PURIFICATION AND PROPERTIES OF ADRENAL ACID PROTEINASE

PAMELA E. E. TODD AND V. M. TRIKOJUS
Department of Biochemistry, University of Melbourne, Parkville, N.2, Victoria (Australia)
(Received April 13th, 1960)

SUMMARY

The proteinase activities at pH 3.5 (haemoglobin substrate) of extracts of a number of bovine tissues, including endocrine glands, have been compared. All endocrine extracts tested were highly active at this pH.

Extracts of thyroid and of adrenal glands, when tested for proteinase activity (haemoglobin substrate) over a range of pH values, showed two pH optima, one in the acid and one in the alkaline range, thus resembling extracts of pituitary glands[1].

A proteinase optimally active between pH 3.5 and pH 4.0 has been purified approx. 70-fold from extracts of bovine adrenal glands and some of its properties are reported. The enzyme had no action on any of the synthetic peptide substrates tested.

INTRODUCTION

Studies of the proteolytic systems of the endocrine glands have been reported for the pituitary by ADAMS AND SMITH[1] and for the thyroid by MCQUILLAN AND TRIKOJUS[2] and LAVER AND TRIKOJUS[3,4]. Extracts of both glands exhibited proteinase activity optimal between pH 3 and pH 4 and, in addition, pituitary extracts showed a second peak of activity in the alkaline range. In the present investigations a comparison of the acid proteinase content of extracts of beef endocrine glands, muscle and liver revealed a relatively high activity in adrenal tissue. Extracts of adrenal and of thyroid glands, when tested against haemoglobin over a range of pH values, showed the same pattern of activity as found by ADAMS AND SMITH[1] for anterior pituitary extracts. The enzyme active at acid pH, present in adrenal glands, has been purified approx. 70-fold and some of its properties have been studied.

MATERIALS AND METHODS

Bovine glands, liver and muscle were obtained fresh frozen from the abattoirs, and dissected free from fat and connective tissue.

Chemicals used were of A.R. quality. Acetone was freshly distilled over NaOH before use.

Proteolytic activity was assayed by the method of ANSON[5], with minor modifications. One unit of proteinase activity refers to the amount of enzyme required to

liberate 10^{-4} mequiv. tyrosine in 30 min at 37° with haemoglobin as substrate (pH 3.5).

For the determination of the pH-activity curves of the purified proteinase and of saline extracts of adrenal and of thyroid glands, urea-denatured haemoglobin and KUNITZ's[6] modification of ANSON's method was used.

Protein-nitrogen was determined by the micro-Kjeldahl procedure, with selenium as catalyst. Before digestion, samples were submitted to prolonged dialysis at 2°.

Paper chromatography was carried out using No. 1 Whatman filter paper at constant temperature (25°) with butanol–acetic acid–water (4:1:1) as the descending phase. (For benzoyl-L-arginine amide, L-leucine amide and glycyl-L-proline the descending phase was *n*-propanol–water (4:1)). Samples (5 μl) were applied to the paper in a single application using a platinum loop. The chromatograms were allowed to run for 8 or 16 h, then dried and developed with ninhydrin. (Chromatograms involving benzoyl-L-arginine amide were developed using SAKAGUCHI's reagents.)

EXPERIMENTAL

Proteinase activity of beef tissue extracts

The extracts were made by blending the tissue with 0.9% saline (3 ml/g) for 1 min in a Waring blendor at 2°. After the addition of toluol (0.1 ml/100 ml extract) the mixtures were allowed to stand overnight in the cold room and then centrifuged at 3,000 × *g* for 2 h. The supernatants were filtered through cotton wool and dialysed at 2° for 48 h against four changes of distilled water. Duplicate nitrogen and proteinase determinations were carried out on these solutions.

The results are set out in Table I and show that all the extracts tested were active at pH 3.5. The number of units/g of tissue is much lower in muscle than in the other extracts. The concentration of activity in the extracted protein (units/mg N) is highest in extracts of adrenal and ovary.

