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Cowper's Gland Secretion in Rat Semen Coagulation

I. Isolation and Amino Acid Analysis of the Seminal Vesicle Substrate

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The seminal vesicle substrate coagulated by an acetone extract of Cowper's gland secretion was isolated by chemical fractionation and DEAE-cellulose column chromatography and defined as the basic protein fraction of seminal vesicle secretion. Secretion from the coagulating gland at pH 7.2 also coagulated this basic protein fraction. Amino acid analysis of the basic protein showed that the molecule is characterized by a high lysine and glutamate content, a low tryptophan and methionine content, and by the absence of proline, hydroxyproline, and cysteine. The molecule has a total of 326 amino acid residues and a minimal molecular weight of 37,905. These data confirm previously published investigations by Mányai *et al.* (1965) on the amino acid composition of the basic protein extracted from the seminal vesicle secretion of the rat. An analysis of the washed coagulum formed by the acetone extract of Cowper's gland secretion yielded an entirely similar amino acid pattern and molecular weight when the clotted protein was compared to substrate. In a series of experiments, the secretion of the coagulating gland was shown to potentiate the coagulating reaction between Cowper's gland secretion extract and the basic substrate from the seminal vesicles. It was concluded that (a) both Cowper's and coagulating gland secretion clot the same seminal vesicle substrate and (b) that this substrate is the basic protein fraction of seminal vesicle secretion.

In the rat, a substrate extracted from the secretion of the seminal vesicles, which coagulates under the action of vesiculase, an enzyme secreted by the coagulating glands, has been isolated and identified chemically as a basic protein (Mányai, 1964). The minimal molecular weight of this protein, based on amino acid analysis, was calculated to be 40,850 (Mányai *et al.*, 1965). The basic protein is characterized by a high lysine and glutamate content, a low tryptophan and methionine content, and by the absence of proline and cysteine. In the guinea pig, the precursor of the seminal clot is a basic protein with a molecular weight of only 17,900 (Notides and Williams-Ashman, 1967). Amino acid analysis of the seminal vesicle substrate of the guinea pig showed that the protein lacked tryrosine, proline, cysteine, hydroxyproline, and hydroxylysine, but, as in the rat, the content of glutamate and lysine was high. Despite these differences in amino acid composition and molecular weight of the seminal vesicle substrate extracted from rats and guinea pigs, the basic protein from either animal is clotted by both homologous and heterologous vesiculase (Mányai, 1964; Hart and Greenstein, 1968).

Coagulating gland secretion not only initiates the coagulation of seminal vesicle fluid but also potentiates the coagulation reaction brought about by Cowper's gland secretion

(Hart, 1969). The potentiating effect observed is thought to result from prior activation of the substrate of seminal vesicle fluid by coagulating gland secretion. However, such activation is not required in order for Cowper's gland secretion to clot seminal vesicle secretion (Hart, 1968; Greenstein and Hart, 1964). Cowper's gland secretion from the rat, mouse, and hamster coagulates homologous or heterologous seminal vesicle secretion. In contrast to the vesicular substrate–vesiculase reaction, however, guinea pig Cowper's gland secretion is inactive and the seminal vesicle secretion of this species is not clotted by heterologous Cowper's gland secretion (Hart and Greenstein, 1968).

Previous studies on rat accessory gland fluid indicated that the seminal vesicle substrate for Cowper's gland secretion differed from that of vesiculase (Hart and Greenstein, 1968). This study was undertaken to isolate and analyze the protein substrate for Cowper's gland secretion and to ascertain whether coagulating gland secretion would clot this protein. The potentiating activity of coagulating gland secretion on this clotting process is also discussed.

MATERIALS AND METHODS

Preparation of Accessory Gland Secretions. The preparation and quantitation of the accessory gland secretions and the *in vitro* technique for measuring seminal vesicle coagulation have been described (Hart and Greenstein, 1968; Hart, 1969). Except where indicated otherwise, all reactions were buffered with phosphate (0.07M) at pH 5.9 and incubated at room temperature.

Extraction of the Active Molecule from Cowper's Gland Secretion. A partial purification of the active molecule in Cowper's gland secretion (pH 5.9, phosphate buffer 0.07M, room temperature) was achieved by the addition of redistilled acetone to a final concentration of 66% (v/v). The precipitate obtained by this method was washed three times with 100% ethanol and dried under an infrared lamp. The powder was then solublized in a phosphate buffer, pH 5.9, and tested for activity. The overall purification obtained by this procedure was 4-fold, and all subsequent studies were carried out with the extract obtained by acetone precipitation (66%, v/v) and washed as described above.

