

# Cathepsin S from bovine spleen

## Purification, distribution, intracellular localization and action on proteins

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Cathepsin S was detected in bovine kidney, spleen, lymph nodes and lung by immunochemical methods. The immunostaining of cathepsin S in kidney was concentrated to the cells of the proximal tubule, where the enzyme was present in cytoplasmic granules. The purification method for cathepsin S from bovine spleen involved  $(\text{NH}_4)_2\text{SO}_4$  fractionation, chromatography on CM-Sephadex C-50, gel filtration on Sephacryl S-200 and chromatofocusing (pH 8.0–6.0). The enzyme was partially destroyed by autolysis of the homogenate at pH 4.2. The isoelectric point of cathepsin S was 7.0. Cathepsin S was found to hydrolyse proteins at a similar rate to cathepsin L below pH 7.0. At pH values of 7.0–7.5 cathepsin S retained most of its activity, whereas cathepsin L was completely inactive.

## INTRODUCTION

Cathepsin S, a cysteine proteinase, has been purified from bovine lymph nodes (Turnšek *et al.*, 1975), bovine spleen (Ločnikar *et al.*, 1981; Kirschke *et al.*, 1986) and rabbit spleen (Maciewicz & Etherington, 1985, 1988), and it has been shown that this enzyme has some similarities to cathepsin L, but is clearly a different protein (Kirschke *et al.*, 1984, 1986; Maciewicz & Etherington, 1985).

Cathepsin S is classified as a lysosomal cysteine proteinase similar to cathepsins B, L and H, although the lysosomal location of cathepsin S has not been proved yet. Kregar *et al.* (1981) reported on the presence of cathepsin S in lysosomes of rat liver, where the enzyme was identified by pepstatin-insensitive haemoglobin-hydrolysing activity at pH 3.5. This cannot be taken as proof of the lysosomal location of cathepsin S, because the activity measured could not be discriminated from that of cathepsin L. Cathepsin S has not been detected in liver yet, but only in lymph nodes and spleen.

The purpose of the present work was to determine the intracellular localization and distribution in organs of cathepsin S, to purify the enzyme, and to test its action on proteins and its stability at different pH values and to compare the properties of the bovine enzyme with those of cathepsin S from rabbit spleen described by Maciewicz & Etherington (1985, 1988). In this study we also present the location of cathepsin S in the kidney, an organ in which immunochemical staining was particularly strong.

## EXPERIMENTAL

### Materials

Z-Arg-Arg-NHMec and Arg-NHMec were obtained from Bachem (Bubendorf, Switzerland). CM-Sephadex C-50, phenyl-Sepharose CL-4B, concanavalin A–

Sepharose, Sephacryl S-200 HR, Polybuffer exchanger PBE 94 and Polybuffer 96 were from Pharmacia (Uppsala, Sweden). SDS, acrylamide, *NN'*-methylene-bisacrylamide, Servalyt Precotes 3–10, Serva Blue G and Amberlite CG 50II were purchased from Serva (Heidelberg, Federal Republic of Germany). L-3-Carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) was from Sigma (Deisenhofen, Federal Republic of Germany). Nitrocellulose (Hybond C) was from Amersham-Buchler (Braunschweig, Federal Republic of Germany). The horseradish-peroxidase-conjugated rabbit anti-peroxidase antibody complex was prepared by Dr. Richter (Karl-Marx University, Leipzig, German Democratic Republic). Cathepsin L (EC 3.4.22.15) was purified from rat liver lysosomes as described previously (Kirschke *et al.*, 1977).

### Enzyme preparation

A 300 g batch of bovine spleen was homogenized in 2 vol. of 50 mM-sodium acetate buffer, pH 4.2, containing 1 mM-EDTA and 150 mM-NaCl. After centrifugation, a 20–75% satn.  $(\text{NH}_4)_2\text{SO}_4$  fraction of the supernatant was dialysed overnight against 20 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA and then applied to a column (2.6 cm × 28 cm) of CM-Sephadex C-50 equilibrated in the same buffer. The column was washed with 4 bed volumes of the buffer and then with 400 ml of 300 mM-NaCl in the buffer.

