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Purification and Characterization of Dipeptidyl Peptidase I from Human Spleen[1]

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The lysosomal hydrolase, dipeptidyl peptidase I (DPPI), was purified from human spleen and its enzymatic activity characterized. The enzyme was purified to apparent homogeneity by a combination of differential pH solubility, heat-treatment, affinity chromatography on concanavalin A-agarose and *p*-hydroxymercuribenzoate-agarose, and gel filtration chromatography on Sephacryl S-300. This procedure resulted in a 1100-fold purification of DPPI protein with a yield of approximately 2% of the total DPPI activity. The enzyme was characterized as a glycoprotein with a p*I* of 5.4, a molecular mass of 200,000 Da as determined by gel filtration under nondenaturing conditions, and a subunit size of 24,000 Da. Amino acid sequence analysis of peptides isolated from cyanogen bromide and trypsin digests of the 24,000-Da subunit revealed extensive sequence similarity between human and rat DPPI. Purified DPPI exhibited both hydrolytic and transpeptidase (polymerase) activity. DPPI exhibited activity against a variety of dipeptide substrates including peptides with either nonpolar or polar residues in the P_1 position. In contrast to the reported substrate specificity of bovine and murine DPPI, the human enzyme exhibited a modest preference for peptides with nonpolar residues in the P_1 position. DPPI content was found to be highest among cytotoxic lymphocytes and myeloid cells. The high level of DPPI expression in these cell populations correlates with their sensitivity to the toxic effects of leucyl-leucine methyl ester, a substrate for DPPI.

Dipeptidyl peptidase I (DPPI,[3] previously known as cathepsin C) is a lysosomal hydrolase capable of sequentially removing dipeptides from the amino-terminus of suitable substrates (1–10). While early studies demonstrated that DPPI-like activity is present in a variety of tissues, the highest level of activity was observed in lymphoid tissues such as spleen (11). Recently, DPPI activity has been reported to be present at significantly higher levels in cytotoxic lymphocytes and myeloid cells than in B cells or T helper cells (12–16). Moreover, DPPI has been shown to catalyze the polymerization of L-leucyl-leucine methyl ester (Leu–Leu-OMe), which selectively kills cytotoxic lymphocytes and myeloid cells that contain high concentrations of DPPI, while having no adverse effect on T helper cells, B cells, or a variety of other cell types of non-bone marrow origin. These results suggest that DPPI is a useful marker for cells with cytolytic potential, although its function in these cells remains to be elucidated.

Studies exploring the mechanism of selective Leu–Leu-OMe toxicity for DPPI-enriched cytotoxic lymphocytes and myeloid cells have been performed with human leukocytes. However, previous structural and functional characterization of DPPI has been limited to partially purified preparations obtained from lymphoid tissues of other species or more recently to enzyme isolated from rat liver.

MATERIALS AND METHODS

Dipeptidyl peptidase I assay. DPPI was routinely assayed by the hydrolysis of glycylphenylalanyl-β-naphthylamide in an assay mixture containing 100 μM substrate, 50 mM sodium acetate–acetic acid, pH 5.5, 30 mM NaCl, 1 mM DTT, and 0.5 mM EDTA in a volume of 600 μl.

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[3] Abbreviations used: DPPI, dipeptidyl peptidase I; Leu–Leu-OMe, leucyl-leucine methyl ester; -βNA, -β-naphthylamide; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; -MNA, -4-methoxy-β-naphthylamide; PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; BLT, benzyloxycarbonyl-L-lysine thiobenzyl ester; pHMB, *p*-hydroxymercuribenzoic acid; CBZ, benzyloxycarbonyl; AMC, 7-amino-4-methylcoumarin; AFU, arbitrary fluorescence unit; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; FITC, fluorescein isothiocyanate.

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After incubation for 20 min at 37°C, the reaction was stopped by the addition of 500 μl of 50 mM glycine–NaOH, pH 10.5. Substrate hydrolysis was monitored by the fluorescence of β-naphthylamine at 335 nm excitation, 405 nm emission with enzyme activity expressed as nanomoles β-naphthylamine released per minute. The selection of DPPI-enriched fractions during the purification of the enzyme was based on the functional activity determined by this assay. Data presented represent the average of duplicate determinations. The variation determined from multiple independent replicates was always less than 5%.

The purification of dipeptidyl peptidase I from human spleen. Cadaveric spleen was collected within 12 h postmortem and stored at −70°C until use. Approximately 200 g spleen was allowed to thaw and the tissue was trimmed of excess fat and minced in an acidic hypotonic homogenization buffer (10 mM sodium acetate–acetic acid, pH 4.0). The ratio of this buffer to tissue was 5 ml/g. The tissue was homogenized in an Oster blender at high speed for 15 s. The homogenate was centrifuged at 13,000*g* for 20 min. The supernatant fluid was saved and the pellet was reextracted with the same buffer containing 0.1% Triton X-100 (2.5 ml/g). After centrifugation, the supernates were combined and heated to 55–60°C. After 30 min, the extract was cooled to room temperature and adjusted to pH 7.5 with 1 M Tris base. Precipitate that formed during heating and pH adjustment was removed by filtration under gentle vacuum. The soluble sample was chromatographed on a concanavalin A-agarose column equilibrated with phosphate-buffered saline (PBS). The column was washed extensively with PBS before the elution of bound protein. Protein was eluted with 500 mM α-methylmannopyranoside in PBS. The eluted material from the concanavalin A-agarose was applied to a *p*-hydroxymercuribenzoate-agarose column equilibrated in PBS. After unbound protein was washed through the column, the column was washed extensively with 10 mM sodium phosphate, pH 7.0. A fraction of the bound protein was eluted in 10 mM sodium phosphate containing 50 mM β-mercaptoethanol. Other sulfhydryl-containing proteins were eluted in PBS containing 50 mM β-mercaptoethanol. Material from this second elution was diluted fivefold with 10 mM sodium phosphate, pH 7.0, and chromatographed on a 1.5-ml DEAE-Sephacel column. Bound protein was eluted with 10× PBS and directly fractionated by chromatography on a Sephacryl S-300 gel filtration column equilibrated with 250 mM NaCl, 0.02% sodium azide. Gel filtration column fractions were monitored by SDS–PAGE using 15% polyacrylamide gels, in addition to assays of protein and dipeptidyl peptidase activity. This procedure yields approximately 100 μg of apparently homogeneous DPPI per 100 g tissue.

