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PURIFICATION AND PROPERTIES OF CATHEPSIN D FROM HUMAN ERYTHROCYTES

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SUMMARY

1. The cathepsin from human erythrocytes has been purified 2000–10 000-fold by *n*-butanol treatment, column chromatography on DEAE-Sephadex A-50, and gel filtration on Sephadex G-100. The purified enzyme was homogeneous according to disc electrophoresis and was free of any other proteolytic activity demonstrated in human erythrocytes.

2. Using urea-denatured haemoglobin as substrate, the enzyme is most active at pH 2.8. It shows little action on acid-denatured albumin and no action at all on the synthetic substrates for the cathepsins A, B and C. The B chain of bovine insulin is preferentially hydrolysed at the peptide bonds Leu^{15}–Tyr^{16} and Phe^{24}–Phe^{25}.

3. The enzyme, which has a molecular weight of about 55 000, is not significantly influenced by EDTA and metal ions. The same holds true for Hg^{2+}, *p*-chloromercuribenzoate (PCMB) and most of the thiol compounds. Only 10 mM dithiothreitol causes 90% inhibition suggesting an essential disulphide group. At pH 2.8 an essential serine would not be in a reactive state. The properties of the enzyme suggest the classification as a cathepsin D (EC 3.4.23.5).

INTRODUCTION

During the maturation and ageing of erythrocytes, a large fraction of the stroma nitrogen disappears [1–3]. This finding suggests the participation of proteolytic enzymes in these processes. Indeed, in rabbit reticulocytes after phenylhydrazine treatment high activities of cathepsin A and another acid proteinase have been demonstrated [2]. During the maturation of the cells the cathepsin A disappears rapidly, whereas the acid proteinase persists at a relatively low level of activity. In human erythrocytes separated into young and old cells a similar though less marked difference in acid proteinase activity has been found [4]. The acid proteinase has been extracted from crude stroma suspensions of lysed human erythrocytes by Morrison and Neurath [5]. In the present communication the purification and characterization of this enzyme is reported.

Abbreviations: Z-, benzyloxycarbonyl; PCMB, *p*-chloromercuribenzoate; DFP, diisopropylphosphorofluoridate.

MATERIALS AND METHODS

Reagents and sources of supply

Infukoll (dextrane solution) was purchased from VEB Serumwerk Bernburg. Bovine serum albumin and human serum albumin were obtained from the Forschungsinstitut für Impfstoffe, Dessau. Leucine aminopeptidase (EC 3.4.1.1) and Contrykal were products of VEB Arzneimittelwerk Dresden. DL-Leucine hydrazide and DL-alanine hydrazide were prepared by Reaclin-Chemie, Leipzig. Benzyloxycarbonyl(Z)-α-L-glutamyl-L-tyrosine, *N*-benzoyl-DL-arginine-*p*-nitroanilide, N^{α}-(3-carboxypropionyl)-L-phenylalanine-*p*-nitroanilide and myoglobin were obtained from Schuchardt, München. DEAE-Sephadex A-50, Sephadex G-100, Sephadex G-200 and Blue Dextran 2000 were products of Pharmacia, Uppsala, Sweden. Crystalline soybean trypsin inhibitor and trypsin were obtained from Fluka, Buchs, Switzerland and chymotrypsin from Spofa, Praha, C.S.S.R. Diisopropylphosphorofluoridate (DFP) was purchased from Arco-Chemie, West-Berlin, Coomassie Brillant Blue R 250 and *p*-chloromercuribenzoate (PCMB) from Serva, Heidelberg, and cytochrome *c* from Koch–Light, Colnbrook, Buckinghamshire, Great Britain. Dithiothreitol was a product of Calbiochem, Luzern, Switzerland, 2-mercaptoethanol of Ferak, West-Berlin, and Trasylol of Bayer, Leverkusen. The oxidized B chain of insulin was purchased from Mann Research, New York, U.S.A. The other chemicals were of analytical grade.

