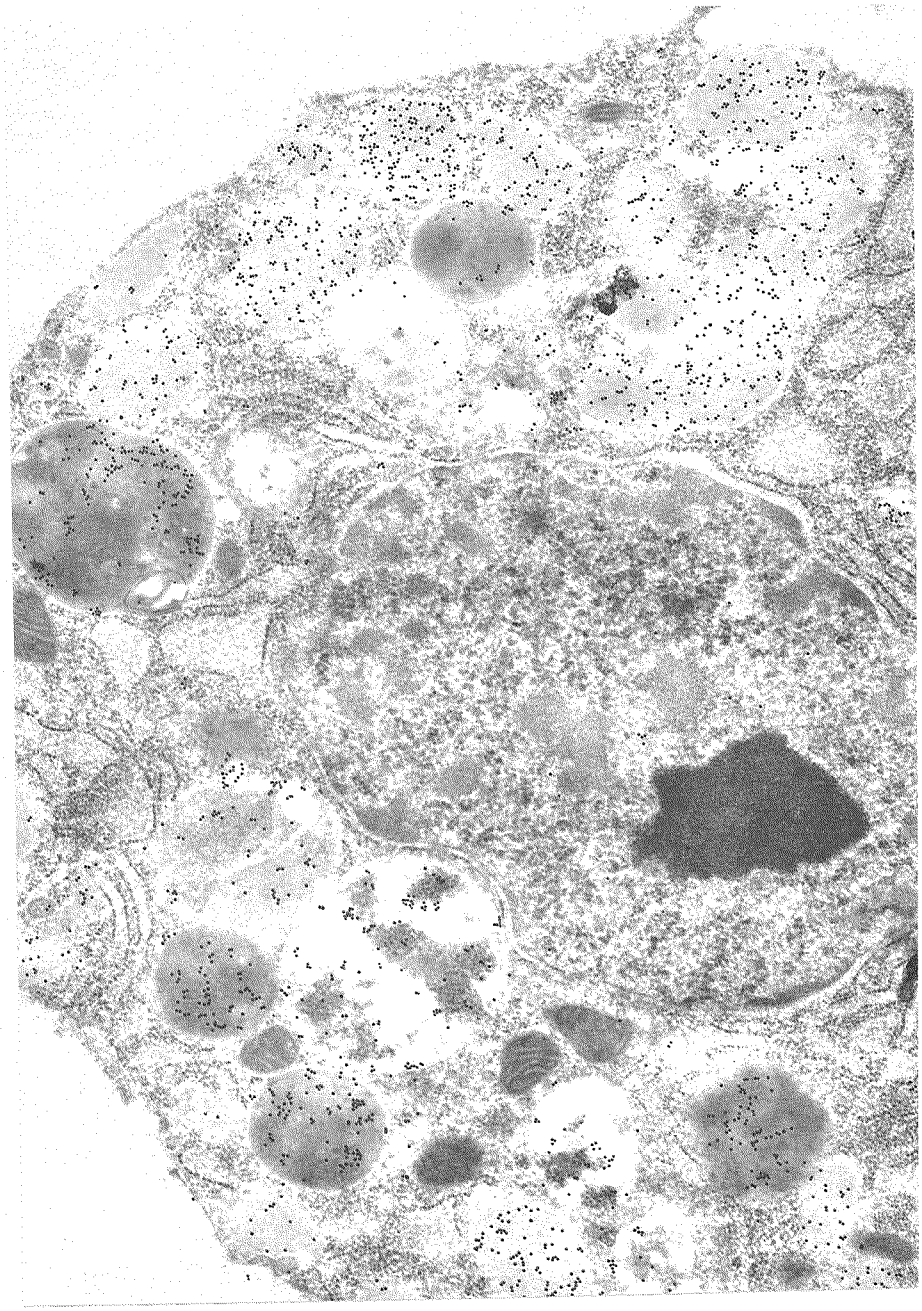

Fig. 3. 4.

Part of granulocyte. x 20,000.

