Purification of a unique glycoprotein that enhances phenol oxidase activity in scorpion (*Heterometrus bengalensis*) haemolymph

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A monomeric glycoprotein (SGP) of M_r 32000 was isolated to purity from scorpion (*Heterometrus bengalensis*) haemolymph by $(\text{NH}_4)_2\text{SO}_4$ fractionation, chromatofocusing and h.p.l.c. The homogeneity of SGP is confirmed by polyacrylamide-gel electrophoresis. SGP is soluble in 100 % -satd. $(\text{NH}_4)_2\text{SO}_4$ solution. Needle-shaped crystals of SGP were obtained in an aqueous environment. The glycan part of the molecule contains arabinose, which does not commonly occur in animal glycoproteins. Amino acid analysis demonstrated a preponderance of glycine, tyrosine and glutamic acid. SGP enhances phenol oxidase (EC 1.14.18.1) activity.

INTRODUCTION

Melanization, i.e deposition of melanin around foreign objects, accompanies the defence reactions of arthropods. The enzyme phenol oxidase (EC 1.14.18.1) is responsible for melanin biosynthesis. The importance of phenol oxidase in protection of insects and other arthropods against invading micro-organisms and parasites is well documented (Nappi, 1973; Ratcliffe *et al.* 1984). While working on an erythroagglutinin (Basu *et al.*, 1984), haemocyanin (Datta *et al.*, 1985) and true acetylcholinesterase (EC 3.1.1.7) (Datta *et al.*, 1988) in the haemolymph of the scorpion *Heterometrus bengalensis*, we have observed a unique glycoprotein (SGP) that can enhance the phenol oxidase reaction. This glycoprotein is soluble in 100 % -satd. $(\text{NH}_4)_2\text{SO}_4$ solution. In the present paper we report the purification of SGP, and its characterization and its crystallization. The biological role of SGP in the activity of phenol oxidase isolated from scorpion haemolymph was also studied.

MATERIALS AND METHODS

Phenylmethanesulphonyl fluoride, trypsin (type III), dopamine (2,4-dihydroxyphenethylamine), *N*- α -benzoyl-DL-arginine *p*-nitroanilide, α -*N*-benzoyl-L-arginine ethyl ester, Alcian Blue, MW-SDS-70 M_r marker kit, standard amino acids and phenyl isothiocyanate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *p*-Chloromercuribenzoate was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. PBE-94 and Polybuffer 74/HCl were from Pharmacia Fine Chemicals, Uppsala, Sweden. Servalyte-Precotes, pH 3–10, and human serum as standard were supplied by Serva, Heidelberg, Germany. All other chemicals used were of analytical grade.

The scorpion *H. bengalensis* belongs to the Phylum Arthropoda and Subphylum Chelicerata. The animals were procured (Basu *et al.*, 1984), and the identification of the animal was facilitated by the Zoological Survey of India. Bleeding was performed by cutting of legs. An average of 0.5 ml of haemolymph from each animal was

collected. The plasma was separated and dialysed as described previously (Basu *et al.*, 1984). Dialysed plasma (13 g of protein) was diluted (1:1, v/v) with 50 mM-Tris/HCl buffer, pH 7.2, containing 100 mM-NaCl, and made 60 % saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The mixture was stirred for 2 h, kept overnight and centrifuged at 27000 *g* for 60 min. The supernatant was then made 100 % saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The solution was centrifuged as described above and the supernatant (pH 4.8) was extensively dialysed against double-distilled water to achieve the complete removal of $(\text{NH}_4)_2\text{SO}_4$ as tested by Nessler's reagent. After dialysis the solution inside the bag was concentrated by freeze-drying to obtain 100 % -satd. $(\text{NH}_4)_2\text{SO}_4$ -soluble glycoprotein (SGP). All preparative operations were performed at 4–10 °C. The total protein content (7.5 mg) was determined by a micro biuret method (Itzhaki & Gill, 1964).

SGP, thus obtained, was applied to chromatofocusing column (20 cm $\times$ 1 cm) of PBE-94. The column was equilibrated with 25 mM-imidazole/HCl buffer, pH 7.4, and SGP solution (containing 1.5 mg) that had previously been dialysed against the same buffer was applied to the column. The sample was eluted (0.5 ml/tube; 20 ml/h) with Polybuffer 74/HCl (1:8 dilution), pH 4.0. The eluted fractions were monitored spectrophotometrically at 280 nm.

