# Characterization of a Structural Glycoprotein from Bovine Ligamentum Nuchae Exhibiting Dual Amine Oxidase Activity†

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ABSTRACT: A structural glycoprotein has been extracted from bovine ligamentum nuchae by using 5 M guanidine hydrochloride containing a disulfide bond reducing agent and purified by preparative gel electrophoresis. The isolated material appeared to be monodisperse, with a molecular weight of ~34 000, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by analytical ultracentrifugation. It contains 10% carbohydrate comprising mannose, *N*-acetylglucosamine, galactose, and sialic acid in a 6:5:3:3 molar ratio. The glycoprotein has been assayed for peptidyl-lysine oxidase activity by using [$^3$H]lysine–aortic elastin, prepared from 15- to 17-day-old chick embryos, as a substrate. In the absence of free lysine, the specific activity of the preparation over a 2-h incubation was $\sim 60 \times 10^4$ dpm/mg of purified protein. Addition of 10 mM lysine resulted in an ~50% decrease in the specific activity. Free lysine was shown to act as a substrate for the glycoprotein preparation as indicated by control experiments using [$^3$H]lysine in place of the aortic substrate. These results demonstrate that the glycoprotein exhibits a dual amine oxidase activity. In the presence of 0.27 mM β-aminopropionitrile fumarate, a concentration which completely inhibits peptidyl-lysine oxidase activity in other lysyl oxidases, the glycoprotein preparation was inhibited by ~14%. In the absence of 5 M guanidine hydrochloride and a reducing agent, the glycoprotein undergoes aggregation which in the presence of copper ions results in the formation of cylindrical tactoids, the diameter of which (11 nm) corresponds closely to that of the fibrils which in the majority of connective tissue matrices constitute the microfibrillar component mainly associated with elastic fibers.

Connective tissue matrices have been reported to contain a glycoprotein component, usually referred to as "structural glycoprotein" (SGP),[1] which can be solubilized only with chaotropic solutions containing a reducing agent (Anderson, 1976; Bach & Bentley, 1980). SGP has been most extensively investigated in aorta where it has been claimed to account for a large, albeit variable, proportion of the tissue dry weight and has been tentatively implicated in both the morphogenesis of the connective tissue stroma (Robert et al., 1971a) and the pathogenesis of atherosclerosis (Robert et al., 1971b; McCullagh et al., 1973; Ouzilou et al., 1973). The most fully characterized SGP preparation isolated so far from this anatomical structure has been reported to behave on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis as a single $M_r$ 35 000 species and to contain mannose, galactose, *N*-acetylglucosamine, and sialic acid in a 4:3:3:1 molar ratio (Moczar et al., 1977). However, the existence of SGP as a major component of the aortic wall has been questioned by Bach & Bentley (1980), who showed that most of the $M_r$ 35 000 material previously considered to be SGP may well have been actin.

In order to overcome any possibility of gross contamination by the latter protein, in the present study we have turned to bovine ligamentum nuchae as the source material for the isolation and characterization of SGP.

Our final preparation behaved as an apparently homogeneous glycoprotein, containing 10% oligosaccharide and exhibiting a molecular weight of ~34 000. The similarity in amino acid composition and molecular weight between this glycoprotein and lysyl oxidases isolated from bovine tissues (Jordan et al., 1977; Kagan et al., 1979) suggests that these proteins may be functionally related. Enzyme activity studies carried out by using [$^3$H]lysine–embryonic chick aortae and free [$^3$H]lysine as substrates showed that ligamentum nuchae SGP exhibited dual amine oxidase activity. For an assessment of whether SGP forms structural elements in connective tissue matrices, attempts were made to induce its aggregation in vitro. If exposed to copper ions, SGP was found capable of forming fibrils of about 11 nm in diameter and therefore of the same size as the microfibrils associated mainly with elastic fibers in both embryonic and mature tissues (Greenlee et al., 1966).

## Experimental Procedures

*Materials.* All reagents (AnalaR or Aristar grade) were obtained from BDH Chemicals, Ltd. Collagenase (EC 3.4.24.3; from *Clostridium histolyticum* type I), proteolytic inhibitors, molecular weight marker proteins, carbohydrate standards, and guanidine hydrochloride were purchased from Sigma. Guanidine hydrochloride was further purified according to Nozaki (1972). Sodium dodecyl sulfate was recrystallized from ethanol until a critical micelle concentration of 2.13 g/L was attained (Birdi, 1976).