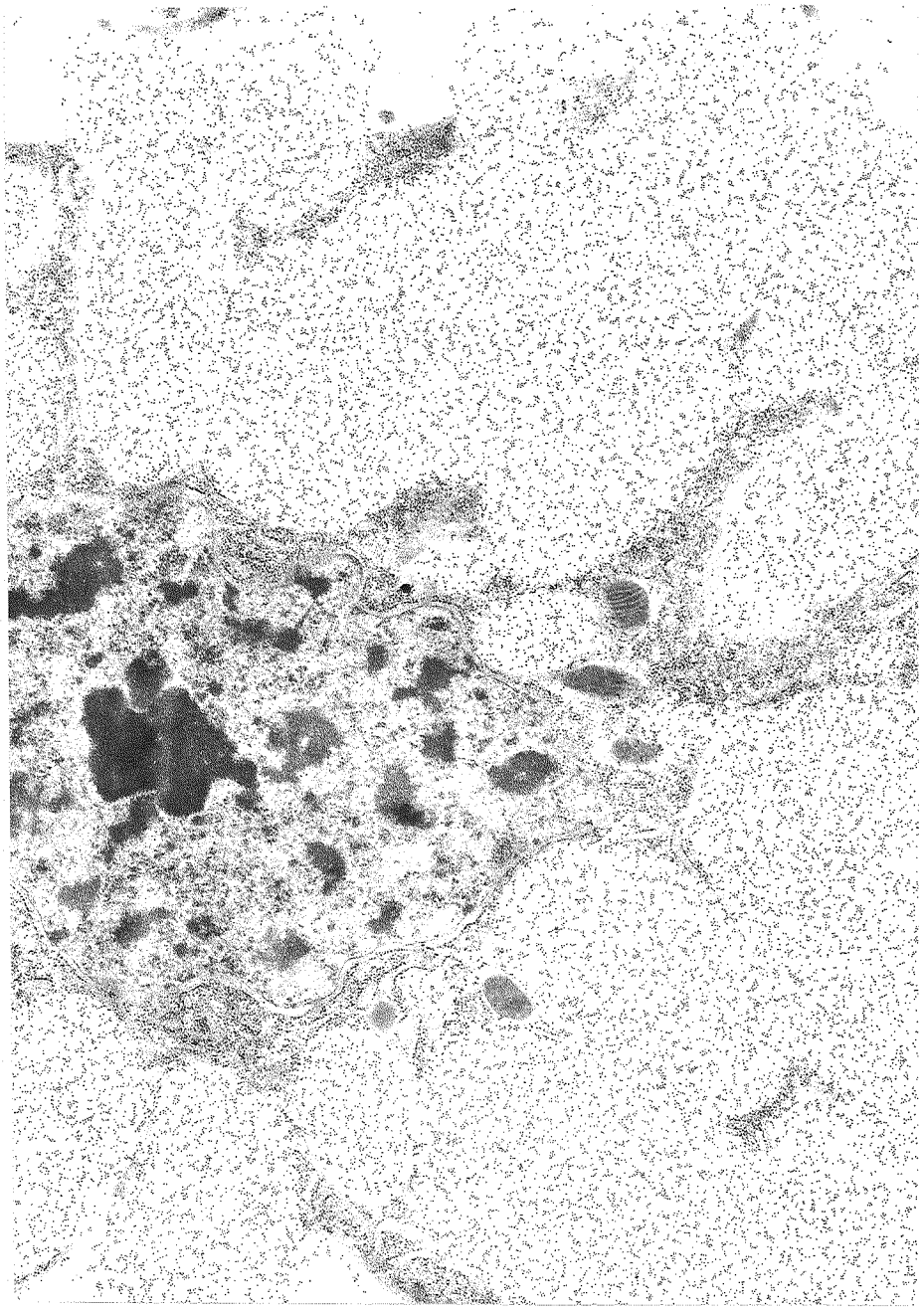

Fig. 3. 5.

Part of spherulocyte. x 12,000.

