ISOLATION AND SOME CHEMICAL PROPERTIES OF ASPERMATOGENIC SUBSTANCE FROM BULL SEMINAL VESICLE FLUID

J. DOSTÁL AND J. MATOUŠEK

*Laboratory of Animal Genetics, The Czechoslovak Academy of Sciences,
Liběchov, Czechoslovakia*

(Received 22nd March 1972)

Summary. Aspermatogenic substance (AS) was isolated from bull seminal vesicle fluid by precipitation with acetic acid and ammonium sulphate, and subjected to CM Sephadex C-50 and Sephadex G-100 column chromatography. The molecular weight of the monomer of AS was 22,000 and of the dimer was 44,000 as determined by gel filtration. The purity of the AS was controlled by disc electrophoresis in acrylamide gel, starch-gel electrophoresis, immunoelectrophoresis in agar gel, by peak homogeneity at the last step of separation and by the ultra-centrifugation pattern. The N-terminal amino acid was lysine. The absorbance of 0.1% AS at 280 nm in pH 5.5 buffer was 0.510. The s value obtained for 0.5% AS solution was 2.7.

INTRODUCTION

In 1966, Matoušek reported impairment of the spermatogenic process in rams and rabbits following intramuscular and subcutaneous administration of bull seminal vesicle fluid. Considerable degenerative changes were recorded in the testes of guinea-pigs, rabbits and rams following a single intratesticular injection of this fluid without adjuvant: the index weight of the testes decreased, there was disturbance of spermatogenesis and, in more severe cases, a distortion and contraction of the seminiferous tubules (Matoušek, 1969). Antibodies produced by animals which were immunized subcutaneously did not cause sperm-agglutination, immobilization or spermatotoxic reactions. No circulating antibodies could be found in guinea-pigs and rabbits which had been immunized by a single intratesticular injection.

An inhibition effect of bull seminal vesicle fluid affecting the fertility of female mice and hens was observed by Matoušek & Petrovská (1969). The first attempt to characterize an aspermatogenic substance in the seminal vesicle fluid of bulls was carried out by Matoušek, Staněk & Veselský (1972) and its antiembryonic effect on mice was recorded by Matoušek (1973).

Purification of the substance from bull seminal vesicle fluid which caused aspermatogenesis in the males of some species and embryonic mortality in female mice was previously described by Dostál & Matoušek (1972).

The present report deals with the isolation and properties of aspermatogenic substance (AS) from bull seminal vesicle fluid.

MATERIALS AND METHODS

Collection of bull seminal vesicle fluid

Bull seminal vesicles were obtained from mature slaughtered bulls. The seminal vesicle fluid was collected as described by Matoušek (1969).

Isolation of bull seminal vesicle aspermatogenic substance

In a method devised for the isolation of AS from bull seminal vesicle fluid, 1 vol. of the bull seminal vesicle fluid was diluted by 2.5 vol. of 2% acetic acid. The protein precipitate was removed by centrifugation and the supernatant was adjusted with solid ammonium sulphate to 3 M-concentration. It was then dialysed against water and freeze-dried. The protein material obtained (BS) was further purified on a CM Sephadex C-50 column in 0.05 M-sodium phosphate buffer, pH 8.0, using a linear gradient of 0.1 to 0.5 M-NaCl. The major aspermatogenic activity peak (AS_{CM}) was liberated at a concentration of 0.36 M-NaCl. This fraction was applied on a column of Sephadex G-100 equilibrated with 0.1 M-tris-hydrochloric acid buffer, pH 7.5.

Electrophoresis

Disc electrophoresis (Ornstein, 1964) constituted a control technique for the separation procedures and was carried out on an apparatus devised by Davis (1964). For the separation of 0.3 mg protein, the standard conditions for electrophoresis at pH 9.4 and 4.3 were chosen according to instructions provided by Canalco (U.S.A.).

Starch-gel electrophoresis was performed using 27 g hydrolysed potato starch, 32 g urea and 100 ml buffer, pH 1.7, according to the method described by Aschaffenburg (1966).

