Purification and biochemical characterization of three major acidic proteins (BSP-A₁, BSP-A₂ and BSP-A₃) from bovine seminal plasma

P. MANJUNATH* and M. R. SAIRAM

Reproduction Research Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec H2W 1R7, Canada

Three major acidic proteins of bovine seminal plasma, BSP-A₁, BSP-A₂ and BSP-A₃, were purified to homogeneity, by employing fast protein liquid chromatography, gel filtration and h.p.l.c. The proteins were purified on the basis of their stimulatory effect on the basal release of gonadotropins by rat anterior-pituitary cells in culture. All three proteins migrated as distinct single bands in the presence or absence of 2-mercaptoethanol in SDS/polyacrylamide-gel electrophoresis. Their M_r values were estimated to be between 15000 and 16500 by SDS/polyacrylamide-gel electrophoresis. Similar M_r estimates were obtained when they were subjected to gel filtration on a calibrated column of Sephadex G-75 equilibrated in 0.05 M-acetic acid, pH 3.0. However, BSP-A₁ and BSP-A₂ were eluted as aggregated molecules (M_r 60000–120000) during gel filtration on Sephadex G-200 equilibrated in 0.05 M-NH₄HCO₃, pH 8.5, or phosphate buffer, pH 7.0, containing 0.15 M-NaCl. In the presence of 8 M-urea both BSP-A₁ and BSP-A₂ were eluted at positions corresponding to M_r values of 17000–20000. BSP-A₁ and BSP-A₂ had an identical amino acid composition, which differed largely from that of BSP-A₃. All three proteins contained aspartic acid as the *N*-terminal residue, and cysteine was identified as the *C*-terminal residue. BSP-A₁ and BSP-A₂ are glycoproteins containing galactosamine, sialic acid and neutral sugars, but BSP-A₃ did not contain any covalently attached sugars. Whereas BSP-A₂ and BSP-A₃ were eluted unadsorbed, BSP-A₁ bound to wheat-germ lectin–Sephadex 6MB and could be eluted by the competing sugar *N*-acetyl-D-glucosamine. Treatment of BSP-A₁ and BSP-A₂ with trypsin resulted in complete loss of gonadotropin-release activity, but BSP-A₃ retained full activity. Antibody raised against BSP-A₁ did not cross-react with BSP-A₃, or vice versa. All these properties indicated marked structural differences between BSP-A₃ and BSP-A₁ (or BSP-A₂). On the basis of amino acid composition it was concluded that BSP-A₁, BSP-A₂ and BSP-A₃ are the same as the gonadostatins [Esch, Ling, Bohlen, Ying & Guillemin (1983) *Biochem. Biophys. Res. Commun.* **113**, 861–867].

INTRODUCTION

The seminal plasma, which serves as a vehicle for ejaculated spermatozoa, is a complex mixture of secretions of the testes and the accessory glands (Mann, 1975). Despite its great physiological significance as the carrier of sperms to the female reproductive tract, the functions of the various proteins and polypeptides in the seminal plasma are still poorly understood. Defining the molecular characteristics, including structure, is an essential step in this direction. Several androgen-dependent proteins have served as useful markers of prostatic function (Heyns *et al.*, 1978; Lea *et al.*, 1979; Wilson & French, 1980; Chen *et al.*, 1982; Isaacs & Shaper, 1983) and seminal-vesicle activity (Ostrowski *et al.*, 1979; Yu-Ching *et al.*, 1980). The amino acid sequences of several seminal plasma proteins have been elucidated in recent years (Esch *et al.*, 1983a; Seidah *et al.*, 1984a,b; Akiyama *et al.*, 1985; Li *et al.*, 1985).

Following a report (Franchimont *et al.*, 1975) that bull seminal plasma contained a follicle-stimulating-hormone- (follicle-stimulating-hormone-) inhibiting factor ‘inhibin’, our laboratory became interested in examining in detail the molecular nature of these substances. During the course of these investigations, it became clear that both basic

proteins (Sairam *et al.*, 1981; Manjunath, 1984a) as well as acidic proteins (Manjunath 1984b) influenced gonadotropin secretion from pituitaries incubated *in vitro*. The partial purification and some characteristics of three acidic proteins of bull seminal plasma, which were designated BSP-A₁, BSP-A₂ and BSP-A₃, were reported (Manjunath, 1984b; Manjunath & Sairam, 1985). These proteins released gonadotropins spontaneously in a manner similar to luteinizing-hormone-releasing hormone (luliberin) in rat pituitary-cell cultures in synthetic medium. However, in the presence of medium containing calf serum gonadotropin release did not occur. In the presence of luteinizing-hormone-releasing hormone a bimodal secretion of both gonadotropins [follicle-stimulating hormone and luteinizing hormone (luteotropin)] was seen, i.e. inhibition at lower concentrations and stimulation at higher concentrations. On the basis of amino acid compositions we speculated that these proteins may be similar to the so-called ‘gonadostatins’, BSP I, BSP II and BSP III, of bull seminal plasma (Esch *et al.*, 1983b). These three proteins were isolated by using gel filtration, partition chromatography and h.p.l.c. BSP II and BSP III have identical amino acid compositions and are collectively named PDC-109 protein. BSP I has a different amino acid composition, suggesting a unique

* To whom correspondence and reprint requests should be addressed, at present address: Maisonneuve Hospital Research Centre, 5415 Boulevard de l'Assomption, Montreal, Quebec H1T 2M4, Canada.

structure for this protein. The primary structure of PDC-109 has been determined. It contains 109 amino acid residues with two structurally similar domains, A and B, of 38 and 41 amino acid residues respectively, each containing two disulphide bridges (Esch *et al.*, 1983a). No structural studies were provided by Esch and co-workers or by any other workers on gonadostatin BSP I.