Isolation of erythrocytes and stroma fractions

Starting from heparinized blood of healthy donors the leukocytes were separated according to the method of Wildy and Ridley [6]. The erythrocytes were washed two times in 0.154 M NaCl solution.

Haemoglobin-free ghosts were prepared according to Dodge et al. [7].

Lipid-free stroma was prepared according to Rosenberg and Guidotti [8].

Butanol extraction

Purified packed erythrocytes were suspended in two volumes of isotonic acetate buffer, pH 5.1, and nine volumes *n*-butanol were added at 37 °C under stirring. The precipitated haemoglobin was separated by centrifugation [9]. From the remaining solution the buffer phase was isolated in a separating funnel and extracted with diethyl ether at 4 °C in order to remove traces of *n*-butanol.

The presence of particulate matter was excluded by centrifugation of the enzyme solution for 10 h at 80 000 × *g* (VAC 40, Janetzki, Leipzig-Engelsdorf).

Concentration of enzyme solutions

Previous to column chromatography the enzyme solutions were concentrated to a volume of about 3 ml using Sephadex G-10 and the centrifugation technique described by Reichelt [9].

Chromatography

Column chromatography of the enzyme solution was carried out using (a) DEAE-Sephadex A 50, 0.1 M Tris–HCl buffer, pH 7.8, and a linear NaCl gradient; (b) Sephadex G-100 and 0.05 M phosphate buffer, pH 7.4.

The hydrolysate of the B chain of insulin was purified by chromatography on a Sephadex G-25 column (1 cm × 100 cm) using 1% collidine–1% pyridine–acetate buffer, pH 8.2, containing 0.01% Triton X-100. The peptides of the B chain were separated on a column (1.5 cm × 100 cm) of Dowex 1-X2 (200–400 mesh) according to the method of Rudloff and Braunitzer [10] using a peristaltic pump (Varioperpex, LKB). The separated peptides were checked for homogeneity by descending paper chromatography using FN 13 filter paper (VEB Spezialfabrik, Niederschlag) and *n*-butanol–pyridine–acetic acid–water (30:20:6:24, by vol.) as solvent system. The spots were detected with ninhydrin. All chromatography was carried out at room temperature.

Amino acid analysis

The course of the hydrolysis of the B chain of bovine insulin was followed using the ninhydrin method of Moore et al. [11].

Samples of the isolated peptide solutions (1.0 ml, containing 1.5–2.0 μmoles) were hydrolysed under vacuum at 110 °C for 24 h with conc. HCl (1.5 ml) and with 2.5 mM norleucine solution (0.5 ml) as an internal standard. The hydrolysate was concentrated by rotary evaporation at 37 °C and the residue was dissolved in 5 ml of sodium citrate–HCl buffer (0.2 M HCl, pH 2.2); of this, 1 ml was used for amino acid analysis. The analyses were performed using an automatic amino acid analyzer (Unichrom, Beckman, Munich) according to the method of Spackman et al. [12].

Disc electrophoresis

Samples (0.1 ml, containing about 30 μg protein) were run on 7.5% acrylamide in Tris–glycine buffer (0.02 M Tris, 0.2 M glycine) pH 8.3, using 30 V (8 mA) for 2 h at a length of 65 mm. The gels were fixed in 20% sulphosalicylic acid for 8 h; protein staining was done with a 0.05% solution of Coomassie Brillant Blue R 250 in 12.5% trichloroacetic acid [13].

Determination of molecular weight

The molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column (65 cm × 2 cm) using 0.15 M NaCl in 0.01 M Tris buffer, pH 7.0, as eluent.

The dead volume (V_0) was determined by means of Dextran blue 2000.

Protein determinations

Protein was estimated by (a) measurement of the absorbance of eluates from chromatography columns at 280 nm by means of a Uvicord II (LKB 8300), (b) the method of Lowry et al. [14] with reference to a standard curve prepared with crystalline bovine serum albumin.