The immunoelectrophoretic method described by Scheidegger (1955) was used with tris-boric acid-EDTA buffer, pH 8.9, in a 1% solution of Bacto agar Difco.

Preparation of antiserum

Four rabbits were immunized over a period of 3 weeks by twice weekly subcutaneous or intramuscular injections into the cervical region of the dialysed and freeze-dried supernatant (BS) obtained by treatment of bull seminal vesicle fluid with 2% acetic acid and 3 M-ammonium sulphate. The amount per injection was 10 mg in 1 ml 0.9% NaCl. Blood was collected from the animals 7 days after the last injection.

Molecular weight determination

The molecular weight of AS was estimated by the gel filtration method (Andrews, 1964) on Sephadex G-75 in 0.1 M-citric acid-sodium phosphate, pH 3.5, in 0.1 M-sodium acetate-acetic acid, pH 5.5, in 0.1 M-tris-hydrochloric acid, pH 7.6, in 0.05 M-boric acid-0.05 M-potassium hydroxide-0.025 M-

sodium hydroxide, pH 9.1, and in 0.05% ammonium hydroxide, pH 10.5; and on Sephadex G-100 in 0.1 M-sodium acetate-acetic acid-8 M-urea, pH 5.5. As reference proteins, ovalbumin, chymotrypsinogen and ribonuclease (Koch-Light Laboratories Ltd, England) were used.

Oxidation cleavage

The oxidation of AS was carried out at 0° C with performic acid according to the method of Keil & Šormová (1959). At the end of the reaction, the performic acid was removed by freeze-drying after the addition of water. Oxidized samples of AS are designated AS-O.

Reduction cleavage

Reduction of disulphide bridges in AS was performed using β -mercaptoethanol in 8 M-urea at laboratory temperature according to the method described by Needleman (1970). Reduced samples of AS are designated AS-R.

Amino acid analysis

Samples of 20 mg AS and of 20 mg AS-O were subjected to acid hydrolysis in 5.7 N-HCl at 105° C for 24 hr. The amino acid analysis was performed on a Mikrotechna Praha AAA Analyzer. The results of four analyses were averaged and the extrapolated values were used for calculation of residue numbers.

Tryptophane was estimated using a colorimetric procedure with *p*-dimethylaminobenzaldehyde (DAB), as described by Needleman (1970).

Thiol-group estimation was performed using the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) method, as described by Needleman (1970).

The N-terminal amino acid determination

The modified method of Sanger (1952) was used for the determination of N-terminal amino acid, dissolving 20 mg AS in 1 ml 1% sodium bicarbonate and adding 2 ml 2% 2,4-dinitrofluorobenzene (DNFB) in ethanol. The reaction mixture was stirred at laboratory temperature in the dark for 3 hr. The addition of a few drops of HCl stopped the reaction. Excessive DNFB was extracted with ether and the sample was freeze-dried. Dry DNP-protein was hydrolysed in 5.7 N-HCl for 16 hr at 105° C in nitrogen. The hydrolysed sample was diluted twice with water and DNP-amino acid was extracted by ether. The ether phase was evaporated in a water bath and the DNP-amino acid was compared with the standards on silica gel sheets ('Silufol', Kavalier, Sávava, Czechoslovakia) in toluene:chloroethanol:pyridine:0.8 M-ammonium hydroxide (50:30:15:30) and 1.5 M-phosphate buffer, pH 6.0.

To check the results, the extracted DNP-amino acid was hydrolysed in ammonium hydroxide at 105° C for 14 hr in nitrogen, and free N-terminal amino acid was determined by paper chromatography in butanol:acetic acid:water (4:1:5).

Absorption spectrum analysis

The samples of 0.1% AS in 0.1 N-NaOH, in sodium acetate-acetic acid buffer, pH 5.5, ionic strength 0.1, and in 2% acetic acid, were measured in an Opton spectrophotometer at 220 to 320 nm.

Ultracentrifugation

Sedimentation analysis of a solution of AS in 0.1 M-sodium acetate buffer, pH 5.5, was performed on a Spinco model E apparatus. The frames were observed at 8-min intervals after reaching 56,100 rev/min (26,500 g). The s value was calculated for the 0.5% protein solution.