The work described in the present paper clearly indicates that the BSP-A₁, BSP-A₂ and BSP-A₃ that we have isolated on the basis of gonadotropin-release activity are identical with the gonadostatins isolated on the basis of their antagonistic action on luteinizing-hormone-releasing hormone. BSP-A₁ and BSP-A₂ in our nomenclature correspond to PDC-109 (BSP II and BSP III), and BSP-A₃ corresponds to BSP I. The present study also revealed that BSP-A₁ and BSP-A₂ are glycosylated and that they are identical polypeptides but containing different amounts of carbohydrate. Furthermore, comparative studies between BSP-A₁ (or BSP-A₂) and BSP-A₃ revealed differences in elution characteristics under various conditions, M_r , amino acid and carbohydrate compositions, stability towards trypsin and immunochemical properties. The present paper also describes a simplified method of purification for obtaining large amounts of these proteins. A subsequent paper describes the structural differences between BSP-A₃ and PDC-109 (Seidah *et al.*, 1987).

MATERIALS AND METHODS

Materials

Sephadex gels (G-75, G-100 and G-200), concanavalin A-Sepharose, wheat-germ lectin-Sepharose 6MB and Mono S (cation-exchange) and Mono Q (anion-exchange) columns were purchased from Pharmacia Fine Chemicals (Dorval, Quebec, Canada). Sequencer-grade anhydrous heptafluorobutyric acid was purchased from Beckman (Montreal, Quebec, Canada). Trifluoroacetic acid was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). μ Bondapak phenyl and μ Bondapak C₁₈ columns were purchased from Waters Millipore (Milford, MA, U.S.A.) M_r marker proteins were purchased from Pharmacia. Reagents used for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Other reagents and chemicals used were of analytical grade.

Collection of the ejaculate and preparation of the crude bovine seminal-plasma powder

Ejaculates from adult Holstein bulls were collected and processed as described previously (Manjunath, 1984b). In brief, the fresh or thawed ejaculates were centrifuged to separate spermatozoa. To the clear spermatozoa-free extract 8 vol. of cold (-20°C) ethanol was added with constant stirring. The solution was left stirring for 2 h and centrifuged to separate the precipitate. The precipitate was dispersed in cold ethanol and centrifuged again. The process was repeated two more times to remove all steroids and other lipid materials present in the seminal plasma. The alcohol precipitate was then dispersed in distilled water and freeze-dried. This freeze-dried powder, designated crude bovine seminal-plasma powder, was stored at -20°C until further purification.

Fig. 1. Gel-filtration pattern of unadsorbed fraction from the Mono S column on Sephadex G-75

Unadsorbed fraction from the Mono S column was loaded in 5 ml on a Sephadex G-75 (superfine grade) column ($2.5\text{ cm} \times 108\text{ cm}$) equilibrated in 0.05 M-acetic acid at 4°C . Elution was carried out with same solvent. Fractions (3.8 ml) were collected at a rate of 15 ml/h. Fractions 100–136 were pooled and freeze-dried (yield approx. 85 mg). Arrows with designations a, b and c indicate elution positions for bovine serum albumin (M_r 67000), ovalbumin (M_r 45000) and lysozyme (M_r 14300) respectively.

Purification

Chromatography on Mono S. Chromatography on a Mono S column essentially removes some of the basic proteins that would otherwise be co-eluted with active proteins in subsequent steps (gel filtrations). About 100 mg of crude bovine seminal-plasma powder was dissolved in 15 ml of 0.05 M-sodium phosphate buffer, pH 7.1, and passed through a Mono S column attached to a fast protein liquid chromatography system. The unadsorbed active fraction from two runs was concentrated to 5 ml by passage through an ultrafiltration cell equipped with a UM-2 membrane. The concentrate was then subjected to gel filtration as described in the next step.

Chromatography on Sephadex G-75. The protein concentrate from the previous step was chromatographed on a column of Sephadex G-75 equilibrated in 0.05 M-acetic acid, pH 3.0 (Fig. 1). Fractions (100–136), eluted at positions corresponding to M_r approx. 15000–20000, contained most of the stimulatory activity (Manjunath, 1984b). These fractions were pooled and freeze-dried (designated G-75 IV).