Enzyme assays

Proteinase activities were estimated according to Anson [15]. Urea-denatured human haemoglobin was prepared from fresh stroma-free haemolysates [16]. Proteinase activities in haemolysates were determined using the endogenous haemoglobin as substrate. The general assay conditions, unless otherwise stated, were as follows: substrate, urea-denatured human haemoglobin, pH 2.8 (established by titration with

0.1 M HCl); final substrate concentration, 5 mg/ml; incubation temperature, 37 °C; incubation time, 60 min. The enzyme activity was computed with reference to a tyrosine standard curve and expressed as nmoles tyrosine/min per ml. If the Folin reaction was influenced by effectors, the absorbance at 280 nm was measured [17] using the spectralphotometer VSU 1 (Zeiss, Jena).

The activity of cathepsin A was estimated by quantitative paper chromatography using Z-L-glutamyl-L-tyrosine as substrate [18]. Activities of cathepsins B and C were measured kinetically using as substrates N^{α}-benzoyl-DL-arginine-*p*-nitroanilide and N^{α}-(3-carboxypropionyl)-L-phenylalanine-*p*-nitroanilide, respectively [19]. The final concentration of these substrates was 1 mM.

Leucine aminopeptidase and alanine aminopeptidase activities were determined according to Haschen et al. [20] and Farr et al. [21] using as substrates leucine hydrazide and alanine hydrazide, respectively.

Dipeptidase activities were assayed under the conditions specified by Haschen et al. [16] and estimated by a newly developed colorimetric procedure, based on the measurement with 8-hydroxyquinoline of the copper bound in the copper–dipeptide complexes [22].

RESULTS

After careful separation of leukocytes the washed erythrocyte suspension was subjected to butanol extraction. This step afforded (1) a cautious haemolysis of the erythrocytes, (2) the liberation of stroma-bound enzyme material [23], (3) the inactivation of several dipeptidases [24], and (4) a nearly complete precipitation of

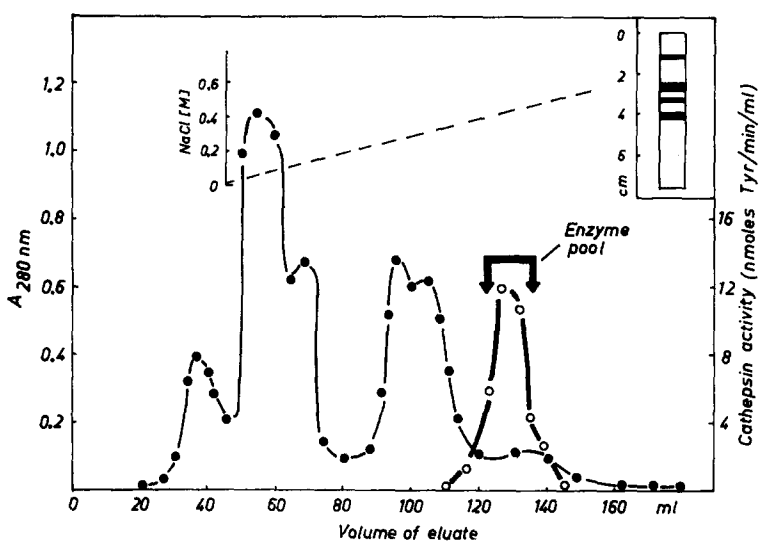

Fig. 1. Chromatography of partially purified cathepsin on DEAE-Sephadex A-50: 3 ml of concentrated enzyme solution and 2 ml of Tris–HCl buffer (0.1 M, pH 7.8) were mixed, applied to column (25 cm × 2 cm) and eluted with a linear NaCl gradient as indicated (mixture of 2 M NaCl and 0.1 M Tris–HCl buffer pH 7.8). Flow rate, 9 ml/h; fraction volume, 3 ml. ●—● $A_{280\ nm}$; ○—○ cathepsin activity. Disc-gel electrophoretic pattern of enzyme pool in the upper right-hand part of the figure; experimental conditions as in Materials and Methods.