Partial purification of DPPI from human lymphoid cells and myeloid cell lines. In order to confirm previous reports on the subcellular distribution of DPPI (17), we isolated the enzyme from a subline (SP4) of a previously described cytotoxic T cell clone [NP4 cells (18)] and the myelomonocytic cell line U-937 (19). DPPI preparations from cultured cells started with at least 10^8 total cells. After the cells were washed by centrifugation and resuspended in saline, they were homogenized in a neutral hypotonic lysis buffer (0.1× PBS, 1 mM $MgCl_2$) and fractionated on Percoll gradients. Percoll gradients were prepared with equal volumes of 39% Percoll and 90% Percoll and centrifuged at 13,500 rpm for 60 min. Gradients were fractionated into 1-ml aliquots and assayed for DPPI activity. Fractions containing DPPI activity were diluted with 10 mM sodium acetate–acetic acid, pH 4.0, and centrifuged to remove insoluble material. After pH adjustment, soluble protein was applied to a concanavalin A-agarose column. DPPI was further purified by the procedures described above with whole spleen.

Determination of isoelectric point. The isoelectric point of human DPPI was determined during preparative isoelectric focusing with a Bio-Rad Rotophor apparatus. The sample loaded into the electrophoresis cell was a 50-fold dilution of whole spleen extract in water containing 1% pH 3–10 ampholytes.

Determination of pH optimum. The pH optima for hydrolysis of synthetic peptide substrates were determined at 100 μM substrate concentration in assays containing the standard concentrations of NaCl, EDTA, and DTT. The pH of the incubations was controlled by 100 mM buffer (acetic acid–sodium acetate, pH 4.0–5.5; sodium phosphate, pH 5.7–8.0).

Erythrocyte lysis assay of Leu-Leu-OMe polymerization. Freshly isolated, normal human red cells were labeled with ^{51}Cr and were incubated for 4 h in pH 7.4 PBS at 37°C in the presence of Leu-Leu-OMe with and without DPPI and percentage specific lysis was calculated as previously described (12).

Other methods. Protein was assayed by absorbance at 280 nm as well as the BCA (20) or *o*-phthalaldehyde methods using reagents purchased from Pierce Chemical Co.

SDS–PAGE was performed by the method of Laemmli in 15% polyacrylamide gels (21). Proteins were stained with Coomassie brilliant blue or periodic acid–Schiff's reagent. Electrophoresis under nondenaturing conditions was performed in 5% polyacrylamide slab gel (375 mM Tris–HCl, pH 8.0) at 4°C. Samples were transferred to nitrocellulose prior to staining for DPPI activity. Nitrocellulose strips were incubated in DPPI assay buffer containing 100 μM Gly-Phe-MNA for 20 min at 37°C prior to coupling to hexazotized *p*-rosaniline.

Cathepsin B was assayed by the hydrolysis of Z–Arg–Arg-β-naphthylamide (22). BLT esterase was assayed by the method of Colman and Green (23). Leukocyte elastase was assayed by the method of Baugh and Travis (24).

Amino acid sequence analysis was performed by automated Edman degradation on a Model 470A amino acid sequencer as described previously (25).

RESULTS

Purification of DPPI from Human Spleen

On the basis of the previously reported distribution of DPPI activity in a variety of mammalian species, human spleen was selected as the starting material for the purification of DPPI.

The total DPPI activity obtained after homogenization of spleen at neutral and acidic pH was found to be identical. However, since homogenization at neutral pH solubilized 2–2.5 times more protein than at pH 4, spleen was routinely homogenized in the acidic buffer. A second extraction of the insoluble material pelleted after acidic homogenization increased the level of total DPPI activity by up to 30% in various preparations.

As previously reported for other species (7, 26), heat-treatment of tissue extracts at 60°C for up to 45 min was found to have no apparent effect on DPPI activity. For enzyme purification, tissue extracts were routinely exposed to heat-treatment for 30 min. Since little visible precipitate formed during heat-treatment, the unfiltered extracts were adjusted to pH 7.5 by the addition of 1 M Tris base. Visible precipitate formed upon addition of Tris base and was removed by filtration under gentle vacuum. The combination of heat-treatment and pH adjustment results in the removal of approximately 25% of the original protein with a negligible loss of DPPI activity (Table I).

The soluble protein fraction was loaded directly on a concanavalin A-agarose column. The vast majority of protein but no DPPI activity appeared in the flowthrough fractions. After extensive washing with PBS, bound protein was eluted by the addition of α-methylmannoside. Approximately 50% of the applied activity was recovered with a 70-fold increase in specific activity.