Chromatography of Sephadex G-200. About 85 mg of freeze-dried protein from the previous step was dissolved in 5 ml of 0.05 M- NH_4HCO_3 and chromatographed on a Sephadex G-200 column equilibrated in 0.05 M- NH_4HCO_3 (Fig. 2). Most of the stimulatory activity of gonadotropin release was eluted in fraction II (designated

Fig. 2. Gel-filtration pattern of fraction G-75 IV on Sephadex G-200

An 85 mg portion fraction G-75 IV was dissolved in 5 ml of 0.05 M-NH₄HCO₃, pH 8.5, and loaded on a Sephadex G-200 column (2.5 cm × 110 cm) previously equilibrated in 0.05 M-NH₄HCO₃ at 4 °C, and elution was carried out with same solvent. Fractions (3.5 ml) were collected at a rate of 20 ml/h. Fractions were pooled as indicated and freeze-dried (yield 45.6 mg of fraction G-200 II and 15.1 mg of fraction G-200 IV). Arrows with designations a, b and c indicate elution positions for bovine serum albumin (M_r 67000), ovalbumin (M_r 45000) and myoglobin (M_r 17000) respectively.

G-200 II) and fraction IV (designated G-200 IV). Fraction G-200 II was eluted at positions corresponding to M_r 60000–120000, and fraction G-200 IV at a position corresponding to M_r approx. 20000. Fractions G-200 II and G-200 IV were pooled separately, freeze-dried and used for further purification by h.p.l.c.

H.p.l.c. Fractions G-200 II and G-200 IV still contained 5–10% of other contaminating proteins. These could be removed by performing reverse-phase h.p.l.c. This was carried out in a Waters Associates h.p.l.c. apparatus equipped with model 510 pumps, a model 440 fixed-wavelength detector, a model U6K loop injector and a reverse-phase μ Bondapak phenyl column (7.8 mm internal diam. × 30 cm). The solvent system used consisted of solvent A [0.13% (v/v) heptafluorobutyric acid in water] and solvent B [0.13% (v/v) heptafluorobutyric acid in acetonitrile]. Typical elution conditions for proteins are indicated in Fig. 3. Proteins were monitored at 280 nm. Fraction G-200 II upon chromatography always gave two active fractions, designated BSP-A₁ (bovine seminal-plasma acidic protein 1) and BSP-A₂ (Fig. 3a).

Fraction G-200 IV always gave one major active fraction, designated BSP-A₃ (Fig. 3b), and a minor active fraction, which corresponds to BSP-A₂. All three active fractions were freeze-dried separately. To remove any traces of adsorbed heptafluorobutyric acid, freeze-dried proteins were dialysed against 0.05 M-NH₄HCO₃ and freeze-dried again. Each of the above fractions was homogeneous electrophoretically (see the Results section). Use of trifluoroacetic acid in place of heptafluorobutyric acid in solvents resulted in a poor resolution of BSP-A₁ and BSP-A₂. Further, it was noted that the use of a μ Bondapak C₁₈ column resulted in serious losses, as these proteins bound strongly to the column.

M_r determination

The M_r values of BSP-A₁, BSP-A₂ and BSP-A₃ were estimated by SDS/polyacrylamide-gel electrophoresis and by gel filtration. The SDS/polyacrylamide-gel electrophoresis run was carried out in accordance with Laemmli (1970). A low- M_r calibration kit (Pharmacia) containing phosphorylase b (M_r 94000), serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), trypsin inhibitor (M_r 20100) and α -

Fig. 3. Reverse-phase h.p.l.c. patterns of fractions G-200 II (a) and G-200 IV (b)

About 200–300 μ g of protein in 100 μ l of solvent A was loaded on a μ Bondapak phenyl column equilibrated in 45% (v/v) solvent B. After 5 min run at 45% solvent B, adsorbed peptides were eluted at room temperature by using a linear gradient of 45% solvent B to 60% solvent B in 30 min, at a flow rate of 1 ml/min (----). —, A_{280} . Two active fractions separated from fraction G-200 II (a) on h.p.l.c. were designated BSP-A₁ (R_F 20.1 ± 0.1 min) and BSP-A₂ (R_F 21.3 ± 0.1 min). The major active formation obtained from fraction G-200 IV (b) was designated BSP-A₃ (R_F 22.9 ± 0.1 min).

lactalbumin (M_r 14400) as marker proteins was used for calibration.

M_r determination by gel filtration was carried out under three different conditions: (a) a Sephadex G-200 column (2.5 cm $\times$ 110 cm) equilibrated with 0.05 M- NH_4HCO_3 , pH 8.5, (b) a Sephadex G-100 column (2.5 cm $\times$ 110 cm) equilibrated with 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, and (c) a Sephadex G-75 column (2.5 cm $\times$ 110 cm) equilibrated with 0.05 M-acetic acid, pH 3.0, were used. Bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), myoglobin (M_r 17200) and lysozyme (M_r 14300) were used for calibration.