The fraction eluted with 300 mM-NaCl from the CM-Sephadex column was concentrated by ultrafiltration on an YM-10 membrane (Amicon) and applied to a column (2.5 cm × 90 cm) of Sephacryl S-200 equilibrated in 100 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA and 500 mM-NaCl. Active fractions in the  $M_r$  range 15000–30000 were combined, concentrated and equilibrated with 25 mM-Tris/acetate buffer, pH 6.0,

Abbreviations used: Z-, benzyloxycarbonyl-; Bz-, benzoyl-; -NHMec, 7-(4-methyl)coumarylamide; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane.

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and then applied to a column (0.9 cm × 28 cm) of chromatofocusing gel (PSE 94) equilibrated with 25 mM-Tris/acetate buffer, pH 8.3. The eluent was Polybuffer 96 (diluted 1:12 with water), pH 6.0.

Polybuffer was removed from the active fractions by hydrophobic chromatography on phenyl-Sepharose (0.9 cm × 4 cm) with 25% (w/v)  $(\text{NH}_4)_2\text{SO}_4$  in 20 mM-sodium malonate buffer, pH 5.0, containing 1 mM-EDTA. The enzyme was eluted with 10% (v/v) ethylene glycol in 20 mM-sodium malonate buffer, pH 5.0. Subsequently the sample was run on a column (0.9 cm × 15 cm) of CM-Sephadex C-50 equilibrated with 20 mM-sodium malonate buffer, pH 5.0, containing 1 mM-EDTA. Cathepsin S was eluted at about 200 mM-NaCl during a linear gradient (0–300 mM-NaCl) in the buffer.

### Enzyme assays

Cathepsin S was routinely assayed in column fractions at pH 7.5: a 60 min preincubation at 40 °C in 100 mM-sodium phosphate buffer containing 2 mM-dithiothreitol and 2 mM-EDTA was followed by a 10 min incubation at 40 °C with 5  $\mu\text{M}$ -Z-Phe-Arg-NHMec as substrate. Substrate hydrolysis was stopped by 100 mM-chloroacetate, pH 4.3, as described by Barrett & Kirschke (1981).

Activities of cathepsins B, L and H were determined with Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec and Arg-NHMec respectively as substrates as described previously (Barrett & Kirschke, 1981).

Enzyme activities on proteins in the presence of 1 mM-dithiothreitol and 1 mM-EDTA were measured as follows.

Azo-casein (0.5%, w/v) in the presence or in the absence of urea (3 M) was incubated for usually 15 min at 40 °C with the enzyme fractions. The reactions (in 0.2 ml) were stopped by 0.2 ml of 10% (w/v) trichloroacetic acid.

Haemoglobin and human serum albumin were labelled with [ $^{14}\text{C}$ ]acetic anhydride as previously described (Wiederanders *et al.*, 1989). The labelled protein was diluted with unlabelled protein to give a final concentration of 0.05% (w/v) and about 8000 d.p.m./assay. After incubation for 30 min at 40 °C the radioactivity in a sample of the trichloroacetic acid (5%, w/v) supernatant was measured in a liquid-scintillation counter.

Hydrolysis of insoluble collagen was determined as described by Etherington (1974) except that the incubation was terminated after 30 min at 30 °C by addition of E-64 (0.5  $\mu\text{M}$ ). Liberated hydroxyproline was measured by using a micro-modification of the procedure described by Woessner (1961).

One unit of enzyme activity corresponds to 1  $\mu\text{mol}$  of synthetic substrate or 1 mg of protein, as appropriate, degraded in 1 min. One unit was also defined as the enzyme activity liberating 1  $\mu\text{mol}$  of hydroxyproline from insoluble tendon collagen in 1 min at 30 °C (Etherington, 1974).

