Biochimica et Biophysica Acta, 341 (1974) 99–111

BBA 67169

IDENTIFICATION AND SEPARATION OF LYSOSOMAL CARBOXYPEPTIDASES

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(Received October 15th, 1973)

SUMMARY

1. Four distinct carboxypeptidases (EC 3.4.12.–) were shown to exist in rat liver lysosomes: cathepsin A, catheptic carboxypeptidase B, catheptic carboxypeptidase C, and catheptic carboxypeptidase G. These carboxypeptidases were distinguished on the basis of substrate specificity, thiol requirement, and separation by Sephadex G-100 column chromatography. The K_m and pH optimum for each carboxypeptidase were determined.

2. From the Sephadex G-100 elution profile, two peaks of cathepsin A activity were found. The major cathepsin A peak, cathepsin A1, was further purified by ion-exchange chromatography on DEAE-cellulose.

3. The highly purified cathepsin A1 fraction hydrolyzed *N*-benzyloxycarbonyl (Cbz)–Glu–Phe, Cbz–Gly–Phe, and Ac–Phe–Tyr, which suggests that these hydrolytic activities were due to the same enzyme.

4. Catheptic carboxypeptidase B and catheptic carboxypeptidase G eluted from the Sephadex G-100 column along with cathepsin B2. This suggests that cathepsin B2 may have carboxypeptidase activity.

5. The purification of the lysosomal carboxypeptidases over homogenate activities was: cathepsin A1, 1280-fold; catheptic carboxypeptidase B, 220-fold; and catheptic carboxypeptidase G, 920-fold. Catheptic carboxypeptidase C was purified 16-fold over light-mitochondrial supernatant activity.

INTRODUCTION

Several lysosomal carboxypeptidases (EC 3.4.12.–) active in the acid pH region have been described in the literature, including cathepsin A [1–3], catheptic carboxypeptidase A [4, 5], catheptic carboxypeptidase B [4–6], catheptic carboxypeptidase C [7], *N*-acetyl-L-phenylalanyl-L-tyrosine (APAT) hydrolase [8], a tripeptide carboxypeptidase [9, 10], angiotensinase [11, 12], and bradykininase [6]. Another enzyme, peptidyldipeptide hydrolase or angiotensin-converting enzyme, is localized in pinocytotic vesicles of lung vascular endothelium [13], which indicates a possible lysosomal origin. The lysosomal localization of some of these enzymes has not been determined unequivocally. These enzymes may not be distinct entities, since the substrate specificities have not been determined. Only cathepsin A has been purified and charac-

terized to any extent [1–3]. Therefore, the existence of additional, unknown lysosomal carboxypeptidases is a distinct possibility.

This study presents the results of a systematic search for carboxypeptidase activity in rat liver lysosomes, and separation and some characterization of these carboxypeptidases.

METHODS AND MATERIALS

Tissue fractionation

Rat liver lysosomes were obtained according to the method of Ragab et al. [14]. The lysosomal soluble fraction used as a source of carboxypeptidases was prepared as described earlier [15]. For subcellular-localization studies, rat livers were homogenized and fractionated into subcellular components according to the method of de Duve et al. [16]. For the chromatographic separation of the carboxypeptidases, the starting material was the light-mitochondrial fraction described by Ragab et al. [14].

Assay of lysosomal carboxypeptidases

The lysosomal carboxypeptidases were assayed in 0.5-ml volumes that included 50–100 μl of a lysosomal soluble fraction at 37 °C under the following conditions: (i) cathepsin A with either 15 mM *N*-benzyloxycarbonyl(Cbz)-α-L-glutamyl–L-phenylalanine in 50 mM citrate–phosphate buffer (pH 5.5) for 30 min or 25 mM Cbz-glycyl-L-phenylalanine in 50 mM citrate–phosphate buffer (pH 5.8) for 30 min; (ii) Ac-Phe–Tyr hydrolase with 10 mM *N*-acetyl-v-phenylalanyl-v-tyrosine in 50 mM citrate–phosphate buffer (pH 5.8) for 60 min; (iii) catheptic carboxypeptidase A with 15 mM Cbz-α-L-glutamyl-L-tyrosine and 40 mM dithioerythritol in 100 mM citrate–phosphate buffer (pH 3.5) for 60 min; (iv) catheptic carboxypeptidase B with 60 mM hippuryl-arginine and 60 mM dithioerythritol in 50 mM citrate–phosphate buffer (pH 5.5) for 30 min; (v) catheptic carboxypeptidase C with 12 mM Cbz-L-prolyl-L-phenylalanine in 20 mM citrate–phosphate buffer (pH 5.0) for 60 min; and (vi) catheptic carboxypeptidase G with 25 mM Cbz-glycyl-α-L-glutamic acid and 40 mM dithioerythritol in 50 mM citrate–phosphate buffer (pH 5.0) for 60 min. Peptidyldipeptide hydrolase activity was determined with the conditions described by Cushman and Cheung [17]. All reactions were stopped by the addition of 0.5 ml of 10% trichloroacetic acid. After centrifugation in a clinical centrifuge to remove any precipitate, a portion of the supernatant was suitably diluted with deionized water. The concentration of amino acid liberated was determined by the fluorometric method of Taylor and Tappel [15], with the appropriate amino acid as a standard.

