Characterization of two dipeptidases purified from hepatic schistosome egg granulomas in mice

Leukotriene D₄ hydrolases of granulomatous tissue

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Extracts prepared from tissue with granulomatous inflammation experimentally produced in liver of CBA-strain mice showed increased hydrolysis of leukotriene D₄ (LTD₄), Leu-Leu and Ala-Gly as compared with normal hepatic cells. Two dipeptidases, Leu-Leu dipeptidase and Ala-Gly dipeptidase, were purified from hepatic granulomas, and quantitative conversion of LTD₄ into leukotriene E₄ (LTE₄) by both enzymes was demonstrated. *M_r* values of the purified enzymes were 178 000 for Leu-Leu dipeptidase and 183 000 for Ala-Gly dipeptidase. The enzymes showed homogeneity, appearing as a single band on SDS/PAGE, and the *M_r* values of the subunits were 56 000 and 57 000 for Leu-Leu and Ala-Gly dipeptidase respectively. The amino acid compositions of the two enzymes differed considerably from each other. The activity of Leu-Leu dipeptidase was inhibited by bestatin and captopril and stabilized with MnCl₂. The *K_m* for LTD₄ was 25 μM with a *V_{max}* of 49.0 μmol/min per mg. In contrast, the activity of Ala-Gly dipeptidase was inhibited by cilastatin, cytinylglycine, EDTA and dithiothreitol, and also by captopril. The *K_m* for LTD₄ was 5.3 μM with a *V_{max}* of 50.4 μmol/min per mg. The findings indicate that the conversion of LTD₄ into LTE₄ by microsomal dipeptidases is elevated during granulomatous tissue reaction. This enzyme activity may become useful for biochemical quantification of the pathological tissue reaction that occurs in organized granulomas.

INTRODUCTION

Leukotriene D₄ [slow-reacting substance (SRS)-Cys-Gly; LTD₄], a highly bioactive component of slow-reacting substance, is known to be transformed into the less active leukotriene E₄ (SRS-Cys; LTE₄). The reaction has been shown to be catalysed by peptidases such as microsomal alanine aminopeptidase (Sok *et al.*, 1980), lysosomal arginine aminopeptidase (Yokota *et al.*, 1983) and microsomal dipeptidase (Kozak & Tate, 1982). Since increases in leukotriene concentration are reported to be associated with up-regulation of inflammatory conditions, including granulomatous tissue reactions (Brain *et al.*, 1984*a, b*; Chensue *et al.*, 1985), and leukotriene C₄ and LTD₄ have been shown to be mitogens (Morelli *et al.*, 1989), we questioned whether conversion of LTD₄ into LTE₄ is also associated with granulomatous tissue reaction. In order to investigate this possibility, we measured conversion of LTD₄ into LTE₄ as well as microsomal dipeptidase activity in crude extracts of mouse skin at different times after granulomatous tissue reaction elicited by inoculation of a granulomatous stimulus by the method of Okamoto *et al.* (1987). An increase in dipeptidase activity, analysed by measuring hydrolysis of Leu-Leu and Ala-Gly, corresponded to the time course of the inflammatory process, and formation of LTE₄ from LTD₄ by the skin granuloma tissue extract was detected (Sato *et al.*, 1991). Furthermore, the Leu-Leu and Ala-Gly dipeptidases detected in the granulomatous tissue reaction showed different inhibitor profiles, suggesting the possibility that at least two dipeptidases may function simultaneously in this pathological condition. Purification and characterization of these dipeptidases were, however, not possible from the skin granulomatous inflammation, because of limited amounts of tissue.

Hypersensitive granulomas develop in liver of mice infected

with *Schistosoma mansoni* (Hsu *et al.*, 1972; Epstein *et al.*, 1979). In this study we report purification and characterization of two dipeptidases that preferentially hydrolyse Leu-Leu or Ala-Gly from hepatic granulomas. Both dipeptidases formed LTE₄ from LTD₄ in a quantitative manner. Inhibition profiles of the two purified dipeptidases from hepatic granulomas showed a trend similar to those of the crude enzymes from skin granulomas, confirming that the specific dipeptidase activities are associated with granulomatous inflammation. Although Leu-Leu and Ala-Gly dipeptidases differ from each other in enzymic properties, both dipeptidases appear to belong to the class of microsomal dipeptidases.

