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PURIFICATION AND ENZYMATIC PROPERTIES OF AN L-LEUCINE AMINOPEPTIDASE FROM SWINE LIVER

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An L-leucine aminopeptidase (α-aminoacyl-peptide hydrolase (cytosol), EC 3 4 11 1), having a specificity toward the substrate L-leucine amide, but not toward L-leucyl β-naphthylamide or L-leucyl *p*-nitroanilide, has been purified 332-fold from swine liver, with a yield of 8 6% This is the first purification of this enzyme from hepatic tissue The purified enzyme submitted to analytical electrophoresis on cellulose acetate strips or in polyacrylamide gel showed a single band after staining with Ponceau S Red dye or Amido black, respectively Purified swine liver L-leucine aminopeptidase, a cytosol enzyme, exhibited a molecular weight of 268 000 ± 50 000 by gel filtration It hydrolyzed L-leucine amide substrate and L-leucyl peptides It was activated by Mg^{2+} and Mn^{2+} and inhibited by Co^{2+} and Zn^{2+} The optimum pH was 10 It was rather sensitive to heat elevation Swine liver L-leucine aminopeptidase was inhibited by EDTA, citric acid, isocaproic acid, dodecylamine, aliphatic alcohols and *p*-chloromercuribenzoate but unaffected by monoiodoacetic acid and diisopropyl fluorophosphate

Introduction

The L-leucine aminopeptidases (α-aminoacyl-peptide hydrolase (cytosol), EC 3 4 11 1) are zinc containing α-aminoacyl-peptide hydrolases of broad specificity They hydrolyse most L-peptides, splitting off an N-terminal residue possessing a free α-amino group L-Leucine amide is recognized as a good substrate for these enzymes

L-Leucine aminopeptidases have been isolated and purified from several animal organs from hog kidney by Spackman et al [1], Smith and Hill [2] and by Hanson and Hutter [3] Hanson et al [4] also purified and crystallized a L-leucine aminopeptidase from bovine-eye lens tissue

At present, little information has been reported concerning liver L-leucine aminopeptidase Tamura et al [5] demonstrated the presence of L-leucine amide splitting activities in bromelain-treated rat liver and in rat plasma after hepatobiliary damage, but no purification was attempted

An L-leucyl-glycine splitting activity [6] has also been reported in human serum, particularly during hepatic disease states

In preceding papers, we described two L-leucine aminopeptidase activities in human sera One activity present in normal and pathological sera, has an optimal velocity at pH 7, and is not activated by Mg^{2+} [7] The other, present only in hepatic diseases, exhibits an optimal activity at pH 9 and is strongly activated by Mg^{2+} and Mn^{2+} [8]

In the present work, we describe the purification of an L-leucine aminopeptidase from swine liver and we present a study of its enzymatic properties This enzyme is significantly different from the human liver naphthylamidase partially purified by Smith et al [9]

Materials and Methods

Substrates and effectors

L-Leucine amide hydrochloride (pure A R) was

obtained from Koch-Light, L-phenylalanine amide, L-tyrosine amide, L-leucyl-glycyl-glycine and L-leucyl-glycine from Schwarz Mann, glycine, L-leucine, glycine amide hydrochloride, *N*-valeramide, acetamide, succinimide, nicotinamide, oxamide, benzamide, butyramide, propionamide, monoiodoacetic acid, natrium *p*-chloromercuribenzoate and diisopropyl fluorophosphate from Fluka, reduced glutathione, $MgCl_2$ 6 H_2O, EDTA 2 H_2O Titriplex III, citric acid H_2O, $CoSO_4$, $ZnSO_4$ and $MnCl_2$ from Merck, isocaproic acid from Schuchardt, dodecylamine and alcohols from Prolabo, and proteins of various molecular weights from Boehringer ("Combithek")

Enzyme assay

The L-leucine aminopeptidase standard assay was performed by following the hydrolysis of L-leucine amide substrate according to a method proposed by our laboratory for use with human sera [8] 50-μl aliquots were mixed with 1 ml 10 mM L-leucine amide/10 mM sodium borate buffer (pH 10)/1 5 mM $MgCl_2$ and incubated for 15 min at 37°C The ammonia liberated was measured colorimetrically, at the rate of 60 samples/h, with phenol hypochlorite by the method of Berthelot [10] adapted to the Technicon I Auto Analyzer with automatic dialysis against 0 15 M NaCl