### Enzyme and protein concentration

The concentration of the cysteine proteinases was determined by active-site titration with E-64 and with Z-Phe-Arg-NHMec as substrate (Barrett & Kirschke, 1981). Protein was measured by a micro-modification of the Lowry method (Langner *et al.*, 1971), with bovine serum albumin as standard.

### Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed with separating gels of 12.5% polyacrylamide (2.6% of this total concentration being methylenebisacrylamide) in a buffer system described by Bury (1981).

### Immunological methods

**Preparation of an antibody to cathepsin S.** An antiserum against cathepsin S from bovine spleen was raised in rabbits (Wiederanders & Kirschke, 1986). The IgG fraction (7.5 mg/ml) of the antiserum was prepared by the method of Steinbuch & Audran (1969).

**Immunoblotting.** Blotting of SDS/polyacrylamide gels after electrophoresis was performed by the procedure of Burnette (1981). The quenched nitrocellulose was treated with monospecific antibodies (immunoglobulin fraction), and the immunoreactive bands were detected by using the peroxidase-anti-peroxidase antibody technique as described previously (Kirschke *et al.*, 1986).

**Immunohistochemistry.** Samples of kidney tissue were removed immediately after the animals were killed. These were fixed for 4 h (4% formaldehyde in iso-osmotic sucrose, pH 7.4, then washed overnight in iso-osmotic sucrose at 4 °C). Samples were embedded in paraffin wax and sectioned on a sledge microtome. The sections were treated with 0.1% (w/v) pepsin in 10 mM-HCl for 1 h.

The avidin-biotin-peroxidase-complex technique (Hsu *et al.*, 1981) was used with some modifications (Rinne *et al.*, 1986). Controls for the immunohistochemical test were performed as described previously (Rinne *et al.*, 1986).

### $M_r$ determination

Columns of Ultrogel AcA-54 (1.6 cm × 90 cm) and Sephacryl S-200 (2.5 cm × 90 cm) were equilibrated with 100 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA and 500 mM-NaCl, and calibrated with Dextran Blue, bovine serum albumin, carbonic anhydrase, chymotrypsinogen and soya-bean trypsin inhibitor.

### Isoelectric focusing

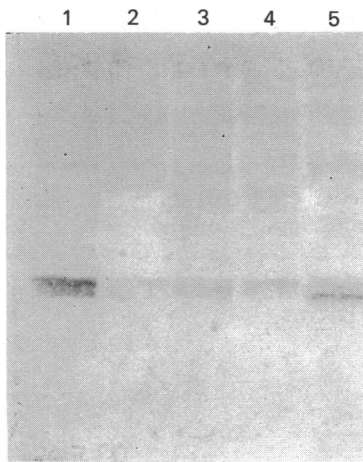
The isoelectric point of cathepsin S was determined on Serva Precotes pH 3–10 in a Pharmacia apparatus in accordance with the recommendations of the manufacturer.

After focusing the gel was stained for protein with Serva Blue G or examined for enzyme activity. A strip of gel was cut into 0.5 cm pieces, each of which was eluted overnight in 250  $\mu\text{l}$  of 100 mM-sodium phosphate buffer, pH 6.0. The activity of cathepsin S was determined in the eluates with Z-Phe-Arg-NHMec as substrate.

