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THE PURIFICATION OF A BOVINE KIDNEY ENZYME WHICH CLEAVES MELANOCYTE-STIMULATING HORMONE-RELEASE INHIBITING FACTOR

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Summary

An enzyme which catalyzes the hydrolysis of L-prolyl-L-leucylglycinamide, the factor which inhibits the release of melanocyte-stimulating hormone, was purified 189-fold from bovine kidney in a 5% yield. The molecular weight of the enzyme on gel filtration was estimated to be 300 000 and its isoelectric point was found to be pH 4.1. The single component seen on sodium dodecyl sulphate-gel electrophoresis was estimated to have a molecular weight of 56 000, indicating that the native enzyme may be a pentamer or hexamer. The enzyme could clearly be distinguished from other prolyl-cleaving enzymes.

Introduction

It has been known for some time that the hypothalamus controls the release of melanocyte-stimulating hormone via secretion of melanocyte-stimulating hormone-release inhibiting factor [1]. The factor responsible for such activity was isolated from bovine hypothalami and its structure was found to be L-prolyl-L-leucylglycinamide [2]. Studies with radioactive-labelled inhibiting factor have shown that it is accumulated and metabolized in a number of tissues, including kidney [3]. This paper reports on the isolation and some of the properties of an enzyme of bovine kidney which cleaves inhibiting factor.

Materials and Methods

Chemicals. All chemicals used were of analytical grade and were obtained from Sigma London Chemical Co. Ltd., Poole, England, or B.D.H. Chemicals Ltd., Poole, England.

Substrates. All substrates were purchased from Sigma London Chemical Co. Ltd., with the exception of L-prolyl-L-leucinamide which was obtained from Vega-fox Biochemicals, Arizona, U.S.A.

Chromatography resins. The gel filtration resin, Ultrogel AcA34, was obtained from LKB Instruments Ltd., South Croydon, England. The microgranular anion-exchange resin, Whatman DE-52, was bought from Whatman, Maidstone, England.

Analytical electrophoresis. Electrophoresis at pH 3.8 and pH 9.4 was carried out on 5% and 10% polyacrylamide gels according to the method of Davis [4]. The gels were stained with Amido Black 10B to detect protein components and were stained for carbohydrate using the method of Clarke [5]. Electrofocusing in pH range 3.5—9.5 was performed on commercial thin-layer gels according to the manufacturer's instructions (LKB Instruments Ltd.). The pH gradient was measured with the aid of an antimony electrode (Activation Glass Ltd., Fife, Scotland). The gels were stained for protein with Coomassie Brilliant Blue R250 following the procedure of Otavsky and Drysdale [6]. Sodium dodecyl sulphate (SDS) gel electrophoresis was performed on 3.3% polyacrylamide gels according to the method of Weber et al. [7] using B.D.H. molecular weight markers (mol. wt. range 53 000—265 000).

Protein concentration. Concentration of protein solutions was carried out by ultrafiltration under nitrogen using Diaflo membranes of the type PM 10 in ultrafiltration cells of various volumes (Amicon Ltd., High Wycombe, England). De-salting and dialysis of fractions was achieved using hollow fibre devices of the type of Bio-Fibre 50 according to the manufacturer's instructions (Bio-Rad Laboratories Ltd., Bromley, England). The concentration of protein in fractions was estimated by the method of Warburg and Christian [8].

Enzyme assays. All enzyme assays were performed at 37°C. Incubations were carried out in 0.05 M Tris-HCl (pH 7.75)/1 mM $MnCl_2$/2 mg/ml bovine serum albumin. Activity towards peptides containing N-terminal proline was determined as detailed by Akrawi and Bailey [9] using the method of Mayer and Nordwig [10]. Activity towards glycyl-L-prolylalanine was measured by the method of Eccleston [11]. In all cases one unit of enzymic activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol substrate per min at 37°C.

Purification procedures. The isolation of the enzyme was carried out at 4°C but the buffers were made at 20°C and therefore all records of pH refer to the latter temperature.