EXPERIMENTAL

Materials

Female CBA-strain mice (5–6 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Q-Sepharose Fast Flow, Sephacryl S-300 superfine and concanavalin A (Con A)-Sepharose were obtained from Pharmacia Biotechnology Inc. (Piscataway, NJ, U.S.A.). LTD₄, Leu-Leu and Ala-Gly were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Hepatic granulomas

Mice were injected subcutaneously with 75 cercariae of the Puerto Rican strain of *Schistosoma mansoni*. They were killed and the livers were removed 9 weeks later. The granuloma-rich fraction was separated from hepatic cells by the method of Nishimura *et al.* (1985). The granulomas were pooled, quickly frozen in liquid nitrogen and stored at –70 °C. Livers from age-matched non-infected mice also were stored at –70 °C for extraction of normal liver enzymes.

Abbreviations used: LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; Con A, concanavalin A; PVDF, poly(vinylidene difluoride).

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Enzyme assays

Leu-Leu hydrolysis was measured by the method of Sugiura *et al.* (1978). The standard assay mixture contained 4 μ mol of Leu-Leu in 1 ml of colour reagent in which 8 mg of 4-aminoantipyrine, 20 μ l of *NN*-dimethylaniline and 300 units of peroxidase were dissolved in 100 ml of 150 mM-Tris/HCl, pH 8.0, and 0.1 ml of enzyme solution. The reaction was carried out at 37 °C for 20 min and stopped by the addition of 1 ml of 0.1 M-acetic acid. The absorbance was measured at 550 nm. One unit of dipeptidase activity is defined as 1 μ mol of Leu-Leu hydrolysed/min at 37 °C.

Ala-Gly dipeptidase was measured by the ninhydrin method (Kozak & Tate, 1982). A reaction mixture of 0.5 ml consisting of 50 mM-Tris/HCl buffer, pH 8.0, enzyme solution and 0.4 mM-Ala-Gly was incubated at 37 °C for 10–20 min. The reaction was stopped by addition of 0.25 ml of 1 M-acetate buffer pH 5.3. Amino acid release from the substrate was determined by the ninhydrin method. One unit of Ala-Gly dipeptidase activity was defined as the amount needed to liberate 1 μ mol of amino acid from the substrate/min at 37 °C. The standard assay for other dipeptides and tripeptides was performed in the same way. The catalytic activity of the enzymes on amino acid- β -naphthylamide derivatives was measured by the method of Ito *et al.* (1984). One unit of enzyme activity was defined as that which hydrolysed 1 μ mol of substrate/min. Protein was determined by the method of Lowry *et al.* (1951) using BSA as a standard.

H.p.l.c. assay of conversion of LTD₄ into LTE₄

The method described by Campbell *et al.* (1990) was used. The reaction mixture of 100 μ l, consisting of 50 mM-Tris/HCl buffer (pH 8.0), sample solution and 5 μ M-LTD₄, was incubated at 37 °C for 5 min. The reaction was stopped by addition of 5 μ l of 0.5% (v/v) acetic acid, and the mixture was stored on ice. Samples (20 μ l) were injected manually into a C₁₈ reversed-phase column (Nucleosil C₁₈ 5 μ m 4.2 ml bed vol.; Alltech, Deersfield, IL, U.S.A.) with a Hamilton no. 702 sample injector. The column was eluted isocratically with methanol/water/acetic acid (69:31:1, by vol.) which had been adjusted to pH 5.7 with NH₃. The flow rate was 1.0 ml/min, and the leukotrienes were detected with a u.v. spectrophotometric detector (Shimadzu SPD-6AV) at 280 nm. One unit of dipeptidase activity was defined as the amount of enzyme that hydrolysed 1 μ mol of LTD₄/min.