## RESULTS AND DISCUSSION

### Distribution in organs and intracellular location

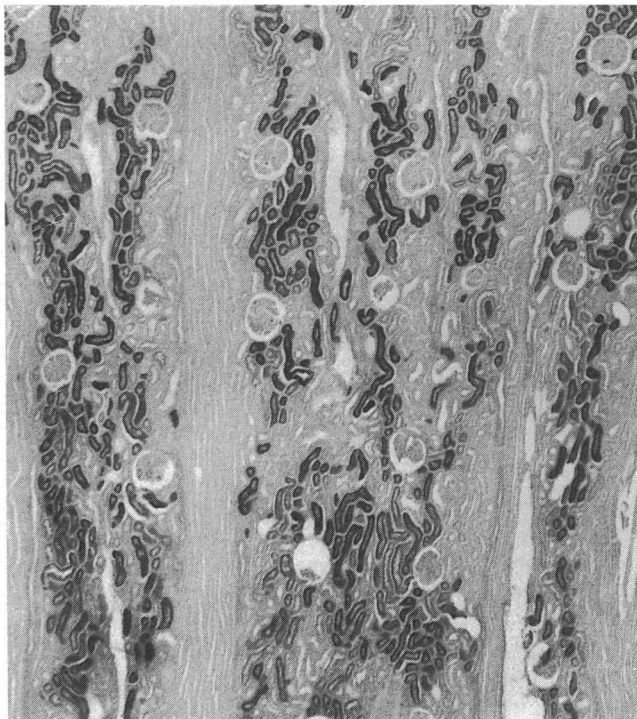
Extracts (water) of several bovine organs (heart muscle, skeletal muscle, liver, lung, spleen, kidney, intestine, stomach and lymph nodes) were checked for the presence of cathepsin S by immunoblotting. Cathepsin S immunoreactive material ( $M_r$  24000 and 20000–21000) could only be detected in kidney, spleen, lymph nodes and lung by this method (Fig. 1). Proteins of  $M_r$



**Fig. 1. Immunoblotting of bovine organ homogenates**

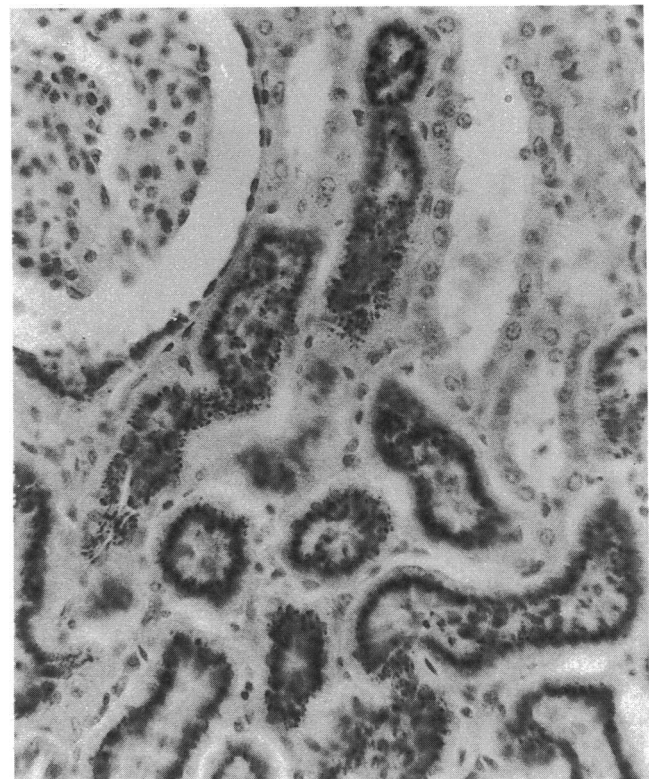
Portions (about 1 mg of protein each) of the organ extracts were subjected to SDS/12.5% polyacrylamide-gel electrophoresis and then blotted on to nitrocellulose. The reaction of the antigens with a monospecific polyclonal antibody to cathepsin S was developed by the peroxidase-anti-peroxidase antibody technique. Lane 1, kidney; lane 2, spleen; lane 3, intestinal lymph node; lane 4, cervical lymph node; lane 5, lung.

20000–21000 were present in these organs that reacted with a monospecific antibody (not affinity-purified) to the enzyme ( $M_r$  24000). The former are probably proteolysis products of cathepsin S, because the immunoreaction could be quenched by the pure enzyme,



**Fig. 2. Location of cathepsin S in kidney cortex**

Strong cathepsin S immunoreactivity can be seen in the cells of proximal tubules. Magnification  $\times 75$ .