The hydrolysis of two substrates, L-leucyl *p*-nitroanilide and L-leucyl β-naphthylamide were determined colorimetrically L-Leucyl *p*-nitroanilide hydrolase activity was determined at 25°C and pH 7 2 at 405 nm, according to Nagel et al [11] using "Test combination L A P", manufactured by Boehringer Mannheim GmbH Diagnostica L-Leucyl β-naphthylamide hydrolase activity was determined at 37°C and pH 7 0 by the procedure of Arst et al [12] liberated β-naphthylamide was converted into an azo die and condensed with naphthylethylene diamine dihydrochloride The absorbancy at 560 nm was recorded Other substrates were tested replacing L-leucine amide in our standard conditions by various amides and peptides at the same concentration

Ammonia liberated from amides was measured by the method of Berthelot [10] and the amino acids liberated from the peptides were analyzed by electrophoresis or chromatography Electrophoresis of amino acids was performed on Whatman No 1 paper sheet in 12 ml pyridine/40 ml acetic acid to 2 l with H_2O (pH 3 9) The running time was 3 h at 450 V The amino acids, glycine and L-leucine, were visualized with 1% ninhydrin reagent One-dimensional descending paper chromatography of amino acids was performed with butanol/acetic acid/water (4 1 5, v/v) for 20 h at 23°C on Whatman No 1 paper and stained with 1% ninhydrin reagent

Metal activation

Four metal ions (Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}) were tested independently according to standard conditions of L-leucine aminopeptidase assay as described above, with the exception that 1 5 mM Mg^{2+} was replaced for each studied bivalent cation by 1 mM concentration, because higher molarities at pH 10 could lead to Zn^{2+} or manganese hydroxide precipitation, perturbing enzyme assay by Berthelot's reaction

Inhibitors

After mixing at various concentrations with the L-leucine amide substrate (Table III), the L-leucine aminopeptidase activity was determined under our standard conditions

Influence of aliphatic alcohols

Influence of methanol, ethanol, *n*-propanol, *n*-butanol, isopropanol and secondary butanol was studied, using our standard assay for L-leucine aminopeptidase activity, after 30 min preincubation at 37°C of a 50% mixture (v/v) of alcohol/enzyme

Protein determination

Protein was determined by the method of Lowry et al [13] which we adapted for a Technicon I Auto Analyzer, equipped with a 625 nm interference filter We used bovine serum albumin as standard

Molecular sieve of proteins by gel filtration on Sephadex G-200

Sephadex G-200 (Pharmacia Sweden Fine Chemicals) was preswollen for 5 days at 4°C in a 25 mM sodium borate buffer (pH 8) and poured into a 1 × 0 01 meter glass column and allowed to sediment by free flow, until the height of the gel reached 60 cm

Protein solutions of various molecular weights

from Boehringer ("Combithek") and purified swine liver L-leucine aminopeptidase were deposited at the top of the column and elutions were performed with 25 mM sodium borate buffer (pH 8) [14] The molecular weight was calculated by determining the ratio of the elution volume of aminopeptidase to the void volume of the column, after calibrating the column with the following pure proteins of known molecular weight, chymotrypsinogen (M_r 25 000), Bovine serum albumin (68 000), Aldolase (158 000), Catalase (240 000), Ferritin (450 000)

Metal content

Zn^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+} were measured by atomic absorption spectroscopy and protein by the method of Lowry et al [13]

pH activity studies

These studies were made at the same time with a purified enzyme and a swine-liver crude homogenate, according to the standard conditions, except for pH, which varied from 7 to 12 Moreover similar experiments were performed without the addition of Mg^{2+}

Heat denaturation of enzyme

Swine liver enzyme samples were submitted to preincubation at 5°C, intervals from 50–75°C Aliquots taken out after 1, 2, 3, 5, 10, 15 and 20 min were analyzed for L-leucine aminopeptidase activity in standard conditions either with or without 1 5 mM Mg^{2+}