Amino acid analysis

Amino acid analysis in a Beckman model 121 MB amino acid analyser, with single-column methodology, was performed in accordance with Fauconnet & Rochemont (1978) under the conditions described previously (Manjunath & Sairam, 1982). Protein samples for analysis were hydrolysed at 110 °C in 6 M-HCl for 22–24 h in sealed tubes under vacuum. Cystine was determined either as cysteic acid after performic acid oxidation of proteins or as carboxymethylcysteine upon carboxymethylation and hydrolysis of the protein. For tryptophan determination proteins were hydrolysed in 6 M-HCl containing 2% (v/v) mercaptoacetic acid (Matsubara & Sasaki, 1969).

Carbohydrate analysis

Amino sugars were determined by using the amino acid analyser. The samples were hydrolysed in the presence of 4 M-HCl at 110 °C for 4 h. Neutral sugars were determined by the phenol/ H_2SO_4 reaction, by following the modification described by McKelvy & Lee (1969). Glucose was used as standard. Sialic acid was determined after acid hydrolysis of protein in 0.04 M-HCl at 80 °C for 1 h by using the modified fluorimetric assay (Hammond & Papermaster, 1976).

N-Terminal and C-terminal analysis

N-Terminal amino acid was determined by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride method (Gray, 1972). Identification of the dansylated N-terminal amino acid was accomplished by t.l.c. on polyamide sheets (Hayashi, 1977). Co-migration with authentic dansyl-amino acid was used to confirm identification.

C-Terminal amino acid was determined by digestion of performic acid-oxidized or carboxymethylated protein with carboxypeptidase Y in 100 mM-pyridine/acetate buffer, pH 6.0, for about 15 h. The released residue was identified by analysing the hydrolysate on the amino acid analyser.

Preparation of antisera

About 200 μg of purified protein in 0.5 ml of 0.9% NaCl was emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, U.S.A.) and injected subcutaneously at multiple sites on the back of the male rabbits. Injections were repeated at 10-day intervals over a period of 50 days. The animals were bled from the marginal ear vein 15 days after the last injection. The blood was allowed to clot at room temperature for 3 h and the serum was separated by centrifugation. Antisera were stored at -20°C until use.

Fig. 4. SDS/polyacrylamide-gel-electrophoresis patterns of BSP-A₁, BSP-A₂ and BSP-A₃ under reducing conditions

About 10 μg of reduced and denatured protein was loaded per slot on a 10% gel slab. The gel was stained with Coomassie Brilliant Blue R250. Lane 1, calibration standards (they are from top to bottom phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α -lactalbumin); lane 2, BSP-A₃; lane 3, BSP-A₂; lane 4, BSP-A₁.

Double-radial-immunodiffusion analysis

Double-radial-immunodiffusion analysis was performed at 4 °C, by using 10 μl -capacity wells, in 1.5% purified agar (Difco Laboratories, Detroit, MI, U.S.A.) in 20 mM-phosphate buffer, pH 7.5, containing 0.15 M-NaCl and 0.01% NaN_3 . After 16–20 h of diffusion, white precipitin bands became visible and plates were photographed under dark-ground illumination.

RESULTS

Purification

Initially, studies were conducted to determine the behaviour of these proteins under the conditions of various purification methods (Manjunath, 1984b). These included fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ or cold ethanol, gel filtration with different eluents such as 0.05 M- NH_4HCO_3 , pH 8.5, 0.05 M-acetic acid, pH 3.0, and 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and with or without 8 M-urea, fast protein liquid chromatography with Mono S and Mono Q ion-exchange columns and chromatography on concanavalin A-Sepharose and wheat-germ lectin-Sepharose.

These studies revealed the following information. (i) These proteins aggregate to form various molecular forms ranging in M_r from 20000 to 120000 at or above

Table 1. Amino acid composition of BSP-A₁, BSP-A₂ and BSP-A₃ and comparison with gonadostatins (PDC-109 protein and BSP I)

Results for BSP-A₁, BSP-A₂ and BSP-A₃ are means for eight different analyses on 24 h hydrolysates

Amino acid	Amino acid composition (residues/molecule)				
	BSP-A ₁ *	BSP-A ₂ *	PDC-109†	BSP-A ₃ ‡	BSP I§
Lys	8.2	8.3	8	12.6	12.3
His	2.1	2.2	2	1.0	1.0
Arg	5.3	5.5	5	1.4	1.2
Asp	12.0	12.0	12	17.0	13.7
Thr	4.6	4.6	5	5.8	5.2
Ser	6.0	6.2	7	8.5	8.3
Glu	10.6	10.7	10	7.7	7.3
Pro	6.7	6.8	7	3.6	4.9
Gly	6.8	7.0	7	7.7	6.8
Ala	4.6	4.5	5	3.9	4.1
Cys	7.9	7.7	8	7.9	6.9
Val	6.3	6.2	6	4.1	3.3
Met	1.9	1.9	2	1.0	1.0
Ile	1.9	2.0	2	4.9	4.4
Leu	4.0	4.0	4	7.4	7.1
Tyr	8.1	8.3	8	10.0	10.1
Phe	6.0	6.0	6	7.8	6.9
Trp	4.4¶	3.9¶	5	5.0¶	4.3

* Values calculated assuming 12 residues of aspartic acid/molecule of protein (M_r 12 774).