haemoglobin. The resulting enzyme solution was concentrated and subjected to ion-exchange chromatography on DEAE-Sephadex A-50. A typical chromatogram is shown in Fig. 1. After previous separation of the bulk of foreign protein and residual activities of leukocytic proteases (not shown in the figure) the red cell cathepsin is eluted at the relatively high ionic strength of 0.45. This step, associated with a purification factor of about 80, was very effective. Disc electrophoresis of the enzyme pool showed four protein bands. The enzyme pool was concentrated to 3 ml and subjected to gel chromatography on a column of Sephadex G-100 (Fig. 2). This step resulted

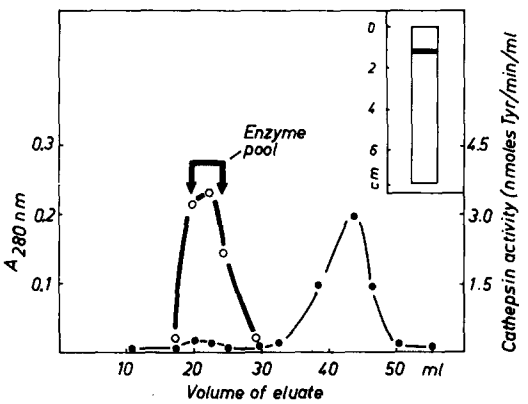

Fig. 2. Chromatography of partially purified cathepsin on Sephadex G-100: 2 ml of concentrated (Sephadex G-100, see Materials and Methods) enzyme pool from chromatography on DEAE-Sephadex A-50 applied to a column (K 15/30, Pharmacia Uppsala, Sweden) and eluted with phosphate buffer (0.05 M, pH 7.4). Flow rate, 8 ml/h; fraction volume, 2 ml. ●—●, $A_{280\,nm}$; ○—○, cathepsin activity. Disc-gel electrophoretic pattern of enzyme pool in the upper right-hand part of the figure.

in (1) the removal of NaCl, (2) an exchange of buffer, and (3) the separation of low-molecular proteins. Only one single protein band is visible after disc electrophoresis of the protein pool. The yields and purification factors of a typical experiment are shown in Table I. The highest purification factor obtained in a single peak fraction amounted to 10 000. For the subsequent investigations an enzyme pool with a purification factor of 2000 and an activity of 200 nmoles/min per mg protein was used.

Properties of the erythrocyte cathepsin

(1) Hydrolysis of different substrates

The hydrolysis of several protein substrates is given in Table II.

Synthetic substrates which, according to Bergmann [25], are appropriate for the characterization of cathepsins A, B and C, were not hydrolysed.

The same was true of the following peptides: glycyl–glycine, glycyl–L-leucine, glycyl–glycyl–glycine, L-leucyl–glycyl–glycine, glycyl–L-tyrosine, glycyl–D-leucine, glycyl–L-proline, L-prolyl–glycine, DL-leucine hydrazide, and DL-alanine hydrazide. Thus a contamination of the enzyme preparation by exopeptidases, which are present in considerable quantities in human erythrocytes [16], could be excluded.

