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Composition of Salivary Gland Extracts from the Leech *Haementeria ghilianii*[1] (41270)

ANDREI Z. BUDZYNSKI,* STEPHANIE A. OLEXA,* AND ROY T. SAWYER†

**Thrombosis Research Center and Department of Biochemistry, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140, and †Department of Molecular Biology, University of California, Berkeley, California 94720*

Abstract. The giant leech, *Haementeria ghilianii*, originating from French Guyana, has two pairs of salivary glands. From the anterior and posterior glands, approximately 1.5 and 0.3 mg of protein per leech, respectively, have been extracted. Electrophoretic analysis showed that the protein composition of the anterior and posterior gland extracts is completely different. However, both gland extracts contain fibrinolytic activity associated with only one electrophoretic band. The fractionation of salivary gland homogenates by differential ultracentrifugation showed that the fibrinolytic activity is found entirely in the cytosol fraction.

Leeches have been used for bloodletting since antiquity. This form of therapy reached its greatest popularity in Europe in the 19th century. The trade with leeches *Hirudo medicinalis* was enormous (1) and led to the near extinction of the species through its original range from western and southern Europe to the Ural Mountains. The practice of leeching was generally abandoned by the end of the past century. Interest in the physiologically active substances present in leech saliva has recently been renewed. An anticoagulant was discovered which allows the ingested blood to remain liquid in the leech gut (2, 3). The substance, hirudin, was isolated and characterized as an inhibitor of thrombin-catalyzed conversion of fibrinogen to fibrin (4). The recent interest in the mechanism of action of anticoagulants stems from their use as specific tools for the investigation of blood coagulation and potentially as therapeutic agents in thrombotic disorders.

The world's largest known leech species, *Haementeria ghilianii*, which has been reported to reach a length of up to 50 cm, occurs in Brazil and French Guyana (5–7). Live specimens collected in French Guyana have been established as a continuously breeding colony at the University of California, Berkeley (8). We have used animals from this colony to obtain and quantify soluble material from salivary glands of *H. ghilianii*, to characterize the electrophoretic composition of the gland extracts, and to demonstrate the presence of fibrinolytic activity in the extracts.

Materials and Methods. *Reagents.* All reagents were of analytical purity grade. Human fibrinogen (grade L; A. B. Kabi, Stockholm) was used either without purification or after affinity chromatography on a lysine-Sepharose column (Pharmacia, Piscataway, N.J.) in order to remove plasminogen (9). Human α-thrombin was kindly provided by Dr. John W. Fenton, II (New York State, Department of Health, Albany, N.Y.).

Salivary gland extracts. Specimens of *H. ghilianii*, collected in a French Guyana swamp, were bred at the University of California, Berkeley (8). The salivary glands of *H.* ghilianii are located at the proboscis base and encapsulated by a thin membrane (Fig. 1). There are two bilateral pairs of glands: the anterior glands (AG), are large (20 mm long) and contain predominantly large cells (about 0.7 mm in diameter); the posterior glands (PG) are small (5 mm long) and contain mostly very small cells (about 0.01 mm in diameter). The