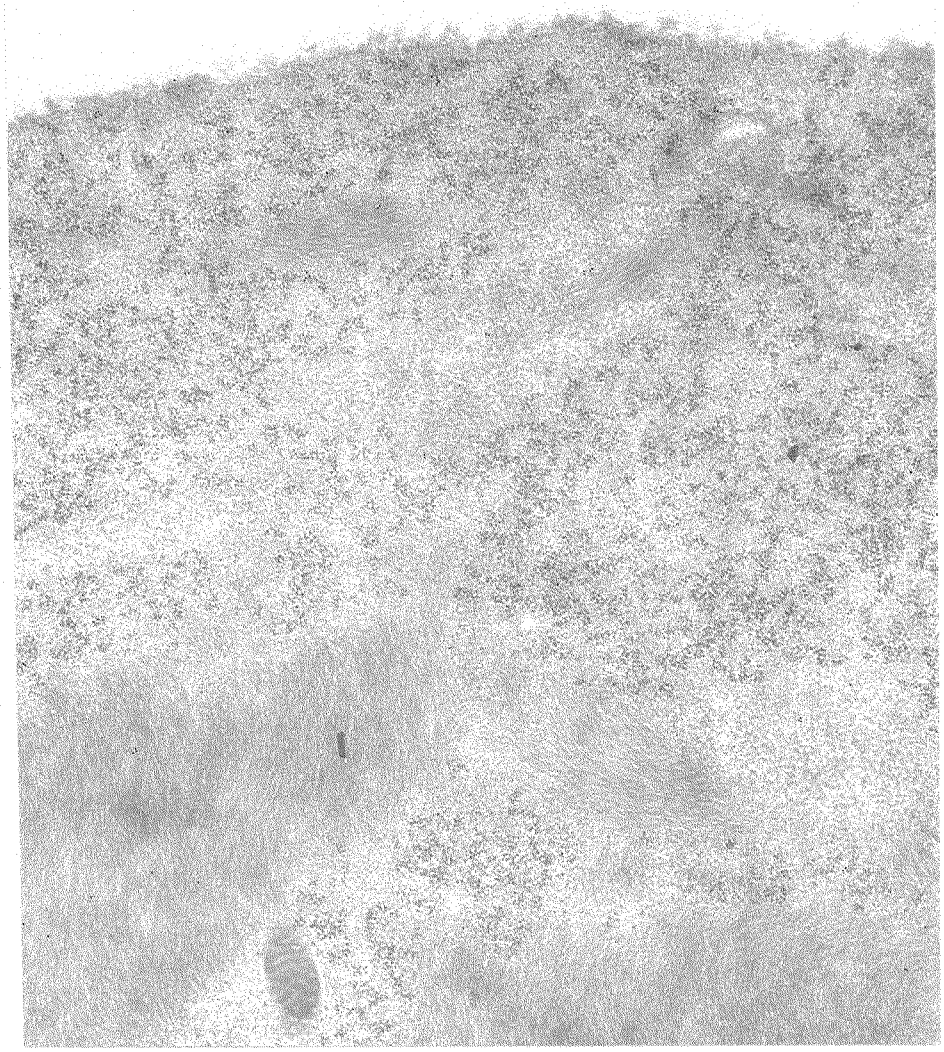

Fig. 3. 6.

Part of oenocytoid. x 12,000.

Table 3. 1.

Labeling densities of gold colloid particles on hemocyte sections.