Step 1: Homogenization. Bovine kidneys weighing 300—500 g were removed from freshly slaughtered animals at a local abattoir. Adipose and connective tissue were carefully disected from each kidney. The cortex was cut into small pieces which were thoroughly washed with isotonic saline to remove blood and then dried by pressing between pads of filter paper. 75-g segments of cortex were homogenized in 300 ml 0.05 M Tris-HCl buffer (pH 7.75)/0.25 M sucrose/1 mM $MnCl_2$/1.5 mM succinic acid for 2 min in a Waring blendor. The homogenate was centrifuged at 23 000 × *g* for 3 h 20 min (R_{av} 10.5 cm) in an MSE High Speed 25 centrifuge.

Step 2: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the supernatant to produce 40% saturation. After the precipitate had been removed by centrifugation further $(NH_4)_2SO_4$ was added to the solution to give 70% saturation. The resulting precipitate, after collection by centrifugation, was dissolved in 60 ml 0.05 M Tris-HCl buffer (pH 7.75)/1 mM $MnCl_2$ and 1.5 mM succinic

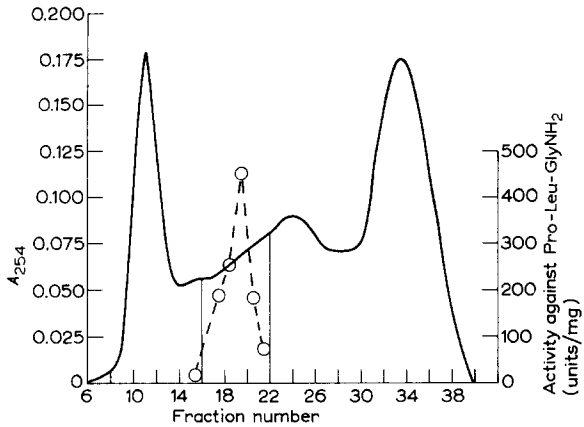

Fig. 1. Gel filtration on Ultrogel AcA34 (step 3). ———, absorbance at 254 nm; o- - - - -o, activity against L-prolyl-L-leucylglycinamide.

acid. The salt was removed by dialysis against the same buffer using the hollow fibre system.

Step 3: Gel filtration on Ultrogel AcA34 resin. The material from step 2 was processed by gel filtration in eight batches. Each batch was performed in an identical manner. 13 ml of the fraction was applied to the column (2.6 X 85 cm) of resin and was eluted by the Tris buffer (flow rate, 24 ml/h; 12-ml fractions). The fractions which were active towards L-prolyl-L-leucylglycinamide were combined as indicated on the representative elution profile (Fig. 1).

The active material from all of the batches was concentrated to 40 ml by ultrafiltration. Gel filtration was also carried out for a series of standard proteins under identical conditions to those used in the separation process in order to estimate the molecular weight of the enzyme by the method of Andrew [12]. The standard proteins were: xanthine oxidase (M_r = 275 000), bovine γ-globulin (M_r = 205 000), aldolase (M_r = 150 000), lactate dehydrogenase (M_r = 130 000), bovine serum albumin (M_r = 67 000).

Step 4: Anion-exchange chromatography on DEAE-cellulose. 10 ml of the

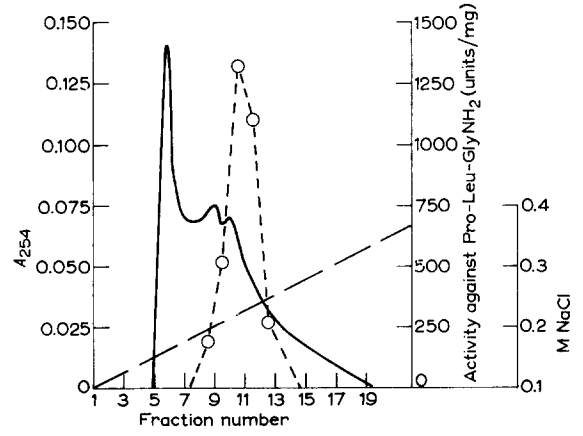

Fig. 2. Anion-exchange chromatography on DEAE-cellulose (step 4). ———, absorbance at 254 nm; o- - - - -o, activity against L-prolyl-L-leucylglycinamide; - - - - - -, NaCl concentration.