Assay of other lysosomal proteases and peptidases

Cathepsin B1 was analyzed by the method of de Lumen and Tappel [18]. Cathepsin B2 was assayed with the reaction conditions described by de Lumen and Tappel [19]. The liberated NH_4^+ ions were measured by the ninhydrin procedure of Moore and Stein [20]. Cathepsin C was assayed by the method of Metrione et al. [21] under the reaction conditions described by de Lumen and Tappel [19]. Cathepsin D activity was measured according to the procedure of Misaka and Tappel [22]. Insulin B-chain hydrolysis was assayed in 0.5-ml reaction volumes that contained 2.5 mg of insulin B chain and 40 mM dithioerythritol in 50 mM sodium acetate buffer (pH 5.0)

and appropriate amounts of various column fractions. The reactions were carried out at 37 °C for 30 min and stopped with 0.5 ml of 10% trichloroacetic acid. After centrifugation in a clinical centrifuge, a portion of the supernatant was removed and the amino acid concentration was determined by the ninhydrin method [20]. Protein was determined according to the method of Lowry et al. [23] as modified by Miller [24]. Acid phosphatase was determined by the method of Gianetto and de Duve [25].

Separation of lysosomal carboxypeptidases

Light-mitochondrial fractions were frozen and thawed 5 times, centrifuged at 9500 × *g* for 20 min, and the supernatant was dialyzed overnight at 4 °C against 50 mM sodium acetate buffer (pH 5.3). The dialyzed preparation was centrifuged at 9500 × *g* for 20 min and the supernatant was further centrifuged at 100 000 × *g* for 60 min in a Beckman model L ultracentrifuge to obtain the light-mitochondrial supernatant fraction. An $(NH_4)_2SO_4$ fractionation was carried out on the light-mitochondrial supernatant fraction with the 30–70% $(NH_4)_2SO_4$ fraction being retained. The 30–70% $(NH_4)_2SO_4$ fraction was dialyzed overnight against four changes of 5 mM sodium acetate buffer (pH 5.3) that contained 250 mM sucrose, 20 mM 2-mercaptoethanol, and 150 mM NaCl. This dialyzed fraction was placed on a 5.0 cm × 90 cm Sephadex G-100 (Pharmacia) column and eluted with 5 mM sodium acetate buffer (pH 5.3) that contained 250 mM sucrose, 20 mM 2-mercaptoethanol, and 150 mM NaCl. The column was eluted at a flow rate of approx. 1.0 ml/min and fractions were collected at 10-min intervals. The column fractions were analyzed for the activities of cathepsin A, catheptic carboxypeptidase A, catheptic carboxypeptidase B, catheptic carboxypeptidase C, catheptic carboxypeptidase G, Ac-Phe–Tyr hydrolase, cathepsin B1, cathepsin B2, cathepsin C, and cathepsin D, and for insulin B-chain hydrolysis. Protein was monitored by absorbance at 280 nm.

The major cathepsin A peak was concentrated in an Amicon Diaflo apparatus with a PM-30 membrane. The concentrated fraction was purified further by ion-exchange chromatography on a 0.9 cm × 30 cm Whatman DE-32 cellulose column. Initial elution of unbound protein with 65 ml of 5 mM sodium acetate buffer (pH 5.3) that contained 250 mM sucrose, 20 mM 2-mercaptoethanol, and 30 mM NaCl was followed by elution with 200 ml of a linear gradient of 30–400 mM NaCl. The column was pumped at a flow rate of 0.5 ml/min, and fractions were collected at 7-min intervals. The fractions were analyzed for the activities of cathepsin A, Ac-Phe–Tyr hydrolase, catheptic carboxypeptidase C, and cathepsin C. Protein was monitored by absorbance at 280 nm.