K_m determination

Kinetics of swine liver L-leucine aminopeptidase activity were determined in standard conditions, the L-leucine amide concentration varying from 1 to 40 mM

Preparation of the crude homogenate

Fresh hog liver obtained from a slaughter house was divided into portions each weighing about 60 g, frozen and maintained at −20°C At this temperature the tissue could be stored for several months without loss of L-leucine aminopeptidase activity After thawing, 5 g samples of hog liver were homogenized for 20 s in 25 ml isotonic NaCl in an Ultraturrax homogenizer This procedure was repeated until 60 g liver homogenate were suspended in 300 ml NaCl The entire suspension was centrifuged at 17 000 rev / min (23 000 × *g*) for 15 min at 4°C

Acetone precipitation

To 1 vol centrifuged supernatant 1 5 vol 60% aqueous acetone solution at −20°C was added The mixture was maintained for 30 min at the same temperature and centrifuged at 9 000 rev /min (8 000 × *g*) for 10 min The resultant supernatant was discarded and the pellet was resuspended in a volume of water corresponding to one-fourth of the initial supernatant, homogenized and centrifuged for 10 min at 9 000 rev /min (8 000 × *g*) at 4°C

DEAE-Sephacel column chromatography

Chromatography was performed at 4°C DEAE-Sephacel (bead-formed, preswollen cellulose ion exchanger) (Pharmacia Fine Chemicals, Uppsala, Sweden) washed by successive decantations with 0 5 M sodium borate buffer (pH 8) and equilibrated with 25 mM sodium borate buffer (pH 8) was then poured into a 5 × 18 cm chromatography glass column and washed with equilibration buffer at free flow for 48 h A 60-ml aliquot of the acetone-precipitated enzyme solution was introduced into the column Elution was performed by washing with 330 ml of a 25 mM sodium borate buffer (pH 8) over a 24-h period, followed by a 50–300 mM sodium chloride gradient (330 ml) developed over a 24 h period employing an Ultrograd LKB gradient mixer All fractions contained 4 6 ml

Preparative Cellogel block electrophoresis

Cellogel blocks for electrophoresis (Cellogel Chemetron Milano) (4 × 17 cm) were immersed overnight in a 80 mM veronal buffer (pH 8 4) previous to use Solutions to be analyzed (0 5 ml) were applied to a perforated deposit line 2 5 cm from the cathodic extremity of the Cellogel block Electrophoresis was performed for 5 h with a 25 mA constant current in each block One block was stained with Amido black in order to visualize protein Other blocks were cut into ten fractions and elution performed by extruding the liquid through a specially designed SPFU Chemetron press syringe

Analytical electrophoresis

(a) Cellulose acetate strip analytical electro-

phoresis 2 or 3 μl of sample were deposited with the aid of an applicator on strips of Cellogel (Cellogel Chemetron Milano) (2 5 × 17 cm) 40 mM veronal (pH 9 2) was the buffer used for electrophoresis Electrophoresis was carried out at 200 V for 75 min The bands were stained with Ponceau S Red dye and the background clarified by rinsing the sheets three times with a 5% acetic acid solution

(b) Polyacrylamide gel-disc electrophoresis (Research disc electrophoresis equipment - Model 1200 Canalco) About 5 μl of sample (2 75 g protein/l) to be analyzed were mixed with the loading gel The stacking gel in 0 5 M Tris buffer (pH 6 6–6 8), and the separating gel in 1 5 M Tris buffer (pH 8 8–9) were both 5% cross-linked Electrophoresis was performed for 40 min in 25 mM Tris/0 19 M glycine buffer (pH 8 2–8 4) with a 5 mA/tube constant current Gels were stained with Amido black and then destained for 90 min at 10 mA/tube in an 7% acetic acid bath

Results

Pig liver was fractionated into various cellular components according to the method of Appelmans et al [15] in order to determine the cellular location of L-leucine aminopeptidase activity The results (Table I) demonstrated that the swine liver L-leucine aminopeptidase activity is almost exclusively located