TEXT-FIG. 1. Disc electrophoresis in acrylamide gel at pH 9.4 and 4.3 of bull seminal vesicle fluid (SVF), the supernatant fraction (BS) after precipitation of SVF with acetic acid and ammonium sulphate, the aspermatogenic fraction after CM Sephadex C-50 column chromatography (AS_{Cm}) and of the finally purified fraction after Sephadex G-100 column chromatography (AS).

Heat precipitation

Buffers of pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and ionic strength 0.1 were prepared according to the method of Miller & Golder (1950). Five-ml samples of 0.5% AS in the prepared buffers were heated in a water bath while being constantly stirred. The temperature at which protein precipitation occurred was measured.

Aspermatogenic activity test

The test for aspermatogenic activity was described by Dostál & Matoušek

(1972). This involved the injection of 0.02 ml of the dialysed and freeze-dried material (10 mg/ml 0.9% NaCl) into the left testes of four males. On the 10th day after the injection, the mice were killed and both their testes were weighed. The right testes were used as controls. The degree of testicular damage was determined histologically (Matoušek, 1969).

RESULTS

The active fraction of purified bull seminal vesicle fluid was shown to be free of contaminating protein on disc electrophoresis (Text-fig. 1), starch-gel electrophoresis (Text-fig. 2) and immunoelectrophoresis. The isolation procedure and

TEXT-FIG. 2. Starch-gel electrophoresis of bull seminal vesicle fluid and its fractions. For abbreviations, see the legend to Text-fig. 1.

chromatographic homogeneity of the AS peak on Sephadex G-100 are illustrated in Text-fig. 3.

The molecular weight of AS was estimated by gel filtration as approximately 22,000 for the monomer and 44,000 for the dimer (Text-fig. 4).

Lysine (di-DNP-lysine) was determined as the N-terminal amino acid of AS by means of thin-layer chromatography.

After splitting of extracted DNP-amino acid, three spots only were detected on paper chromatography. The spots were designated lysine, di-DNP-lysine and DNFB.

The results of AS amino acid analysis are given in Table 1. No tryptophane

TEXT-FIG. 3. The isolation procedure for aspermatogenic substance (AS) from bull seminal vesicle fluid.

residue was found by the DAB method and absorption analysis of AS solution in 0.1 N-NaOH. The half-cystine content was calculated from the value for cysteic acid in AS-O samples and agreed well with the value for half-cystine in native AS samples. The contents of basic, dicarboxylic and sulphur-containing amino acids were about 18.1%, 19.1% and 11.0% of the total residues, respec-

TEXT-FIG. 4. Molecular weight determination of bull seminal vesicle fluid aspermatogenic substance (interrupted lines) on a 1.5 x 35-cm column of Sephadex G-75 equilibrated by buffers of pH 3.5 (a), pH 5.5 (b), pH 7.6 (c), pH 9.1 (d) and pH 10.5 (e); and on a 3 x 50-cm column of Sephadex G-100 in 8 M-urea buffer of pH 5.5 (f). For the composition of the buffers, see text. The reference proteins used were ribonuclease (Ri), chymotrypsinogen (Ch) and ovalbumin (Ov).

tively. The content of aromatic amino acids (tyrosine and phenylalanine) was about 5%. Samples of AS did not contain any thiol groups as determined by the DTNB method.

Absorption spectrum analysis of a 0.1% solution of AS (calculated on dry weight) in sodium acetate-acetic acid buffer, pH 5.5 and ionic strength 0.1, gave an absorption maximum at 278 to 280 nm and an absorption minimum at 254 to 256 nm. The absorbance of this solution measured at 280 nm was 0.510. The shift of the absorption maximum and minimum of the 0.1% solution of AS in 0.1 N-NaOH and the small difference in the absorption curve of the 0.1% solution of AS in 2% acetic acid (Text-fig. 5) confirmed the estimated content of tyrosine residues.