**Fig. 3. Location of cathepsin S in proximal-tubule cells**

The immunoreaction in the proximal tubules is granular (lysosomal). In the Bowman's capsule only a weak reaction can be seen. Magnification  $\times 340$ .

and double-diffusion tests (Ouchterlony) revealed partial immunological identity of the enzyme ( $M_r$  24000) and the proteins ( $M_r$  20000–21000). Immunoblots show that kidney seems to be especially rich in cathepsin S. The detection of cathepsin S in kidney disproves our suggestion (Kirschke *et al.*, 1986) that this enzyme occurs only in lymphoid tissues.

The location of cathepsin S (and the proteolysis products) in kidney was examined by immunohistochemical methods. The cells of the proximal tubules showed strong immunoreactivity for cathepsin S, and the reaction was in the cytoplasmic granules, suggestive of lysosomal location (Figs. 2 and 3). The preliminary results of our immuno-electron-microscopic investigation support this view.

The cells of the distal tubules showed only very weak immunoreactivity. Some cells of the collecting tubules showed a strong reaction. A few cells of Bowman's capsule reacted weakly.

The location of cathepsin S seems to be identical with the location of cathepsins L and H in kidney (Rinne *et al.*, 1986).

Apart from the localization in kidney cathepsin S has been detected in cells of the reticuloendothelial system (e.g. in sessile macrophages of lung).

Further evidence for the lysosomal localization of cathepsin S was the presence of the enzyme in a lysosomal extract obtained by the cell fractionation procedure of bovine spleen that was originally described for rat liver (Bohley *et al.*, 1969). The resulting lysosomal mito-

**Table 1. Purification of cathepsin S**

Enzyme fractions were incubated for 1 h at 40 °C and pH 7.5 in the presence of dithiothreitol and EDTA. Subsequently the activity was determined with Z-Phe-Arg-NHMec (5  $\mu$ M) in stopped assays.

	Total protein (g)	Total activity (units)	Specific activity (munits/mg)	Purification (fold)	Yield (%)
Homogenate	52.3	9.99	0.19	1	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	7.8	5.90	0.76	4	59
CM-Sephadex C-50 chromatography	2.6	3.20	1.19	6	32
Sephacryl S-200 chromatography	0.070	0.71	8.6	45	7
Chromatofocusing	0.015	0.62	41.6	219	6.2
CM-Sephadex C-50 chromatography	0.001	0.58	582	3063	5.8

chondrial pellet was washed several times with 250 mM-sucrose and then homogenized with 2.5 vol. (v/v) water. After centrifugation the supernatant was nearly free of contaminating cytosolic and mitochondrial proteins (Bohley *et al.*, 1969). Cathepsin S was identified in this lysosomal extract by precipitation with the antibody in Ouchterlony double-diffusion tests and by immunoblotting.

#### Purification

Improvement of the purification procedure used previously (Kirschke *et al.*, 1986) resulted in a high yield of cathepsin S (Table 1). This was partly due to omission

of acid autolysis of the homogenate (see below under 'Stability'). Use of a lysosomal extract as starting material for the purification of cathepsin S was not advantageous because of a very low yield of lysosomes obtained by cell fractionation of spleen homogenate by the procedure described for liver (Bohley *et al.*, 1969).

Preincubation of the enzyme fractions at 40 °C and pH 7.5 over a period of 60 min allowed us to determine the activity of cathepsin S in the presence of cathepsin L and cathepsin B with Z-Phe-Arg-NHMec as substrate (Fig. 4). But in addition to cathepsin S we measured by this procedure the activity of a cysteine proteinase ( $M_r$  about 60000) that was separated from cathepsin S by gel filtration. Cathepsins S and B emerged together from CM-Sephadex C-50 and Sephacryl S-200 columns. By chromatofocusing at pH 8.0–6.0 cathepsin S was eluted at pH 7.1–6.8, whereas the main part of cathepsin B remained bound to the column. (The latter could be eluted by acetate buffer, pH 4.5.) Separation of cathepsins S and B was also obtained by using an Amberlite CG-50II column at pH 5.3 (results not shown) as described by Etherington (1976) for separation of cathepsins B and N and by Maciewicz & Etherington (1985) for separation of cathepsins B, N and S.