*Preparation of SGP.* Ligamentum nuchae from 3-year-old cattle was freed from adhering tissue and minced at 4 °C. Aliquots (250 g) were stirred at 4 °C for two 24-h periods in 1% (w/v) NaCl containing 25 mM EDTA, 10 mM *N*-ethylmaleimide, and 1 mM phenylmethanesulfonyl fluoride. The concentration of phenylmethanesulfonyl fluoride in this and subsequent manipulations was maintained by addition of fresh reagent every 12 h. The tissue was then washed with distilled $H_2O$ and defatted with chloroform–methanol (1:3 v/v), followed by a second treatment with the same solvents (1:2 v/v).

The dried material was suspended in 1 L of 5 M guanidine hydrochloride–0.1 M Tris (pH 7.4) containing 25 mM EDTA, 10 mM *N*-ethylmaleimide, and 1 mM phenylmethanesulfonyl fluoride and extracted for 24 h at 4 °C with continuous stirring. The residue was collected by centrifugation (2000*g* for 2 h) and washed extensively with 5 M guanidine hydro-

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[1] Abbreviations used: SGP, structural glycoprotein; $NaDodSO_4$, sodium dodecyl sulfate; BAPN, β-aminopropionitrile; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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chloride–0.1 M Tris (pH 7.4) containing 25 mM EDTA and 1 mM phenylmethanesulfonyl fluoride prior to a further extraction at 37 °C, under nitrogen, with 1 L of the same buffer solution containing 20 mL of 2-mercaptoethanol and adjusted to pH 8.5 with *N,N,N′,N′*-tetramethylethylenediamine. After 24 h, this solution was centrifuged at 23000*g* for 30 min, under nitrogen, and the supernatant was slowly added to 3 volumes of absolute ethanol at 4 °C. Following a 12-h period, the resultant precipitate was collected by centrifugation at 4 °C, dried by serial washing with ethanol, acetone, and ether, and stored at −10 °C until used.

In the preparation of SGP for enzyme assays, the extraction was carried out essentially as described above but in the absence of protease inhibitors.

Aliquots (200 mg) of both crude SGP preparations were dissolved in 20 mL of a solution containing 8 M urea, 0.1 M Tris, and 3% (v/v) 2-mercaptoethanol (pH 7.4), under nitrogen. The suspension was left for 12 h before centrifugation at 38000*g* for 30 min. The supernatant was filtered through a Diaflo XM-100 membrane (Amicon-Holland) under a nitrogen pressure of 2 kg/cm$^2$ prior to dialysis against distilled $H_2O$ carried out by using Visking 18/32 tubing, acetylated according to Craig & Konigsberg (1961). The resulting precipitates were freeze-dried, and 20-mg aliquots were suspended in 5 mL of 8 M urea–0.1 M Tris–3% (v/v) 2-mercaptoethanol (pH 7.4) containing 60 mg of $NaDodSO_4$. After 12 h, the suspensions were centrifuged at 38000*g* for 30 min and the supernatants subjected to preparative polyacrylamide gel electrophoresis on an 8% acrylamide column (6 × 7 cm) using a current of 0.23 mA and 63 V. The gel buffer contained 0.1 M Tris, 8 M urea, and 1% $NaDodSO_4$ (pH 7.4); the tank buffer contained 0.1 M Tris and 0.2% $NaDodSO_4$ (pH 8.0). Fractions (3 mL) were collected at hourly intervals, and the absorbance was monitored at 280 nm. Fractions 27–33 (see Figure 2) were pooled, dialyzed exhaustively against distilled $H_2O$, and lyophilized. Bound $NaDodSO_4$ was removed by the method of Pitt-Rivers & Ambesi Impiombato (1968). The resulting precipitates were dissolved in, and dialyzed against, serial changes of 8 M urea–3% (v/v) 2-mercaptoethanol–0.1 M Tris (pH 7.4) and finally dialyzed against distilled $H_2O$. The precipitates that formed were collected by centrifugation and freeze-dried. The freeze-dried product will be referred to a ligamentum nuchae SGP.