H.p.l.c. on a Waters Associates (Milford, MA, U.S.A.) model 440 apparatus was used for further analysis of the protein. The protein solution obtained from the chromatofocusing column was dialysed against phosphate-buffered saline (0.15 M-NaCl/8.0 mM-sodium phosphate buffer, pH 7.2) and a portion containing 25 μ g of protein was applied to the h.p.l.c. column (Protein Pak I 125) previously equilibrated with phosphate-buffered saline. The elution was carried out at a flow rate of 0.8 ml/min. The absorbance at 280 nm was monitored.

Servalyte-Precotes, pH 3–10, and human serum as standard were used for determination of the pI of the purified SGP.

Polyacrylamide-gel electrophoresis (Davis, 1964) in Tris/glycine buffer, pH 8.3, was performed to determine

the homogeneity of the purified SGP. The glycan part of the glycoprotein was stained by Alcian Blue (Wardi & Michos, 1972) and periodic Acid/Schiff reagents (Zaccharius *et al.*, 1969).

The M_r of the purified SGP was determined (Andrews, 1970) by gel filtration on a Bio-Gel P-150 column (80 cm $\times$ 1.5 cm; 2 ml/tube; flow rate 15 ml/h) calibrated with bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), trypsinogen (M_r 24000) and β -lactoglobulin (M_r 18500). The M_r of the purified glycoprotein was also determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) in the presence of 2-mercaptoethanol, with calibration with standard M_r markers (MW-SDS-70) from Sigma Chemical Co.

Amino acid analysis of the purified SGP was carried out according to the method of Heinrikson & Meredith (1984). A 20 μ g portion of SGP was hydrolysed in 6 M-HCl for 24 h at 110 $^{\circ}$ C in a sealed tube. The sample was evaporated to dryness and dissolved in 100 μ l of coupling buffer (acetonitrile/pyridine/triethylamine/water, 10:5:2:3, by vol.). Phenylthiocarbamoyl derivatives of the hydrolysate of SGP were prepared by the addition of 5 μ l of phenyl isothiocyanate. The modified amino acids dissolved in 250 μ l of 50 mM-ammonium acetate were analysed by h.p.l.c. on a C_{18} reverse-phase column (25 cm $\times$ 0.46 cm). Standard amino acids were obtained from Sigma Chemical Co., and the amino acid derivatives were prepared in an identical manner.

The carbohydrate content was determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956) with mannose as standard. Sugars of SGP were determined by g.l.c. (Sloneker, 1972) on a Hewlett Packard 5730A gas chromatograph with flame ionization detector by using column of 3% EC NSS-M on GasChrom Q (100–120 mesh) at 190 $^{\circ}$ C for alditol acetates of sugars. The glycoprotein was hydrolysed in 2 M-trifluoroacetic acid in a sealed tube for 2 h at 120 $^{\circ}$ C and the acid was removed under vacuum. Traces of acid were removed by co-distillation with water. For quantitative determination, D-allose (100 μ g) was added after hydrolysis as an internal standard and sugars were quantified from peak areas after appropriate corrections. SGP was also analysed for sialic acid (Aminoff, 1961).

The phenol oxidase (EC 1.14.18.1) was isolated from the scorpion haemolymph as described below. The haemolymph was centrifuged at 5000 g for 15 min to sediment the cells and other materials. The haemolymph was then dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 6 mM- $CaCl_2$ and 16 mM- $MgCl_2$ in the cold. The dialysed haemolymph was then ultracentrifuged at 120000 g for 3 h. This resulted in a thick blue pellet and a clear supernatant. The pellet fraction showed most of the phenol oxidase activity. The pellet was suspended in 50 mM-Tris/HCl buffer, pH 7.5 (buffer A). The pellet suspension was then applied on a Bio-Gel P-200 column (1.5 cm $\times$ 66 cm) previously equilibrated with buffer A. Active elution was done with the same equilibrating buffer at a flow rate of 30 ml/h. Fractions from the Bio-Gel P-200 column chromatography showing phenol oxidase activity were combined and further applied on a DEAE-Sephadex A-50 ion-exchange column (1.5 cm $\times$ 35 cm). The unbound fractions were washed out with buffer A. Active elution was done with a linear gradient of 0.1–0.6 M-NaCl in buffer A, obtained by using a continuous gradient mixture in a total volume of 300 ml. The elution was done at a flow rate of 12 ml/h,

and a 3 ml fraction was collected in each tube. The fractions showing phenol oxidase activity were combined and dialysed against buffer A with several changes, and the enzyme protein was concentrated by freeze-drying. The concentrated enzyme was taken as the isolated phenol oxidase preparation.