The material eluted from the lectin-affinity column was applied directly to a mercurial-affinity column. The majority of applied protein, but no DPPI activity, appeared in the flowthrough fraction. The column was washed extensively with PBS and then with 10 column volumes of 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, to remove unbound protein. Bound protein was then eluted from the column in a two-step process. Washing the column with 50 mM β-mercaptoethanol in 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, eluted a portion of the bound protein but little, if any, active DPPI. A second wash with PBS containing 50 mM β-mercaptoethanol eluted the majority of DPPI (Fig. 1).

DPPI eluted from the mercurial affinity chromatography was diluted with 5 vol of 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, to decrease the ionic strength of the sample and chromatographed on a column of DEAE-Sephacel. DEAE-Sephacel chromatography resulted in an almost twofold increase in specific activity.

Previously published methods for the partial purification of DPPI have used Sephadex G-200 or Sephacryl S-200 for gel filtration chromatography. Neither of these gels yielded adequate resolution of DPPI from other high-molecular-weight proteins (data not shown). However, such resolution was achieved when DPPI was chromatographed on a Sephacryl S-300 gel filtration column. In the individual Sephacryl S-300 fractions, DPPI activity was correlated with the presence of a protein band determined by SDS–PAGE corresponding to a molecular weight of 24,000 (Fig. 2). Furthermore, in fractions corresponding to the peak of DPPI activity, this was the only protein band observed after SDS–PAGE. This protein band stained positively with periodic acid–Schiff's reagent, confirming the presence of carbohydrate (data not shown). Polyacrylamide gel electrophoresis under nondenaturing conditions demonstrated a single protein band in the Sephacryl S-300 pool. After transfer of this protein to nitrocellulose, DPPI activity staining demonstrated that it was associated with the enzymatic activity. SDS–PAGE analysis of the protein band isolated in the nondenaturing gel system demonstrated a single 24,000-Da protein (data not shown). The 24,000-Da protein was also correlated with DPPI activity by SDS–PAGE analysis of fractions obtained by preparative isoelectric focusing (data not shown).

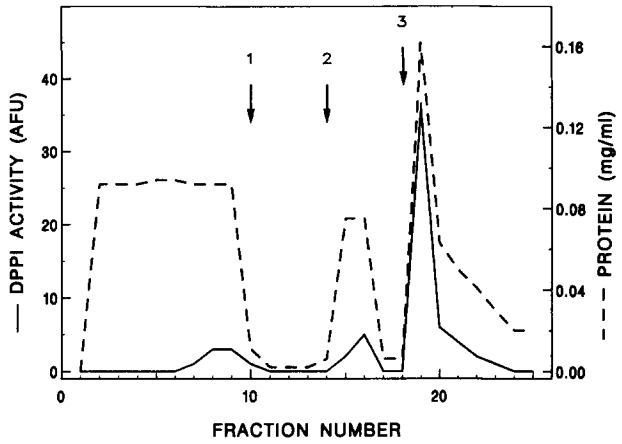

FIG. 1. Affinity chromatography of human DPPI on *p*-hydroxymercuribenzoate-agarose. Protein fractionated by lectin-affinity chromatography was chromatographed on pHMB-agarose equilibrated with PBS. After loading the sample, the column was washed and bound protein was eluted, as indicated by the arrows, with (1) 10 mM sodium phosphate, pH 7.0; (2) 10 mM sodium phosphate, pH 7.0, 50 mM β-mercaptoethanol; and (3) PBS containing 50 mM β-mercaptoethanol. Fractions were assayed for DPPI activity and protein (*o*-phthalaldehyde method).

Comparison of the relative elution position of DPPI during gel filtration with proteins of known molecular weight indicated that DPPI had an estimated molecular mass of 200,000 Da (data not shown). The elution position of DPPI was not altered by inclusion of 1 M NaCl in the column buffer, suggesting that the high molecular weight of the enzyme is not the result of protein aggregation. The purification scheme detailed here has resulted in a more than 1000-fold purification of DPPI from the acidic extract of human spleen (Table I). Approximately 100 μg of purified DPPI was obtained per 100 g wet wt of tissue. Of note, tissue has been stored frozen for up to a year without noticeable loss of DPPI yield. Purified DPPI has

TABLE I

Purification of Human Dipeptidyl Peptidase I

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Yield (%)	Enrichment (-fold)
Extract	12,000	38,000	3	100	1
Heat-treated/pH adjusted	9,600	36,000	4	93	1
Concanavalin A-agarose	89	19,000	210	50	70
pHMB-agarose	6.7	7,100	1100	19	370
DEAE-Sephacel	2.0	3,700	1800	10	600
Sephacryl S-300	0.2	640	3200	1.7	1100

Note. Human dipeptidyl peptidase I was purified from cadaveric spleen (195 g wet wt) as described under Materials and Methods. Enzyme activity was assayed by the hydrolysis of glycylphenylalanyl-β-naphthylamide and is expressed as nmol β-naphthylamine released per minute.