Gel electrophoresis was done by a modification of the procedure described by Davis [26]. The running-gel concentration was 7% acrylamide buffered at pH 8.9, the stacking-gel concentration was 2.5% acrylamide buffered at pH 6.7, and the reservoir buffer was 50 mM Tris–glycine (pH 8.3). The sample (25–80 μg of protein in 10% sucrose) was layered directly onto the stacking gel. Electrophoresis was run for 45 min at 4 °C with a current of 5 mA per tube. Staining with Coomassie Blue and destaining were done by the method of Fishbein [27].

Sources of substrates and reagents

Cbz-Gly–Arg, Cbz-Gly–L-Phe, Cbz-Gly–D-Phe, Cbz-Gly–Tyr, Cbz-Gly–Glu, Cbz-Gly–Trp, Cbz-Gly–Gly, Cbz-Gly–His, Cbz-Phe–Gly, Cbz-Gln–Gly, Cbz-Pro–

Gly, Cbz-Ser–Gly, Cbz-Met–Gly, Cbz-Glu–Gly, Cbz-Trp–Gly, Cbz-Leu–Gly, Cbz-His–Gly, and Ac-Phe–Tyr were obtained from Fox Chemical Co., Los Angeles, Calif.; Cbz-Gly–Met, Cbz-Gly–Leu, Cbz-Gly–Ser, Cbz-Gly–Sar, Cbz-β-Ala–Gly, Cbz-Pro–Phe, Cbz-Glu–Phe, and hippuryl-Arg from Cyclo Chemical Corp., Los Angeles, Calif.; Cbz-Glu–Tyr, Cbz-Glu–Phe, Bz-Arg–amide, and Bz-Arg–β-naphthylamide from Schwarz–Mann, Orangeburg, N. J.; Gly–Tyr $NH_2 \cdot HCl$ from Sigma Chemical Co., St. Louis, Mo.; and hippuryl-His–Leu from Research Plus Laboratories, Inc., Denville, N. J. Sources of reagents for fluorometric amino acid analysis have been given previously [15]. Ninhydrin and hydrindantin were obtained from Eastman Organic, Rochester, N. Y. and Sigma Chemical Co., respectively.

RESULTS

Identification of lysosomal carboxypeptidase activity

Table I gives the rate of hydrolysis of a number of N-blocked dipeptides by a rat liver lysosomal soluble fraction in the presence and absence of the sulfhydryl activator, dithioerythritol. In the absence of sulfhydryl activator, N-blocked dipeptides with bulky or hydrophobic amino acids in the C-terminal or penultimate positions were hydrolyzed most rapidly. With this group of substrates, sulfhydryl activation was found only with Cbz-Glu–Phe, Cbz-Gly–Trp, and Cbz-Gly–Phe, which indicates the possible presence of a sulfhydryl-activated enzyme with specificity for hydrophobic amino acids. With sulfhydryl activator present, large increases in the rates of hydrolysis of N-blocked dipeptides with basic amino acids or acidic amino acids occurred. Some N-blocked dipeptides, such as Cbz-Gly–D-Phe, Cbz-Pro–Gly, and Cbz-Ser–Gly, were not hydrolyzed at an appreciable rate. This evidence suggests the presence of two or three lysosomal carboxypeptidases. One carboxypeptidase has specificity for hydrophobic amino acids and is not activated by sulfhydryl compounds. Two sulfhydryl-activated carboxypeptidases, one with specificity for basic amino acids and the other with specificity for acidic amino acids, may exist. However, these two carboxypeptidases may not be distinguished merely on the basis of substrate specificity. Also, it should be pointed out that these model substrates are not necessarily specific for carboxypeptidases, and some of the activity detected could be that of endopeptidases.

The presence of other carboxypeptidases was not eliminated by this experiment. Catheptic carboxypeptidase C activity in the lysosomal soluble fraction was demonstrated with Cbz-Pro–Phe as the substrate. There was no evidence for the existence of peptidyldipeptide hydrolase in rat liver lysosomes. In agreement with the results of Mellors [5], catheptic carboxypeptidase A even in crude preparation was unstable after several hours of storage at 0 °C.