Phenol oxidase activity was assayed according to the method of Sugumaran *et al.* (1985). The reaction mixture contained 5 mM-dopamine, 25 mM-sodium phosphate buffer, pH 6.0, and a known amount of enzyme protein in a total reaction volume of 1 ml. After incubation at 25 $^{\circ}$ C for 20 min the product dopachrome was measured at 410 nm.

The esterase-like and amidase-like activities of SGP with 1 mM- α -N-benzyl-L-arginine ethyl ester (Schwert & Takenaka, 1955) and 0.5 mM- N - α -benzoyl-DL-arginine p -nitroanilide (Yokosawa *et al.*, 1985) were examined. Trypsin (1 μ g) and SGP (50 μ g) were used for the control and experimental reactions respectively.

RESULTS AND DISCUSSION

The elution profile of SGP from chromatofocusing showed a single peak at pH 5.72 (Fig. 1). The protein was further analysed by h.p.l.c., which also showed a single peak (Fig. 2). The isoelectric point (pI) of SGP determined by Servalyte-Precotes was found to be 5.8.

Fig. 1. Chromatofocusing elution profile of SGP

For experimental details see the text. —, A_{280} ; ----, pH.

Fig. 2. H.p.l.c. elution profile of SGP

For experimental details see the text.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of SGP at pH 8.3

A constant current of 3 mA/gel (7.5 %) was used and the run was conducted for 6 h at 14 °C. A 50 µg portion of SGP was used in each gel tube. Lane 1, SDS-treated SGP; lane 2, SDS + 2-mercaptoethanol-treated SGP; lane 3, SDS-treated M_r markers.

Polyacrylamide-gel electrophoresis indicated the purity of the protein. Alcian Blue staining and periodic acid/Schiff staining indicated that SGP is a glycoprotein. The M_r of SGP determined by the gel-filtration method was 30000. SDS/polyacrylamide-gel electrophoresis with or without 2-mercaptoethanol revealed only one band (Fig. 3). SDS/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol indicated an M_r of 32000 for SGP. The results of amino acid analysis of the purified glycoprotein were calculated as mol/100 mol (Table 1). Glycine, glutamic acid and tyrosine were higher in content with respect to other amino acids; cysteine was not detected. SGP contained 50 % neutral sugar according to the method of Dubois *et al.* (1956). Carbohydrate analysis (Sloneker, 1972) indicated the occurrence of glucose, mannose, galactose, fucose and arabinose. From Table 1 it was observed that contents of glucose and arabinose were higher in comparison with other sugars. Sialic acid was not found in SGP under the conditions of our experiments.

Phenol oxidase activity was determined in the haemolymph of the scorpion *H. bengalensis* and in the isolated phenol oxidase preparation. The presence of the enzyme both in haemolymph and in the isolated preparation was confirmed by inhibition of the enzyme activity with its specific inhibitor thiourea (Ashida & Yoshida, 1988). Table 2 indicates that phenol oxidase activity in both haemolymph and the isolated phenol oxidase preparation was enhanced by SGP. The ester substrate *N*- α -benzoyl-L-arginine ethyl ester and the amide substrate *N*- α -benzoyl-DL-arginine *p*-nitroanilide for trypsin were not hydrolysed by the purified SGP. Thus it was inferred that SGP has no amidase or esterase activity against these substrates. Phenol oxidase in insect haemolymph usually occurs in a pro-phenol oxidase form that can be activated either by an activator present in haemolymph/cuticle or immediately after bleeding of

Table 1. Amino acid and sugar analyses of SGP

Amino acid	Composition (mol of residue/ 100 mol of residues)
Aspartic acid	2.86
Glutamic acid	10.10
Serine	6.64
Glycine	16.13
Histidine	2.37
Arginine	1.45
Threonine	4.95
Alanine	4.45
Proline	7.42
Tyrosine	12.91
Valine	7.94
Methionine	5.90
Cysteine	Nil
Isoleucine	2.90
Leucine	3.66
Phenylalanine	2.29
Lysine	8.03

Neutral sugar	Composition (mol/mol of SGP)
Mannose	1.0
Fucose	8.0
Galactose	4.0
Arabinose	37.0
Glucose	41.0

Table 2. Phenol oxidase activity

Haemolymph was used after centrifugation at 5000 *g* for 15 min. Results are expressed as the mean value for four experiments in each case.