The crude preparations and the fractions collected after preparative gel electrophoresis were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis according to the procedure of Weber et al. (1972), using gels of varying porosity (5%, 7.5%, and 10% final acrylamide concentrations). The standard proteins employed as molecular weight markers were lysozyme, trypsin, actin, and bovine serum albumin. Gels were stained with Coomassie brilliant blue (Weber et al., 1972) and by the periodic acid–Schiff procedure (Furlan et al., 1975).

An aliquot of SGP was digested with collagenase purified by affinity chromatography, as detailed elsewhere (Serafini-Fracassini et al., 1975).

*Amino Acid Analysis.* Hydrolyses were carried out, under nitrogen, in constant-boiling HCl (5 mL/mg of protein) containing 0.01 M thioglycolic acid (Sanger & Thompson, 1963) at 110 °C. Analyses were performed on a single-column Locarte amino acid analyzer. Tryptophan was determined colorimetrically (Spies & Chambers, 1949).

*Carbohydrate Analysis.* Neutral sugars, hexosamines, and sialic acid were quantified by gas–liquid chromatography following the procedure of Bhatti et al. (1970). The hexosamine content was also determined on the amino acid analyzer after a 6-h hydrolysis at 90 °C, under nitrogen, in 4 N HCl, while sialic acid was characterized and quantified according to the colorimetric procedure of Jourdian et al. (1971).

*Analytical Ultracentrifugation.* For molecular weight determinations, SGP and collagenase-treated SGP were S-carboxymethylated according to the procedures of Crestfield et al. (1963), and aliquots were dissolved in 5 M guanidine hydrochloride–0.1 M Tris (pH 7.4) prior to exhaustive dialysis against several changes of the same buffer. Determination of molecular weight moments at two different loading concentrations was performed at 20 °C in a Spinco Model E analytical ultracentrifuge equipped with an ANH titanium rotor, using a 12-mm double-sector cell and employing the meniscus-depletion technique of Yphantis (1964) as described by Chervenka (1970). Photographs were taken at 24-h intervals by using interference optics. Fringe displacements, measured with a two-dimensional microcomparator, were analyzed according to the procedure of Roark & Yphantis (1969) by using a computer program kindly supplied by Dr. Roark.

*Preparation of SGP for Enzyme Assays.* An aliquot (3 mg) of SGP was dissolved in 1 mL of 8 M urea–3% (v/v) 2-mercaptoethanol–0.1 M Tris (pH 7.4) and dialyzed exhaustively against distilled $H_2O$ followed by dialysis against 10 mM $CuCl_2$ for 48 h and finally against distilled $H_2O$. The content of the dialysis sac was made 8 M with respect to urea and 3% (v/v) with respect to 2-mercaptoethanol prior to dialysis against 0.1 M $NaH_2PO_4$–0.15 M NaCl (pH 7.7) for 36 h to dissolve the precipitated glycoprotein. This preparation was immediately used for enzyme assays since the solution became opalescent in approximately 24 h.

An aliquot (4.5 mg) of SGP, dissolved in 8 M urea–3% (v/v) 2-mercaptoethanol–0.1 M Tris (pH 7.4), was applied to a Sephadex G-75 column (1.6 × 64 cm) equilibrated and eluted with 8 M urea–0.1 M Tris (pH 7.4). The absorbance at 280 nm was recorded, and 1-mL fractions were collected. These were prepared for enzyme assays as described above.

*Preparation of [$^3$H]Lysine-Labeled Elastin Substrate.* The [$^3$H]lysine-labeled aortic protein substrate was prepared by organ culture employing the method of Pinnell & Martin (1968). Twenty-five aortas were removed from 15- to 17-day-old chick embryos, cut into small pieces, and added to 10 mL of Eagle's minimal essential medium, lacking lysine and glutamine but supplemented with 50 μg/mL β-aminopropionitrile fumarate (BAPN),[1] 50 μg/mL ascorbic acid, and 50 μg/mL kanamycin. The culture vessel was gassed with $O_2$–$CO_2$ (95:5 v/v) and the incubation carried out at 37 °C with constant agitation for 48 h. Prior to the start of each incubation, the medium was further supplemented by addition of 250 μCi of L-[4,5-$^3$H]lysine monohydrochloride (specific activity 76 Ci/mmol). At the end of this incubation, the aortas were rinsed in distilled $H_2O$, lyophilized, and stored at 4 °C until used. The lyophilized aortas were then homogenized in 5 mL of 0.15 M NaCl by using an Ultra Turrax homogenizer. The insoluble material, collected by centrifugation at 17500*g* for 10 min, was extracted twice with 0.15 M NaCl, once with 1 M HCl in order to inactivate endogenous enzymes (Kagan et al., 1974), once with 0.1 M $NaH_2PO_4$–0.15 M NaCl (pH 7.7), and twice with 0.15 M NaCl. The final pellet was resuspended in 0.1 M $NaH_2PO_4$–0.15 M NaCl (pH 7.7).