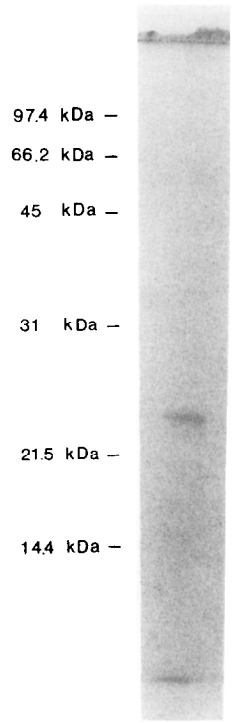

FIG. 2. SDS–PAGE analysis of purified human DPPI. Purified human DPPI (4 μg) was subjected to SDS–PAGE and visualized by staining with Coomassie brilliant blue. Molecular mass standards included phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. The estimated molecular mass of the DPPI subunit is 24 kDa.

also been stored frozen and at 4°C for months with no significant loss of activity.

Amino Acid Sequence Analysis of Purified Human DPPI

The 24,000-Da subunit isolated by SDS–PAGE was transferred to PVDF paper and subjected to N-terminal sequence analysis by Edman degradation. Based on the failure of N-terminal sequencing, we concluded that the 24,000-Da subunit was blocked. Therefore, the protein was digested with TPCK-treated trypsin or cyanogen bromide. The peptides generated by the digestion were resolved by reverse-phase HPLC and analyzed by Edman degradation. The results are shown in Fig. 3. The sequences of the peptides of human DPPI are shown to align with similar sequences of the rat enzyme obtained by amino acid sequencing and deduced from cDNA clones (34). On the basis of the size of the 24,000-Da subunit, approximately 45% of the human subunit has been sequenced. Compared to the amino acid sequence deduced from the rat cDNA clone there is greater than 85% identity at the protein level between the segments that have been sequenced.

Characterization of Human DPPI

Isoelectric focusing in a gradient of pH from 3 to 10 resolved the DPPI activity into a single peak centered at pH 5.4 (data not shown). DPPI remained soluble at its isoelectric point and was enzymatically stable throughout the procedure.

The specificity of human DPPI was studied using a variety of synthetic peptide substrates (Table II, Fig. 4). Substrates with blocked amino-termini, with proline at the P_1 position, arginine at the P_2, or containing only single amino acids, were not hydrolyzed. DPPI was found to hydrolyze peptides with both polar and nonpolar side chains. The enzyme was able to hydrolyze an unblocked tetrapeptide nitroanilide but did not hydrolyze an unblocked tripeptide nitroanilide or chemically blocked peptide substrates of similar composition. These results support the conclusion that DPPI does not express endoproteolytic activity. Furthermore, purified DPPI did not

AMINO ACID SEQUENCE ANALYSIS OF HUMAN DPPI

A. AMINO ACID SEQUENCES OF PEPTIDES ISOLATED FROM HUMAN DPPI

PEPTIDE	AMINO ACID SEQUENCE
A	XLPTSXDVR
B	NVHGINFVSPVR
C	NQASCGSCYSFASMGMLEAR
D	IRILTXNSQTPILSPQEVVS
E	YAQDFGLVEEASFPYTXXD
F	YYSSEYHYVGGFYGGMNEALMK
G	LELVRHGPMAVAFEYVYD
H	GMLEARIR
I	AVAFEYVYDFLHY

B. SEQUENCE COMPARISON OF HUMAN AND RAT DPPI

H	XLPTSXDVRNV	HGINFVSPVR	NQASCGSCYS	FASMGMLEAR	IRILTXNSQT
R	LPESWDWRNV	RGINFVSPVR	NQESCSGCYS	FASLGMLEAR	IRILTNNSQT
H	PILSPQEVVS	----------	---------Y	AQDFGLVEEA	SFPYTXXD--
R	PILSPQEVVS	CSPYAQGCDG	GFPYLIAGKY	AQDFGVVEEN	CFPYTATDAP
H	---------Y	YSSEYHYVGG	FYGGMNEALM	KLELVRHGPM	AVAFEYVYDF
R	CKPKENCLRY	YSSEYYYVGG	FYGGCNEALM	KLELVKHGPM	AVAFEVHDDF
H	LHY-------	----------	----------	----------	----------
R	LHYHSGIYHH	TGLSDPFNPF	ELTNHAVLIV	GYGKDPVTGL	DYWIVKNSWG
H	----------	----------	----------	---	
R	SQWGESGYFR	LRRGTDECAI	ESIAMAAIPI	PKL	

FIG. 3. Sequence comparison of human and rat DPPI. (A) Purified human DPPI sequence was obtained by Edman degradation of peptides obtained after digestion of the isolated 24,000-Da subunit with trypsin (A–G) and cyanogen bromide (H and I). (B) The peptide sequences have been aligned with the deduced amino acid sequence of rat DPPI obtained by analysis of a cDNA clone (34). Amino acids are designated by the single letter code. The letter "X" indicates the position of a residue unidentified by Edman sequencing.