System	Activity (A_{410})
Haemolymph (1.6 mg)	0.17
Haemolymph (1.6 mg) + SGP (0.05 mg)	0.56
Phenol oxidase prepn. (0.10 mg)	0.11
Phenol oxidase prepn. (0.10 mg) + SGP (0.05 mg)	0.27

the animal (Leonard *et al.*, 1985). Sugumaran *et al.* (1985) observed that spontaneous activation of the enzyme upon bleeding of *Sarcophaga bullata* and *Manduca sexta* was rarely witnessed, although haemolymph contained all three components, namely pro-phenol oxidase, activator and phenol oxidase substrate. The natural activator so far reported has the characteristics of protein and seems to act as an enzyme (Ashida, 1971). Dohke (1973) has purified such an activator from *Bombyx mori* cuticle and characterized it to be a serine proteinase. It has also been reported that two serine proteinases activated inactive pro-phenol oxidase to the active phenol oxidase in crayfish plasma (Hergenhausen *et al.*, 1987) for the biosynthesis of melanin. Fig. 4 shows the time course of the reaction. The reaction of phenol oxidase enzyme is instantaneous. No change was observed when pre-

Fig. 4. Time course of phenol oxidase reaction

For experimental details see the text. ○, Haemolymph (1.6 mg); ●, haemolymph (1.6 mg)+SGP (0.05 mg); △, phenol oxidase prepn. (0.1 mg); ▲, phenol oxidase prepn. (0.1 mg)+SGP (0.05 mg).

incubation of enzyme with SGP before substrate addition was varied. This observation indicated that SGP activates the phenol oxidase itself, not pro-phenol oxidase. Simpson *et al.* (1987) have reported the presence of phenol oxidase in the shrimp's head. SGP in the presence of phenylmethanesulphonyl chloride and *p*-chloromercuribenzoate, which are inhibitors of the activator of pro-phenol oxidase to phenol oxidase (Dohke, 1973), was devoid of any effect.

Arthropods lack immunoglobulins. They are, however, able to protect themselves against parasites and micro-organisms and to remove non-self particles from the circulation. The mechanism by which an arthropod can discriminate self from non-self is far from clear. The enzyme phenol oxidase has an important role in the synthesis of melanin. Deposition of melanin takes place around non-self substances, which are subsequently phagocytosed, and thus this constitutes an important component of arthropod immunity (Ratcliffe *et al.*, 1985). Undesired melanin synthesis is, however, fatal for the animal. Since phenol oxidase activity was observed in the haemolymph and SGP enhanced the enzyme activity *in vitro*, it may be suggested that one or more physiological mechanisms must exist to control the action of SGP *in vivo*. Sugumaran *et al.* (1985) and Hergenbahn *et al.* (1987) reported that endogenous proteinase inhibitors prevented the action of activator and thus inhibited unwanted activation of phenol oxidase enzyme in insect haemolymph. Co-occurrence of SGP in the scorpion haemolymph along with the phenol oxidase enzyme and substrate *in vivo* may regulate melanin synthesis according to the animal's need.

Sialic acid was not detected in SGP, which had an M_r of 30000 and pI of 5.8. SGP formed needle-shaped crystals in an aqueous environment (Fig. 5). The identity of the SGP crystal was confirmed by the activation of phenol oxidase activity. The presence of arabinose in SGP is interesting, since arabinose does not commonly occur in animal glycoproteins (Sharon & Lis, 1982).

Fig. 5. Crystals of SGP

The photomicrograph was taken with a Leitz microscope ($\times 400$). A 20 ml portion of SGP solution containing 2 mg of protein was concentrated under vacuum at 0–4 °C until aggregates were noticed; on standing, needle-shaped crystals appeared.

The identification of the glycoprotein SGP in the haemolymph of *H. bengalensis* prompted us to examine the presence of similar glycoproteins in the haemolymph of a marine relative, the Indian king-crab *Carcinoscorpius rotundicauda* (Ellerton *et al.*, 1983), belonging to the Subphylum Chelicerata. A 100 %-satd.-(NH₄)₂SO₄-soluble proteinaceous component was found in the haemolymph of *C. rotundicauda*, but further work could not be pursued as the animal is now protected in India. In conclusion, we propose that SGP-like proteins may have a role in controlling the melanization process in the animals belonging to the Subphylum Chelicerata.

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