The proteinase activity of saline extracts of adrenal and thyroid glands over a range of pH values is shown in Fig. 1. For comparison, the curve obtained from extracts of anterior pituitary glands by ADAMS AND SMITH[1] is included. In each case there are two distinct peaks of activity—one between pH 3.5 and pH 4.0, the other between pH 8.5 and pH 9.5.

TABLE I

COMPARISON OF PROTEINASE ACTIVITY OF DIALYSED SALINE EXTRACTS OF BEEF TISSUE

Substrate: acid-denatured haemoglobin. Incubation: 30 min, 37°, pH 3.5.

	Muscle	*Pituitary*		*Thyroid*	*Adrenal*	*Ovary*		*Liver*
		Anterior	*Posterior*			*Total*	*(Corp. lut.)*	
Units*/g tissue wet wt.	22	270	230	300	320	355	(85)	500
Units/mg N in extract	6	41	33	16	57	77	(18)	41
N (mg) extracted/g tissue wet wt.	3.8	6.5	6.9	19	5.6	4.6	(4.6)	12.3

* For definition see MATERIALS AND METHODS.

Purification of the adrenal proteinase optimally active between pH 3.5 and pH 4.0

Initial experiments followed the method adopted by LAVER AND TRIKOJUS[3] for thyroid proteinase in which the bulk of contaminating protein is removed by precipitation at pH 3.5, further purification being effected by fractionation with

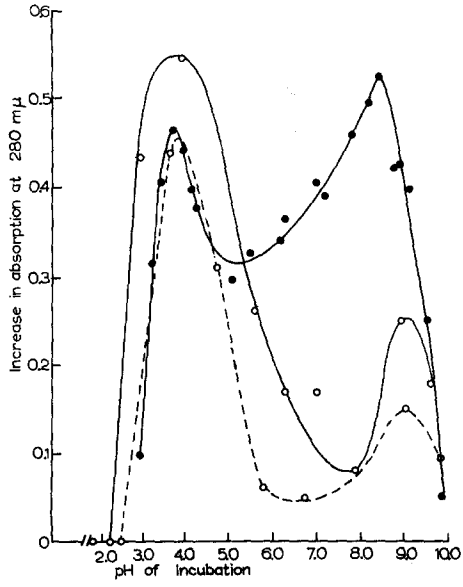

Fig. 1. Variation with pH in proteinase activity of saline extracts of bovine thyroid and adrenal glands. The curve obtained by ADAMS AND SMITH[1] with extracts of hog anterior pituitary glands is included for comparison. Substrate: bovine haemoglobin. Incubation: 30 min, 37°. O—O, adrenal; O- - -O, thyroid; ●—●, pituitary (ADAMS AND SMITH[1]).

acetone. However, with adrenal extracts the final products were only about 0.1 as active as those from thyroid tissue. By including a step involving incubation of the extracts at a pH value within the optimal range of the adrenal enzyme, potency was increased without reduction in the total enzymic activity. Material so treated was also much more susceptible to further purification by acetone fractionation. The procedure adopted was as follows (see also Table II):

Step 1

The dissected glands (1,000 g) were blended at 0° with saline (0.9 %; 3,000 ml) for 1 min in a Waring blendor, toluol (1 ml) was added and the suspension transferred to the cold room overnight (saline extract; Table II). Cold acetone (1,000 ml) was then added slowly with stirring and the mixture centrifuged at 3,000 × *g* (1 h). The addition of acetone increased the number of units extracted (probably due to the removal of fatty material) and it also facilitated centrifugation. (This was also found to be the case in extracting thyroid tissue for acid proteinase). The residue was re-blended for 0.25 min with cold saline (300 ml) plus acetone (to 20 % v/v) and centrifuged. The supernatants were combined to form the saline–acetone extract (Table II).

Step 2

The extract was gently stirred in an ice bath and pre-cooled acetone (below

—10°) gradually added to 80 % (v/v). After 30 min the clear supernatant was siphoned off as far as possible and the remaining suspension rapidly filtered at 2° with light suction. The precipitate was washed with cold 80 % acetone (750 ml) and acetone (2 × 250 ml) and immediately transferred to water (2,000 ml) with hand stirring.