By means of the last step of purification (chromatography on CM-Sephadex C-50) contaminating proteins in the range  $M_r$  10000–20000 were removed. Cathepsin S was electrophoretically pure (Kirschke *et al.*, 1986) and contained 30–40% active enzyme molecules as revealed by titration.

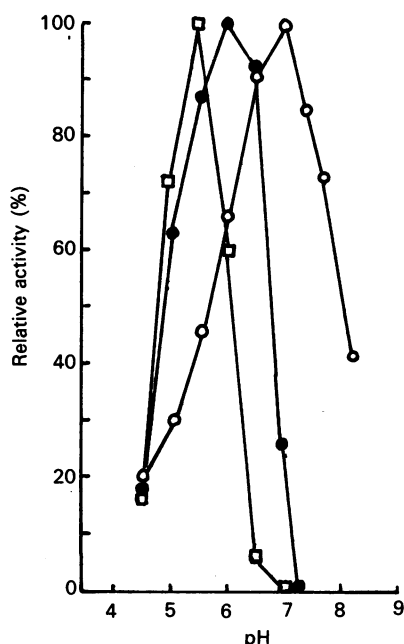
#### Stability

Cathepsin S (12–40  $\mu$ M) was stored for up to 8 months in 100 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA at –10 °C without loss of activity.

Storage in the presence of 0.5 mM-Hg<sup>2+</sup> and 1 mM-EDTA resulted in a rapid decrease of about 50% of the activity of cathepsin S, which then remained unchanged for 3 months at –10 °C.

Preincubation of cathepsin S (1 nM) with 2 mM-dithiothreitol and 2 mM-EDTA at 40 °C before assay with Z-Phe-Arg-NHMec resulted in an increase of the activity. The amount of activation was obviously dependent on the preparation method, because it was different for each of the batches of cathepsin S that we have prepared.

No loss of activity could be observed after 240 min at



**Fig. 4. Effect of pH on Z-Phe-Arg-NHMec hydrolysis by cathepsins S, L and B**

Cathepsin L (□), cathepsin B (●) and cathepsin S (○) were incubated for 1 h at 40 °C in the presence of 2 mM-dithiothreitol and 2 mM-EDTA. Subsequently Z-Phe-Arg-NHMec was added (5  $\mu$ M final concn.) and the incubation was continued for 10 min. Buffers were sodium acetate (pH 4.5–5.5), sodium phosphate (pH 6.0–7.5) and Tris/HCl (pH 7.5–8.5).

**Table 2. Hydrolysis of proteins by cathepsins S and L**

Values are expressed as percentages of the activity towards  $^{14}\text{C}$ -labelled haemoglobin (units/ $\mu\text{mol}$  in parentheses). Incubation conditions were as described under 'Enzyme assays' in the Experimental section. Enzyme concentrations in the incubation mixtures were:  $^{14}\text{C}$ -labelled proteins, 200 nM-cathepsins S and L; azo-casein, 31 nM-cathepsins S and L; collagen, 100 nM-cathepsin S and 43 nM-cathepsin L.

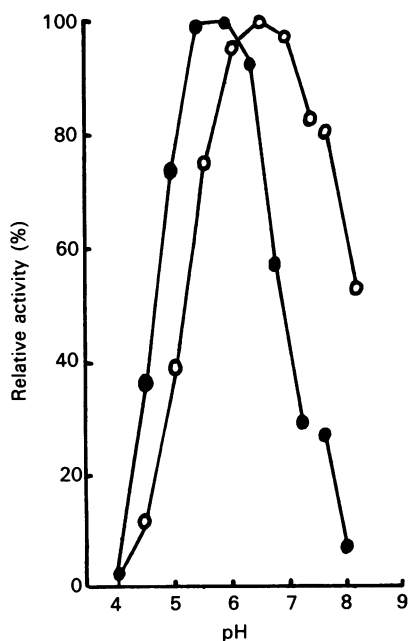
Substrate	pH	Activity (%)	
		Cathepsin S	Cathepsin L
$^{14}\text{C}$ -labelled haemoglobin	5.0	100 (43.5)	100 (44.3)
$^{14}\text{C}$ -labelled haemoglobin	7.5	98	0
$^{14}\text{C}$ -labelled albumin	5.0	78	56
$^{14}\text{C}$ -labelled albumin	7.5	83	0
Azo-casein	6.0	878	664
Azo-casein	7.5	800	0
Azo-casein, urea present	5.5	1782	1603
Azo-casein, urea present	7.5	538	0
Collagen (insoluble)	3.5	462	1284