TABLE I

CELLULAR LOCATION OF SWINE LIVER L-LEUCINE AMINOPEPTIDASE

	Total enzymatic activity (I U)	Activity (%)
Step I		
600 × *g* 10 min (nuclei)	27	8 8
Step II		
20 000 × *g* 10 min (mitochondria)	12	3 9
Step III		
105 000 × *g* 60 min		
Pellet III (ribosomes)	3 3	1 1
Supernatant III		
Cytosol	264	86 2

in 105 000 × *g* supernatant corresponding to the cytosol

Purification of swine liver L-*leucine aminopeptidase*

The enzyme was precipitated from the supernatant fraction obtained from the crude homogenate by adding 60% aqueous acetone solution as described At lower percentages, the full precipitation of the enzyme was not obtained The yield was not improved by increasing the acetone percentage over 60%, but the specific activity decreased The acetone-precipitated enzyme preparation exhibited a 11 3-fold purification and a 58 7% yield, starting with the crude homogenate (Table II)

The enzyme was further purified from the acetone-precipitated enriched preparation by chromatography on DEAE-Sephacel as shown in Fig 1 Elution was carried out initially with a 25 mM sodium borate buffer (pH 8) separating out a protein peak without any L-leucine aminopeptidase activity Then, using a 50–300 mM NaCl gradient, a second protein peak was eluted, just in the front of the L-leucine aminopeptidase activity Activities toward L-leucyl β-naphthylamide and L-leucyl *p*-nitroanilide substrates were also evidenced, but these activities were very weak and shifted to the back side of the L-leucine aminopeptidase peak (Fig 1) The fractions between 813 and 888 ml contained almost the entire activity toward the L-leucine amide substrate These fractions were pooled, dialyzed overnight against deionized water at 4°C, concentrated with a rotatory evaporator at 30°C and reduced to a volume of 2 5 ml

A 0 5 ml sample of the preceding solution was applied on five Cellogel blocks and submitted to preparative electrophoresis After 5 h, a coloured zone could be seen on the anionic side followed by a differentiated streak from the origin to the preceding spot These zones could be stained by Amido black, showing protein heterogeneity The block was cut into ten fractions starting from the deposit line to the anodic side After elution, the L-leucine aminopeptidase activity was found in fractions 6, 7 and 8, which migrated faster than the main protein fraction Fraction 8 exhibited the highest enzymatic activity (spec act 338 7) and the yield was 8 6% starting from the crude homogenate (Table II)

Cellulose acetate strip analytical electrophoresis

TABLE II

PURIFICATION OF L-LEUCINE AMINOPEPTIDASE FROM PIG LIVER

	Enzymatic Activity (I U /ml)	Protein (mg/ml)	Specific Activity (I U /mg)	Purification	Yield (%)
Homogenate	27 44	26 92	1 02	1	100
Step 1 (supernatant, 23 000 $\times g$ for 15 min)	32 76	21 5	1 52	1 5	
Step 2 (acetone precipitation)	83 86	7 27	11 53	11 3	58 7
Step 3 (DEAE-Sephacel chromatography	38	1 58	24 05	23 5	36 5
Step 4 (preparative Cellogel block electrophoresis)					
Fraction no 6	11 34	0 78	14 53	14 2	0 3
Fraction no 7	72 45	0 42	172 5	169 1	3 6
Fraction no 8	105	0 31	338 7	332	8 6

performed as a control of purity, showed by staining with Ponceau S Red dye, that fraction 7 still contained an important part of the impurities, while in fraction 8 a single coloured band could be observed After elution this band was active toward L-leucine amide substrate Polyacrylamide gel electrophoresis also showed a single band after staining with Amido black, but enzymatic activity was inhibited by TEMED, necessary for gel preparation, and could not be demonstrated (Fig 2)