TABLE II

Substrate Specificity of Human DPPI

Substrate peptide						Substrate concentration	
P_5	P_4	P_3	P_2	P_1		100 μM	500 μM
				Ala-	βNA	0	0
				Arg-	βNA	0	0
				Gly-	βNA	0	0
				Leu-	βNA	0	0
				Phe-	βNA	0	0
				Pro-	βNA	0	0
			CBZ-	Arg-	βNA	0	0
			CBZ-	Phe-	βNA	0	0
			Arg-	Arg-	βNA	0	0
			Asp-	Ala-	βNA	250	1000
			Gly-	Phe-	βNA	2380	4380
			Gly-	Pro-	βNA	0	0
			Ser-	Tyr-	βNA	1750	2000
		CBZ-	Arg-	Arg-	βNA	0	0
			Gly-	Arg-	MNA	2500	4750
			Gly-	Phe-	MNA	3500	5750
			Gly-	Pro-	MNA	0	0
			Ser-	Tyr-	MNA	1000	1750
	CBZ-	Val-	Leu-	Arg-	MNA	0	0
			Ala-	Ala-	pNA	380	1000
			Gly-	Phe-	pNA	120	380
	Suc-	Ala-	Ala-	Ala-	pNA	0	0
	Ala-	Ala-	Val-	Ala-	pNA	120	120
Suc-	Ala-	Ala-	Pro-	Phe-	pNA	0	0
			CBZ-	Lys-	AMC	0	0
			Gly-	Phe-	AMC	3500	6120
		Pro-	Phe-	Arg-	AMC	0	0
	CBZ-	Gly-	Pro-	Arg-	AMC	0	0

Note. Peptides were tested for susceptibility to hydrolysis by purified human dipeptidyl peptidase I (0.4 μg) under standard assay conditions. Results are expressed as nmol βNA, MNA, pNA, or AMC released per minute per milligram protein.

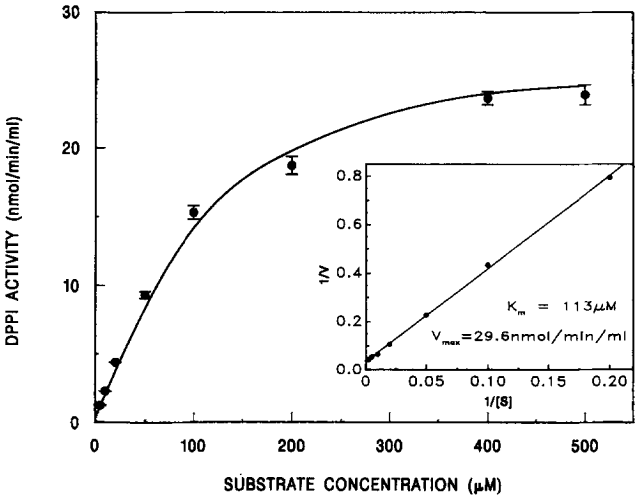

FIG. 4. Concentration dependence of glycylphenylalanyl-β-naphthylamide hydrolysis by purified human DPPI. Purified human DPPI was assayed for the hydrolysis of synthetic peptide over a 25-fold range of substrate concentrations. The inset shows the double reciprocal plot used for the determination of kinetic constants for this preparation of enzyme.

exhibit endoproteolytic activity as assayed by the ability to generate acid soluble peptides from FITC-casein (data not shown). The amino-terminal sequence of bovine α-casein, Arg–Pro, prohibits the processive cleavage of amino-terminal dipeptides from this protein and therefore should increase the ability to specifically detect endoproteolytic activity, if present.

Purified human DPPI was noted to hydrolyze fluorogenic peptides over a broad acidic pH range (Fig. 5). A similar pH profile was observed with each of the susceptible peptide substrates tested. Maximal peptidase activity occurs over the range of pH 4.5 to 6.8.

DPPI has been demonstrated to be able to polymerize dipeptide ester and amide substrates at neutral to mildly alkaline pH (1–3, 7). Previously described assays of the polymerizing activity of bovine DPPI have been based on the formation of dipeptide hydroxamates (7) or the formation of insoluble products from hydrophobic dipeptide esters (2). More recently, studies performed in our laboratory have demonstrated that $(Leu–Leu)_n$-OMe polymers where $n \geq 3$ or polymers of Leu–Leu-OMe or other hydrophobic dipeptide esters generated by the action of partially purified bovine DPPI possess membranolytic activity. A hemolytic assay based on these observations has been developed (12). While this is only a qualitative assay of the polymerase activity of DPPI, this assay, unlike those based on hydroxamate or precipitin formation, has been shown to detect DPPI transpeptidase activity with low (10^{-4} to 10^{-3} M) concentrations of dipeptide ester substrate. In the experiment detailed in Fig. 6, the poly-

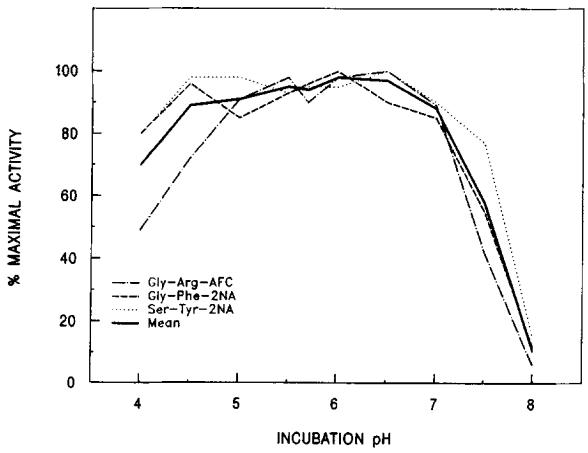

FIG. 5. Effect of pH on the rate of substrate hydrolysis. Purified human DPPI was assayed for the hydrolysis of synthetic peptide substrates over a pH range of 4 to 8. Assay buffers used were: acetic acid/sodium acetate for pH 4.0 to 5.5; sodium phosphate mono/dibasic for pH 5.7 to 8.0.