Effect of substrate concentration and pH on lysosomal carboxypeptidases

Table II gives the pH optima, the maximal specific activity at each pH optimum, and the K_m values for several classes of lysosomal carboxypeptidases. The highest rate of hydrolysis occurred in the acid pH range, particularly between pH 5.0 and 6.0 for all the carboxypeptidases. No activity at pH 3.5 was found for catheptic carboxypeptidase A, which indicates this enzyme was not present in these liver lysosomal preparations. The pH 5.5 optimum for catheptic carboxypeptidase A was likely

TABLE I

IDENTIFICATION OF LYSOSOMAL CARBOXYPEPTIDASE ACTIVITY

The 0.5-ml reaction mixtures consisted of 25 mM N-blocked dipeptide, except with Ac-Phe–Tyr, Cbz-Glu–Phe, and Cbz-Glu–Tyr where 10 mM, 15 mM, and 15 mM substrate concentrations were used, respectively, in 50 mM citrate–phosphate buffer (pH 5.5). Reactions were carried out for 60 min at 37 °C. Hydrolysis was determined by the fluorometric method described in Methods and Materials.

Dipeptide derivative	Specific activity (nmoles amino acid/min per mg protein)	
	−SH	+SH*
Cbz-Gly–L-Phe	160	217
Cbz-Gly–D-Phe	10	2
Cbz-Glu–Phe	80	179
Cbz-Glu–Tyr	91	88
Cbz-Gly–Tyr	85	117
Ac-Phe–Tyr	196	129
Cbz-Gly–Trp	27	113
Cbz-Gly–Met	143	167
Cbz-Gly–Leu	196	204
Cbz-Gly–His	10	86
Cbz-Gly–Arg	9	43
Bz-Gly–Arg	10	93
Cbz-Gly–Glu	11	87
Cbz-Gly–Ser	30	130
Cbz-Gly–Gly	1	27
Cbz-Sar–Gly	2	2
Cbz-Pro–Gly	0	0
Cbz-Phe–Gly	246	238
Cbz-Tyr–Gly	105	93
Cbz-Leu–Gly	71	71
Cbz-Met–Gly	97	111
Cbz-Trp–Gly	52	52
Cbz-His–Gly	14	14
Cbz-Gln–Gly	6	14
Cbz-Glu–Gly	0	14
Cbz-Ser–Gly	0	4

* 40 mM dithioerythritol was included in the reaction mixtures.

owing to the action of cathepsin A, which readily hydrolyzes Cbz-Glu–Tyr [1]. The activity at pH 7.0 with Cbz-Gly–Glu may reflect contamination of the lysosomal soluble fraction with microsomal fragments that are known to possess neutral protease and peptidase activity [28]. The K_m values for the lysosomal carboxypeptidases ranged from 1.8 to 14.6 mM. From these data, it appears that lysosomal soluble fractions possess considerable capacity for carboxypeptidase-like activity. K_m and V values on impure enzyme preparations are approximations of actual values. For accurate K_m and V values, each of the carboxypeptidases will have to be purified.

TABLE II

pH OPTIMA AND K_m VALUES FOR LYSOSOMAL CARBOXYPEPTIDASES

Enzyme	pH optimum*	Maximal specific activity (nmoles amino acid/min per mg protein)	K_m** (mM)
Cathepsin A (Cbz-Glu–Phe)	5.0–5.5	376	7.4
Cathepsin A (Cbz-Gly–Phe)	5.8	479	5.4
Ac-Phe–Tyr hydrolase	5.8	62	12.0
Catheptic carboxypeptidase A	5.5	541***	—
Catheptic carboxypeptidase B	5.5	306	10.3
Catheptic carboxypeptidase C	5.0	81	1.8
Catheptic carboxypeptidase G	5.0	88	14.6
	7.0	36***	—

* pH-optima determinations were made by the assays described in Methods and Materials with 50 mM citrate–phosphate buffer (pH 3.0–5.8); 50 mM potassium phosphate buffer (pH 6.0–8.5); and 50 mM carbonate buffer (pH 9.0–10.5).

** K_m determinations were made with the Lineweaver–Burk plot using linear-regression analysis.

*** In the cases where K_m values are not given, the activities given are initial reaction rates.

Effect of enzyme concentration and time of hydrolysis on lysosomal carboxypeptidase activity

The effects of enzyme concentration and time of hydrolysis were determined for cathepsin A with Cbz-Glu–Phe and Cbz-Gly–Phe, catheptic carboxypeptidase G, catheptic carboxypeptidase C, catheptic carboxypeptidase B, and Ac-Phe–Tyr hydrolase with the assay conditions described in Methods and Materials. With the exception of Ac-Phe–Tyr hydrolase, the rate of hydrolysis was proportional to enzyme concentration in the range of 0–300 μg of protein, and linear with time of hydrolysis up to the time limit of 60 min normally used for assay. For Ac-Phe–Tyr hydrolase, solubility limitations of Ac-Phe–Tyr caused a non-linear rate of hydrolysis with increasing enzyme concentration and increasing time of hydrolysis. The data on hydrolysis of this substrate are useful only in qualitative terms.

