Purification and Characterization of a Sulfated Glycoprotein Secreted by Sertoli Cells†

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Received April 11, 1986; Revised Manuscript Received July 3, 1986

ABSTRACT: Sulfated glycoprotein 2 (SGP-2), the major secretion product of Sertoli cells, was purified from cell culture medium by reverse-phase high-performance liquid chromatography. The native protein consists of disulfide-linked monomers of 41 000 and 29 000 daltons which have a strong tendency to associate into multimers. The purified SGP-2 was subjected to amino acid analysis and contained high levels of Asx (11.1%), Glx (15.1%), and leucine (11.5%). The oligosaccharides on the purified SGP-2 were analyzed to determine the monosaccharide compositions and the molecular weights of the intact carbohydrate moieties. SGP-2 was shown to be 23.7% carbohydrate and consisted of 1% fucose, 3.5% mannose, 4.1% galactose, 7.1% *N*-acetylglucosamine, and 8.0% *N*-acetylneuraminic acid. No *N*-acetylgalactosamine was detected. When the SGP-2 was digested with proteases, the intact oligosaccharides were chromatographed over a Bio-Gel P-6 column and found to elute in a single symmetrical peak of approximately 3300 g/mol. On the basis of these results, the oligosaccharides on SGP-2 were proposed to consist of triantennary chains similar to those found on fetuin. When the $^{35}SO_4^{2-}$-labeled SGP-2 was digested with Pronase, the free amino acids could be separated by chromatography from the oligosaccharide. The $^{35}SO_4^{2-}$ was shown to be associated with the oligosaccharide portion of SGP-2.

Sertoli cells are the epithelial somatic cells of the seminiferous tubules of the testis. These cells interact directly with the developing germinal cells and provide physical and biochemical support (Fawcett, 1975; Fritz, 1973). Adjacent Sertoli cells partition the seminiferous tubules by means of tight junctions into a basal compartment containing spermatogonia and spermatocytes and into an adluminal compartment containing meiotic cells and spermatids (Fawcett, 1975). This latter compartment has a unique environment determined to a large extent by the secretions of Sertoli cells (Waites, 1977). Among the secretory products of the Sertoli cells are a number of glycoproteins some of which bind specifically to developing germinal cells or to released spermatozoa (Kissinger et al., 1982; Skinner & Griswold, 1980, 1983; Sylvester et al., 1984).

Sertoli cells cultured under serum-free conditions secrete a number of glycoproteins into the culture medium. The total spectrum of [^{35}S]methionine-labeled secreted proteins has been analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE) followed by fluorography (Kissinger et al., 1982). One of these secretion products has been identified as testicular transferrin which appears to function to transport iron from the basal compartment of the tubules to the spermatids in the adluminal compartment (Skinner & Griswold, 1980; Huggenvik et al., 1984). Two additional major secretion products are sulfated glycoproteins designated sulfated glycoproteins 1 (SGP-1) and 2 (SGP-2). In previous publications, these proteins were referred to as band 4 and dimeric acid glycoprotein (DAG), respectively (Sylvester et al., 1984). SGP-1 was shown to be a glycoprotein of 70 000 daltons by sodium dodecyl sulfate (SDS) gel electrophoresis. SGP-2, which was the major Sertoli cell secretion product, was shown by SDS gel electrophoresis to be a disulfide-linked heterodimer of 65 000–70 000 daltons with subunits of 41 000 and 29 000 daltons (Kissinger et al., 1982). Both subunits of SGP-2 exhibited a large degree of charge heterogeneity over several pH units on two-dimensional gel electrophoresis (Kissinger et al., 1982). Additional studies revealed that SGP-2 was extensively sulfated, but the location of the sulfate residues was not determined (Sylvester et al., 1984). SGP-2 was shown to be secreted into the lumen of the seminiferous tubule and to bind the surface of spermatozoa in a defined pattern and SGP-2 was also found to be a major product of the principal cells of the caput epididymis (Sylvester et al., 1984).

This report describes the purification and partial biochemical characterization of SGP-2 isolated from cultured Sertoli cells. This characterization includes an analysis of the amino acid and oligosaccharide compositions, an analysis of the molecular weight under nondenaturing conditions, and the localization of the sulfate to the oligosaccharide moiety.

Materials and Methods

Cell Culture. Sertoli cells from 20-day-old rats were prepared and cultured essentially as previously described (Dorrington & Fritz, 1975; Wilson & Griswold, 1979). Spent medium from these cultures was collected every 3 days after the initial 2-day incubation, centrifuged to remove cellular debris, and stored at −20 °C. A total of three medium collections were made from each series of cultures. The culture medium contained 0.7 mM testosterone and 0.1 mM dibutyryl cyclic AMP throughout the experiment. For ease in detection of proteins, the secreted proteins were routinely labeled with [^{35}S]methionine (Kissinger et al., 1982). Occasionally, SGP-2 was labeled with [^{3}H]glucosamine or [^{3}H]mannose by incubating the isotope with cultured Sertoli cells for 24 h.