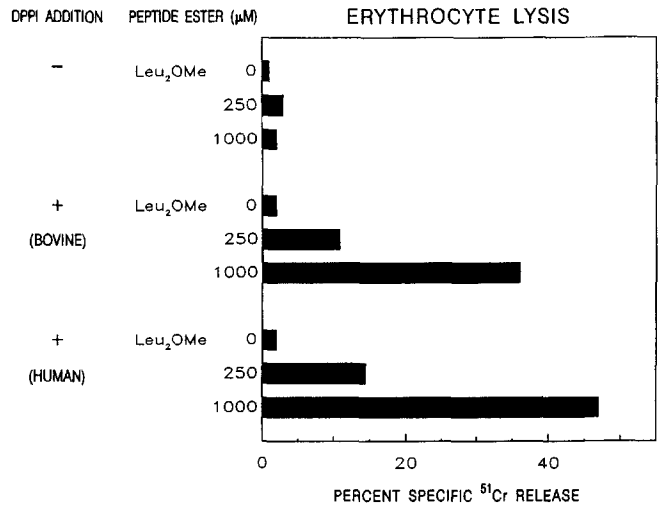

FIG. 6. DPPI-catalyzed polymerization of Leu–Leu-OMe and lysis of red blood cells. ^{51}Cr-labeled human red blood cells were incubated in the presence of the indicated additives for 4 h at 37°C and assayed for the percentage specific lysis. Equivalent levels of purified human DPPI (1 μg) and partially purified bovine DPPI (Boehringer-Mannheim) were added to the polymerization assays based on hydrolytic assays using Gly–Phe-βNA.

merizing (transpeptidase) activity of purified human DPPI was demonstrated by the ability to form membranolytic products from leucyl-leucine methyl ester.

In additional studies, the inhibitor profile of human DPPI was found to be consistent with the classification of the enzyme as a cysteine peptidase (Table III). Thus, DPPI activity was potently inhibited by mersalyl acid, iodoacetic acid, and cystatin, three general inhibitors of cysteine peptidases. Furthermore, DPPI activity was inhibited by dithiodipyridine at low pH. Under these conditions, this reagent is considered a specific inhibitor of enzymes with active site cysteine residues (30). DPPI activity was also inhibited by *N*-ethylmaleimide. However, under the conditions used for screening inhibitors (pH 5.5) the effect of *N*-ethylmaleimide was not complete. At pH values above 6.5, the potency of this inhibitor increased as expected (data not shown). Human DPPI activity was also potently inhibited by Gly–Phe-diazomethane, an inhibitor specifically designed for this activity (31–33). Surprisingly, DPPI was also potently inhibited by both TLCK and TPCK. Whereas these inhibitors affect a variety of serine and cysteine peptidases, including papain (33), they have not been reported to be inhibitors of DPPI previously. Based on the substrate requirements for an unblocked amino-terminus and dipeptide length, inhibition of DPPI by TLCK and TPCK was unexpected. The general serine peptidase inhibitor PMSF, the aminopeptidase inhibitor bestatin, and metal chelators had no effect on DPPI activity.

TABLE III

Effect of Protease Inhibitors on DPPI Activity

Inhibitor	Concentration	Percentage control activity
PMSF	1 mM	98
TLCK	1 mM	5
TPCK	1 mM	10
1,10-Phenanthroline	1 mM	98
Bestatin	500 μg/ml	103
Cystatin	50 μg/ml	32
N-Ethylmaleimide	1 mM	63
Gly–Phe-diazomethane	20 μM	12
Iodoacetic acid	1 mM	10
Mersalyl acid	1 mM	3
2,2′-Dithiodipyridine	400 μM	9

Note. Purified human dipeptidyl peptidase I (0.4 μg) was preincubated with each of the inhibitors at the stated concentration for 15 min at 37°C. Each sample was diluted three-fold with assay buffer containing substrate and incubated for an additional 20 min.

TABLE IV

Dipeptidyl Peptidase I Activity in Isolated Human Cells

Cell type	Dipeptidyl peptidase I activity
Natural killer cell ($CD16^+$)	42
Polymorphonuclear leukocytes	18
Monocytes	16
$CD8^+$T cells	10
$CD4^+$T cells	3
B cells	2
Endothelial cells	2
Fibroblasts	2

Note. Human umbilical vein endothelial cells and peripheral blood leukocytes were isolated as previously described (39). Cell extracts were prepared by sonication in normal saline and assayed for DPPI activity. DPPI activity is expressed as nmol β-NA released/min/mg soluble protein. Specificity of assay for DPPI activity was based on comparison of the hydrolysis of Gly–Phe-βNA and Phe-βNA under standard assay conditions and inhibition of Gly–Phe-βNA hydrolysis on Gly–Phe-CHN_2.

Lysosomal Localization of DPPI in Extracts of Human Lymphocytes and Myeloid Cells

Surveys of a variety of cell types demonstrated that although each cell type exhibited some hydrolytic activity against Gly–Phe-βNA, the highest levels of Gly–Phe-βNA hydrolytic activity were observed in cells of bone marrow origin (Table IV). Specifically, the levels of DPPI activity were 10- to 20-fold higher among cytotoxic lymphocytes ($CD16^+$ NK cells and $CD8^+$ T cells) and myeloid cells when compared to noncytotoxic lymphocytes, endothelial cells, and fibroblasts.