Extraction of Seminal Vesicle Substrate. The extraction of the seminal vesicle substrate was carried out at 4C as described by Mányai (1964) for the isolation of the basic protein fraction with the following modifications. The seminal vesicle secretion was diluted with 2–3 vol of a solution containing 0.15M NaCl + 0.01M borate buffer, pH 8.0, and centrifuged at 4000*g* for 10 min. The pH of the supernatant fluid was adjusted to 9.7 by the addition of an equal volume of 0.1M borate buffer, pH 9.7, and the basic protein fraction was precipitated by the addition of glycerol to a final concentration of 20% (v/v). The solution was stored at 0C for 30 min, followed by centrifugation (4000*g*). The precipitate was washed five times with 0.1M borate buffer, pH 9.7, twice with ethanol (100%), and three times with distilled water. The insoluble protein was then freeze-dried and stored at −20C. Paper electrophoresis (Hart and Greenstein, 1968) of all samples extracted by this method displayed only a single basic protein component (Fig. 1). The fractionation of the basic protein by DEAE-cellulose column chromatography was carried out at 4C as described by Mányai (1964).

Amino Acid Analysis. Samples of basic protein (3–10 mg) isolated from seminal vesicle secretion by the glycerol method were hydrolyzed in 3–10 ml of 6N HCl in sealed tubes under vacuum. The tubes were heated to 110 ± 2C for 22, 48, and 72 hr. The samples were evaporated to dryness on a rotary evaporator and dissolved in citrate buffer at pH 2.8. The hydrolysates were then subjected to automatic amino acid analysis using a Phoenix Precision instrument (model K-800) (Moore *et al.*, 1958). Corrections for hydrolytic losses were made as described by Boyer and Talalay (1966). Tryptophan and amide-N was determined according to the method of Goodwin and Morton (1946) and Bent and Morton (1964), respectively.

RESULTS

Identification of Seminal Vesicle Substrate

Since the procedure for the extraction and purification of the basic protein substrate for vesiculase has been established (see Methods), the ability of Cowper's extract (CE) to coagulate this seminal vesicle fraction was tested first. The addition of CE to a solution of the basic protein resulted in the initiation of a coagulation reaction as judged by the precipitation of substrate (Table 1). On the

other hand, the protein fractions which remained in solution after glycerol precipitation of the basic protein fraction (see Methods) were not clotted by CE (Table 1). In order to determine the purity of the extracted basic protein fraction, the extract was subjected to paper electrophoresis. Figure 1 shows that only one protein fraction could be detected by this technique. As a further test to verify the observations made on extracted seminal vesicle fluid, the secretion from the seminal vesicle was fractionated by DEAE–cellulose column chromatography as described by Mányai (1964). The addition of CE to the various fractions obtained by this method resulted in the formation of a precipitate only in the fraction which ran through the column without being bound to it. This fraction is the basic protein component of seminal vesicle secretion (Mányai, 1964).

The activity of coagulating gland secretion in clotting the basic protein extracted by the glycerol method was likewise determined by mixing the secretion with a solution containing the basic protein fraction (phosphate buffer, pH 7.2, 0.2M urea). The isolated basic protein was immediately clotted by vesiculase.

TABLE 1

EFFECT OF CE AND COAGULATING GLAND SECRETION ON THE COAGULATION OF THE BASIC PROTEIN EXTRACTED FROM THE SEMINAL VESICLES

Secretion incubated	Secretion added after incubation[a]	OD change
Cowper's extract	Basic protein	0.56 ± 0.02[b]
Cowper's extract	Supernatant	0.00
Coagulating gland secretion	Basic protein	0.00
Coagulating gland secretion plus basic protein	Cowper's extract	1.8 ± 0.11

[a] The concentration of the basic protein was 2.5 mg protein per reaction; Cowper's extract concentration was 80 μg protein/reaction, and coagulating secretion concentration was 200 μg protein per reaction. Supernatant refers to the nonglycerol precipitated proteins (see Methods, concentration 20 mg protein per reaction). The final reaction volume was 2.6 ml, and the reactions were buffered with phosphate (0.07 M) at pH 5.9.

[b] Values represent mean ± standard deviation of five experiments conducted on extracted accessory fluid. Coagulating gland secretion was prepared fresh for each experiment.