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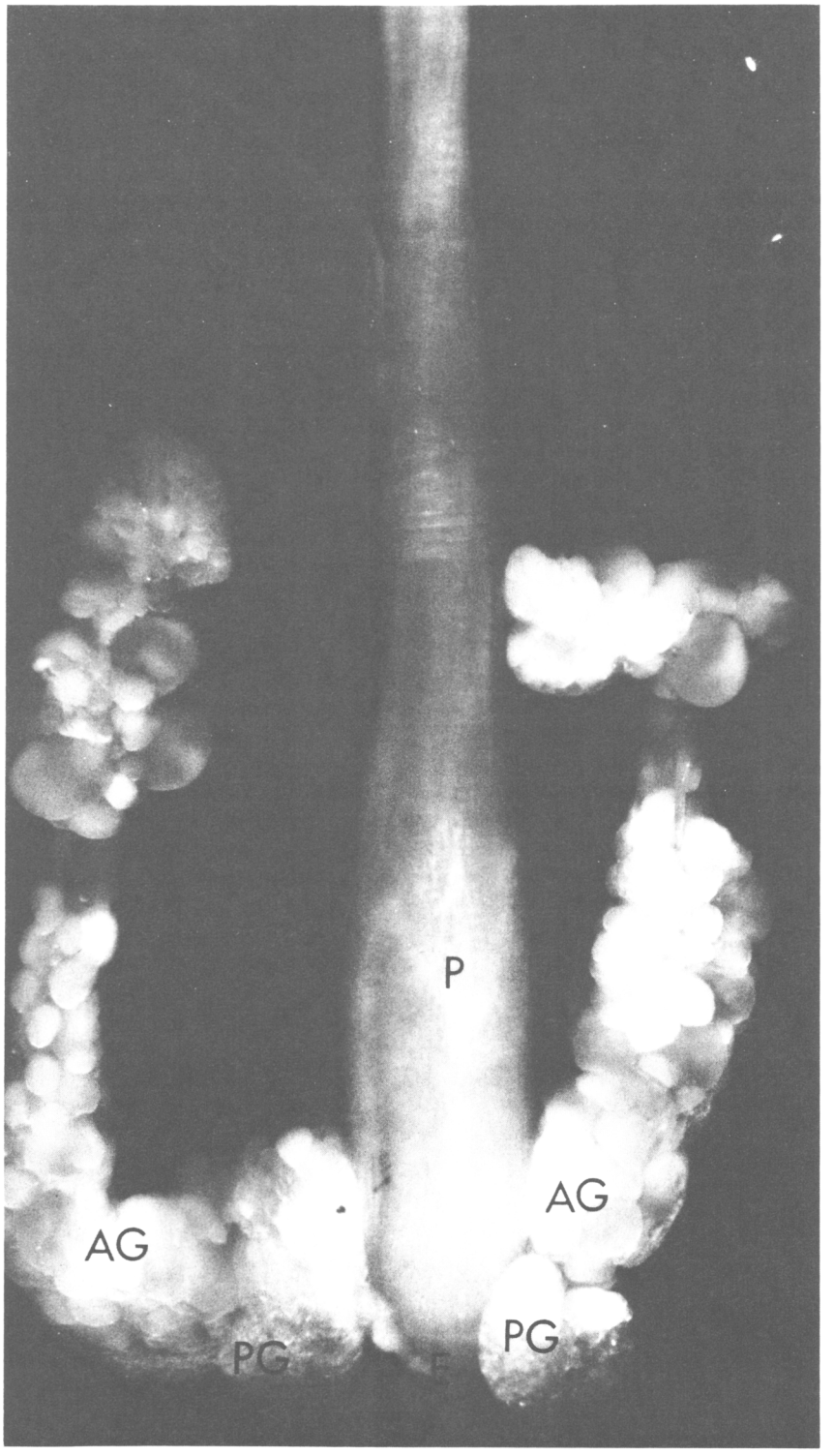

FIG. 1. Dissected salivary glands of *H. ghilianii*. AG, anterior glands; PG, posterior glands; P, proboscis; E, esophagus. (Photograph courtesy, David Weisblat.)

leeches had not been allowed to feed for several months prior to dissection, to increase the concentration of salivary enzymes (10). For the dissection, ether-anesthetized leeches were opened by a longitudinal incision on the dorsal midline, and the anterior and posterior salivary glands were removed separately without any surrounding tissue. The glands were freeze-dried and stored at −80°.

To prepare a salivary extract, the anterior or posterior glands obtained from 10 leeches were placed into 0.8 or 0.3 ml of 0.15 *M* Tris−HCl buffer, pH 7.8, respectively. The tissue was homogenized manually in a Potter−Elvehjem-type Pyrex tissue grinder and centrifuged at 8500*g* for 10 min, and the supernate was collected. The pellets were extracted two more times with the same buffer. The combined supernates constituted the salivary gland extracts and were stored at −80° for up to 6 months without any sign of deterioration.

Protein concentration was determined by three methods: (i) a micromethod utilizing a Biuret reagent (11), (ii) spectrophotometry at 630 nm of amido black-stained TCA[2]-precipitated material (12), and (iii) differential spectrophotometry by measuring the absorbance shift at 595 nm in solution in the presence of Coomassie brilliant blue G-250 (13). All three techniques were standarized with human albumin and fibrinogen.

Fibrin strips were prepared for the determination of fibrinolytic activity. Strips (63 × 26 mm) of cellulose acetate (Gelman Instrument Co., Ann Arbor, Mich.) were immersed in a human fibrinogen solution (1 mg/ml), blotted, immersed in a thrombin solution (0.25 units/ml), blotted, incubated in a humid chamber for 1 hr, and stored in 0.15 *M* Tris−HCl buffer, pH 7.8, in a cold room. Just before the use, a strip was blotted. A 1-μl of the sample was applied and incubated in a humid chamber at 37° for 2 hr. After incubation, the strip was soaked in buffer, rinsed with water, stained with Coomassie brilliant blue R-250 and destained with 10% acetic acid. Lysis of fibrin is shown on the strip by a colorless circle on a blue background. The logarithm of an enzyme concentration in the sample is directly proportional to the logarithm of the square of the circle diameter.