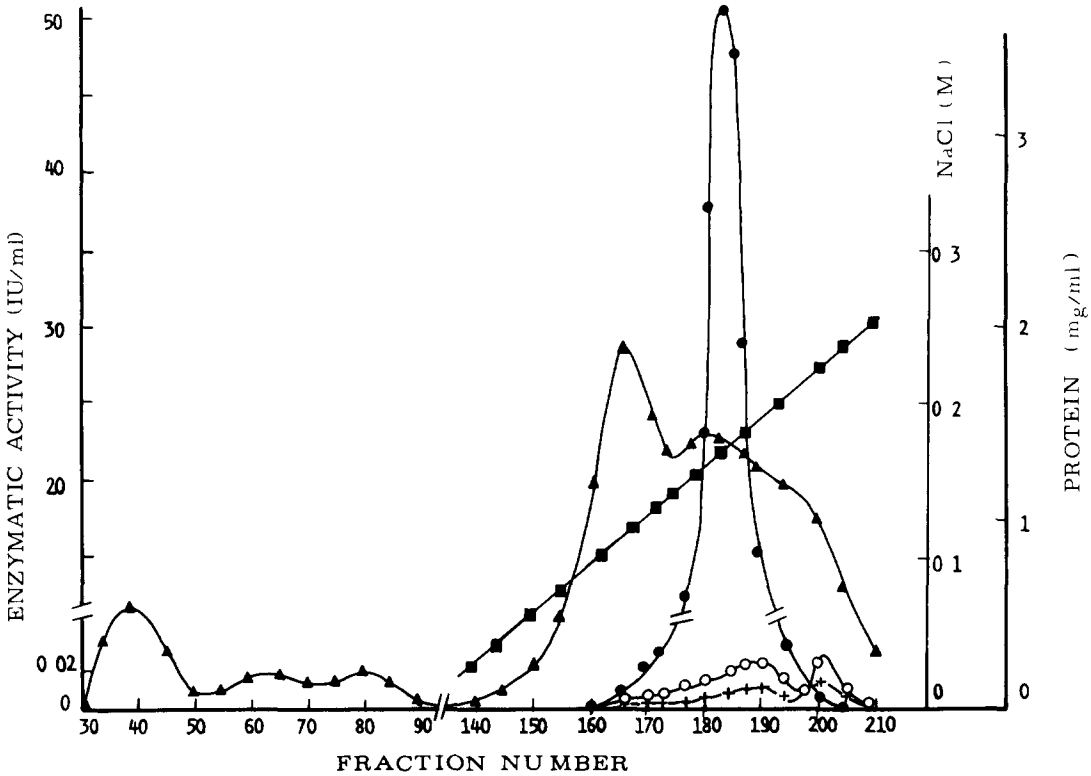

Fig 1 Ion-exchange chromatography on DEAE-Sephacel of swine liver L-leucine aminopeptidase which has been partially purified by precipitation with acetone Elution is performed at first with a 25 mM sodium borate buffer (pH 8) then with 50–300 mM NaCl gradient (■———■) In each fraction, the level of protein (▲———▲) and the splitting activities toward L-leucine amide (●———●), L-leucyl *p*-nitroanilide (+———+) and L-leucyl β-naphthylamide (———) substrates are determined All fractions contained 4 6 ml

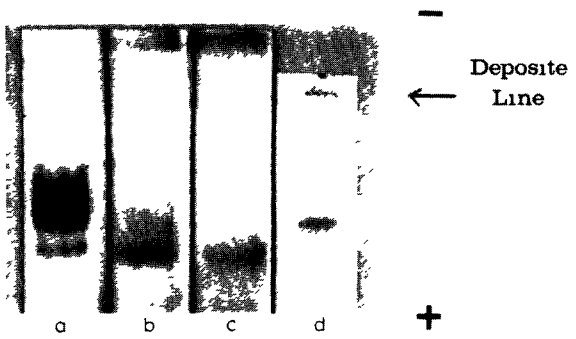

Fig 2 Electrophoretic control at various stages of purification of swine liver L-leucine aminopeptidase on cellulose acetate strips (a–c) and on polyacrylamide gel (d) a, pooled active fractions from DEAE-Sephacel chromatography, b, partially purified (fraction No 7 of Cellogel preparation block electrophoresis), c and d, purified enzyme preparation (fraction No 8)

Enzymatic properties

Substrate specificity The enzyme was highly specific for L-leucine amide with the exception of some aromatic amino acid amides L-tyrosine amide and L-phenylalanine amide (respective activity 4 and 5 7%, relative to leucine amide splitting activity = 100%) Other amides studied (propionamide, butyramide, glycinamide-HCl, *n*-valeramide, L-asparagine-HCl, succinimide, nicotinamide, benzamide, acetamide, L-glutamine and oxamide) were not split