Effect of sulfhydryl compounds on catheptic carboxypeptidase B and catheptic carboxypeptidase G

Sulfhydryl activator was required for the optimal rate of activity of catheptic carboxypeptidase B and of catheptic carboxypeptidase G. In studies of sulfhydryl activation, maximum activation occurred at 4 mM dithioerythritol for catheptic carboxypeptidase B and catheptic carboxypeptidase G.

Subcellular distribution of the acidic carboxypeptidases

The distribution of the acidic carboxypeptidases and acid phosphatase among the subcellular fractions is shown in Fig. 1. The carboxypeptidases exhibited the highest relative specific activity in the light-mitochondrial fraction, as did the lysosomal marker enzyme, acid phosphatase. Catheptic carboxypeptidase B, catheptic carboxypeptidase G, and Ac-Phe–Tyr hydrolase had appreciable activities in the soluble fraction, which also indicates either the presence of similar peptidases in the soluble fraction or some specific release of these enzymes from the lysosomes during homo-

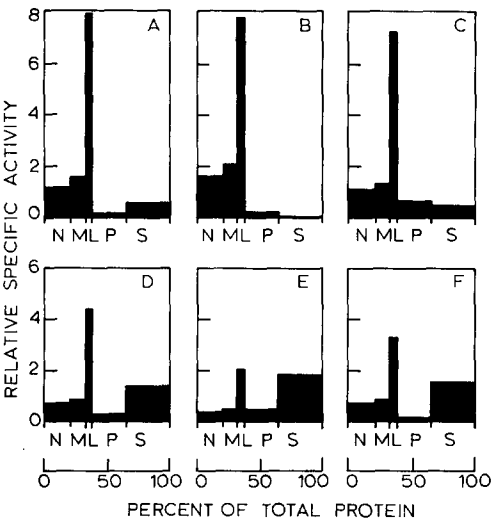

Fig. 1. Subcellular distribution of A, cathepsin A (Cbz-Glu–Phe); B, cathepsin A (Cbz-Gly–Phe); C, acid phosphatase; D, catheptic carboxypeptidase B; E, catheptic carboxypeptidase G; and F, Ac-Phe–Tyr hydrolase. The fractions are: N, nuclear; M, heavy-mitochondrial; L, light-mitochondrial-lysosomal; P, microsomal; and S, soluble. Relative specific activity is defined as percent total activity/percent total protein.

genization and preparation of the subcellular fractions. Another possible explanation would be adsorption of soluble carboxypeptidases to lysosomes, although the presence of acidic carboxypeptidases in the soluble fraction would be difficult to rationalize. The distribution profile of catheptic carboxypeptidase C is not given because the activity of this enzyme could not be determined in the homogenate or the light-mitochondrial fraction, perhaps owing to the presence of an inhibitor.

Separation of lysosomal carboxypeptidases

Chromatography of the 30–70% $(NH_4)_2SO_4$ fraction on Sephadex G-100 resulted in the separation of the major classes of lysosomal carboxypeptidases, as shown in Fig. 2. Two peaks of cathepsin A were eluted, one in the void volume and a more active peak centered in Fractions 86–93. Ac-Phe–Tyr hydrolase eluted from the column in a similar manner, which suggests that the carboxypeptidase substrates Cbz-Glu–Phe, Cbz-Gly–Phe, and Ac-Phe–Tyr are hydrolyzed by the same enzyme. Catheptic carboxypeptidase C eluted between the void volume and the major cathepsin A peak. In accordance with the results of McDonald et al. [7], cathepsin C and catheptic carboxypeptidase C were almost inseparable on Sephadex G-100. Another peak of carboxypeptidase activity that contained catheptic carboxypeptidase B and catheptic carboxypeptidase G eluted after the major cathepsin A peak and coincidentally with the highest activity peak of cathepsin B2. Insulin B-chain hydrolysis, included as a marker for general peptidase activity, was localized in the region of cathepsin C, cathepsin D and cathepsin A elution. Catheptic carboxypeptidase A activity was not found in any fraction beyond the light-mitochondrial supernatant.