Purification of dipeptidases from hepatic granulomas

A crude extract of 3 g of isolated hepatic granulomas after 9 weeks infection was homogenized in a mixture of water and butan-1-ol with proportions of tissue/water/butan-1-ol of 1:3:1 (w/v/v) (1 weight/3 vol/1 vol) and centrifuged at 10000 *g* for 10 min. Proteins in the supernatant were precipitated with 60% acetone at 0 °C and dissolved in 50 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MnCl₂. The enzymes were precipitated between 40 and 60%-satd. (NH₄)₂SO₄ and dialysed against 10 litres of 50 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MnCl₂ for 20 h at 4 °C. These proteins were applied to a Q-Sepharose Fast Flow column (38 ml bed vol.) equilibrated with 50 mM-Tris/HCl buffer (pH 8.0) containing 0.1 mM-MnCl₂. After the column had been washed with 50 ml of the same buffer, the enzymes were eluted with a linear concentration gradient from 0 to 0.3 M-NaCl. Hydrolysis of Leu-Leu and Ala-Gly was detected in separate fractions. Each active fraction was concentrated through a membrane filter (Amicon XM-50) and adjusted to 1.5 ml with 50 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MnCl₂ and 50 mM-NaCl. They were applied to a Sephacryl S-300 column (160 ml bed vol.) equilibrated with 50 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MnCl₂ and 50 mM-NaCl. The enzyme fractions were

again separately concentrated through the membrane filter, and applied to a Con A-Sepharose column (5 ml bed vol.) equilibrated with 50 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MnCl₂. Leu-Leu dipeptidase was not absorbed and appeared in the eluate, whereas Ala-Gly dipeptidase was absorbed on the column and collected by washing with the same buffer containing 20 mg of methyl α -glucoside/ml. The dipeptidases were further purified by h.p.l.c. (Bio-Rad Bio-Sil SEC-250 columns; 27 ml bed vol.) equilibrated with 50 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MnCl₂ and 200 mM-NaCl, and used as a purified enzyme.

SDS/PAGE

The method of Laemmli (1970) was used with 12.5% (w/v) polyacrylamide and a constant current of 20 mA per slab gel. Proteins were detected with Coomassie Brilliant Blue R-250.

Amino acid composition analysis

The proteins for amino acid analysis were prepared by the method of Ploug *et al.* (1989). SDS/PAGE of purified enzymes was performed. The proteins were transblotted on a poly(vinylidene difluoride) (PVDF) membrane. A single band corresponding to each enzyme was cut from the PVDF membrane, and amino acid composition analysis was carried out at the Protein and Nucleic Acid Facility, Beckman Center, Stanford University Medical Center (Stanford, CA, U.S.A.).

RESULTS AND DISCUSSION

Purification of dipeptidases from hepatic granulomas

The hydrolysis of Leu-Leu, Ala-Gly and LTD₄ by crude extracts prepared from hepatic granulomas developed 9 weeks after infection with *Schistosoma mansoni* was compared with that by normal liver crude extract (Table 1); it was increased 4.9-, 3.2- and 3.2-fold respectively. Campbell *et al.* (1990) reported time-dependent release of dipeptidase from lung membrane preparations, by digestion with phosphatidylinositol-specific phospholipase C purified from *Bacillus thuringiensis*. In a separate experiment we tried to extract the enzyme, using phospholipase C. The membrane preparation was collected and incubated with phospholipase C (0.05 unit/ml) at 37 °C for 1 h, but recovery of dipeptidase activity from the granulomatous tissues was extremely low as compared with the activity of the enzymes extracted by the method of Ito *et al.* (1983) using butan-1-ol treatment as in this study.

The methodology used for purification of the enzymes was essentially as described by Sugiura *et al.* (1978) for human kidney, although DEAE-cellulose chromatography was replaced by Q-Sepharose Fast Flow column chromatography, which readily separated Leu-Leu and Ala-Gly dipeptidase activities (Fig. 1). The two dipeptidases were then separately purified by Sephacryl S-300 column chromatography followed by ConA-

Table 1. Dipeptidase activity detected in granulomatous and normal tissues

The substrate concentrations are given in parentheses. The enzyme activities (m-units/mg of protein) were calculated from three samples for each granulomatous tissue and normal tissue and expressed as means $\pm$ S.E.M.

Substrate	Hepatic granuloma	Normal liver
Leu-Leu (0.4 mM)	152.1 $\pm$ 1.7	31.3 $\pm$ 0.6
Ala-Gly (0.5 mM)	281.0 $\pm$ 5.1	86.7 $\pm$ 0.5
LTD ₄ (5 μ M)	346.3 $\pm$ 0.5	108.7 $\pm$ 0.9

Fig. 1. Q-Sepharose Fast Flow chromatography of hepatic granuloma dipeptidases

The protein fractionated with $(\text{NH}_4)_2\text{SO}_4$ was dialysed and applied to a column as detailed in the Experimental section. Bound proteins were eluted with a linear gradient of 0–0.3 M-NaCl (----), and 3.3 ml fractions collected and assayed for hydrolysis of Leu-Leu (○) and Ala-Gly (●). ○---○, A_{280} .

