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A Specific Antiserum to Lysosomal Cathepsin D

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Summary. A specific antiserum to adult chicken liver cathepsin D was prepared by injecting rabbits with precipitin lines formed in agar gel between a purified enzyme preparation and a polyvalent antiserum. The specific antiserum was used to show the identity of cathepsin D from liver with that from spleen, heart, kidney, testis and brain of the adult, and from limb bones of the embryo chicken.

INTRODUCTION

A specific antiserum directed against a lysosomal enzyme should be a powerful tool with which to investigate the role of that enzyme in tissue physiology. Organ cultures of embryonic limb bones of chicken (*Gallus domesticus*) have provided an *in vitro* model suitable for the study of cartilage breakdown (for review see Dingle, 1968), and Lucy, Dingle and Fell (1961) obtained evidence that a lysosomal protease is concerned in this process. Thus, an antiserum prepared against the chicken lysosomal protease cathepsin D might be useful in examining the involvement of that particular enzyme in the degradation of cartilage matrix.

Antisera to enzymes have frequently been raised and proteolytic enzymes are no exception. For example, pepsin, trypsin, chymotrypsin and papain have all been successfully used as antigens (see reviews Cinader, 1967; Arnon, 1967). Trouet (1965), in a preliminary communication, reported the preparation of an antiserum to a crude lysosomal extract which reacted with more than one enzyme; Glynn and Parkham (1964) prepared an antiserum which reacted with rat lysozymes.

When this work began, a crude enzyme (preparation A) was available, to which a polyvalent antiserum had been raised; there was also ready a more highly purified enzyme (preparation B), which still contained other proteins in addition to the enzyme (Barrett, personal communication).

This paper describes how these materials have been used to prepare a specific antiserum against cathepsin D from adult chicken liver, and how the identity of cathepsin D from several tissues of the adult chicken, and from limb bones of embryonic chicken has been established by means of the antiserum. The inhibitory action of the antiserum on the hydrolysis by lysosomal enzyme preparations of haemoglobin and chondromucoprotein (Barrett, Weston and Dingle, unpublished observations) and on the autolytic degradation of cartilage is reported elsewhere (Weston, Barrett and Dingle, 1969).

MATERIALS

Chicken tissues

Chicken livers were obtained from SAPPA Cobb chickens from J. Sainsbury's Poultry Packing Station, Bury St Edmunds; these birds are from a cross of Cornish cock birds with

White Rock Females. Eggs were obtained from Chivers Ltd, Histon, and were from a cross of Rhode Island Red and Light Sussex. Extracts of several tissues were made from adult cocks, both Rhode Island Red and Light Sussex, obtained from Chivers Ltd, Histon.

Enzyme preparations

Three enzyme preparations were available.

Preparation A: a crude preparation (90 units/ml, 1 mg protein/ml) made from chicken liver by the method described by Barrett (1967) for rabbit liver, the Sephadex G-100 separation being omitted.

Preparation B: a more purified preparation (800 units/ml, 1 mg protein/ml).

Preparation C: a highly purified preparation (450 units/ml, 0·44 mg protein/ml). Preparations B and C were obtained by a newly developed procedure (Barrett, personal communication).

Antiserum to the crude enzyme (preparation A)

New Zealand White rabbits were immunized with intraperitoneal injections of sterile enzyme preparation A (5 ml) on days 0, 1 and 3, followed by intramuscular injections of the same preparation with Freund's complete adjuvant (5 ml of 1 : 1 emulsion, v/v) on days 14, 17, 28, 42, 56 and 77. Blood was taken from the marginal ear vein on day 98 and examined for precipitating antibodies in gel diffusion analysis.

Miscellaneous materials

Bee venom was kindly donated by Mr J. Palmer, Codsall, Staffordshire. Freund's complete adjuvant and Special Agar Noble were Difco products bought through Baird & Tatlock Ltd; bovine haemoglobin was obtained from Armour Pharmaceutical Co. Ltd. Eastbourne; Triton X-100 from Lennig Chemicals Ltd; Naphthalene black TS from G. T. Gurr Ltd; and normal rabbit serum from Burroughs Wellcome Ltd.

METHODS

Cathepsin D assay Analysis

As described by Barrett (1967), except that all volumes were reduced to 60 per cent of those used by that author.

Immunoelectrophoresis

Agar plates were prepared with 1·5 per cent Special Agar Noble in barbiturate buffer (pH 8·6, $I = 0{\cdot}045$) with 11 ml of solution on a glass slide 8×8 cm. The capacity of the antigen well was 5 μl, and of the antiserum trough 150 μl. Electrophoresis was for 5 hours at 8 mA, 20 V/cm.

Gel diffusion analysis

The methods of Ouchterlony (see review 1967) were used, with 1·5 per cent agar in sodium phosphate buffer (0·02 M, pH 7·2) including 0·15 M NaCl.