Fractions 86–93, which contained the major peak of cathepsin A, were com-

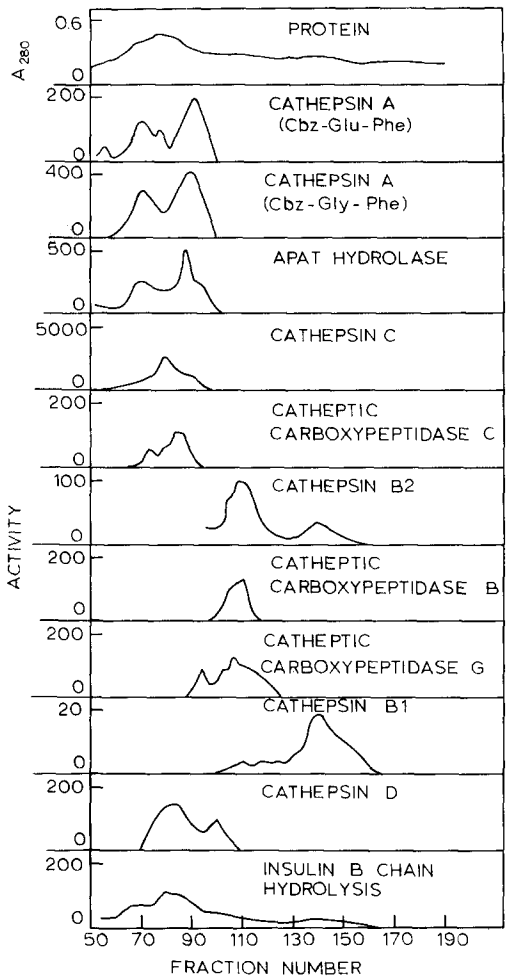

Fig. 2. Sephadex G-100 chromatography of the 30–70% $(NH_4)_2SO_4$ fraction of soluble rat liver lysosomes. The elution buffer was 5 mM acetate (pH 5.3) with 250 mM sucrose, 20 mM 2-mercaptoethanol, and 150 mM NaCl. Protein was monitored by absorbance at 280 nm. Activities are expressed as nmoles amino acid released per min per ml except that cathepsin C is expressed as nmoles tyrosine hydroxamate equivalents produced per min per ml, cathepsin B1 as nmoles β-naphthylamine released per min per ml, and cathepsin B2 as nmoles NH_3 released per min per ml.

bined, concentrated, and subjected to ion-exchange chromatography on DEAE-cellulose. The elution patterns are shown in Fig. 3. Cathepsin A activity against Cbz-Glu–Phe and Cbz-Gly–Phe, along with Ac-Phe–Tyr hydrolase, was eluted by 150 mM NaCl in a peak with high specific activity. The known peptidases that contaminated the cathepsin A peak from the Sephadex G-100 column fractionation were eluted in the initial buffer wash of the DEAE-cellulose column and were well separated from cathepsin A. The cathepsin A peak from DEAE-cellulose chromatography showed one major band and three minor bands when subjected to disc-gel electrophoresis at pH 8.9. Partially purified cathepsin A from Sephadex G-100 chromato-

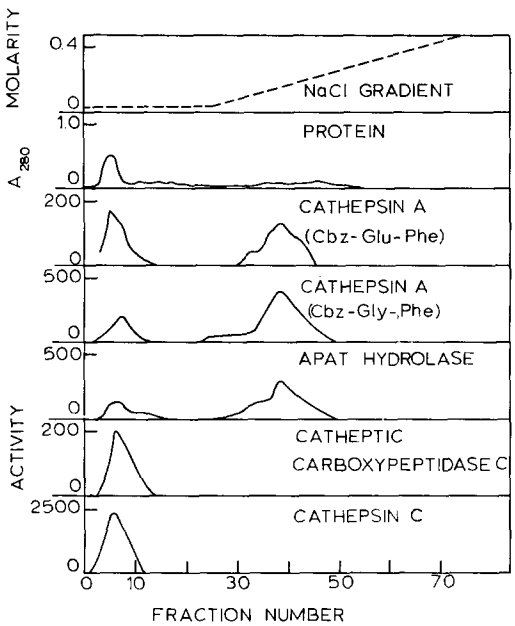

Fig. 3. DEAE-cellulose chromatography of pooled, concentrated Fractions 86–93 from the Sephadex G-100 separation. A linear NaCl gradient in 5 mM sodium acetate buffer (pH 5.3) with 250 mM sucrose and 20 mM 2-mercaptoethanol was used to elute the protein, which was monitored by 280 nm absorbance. Activities are defined as in Fig. 2.

graphy (Fractions 86–93) did not show distinct bands on electrophoresis. Electrophoresis shows that the cathepsin A peak from DEAE-cellulose chromatography was not homogeneous; however, it was not contaminated with other known proteases and peptidases.