*Enzyme Assay.* Enzyme reactions were carried out in 1-mL ReactiVials fitted with Teflon-coated triangular stirring bars (Pierce Chemical Co.). The normal assay system was comprised of 0.02 mL of [$^3$H]lysine-labeled aortic substrate

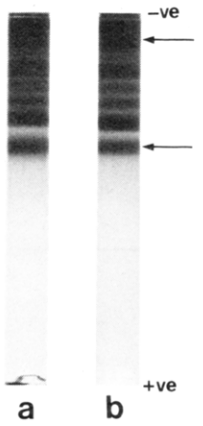


FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of crude 5 M guanidine hydrochloride–2-mercaptoethanol extracts of ligamentum nuchae carried out in the presence (a) and absence (b) of protease inhibitors. Gels were stained with Coomassie brilliant blue. The arrows indicate the periodic acid–Schiff-positive bands.

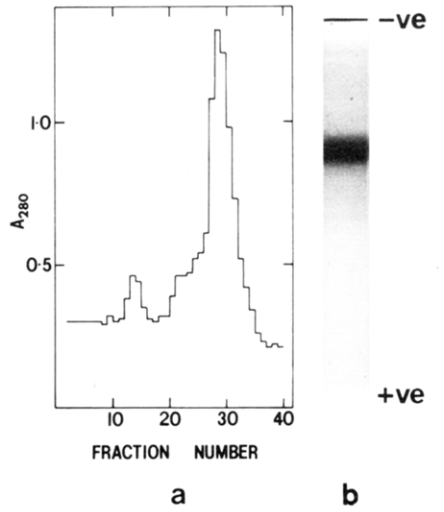


FIGURE 2: (a) Absorbance profile of the leading fractions from a preparative gel electrophoretic separation of a crude extract of ligamentum nuchae. (b) $NaDodSO_4$–polyacrylamide gel electrophoresis of pooled fractions (27–33) obtained from the preparative gel electrophoretic separation described in (a).

(containing ~280 000 dpm) or [$^3$H]lysine (~220 000 dpm), 0.02 mL of SGP preparation (containing 24–41 μg of protein), and 0.5 mL of 0.1 M $NaH_2PO_4$–0.15 M NaCl buffer (pH 7.7). β-Aminopropionitrile fumarate (final concentration 50–1000 μg/mL) and/or lysine (final concentration 0.01 M) was added to certain assay tubes before incubation, as stated in the text. Reaction mixtures were incubated at 37 °C with continuous stirring for 2 h. In all vials, the air space above the reaction mixture was exchanged with 100% $O_2$ for 1 min before capping (Siegel et al., 1970). The tritium released during the assay was determined by a microdistillation procedure (Misiorowski et al., 1976). The efficiency of distillation was determined by using tritiated $H_2O$ and found to be 83–90%. Radioactivity in the distillates was quantified after addition of 5 mL of Packard Picofluor-30 to the frozen distillate by using a Packard "Prias" liquid scintillation spectrometer. A computer program was used to calculate disintegrations per minute from the observed counts per minute with a calibrated external standard. Each assay was performed at least in duplicate, employing appropriate controls containing either substrate alone or substrate plus boiled enzyme.