Cell type and part of the cell	Number of gold particles/0.25 μm^2 (n) control	experiment
prohemocyte		
nucleus	0.20 ±0.13 (18)	0.19 ± 0.13 (18)
cytoplasm	0.19 ±0.11 (15)	0.18 ± 0.11 (13)
plasmatocyte		
nucleus	0.20 ±0.12 (15)	0.19 ± 0.11 (15)
cytoplasm	0.22 ±0.13 (16)	0.26 ± 0.16 (20)
granulocyte		
nucleus	0.28 ±0.19 (15)	0.44 ± 0.20 (48)
granule	0.30 ±0.25 (15)	51.01 ±22.13 (75)
cytoplasm*	0.31 ±0.22 (15)	2.52 ± 1.25 (63)
spherulocyte		
nucleus	0.41 ±0.25 (15)	15.23 ± 9.50 (47)
spherule	1.20 ±0.65 (15)	265.26 ±50.12 (88)
cytoplasm*	0.88 ±0.45 (10)	30.26 ±10.02 (15)
oenocytoid		
nucleus	0.20 ±0.12 (10)	0.21 ± 0.13 (15)
cytoplasm	0.19 ±0.18 (10)	0.20 ± 0.13 (15)

* Here, the term cytoplasms, on the granulocyte and spherulocyte mean the areas except those occupied by the granules and the spherules, respectively.

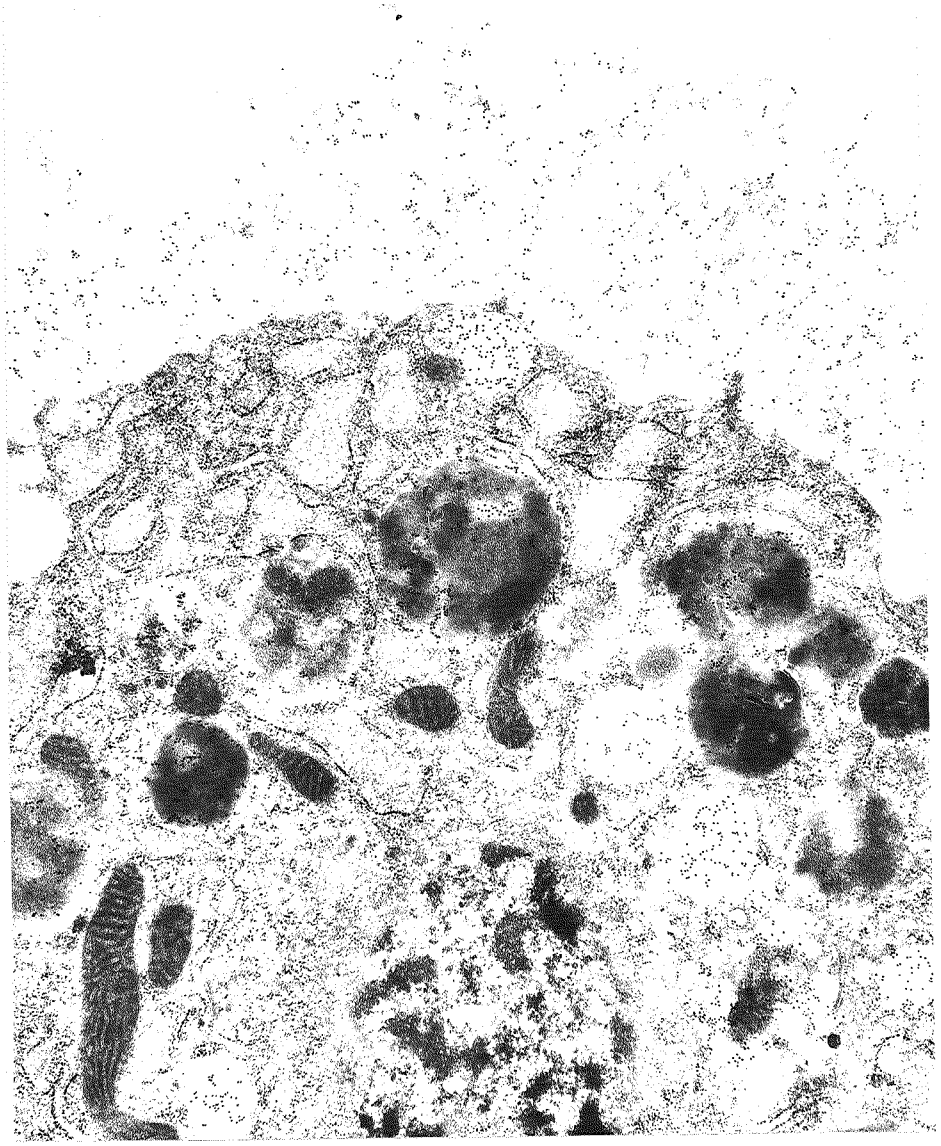

Fig. 3. 7.