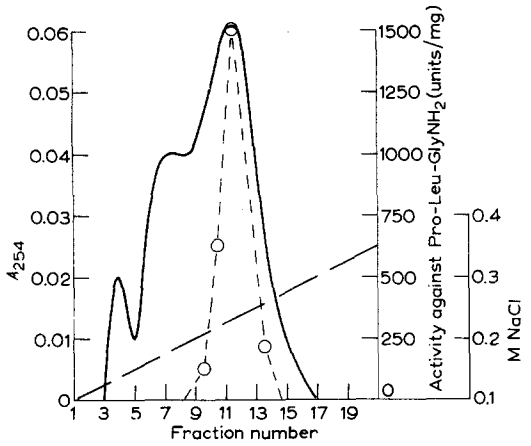

Fig. 3. Re-chromatography on DEAE-cellulose (step 5). ———, absorbance at 254 nm; o- - - - -o, activity against L-prolyl-L-leucylglycinamide; - - - - - -, NaCl concentration.

concentrated sample from step 3 was dialyzed for 20 h against 500 ml 0.005 M Tris-HCl buffer (pH 7.75)/1 mM $MgCl_2$/1.5 mM succinic acid. The dialyzed sample was applied to a column (2.6 X 11 cm) of Whatman DE-52 resin which had been equilibrated against the same buffer. The column was washed with the buffer (flow rate, 30 ml/h; 10-ml fractions); no ultraviolet-absorbing substances were eluted. The absorbed material was removed by application of a linear salt gradient (0.1—0.4 M NaCl in Tris buffer, total volume 200 ml). All the material from step 3 was processed in an identical manner (Fig. 2)

Step 5: Re-chromatography on DEAE-cellulose. The two most active fractions (10 and 11 on Fig. 2) from step 4 were combined and were concentrated to 6 ml by ultrafiltration. The solution was subjected to chromatography on a column (2.6 X 9.5 cm) of Whatman DE-52 resin using identical conditions to those employed in step 4. Fraction 11 was found to have maximum enzymic activity (Fig. 3).

In steps 4 and 5, the fractions were tested for enzymic activity after a 15 min pre-incubation with 1 mM $MnCl_2$ at 20°C.

Results

The results of the purification procedure are summarized in Table I. Gel filtration on Ultrogel AcA34 resin proved to be the major purification step, presumably because of the large size of the enzyme compared to the other kidney constituents. By comparison with the behaviour of standard proteins on the same resin, the molecular weight of the enzyme was estimated to be 300 000.

The fraction of maximum enzymic activity produced by the 5-step procedure (fraction 11 on Fig. 3) showed only one component on electrophoresis on 10% polyacrylamide gels at both pH 3.8 and pH 9.4, even at loadings of up to 100 μg protein. It was thought possible that the large size of the molecule, as suggested by the gel filtration experiment, might prevents its passage into the

TABLE I

PURIFICATION OF INHIBITING FACTOR-CLEAVING ENZYME FROM BOVINE KIDNEY

Enzymic activity was measured with 50 mM Pro-Leu-Gly-NH_2 as substrate.

Fraction	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield of activity (%)	Purification (*n*-fold)
Supernatant	7560	7.9	59 440	(100)	(1)
$(NH_4)_2SO_4$	2100	21.0	44 226	74.4	2.7
Gel filtration	80	341.7	27 336	46.0	43.2
Anion-exchange fractions 11 and 12	20.6	913	18 810	31.7	115.5
Re-chromatography fraction 11	2.0	1494	2 988	5.0	189

gel and that the single protein band might then represent an impurity. However, only a single band could be detected when the electrophoresis was repeated using 5% polyacrylamide gels. Furthermore, for both sets of gels, no protein band could be detected at the top of the gel. Also, gel electrofocusing of fraction 11 revealed the presence of only one component. The isoelectric point was found to be pH 4.1. Furthermore, only one component could be detected on SDS gel electrophoresis after treatment with mercaptoethanol and SDS. The molecular weight of that component was estimated to be 56 000. Bearing in mind the behaviour on gel filtration, the native enzyme may be composed of five or six identical subunits. Thus it is believed that fraction 11 represents the pure enzyme. One band could be seen on staining the polyacrylamide gels after electrophoresis, according to the procedure of Clarke [5], indicating the presence of carbohydrate and suggesting that the enzyme is a glycoprotein. The results of a survey of the specificity of the pure enzyme towards proline-containing substrates are recorded in Table II. The enzyme was very stable: it showed the same level of activity against the substrates (Table II) in spite of repeated freezing and thawing over a period of 8 weeks.