Extracts of lymphoid and myeloid cells were fractionated on Percoll gradients under isotonic conditions and assayed for hydrolytic activity against Gly–Phe-βNA at

pH 5.5. In all cases, this hydrolytic activity was restricted to the lysosomal fraction of the gradient (Fig. 7). In extracts prepared from the $CD8^+$ SP4 cytotoxic T cell clone, Gly–Phe-βNA hydrolytic activity was found to be coincident with BLT esterase activity (Fig. 7A) and cathepsin B (data not shown) in Percoll gradient fractions. In extracts prepared from the myelomonocytic leukemia cell line, U-937, DPPI activity was coincident with the lysosomal/granule fraction containing elastase activity (Fig. 7B). No differences in the chromatographic behavior of the Gly–Phe-βNA hydrolytic activity isolated from spleen, SP4 cells, or U-937 cells were observed during gel filtration, lectin- and mercurial-affinity, or ion-exchange chromatography (data not shown), thus indicating that a single enzyme (DPPI) mediated all such activity.

DISCUSSION

The lysosomal hydrolase dipeptidyl peptidase I has been purified to apparent homogeneity from human spleen. The purification scheme takes advantage of the solubility of DPPI under a range of pH from 4 to 7.5 and the stability of the activity upon exposure to heat. Additionally, the purification uses two techniques, lectin-affinity and mercurial-affinity chromatography, that have not previously been applied to this enzyme. Finally, the enzyme is concentrated on a high capacity anion-exchange resin and resolved by gel filtration. This scheme results in over a 1000-fold enrichment of DPPI-specific activity with a yield of 100 μg of pure protein per 100 g tissue.

Many previous attempts have been made to purify DPPI from various sources (1–9). Most of these attempts were carried out before the development of currently available chromatographic techniques. Therefore, these early purification schemes relied heavily on methods such as heat-treatment, ammonium sulfate fractionation, and solvent precipitation of extracts. Partially purified bovine DPPI prepared by these methods was then treated with reagents such as DFP and EDTA to inactivate other peptidases that were not resolved from DPPI (4–7). Despite the lack of homogeneous preparations, DPPI has been characterized both structurally and functionally. For example, sedimentation analysis demonstrated the unusually high molecular weight of this lysosomal enzyme (6–8). Furthermore, the subunit size was suggested to be 24,000 Da based on sedimentation of DPPI in the presence of denaturants (8). Disparate results have been reported, however. Thus, it has been suggested that DPPI purified from rat liver is composed of 18,500-molecular-weight subunits (35). Furthermore, data have been presented suggesting that DPPI is synthesized as a larger (92,500 Da) precursor (36, 37). This conclusion, however, is based on immunoprecipitation experiments using an antibody that did not specifically adsorb DPPI (36, 37). Since the preparation of DPPI (cathepsin C) used to elicit the antibodies was not homogeneous, the immunoreactive proteins may be unrelated to DPPI. Recent cloning of the cDNA for rat DPPI predicts an initial translation product of 52,000 Da that is processed to a size of 26,000 Da (34). Based on the sequencing of two amino-termini, Ishidoh *et al.* (34) suggest that this subunit is further processed to yield an 18,400-Da heavy chain and a 7700-Da light chain. In contrast, we have not observed these smaller peptides after SDS–PAGE or upon amino-terminal sequencing. Therefore, we conclude that the native human enzyme is a 200,000-Da protein and is composed of 24,000-Da subunits.

In the current report, human spleen DPPI was resolved in a single broad peak with a p*I* of 5.4. In previous studies, human fibroblast cell sonicates, resolved on isoelectric focusing gels, yielded four peaks of DPPI activity with p*I*s of 6.2, 6.4, 6.6, and 7.2 (29). In this same report, bovine DPPI was reported to focus into two peaks with p*I*s of 6.4 and 6.6 (29). These apparent differences in isoelectric focusing profiles may relate to protein isoenzyme forms of DPPI in different cell types, to subtle differences in carbohydrate modification, or to the enhanced ability to focus this protein in solution versus the previously employed polyacrylamide gels. Interestingly, one previous investigator concluded that bovine spleen DPPI had a p*I* of 5.4 based on electrophoresis-convection experiments (7), a finding that is consistent with the results reported here using human spleen DPPI. The purification method described here should make it possible to compare the properties of DPPI isolated from other tissues and resolve these issues.

Human DPPI has a broad substrate range that can be summarized as follows. Susceptible synthetic peptide substrates contain a minimum of two amino acid residues and an unmodified amino-terminus. While DPPI cleaves substrates composed of both polar and hydrophobic amino acids, proline cannot occupy the P_1 position of the substrate. Additionally, a basic amino acid, such as arginine, in the P_2 position blocks hydrolysis. K_m values of the substrates tested averaged in the 100–200 μM range. Gly–Phe-MNA and Gly–Phe-AMC are cleaved at slightly higher rates than Gly–Phe-βNA, the standard substrate for DPPI assays. Surprisingly, Gly–Phe-MNA was cleaved at a higher rate than Gly–Arg-MNA. Previous reports with partially purified murine and bovine DPPI demonstrated that Gly–Phe-βNA was cleaved at only 5% of the rate of hydrolysis of Gly–Arg-βNA (4, 9). This contrast in substrate preference may reflect species variation in individual residues that combine to form the peptide binding pocket of the enzyme. Additionally, it is difficult to compare some of the previously published data with the current results because of the wide variety of assay protocols used to study DPPI (cathepsin C). In particular, different groups used substrates with disparate peptide composition as well as a variety of leaving groups to assay the hydrolytic activity of DPPI. While the data presented here demonstrate that DPPI can accept a variety of pep-