The resulting purification of the lysosomal carboxypeptidases is shown in Table III. After DEAE-cellulose chromatography, there was a 310-fold purification of cathepsin A over the homogenate with Cbz-Glu–Phe as the substrate and 1280-fold purification with Cbz-Gly–Phe as the substrate, while there was a 2130-fold purification of Ac-Phe–Tyr hydrolase. Through the Sephadex G-100 purification step, the other carboxypeptidases had been purified as follows: catheptic carboxypeptidase B, 220-fold over homogenate; catheptic carboxypeptidase G, 920-fold over homogenate; and catheptic carboxypeptidase C, 16-fold over light-mitochondrial supernatant.

DISCUSSION

Lysosomes are well known to contain endopeptidases, such as cathepsin D and cathepsin B1, that hydrolyze proteins to yield peptide products of various sizes. The presence of lysosomal exopeptidases to hydrolyze further the products of endopeptidase action is less well substantiated, although cathepsin C, a dipeptidylpeptide hydrolase, has been extensively purified and characterized [29]. The only previously known lysosomal carboxypeptidase was cathepsin A [2]. This study established the

TABLE III

PURIFICATION OF LYSOSOMAL CARBOXYPEPTIDASES

Details are described under Methods and Materials.

Enzyme and fraction	Total protein (mg)	Total activity (nmoles product/min)	Specific activity (nmoles product/min per mg protein)	Yield (percent)	Purification (-fold)
Cathepsin A (Cbz-Glu–Phe)					
Homogenate	235 000	$4.2 \cdot 10^6$	17.7	100	1
Light-mitochondrial supernatant	520	$1.5 \cdot 10^5$	287	3.6	16
30–70% $(NH_4)_2SO_4$ fraction	100	$1.3 \cdot 10^5$	1290	3.1	73
Sephadex G-100 fraction Nos 86–93	5.5	$1.5 \cdot 10^4$	2830	0.4	160
DEAE-cellulose fraction Nos 37–42	0.41	$2.3 \cdot 10^3$	5440	0.06	310
Cathepsin A (Cbz-Gly–Phe)					
Homogenate	235 000	$3.1 \cdot 10^6$	13.2	100	1
Light-mitochondrial supernatant	520	$1.9 \cdot 10^5$	360	6.0	27
30–70% $(NH_4)_2SO_4$ fraction	100	$1.2 \cdot 10^5$	1170	3.8	89
Sephadex G-100 fraction Nos 86–93	5.5	$2.9 \cdot 10^4$	5290	0.9	400
DEAE-cellulose fraction Nos 37–42	0.41	$6.9 \cdot 10^3$	16 800	0.2	1280
Ac-Phe–Tyr Hydrolase					
Homogenate	235 000	$1.2 \cdot 10^6$	5.2	100	1
Light-mitochondrial supernatant	520	$1.2 \cdot 10^5$	230	10	44
30–70% $(NH_4)_2SO_4$ fraction	100	$6.6 \cdot 10^4$	660	5.4	130
Sephadex G-100 fraction Nos 86–93	5.5	$1.9 \cdot 10^4$	3500	1.6	680
DEAE-cellulose fraction Nos 37–42	0.41	$4.6 \cdot 10^3$	11 100	0.4	2130
Catheptic carboxypeptidase B					
Homogenate	235 000	$2.2 \cdot 10^6$	9.4	100	1
Light-mitochondrial supernatant	520	$1.1 \cdot 10^5$	215	5.1	23
30–70% $(NH_4)_2SO_4$ fraction	100	$5.1 \cdot 10^4$	510	2.3	54
Sephadex G-100 fraction No. 110	0.6	$2.0 \cdot 10^4$	2100	0.9	220
Catheptic carboxypeptidase C					
Homogenate	235 000	–	–	–	–
Light-mitochondrial supernatant	520	$4.9 \cdot 10^4$	93	–	1
30–70% $(NH_4)_2SO_4$ fraction	100	$3.2 \cdot 10^4$	320	–	3
Sephadex G-100 fraction No. 85	0.8	$1.1 \cdot 10^3$	1460	–	16
Catheptic carboxypeptidase G					
Homogenate	235 000	$5.2 \cdot 10^5$	2.2	100	1
Light-mitochondrial supernatant	520	$3.6 \cdot 10^4$	68	6.9	31
30–70% $(NH_4)_2SO_4$ fraction	100	$5.3 \cdot 10^4$	530	10	240
Sephadex G-100 fraction No. 106	0.6	$1.26 \cdot 10^3$	2000	0.2	920

presence of a wide variety of carboxypeptidase activities in lysosomes. These carboxypeptidases have been distinguished on the basis of substrate specificity, sulfhydryl activation, and separability by column chromatography. At least four different carboxypeptidases are localized in rat liver lysosomes.