Step 3

The cold slightly turbid solution was brought to pH 4.5 with 2 *M* HCl and allowed to stand for 10 min at 2°. The insoluble material was removed by centrifuging, washed with cold water adjusted to pH 4.5 (250 ml) and discarded.

Step 4

The supernatant and washing were combined and pre-cooled acetone (below —10°) gradually added to 80 % (v/v) as in *Step 2*. After standing for 30 min, the clear supernatant was siphoned off and the remaining suspension centrifuged. The residue was dissolved in water (350 ml) and dialysed. Insoluble material was removed by centrifuging, washed once with water and then discarded.

Step 5

The clear supernatant (including washing: 1,140 ml) from *Step 4* was adjusted to pH 3.9 by the gradual addition of NaAc buffer (0.5 *M*, pH 3.6; 47.5 ml). Toluol (1 ml) was added and the solution was gently shaken in two heat-sterilized stoppered bottles at 37° for 5 h. After standing overnight at 2° the solution was dialysed. In early preparations this solution was freeze dried and then, prior to acetone fractionation, dissolved in NH_4Ac buffer (0.02 *M*, pH 5.0) to give a 1 % solution. However, in subsequent preparations it was found more convenient to avoid freeze drying and to bring the solution to 80 % (v/v) with acetone and dissolve the precipitate in water (300 ml); after removal of the acetone by dialysis the solution was brought to pH 5.0 by the addition of 0.2 times its volume of NH_4Ac buffer (0.1 *M*, pH 5.0). In this way, the recovery of enzyme during the subsequent fractionation procedure was increased.

Acetone fractionation

The buffered solution prepared above was brought successively to 40 %, 55 % and 80 % (v/v) with acetone. The precipitates at each concentration of acetone (Fractions I, II and III respectively) were collected by centrifuging, washed once with the appropriate concentration of acetone, dissolved in water (100 ml, 30 ml and 30 ml respectively) and dialysed. Fractions I and III were freeze dried. Fraction II was brought to pH 5.0 by the addition of 0.1 times its volume of NH_4Ac buffer (0.2 *M*, pH 5.0) and refractionated with acetone. Precipitates were collected at 40 %, 60 % and 80 % acetone (v/v) (Fractions IIa, IIb and IIc respectively), dissolved in water (30 ml), dialysed and freeze dried. From Table II, which shows the recovery and purification during a typical preparation, it is seen that acetone fractionation can lead to appreciable further purification of the enzyme, Fraction IIb having the highest potency and representing a purification factor of 23 and a recovery, based on a saline-acetone extract, of 49 %.

Adsorption on DEAE cellulose

Further purification of this material was effected in the following way (*cf.* ref. 7). A sample of Fraction IIb (400 mg; 300 units/mg) was dissolved in 200 ml

CO_2-free water. DEAE cellulose (Brown Type-20, 2 g) was added and the suspension stirred gently for 15 min at 0° and then centrifuged. The precipitate was washed with 50 ml CO_2-free water. The combined supernatant and washing was freeze dried and found inactive. The precipitate was resuspended in 200 ml CO_2-saturated water and stirred gently for 15 min at 0° in an atmosphere of CO_2. The suspension was centrifuged and the precipitate washed with 100 ml and then with 50 ml CO_2-saturated water. The combined supernatant and washings was freeze dried.

This material (Fraction C1; 168 mg) assayed at 550 units/mg. Fraction C1 was dissolved in water (5 ml) and applied to a column of DEAE cellulose (1.5 cm in diameter, 12 cm high) equilibrated with CO_2-free water. The column was eluted first with CO_2-free water. After 25 × 2 ml fractions had been collected the eluent was changed to NH_4Ac buffer (0.02 *M*, pH 5.0). A main protein peak appeared between tubes 50 and 60. These fractions were pooled, dialysed and freeze dried. This material (Fraction C2; 80 mg) assayed at 880 units/mg.

The overall purification and recovery data are shown in Table II.

pH-Activity curve

Fig. 2 shows that the purified enzyme has only one peak of activity, *i.e.* between pH 3.5 and pH 4.0, when tested with both bovine haemoglobin and bovine serum albumin. Solutions containing four times this concentration of the purified enzyme showed no activity in the alkaline range.