† Values from sequence data (Esch *et al.* 1983a).

‡ Values calculated assuming 17 residues of aspartic acid/molecule of protein (M_r 13 403) (Seidah *et al.*, 1987).

§ Values from Esch *et al.* (1983a).

|| Determined after performic acid oxidation or carboxymethylation.

¶ Determined by hydrolysing protein in 6 M-HCl containing 2% mercaptoacetic acid (Matsubara & Sasaki, 1969).

pH 7.0, even in the presence of high salt concentration such as 0.15 M-NaCl in 0.05 M-phosphate buffer, pH 7.0; 8 M-urea or acidic conditions (pH 3.0) convert these proteins into monomeric forms. (ii) They appear to be acidic hydrophobic proteins. (iii) The presence of carbohydrate could be one of the reasons for their different molecular forms. (iv) Their tendency to adsorb strongly on other proteins affects the solubility and elution characteristics during gel filtration and ion-exchange chromatography. On the basis of these data, a simplified four-step purification method was developed. Our present procedure involves (a) chromatography on a Mono S column, (b) gel filtration with 0.05 M-acetic acid, pH 3.0, (c) gel filtration with 0.05 M-NH₄HCO₃, pH 8.5, and (d) h.p.l.c. on a μ Bondapak phenyl column. The third step in purification is particularly important because under the conditions employed in this step both BSP-A₁ and BSP-A₂ self-associate to form larger aggregates and are eluted earlier than BSP-A₃. Inclusion of this step before h.p.l.c. is quite beneficial because both BSP-A₂ and BSP-A₃ are eluted very close to each other during h.p.l.c. and therefore are difficult to obtain in pure form.

The purification procedure described in the Materials and methods section is simple, reproducible and useful for obtaining large amounts of these proteins. From

200 mg of crude bovine seminal-plasma powder (approximately equal to 3 ml of pooled bull seminal plasma) about 45 $\pm$ 5 mg (20–25%) of BSP-A₁ and BSP-A₂ and 17 $\pm$ 3 mg (7–10%) of BSP-A₃ were obtained after the third step of purification. Thus these three proteins constitute more than 30% of the total protein contained in bull seminal plasma.

SDS/polyacrylamide-gel electrophoresis and M_r

Fig. 4 shows the SDS/polyacrylamide-gel-electrophoretic pattern under reducing conditions. All three peptides migrated as single distinct bands. In the absence of reducing agents, a similar pattern (not shown) was obtained, but the proteins stained poorly. These results clearly indicate that they are single-chain polypeptides. The M_r values, as calculated by the use of marker proteins, were 16500, 16000 and 15000 for BSP-A₁, BSP-A₂ and BSP-A₃ respectively.

Similar estimates of about 17000–20000 were obtained when the M_r values of these polypeptides were determined on a calibrated column of Sephadex G-75 equilibrated in 0.05 M-acetic acid (Fig. 1). When M_r estimations were carried out on a Sephadex G-200 column equilibrated in 0.05 M-NH₄HCO₃ (Fig. 2) or on a Sephadex G-100 column (2.5 cm $\times$ 110 cm) equilibrated in 0.05 M-phosphate buffer, pH 7.0, containing 0.15 M-NaCl, quite disparate results were obtained. Whereas BSP-A₃ gave a value of about 20000, BSP-A₁ and BSP-A₂ gave M_r estimates ranging from 60000 to 120000. When phosphate buffer containing 8 M-urea was used, both BSP-A₁ and BSP-A₂ were eluted at a region corresponding to an M_r of 20000.

Amino acid composition

Table 1 shows the amino acid compositions of BSP-A₁, BSP-A₂ and BSP-A₃ and compares them with those of gonadostatins (Esch *et al.*, 1983a). BSP-A₁ and BSP-A₂ showed an identical amino acid composition but this differed from that of BSP-A₃. Differences were noticed in the contents of almost all amino acids, notably in basic amino acids as well as glutamic acid, proline, valine, methionine, isoleucine and leucine. All three proteins contained about four or five residues of tryptophan. Further, the amino acid composition of BSP-A₁ and BSP-A₂ was identical with that of PDC-109 proteins (BSP II and BSP III), and BSP-A₃ appears to be similar in amino acid composition to BSP I (Esch *et al.*, 1983a). The composition of BSP-A₃ is nearly identical with values obtained from sequence data (Seidah *et al.*, 1987).