Cathepsin A has been defined classically by its ability to hydrolyze Cbz-Glu–Phe [30]. However, the evidence from these experiments suggests that cathepsin A may be responsible for the hydrolysis of Cbz-Gly–Phe, Ac-Phe–Tyr, and the hydrolysis of model substrates that contain hydrophobic amino acids. Two peaks of cathepsin A activity were eluted from the Sephadex G-100 column, which suggests the presence of two forms of cathepsin A in rat liver lysosomes. The major cathepsin A peak was purified further by ion-exchange chromatography. This highly purified cathepsin A fraction maintained activity toward Cbz-Glu–Phe, Cbz-Gly–Phe, and Ac-Phe–Tyr. However, it should be pointed out that the yield of total activity through the purification scheme for these three hydrolase activities varied. This indicates that the multiple forms of cathepsin A likely have different specificities toward these three substrates. The presence of more than two forms of cathepsin A is possible. The Cbz-Glu–Phe hydrolase activity is unstable compared with Cbz-Gly–Phe or Ac-Phe–Tyr hydrolase activity, which suggests the possible presence of an unstable enzyme with high specificity for Cbz-Glu–Phe. The question of multiple forms of cathepsin A will require more research for proper definition. Further work is also needed to establish unequivocally that the activity toward Cbz-Glu–Phe, Cbz-Gly–Phe, and Ac-Phe–Tyr is due to the same enzyme. However, since at least two forms are obvious, we suggest the terminology cathepsin A1 for the highly purified cathepsin A preparation described here. Cathepsin A1 has been further characterized [31].

A second type of lysosomal carboxypeptidase, termed catheptic carboxypeptidase B, was identified in this study. This carboxypeptidase is responsible for the sulfhydryl-dependent hydrolysis of N-blocked dipeptides that have basic amino acids in the C-terminal position. Catheptic carboxypeptidase B has been previously identified in beef spleen lysosomal preparations [4], and it has been implicated in the partial hydrolysis of bradykinin [6]. This study established the localization of a similar enzyme in rat liver lysosomes. In this work catheptic carboxypeptidase B could not be separated from cathepsin B2 and catheptic carboxypeptidase G by Sephadex G-100 chromatography. Since the discovery of cathepsin B1 as the major papain-like endopeptidase in lysosomes [32], the function of cathepsin B2 has been unclear. These initial chromatographic separation experiments suggest that cathepsin B2 may have carboxypeptidase B-like activity. The presence in the same fraction with cathepsin B2 of the sulfhydryl-dependent catheptic carboxypeptidase G, defined by its specificity for acidic amino acids, may suggest that cathepsin B2 has activity of this type also. Work is proceeding to further characterize these three activities.

The fourth type of carboxypeptidase present in lysosomes is catheptic carboxypeptidase C, which was first described by McDonald et al. [7] as a contaminant in their purified cathepsin C preparations. This carboxypeptidase does not hydrolyze all Cbz-Pro–X substrates since Cbz-Pro–Gly was not hydrolyzed by rat liver lysosomal soluble fraction. Catheptic carboxypeptidase C is probably identical with an enzyme known as angiotensinase [11] or angiotensinase C [12]. We suggest that catheptic carboxypeptidase C is a better name, since the enzyme would likely cleave the terminal Pro–Phe bond in peptides other than angiotensin II.

The sulfhydryl-dependent catheptic carboxypeptidase A that has been reported in beef spleen lysosomal preparations [4] was found also in rat liver lysosomes. However, this enzyme was not stable during purification and was not studied.

The presence of carboxypeptidase activity in lysosomes has great significance

in explaining the rate and extent of protein hydrolysis by lysosomes. Coffey and de Duve [33] noted that after extensive hydrolysis of globin by lysosomes the major products were dipeptides and amino acids. The dipeptides arise from cathepsin C action, while the amino acids are likely products of other carboxypeptidase, aminopeptidase, and dipeptidase action. The rate of protein hydrolysis in lysosomes should be increased by the presence of exopeptidases that act in concert with endopeptidases. It has been shown that cathepsins A, B, and D can act in a concerted manner in the hydrolysis of hemoglobin and insulin [34]. Much research is needed to determine the nature and specificity of these lysosomal carboxypeptidases and the presence of other lysosomal exopeptidases and dipeptidases.

ACKNOWLEDGMENT

This investigation was supported by United States Public Health Service Research Grant AM 06424 from the National Institute of Arthritis and Metabolic Diseases.

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