*Electron Microscopy*. An SGP preparation which had been dialyzed against 10 mM $CuCl_2$ was dissolved in 8 M urea–0.1 M Tris (pH 7.4) containing 3% (v/v) 2-mercaptoethanol and dialyzed against distilled $H_2O$ at 4 °C. The precipitate that formed after ~24 h was suspended in 12 mM uranyl formate (pH 2.2) and sprayed on carbon-coated grids. An aliquot of the precipitate in the form of a pellet was fixed in 4% paraformaldehyde–5% glutaraldehyde in 0.08 M sodium cacodylate (pH 7.3), postfixed with 1% osmium tetroxide in 0.08 M sodium cacodylate (pH 7.3), dehydrated in ethanol, and embedded in Araldite. Ultrathin sections were cut on a Reichardt OMU2 ultramicrotome and stained with 2% uranyl acetate in 50% ethanol for 25 min followed by 0.4% lead citrate in 0.1 M NaOH for 5 min. Grids were examined in an AEI–EM6B electron microscope. Micrographs were taken at an instrument magnification of 40 000, calibrated by using beef liver catalase crystals (Wrigley, 1968).

## Results

*Characterization of SGP*. Polyacrylamide gel electrophoresis in $NaDodSO_4$ (Figure 1) showed that the guanidine hydrochloride–mercaptoethanol extracts of bovine ligamentum nuchae contain a complex mixture of proteins similar to those obtained, under analogous experimental conditions by Bach & Bentley (1980), from bovine thoracic aorta. Preparations from both tissues showed a prominent component, exhibiting a mobility comparable to that of actin, which in ligament extracts generates the major periodic acid–Schiff-positive band on polyacrylamide gel electrophoresis and accounts for ~0.1% of the tissue dry weight. The identity of the polyacrylamide gel electrophoresis banding patterns of ligament preparations obtained in the presence and absence of protease inhibitors suggests that any significant proteolytic activity is absent in such extracts.

Preparative gel electrophoresis preceded by ultrafiltration proved to be an effective method of isolating a homogeneous preparation of the major periodic acid–Schiff-positive component from ligament, as indicated by the absorbance elution profile (Figure 2a) and by the polyacrylamide gel analysis of the pooled fractions 27–33 (Figure 2b).

Molecular weight determination of this SGP preparation carried out by disc gel electrophoresis on gels of varying porosity (Segrest & Jackson, 1972) yielded an apparent molecular weight of 35 000.

The compositional data of SGP are reported in Table I (column a). The chromogen (at 630 nm) produced by periodate oxidation of SGP was stable for 100 min at 37 °C. This suggested that sialic acid was present as *N*-glycolylneuraminic acid (Jourdian et al., 1971). The correlation of the amino acid and sugar analyses was effected by using the *N*-acetylglucosamine content as an internal standard. Analytical data were employed in the evaluation of the minimum molecular weight of SGP according to Black & Hogness (1969), using the computer program of Bryce (1979) modified to account for sugar residues. In the range 25 000–45 000, the minimum, in the profile, of the fraction of maximum deviation corresponded to a molecular weight of 34 400. The refined integer fit of amino acid and sugar residues obtained at this value is listed in Table I (column b). It should be noted that galactose and sialic acid values showed the greatest deviation from computed data and that they have been rounded off to the next highest integer.

In equilibrium sedimentation experiments, molecular weights were calculated from the corresponding reduced molecular weights (Yphantis, 1964). A value of 0.706 mL/g, as calculated from analytical data (Gibbons, 1966; Zamyatnin, 1972), was assigned to $\bar{v}$. The standard apparent point-average molecular weight moments ($n$, w, and $z$) converged at a van-

Table I: Composition of Ligamentum Nuchae SGP

| | (a) analytical results[a] | (b) refined integer fit from analytical data ($M_r$ 34 400) |
|---|---|---|
| hydroxyproline | 0.0 | |
| aspartic acid | 118.8 | 33 |
| threonine | 44.2 | 12 |
| serine | 60.8 | 17 |
| glutamic acid | 103.3 | 29 |
| proline | 47.0 | 13 |
| glycine | 111.7 | 31 |
| alanine | 72.3 | 20 |
| cysteine | 27.0 | 8 |
| valine | 56.4 | 16 |
| methionine | 14.1 | 4 |
| isoleucine | 33.1 | 9 |
| leucine | 83.9 | 23 |
| tyrosine | 43.3 | 12 |
| phenylalanine | 48.2 | 13 |
| tryptophan | 16.8 | 5 |
| lysine | 47.1 | 13 |
| histidine | 17.5 | 5 |
| arginine | 54.5 | 15 |
| total | 1000.0 | |
| *N*-acetyl glucosamine | 17.6 | 5 |
| galactose | 9.0 | 3 |
| mannose | 22.0 | 6 |
| sialic acid | 10.0 | 3 |