Degranulated granulocyte. Gold colloid particles are seen in both the coagulum around the cell and the inside of the cell. x 12,000.

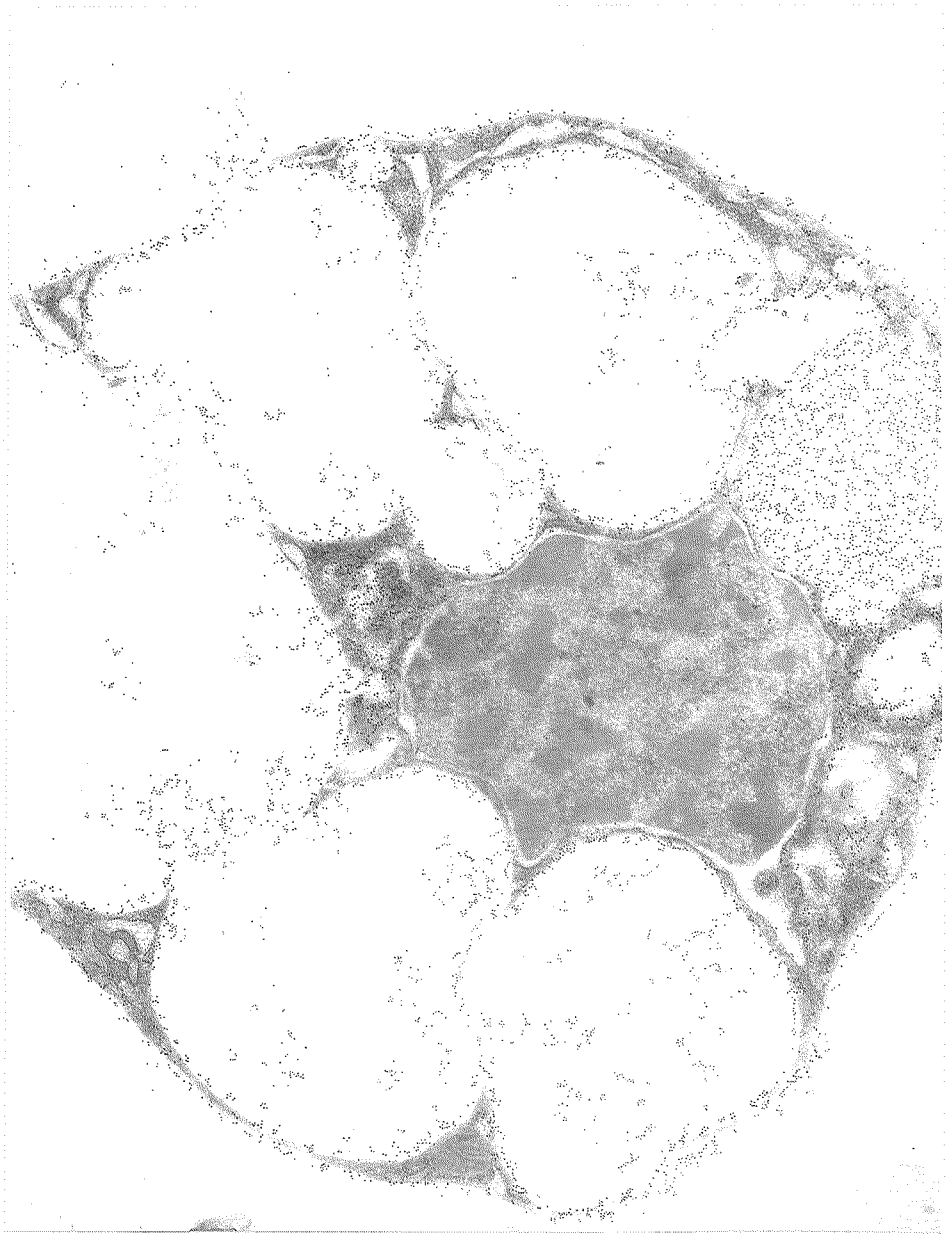

Fig. 3. 8.