Examination of precipitin lines for cathepsin D activity

The method of Uriel (1963) was tried; major changes were made and the following procedure finally adopted. The immunoelectrophoresis or gel diffusion plate containing the precipitin lines which were to be examined for enzyme activity, was washed in 0·15 M

NaCl repeatedly in the cold for 2 days, then in water twice for 2 hours and dried at 37°. A second agar plate was poured as for immunoelectrophoresis and immersed in sodium formate buffer (pH 3·0, 0·25 M) at 4° for 16 hours, and then in 0·5 per cent haemoglobin solution in the same buffer at 20° for 30 minutes. The haemoglobin substrate plate was rinsed free of excess haemoglobin solution and placed in contact with the dried immunoelectrophoresis plate. The plates were incubated together at 37° for 2·5 hours. The substrate plate was discarded and the original immunoelectrophoresis plate fixed for 10 minutes in ethanol–water–acetic acid (50 : 45 : 5 by volume). The plate was then stained for approximately 5 minutes in Napthalene black TS (0·1 per cent in 3 per cent, w/v trichloroacetic acid) and destained with 0·3 N acetic acid. Enzymic activity was seen as a colourless line which contrasted with the blue background due to haemoglobin fixed in the agar, and with any other precipitin lines which stained dark blue (Fig. 1). If the precipitin line containing the enzyme was heavy, then the final appearance was of a thick colourless line with the precipitin line stained blue running down the middle.

Preparation of extract from embryonic limb bones and use of hyaluronidase

Limb bone rudiments (1·1 g) were dissected from thirty-six chick embryos (10 days old) and extracted by gentle agitation in Triton X-100 (2 ml, 0·1 per cent) at 0° for 16 hours. The extract was concentrated four-fold by freeze drying and reconstituted in water, but was too viscous for successful gel diffusion analysis. The extract (500 μl) was mixed with sodium phosphate buffer (25 μl, 0·1 M, pH 6·9). Bee venom (25 μl, 1·2 mg/ml) in water was added as a source of hyaluronidase, and the mixture incubated at 37° for 18 hours, and then freeze dried. The solid was dissolved in 100 μl of water.

Precipitation of enzymic activity with antiserum

The specific antiserum, and normal rabbit serum for the controls, were diluted with sodium phosphate buffer (pH 7·1, 0·02 M) including 0·15 M NaCl (one part serum+three parts buffer). From this mixture 200 μl was added to 200 μl of enzyme solution in a conical Dryer tube. The enzyme concentration was sufficient to give an increase of 0·24 in E 280 mμ/1 cm in the final assay. The tubes were capped with Parafilm and after thorough mixing incubated at 37° for 1 hour and then at 0° for 16 hours. After centrifugation at 1000 ***g*** for 5 minutes the supernatant (300 μl) was assayed for cathepsin D activity.

Immunization procedure for specific antiserum

The enzyme preparation B was run in immunoelectrophoresis, the polyvalent antiserum being used to develop the plate. Only a single line developed, and this was shown to contain cathepsin D activity (see above). A number of immunoelectrophoresis plates giving only the single precipitin line were prepared, and were then washed with repeated changes of 0·15 M NaCl at 4° over 4 days; the lines were cut out with a scalpel and pooled in a dry syringe barrel. For injections on day 0 and day 28, seven precipitin lines were suspended in 0·15 M NaCl (0·8 ml) and Freund's complete adjuvant (0·8 ml) was drawn into the syringe. The mixture was emulsified by repeated passage through a serum 1 needle into a second syringe, a device described by Berlin and McKinney (1958). The emulsion (0·75 ml) was injected into each shoulder subcutaneously on day 0, and into each footpad on day 28. New Zealand White and Sandy Half Lop rabbits were immunized. A suspension of seven precipitin lines in 0·15 M NaCl (1 ml) was passed through the emulsifying device to break up the particles and then injected intravenously on day 39. Blood was taken from the marginal ear vein on day 48 and examined for precipitating antibodies.

RESULTS

PREPARATION OF THE POLYVALENT ANTISERUM

The course of injections of the crude liver cathepsin D (preparation A) resulted in the production of a polyvalent antiserum which showed at least five precipitin lines when examined by immunoelectrophoresis, with the crude enzyme (preparation A) in the antigen well.

DEMONSTRATION OF PROTEOLYTIC ACTIVITY IN PRECIPITIN LINES

An essential step in this work was to identify the precipitin line which contained the cathepsin D. At first the technique described by Uriel (1963) was followed in which an agar plate, containing haemoglobin as substrate, is incubated in contact with the plate containing the proteolytic enzyme in one of the precipitin lines; enzymic activity is observed as a colourless line in the fixed and stained haemoglobin containing plate. It was then found that if the original gel diffusion plate was stained after contact with the substrate plate, the line that contained the enzyme was colourless, in sharp contrast to the blue background, whereas all other lines were stained dark blue (Fig. 1); the background colour being due to absorption of haemoglobin from the substrate plate. The newly developed technique was found to be reproducible and was used to define which precipitin lines contained cathepsin D.