Swine liver L-leucine aminopeptidase hydrolysing activity on chromogenic substrates was very low compared to L-leucine amide In our experience, the activities toward substrates L-leucyl β-naphthylamide, L-leucyl *p*-nitroanilide and L-leucine amide were, respectively, 0 054 I U /ml, 0 027 I U /ml and 105 I U /ml in the purified L-leucine aminopeptidase from swine liver, and 0 76 I U /ml, 0 245 I U /ml and 27 44 I U /ml in crude homogenate Thus, the ratios of activities toward L-leucyl β-naphthylamide or L-leucyl *p*-nitroanilide/activity toward L-leucine amide were, respectively, 0 00051 and 0 00025 with the purified preparation and 0 027 and 0 0089 with a crude swine liver homogenate Thus, after purification, and in comparison with the starting material, these ratios fell to 1 88 and 2 8% of their initial values, respectively, with L-leucyl β-napthylamide and L-leucyl *p*-nitroanilide

L-Leucyl-glycine dipeptide was completely hydrolyzed into free leucine and glycine, while L-leucyl-glycyl-glycine tripeptide was split into free leucine and glycyl-glycine dipeptide, and reduced glutathione remained unmodified

Metal activation Our results indicated that swine liver L-leucine aminopeptidase activity was enhanced by 1 mM Mg^{2+} or Mn^{2+} concentration and was inhibited by 1 mM Zn^{2+} or Co^{2+} (280, 354, 28 and 60%, respectively, relative to enzyme lacking ions = 100%) Although Mn^{2+} activation was more effective, we preferred to use Mg^{2+} to avoid any precipitation This was all the more interesting since the curve of the enzymatic activity plotted against Mg^{2+} molarity showed that the 1 5 mM concentration retained for our standard assay, maximum velocity was obtained for swine liver L-leucine aminopeptidase activity

Inhibitors The metal chelators EDTA and citric acid were inhibitors of the enzyme (Table III) Diisopropyl fluorophosphate did not inhibit the enzyme nor did monoiodoacetic acid However 1 mM sodium *p*-chloromercuribenzoate produced strong inhibition (Table III) It is obvious that nonpolar hydrophobic hydrocarbon chains can compete with L-leucine amide substrate to occupy L-leucine amino-

TABLE III

EFFECT OF VARIOUS INHIBITORS ON L-LEUCINE AMINOPEPTIDASE ACTIVITY

Reagent	Concentration (mM)	Relative activity	
		without any Mg^{2+}	with 1 5 mM Mg^{2+}
None		100	280
Natrium *p*-chloro mercuribenzoate	1	15	33 5
Iodoacetic acid	10	97	280
Diisopropyl-fluorophosphate	10	97 5	269
Dodecylamine	10	50	168
Isocaproic acid	100	86	196
	200	30	140
EDTA	100	25	–
	200	0	–
Citric acid	100	31	–
	200	6	–

TABLE IV

INHIBITION OF L-LEUCINE AMINOPEPTIDASE ACTIVITY BY VARIOUS ALCOHOLS

The l-leucine aminopeptidase activity was tested after 30 min preincubation at 37°C of a 50% mixture (v/v) of alcohol and enzyme solution

Alcohol	Relative activity
None	100
Methanol	80 5
Ethanol	78
1-Propanol	47 3
2-Propanol	62 2
1-Butanol	38 1
2-Butanol	68

peptidase active sites Indeed, we observed substantial inhibition of swine liver L-leucine aminopeptidase activity in the presence of 100 and 200 mM isocaproic acid or of 10 mM dodecylamine Inhibition of L-leucine aminopeptidase with aliphatic alcohols increased with length of hydrocarbon chain (Table IV) Branched chain aliphatic alcohols produced less inhibition than straight chain alcohols

Molecular weight The elution volume of liver L-leucine aminopeptidase from Sephadex G-200 was between those of catalase (M_r 240 000) and ferritin (M_r 450 000) This elution volume coincided with an M_r of 268 000 ± 50 000