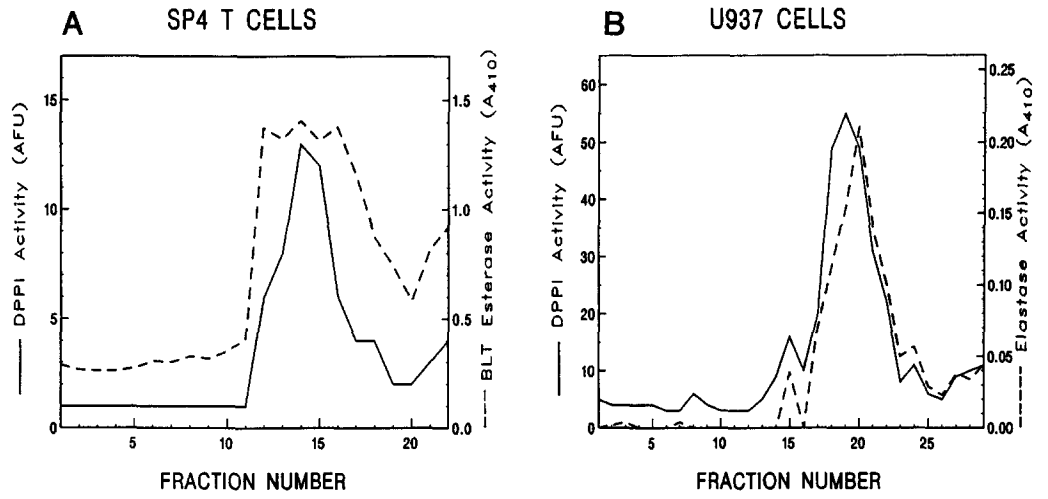

FIG. 7. Localization of DPPI activity to the lysosomal fraction of human lymphocytes and myeloid cells. Cell extracts were prepared from a human T lymphocyte clone (SP4 cells, A) and a myeloid cell line (U-937 cells, B) and fractionated on Percoll gradients (39–90%). Fractions were assayed for DPPI, BLT esterase (A), and elastase (B). The specificity of the assay for determining DPPI activity in cell extracts and Percoll gradient fractions was based on the comparison of the hydrolysis of Gly–Phe-βNA and Phe-βNA and inhibition of the hydrolysis of Gly–Phe-βNA by Gly–Phe-CHN_2. The chromatographic behavior of DPPI obtained from these cell lines was compared to DPPI purified from spleen as described in the text.

tide substrates, they also demonstrate that both the peptide and the leaving group influence the rate of substrate hydrolysis. Further complexity is caused by the tendency of early workers to study only the transpeptidation reaction catalyzed by DPPI (35, 38).

The transpeptidation assays are relatively insensitive when compared to the assays of peptide hydrolysis. The transpeptidase assay utilized in the current studies is a qualitative assay based on the recently demonstrated ability of leucine polymers larger than six residues to lyse human red blood cells (12). While this hemolytic assay is similar in principle to qualitative assays based on the transpeptidation of hydrophobic dipeptides to form insoluble polymers, it has proven to be more sensitive for the detection of transpeptidase activity with low (10^{-4} to 10^{-3} M) concentrations of substrate.

Recently, Ishidoh *et al.* (34) reported the independent purification of rat liver DPPI to near homogeneity and the cloning of a cDNA from a rat kidney cDNA library. The amino acid sequences of nine peptides isolated from cyanogen bromide and tryptic digests of the human DPPI subunit demonstrate a high degree of similarity between the protein isolated from both species. The sequence identity between the two species includes the tyrosine residue adjacent to the active site cysteine. Interestingly, this is distinct from the well-conserved tryptophan residue present in other members of the papain protease family. Ishidoh *et al.* (34) suggest that this residue may participate in substrate specificity since DPPI is the only member of the papain family that is limited to exopeptidase activity. The analysis of rat liver DPPI demonstrated the amino-terminal sequence of the 18,400-Da heavy chain consists of a mixture of LPESWDWRNVRGINFV and an N-terminal dipeptide extension of this peptide LSLPESQDYR. In contrast, the human enzyme appears to be chemically blocked at the amino-terminus based upon the resistance of the intact subunit to Edman degradation. A tryptic peptide that was nearly identical in sequence to the rat peptide was isolated from the human enzyme. However, the human peptide is preceded by an unidentifiable residue (designated by the "X" in Fig. 3).

DPPI activity has been demonstrated previously in most tissues that have been assayed for this activity. Tissue surveys in rat, pig, and cow have demonstrated higher DPPI activity in spleen and liver than other tissues (4, 11). Based on these findings, human spleen was chosen for the starting material for the purification of DPPI described here. Additional studies have demonstrated that DPPI activity is highest in lymphocytes with cytolytic potential and myeloid cells. DPPI content is up to 20-fold higher in these cell types than in fibroblasts. DPPI therefore is a novel phenotypic marker for cytolytic lymphocytes and myeloid cells. The degree to which expression of DPPI in tissue macrophages accounts for DPPI activity detected in the liver or other nonlymphoid organs remains to be determined. Subcellular fractionation of lymphocytes and myeloid cells localized all of the DPPI activity to the lysosomal fraction. The lysosomal granules of these cells have been noted previously to play major roles in their specialized effector functions. Thus, while previous studies have suggested that DPPI may play a role in the degradation of cellular proteins, the enrichment of this enzyme in the lysosomal granules of cytotoxic lymphocytes and myeloid cells suggests that DPPI may play a more specific role in these immune effector cells.

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