Polyacrylamide gel electrophoresis was carried out in cylindrical tubes (0.5 × 9 cm) in 7% gels containing 0.1% SDS (14). Samples (10 μg) were applied either untreated or after reduction with 3% β-mercaptoethanol at 90° for 5 min. The gels were stained with Coomassie brilliant blue R-250 (15). Plasma fibronectin chains (MW 220,000), fibrin γ-chain dimer (MW 94,000), fibrinogen chains (Aα MW 68,000 Bβ MW 58,000, γ MW 47,000), ovalbumin (MW 43,000), α-chymotrypsinogen (MW 25,700), and lysozyme (MW 14,300) were used as standards of known molecular weight.

Cellulose acetate electrophoresis was carried out in a Microzone apparatus (Beckman, Palo Alto, Calif.). A 0.075 *M* sodium Veronal buffer, pH 8.6, was used. Approximately 5 μg of protein was applied per track and electrophoresed at 250 V and 6 mA for 20 min. The membrane was stained with 0.2% solution of Ponceau S and destained in 5% acetic acid. Dry stained membrane was made transparent in a methanol: acetic acid (3:1) mixture and scanned in an automated densitometer (CDS 100F, Beckman).

Preparative ultracentrifugation was done in a swinging-bucket rotor in a Model L3-50 centrifuge (Beckman).

Results and Discussion. *Salivary gland extracts.* The amount of protein extracted from the anterior and posterior glands obtained from 60 leeches was in good agreement as determined by three different methods (Table I). Approximately 1.5 or 0.3 mg of protein was extracted per leech from the pair of anterior or the pair of posterior glands, respectively. The absorption coefficients at 280 nm, pH 7.8, 1 mg/ml, were 1.27 and 1.43 for the anterior and posterior extracts, respectively. The different values of the coefficients were indicative of the dissimilarity of proteins from the two glands. The large size of *H. ghilianii* (8) assures enough material for purification of substantial amounts of salivary proteins as opposed to a minute quantity recovered from *H. medicinalis* (16).

Composition of the salivary gland extracts. In order to examine whether the

[2] Abbreviations used: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

TABLE I. EXTRACTION OF PROTEINS FROM THE ANTERIOR AND POSTERIOR SALIVARY GLANDS OF *H. ghilianii*[a]

Gland	Extract	Volume (ml)	A_{280}	Protein concentration[b] (mg/ml)				Protein extracted per leech (mg)
				Biuret	Amido black	Coomassie blue	Mean	
Anterior	1	2.0	32.8					
	2	2.2	16.3					
	3	2.3	5.3					
	Total	6.5	17.2	12.1	14.7	13.8	13.5	1.46
Posterior	1	1.0	24.7					
	2	0.8	2.7					
	3	0.6	0.2					
	Total	2.4	11.3	7.28	6.39	10.0	7.89	0.32

[a] The data represent extraction of glands from 60 leeches.
[b] Protein concentration in gland extracts was determined by three different methods (11–13).

anterior and posterior salivary glands are related in their composition, proteins present in the gland extracts were characterized by polyacrylamide gel electrophoresis. The patterns presented in Fig. 2 show that the protein compositions of the anterior and posterior gland extracts are completely different both in nonreduced and reduced conditions. Most of the proteins contain disulfide bonds as inferred from the change in electrophoretic mobility after reduction. The predominant protein in the anterior gland extract has a MW 37,000 and it is affected by reduction. In the posterior gland