The hydrolysis of native and lipid-free stroma protein by purified cathepsin was tested at pH 2.8 and 7.0 at a temperature of 37 °C. Three methods were used for

TABLE I

SUMMARY OF THE PURIFICATION OF CATHEPSIN FROM HUMAN ERYTHROCYTES

Stage of purification	Blood cells (1000/mm³)			Specific enzyme activity (nmoles Tyr/min per mg protein)	Yield (%)	Purification factor
	Erythro-cytes	Leuko-cytes	Reticulo-cytes			
1. Blood	4500	8	49	—	—	—
2. Suspension of erythrocytes	3900	0.1	4	—	—	—
3. Haemolysate	—	—	—	0.1	(100)	(1)
4. *n*-Butanol extraction	—	—	—	1.1	7	11
5. Chromatography on DEAE-Sephadex A-50	—	—	—	81.0	4	810
6. Chromatography on Sephadex G-100	—	—	—	200.0	3	2000

TABLE II

HYDROLYSIS OF PROTEIN SUBSTRATES BY PURIFIED CATHEPSIN

Experimental conditions: Final substrate concentration 5 mg/ml; incubation for 60 min at pH 2.8 and 37 °C. The Folin–Ciocalteu reaction was standardized against appropriate tyrosine solutions according to the original Anson method [15].

Substrate	Relative degree of hydrolysis (%)
Human haemoglobin urea denatured	100
Bovine haemoglobin denatured*	89
Human haemoglobin acid denatured	63
Bovine insulin**, acid denatured	37
Casein (Hammarsten), acid denatured	27
Bovine albumin***, acid denatured	25
Human γ-globulin†, acid denatured	10
Human albumin†, acid denatured	10

* SERVA, Heidelberg.
** VEB Berlin-Chemie.
*** Behring-Werke, Marburg.
† Forschungsinstitut für Impfstoffe Dessau.