C-Terminal and N-terminal amino acid analysis

Treatment of performic acid-oxidized or reduced and carboxymethylated BSP-A₁ and BSP-A₂ with carboxypeptidase Y for 2–4 h resulted in the release of cysteic acid or carboxymethylcysteine as first amino acid, indicating the presence of cysteine at the C-terminus of both proteins. Similar treatment of performic acid-oxidized or reduced and carboxymethylated BSP-A₃ did not release any amino acid. Increase in either incubation time or enzyme concentration resulted in the release of a number of amino acids, including cysteic acid or carboxymethylcysteine, probably due to the presence of endopeptidase activity in commercial carboxypeptidase Y used in the present investigation. Thus unambiguous identification of the C-terminal amino acid of BSP-A₃ by carboxypeptidase Y digestion was not possible. However,

Table 2. Carbohydrate compositions of BSP-A₁, BSP-A₂ and BSP-A₃

Component	Carbohydrate composition (g/100 g of glycoprotein)		
	BSP-A ₁	BSP-A ₂	BSP-A ₃
Neutral sugars*	1-2	0.0	0.0
Amino sugars†			
Glucosamine	0.0	0.0	0.0
Galactosamine	1-2	0.6-1	0.0
Sialic acid‡	1.6-2.5	0.6-1	0.0
Total	3.6-6.5	1.2-2	

* Determined by phenol/H₂SO₄ reaction.

† Determined on amino acid analyser.

‡ Determined by microfluorimetric assay (Hammond & Papermaster, 1976).

Fig. 5. Effect of trypsin on follicle-stimulating-hormone-release activity of BSP-A₁, BSP-A₂ and BSP-A₃

Protein solutions (1 mg/ml in 0.05 M-NH₄HCO₃) containing trypsin (enzyme/substrate ratio 1:100, w/w) were incubated at 37 °C. Portions of digest were withdrawn at intervals up to 6 h, mixed immediately with lima-bean trypsin inhibitor (8-fold excess over trypsin concentration), freeze-dried and tested for follicle-stimulating-hormone-release activity on pituitary-cell cultures as described previously (Manjunath, 1984b), and compared with controls (100%). Controls were treated similarly except that trypsin was added after adding trypsin inhibitor.

sequence analysis indicated cysteine as the C-terminal amino acid in this protein (Seidah *et al.*, 1987). All three proteins contained the same N-terminal amino acid, namely aspartic acid. Similar terminal residues were shown to be present in BSP II and BSP III (PDC-109 protein) isolated by Esch *et al.* (1983a), who, however, did not analyse the terminal residues of BSP I, which is the same as BSP-A₃ of the present study.

Fig. 6. Double-radial-immunodiffusion analysis of BSP-A₁, BSP-A₂ and BSP-A₃ with (a) anti-BSP-A₁ serum and (b) anti-BSP-A₃ serum

Wells 1, 2, 3 and 4 contained respectively about 2 µg of BSP-A₁, 2 µg of BSP-A₂, 2 µg of BSP-A₃ and phosphate-buffered saline (control). In (a) the central well contained 10 µl of anti-BSP-A₁ serum and in (b) the central well contained 10 µl of anti-BSP-A₃ serum.

Carbohydrate content

The detection of galactosamine during amino acid analysis prompted examination of their carbohydrate compositions. Carbohydrate analysis (Table 2) showed that BSP-A₁ and BSP-A₂ are glycoproteins and that BSP-A₃ did not contain any covalently bound carbohydrate. BSP-A₁ contained higher amounts of carbohydrate (3.6-6.5%) than BSP-A₂ (1.2-2%). Neutral sugars were absent from BSP-A₂.

Binding to wheat-germ lectin-Sepharose

The results on carbohydrate analysis prompted us to check for binding to wheat-germ lectin-Sepharose. Over 90% of BSP-A₁ was bound to the column, and the bound protein could be eluted by the competing sugar N-acetyl-D-glucosamine. In contrast, when BSP-A₂ and BSP-A₃ were chromatographed under the same conditions, both of them were eluted unadsorbed. Thus lectin binding studies and sugar analysis, together with amino acid analysis, indicated the following: BSP-A₁ and BSP-A₂ are same protein moieties containing different amounts of sugar and therefore separable on h.p.l.c. BSP-A₁ is the more extensively glycosylated and consequently bound to lectin, whereas the partially glycosylated BSP-A₂ did not interact with lectin; BSP-A₃ is devoid of covalently bound carbohydrate and therefore did not interact with lectin.

Treatment with trypsin

Treatment of BSP-A₁ and BSP-A₂ with trypsin resulted in complete loss of gonadotropin-release activity in about 30 min (Fig. 5). BSP-A₃ under similar conditions retained full activity even after 6 h. In fact, there was a slight increase in gonadotropin-release activity with this protein. These results clearly indicate the probability of structural differences between BSP-A₃ and BSP-A₁ or BSP-A₂.

Double-radial-immunodiffusion studies

This immunochemical technique was used in the present study to investigate whether BSP-A₁, BSP-A₂ and

BSP-A₃ are immunologically related. As shown in Fig. 6(a), BSP-A₁ and BSP-A₂ cross-reacted with anti-BSP-A₁ serum, and when they were placed in adjacent wells the precipitin line obtained was continuous (reaction of identity). No precipitin line was formed with BSP-A₃, indicating non-identity with BSP-A₁. With an antiserum to BSP-A₃ a sharp single precipitin line was seen with BSP-A₃, but BSP-A₁ and BSP-A₂ failed to cross-react (Fig. 6b). Thus the antigenic determinants for BSP-A₃ are different from those of BSP-A₁ and BSP-A₂. Similar conclusions have been drawn from the radioimmunoassay of these proteins.