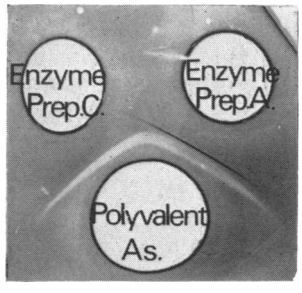

FIG. 1. Technique for showing cathepsin D activity in precipitin lines. The colourless line indicates the presence of cathepsin D; the stained line is another precipitin line with no proteolytic activity. The background staining is due to undigested haemoglobin.

PREPARATION OF THE SPECIFIC ANTISERUM

In immunoelectrophoresis plates the polyvalent antiserum developed only a single precipitin line with the purified enzyme (preparation B) in the antigen well. The line was shown to contain cathepsin D activity, and such precipitin lines were injected into rabbits to produce a specific antiserum.

Out of seven rabbits which received an injection course of precipitin lines, three produced a precipitating antiserum and two of these were completely specific. The gel diffusion plate (Fig. 2) shows the single precipitin line developed between one of the specific antisera and the crude enzyme (preparation A) in contrast to the several lines developed by the polyvalent antiserum. In repeated experiments with various concentrations of a crude liver homogenate the single precipitin line was observed, proving the specificity

of the antiserum within the known limitations of the method. The precipitin line was examined by incubation with a haemoglobin substrate plate and proved to contain cathepsin D activity.

At high concentrations of antigen double lines sometimes developed. Use of a haemoglobin substrate plate showed that both lines contained cathepsin D activity.

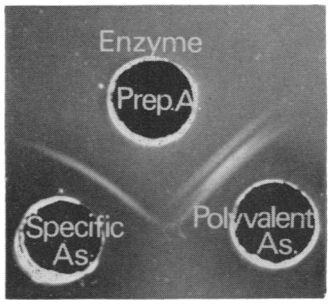

FIG. 2. Gel diffusion analysis to compare the reactions of specific and polyvalent antisera with a crude preparation containing cathepsin D.

IMMUNOLOGICAL IDENTITY OF CATHEPSIN D FROM TISSUES OF THE ADULT

An adult cock bird (Rhode Island Red) was killed by cervical dislocation. The kidney, heart, brain, testes, liver and spleen were removed immediately and homogenized at 0° (one part tissue: four parts 0·1 per cent Triton X-100). The homogenates were centrifuged (9500 ***g***, 30 minutes) and the supernatants assayed for cathepsin D activity (Table 1). The solutions were adjusted to the same enzyme concentration (16 units/ml), ultrafiltration being used as the method of concentration.

TABLE 1

LEVEL OF CATHEPSIN D ACTIVITY FOUND IN TISSUE EXTRACTS

	Cathepsin D (units/ml)
Adult liver	30
Adult spleen	27
Adult kidney	30
Adult testis	12
Adult brain	8
Adult heart	6
Embryonic limb bone*	4

Assay was on a homogenate of one part tissue+four parts Triton X-100 (0·1 per cent)

*Not homogenized: extract was made by gentle agitation of limb bones in Triton X-100.

The extract from each tissue was compared with that from liver by gel diffusion analysis using the specific antiserum. For each of the seven tissues the precipitin lines showed a reaction of complete identity with the line given by the liver; no spurs were observed (Fig. 3a and b). The gel diffusion plates were examined by incubation with a haemoglobin substrate plate and the precipitin lines proved to contain cathepsin D activity. The same experiment was done with a Light Sussex cock bird as the source of the tissues; this gave exactly the same results.

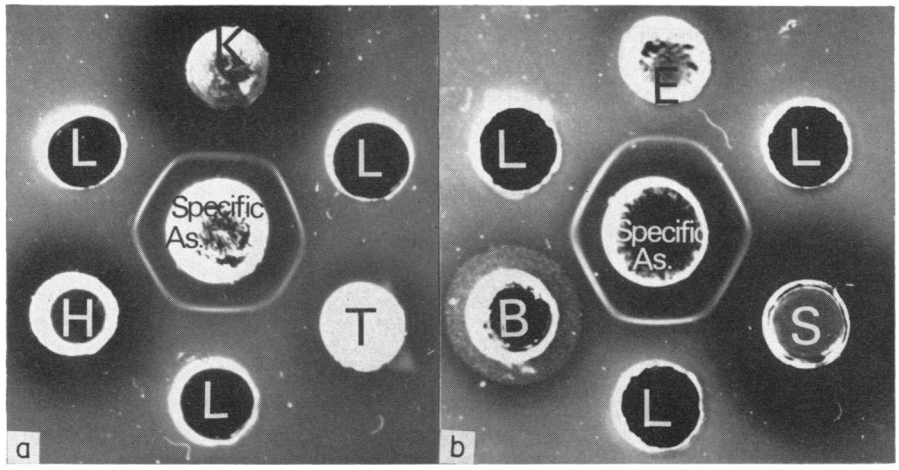

FIG. 3. Reaction of identity of extracts from various tissues. L = Liver; K = kidney; B = brain; H = heart, S = spleen; T = testis; E = embryonic limb bone. In the central well is the specific antiserum.