Preparation of Secreted Proteins. Frozen spent medium was thawed and concentrated by pressure filtration over Amicon YM-10 ultrafilters. Each liter of spent medium was concentrated to approximately 20 mL and centrifuged. The concentrated secretion products were desalted by passage over a Bio-Rad P-6 gel filtration column equilibrated in distilled H_2O. Fractions containing the glycoproteins were pooled, lyophilized, and stored at −20 °C.

Separation by High-Performance Liquid Chromatography (HPLC). Lyophilized glycoproteins obtained from cultured

† Supported by Grant HD10808 from NICHHD.
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0006-2960/86/0425-7265$01.50/0

Sertoli cells were solubilized in H_2O. Aliquots of these glycoproteins were applied to a Vydak C-4 reverse-phase HPLC column equilibrated in 76% solvent A (0.1% trifluoroacetic acid) and 24% solvent B (100% acetonitrile–0.1% trifluoroacetic acid). The HPLC system consisted of two Beckman 112 pumps, a Beckman 165 detector, and a Beckman 421 controller. The glycoproteins were eluted from the column by an increasing gradient of solvent B over 47 min from 24% B to 55% B. After 47 min, the gradient increased linearly to 80% B over the next 13 min. The column flow rate was maintained at 1 mL/min, and 1-mL fractions were collected. The absorbance of the effluent was routinely monitored at 214 nm.

Gel Electrophoresis and Fluorography. Pooled fractions from the HPLC effluent were neutralized by the addition of 1 M bicarbonate buffer, pH 7.0. The pooled fractions were dialyzed overnight and lyophilized in 12 × 75 mm glass tubes on a Speed-Vac freeze-drying apparatus. The sample was then solubilized in gel electrophoresis sample buffer and analyzed by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS–PAGE) with 5–15% polyacrylamide gradient gels (Laemmli, 1970). After electrophoresis, the gels were dried and fluorographed or were stained with silver stain and photographed (Skinner & Griswold, 1983; Marshall, 1984). Two-dimensional gel electrophoresis was done by the procedure of O'Farrell (1975) as modified by Kissinger et al. (1982).

Carbohydrate Analysis. All solvents were AR grade, freshly distilled and dried, except acetic anhydride. Methanolic hydrogen chloride was prepared by passing dry hydrogen chloride gas through methanol which was freshly distilled after refluxing several hours in a mixture of magnesium methoxide and anhydrous methanol. Monosaccharide standards were obtained from Sigma Chemical Co. as the finest crystalline grade available except for mannose which was obtained from Baker Chemical Co. and twice recrystallized from 95% ethanol. Fetuin (type IV), ovalbumin (type III, crystalline), and human transferrin (crystalline) were obtained from Sigma Chemical Co.

Methanolysis was performed by a modification of the method described by Chaplin (1982). Aqueous solutions of monosaccharide standards (10–20 μg) or glycoproteins (100–200 μg) were lyophilized in 0.3-mL reaction V vials (Wheaton Scientific, Millville, NJ) and then dried over P_2O_5 for 24 h in vacuo. Dry 0.625 M methanolic hydrogen chloride (320 μL) and dry methyl acetate (80 μL) were then added; the reaction mixture was sealed under argon and heated at 70 °C for 16 h. The hydrogen chloride was removed by adding *tert*-butyl alcohol (75 μL) and evaporated to dryness at 40 °C by using a stream of argon. The amino sugars were re-N-acetylated with methanol (340 μL), pyridine (34 μL), and acetic anhydride (34 μL) at room temperature for 15 min. The reaction mixture was then evaporated under a stream of argon and dried in vacuo as before. One set of monosaccharide standards, a set of glycoprotein standards, and a SGP-2 sample were directly analyzed as described below. To be certain that methanolysis was complete, a second set of samples was cycled through the methanolysis and N-acetylation procedure twice prior to analysis.

Silylation was performed by addition 150 μL of Tri Sil reagent (Pierce Chemical Co., Rockford, IL) directly into the reaction vials and incubation of the mixture at 70 °C for 15 min. Upon cooling to room temperature, the reaction mixtures were evaporated to dryness under a stream of argon. The silylated methyl glycosides were then extracted with 100 μL of hexane, transferred to clean 0.3-mL conical reaction vials, evaporated to dryness, redissolved in 5 μL of hexane, and immediately analyzed by capillary gas chromatography.