Table 1. Amino acid composition of bull seminal vesicle fluid aspermatogenic substance

<i>Amino acid</i>	<i>nmol</i>	<i>Mean no. of residues calculated on 22,000 mol. wt</i>	<i>Nearest integer</i>
Lysine	86.6	25.04	25
Histidine	18.0	5.20	5
Arginine	21.7	6.27	6
Aspartic acid	62.7	18.12	18
Threonine	44.9	12.97	13
Serine	77.4	22.37	22
Glutamic acid	67.5	19.51	20
Proline	28.8	8.32	8
Glycine	40.4	11.68	12
Alanine	50.1	14.48	14
Half-cystine*	55.7	16.10	16
Valine	57.0	16.47	16
Methionine	22.0	6.36	6
Isoleucine	14.7	4.25	4
Leucine	14.9	4.31	4
Tyrosine	17.3	5.00	5
Phenylalanine	18.8	5.43	5
Tryptophane†	0	0	0
Total residues			199

* Determined as cysteic acid and as half-cystine.

† Determined by colorimetric method.

Heat precipitation of AS was found to be pH dependent. The results of this experiment are shown in Table 2. In samples of AS heated to 100° C for 120 min at pH 4.0, the aspermatogenic activity remained unchanged. No aspermatogenic activity was observed after oxidation and reduction cleavage. Electrophoresis of AS-O and AS-R samples (Text-fig. 6) demonstrated a change of total molecular charge. The ultracentrifugation patterns of the 0.5% AS solution in the pH 5.5 buffer showing a homogeneous peak ($s = 2.7$), and the electrophoresis of AS-O and AS-R confirmed the purity of the isolated AS.

When purified AS was prepared, the concentration of AS/ml 0.9% NaCl was gradually lowered from 10 mg to 1 mg in order to find the minimal amount of AS which would cause a 50% decrease in the weight of mouse testes and arrest spermatogenesis within 10 days of the injection. It was found that such an

TEXT-FIG. 5. The absorption spectrum of 0.1 % bull seminal vesicle fluid aspermatogenic substance in 0.1 N-NaOH (OH), in sodium acetate-acetic acid buffer, pH 5.5, ionic strength 0.1, and in 2% acetic acid (H).

effect could be observed when 0.06 to 0.08 mg AS was injected directly into the testis.

DISCUSSION

As determined on Sephadex G-75 at pH 9.1 and 10.5, and on Sephadex G-100 in 8 M-urea buffer at pH 5.5, the molecular weight of AS was shown to be about

Table 2. Heat precipitation of bull seminal vesicle fluid aspermatogenic substance

<i>pH of buffers*</i>	<i>Precipitation at:</i>
4.0	100° C for 120 min, no precipitation
5.0	100° C for 3 min
6.0	Undissolved
7.0	Undissolved
8.0	86° C
9.0	68° C
10.0	66° C

* The ionic strength of the buffers was 0.1.

22,000. When buffers of pH 3.5 and 5.5 without urea were used, the elution patterns of AS corresponded to a molecular weight of 44,000. It seems that the monomer AS (22,000) associates to the dimer (44,000) under certain conditions.

To support this result, the performic acid-oxidized sample (AS-O) was used for the study of its elution behaviour. The elution patterns of AS-O corresponded to a molecular weight of 27,000. While it is clear that the molecular weight of the AS monomer is about 22,000, the dimer molecule is held together

TEXT-FIG. 6. Disc electrophoresis in polyacrylamide gel at pH 9.4 and 4.3 of bull seminal vesicle fluid aspermatogenic substance (AS), performic acid-oxidized aspermatogenic substance (AS-O), and reduced aspermatogenic substance (AS-R).

by relatively weak forces which can easily be dissociated by a change of pH. At pH 7.6, incomplete dissociation probably occurs and the AS behaves as a protein with a molecular weight of 29,000.

The absence of thiol groups in AS, the finding that lysine is the one N-terminal amino acid and the small difference between the molecular weights of AS and AS-O indicates the presence of intrasubunit disulphides only. One polypeptide chain in this case is equal to one AS subunit and to AS monomer.