The tissue extracts were used to quantify the precipitation of cathepsin D by the antiserum used in excess. Mixtures of antiserum with tissue extract were made in micro test tubes (see 'Methods'); after incubation for 1 hour at 37° and then for 16 hours at 0°, the tubes were centrifuged and the supernatant solution was assayed for cathepsin D activity. The results are presented in Table 2. For five of the tissues examined the degree of precipitation was the same; the testis extract gave a low result. A very highly purified solution of the liver enzyme (preparation C) showed the same precipitation characteristics as the crude homogenate.

TABLE 2

CATHEPSIN D ACTIVITY PRECIPITATED BY THE ANTISERUM FROM DIFFERENT TISSUE EXTRACTS

Adult liver	89±2 (6)
Adult heart	88±1 (4)
Adult kidney	93±2 (4)
Adult spleen	92±3 (4)
Adult testis	82±2 (4)
Adult brain	88±6 (4)
Embryonic limb bone	88±4 (2)
Adult liver, purified enzyme (preparation C)	91±3 (2)

The results are given as the percentage (± SD) of the cathepsin D activity precipitated. The number of experiments is shown in parenthesis.

IMMUNOLOGICAL IDENTITY OF EMBRYONIC LIMB BONE AND ADULT LIVER ENZYME

An extract prepared from embryonic limb bones (see 'Methods') was too viscous for successful gel diffusion analysis. After treatment with hyaluronidase using bee venom as a source of the enzyme, the preparation was compared in gel diffusion with the adult chicken liver enzyme. The embryonic limb bone preparation showed a line of complete identity with the adult liver preparation (Fig. 3b). The precipitin lines were examined for cathepsin D activity which proved to be present.

Another extract from embryonic limb bone rudiments was used to quantify the degree of precipitation of enzyme by antiserum (see 'Methods'). This result is presented in Table 2; the same proportion of enzymic activity was precipitable from the limb bone extract as from the adult liver extract.

DISCUSSION

Immunoelectrophoresis of the enzyme (preparation B) developed with the polyvalent antiserum allowed purification of the enzyme prior to injection. Only single, well washed precipitin lines that contained the enzyme, were used for immunization. This technique allowed the preparation of antisera from very small amounts of antigen, the whole injection course requiring approximately 120 μg of enzyme per rabbit. The method was originated by Smith, Gallop and Tozer (1964) and independently by Goudie, Horne and Wilkinson (1966); it has been used by Shivers and James (1967).

The specificity of the final antiserum was consistently demonstrable in gel diffusion analysis against crude homogenates of liver at a variety of concentrations. Single precipitin lines developed which were shown to contain cathepsin D activity. Double lines were sometimes formed, but these developed only in the presence of high concentrations of antigen. The phenomenon is probably the same as that described by Burtin (1954) who reported doubling of lines and even up to four lines, when the antigen was in excess. In the present work when two lines formed they were both found to contain acid protease activity, so this observation does not cast doubt upon the specificity of the antiserum towards cathepsin D alone.

The evidence shows that the cathepsin D activity of all the tissues examined is provided by the same enzyme that occurs in the liver. Thus, precipitin lines of complete identity were observed in gel diffusion analysis (Fig. 3a and b). Furthermore the percentage of cathepsin D activity precipitated in antiserum excess was the same for each of the tissue extracts as for the liver extract (Table 2). The degree of precipitation was approximately 88 per cent, and this was found to be the same with a highly purified sample of cathepsin D (preparation C). Incomplete precipitation of this nature is consistent with the observations of Branster and Cinader (1961) on anti-ribonuclease antisera. A number of authors (Henion and Sutherland, 1957; McGeachin and Reynolds, 1959; Nisselbaum and Bodansky, 1959; Schlamowitz and Bodansky, 1959; Glynn and Parkman, 1964) have used immunological techniques to study a single enzyme in different tissues within one species.

Cathepsin D activity of the embryonic limb bone has been shown to be due to the same enzyme as that found in the adult liver. It is indeed fortunate that antisera raised against the relatively abundant enzyme of adult liver can be utilized for the study of the enzyme of embryonic cartilage (Barrett, Weston and Dingle, unpublished observations; Weston, Barrett and Dingle, 1969).

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