Silylated methyl glycoside was analyzed by injecting 0.5 μL onto a capillary column (30 m × 0.5 nm i.d. fused silica wall coated with DB-1; J&W Scientific Inc., Rancho Condora, CA) with a split ratio of 70:1 and an injection temperature of 270 °C. Chromatography was performed with oxygen-free helium at 24 psi head pressure and a temperature program which consisted of 135 °C for 10 min which was then increased 4 °C/min to 240 °C. Under these conditions, base-line resolution was achieved for all of the isomers of each methyl glycoside. The peaks were quantified by using an electronic integrator (HP-3390, Hewlett Packard, Palo Alto, CA).

Protease Digestion of Glycoproteins. To determine the location of the sulfate moiety, individual samples of pure SGP-2 labeled in vitro with [^{3}H]glucosamine, $^{35}SO_4^{2-}$, or [^{35}S]methionine were dissolved in 0.5 mL of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, containing 1 mg/mL pure unlabeled SGP-2 as carrier and digested with Pronase (Calbiochem, San Diego, CA) as described by Finne and Krusius (1982). The resulting digests were then subjected to gel filtration chromatography on a 20 × 2.5 cm column packed with Bio-Gel P-2, 200–400 mesh (Bio-Rad, Richmond, CA), in 0.1 M pyridine acetate (pH 5.0).

To determine the molecular weight of the oligosaccharide moiety, fetuin, human transferrin, and unlabeled SGP-2 were digested with Pronase as described above except that proportionally larger amounts of protein (10–100 mg) and Pronase were used. The oligosaccharides thus obtained were subjected to further digestion for 24 h with leucine aminopeptidase and then carboxypeptidase A (Sigma Chemical Co., St. Louis, MO). The resulting digests of oligosaccharides were then heated in a boiling water bath for 10 min, the precipitate was removed by centrifugation, and the supernatant was desalted by gel filtration over Bio-Gel P-2.

Oligosaccharides obtained from commercial fetuin, human transferrin, ovalbumin, and unlabeled, purified SGP-2 were radiolabeled by N-acetylation with [2-^{14}C]acetic anhydride (New England Nuclear) as described by Finne and Krusius (1982).

Gel filtration chromatography was performed by using a 100 × 1.6 cm glass column packed with Bio-Gel P-6, 200–400 mesh (Bio-Rad, Richmond, CA), equilibrated with 0.1 M pyridine acetate (pH 5.0) at room temperature (Edge & Spiro, 1984). Samples (0.5–2 mg dissolved in 0.5 mL) were applied and eluted with pyridine acetate buffer at a flow rate of 0.5 mL/min. Fractions (2.9 mL) were monitored for radioactivity by scintillation counting (0.1-mL aliquots) and for carbohydrate by the relative absorbance at 490 nm obtained from the phenol–sulfuric acid assay (0.1-mL aliquots) of DuBois et al. (1956). The nominal molecular weight of SGP-2 oligosaccharide was determined from its elution volume relative to the oligosaccharides obtained from human transferrin and fetuin.

Results

When the pooled culture medium from Sertoli cells was collected and concentrated, the secreted glycoproteins were aggregated and eluted from gel filtration columns as if they had a molecular weight of several hundred thousand (Kissinger et al., 1982). However, if the secreted glycoproteins were labeled to a high specific activity with [^{35}S]methionine and applied in a relatively low concentration directly to a Bio-Rad P-150 column, separation and analysis of components were possible. Each fraction from this column was analyzed by

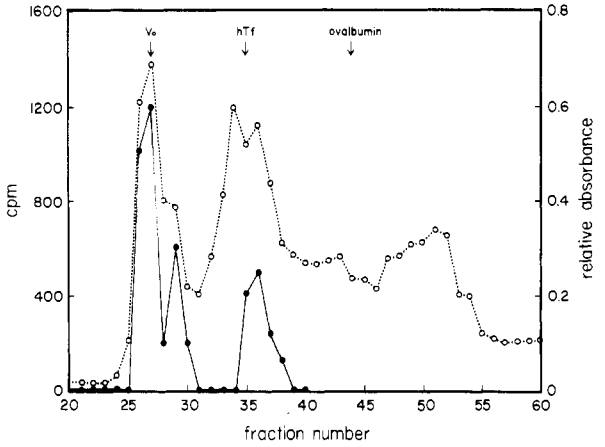

FIGURE 1: Gel filtration elution profile of native SGP-2. Sertoli cell secreted proteins labeled with [^{35}S]methionine were separated by chromatography over a Bio-Gel P-150 column (90 × 1.6 cm) equilibrated in 350 mM phosphate buffer, pH 7.2, 100 mM NaCl, and 5 mM ethylenediaminetetraacetic acid (EDTA). The flow rate of the buffer was adjusted to 0.1 mL/min, and 1.6-mL fractions were collected. Each fraction was dialyzed against H_2O, and a portion was analyzed by polyacrylamide gel electrophoresis and fluorography. The fluorograms were scanned by an LKB XL laser densitometer, and the integrated absorbance of each band in each fraction was determined. The elution profile of the radioactive proteins is plotted along with the absorbance on the fluorogram of the 41-kDa (large) subunit of SGP-2. Human transferrin and ovalbumin eluted at the positions designated in the figure: (○) cpm; (●) relative absorbance.

SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography as described. Under these conditions, SGP-2 is resolved into bands corresponding to the 41 000- (heavy) and 29 000-dalton (light) subunits. These fluorograms were scanned by a densitometer (LKB XL laser densitometer), and the absorbance of the heavy subunit was plotted to show the location of SGP-2 (Figure 1). Identical elution profiles are obtained if the absorbance of the light (29 000 daltons) subunit is used for the plot. SGP-2 eluted from the column in fractions coincident with the void volume and in two retained peaks corresponding to approximate molecular weights of 65 000 and 120 000. It is likely that these three peaks represent SGP-2 in the form of disulfide-linked monomers (M_r 65 000), and as aggregated forms (M_r 120 000 and larger). If the sample was frozen and thawed before the chromatography, very little of the 65 000-dalton molecule was present, and nearly all of the SGP-2 was present as aggregates larger than the exclusion limit of the P-150 matrix. SGP-2 will bind to Sephadex or Sepharose columns and is very difficult to elute. The addition of detergents, or a variety of salts, did not eliminate the tendency of SGP-2 to bind or aggregate. In the experiment shown in Figure 1, SGP-1 was eluted from the P-150 column very close to the void volume and under these conditions probably existed as a noncovalently linked dimer of identical 70 000-dalton monomers (data not shown).

The SGP-2 was purified from the Sertoli cell secreted proteins by reverse-phase high-performance liquid chromatography (HPLC) on Vydak C-4 columns (Figure 2). The elution profile from the column was monitored by the absorbance at 214 nm and by assay of the [^{35}S]methionine in each fraction. Transferrin (fraction 24), SGP-2 (fractions 34–35), and SGP-1 (fractions 48–53) comprised the major UV-absorbing and [^{35}S]methionine-labeled protein. SGP-1, in particular, and SGP-2, to some extent, were strongly retained and eluted in broad peaks at approximately 60% and 49% acetonitrile, respectively.

Fractions from the HPLC effluent were pooled as shown in Figure 3, neutralized with bicarbonate buffer, dialyzed for 24 h vs. H_2O, lyophilized, and analyzed by PAGE and fluorography. SGP-2 was found in pooled fractions K, L, M, and