The relatively low absorbance value of 0.510 for a 0.1% AS solution at pH 5.5 estimated at 280 nm seems to agree with the presence of five tyrosine residues,

five phenylalanine residues and no tryptophane in AS. Since phenylalanine has a low molar extinction and the maximum of absorption is shifted to a lower wave-length (257.5 nm), the absorbance of AS at 280 nm is due to tyrosine only. The shift of the absorption curve of the 0.1% AS solution in 0.1 N-NaOH confirms the results.

Total loss of aspermatogenic activity can be observed after oxidation or reduction cleavage of AS. This finding demonstrates a considerable influence of such chemical modifications on the activity of the AS molecule. Both modifications changed the total molecular charge as shown by alterations in the electrophoretic mobilities of AS-O and AS-R in acrylamide-disc electrophoresis (Text-fig. 6). A large change in the total molecular charge of AS-O is in agreement with the observation of a highly positive charge for native AS.

The aspermatogenic activity and electrophoretic mobility of AS heated to 100° C for 120 min at pH 4.0 both remain unchanged, results which may be taken to demonstrate the stability of the AS molecule.

ACKNOWLEDGMENTS

The authors wish to thank Miss Libuše Zemanová and Miss Jaroslava Janatková for technical collaboration. The authors are indebted to Dr Č. Kysilka for his kind advice concerning the N-terminal amino acid estimation, thiol-group determination and reduction cleavage. For the amino acid analysis, the authors are grateful to Dr J. Procházka from the Health Institute of the Uranium Industry, Příbram. The authors express their thanks to Mr J. Neuman, Institute of Organic Chemistry and Biochemistry, Prague, for sedimentation analysis. The Opton spectrophotometer was kindly presented by the Alexander von Humboldt foundation.

REFERENCES

- ANDREWS, P. (1964) Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem. J.* **91**, 222.
- ASCHAFENBURG, R. (1966) Modified procedure of starch gel electrophoresis for beta-casein phenotyping. *J. Dairy Sci.* **49**, 1285.
- DAVIS, B. T. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404.
- DOSTÁL, J. & MATOUŠEK, J. (1972) Purification of aspermatogenic substance in bull seminal vesicle fluid. *J. Reprod. Fert.* **31**, 273.
- KEIL, B. & ŠORMOVÁ, Z. (1959) *Laboratorní technika biochemie* (Laboratory manual in biochemistry), Czechoslovak Academy of Sciences Press, Praha.
- MATOUŠEK, J. (1966) *Antigenicity of the seminal vesicle fluid in bulls*. In: Xth Int. Congr. Anim. Blood Grps, Paris, p. 513.
- MATOUŠEK, J. (1969) Effects on spermatogenesis in guinea-pigs, rabbits and sheep after their immunization with sexual organ fluids of bulls. *J. Reprod. Fert.* **19**, 63.
- MATOUŠEK, J. (1973) Antiembryonic effect of bull seminal vesicle fluid in mice. *J. Reprod. Fert.* **32**, 175.
- MATOUŠEK, J. & PETROVSKÁ, E. (1969) Antifertilizing effect of the seminal vesicle fluid of bulls on females. *J. Reprod. Fert.* **20**, 189.
- MATOUŠEK, J., STANĚK, R. & VESELSKÝ, L. (1972) First attempt to characterize an aspermatogenic substance in the seminal vesicle fluids of bulls. *J. Reprod. Fert.* **29**, 179.
- MILLER, G. L. & GOLDBERGER, R. H. (1950) Buffers of pH 2-12 for use in electrophoresis. *Archs Biochem.* **29**, 420.
- NEEDLEMAN, S. B. (1970) *Protein sequence determination*. Springer-Verlag, Berlin, Heidelberg and New York.

- ORNSTEIN, L. (1964) Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.* **121**, 366.
- SANGER, F. (1952) *The arrangement of amino acids in proteins*. In: *Advances in Protein Chemistry*, Vol. 7, p. 2. Eds. M. L. Anson, K. Bailey and J. T. Edsall. Academic Press, New York.
- SCHEIDEGGER, J. (1955) Une micro-méthode de l'immunoélectrophorèse. *Int. Archs Allergy appl. Immun.* **7**, 103.