[a] Values, corrected for hydrolytic losses, are expressed as residues per 1000 amino acid residues.

ishing concentration to a value corresponding to a molecular weight of 33 300. Ideal moments were also calculated (Roark & Yphantis, 1969), and values of $M_{y8}$, which is independent of the second, third, and fourth virial coefficients, were found to range throughout the cell from 33 200 ± 1 800 (at $C = 0$) to 34 600 ± 700 (at column base). No appreciable variation of both standard and ideal moments was observed at different loading concentrations.

Standard and ideal molecular weight moments of collagenase-treated SGP converged at a vanishing concentration to a value of $M_r \sim 11\,600$. A partial specific volume of 0.706 mL/g was assumed.

*Electron Microscopy.* The ligamentum nuchae SGP showed a marked tendency to aggregate in the absence of guanidine hydrochloride and mercaptoethanol, giving rise to precipitates that appear amorphous in the electron microscope. When SGP was exposed to copper ions, under conditions outlined under Experimental Procedures, resulting precipitates appeared to

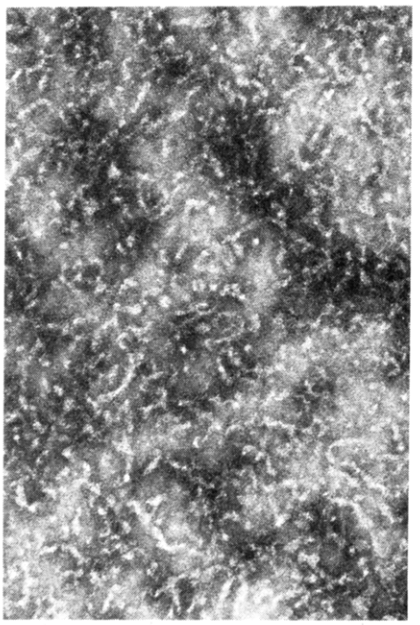

FIGURE 3: Precipitate of SGP, obtained after exposure to copper ions, negatively stained with uranyl formate (109000×).

be constituted by cylindrical tactoids (Figure 3). The diameter of these was measured on thin sections of the embedded precipitate and found to average 11 nm.

*Enzyme Assay for SGP.* The lysyl oxidase assay of Pinnell & Martin (1968) was employed to ascertain whether any enzyme activity was associated with ligamentum nuchae SGP. This assay measures the tritium released from a $^3$H-labeled substrate, containing principally elastin, as the $^3$H-labeled peptidyl lysine residues are converted to allysine. As shown in Table II, SGP preparations catalyze significant tritium release from the [$^3$H]lysine–aortic substrate. However, if the substrate (peptidyl lysine) specificity is not absolute, this tritium release could reflect enzymatic activity towards both peptidyl and free lysine. A high concentration of free lysine (10 mM) would be expected to inhibit any enzyme activity not specific for peptide-bound lysyl residues (Siegel, 1979). In the present experiment, when free lysine at such a concentration was included in the assay, a substantial degree of tritium release (~56% of the initial value) was still observed, suggesting that SGP possesses specific peptidyl-lysine oxidase activity. The fact that a high concentration of free lysine did not achieve full inhibition also suggested that SGP may possess

Table II: Enzyme Assay for SGP[a]

| SGP preparation | SGP added to assay mixture (μg) | age of SGP following final dialysis (days) | substrate (at final concn) | net $^3$H release[b] (dpm) | sp act.[c] × $10^{-4}$ (dpm/mg of protein) |
|---|---|---|---|---|---|
| 1 | 24 | 1 | $^3$H-labeled aorta | 12469 | 59.71 |
| | 24 | 1 | $^3$H-labeled aorta + BAPN (0.27 mM) | 10699 | 51.23 |
| | 24 | 1 | $^3$H-labeled aorta + L-lysine (10 mM) | 6988 | 33.47 |
| | 24 | 4 | $^3$H-labeled aorta | 6187 | 29.63 |
| 2 | 41 | 1 | [$^3$H]lysine + L-lysine (0.1 mM) | 5172 | 14.52 |
| | 30 | 2 | $^3$H-labeled aorta | 4489 | 17.20 |
| | 30 | 2 | $^3$H-labeled aorta + BAPN (0.27 mM) | 3879 | 14.86 |
| | 30 | 2 | $^3$H-labeled aorta + BAPN (2.7 mM) | 2201 | 8.44 |
| | 30 | 2 | $^3$H-labeled aorta + BAPN (5.4 mM) | 1406 | 5.39 |
| | 30 | 2 | $^3$H-labeled aorta + BAPN (27.0 mM) | 1500 | 5.68 |