Table 2. Purification of dipeptidases from hepatic granulomas (3 g)

	Volume (ml)	Total activity (units)		Protein (mg)	Specific activity (units/mg of protein)		Recovery (%)	
		Leu-Leu	Ala-Gly		Leu-Leu	Ala-Gly	Leu-Leu (fold)	Ala-Gly (fold)
Crude extract	7.5	3.53	5.64	26.9	0.136	0.218	100.0 (1.0)	100.0 (1.0)
$(\text{NH}_4)_2\text{SO}_4$ fractionation	4.0	1.99	2.71	8.53	0.233	0.318	56.4 (1.7)	48.0 (1.5)
Q-Sepharose Fast Flow	40.0	2.55	—	1.29	1.97	—	72.2 (14.5)	—
	30.0	—	4.39	0.950	—	4.62	—	77.8 (21.2)
Sephacryl S-300	15.0	1.74	—	0.351	4.96	—	49.3 (36.5)	—
	10.0	—	3.52	0.316	—	11.1	—	62.4 (50.9)
Con A-Sepharose	11.0	1.20	—	0.079	15.2	—	34.0 (111.8)	—
	16.0	—	3.31	0.076	—	43.6	—	58.7 (200.0)
H.p.l.c.	1.5	1.12	—	0.057	19.6	—	31.7 (144.1)	—
SEC-250 column	1.6	—	3.07	0.049	—	62.7	—	54.4 (287.6)

Sephacryl chromatography. Ala-Gly dipeptidase bound to Con A-Sepharose, whereas Leu-Leu dipeptidase did not. Kidney dipeptidases from human (Sugiura *et al.*, 1978) and pig (Littlewood *et al.*, 1989) with enzyme properties similar to the Ala-Gly dipeptidase from granulomas were shown to be glycoproteins. Final purification of the dipeptidases was accomplished by h.p.l.c., and recovery of protein and purification yield of the purified enzymes were calculated (Table 2). The M_r of Leu-Leu dipeptidase was 178 000 and that of Ala-Gly dipeptidase was 183 000.

Substrate specificity

The catalytic activities of the two dipeptidases with various synthetic substrates are compared in Table 3. The most reactive substrates for Leu-Leu dipeptidase and Ala-Gly dipeptidase were Leu-Leu and Ala-Gly respectively. The rate of hydrolysis of other dipeptides was much lower, except for Leu-Leu dipeptidase on His-Leu and Ala-Gly dipeptidase on Ala-Ala. In addition, Leu-Gly was hydrolysed moderately efficiently by both enzymes. The granuloma enzymes did not hydrolyse β -naphthylamide

aminopeptidase substrates, but showed hydrolytic activity for some tripeptides tested in this study. The hydrolytic activity of the two granuloma enzymes with different dipeptide substrates was further contrasted with substrate specificity reported for others from various tissues. Hepatic granuloma Leu-Leu dipeptidase activity is similar to human liver dipeptidase activity (Sugiura *et al.*, 1977; Ito *et al.*, 1987), but Ala-Gly dipeptidase activity appears to be more like the activity reported for kidney dipeptidase from hog (Campbell *et al.*, 1966; Harper *et al.*, 1971), human (Sugiura *et al.*, 1978; Ito *et al.*, 1987) and rat (Kozak & Tate, 1982). The kinetic constants (Table 4) of purified granuloma enzymes towards the most reactive substrates were examined by Lineweaver–Burk plots as well as by an analysis program for the microcomputer (Yamaoka *et al.*, 1981), by using substrate concentrations of 0.1–0.5 mM in 50 mM-Tris/HCl buffer, pH 8.0, at 37 °C. k_{cat}/K_m ($\mu\text{M}^{-1}\cdot\text{s}^{-1}$) was calculated to be 0.13 for Leu-Leu dipeptidase and 0.27 for Ala-Gly dipeptidase with Leu-Leu and Ala-Gly respectively as substrates. Kinetic data for comparison from previous studies are quite limited. Granuloma dipeptidase catalysed hydrolysis of Leu-Leu with a higher K_m

Table 3. Substrate specificity

A final concentration of 0.5 mM-substrate was used in the reaction. Enzymes purified on a Con A-Sepharose column were used as enzyme source. A final concentration of 8 μ M-LTD₄ was used. Abbreviation: β NA, β -naphthylamide.