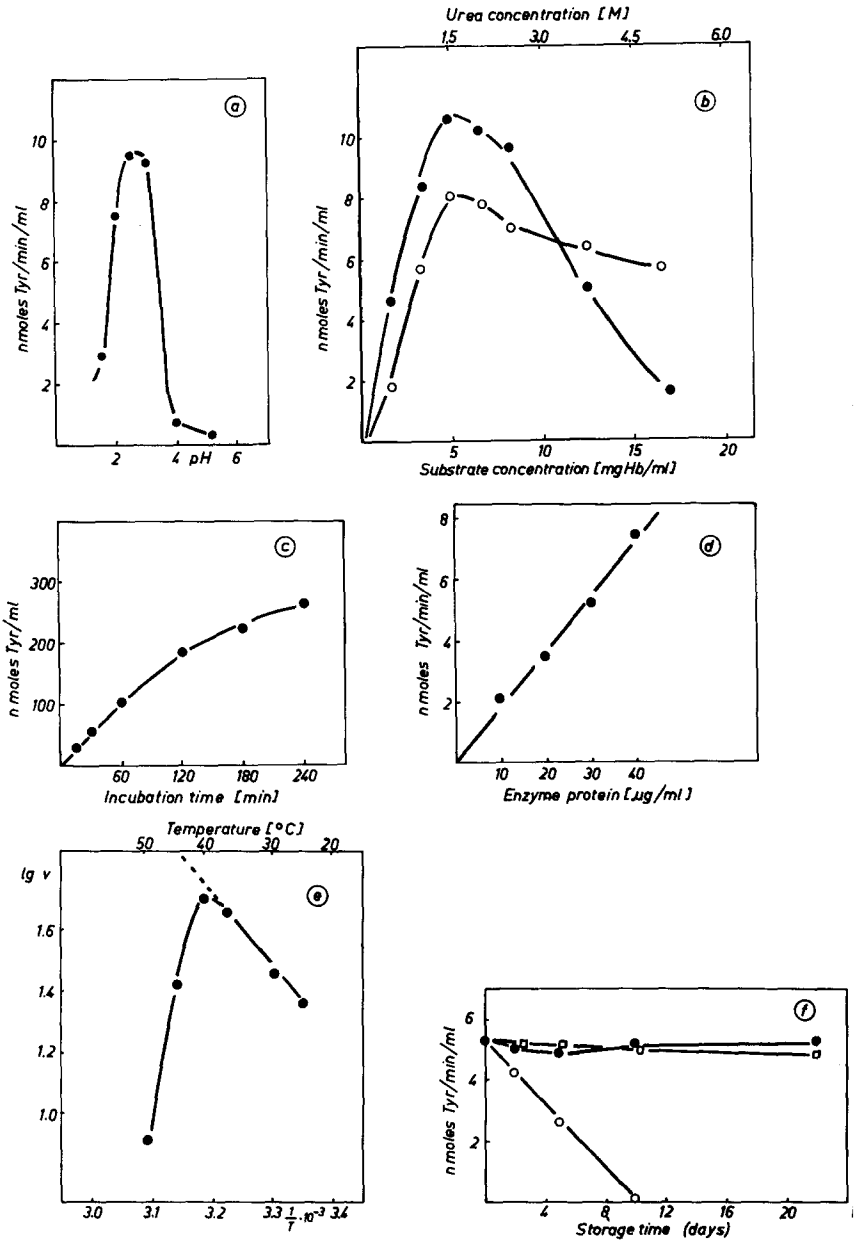

Fig. 3. Cathepsin activity as a function of (a) pH (substrate: urea-denatured human haemoglobin; different pH values established by titration with 0.1 M HCl), (b) substrate concentration (substrates: ●—●, urea-denatured human haemoglobin; ○—○, acid-denatured human haemoglobin. In the case of urea-denatured haemoglobin the relative concentrations of urea and haemoglobin remained constant), (c) incubation time, (d) enzyme concentration, (e) incubation temperature (Arrhenius plot), (f) storage conditions (●—●, −17 °C; □—□, +4 °C; ○—○, +25 °C). General assay conditions, unless otherwise stated, as in Materials and Methods, Enzyme assays, estimation of proteinase activities.

the demonstration of split products: the Folin reaction, as used in the Anson method [15], the ninhydrin procedure of Moore et al. [11], and the Kjeldahl method. The enzyme had no detectable action on stroma protein.

(2) Reaction parameters of the hydrolysis of urea-denatured haemoglobin (Fig. 3)

The purified cathepsin is optimally active at pH 2.8 (Fig. 3a). The substrate is maximally hydrolysed at a substrate concentration of 5 mg/ml. At higher haemoglobin concentrations a progressive decrease in the formation of trichloroacetic acid-soluble peptides is observed (Fig. 3b). This might be due to (1) an inhibition of the enzyme by an excess of substrate, (2) a denaturation of the enzyme by the increasing urea concentrations, and/or (3) the formation of larger, trichloroacetic acid-precipitable peptides at higher substrate concentrations. Suggestions 1 and 2 would be substantiated by the same tendency and, at the same time, the significant difference in the course of both curves. Suggestion 3 is relatively improbable because in the presence of urea one would expect more trichloroacetic acid-soluble peptides than in its absence. The time course of the enzyme reaction was linear up to 60 min (Fig. 3c). Under the conditions stated a linear dependence of enzyme activity on enzyme concentration could be established (Fig. 3d). According to the Arrhenius plot the optimal temperature is 37 °C, while maximal activity is reached at 40 °C. At higher temperatures the enzyme is rapidly inactivated (Fig. 3e). The stability of the enzyme was tested at 25, 4, and −17 °C. At 25 °C the enzyme is completely inactivated within 10 days. At 4 and −17 °C, however, the original activity was maintained for at least 3 weeks (Fig. 3f).

(3) Characterization of the active centre

Metal ions. EDTA in 1 and 10 mM concentrations had no influence at all on enzyme activity. Of the metal ions only higher concentrations of Fe^{2+}, Co^{2+} and Hg^{2+} had a certain inhibitory effect (Table III).

TABLE III

INFLUENCE OF METAL IONS AND EDTA ON CATHEPSIN ACTIVITY

Samples (0.09 ml) of enzyme solution were added to 0.01 ml of effector solution, preincubated at neutral pH for 30 min at 37 °C, mixed with 0.5 ml of urea-denatured human haemoglobin solution to give a final substrate concentration of 5 mg/ml and a final pH of 2.8 and incubated for 60 min at 37 °C. All cations used were in the chloride form.