Spherulocyte with both discharged and non-discharged spherules. In the non-discharged spherule, higher labeling density of gold colloid particles is observed. x 12,000.

Concluding remarks and discussion

The main points discovered in the present studies with respect to β-1,3-glucan recognition protein in the prophenoloxidase (proPO) activating system of the silkworm, hemolymph are as follows: β-1,3-glucan recognition protein and peptidoglycan recognition protein of the proPO activating system exist as separate entities in plasma of the silkworm, Bombyx mori; an assay method for β-1,3-glucan recognition protein and a procedure for the purification of the protein were established; the purified β-1,3-glucan recognition protein has a molecular mass of 62 kDa composed of a single polypeptide and an isoelectric point of pH 4.3; the protein binds specifically to β-1,3-glucan with strong affinity and the binding of β-1,3-glucan recognition protein with β-1,3-glucan causes the triggering of the proPO activating system; the granulocyte and the spherulocyte of silkworm contain the protein in the granules and the spherules, respectively.

The proPO activation system in the silkworm, Bombyx mori is triggered by β-1,3-glucan or peptidoglycan at a very low concentration (10^{-8} g elicitor/ml of plasma)(Yoshida and Ashida, 1986). The proPO activating system in plasma-CPB and plasma-PG prepared as is described in chapter 1 were shown to lack their reactivates to β-1,3-glucan and peptidoglycan, respectively. These plasma preparations could be used as specific reagents to detect the minute amounts of β-1,3-glucan and peptidoglycan that may be present in the plasma.

Recently, Söderhäll et al reported on the purification of a protein from Blaberous craniifer plasma which binds to an

immobilized β-1,3-glucan and which enhances the activation by β-1,3-glucan of proPO in B. craniifer hemocyte lysate (1988). The protein is a glycoprotein with a molecular mass of ca 91,000 and composed of subunits of 63,000 and 52,000 as determined by SDS-PAGE. The amino acid composition of the protein indicates a high content (ca 65 %) of hydrophilic amino acids. No similarity in characteistics has been detected between the protein of B. craniifer and β-1,3-glucan recognition protein of the silkworm, Bombyx mori. The function property of the protein of B. craniifer is reported to enhance the maximal activities of peptidase and PO induced by β-1,3-glucan, whereas β-1,3-glucan recognition protein of B. mori does not enhance the maximal values of PO and BAEEase activities induced through the action of β-1,3-glucan, although the protein is essential for the system to be triggered by β-1,3-glucan. Since the B. craniifer proPO activating system deprived of β-1,3-glucan recognition protein, which corresponds to that of plasma-CPB in silkworm, is not obtained, the reconstruction experiment using the purified C. craniifer protein and the system deprived of β-1,3-glucan recognition protein has not been carried out. Therefore, it can not be concluded at present whether or not the B. craniifer protein is essential for the proPO activating system to be triggered by β-1,3-glucan, although it is probably a factor within the system.

Obviously, much remains to be studied on the physicochemistry of β-1,3-glucan recognition protein and the mode of action of the molecule in the activation of the proPO activating system by β-1,3-glucan. It seems to be desirable to carry out the investigation to answer the following

questions: What structure of β-1,3-glucan is recognized by the protein? ; Does any structural change occur in this protein which binds to β-1,3-glucan? ; What kind of factor does interact with the protein-glucan complex within the proPO activating system? ; Does the amino acid sequence of the binding site of β-1,3-glucan recognition protein have similarity with those of the β-1,3-glucan recognition proteins or receptors in the other biological system? Once these questions can be answered, our understanding of the effects exerted by β-1,3-glucan in various biological systems including insects will advance considerably.

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