Substrate	Specific activity (units/mg of protein)	
	Leu-Leu dipeptidase	Ala-Gly dipeptidase
Leu-Leu	15.5	5.31
Leu-Gly	9.35	17.4
Ala-Gly	2.04	47.3
Gly-Gly	0.46	6.17
Cys-Gly	1.74	2.53
His-Leu	10.5	2.65
Ala-Ala	1.49	32.0
Gly-Ala	2.04	12.0
Leu- β NA	0	0
Asp- β NA	0	0
Gly- β NA	0	0
Ala- β NA	0	0
Ala-Ala-Ala	2.12	8.19
Gly-Ala-Ala	0	0
Leu-Gly-Gly	3.17	6.11
Ala-Pro-Gly	0.40	2.52
LTD ₄	14.2	31.1

value (0.45 mM) than human liver dipeptidase (K_m 0.29 mM), as reported by Sugiura *et al.* (1977), but the values for hydrolysis of Ala-Gly by the granuloma enzyme and rat kidney enzyme (Kozak & Tate, 1982) were comparable at K_m 0.8 mM.

The conversion of LTD₄ into LTE₄ catalysed by Ala-Gly dipeptidase detected by h.p.l.c. is illustrated in Fig. 2. A decrease in the LTD₄ peak was associated with the appearance of an LTE₄ peak, but no other peak was detectable at 280 nm, indicating specificity for the enzyme reaction. The retention times measured were 21.6 min for LTD₄ and 25.7 min for LTE₄. Conversion of LTD₄ into LTE₄ by dipeptidases from various tissue has been reported without kinetic values by Kozak & Tate (1982), Hirota *et al.* (1985, 1986) and Campbell *et al.* (1988). Table 4 summarizes kinetic values for granuloma dipeptidases. The K_m values were

Table 4. Kinetic parameters of dipeptidases with dipeptides and LTD₄ as substrates

K_m and V_{max} were determined by Lineweaver-Burk plots. k_{cat} values were calculated on the basis of M_r values of 178000 for Leu-Leu dipeptidase and 183000 for Ala-Gly dipeptidase.

Substrate...	Leu-Leu dipeptidase		Ala-Gly dipeptidase	
	Leu-Leu	LTD ₄	Ala-Gly	LTD ₄
s (mM)	0.1–0.5	0.004–0.01	0.1–0.5	0.004–0.01
K_m (μ M)	450.0	25.0	830.0	5.3
V_{max} (μ mol/min per mg)	19.4	49.0	73.0	50.4
k_{cat} (s^{-1})	57.6	145.4	222.7	153.3
k_{cat}/k_m (μ M ⁻¹ ·s ⁻¹)	0.13	5.82	0.27	28.9

lower than sheep lung dipeptidase (K_m = 43.6 μ M; Campbell *et al.*, 1990), comparable with that of hepatoma cells (K_m = 6.6 μ M; Huber & Keppler, 1987), but considerably higher than that reported for the enzyme from human polymorphonuclear leucocytes (K_m = 0.95 μ M; Raulf *et al.*, 1987).

Effects of bivalent cations

The percentage residual activity of the two dipeptidases after preincubation with a final concentration of 1 mM of each bivalent cation at 37 °C was calculated, taking the activity without additions as 100 %. A modest activation of Leu-Leu dipeptidase with MnCl₂ (117 %) and Ala-Gly dipeptidase with ZnCl₂ (114 %) was found. MnCl₂ activation has also been reported for human liver (Sugiura *et al.*, 1977) and pancreatic (Ito *et al.*, 1983) enzymes, both of which effectively hydrolyse Leu-Leu. The rate of MnCl₂ activation of the granuloma enzyme was much lower than that seen for the human enzymes. However, MnCl₂ seems to have a 'stabilizing' effect on the granuloma Leu-Leu dipeptidase, as the enzyme activity was rapidly lost during the first and second steps of purification without 0.1 mM-MnCl₂ in the buffers. Whether ZnCl₂ is an activator of Ala-Gly dipeptidases from other tissue sources is unknown, although hog kidney (Campbell *et al.*, 1966) and rat kidney (Kozak & Tate, 1982) enzymes are reported to contain zinc. FeCl₂ inhibited Leu-Leu

Fig. 2. H.p.l.c. profiles of conversion of LTD₄ into LTE₄ catalysed by the Ala-Gly dipeptidase

Only LTD₄ (retention time = 21.6 min) is detected in the 0 min reaction (a), but LTD₄ decreases and LTE₄ (retention time = 25.7 min) increases after 3 min (b) and 5 min (c).