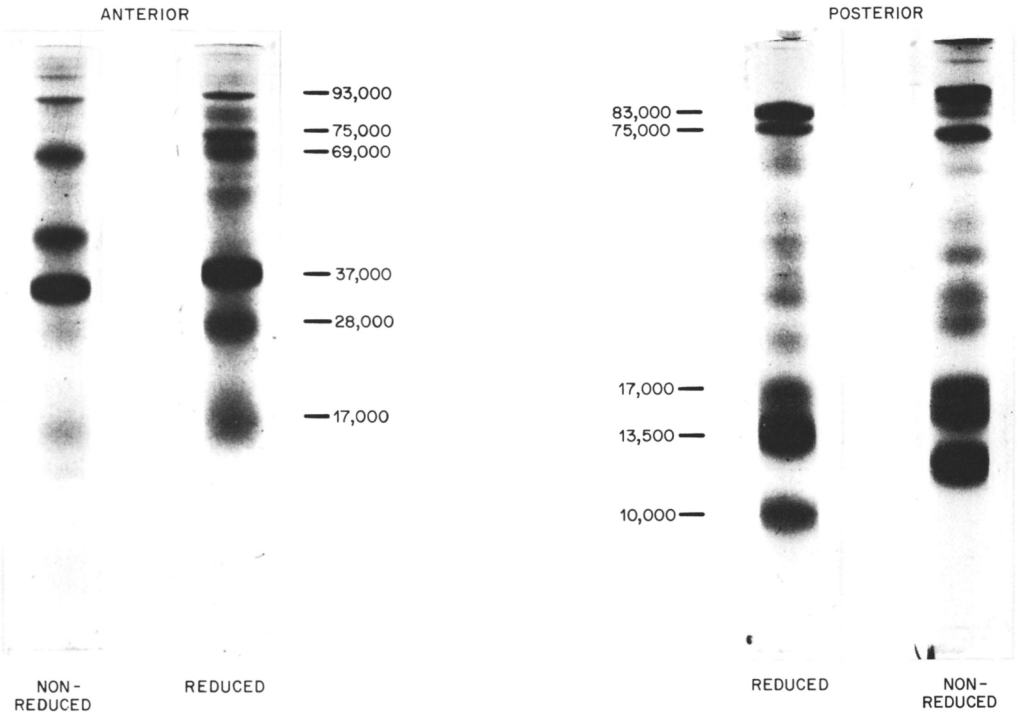

FIG. 2. Protein composition of the anterior and posterior gland extracts. Polyacrylamide gel electrophoretic patterns were obtained under nonreduced (two outer gels) and reduced (two middle gels) conditions. The molecular weights were derived from a calibration line of standard protein markers and are pertinent for the reduced patterns only.

extract, two major species are present with MW 13,500 and 83,000, respectively; both proteins appear to be disulfide bonded.

The extracts were analyzed by electrophoresis on cellulose acetate at pH 8.6 under nondissociating conditions that preserve protein quaternary structure and catalytic activity of enzymes. By this method the protein composition of the anterior and posterior gland extracts was also found to be completely different (Fig. 3A). The anterior gland extract contained a predominant protein with cathodic mobility. The posterior gland extract contained two major bands, one with cathodic and the other with anodic mobility.

It was found that the extracts induced lysis in fibrin strips. The identification of the fibrinolytic activity was done by incubating an unstained cellulose acetate