Activators

The effect of incubation with Ba^{++}, Ca^{++}, Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, Zn^{++}, ascorbic acid, cysteine and KCN on the activity of adrenal acid proteinase was tested. The enzyme was pre-incubated for 2 h at pH 3.5 in the presence of 0.01 *M* concentra-

TABLE II

RECOVERY AND PURIFICATION OF ACID PROTEINASE FROM A SALINE–ACETONE EXTRACT OF ADRENAL GLANDS

Stage of purification	*Units*/mg protein*	*Recovery (% of units in saline–acetone extract)*	*Purification factor*
Saline extract	9**	63***	
After Step 1 (saline–acetone extract)	13**	100§	1
After Step 2	12**	92	1
After Step 4	51**	84	3.9
After Step 5	70**	84	5.4
Acetone fractionation			
Fraction II	—	68	—
Fraction IIb	300	49	23
Adsorption on DEAE cellulose			
Fraction C1	550	39	42
Fraction C2	880	30	68

* For definition see MATERIALS AND METHODS.

** Calculated from $\frac{\text{units/mg N.}}{6.25}$

*** Total units in saline extract, 334,000.

§ Total units in saline-acetone extract, 533,000.

tions of the various substances and the solutions then assayed against haemoglobin in the usual way (pH 3.5). None of these substances had any effect on the activity of the enzyme.

The enzyme was also pre-incubated for 15 min at pH 3.5 with and without diisopropylfluorophosphate (DFP; 10^{-4} *M*) and then assayed against haemoglobin. The DFP was without effect.

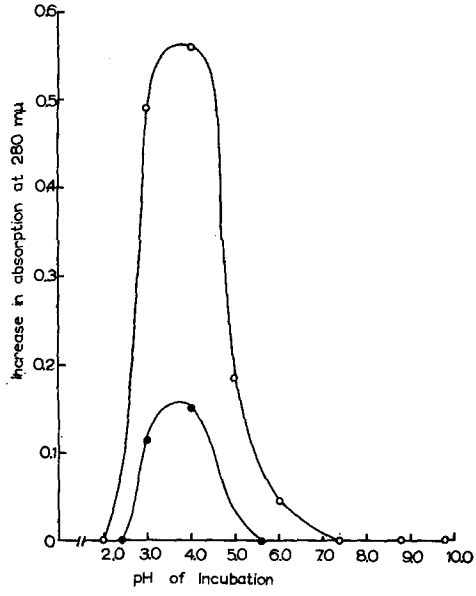

Fig. 2. Variation with pH in proteinase activity of purified adrenal acid proteinase (Fraction C2; Table II). Incubation: 30 min, 37°. O—O, substrate–bovine haemoglobin; ●—●, substrate–bovine serum albumin.

Action on protein substrates

Solutions of purified enzyme containing 20 units/ml were tested against various protein substrates. The substrates (1 g) were dissolved in water, adjusted to pH 1–2 with 2 *M* HCl and allowed to stand for 30 min. The solutions were then adjusted to pH 3.5 with 2 *M* NaOH and made up to 50 ml with water. The activity of the enzyme against these substrates was measured in the usual way. The enzyme was approximately 0.3 as active against bovine serum albumin as against bovine haemoglobin and only about 0.05 as active against human serum albumin. The enzyme apparently had no action on egg albumin, human γ-globulin or porcine thyroglobulin. It should be noted that the method of assay measures only the increase in the number of trichloroacetic acid-soluble fragments produced during hydrolysis which contain those amino acids absorbing at 280 mμ; fragments not absorbing in this region would not have been detected.

Electrophoretic behaviour

Electrophoresis of the purified enzyme on starch gel at pH 8.48 (0.023 *M* borate; 6 V/cm; 4.5 h) revealed the presence of a major band and two minor slower-moving components.

Peptidase activity on various substrates

The action of a solution of purified proteinase on a number of synthetic peptides and peptide derivatives was tested and compared with the action of a dialysed saline-acetone extract (Step 1, Table II). Using bovine haemoglobin as substrate, the solution of the purified enzyme assayed at 360 units/ml and the crude extract at 90 units/ml.