Cation	Residual activity (%)	
	Final effector concn 1 mM	Final effector concn 10 mM
None	100	100
K^+	100	100
NH_4^+	100	100
EDTA	100	100
Na^+	98	95
Zn^{2+}	98	—
Ni^{2+}	96	—
Co^{2+}	85	—
Hg^{2+}	84	54
Fe^{2+}	83	49

TABLE IV

INFLUENCE OF SH-REAGENTS ON CATHEPSIN ACTIVITY

Experimental conditions as described in Table III.

Modifier	Residual activity (%)	
	Final effector concn 10 mM	Final effector concn 1 mM
None	100	100
PCMB	–	81
CN^-	46	60
L-Cysteine	30	95
Ascorbic acid	55	100
2-Mercaptoethanol	88	100
Dithiothreitol	10	60

Thiol reagents. The effect of preincubation with agents which predominantly affect the SH-groups of proteins is shown in Table IV. Under the conditions stated the enzyme was slightly affected by 2-mercaptoethanol, moderately influenced by ascorbic acid, CN^- and L-cysteine, and markedly inhibited by 10 mM dithiothreitol.

Biological inhibitors. Trasylol, Contrykal, soybean trypsin inhibitor, and native human serum were tested in different concentrations. The effects of these inhibitors was relatively poor (about 30% inhibition), especially in comparison with the inhibition of trypsin [22].

(4) Molecular weight

The elution volume (V_e/V_0) of the cathepsin was 1.72. According to the Whitaker plot [32] in Fig. 4 this corresponds to a molecular weight of 55 000.

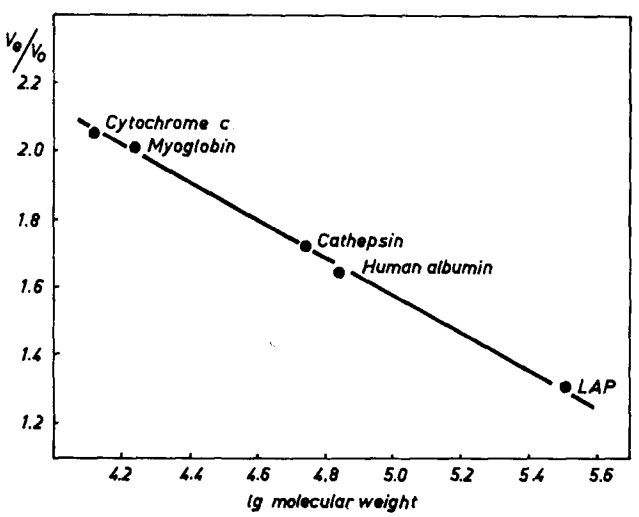

Fig. 4. Determination of molecular weight of purified cathepsin according to Whitaker [32]. Ge filtration performed as described in Materials and Methods. Molecular weights of standard substances: cytochrome *c* 13 000; myoglobin 17 000; human albumin 69 000; leucine aminopeptidase (LAP) 326 000.

TABLE V

AMINO ACID COMPOSITION OF THE PEPTIDES ISOLATED FROM THE CATHEPSIN DIGEST OF THE OXIDIZED B CHAIN OF BOVINE INSULIN

The peptides (0.3–0.4 μmole) were analysed as in Materials and Methods. The figures in parentheses represent the theoretical numbers of the amino acids in the peptide given.