DISCUSSION

In this paper we describe the isolation and characterization of three major acidic proteins, designated BSP-A₁, BSP-A₂ and BSP-A₃, found in bull seminal plasma. These proteins were purified on the basis of their ability to stimulate the release of gonadotropins by rat anterior-pituitary-cell cultures. A detailed report of the effect of these proteins on release of gonadotropin by pituitary-cell cultures has been given previously (Manjunath, 1984b). A comparison of the amino acid compositions of BSP-A₁, BSP-A₂ and BSP-A₃ of the present study with those of the three proteins BSP I, BSP II and BSP III isolated from the same source by Esch *et al.* (1983a) revealed that they are identical. Whereas we isolated these proteins on the basis of their ability to stimulate the release of gonadotropins by pituitary-cell cultures, Esch *et al.* (1983a) isolated the same proteins on the basis of their antagonistic action on luteinizing-hormone-releasing hormone. Although they may have effects on gonadotropin release in pituitary-cell cultures *in vitro*, their physiological significance still remains to be determined. Therefore we have not given any specific name for these proteins and prefer to call them BSP-A₁, BSP-A₂ and BSP-A₃ (based on their order of elution during h.p.l.c.). BSP II and BSP III have an identical amino acid composition and together are called PDC-109 protein (P = protein, D = N-terminal aspartic acid and C = C-terminal cysteine) by Esch *et al.* (1983a), following Mutt's nomenclature. BSP-A₁ and BSP-A₂ of the present investigation correspond to PDC-109 protein, and BSP-A₃ corresponds to BSP I described by Esch *et al.* (1983a).

One important finding of the present investigation is their behaviour during gel filtration in various aqueous media of different pH values (pH 3.0, pH 7.0 and pH 8.5). Whereas BSP-A₃ was eluted at a position corresponding to an M_r of 17000–20000 at all pH values, BSP-A₁ and BSP-A₂ were eluted as aggregated molecules of M_r in the range 60000–120000 at pH 7.0 or above. However, at acidic pH or in 8 M-urea-containing buffer the latter two proteins were eluted at a position corresponding to the M_r region 17000–20000. The apparent M_r (15000–16500) determined for these proteins by SDS/polyacrylamide-gel electrophoresis or gel filtration is higher than the calculated value, which is 12774–13403 by structure analysis (Esch *et al.*, 1983a; Seidah *et al.*, 1987). Such abnormal behaviour could be due to the presence of carbohydrate in BSP-A₁ and BSP-A₂.

The results of the present study indicate that BSP-A₁ and BSP-A₂ are glycoproteins containing galactosamine

and sialic acid. Neutral sugars were present only in BSP-A₁. Further, BSP-A₁ contained higher amounts of carbohydrate as compared with BSP-A₂. In contrast, BSP-A₃ did not contain any covalently bound carbohydrate. Esch *et al.* (1983a) have not reported the presence of any sugars in PDC-109 or BSP I. Thus ours is the first report to indicate the presence of carbohydrate in these proteins. As glucosamine was absent from both BSP-A₁ and BSP-A₂, it is reasonable to speculate that the carbohydrate is linked to the protein in the form of O-glycosidic linkages, probably involving galactosamine with hydroxy groups of threonine/serine residues, as in mucins (Kornfeld & Kornfeld, 1976).

The two peptides BSP-A₁ and BSP-A₂ are identical in amino acid composition but they have different retention times during elution on h.p.l.c. Esch *et al.* (1983a) also obtained similar results with BSP II and BSP III. Whereas they believe that the methionine residues in native BSP II were oxidized to methionine sulfoxide during purification to give rise to BSP III, we believe that the phenomenon is due to different amounts of carbohydrate. BSP-A₁ contained more carbohydrate and therefore was eluted earlier during the h.p.l.c. than was BSP-A₂, which contained less carbohydrate.

It is noteworthy that all three peptides have same N-terminal aspartic acid and same C-terminal cysteine. The amino acid composition of BSP-A₃ is quite different from that of BSP-A₁ (or BSP-A₂), and consequently BSP-A₃ cannot be a product of post-translational processing of BSP-A₁ (or BSP-A₂). This is further substantiated by our immunochemical studies (Fig. 6). Anti-BSP-A₁ serum did not cross-react with BSP-A₃. Similarly, anti-BSP-A₃ serum cross-reacted neither with BSP-A₁ nor with BSP-A₂. Thus antigenic determinants for BSP-A₃ are different from those of BSP-A₁ or BSP-A₂. In addition, BSP-A₃ retained full activity even after 6 h treatment with trypsin, whereas BSP-A₁ and BSP-A₂ lost their activity within 30–60 min. This is another major difference between BSP-A₃ and BSP-A₁ (or BSP-A₂).