In the first set of experiments (Table III) the substrates were dissolved in Na citrate buffer (0.1 *M*, pH 5.1) to give 0.04 *M* solutions. Incubation tubes contained: (a) 30 μl substrate; 60 μl buffer; 30 μl enzyme. (b) 30 μl substrate; 30 μl buffer; 30 μl cysteine (0.016 *M* in buffer); 30 μl enzyme.

In the second set of experiments (Table IV) the substrates were dissolved in water to give 0.04 *M* solutions. Incubation tubes contained: (a) 30 μl substrate; 30 μl Na citrate buffer (0.1 *M*, pH 5.1); 30 μl $MnSO_4$ (0.004 *M*); 30 μl enzyme. (b) 30 μl substrate; 30 μl Tris buffer (0.1 *M*, pH 7.8); 30 μl $MnSO_4$ (0.004 *M*); 30 μl enzyme. ($MnSO_4$ was replaced by 0.004 *M* $CoCl_2$ in experiments with glycyl–glycine and by buffer in experiments with glycyl–glycyl–glycine.)

The mixtures were incubated at 37° in tightly stoppered tubes for 16 h.

Following incubation in the first experiments 30 μl of buffer was added to tubes (a) and 30 μl of 0.02 *M* N-ethylmaleimide[8] (in buffer) to tubes (b). 30 μl of 0.06 *M* N-ethylmaleimide was added to both tubes (a) and (b) when the substrate contained cysteine. No additions were made in the second set of experiments.

Samples (5 μl) of the mixtures were then chromatographed together with appropriate controls.

As can be seen from Table III the saline–acetone extract hydrolysed all the substrates tested in the first set of experiments in the presence or absence of cysteine. In the second set of experiments (Table IV) it hydrolysed all the substrates at alkaline pH and also hydrolysed L-leucine amide, glycyl–L-proline, glycyl–glycyl–glycine, L-leucyl-L-phenylalanine and L–leucyl–L-tyrosine at pH 5.1. The purified enzyme had no action under any of the conditions on any of the substrates tested.

TABLE III

PEPTIDASE ACTIVITY OF ADRENAL EXTRACTS AND PURIFIED ADRENAL ACID PROTEINASE

Incubation: 16 h, 37°, pH 5.1. (1) (2) (3) Cathepsin A, B, C substrates respectively[9]. (4) Carboxypeptidase substrate[10]. (5) Thyroid "cysteinyltyrosinase" substrates[11]. ++++, 100% hydrolysis; ++, approximately 50% hydrolysis; —, no hydrolysis.

Peptide	*Saline-acetone extract*		*Purified proteinase 880 units/mg*	
	No activator	*0.004 M cysteine*	*No activator*	*0.004 M cysteine*
Carbobenzoxy-L-glutamyl-L-phenylalanine (1)	++++	++++	—	—
Carbobenzoxy-L-glutamyl-L-tyrosine (1)	++	++	—	—
L-cysteinyl-L-tyrosine (5)	++	++	—	—
Carbobenzoxy-glycyl-L-phenylalanine (4)	++	++	—	—
Glycyl-L-phenylalanine amide* (3)	++	++	—	—
L-cysteinyl-L-tyrosine amide** (5)	++	++	—	—
Benzoyl-L-arginine amide (2)	++	++	—	—

* Gave glycyl-L-phenylalanine.
** Gave cysteine and tyrosine amide.

TABLE IV

PEPTIDASE ACTIVITY OF ADRENAL EXTRACTS AND PURIFIED ADRENAL ACID PROTEINASE

Incubation: 16 h, 37°, 0.001 *M* $MnSO_4$. (1), leucine aminopeptidase substrates[12]; (2) tripeptidase substrate[13]. ++++, 100% hydrolysis; ++, approximately 50% hydrolysis; +, slight hydrolysis; —, no hydrolysis.