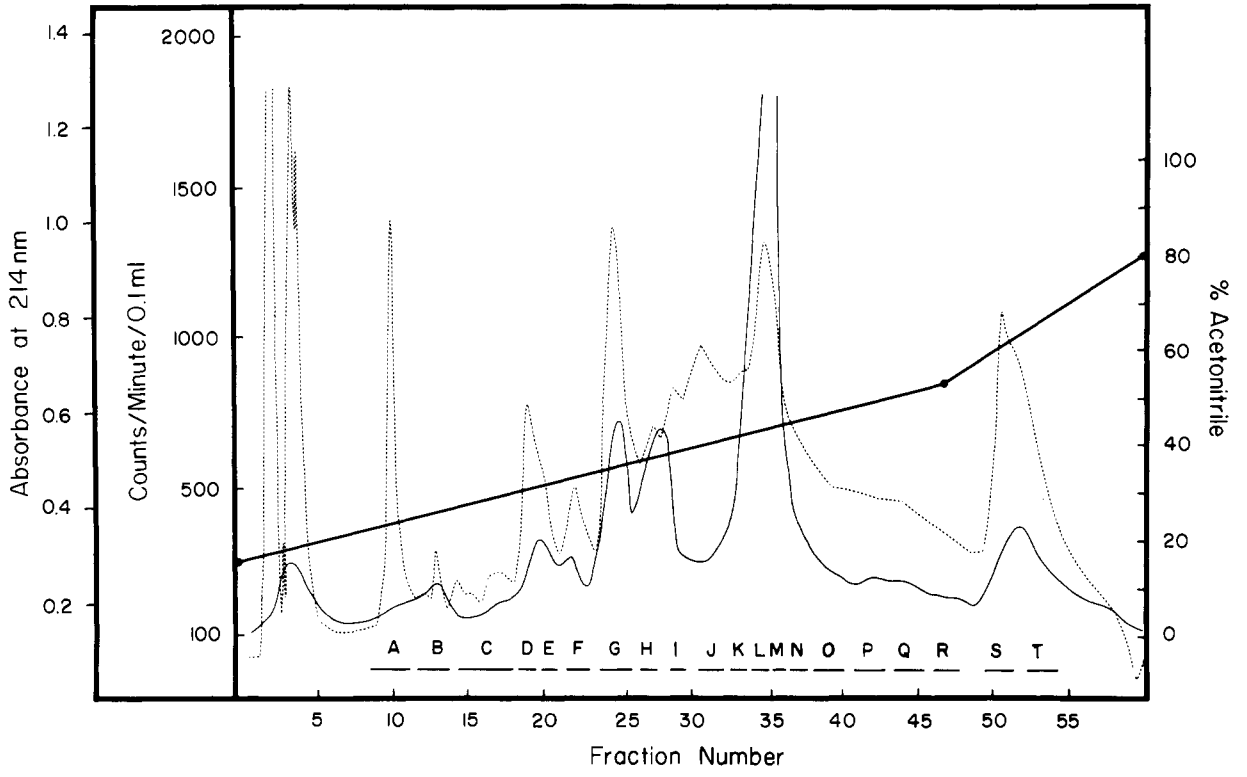

FIGURE 2: Elution profile from reverse-phase high-pressure liquid chromatographic separation of proteins secreted by Sertoli cells. Approximately 1 mg of concentrated lyophilized secreted protein was dissolved in solvent A (2 mL) and applied to a Vydak C-4 reverse-phase column (2.5 × 25 cm), and the programmed gradient was initiated. The flow rate was 1 mL/min, and 1-mL fractions were collected. The radioactivity was determined by counting 100 μL of each fraction in a liquid scintillation counter. Fractions denoted by letters were pooled for further analysis: (···) absorbance; (—) radioactivity.

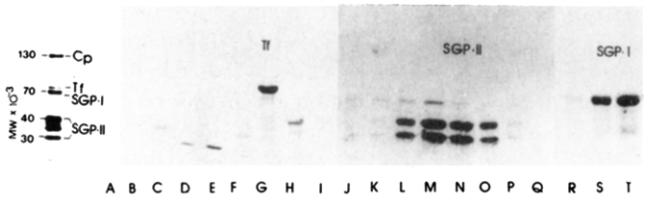

FIGURE 3: Analysis of column fractions. The protein fractions collected as shown in Figure 3 were neutralized, dialyzed, lyophilized, and analyzed by SDS–PAGE. Letters denoting the lanes correspond to the pooled fractions shown in Figure 3. The polyacrylamide gels were dried and fluorographed, and only the fluorograph is shown.

Table I: Amino Acid Composition of SGP-2[a]

amino acid	mol/10² amino acids	amino acid	mol/10² amino acids
Asx	11.1	Met	2.4
Thr	5.0	Ile	3.9
Ser	8.1	Leu	11.5
Glx	15.1	Tyr	2.1
Pro	4.7	Phe	4.7
Gly	5.3	Lys	6.3
Ala	5.4	His	2.8
Val	6.2	Arg	4.6

[a] The value for each amino acid is the mean from a total of three analyses on three different preparations. The samples (100 μg) were hydrolyzed in 6 M HCl at 110 °C for 24 h. *Hydrolytic losses of serine and threonine were not corrected.*

N (Figure 3). These fractions were themselves pooled, dialyzed, and rechromatographed over the same column using an identical mobile phase gradient. The peak which corresponded to SGP-2 was recovered, neutralized, dialyzed, and lyophilized for further analysis. The twice-chromatographed SGP-2 was analyzed by two-dimensional polyacrylamide gel electrophoresis followed by silver stain and was found to be substantially free of impurities (Figure 4). It has been our experience that both SGP-1 and SGP-2 are difficult to stain with a silver stain, and it can be seen in Figure 4 that the large subunit is lightly stained even though 1 μg of protein was used.

If a C-18 reverse-phase HPLC column was used for the purification of secreted glycoproteins, the recovery of SGP-1 and SGP-2 from the column was very low. By comparing the amount of radioactive protein applied to the column and the amount of radioactivity in each fraction, it was determined that the recovery from the Vydak C-4 column was greater than 90%.