In conclusion, two of the proteins, namely BSP-A₁ and BSP-A₂, purified from bovine seminal plasma on the basis of their ability to stimulate release of gonadotropins by rat anterior-pituitary-cell cultures are identical and are same as gonadostatin PDC-109 (Esch *et al.*, 1983a). These two proteins differ in degree of glycosylation. The third member of this family of proteins, namely BSP-A₃, which is the same as gonadostatin BSP I (Esch *et al.*, 1983a), is not glycosylated, and differs in terms of elution characteristics, amino acid composition, stability towards trypsin treatment and antigenicity as compared with BSP-A₁ (or BSP-A₂). All these results therefore indicated structural dissimilarity between BSP-A₃ and BSP-A₁ (or BSP-A₂). A subsequent paper (Seidah *et al.*, 1987) describes the molecular details of such differences between BSP-A₃ and BSP-A₁ (or BSP-A₂).

The technical assistance of Uma J. and Carole Chagnon-Labelle is appreciated. We thank Dr. P. Lamothe, Faculty of Veterinary Medicine, University of Montreal (St.-Hyacinthe, Quebec, Canada), for supply of bull seminal plasma and Ms. Francine De Coste for secretarial assistance. This work was supported in part by grants from the Fonds de la Recherche en Santé du Québec, by the Medical Research Council of Canada and by the Mellon Foundation (New York, NY, U.S.A.). P. M. is a Research Scholar of the Fonds de la Recherche en Santé du Québec.

REFERENCES

- Akiyama, K., Yoshioka, Y., Schmid, K., Ofner, G. D., Troxler, R. F., Tsuda, R. & Hara, M. (1985) *Biochim. Biophys. Acta* **829**, 288–294
- Chen, C., Schilling, K., Hiipakka, R. A., Huang, I.-Y. & Liao, S. (1982) *J. Biol. Chem.* **257**, 116–121
- Esch, F. S., Ling, N. C., Bohlen, P., Ying, S. Y. & Guillemin, R. (1983a) *Biochem. Biophys. Res. Commun.* **113**, 861–867
- Esch, F., Ling, N., Ying, S. Y. & Guillemin, R. (1983b) in *Role of Peptides and Proteins in Control of Reproduction* (McCann, S. M. & Dhindsa, D. M., eds.), pp. 275–290, Elsevier Science Publishing Co., Amsterdam
- Fauconnet, M. & Rochemont, J. (1978) *Anal. Biochem.* **91**, 403–409
- Franchimont, P., Chari, S., Hagelstein, M. T. & Duraiswami (1975) *Nature (London)* **257**, 402–404
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Hammond, K. S. & Papermaster, D. S. (1976) *Anal. Biochem.* **74**, 292–297
- Hayashi, R. (1977) *Methods Enzymol.* **47**, 84–93
- Heyns, W., Peeters, B., Mous, J., Rombauts, W. & De Moor, P. (1978) *Eur. J. Biochem.* **89**, 181–186
- Isaacs, W. B. & Shaper, J. H. (1983) *J. Biol. Chem.* **258**, 6610–6615
- Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* **45**, 217–237
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lea, O. A., Petrusz, P. & French, F. S. (1979) *J. Biol. Chem.* **254**, 6196–6202
- Li, C. H., Hammond, R. G., Jr., Ramasharma, K. & Chung, D. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4041–4044
- Manjunath, P. (1984a) *J. Steroid Biochem.* **20**, Abstr. I5
- Manjunath, P. (1984b) in *Gonadal Proteins and Peptides and their Biological Significance* (Sairam, M. R. & Atkinson, L. E., eds.), pp. 49–61, World Scientific Publishing Co., Singapore
- Manjunath, P. & Sairam, M. R. (1982) *J. Biol. Chem.* **257**, 7109–7115
- Manjunath, P. & Sairam, M. R. (1985) *Abstr. Int. Congr. Biochem. Abstr. TH-197*
- Mann, T. (1975) *Handb. Physiol. Sect. 7: Endocrinol.* **5**, 461–471
- Matsubara, H. & Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun.* **35**, 175–181
- McKelvy, J. F. & Lee, Y. C. (1969) *Arch. Biochem. Biophys.* **132**, 99–110
- Ostrowski, M. C., Kistler, M. K. & Kistler, W. S. (1979) *J. Biol. Chem.* **254**, 383–390
- Sairam, M. R., Ranganathan, M. R., Ramasharma, K. & Lamothe, P. (1981) *Mol. Cell. Endocrinol.* **22**, 231–250
- Seidah, N. G., Ramasharma, K., Sairam, M. R. & Chrétien, M. (1984a) *FEBS Lett.* **167**, 98–102
- Seidah, N. G., Arbatti, N. J., Rochemont, J., Sheth, A. R. & Chrétien, M. (1984b) *FEBS Lett.* **175**, 349–355
- Seidah, N. G., Manjunath, P., Rochemont, J., Sairam, M. R. & Chrétien, M. (1987) *Biochem. J.*, in the press
- Wilson, E. M. & French, F. S. (1980) *J. Biol. Chem.* **255**, 10946–10953
- Yu-Ching, E. P., Silverberg, A. B., Harris, S. E. & Li, S. S. L. (1980) *Int. J. Pept. Protein Res.* **16**, 143–146

Received 1 April 1986/18 August 1986; accepted 30 September 1986