Amino acid	Peptide A	Peptide B	Peptide C
Lys	0.38 (1)	—	—
His	—	—	0.70 (2)
Arg	—	0.35 (1)	—
Cys-O_3H	—	0.33 (1)	0.28 (1)
Asp	—	—	0.24 (1)
Thr	0.33 (1)	—	—
Ser	—	—	0.24 (1)
Glu	—	0.39 (1)	0.52 (2)
Pro	0.32 (1)	—	—
Gly	—	0.78 (2)	0.32 (1)
Ala	0.33 (1)	—	0.29 (1)
Val	0.03	0.39 (1)	0.40 (2)
Ile	—	—	—
Leu	—	0.35 (1)	0.83 (3)
Tyr	0.34 (1)	0.29 (1)	0.01
Phe	0.46 (1)	0.39 (1)	0.28 (1)
Residues	25–30	16–24	1–15

(5) The sensitive bond

It was investigated using the B chain of bovine insulin as substrate. 30 mg of oxidized B chain were dissolved in 5 ml of 0.10 M citrate buffer, pH 2.8. After the addition of 1 ml of purified enzyme solution the mixture was incubated at 37 °C. After 4 h the hydrolysis of the B chain had come to a standstill. To the incubation mixture 2 M NaOH was added until a pH of 8.2 was reached. Purification and chromatographic separation of the incubation mixture resulted in three peptide fractions which, according to paper chromatography, were homogeneous. The amino acid composition of these peptides is given in Table V. The result allows the identification of the peptides and, consequently, the sensitive bonds (Fig. 5). Leu^{15}–Tyr^{16} and Phe^{24}–Phe^{25} are preferentially hydrolysed. The bonds Tyr^{16}–Leu^{17} and Gly^{23}–Phe^{24} appear to be secondary sites of hydrolysis.

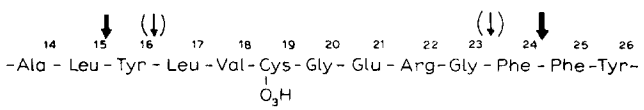

Fig. 5. Cleavage of oxidized B chain of bovine insulin by purified erythrocyte cathepsin. Arrows indicate the sensitive bonds (in parentheses secondary sites of hydrolysis).

DISCUSSION

The properties of the isolated enzyme suggest its classification as a cathepsin D. As for the molecular weight, similar values have been found for the cathepsin D

from human and chicken liver [26] and from bovine uterus [27]. Cathepsin E is a much larger molecule [28]. Similar considerations apply to the pH dependence of the isolated enzyme. For cathepsin D, values ranging from pH 3.0 to 3.5 have been described [26, 27]. Cathepsin E and pepsin would be expected to have a more acid pH optimum. As for the substrate specifity, albumin is poorly hydrolysed by the cathepsin D from human and chicken liver [26] as well as the cathepsin from human erythrocytes which gave values of 5, <1, and 25 and 10% (this paper), respectively, of those obtained with haemoglobin. Cathepsin E and pepsin, on the contrary, are highly active with albumin as substrate [28]. Furthermore, the major points of cleavage of the B chain of insulin are in accordance with those described for the cathepsins D from bovine spleen [29] and bovine uterus [27]. A similar substrate specificity has been described for the acid proteinases from bovine adrenals [30] and the cathepsin from cod muscle [31], especially Component I [32].

An acid and, in addition, a neutral proteinase in human erythrocyte membranes have recently been described by Bernacki and Bosmann [33]. According to own previous experiments [22, 34] neutral proteinase activities in haemolysates and erythrocyte stroma suspensions are a function of the percentage of leukocytes in the starting material. The danger of such a contamination is greater in the case of neutral proteinase because in human leukocytes its activity is about 20 times higher than that of acid proteinase [35]. Therefore, a careful separation of leukocytes is a prerequisite for the isolation of erythrocyte proteinases.

All cathepsins D described so far are of lysosomal origin. But the percentage of reticulocytes, which contain lysosomes, in our starting material (suspension of erythrocytes, Table I) was very low. We think, therefore, that in accordance with Bernacki and Bosmann [33] the described cathepsin D is mainly membrane bound.

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