The purified SGP-2 was subjected to amino acid and carbohydrate analysis. SGP-2 was found to contain 44% hydrophobic amino acids as well as a large percentage of charged amino acids (Table I). The monosaccharide composition of SGP-2 was compared with that of hen ovalbumin, human transferrin, and fetuin (Table II). It was apparent that SGP-2 is heavily glycosylated with 24% of its mass accounted for as carbohydrate of a complex type. The calculated molar ratios

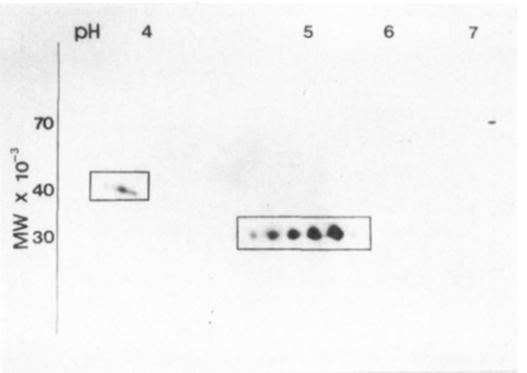

FIGURE 4: Two-dimensional PAGE and silver stain of purified SGP-2. Fractions containing SGP-2 from the HPLC purification described in Figures 4 and 5 were pooled (K–N) and rechromatographed over the same HPLC column using an identical mobile phase gradient. The peak corresponding to SGP-2 was collected, neutralized, dialyzed, and lyophilized. The SGP-2 (1 μg) was then subjectged to two-dimensional PAGE and silver stained. The heavy subunit (41 000 daltons), the light subunit (29 000 daltons), and all of the charge isomers are denoted by boxes.

(i.e., mole percent) normalized to 3 mol of mannose per oligosaccharide are as follows: fucose, 0.9 ± 0.1; mannose, 3.0 ± 0.5; galactose, 3.5 ± 0.3; *N*-acetylglucosamine, 5.0 ± 1.5; *N*-acetylneuraminic acid, 4.0 ± 0.7.

When the oligosaccharide–amino acid complexes generated by extensive proteolysis of SGP-2, human transferrin, and fetuin were subjected to chromatography over Bio-Gel P-6, it was found that the SGP-2–oligosaccharide complexes eluted as a single symmetrical peak just before the complex N-linked triantennary oligosaccharide of fetuin but after the void volume (Figure 5). When the oligosaccharide–amino acid complexes in this peak were subjected to amino acid analysis, the only amino acid detected at unit values was Asp, presumably derived from Asn. Some Thr, Ser, and Glu could be detected at trace levels, but the digestion was essentially complete. The apparent molecular weight for the SGP-2 oligosaccharide–asparagine complex is calculated to be 3300 g/mol.

SGP-2 was labeled in culture with either [^{3}H]glucosamine or [^{3}H]mannose, purified, and analyzed by PAGE and fluorography. The fluorograms showed that both [^{3}H]glucosamine and [^{3}H]mannose were present on both subunits of SGP-2 (data not shown). When the fluorograms were scanned by a densitometer (LKB XL laser densitometer), the ratio of isotope incorporated into the 41 000-dalton subunit to the 29 000-dalton subunit was 2.0 for glucosamine and 1.9 for mannose.

The location of the sulfate moiety was examined after the purified SGP-2 labeled in culture with $^{35}SO_4^{2-}$, [^{35}S]-methionine, or [^{3}H]glucosamine was digested extensively with Pronase. The digested protein was then chromatographed on a Bio-Rad P-2 column which was capable of separating free

Table II: Carbohydrate Composition of SGP-2[a]

carbohydrate	% of total weight			
	ovalbumin	fetuin	human transferrin	SGP-2
fucose	0	0	0	1.0 ● 0.1
mannose	2.4 ± 0.3 (2.4)	2.7 ± 0.8 (3.2)	1.3 ± 0.2 (1.3)	3.5 ± 0.6
galactose	trace	3.2 ± 0.2 (4.1)	1.0 ± 0.1 (0.88)	4.1 ± 0.4
N-acetylgalactosamine	0	1.0 ± 0.4 (1.1)	0	0
N-acetylglucosamine	2.6 ● 0.5 (2.7)	4.7 ± 1.5 (5.5)	2.1 ± 0.4 (2.2)	7.1 ± 2.1
N-acetylneuraminic acid		9.7 ± 3.5 (9.3)	1.8 ± 0.4 (1.6)	8.0 ± 1.4
total carbohydrate by weight				23.7

[a] Values for each sugar in SGP-2, fetuin, human transferrin, and ovalbumin are the mean and standard deviations of eight independent determinations by methanolysis and gas chromatography as described. The values for SGP-2 were determined on three separate preparations. The values in parentheses are those reported by Chaplin (1982) for fetuin and ovalbumin and by Spik et al. (1975) for human transferrin.