Table 5. Inhibition of dipeptidases purified from hepatic granulomas

The activity without the modifier is taken as 100%. Enzymes purified on a Con A-Sepharose column were used. Leu-Leu dipeptidase (4.3 m-units) and Ala-Gly dipeptidase (4.7 m-units) were incubated for 20 min at 37 °C with the potential inhibitors.

Inhibitor (concn.)	Relative activity (%)	
	Leu-Leu dipeptidase	Ala-Gly dipeptidase
Bestatin (5 μ M)	0.15	81.8
Cys-Gly (0.1 mM)	85.2	39.3
Cilastatin (5 μ M)	71.4	43.0
(50 μ M)	62.6	36.0
<i>o</i> -Phenanthroline (0.1 mM)	100.0	66.8
EDTA (0.1 mM)	80.3	45.2
Dithiothreitol (0.1 mM)	100.0	74.3
Captopril (5 μ M)	19.8	100.0
(50 μ M)	0	38.5

dipeptidase (69%) but MnCl₂ (86%) and CaCl₂ (96%) did not. On the other hand, CaCl₂ (67%) and FeCl₂ (64%), but not MgCl₂ (89%), inhibited Ala-Gly dipeptidase activity.

Inhibition profile of hepatic granuloma dipeptidases

The percentage inhibition of the purified enzymes by various chemicals is listed in Table 5. Bestatin is a potent inhibitor of Leu-Leu dipeptidase but a rather weak inhibitor of Ala-Gly dipeptidase. Data reported for effects of bestatin on dipeptidases from different sources are complex. It is a competitive inhibitor of the enzyme from mouse ascites tumour (K_i 2.7 mM; Patterson, 1989), but a non-competitive inhibitor of human kidney enzyme (K_i 4.25 μ M; Campbell *et al.*, 1988). It was found not to inhibit hydrolysis of glycyldehydrophenylalanine by rat kidney and lung dipeptidase (Hirota *et al.*, 1985, 1986), but Littlewood *et al.* (1989) reported inhibition of hydrolysis of the same substrate by the hog kidney enzyme. Bestatin also did not inhibit hydrolysis of LTD₄ by human polymorphonuclear leucocytes (Raulf *et al.*, 1987). Although the precise inhibitory mechanisms of the different enzymes remain to be determined, this inhibitor most clearly differentiates between the two dipeptidases associated with granulomatous inflammation. The effects of Cys-Gly, an inhibitor of the rat kidney enzyme (Kozak & Tate, 1982), were contradictory as it was a much stronger inhibitor of Ala-Gly dipeptidase than of Leu-Leu dipeptidase. The inhibitory activity of cilastatin toward dipeptidase is well established (Köller *et al.*, 1985; Hirota *et al.*, 1985, 1986; Littlewood *et al.*, 1989). It showed a modest inhibition of both granuloma dipeptidases but was more effective with Ala-Gly dipeptidase. Chelators such as *o*-phenanthroline and EDTA showed inhibitory activity toward Ala-Gly dipeptidase, as also reported for human and hog enzymes (Harper *et al.*, 1971; Sugiura *et al.*, 1977, 1978), but did not inhibit Leu-Leu dipeptidase. Similarly dithiothreitol inhibited Ala-Gly dipeptidase but not Leu-Leu dipeptidase.

Captopril is an inhibitor of angiotensin-converting enzyme (Ondetti *et al.*, 1977). Ito *et al.* (1987) tested the inhibitory activity of captopril toward dipeptidase purified from various cell types, since the structure of captopril resembles a dipeptide. In the present study we confirmed that captopril competitively inhibits both granuloma dipeptidases. K_i values were 0.85 μ M and 37 μ M for Leu-Leu and Ala-Gly dipeptidases respectively.