[a] Enzyme activity, determined by $^3$H release (isolated as $^3H_2O$), was quantified by the microdistillation procedure of Misiorowski et al. (1976). Assays were performed at least in duplicate; mean values are quoted. [b] Net $^3$H release in dpm was quantified following subtraction of a blank value (2463 ± 391 dpm), the mean of five determinations. Similar blank values were obtained by using either substrate alone or substrate + boiled enzyme. [c] Specific enzyme activity was defined as the dpm of $^3H_2O$ formed per mg of purified SGP protein per 2-h incubation. Values are corrected for distillation losses.

at least dual substrate specificity.

So that the possibility that free lysine may be oxidized by SGP could be tested, an aliquot of SGP preparation 2 was incubated with [$^3$H]lysine (specific activity of tritium in the mixture ~2 $\mu$Ci/$\mu$mol), tagged with carrier lysine (0.1 mM), and shown to utilize the free amino acid as a substrate.

An additional criterion used to evaluate the presence of lysyl oxidase activity is to study the inhibitory effect of BAPN which is known to irreversibly inhibit the cross-linking of both collagen and elastin in vivo (Gross et al., 1960; Miller et al., 1965, 1967) probably through a mechanism which results in covalent linkage of BAPN to the enzyme (Walsh, 1978). The validity of such a mechanism has not been proven, although evidence is available which shows that BAPN forms a stable complex with lysyl oxidase from chick cartilage (Narayanan et al., 1972). Other workers (Siegel et al., 1970) have shown that the presence of BAPN in enzyme assays at a final concentration of 50 $\mu$g/mL results in complete inhibition. In the present study (using SGP preparation 1) the inclusion in the assay of $\beta$-aminopropionitrile fumarate at a final concentration of 50 $\mu$g/mL (0.27 mM) produced only 14% inhibition of tritium release. A second, apparently less active preparation of SGP (preparation 2), was used to study the effect of increasing BAPN concentrations. A similar reduction in activity to that obtained with preparation 1 was observed at a BAPN concentration of 0.27 mM, while increasing the BAPN concentration produced a maximum 70% inhibition. $\beta$-Aminopropionitrile does not inactivate amine oxidases (Page & Benditt, 1967a) but can decrease their rate of amine metabolism since it can function as a substrate (Bird et al., 1966). Page & Benditt (1967b) found that the activity of amine oxidases was competitively inhibited by BAPN only at levels in the region of 400–1000 $\mu$M, concentrations comparable to those used in this experiment. The evidence that BAPN at low concentrations induces only slight reduction in tritium release may indicate that it is metabolized by the amine oxidase function of SGP, which appears to constitute the major enzymatic activity of the glycoprotein in vitro.

Although calculation of the specific radioactivity of the free lysine substrate presents no difficulty, that of the aortic protein substrate is far more complex, and for this reason, it should be noted that no direct comparison of enzymatic specific activities toward the different substrates can be made.

As mentioned earlier, SGP preparations undergo time-dependent aggregation. This aggregation occurs concomitantly with a reduction of tritium release (~50%) within 4 days, as seen for preparation 1 in table II.

As shown in Figure 4, lysyl and amine oxidase activities are present throughout the single peak, obtained by gel exclusion chromatography, of the SGP preparation.