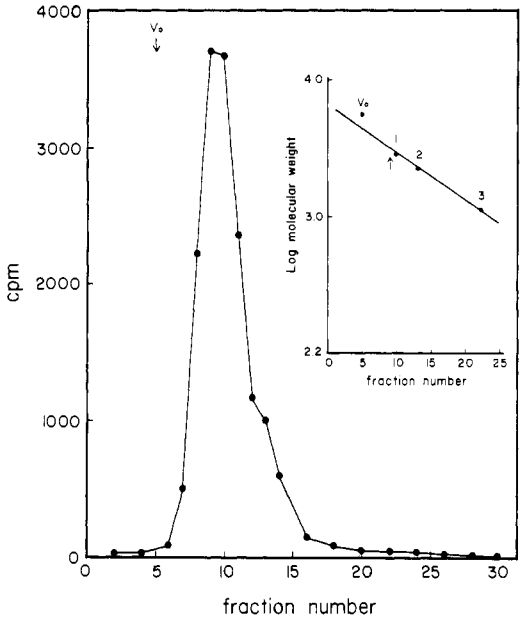

FIGURE 5: Approximate molecular weight of SGP-2 oligosaccharide by gel filtration. Purified SGP-2, fetuin, and human transferrin were digested sequentially with Pronase, leucine aminopeptidase, and carboxypeptidase A. The oligosaccharides from each glycoprotein were radiolabeled by N-acetylation with [2-^{14}C]acetic anhydride (Finne & Krusius, 1982). The oligosaccharides were chromatographed over a Bio-Gel P-6 column equilibrated with 0.1 M pyridine acetate (pH 5.0). Fractions were monitored for radioactivity by scintillation counting and for carbohydrate by the phenol–sulfuric acid assay of DuBois et al. (1956). The nominal molecular weight of the SGP-2 oligosaccharide was determined from its elution volume relative to the oligosaccharides obtained from human transferrin and fetuin (inset): (1) fetuin N linked (2995 daltons); (2) human transferrin (2336 daltons); and (3) fetuin O linked (1050 daltons) (Chaplin, 1982; Spik et al., 1975; Baenziger & Fiete, 1979).

amino acids from the complex oligosaccharide (Figure 6). The $^{35}SO_4^{2-}$ was shown to be associated with the peak which represented the oligosaccharide–asparagine complex and not with the free amino acids.

Discussion

Previous studies have shown that SGP-2 is made by Sertoli cells and becomes localized on the surface of spermatozoa (Sylvester et al., 1984). This report describes a relatively simple method for purification of SGP-2 and the subsequent biochemical characterization of the purified protein. Previous purification methods involved column chromatography in 6 M guanidine hydrochloride and required the reduction of the protein into subunits. The extensive aggregation of the secreted proteins which made conventional chromatography difficult was not a problem in the reverse-phase HPLC procedure. The hydrophobic column matrix and the acetonitrile apparently prevented or reversed the aggregation. The relatively high concentration of acetonitrile required to elute SGP-2 from the C-4 column, the tendency for the protein to aggregate, and the binding to sperm surfaces all suggest that SGP-2 has hydrophobic properties which are not predicted from the overall amino acid composition. While SGP-2 can be found in the medium from cultured Sertoli cells as a 70-kilodalton (kDa) dimer and as multiples of that dimer, SGP-1 can only be found as an apparent noncovalently linked dimer of the previously reported 70-kDa monomer (Kissinger et al., 1982).

The analysis of [^{3}H]glucosamine and [^{3}H]mannose incorporation into SGP-2 showed that oligosaccharides were present on both subunits and approximately twice as much carbohydrate was present on the large subunit as on the small subunit.

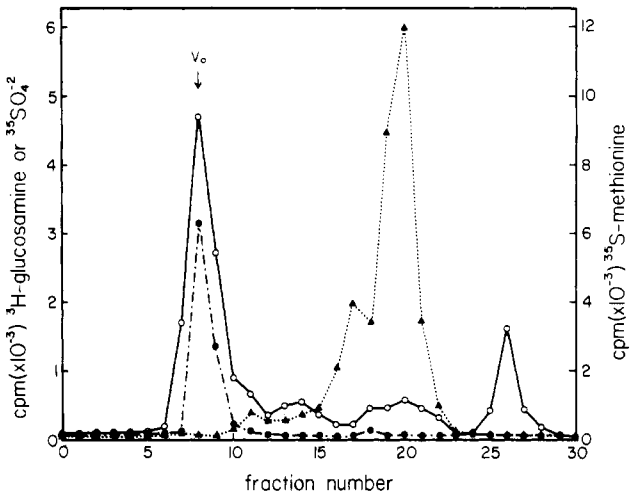

FIGURE 6: Localization of sulfation to the oligosaccharide moiety. Three different preparations of SGP-2 labeled with [^{3}H]glucosamine, $^{35}SO_4^{2-}$, or [^{35}S]methionine were digested with Pronase (Finne & Krusuis, 1982) and passed over a Bio-Rad P-2 column (20 × 2.5 cm). The oligosaccharide containing the [^{3}H]glucosamine (●) and the $^{35}SO_4^{2-}$ (○) eluted in the void volume (V_o) while the free amino acids (▲) were retained by the column.