Molecular properties of granuloma dipeptidases

The M_r values determined by Bio-Sil SEC-250 h.p.l.c. were approx. 178000 and 183000 for Leu-Leu and Ala-Gly di-

Fig. 3. SDS/PAGE of Leu-Leu and Ala-Gly dipeptidases purified from mouse hepatic granulomas

Lane M, marker proteins; lane 1, Leu-Leu dipeptidase (10 μ g of protein); lane 2, Ala-Gly dipeptidase (10 μ g of protein).

Table 6. Amino acid composition of hepatic granuloma dipeptidases

Methionine and tryptophan were detected, but the concentration was very low. N.D., not determined.

Amino acid	Composition (mol. %)	
	Leu-Leu dipeptidase	Ala-Gly dipeptidase
Asp	9.7	13.6
Thr	9.4	7.5
Ser	9.3	6.3
Glu	10.5	9.9
Pro	8.8	7.0
Gly	6.5	3.9
Ala	5.8	7.7
Cys	N.D.	N.D.
Val	9.8	9.3
Met	—	—
Ile	4.1	4.6
Leu	6.3	10.8
Tyr	3.9	2.5
Phe	4.1	3.4
His	2.3	2.8
Lys	7.5	5.9
Arg	2.0	4.7
Trp	—	—

peptidases respectively. SDS/PAGE of both enzymes (Fig. 3) showed apparent homogeneity and the M_r of the subunits of Leu-Leu dipeptidase was calculated to be about 56000 and that of Ala-Gly dipeptidase about 57000. Most dipeptidases from mammalian tissues have been reported to have M_r values higher than 100000 and to be composed of subunits ranging in M_r from 45000 to 100000 (Hooper *et al.*, 1990; Hirota *et al.*, 1985; Sugiura *et al.*, 1978).

Amino acid composition

Amino acid analysis (Table 6) was performed on the excised protein bands from the PVDF membrane in order to ensure purity of the proteins, although we were unable to determine the values of amino acids present at low concentration; methionine values were not included in this study. The amino acid composition of Ala-Gly dipeptidase was comparable with that of human (Adachi *et al.*, 1989) and pig (Campbell, 1970; Heese *et al.*, 1988) kidney dipeptidase. On the other hand, considerable

differences between the compositions of Leu-Leu and Ala-Gly dipeptidases were observed. Leu-Leu dipeptidase contained a higher percentage of threonine and serine and a lower percentage of leucine.

Role of leukotrienes in granulomatous inflammation

Leukotrienes, derived via the lipoxygenase pathway from arachidonic acid, are pro-inflammatory peptidolipids which have been implicated in the pathobiology of a number of human diseases, particularly hypersensitivity (Lewis *et al.*, 1990). Their place in chronic granulomatous inflammation, however, is less clear. In the few studies that have addressed the question, alveolar macrophages from patients with sarcoidosis (Bachwich *et al.*, 1987) or during experimental pulmonary granuloma formation (Shellito & Smezek, 1990) and macrophages from patients with hepatic schistosomiasis (Tripp *et al.*, 1988) produced decreased or normal amounts of the leukotrienes measured and increased amounts of other eicosanoids. Since no-one has assayed the full complement of leukotrienes or done a complete time-course analysis, the conclusion that leukotriene metabolism in granulomas is depressed may be premature. This cautionary note is especially cogent because macrophages possess leukotriene-metabolizing enzymes and their activity is higher than that found in neutrophils or lymphocytes (Nagaoka & Yamashita, 1987a,b). Furthermore our findings of a marked elevation of LTD₄-metabolizing activity in both skin (Sato *et al.*, 1991) and liver granulomas suggest the opposite may be true. It may be possible that, during granulomatous inflammation, eicosanoid metabolism through the leukotriene pathway is elevated but accelerated to its terminus in a down-modulating mode which might be important for maintaining lesional homeostasis and chronicity. Alternatively, the dipeptidases studied by us may have other natural substrates in the granulomatous tissue. A more thorough analysis of the leukotriene pathway is required to resolve this issue. In the meantime, it is clear that LTD₄-metabolizing dipeptidase activity closely follows the course of development of experimental hypersensitivity granulomas in skin, and as such is a candidate to become a useful marker of granulomatous inflammation in the same way as angiotensin-converting enzyme and proline endopeptidase (Iida *et al.*, 1991).

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