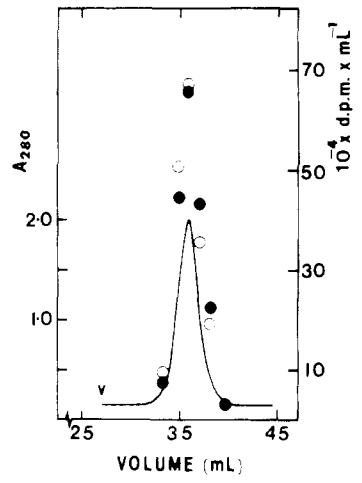


FIGURE 4: Chromatography of SGP on Sephadex G-75 in 8 M urea–0.1 M Tris, pH 7.4. (—) $A_{280}$; (○) enzyme activity assayed by using [$^3$H]lysine–aortic substrate; (●) enzyme activity assayed by using [$^3$H]lysine tagged with 0.1 mM carrier lysine. Tritium released is expressed as dpm per 1-mL fraction with the background subtracted; v, void volume.

## Discussion

Bovine ligamentum nuchae SGP shows a compositional similarity with the microfibrillin preparation isolated from the same tissue by Ross & Bornstein (1969) and considered to represent the constituent or one of the components of the 11-nm microfibrils associated with elastic fibers in that tissue. Discrepancies in the amino acid profiles of the two preparations could reflect proteolytic digestion of the Ross & Bornstein glycoprotein induced by the collagenase treatment that these authors adopted in their extraction procedure. In our hands, in fact, this enzyme appears to cause considerable cleavage of SGP, resulting in the formation of fragment(s) of $M_r$ ~ 12 000. The correlation between SGP and microfibrillin is supported by the electron microscopic observation that SGP can be induced in vitro to acquire a quaternary conformation in forming fibrils which are indistinguishable from the microfibrillar component of the elastic fiber in terms of lateral dimensions.

Comparison of ligamentum nuchae SGP with data published by Moczar et al. (1977) for SGP isolated from pig aorta shows considerable differences which in turn may be attributed to tissue and species specificity. The correlation between these two preparations therefore relies mainly on their similarity in molecular weight.

The presence of a carbohydrate moiety, accounting for ~ 10% of the dry weight of ligamentum nuchae SGP, may affect the mobility of this macromolecule on NaDodSO$_4$–polyacrylamide gel electrophoresis and therefore introduce a margin of error in the determination of its molecular weight. On the other hand, in the determination of molecular weight by ultracentrifugation analysis, the use of guanidine hydrochloride as a solvent may lead, because of selective binding, to a considerable degree of uncertainty in the evaluation of the apparent specific volume. For these reasons, three different approaches were adopted in the assessment of molecular weight. Confidence in the results obtained by these procedures is justified by their close agreement, $M_r$ ~35 000 (NaDodSO$_4$–polyacrylamide gel electrophoresis), ~34 400 (compositional analysis), and ~33 300 (equilibrium ultracentrifugation).

Some degree of similarity in amino acid composition and molecular weight was also observed between ligamentum nuchae SGP and the urea-soluble lysyl oxidase isoenzymes isolated from both bovine ligamentum nuchae (Jordan et al., 1977) and bovine aorta (Kagan et al., 1979). These lysyl oxidases also resemble SGP in their tendency to aggregate in the absence of denaturants, forming multimeric species, which are functionally active (Jordan et al., 1977; Kagan et al., 1979). As SGP exhibits both amine oxidase activity toward free lysine and lysyl oxidase activity toward the aortic elastin substrate, it would appear to belong to a different class of enzyme from the connective tissue lysyl oxidases, which are generally believed to exhibit exclusive specificity for lysyl residues in peptide linkage (Siegel, 1979). In this respect, SGP may therefore be more closely related, from a functional viewpoint, to the diamine oxidase purified from human placenta (Crabbe et al., 1976), belonging to a group of amine oxidases outside of the two main classes (Dixon et al., 1979) which have been isolated from biological sources. The classification of con-

nective tissue lysyl oxidases may, however, need revision in view of the recent observation (Trackman & Kagan, 1979) that one of the lysyl oxidase isoenzymes isolated from bovine aorta is capable of oxidizing nonpeptidyl compounds containing primary amine functions.

Since ligamentum nuchae SGP represents, in quantitative terms, the major component of the ligament which possesses lysyl oxidase activity, it is open to speculation exactly what its biological function might be, particularly in relation to other isoenzymes capable of catalyzing the same type of reaction and to its presence in connective tissue in a highly aggregated form.

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