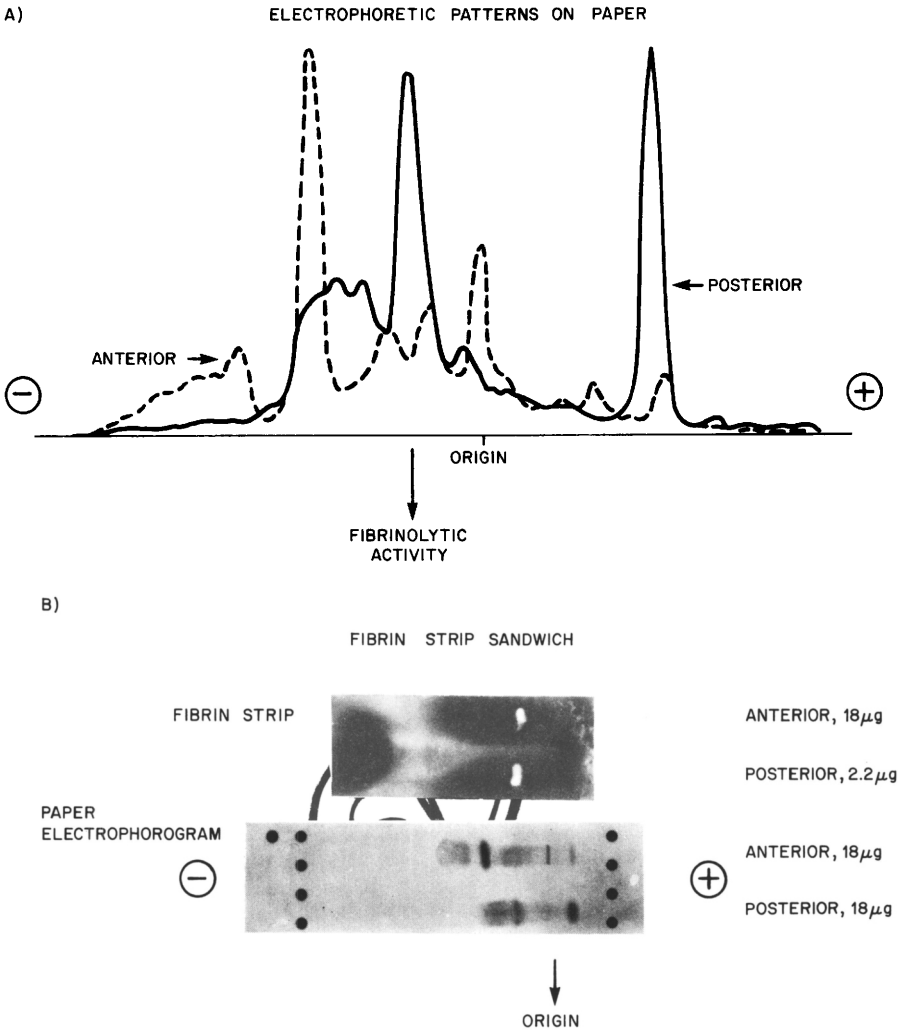

FIG. 3. Paper electrophoresis of the anterior and posterior gland extracts. Cellulose acetate electrophoresis of the extracts was carried out in duplicate in 0.075 *M* sodium Veronal buffer, pH 8.6. After electrophoresis, (A) one specimen was stained, and scanned and the two patterns are shown overlapped with respective origins of sample application. (B) The other, unstained electrophoretic specimen was overlayed with a fibrin strip and incubated at 37° for 2 hr. The fibrin strip was then detached and stained. The positions of the lysis zones indicate the electrophoretic mobility of species with fibrinolytic activity.

TABLE II. ULTRACENTRIFUGAL FRACTIONATION OF HOMOGENATES OF ANTERIOR AND POSTERIOR SALIVARY GLANDS

Sample	Protein		Fibrinolytic activity[a]
	A_{280}/leech	Percentage of initial homogenate	
Homogenate			
Anterior	9.29	100	+
Posterior	0.98	100	+
1000*g* pellet			
Anterior	7.53	81	Trace
Posterior	0.21	21	−
12,000*g* pellet			
Anterior	0.27	3	−
Posterior	0.23	23	−
100,000*g* pellet			
Anterior	0.24	3	−
Posterior	0.11	11	−
Cytosol			
Anterior	1.33	14	+
Posterior	0.56	57	+

[a] Determined on fibrin strips.

electrophorogram overlaid with a fibrin strip (Fig. 3B). Significantly, only one area of lysis was observed having the same electrophoretic mobility in both gland extracts.

To our knowledge, this is the first report demonstrating striking differences in the composition of proteins from the anterior and posterior glands of a leech. It was expected that the two glands with their different biologic functions would contain some different stored enzymes. However, the presence of a similar fibrinolytic activity in both glands was unexpected. The data suggest that the same enzyme may be responsible for this activity.

Ultracentrifugal fractionation of salivary gland extracts. To attempt a first-stage purification of the fibrinolytic activity, homogenates of the anterior and posterior salivary glands were fractionated by differential ultracentrifugation. Four fractions were obtained: a 1000*g* pellet containing particulate matter, a 12,000*g* pellet containing nuclei, granules, mitochondria, and lysozomes, a 100,000*g* pellet containing membrane fragments and ribosomes, and the 100,000*g* supernate containing cytosol. The three pellets were suspended in 0.075 *M* sodium Veronal buffer, pH 8.6. The resuspended pellets, the final supernate, as well as the initial homogenate were tested for protein concentration by the measurement of absorbance at 280 nm. The fibrinolytic activity tested on fibrin strips (Table II) was found exclusively in the cytosol of homogenates of both glands. The fraction of the total protein of the initial homogenate recovered in the cytosol was higher in the posterior gland preparation (57%) than in the anterior gland preparation (14%). That yield was paralleled by the removal of more material in the 1000*g* pellet from the anterior (81%) than from the posterior (21%) gland. Thus, by a one-step differential centrifugation at 100,000*g*, the entire fibrinolytic activity from *H. ghilianii* anterior or posterior salivary glands can be recovered in the soluble fraction.

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