Amino Acid Analysis of the Basic Protein

The amino acid analysis of the basic protein isolated by the glycerol method is shown in Table 2. This protein is characterized by a high lysine and glutamate content, a low tryptophan, methionine, and isoleucine content, and by the absence of proline, hydroxyproline, and cysteine. Tryptophan was the amino acid present in the lowest concentration. When the molar ratio of each amino acid was calculated and these figures adjusted to the nearest integral value (Table 2) the empirical formula of the basic protein is: Asp_{13}, Thr_8, Ser_{34}, Glu_{71}, Gly_{39}, Ala_{12}, Val_{19}, Met_5, $Ileu_3$, Leu_{25}, Tyr_6, Phe_{26}, Lys_{44}, His_{10}, Arg_{10}, and Trp_1 with a total of 326 residues. The total number of amide residues in the molecule was 65 and the calculated minimal molecular weight of the basic protein was 37,905.

In order to demonstrate more directly that the basic protein is the substrate coagulated by Cowper's extract (CE), the extracted basic fraction was clotted by the addition of CE to a solution containing the basic substrate. The coagulum was centrifuged and washed successively five times with 0.1M borate buffer, pH 9.7, five times with distilled water, and then hydrolyzed. The amino acid analysis of the clotted protein is shown in Table 2. The marked similarity in amino acid composition of the coagulated protein to the basic protein fraction is apparent. The coagulum, like the basic protein, lacked proline,

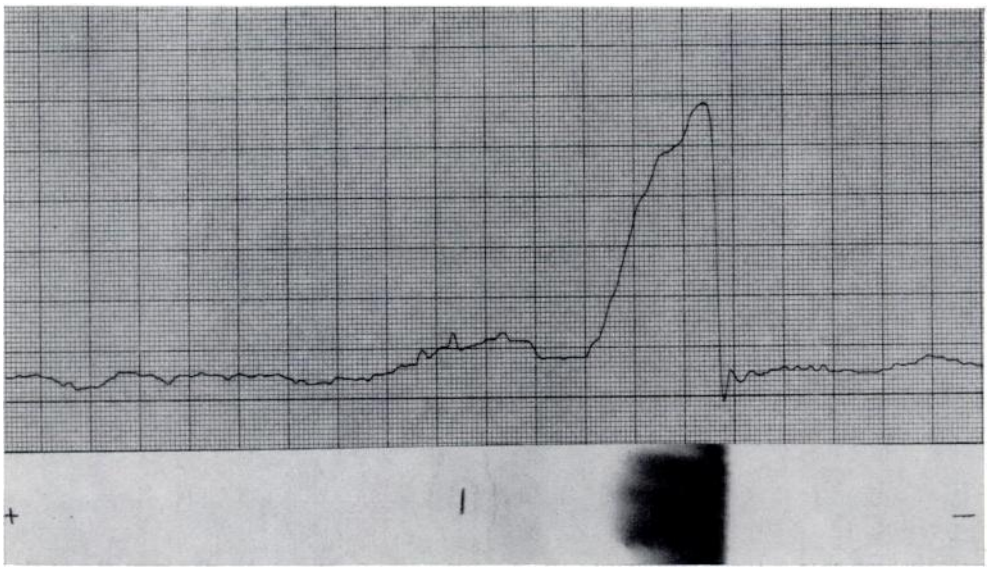

FIG. 1. Paper electrophoresis of the extracted basic protein from the seminal vesicles in a veronal buffer containing 2.5 M urea, pH 8.6, 120 V, 18 hr, stained with bromo-phenol blue and a densitometric trace of the electropherogram. Paper electrophoresis under identical conditions but at pH 10.9 also showed only one protein fraction in the basic protein extract.

hydroxyproline, and cysteine and was rich in the amino acids lysine and glutamate. The minimal molecular weight of the coagulated protein was calculated as 38,127.

It should be noted that the aspartic acid concentration in particular and to a lesser extent the glycine concentration in the coagulum was higher than the concentration of these amino acids in the basic protein. These data suggest that the clotted protein was not washed completely free of CE before analysis. An initial amino acid analysis of CE showed the extract to be high in aspartic acid (490 nmole/mg acetone powder) and glycine (273 nmole/mg acetone powder). The content of these two amino acids plus glutamic acid (215 nmole/mg acetone powder) accounted for 51% of the total number of nmoles of amino acid in the extract.