TABLE II

SPECIFICITY OF INHIBITING FACTOR-CLEAVING ENZYME TOWARDS PROLINE-CONTAINING SUBSTRATES

Substrate (50 mM)	Activity (%)
Pro-Leu-Gly-NH_2	(100)
Pro-Leu-NH_2	30.5
Pro-Leu	1.8
Gly-Pro-Ala	0
Pro-β-Naphthylamide	0
Pro-Phe-Gly-Lys	0
Salmine (5 mM)	0
Pro-Gly-Gly	4.0
Pro-Gly-Gly-NH_2	9.6
Pro-Gly	5.6
Pro-Ala	0
Pro-Met	0
Val-Pro	0

Pre-incubation of the enzyme for 15 min at 20°C with 5 mM EDTA or 5 mM *p*-chloromercuribenzoate resulted in complete loss of activity towards L-prolyl-L-leucylglycinamide, L-prolylglycine and L-prolylleucinamide.

Discussion

The only report of a purification of an enzyme-cleaving L-prolyl-L-leucylglycinamide was that of Nordwig and Mayer [13]. They purified an enzyme from porcine kidney in a yield of 0.6% by an 8-step procedure, achieving an overall purification factor of 200 as compared with the crude homogenate. However, their final preparation was not homogeneous and contained some prolyl dipeptidase (EC 3.4.13.8) as impurity.

In contrast, in this study, an enzyme has been purified 189-fold from bovine kidney by a much simpler 5-step procedure and in a better yield of 5%. The enzyme was found to be homogeneous on analytical electrophoresis and electrofocusing. It is believed that the bovine enzyme possesses an intrinsic ability to catalyze the hydrolysis of L-prolylglycine since, in contrast to the porcine enzyme, such activity was not reduced on repeated freezing and thawing. Furthermore, a complete loss of such activity occurred after incubation with *p*-chloromercuribenzoate whereas only 50% reduction was recorded by Akrawi and Bailey [9] for bovine prolyl dipeptidase. Perhaps, the inability of Mayer and Nordwig to completely separate the inhibiting factor-cleaving enzyme from prolyl dipeptidase was due to the similarity in size of the two enzymes [10].

The enzyme of the present study differs markedly from other prolyl-cleaving enzymes. It can clearly be distinguished from prolyl dipeptidase of bovine kidney [9] in its greater molecular weight, lower isoelectric point, different substrate specificity and much greater stability. In its lack of activity towards salmine and L-prolyl-L-phenylalanylglycyl-L-lysine it differs from proline aminopeptidase (EC 3.4.11.5) [14]. It differs from proline dipeptidase (EC 3.4.13.9) of porcine intestine in molecular weight, isoelectric point and in its lack of activity towards L-valyl-L-proline [15]. The ability of the bovine enzyme to hydrolyze glycyl-L-prolyl-L-alanine, its greater size and lower isoelectric point show that it is different from post-proline dipeptidyl aminopeptidase (EC 3.4.14.1) of lamb kidney [16].

It has been proposed that in mammals the predominant control over the release of melanocyte-stimulating hormone from the intermediate lobe of the pituitary is mediated by the inhibiting factor secreted by the hypothalamus [1]. Also, effects of inhibiting factor outside of the pituitary gland have recently been recorded, indicating that L-prolyl-L-leucylglycinamide may have more than one physiological role [17]. Experiments with radioactive-labelled L-prolyl-L-leucylglycinamide have shown that it is rapidly accumulated by a number of rat organs, including kidney, indicating that those organs are important sites of action and/or inactivation of the tripeptide amide [3].

The enzyme purified in the present study may represent the physiological mechanism for inactivation of the inhibiting factor by bovine kidney.

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