40 °C in the presence of dithiothreitol and EDTA at pH values in the range 5.5–7.0. Some 10–12% of the enzyme activity was lost in 60 min at pH 7.5 and at pH 4.0.

In the absence of dithiothreitol and EDTA cathepsin S was inactivated at pH 4.0 by 70% in 60 min at 40 °C.

#### Catalytic activity on proteins

One property of cathepsin S by which the enzyme differs from all other lysosomal cysteine proteinases has to be emphasized: this is the stability of the active enzyme at neutral pH values.

**Fig. 5. Effect of pH on azo-casein hydrolysis by cathepsin S**

Cathepsin S (31 nM) was incubated with 2 mM-dithiothreitol and 2 mM-EDTA for 10 min at 40 °C. Subsequently azo-casein (○) or azo-casein plus urea (●) was added and the incubation was continued for 15 min at 40 °C. Buffers were sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.5) and Tris/HCl (pH 7.5–8.5).

The assay of cathepsin S in crude fractions containing cathepsins L and B that we used here took advantage of this property of cathepsin S (see Fig. 4).

Cathepsin S from human and rat spleen showed the same stability at neutral pH values as the bovine enzyme. Turk *et al.* (1980) mentioned that cathepsin S from spleen retains some activity above pH 7.0, but experimental details on this important property were not published.

Data on the hydrolysis of proteins by cathepsin S are given in Table 2 and show the following. (1) Cathepsin S degrades several proteins as fast as cathepsin L does (specific activities are in the same order of magnitude), except collagen (see below). (2) Cathepsin S degrades proteins at pH 7.5, where cathepsin L is inactive. The optimum pH for activity was below pH 7.0 for most of the tested proteins, as is shown for azo-casein in Fig. 5. (3) The specific activities (units/ $\mu\text{mol}$  of enzyme) cannot be compared with those reported by Maciewicz & Etherington (1985) for cathepsin S from rabbit spleen. One reason for this may be that the values are dependent on incubation conditions and determination methods (e.g. concentration of trichloroacetic acid, incubation time in assays containing azo-casein and urea, concentrations of enzyme and substrate in the assay, source of proteins), as discussed by Mason *et al.* (1982) and Wiederanders *et al.* (1986).

Cathepsin S from bovine spleen does not degrade collagen as fast as cathepsin L does (36%; Table 2). Maciewicz & Etherington (1985) reported a specific activity of cathepsin S from rabbit spleen that was only 2% of the value for cathepsin L, although the latter (500 mg of collagen degraded/ $\mu\text{mol}$  of cathepsin L in 1 min = 166 mol of collagen/mol of cathepsin L in 1 min) was nearly identical with the specific activity of cathepsin L in Table 2.

#### $M_r$ , pI and glycosylation

Cathepsin S from bovine spleen appeared as a single protein with  $M_r$  about 24000 on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions (Ločnikar *et al.*, 1981; Kirschke *et al.*, 1986). An  $M_r$  of 24000 was also determined by this method for cathepsin S from human and rat spleens.