The Potentiating Activity of Coagulating Gland Secretion on Extracted Substrate

Since coagulating gland secretion has been shown to potentiate the clotting reaction involving Cowper's and seminal vesicle secretion, the potentiating activity of this secretion was tested on the extracted basic protein. Table 1 shows the activity of CE and coagulating gland secretion in clotting the protein fraction. At a reaction pH 5.9 only CE precipitated substrate. When the secretion from the coagulating glands was incubated in a solution containing the extracted basic protein, the amount of coagulation produced upon the addition of CE was much greater than the sum of the coagula produced when CE and coagulating gland secretion were reacted individually with substrate (Table 1). These results are identical to those obtained with crude secretions (Hart, 1969).

An interesting observation that emerged from these studies was that the basic protein substrate after incubation with coagulating gland secretion displayed a distinctly different electrophoretic mobility than the unreacted protein. The reacted substrate in this system remained at the origin, whereas the unreacted protein moved toward the cath-

ode. Whether the observed change in the basic protein, which may represent a polymerization of the molecule, is related to the potentiating effect of coagulating gland secretion on substrate remains to be determined.

DISCUSSION

The similarity of the results reported in this paper on the amino acid composition of the basic protein isolated by the glycerol method from rat seminal vesicle secretion to those published by Mányai *et al.*, (1965) is most striking. The calculated minimal molecular weights of the basic protein are comparable as is the absence of proline, hydroxyproline, and cysteine in the molecule. The fact that the amino acid composition of the clotted basic protein was nearly identical to that of the unreacted protein proves that the clottable substrate for CE is the basic protein and suggests that this molecule is the sole precursor of the coagulum formed by Cowper's gland secretion.

Since the reaction involving CE and substrate does not result in any detectable change in amino acid composition of the clotted basic protein, other than an increase in aspartic acid content, a hydrolytic mechanism of action for CE involving extensive hydrolysis of substrate (greater than 10%) can be ruled out. The possibility that substrate coagulation results from the formation of a polyanion (CE)–basic protein complex which subsequently precipitates is suggested by the basic and acidic nature of substrate and CE, respectively. Amino acid analysis of CE showed that the extract was rich in aspartic and glutamic acid and previous chemical studies reported a high content of sialic acid in Cowper's gland secretion (Hart and Greenstein, 1964; 1968). This interpretation on the mechanism of action of CE is in agreement with kinetic studies which suggested the coagulation reaction was not enzymatic (Hart, 1968). Furthermore, Mányai (1965) has shown that polyanions precipitate the basic protein fraction of rat seminal vesicle secretion.

TABLE 2

AMINO ACID COMPOSITION OF BASIC PROTEIN FROM THE SEMINAL VESICLES BEFORE AND AFTER CLOTTING BY CE

Amino acid residue	Basic protein[a]		Clotted protein[a]
	nmole/mg protein	Nearest integral	nmole/mg protein
Aspartic acid	304	13	485
Threonine	185	8	195
Serine	780	34	701
Glutamic acid	1642	71	1609
Glycine	902	39	1002
Alanine	268	12	254
Valine	440	19	402
Methionine	121	5	115
Isoleucine	80	3	81
Leucine	570	25	585
Tyrosine	134	6	128
Phenylalanine	600	26	635
Lysine	1004	44	940
Histidine	220	10	223
Tryptophan	23	1	23
Arginine	219	10	217
Proline	0	—	0
Cysteine	0	—	0
Hydroxyproline	0	—	0
Amide-N	1499	65	1420

[a] Mean of four separate determinations.

The observation that both CE and vesiculase coagulate the basic protein fraction of seminal vesicle secretion disagrees with data reported by Hart and Greenstein (1968). These authors suggested that the substrate for these two secretions differed. In the above study substrate was not isolated, but rather was judged on the basis of electrophoretic fractionation of seminal vesicle secretion before and after clotting. Obviously this method was not satisfactory for the resolution of the substrate coagulated by Cowper's gland secretion.

The intriguing possibility that arises from the observation that both CE and coagulating gland secretion clot the basic protein fraction of seminal vesicle secretion is that

the potentiating activity of coagulating gland secretion on extracted substrate may be a result of vesiculase acting directly on the basic protein. Present evidence, although far from conclusive, suggests that vesiculase may be a transamidase (Notides and Williams-Ashman, 1967). In blood coagulation, fibrinase, which causes a polymerization of the fibrin molecules, is also a transamidase (Lorand, 1965). Now that the seminal vesicle substrate which participates in semen coagulation has been isolated the speculation that coagulating gland secretion, mediated by vesiculase, may be potentiating the coagulation reaction involving CE by a reaction analogous to the fibrinase system can be tested.

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