The available evidence is consistent with an N linkage for all of the oligosaccharides on SGP-2. First, in previous studies it was shown that when Sertoli cells were cultured in the presence of tunicamycin, an inhibitor of N-glycosylation, the molecular weights of the secreted SGP-2 subunits decreased from 41 000 and 29 000 to 27 000 and 21 000, respectively (Sylvester et al., 1984). This decrease in molecular weights indicated that SGP-2 was approximately 30% carbohydrate which is consistent with the 24% we obtained by chemical analysis in this study. Second, the oligosaccharides all elute from gel filtration columns in a single symmetrical peak of relatively high molecular weight (3300). The presence of small O-linked oligosaccharides is therefore unlikely. Third, the composition of the oligosaccharides was found to be consistent with N-linked oligosaccharides, and no galactosamine, which is commonly associated with O-linked sugars, was detected. Therefore, most of the carbohydrate is clearly N linked, and if any O-linked sugars are present, they must lack galactosamine and must have a molecular weight of 3300.

When the mole percent of each monosaccharide was normalized to three mannose residues per oligosaccharide, the apparent stoichiometry for each chain is one fucose, three mannose, three galactose, five glucosamine, and three sialic acid residues. The data are consistent with an oligosaccharide structure similar to the triantennary structure reported for N-linked fetuin oligosaccharides with the additional complexities of fucosylation and sulfation (Baenziger & Fiete, 1979). However, elucidation of the actual oligosaccharide structure must await further analysis. The relatively small amounts of SGP-2 which can be isolated from the cell cultures have limited the current structural analysis.

In previous studies when the SGP-2 was exhaustively digested with neuraminidase, a shift of both subunits on two-dimensional PAGE to the more basic side of the gel was observed, but the overall charge heterogeneity of the protein was not decreased (Kissinger et al., 1982). The extreme charge heterogeneity observed on two-dimensional gel electrophoresis most likely resulted from differential sulfation of the protein and not from incomplete sialation of the oligosaccharide. Similar results were first demonstrated for the viral G protein of vesicular stomatitis virus (VSV) (Hsu & Kingsbury, 1982). A large number of proteins have been reported to contain

sulfate residues either in the oligosaccharides or as sulfated tyrosines (Huttner, 1982). The location of the sulfate residue on the SGP-2 oligosaccharide was confirmed by the presence of $^{35}SO_4^{2-}$ on the glycopeptides after extensive Pronase digestion of SGP-2. Similar methods were used to localize the point of attachment of sulfate to lutropin (Anumula & Bahl, 1983). On the basis of previous studies, a likely site for the sulfation to occur is on the *N*-acetylglucosamine residues (Anumula & Bahl, 1983; Lian & Horowitz, 1982; Edge & Spiro, 1984). Recent studies on the structural requirements for sulfation of asparagine-linked oligosaccharides of lutropin suggested that the sequence GalNAc(β1–4)GlcNAc(βL2)-Manα was recognized by a specific sulfotransferase which sulfated the GalNAc residue (Green et al., 1986). Since SGP-2 contains no GalNAc, it is likely that a different sulfotransferase is involved.

The function of the sulfated glycoproteins produced by the Sertoli cells is unknown and under investigation. The combination of SGP-1 and SGP-2 constitutes the major Sertoli cell secretion products and represents more than 50% of the mass of the total secreted proteins. The similarity of SGP-2 to a protein F described by Brooks has previously been noted (Sylvester et al., 1984; Brooks, 1983). SGP-2 also has a number of structural similarities to a protein which has been isolated from ovine rete testis fluid. This protein has the capability to aggregate red blood cells and other cell types and was given the name "Clusterin" (Blaschuk et al., 1983; Fritz et al., 1983). Both Clusterin and SGP-2 appear to be disulfide-linked dimers of 30000–40000 daltons, and both proteins appear to aggregate into multimers in the absence of denaturants. Both proteins were found in high concentrations in the tract fluids and localized on sperm surfaces in the testis and epididymis. The overall amino acid compositions of Clusterin and SGP-2 are different, but both are heavily glycosylated and have a p*I* of 3.5–4.0 (Blaschuk et al., 1983; Bluschuk & Fritz, 1984; Kissinger et al., 1982). Whether or not SGP-2 and Clusterin represent the same functional protein isolated from two different species is a question which will be addressed in future investigations.

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