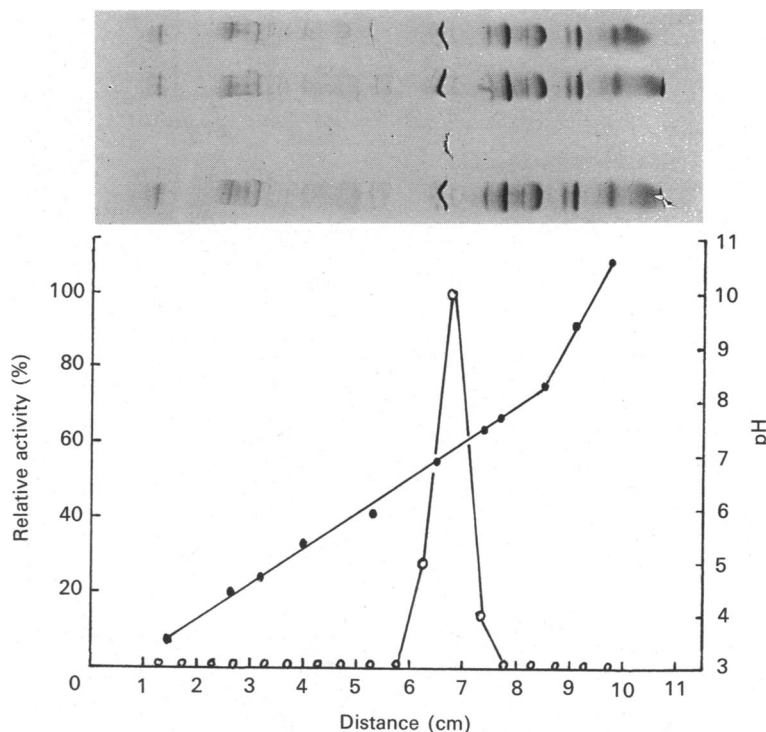


Fig. 6. Isoelectric focusing of cathepsin S

Cathepsin S (10  $\mu$ g) and a standard protein mixture were run on Serva Precotes pH 3–10. pH values (●) were evaluated from the mobility of standard proteins. One strip of the Precote loaded with cathepsin S was cut in pieces and eluted with buffer. The activity of the eluates (○) was determined with Z-Phe-Arg-NHMec as substrate.

Gel-filtration experiments on Ultrogel AcA-54 or Sephacryl S-200 revealed an  $M_r$  for cathepsin S of about 20000. An  $M_r$  of 20000 obtained by gel filtration was described for two enzymes from bovine spleen, cathepsin S (Ločnikar *et al.*, 1981) and cathepsin N (Etherington, 1976). This value is quite different from the  $M_r$  value of 30500 determined for cathepsin S from rabbit spleen (Maciewicz & Etherington, 1985).

Staining of isoelectric-focusing gels for protein showed only one band of cathepsin S at pH 7.0. This was the same position where the activity against Z-Phe-Arg-NHMec was eluted from a gel strip loaded with cathepsin S (Fig. 6). Chromatofocusing (pH 8–6) was one step of the purification procedure. The enzyme was eluted at pH 7.1–6.8 from the polybuffer exchanger. Data on the isoelectric points of bovine spleen cathepsin S in the range 6.3–6.9 have been published by Ločnikar *et al.* (1981). A pI of 7.1 was reported for rabbit spleen cathepsin S (Maciewicz & Etherington, 1985).

Cathepsin S, as a lysosomal enzyme, was expected to contain carbohydrate. The enzyme was found to bind partially to concanavalin A-Sepharose: about 25% of the activity was adsorbed and could be eluted with 100 mM-methyl  $\alpha$ -D-mannoside, and about 75% passed through the gel at pH 6.8.

## CONCLUSIONS

Cathepsin S from bovine spleen can be characterized as follows:  $M_r$  about 24000 (Kirschke *et al.*, 1986); pI 7.0; stable for up to 4 h at 40 °C at pH 5.5–6.5 and catalytically active at pH 5.0–7.5; high activity on

proteins; faster hydrolysis of Bz-Phe-Val-Arg-NHMec than of Z-Phe-Arg-NHMec (Kirschke *et al.*, 1984; Brömme *et al.*, 1989); negligible activity towards Z-Arg-Arg-NHMec.

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