Peptide	*Saline-acetone extract*		*Purified proteinase 880 units/mg*	
	pH 5.1 (citrate)	*pH 7.8 (Tris)*	*pH 5.1 (citrate)*	*pH 7.8 (Tris)*
L-leucine amide (1)	++	++++	—	—
Glycyl-L-proline	++	++++	—	—
Glycyl-L-phenylalanine	—	++++	—	—
Glycyl-L-tyrosine	—	++++	—	—
Glycyl-L-leucine	—	++++	—	—
Glycyl-glycine*	—	++	—	—
Glycyl-glycyl-glycine** (2)	++	++++	—	—
L-alanyl-L-alanine	—	++++	—	—
L-alanyl-L-leucine (1)	+	++++	—	—
L-leucyl-L-phenylalanine (1)	++	++++	—	—
L-leucyl-L-tyrosine (1)	++	++++	—	—

* $CoCl_2$ replaced $MnSO_4$.
** Buffer replaced $MnSO_4$.

DISCUSSION

A comparison of the proteinase activities at pH 3.5 (haemoglobin substrate) of extracts of endocrine glands, liver and muscle showed that, with the exception of muscle, all the extracts had considerable activity. The acid proteinase activities/mg N of thyroid extracts were comparatively low, although presumably proteolytic enzymes are involved in the release of thyroid hormone from thyroglobulin, its stored form in the gland. In contrast, the values for ovary and for adrenal extracts at this pH were high and of the same order as those for liver. Since the hormones of both adrenal and ovary glands are not protein in nature, nor stored in protein form, it would appear that acid proteinases play a general rather than a specific role in cell metabolism.

The pH-activity curves (haemoglobin substrate) of extracts of thyroid and of adrenal glands resembled those of extracts of anterior pituitary glands[1] in that they each showed two pH optima—at pH 3.5 to pH 4.0 and at pH 8.5 to pH 9.5. Also, the peptidase activities of beef adrenal extracts (see Tables III and IV) were similar to those reported for pituitary extracts[1].

The purified adrenal acid proteinase, like both purified thyroid acid proteinase[2-4,14] and pituitary Proteinase I (see ref. 1), was not activated by metal ions or reducing agents nor did it hydrolyse a wide range of synthetic peptide substrates including those for Cathepsins A, B and C (see Table III). On the other hand, pituitary Proteinase I has been found to hydrolyse human γ-globulin[1], whereas the adrenal acid proteinase had apparently no action on this substrate. Additional studies of the action of the three acid proteinases on purified protein substrates may well reveal other specificity characteristics.

ACKNOWLEDGEMENTS

We are indebted to the Nuffield Foundation for a personal grant to one of us (P.E.E.T.) and for assistance towards the costs of the investigation.

REFERENCES

1 E. ADAMS AND E. L. SMITH, *J. Biol. Chem.*, 191 (1951) 651.
2 M. T. MCQUILLAN AND V. M. TRIKOJUS, *Australian J. Biol. Sci.*, 6 (1953) 617.
3 W. G. LAVER AND V. M. TRIKOJUS, *Biochim. Biophys. Acta*, 16 (1955) 592.
4 W. G. LAVER AND V. M. TRIKOJUS, *Biochim. Biophys. Acta*, 20 (1956) 444.
5 M. L. ANSON, *J. Gen. Physiol.*, 22 (1938) 79.
6 M. KUNITZ, *J. Gen. Physiol.*, 30 (1947) 291.
7 M. A. MITZ AND S. S. YANARI, *J. Am. Chem. Soc.*, 78 (1956) 2649.
8 C. S. HANES, F. J. R. HIRD AND F. A. ISHERWOOD, *Nature*, 166 (1950) 288.
9 H. H. TALLAN, M. E. JONES AND J. S. FRUTON, *J. Biol. Chem.*, 194 (1952) 793.
10 M. L. ANSON, *J. Gen. Physiol.*, 20 (1937) 663.
11 R. E. LOUGHLIN AND V. M. TRIKOJUS, *Biochem. J.*, 74 (1960) 39 P.
12 E. L. SMITH AND D. H. SPACKMAN, *J. Biol. Chem.*, 212 (1955) 271.
13 D. ELLIS AND J. S. FRUTON, *J. Biol. Chem.*, 191 (1951) 153.
14 M. T. MCQUILLAN, unpublished results.