Metal content of purified swine liver L-leucine aminopeptidase solution The results indicated Zn^{2+} 11 4 gatom and Mg^{2+} 2 8 gatoms/mol pure enzyme Co^{2+} and Mn^{2+} were absent

pH activity studies No significative difference in pH optima was found between the crude homogenate and the purified enzyme In both cases, a maximum was observed at pH 10 The enzymatic activity diminished as the pH decreased, and became very weak below pH 7 (Fig 3)

Heat denaturation of enzyme Swine liver L-leucine aminopeptidase is rather sensitive to heat elevation it is fully denatured in few minutes at 37°C After a 20 min preincubation at 60°C, only 32% of the initial activity was recovered Even at 50°C, about 20% of enzymatic activity was lost after 15 min heating Furthermore, it was observed that 1 5 mM Mg^{2+} did not significantly influence thermic denaturation

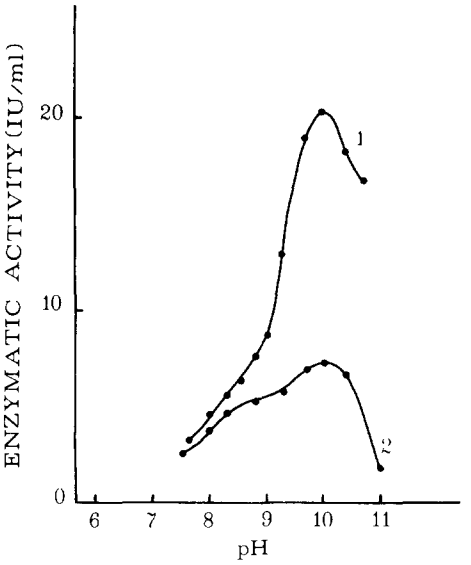

Fig 3 pH dependence of pure swine liver L-leucine aminopeptidase Enzyme preparations were incubated (1) In the presence of 1 5 mM Mg^{2+} and (2) without addition of Mg^{2+}

Influence of L-leucine amide substrate concentration The Michaelis constant calculated from Lineweaver-Burk reciprocal plot was established to be 1 54 10^{-2} M in the presence of 1 5 mM Mg^{2+} at 37°C It was also observed that without Mg^{2+} addition, the Michaelis constant was 2 85 10^{-2} M

Discussion

According to the results described above (Table I), it is obvious that swine liver L-leucine aminopeptidase is essentially located in the 105 000 × *g* supernatant i e , in the cytosol fraction Indeed, only a small proportion of enzyme was found in mitochondrial and microsomal fractions In that, liver enzyme is closed to L-leucine aminopeptidases from swine kidney as described by Smith and Hill [2] and from bovine-eye lens purified by Hanson et al [4] and opposite to microsomal swine kidney aminopeptidase, isolated by Pfleiderer and Celliers [16] Moreover, in human liver, Smith et al [9] partially purified β-naphthyl amino-acyl amidase, the activity of which studied by differential centrifugation was widely distributed among the various cell fractions, so that attempts to localize the enzyme within any one particular fraction were not successful

The determination procedure of L-leucine aminopeptidase activity proposed here is based on the hydrolysis of the L-leucine amide at pH 10 Smith and Hill [2] have demonstrated that this substrate is hydrolyzed by hog kidney L-leucine aminopeptidase as effectively as L-leucyl-glycine dipeptide, and more quickly that other peptides Hanson et al [4] demonstrated that the crystalline L-leucine aminopeptidase from bovine-eye lens tissue, has only a moderate effect upon this substrate, which can also be hydrolyzed by other aminopeptidases Fleischer et al [6] provided evidence to differentiate between human serum L-leucyl β-naphthylamide-hydrolyzing enzymes, and those which hydrolyze L-leucine amide and L-leucyl-glycine As it can be seen on the elution profile of our DEAE-Sephacel column chromatography (Fig 1), hog liver L-leucyl β-naphthylamide and L-leucyl *p*-nitroanilide, splitting activities do not completely co-elute with L-leucine amidase activity

Swine liver L-leucine aminopeptidase activity toward chromogenic substrates is almost insignificant we observed that after purification of the enzyme, the L-leucine amide splitting activity was augmented, while the chromogenic substrates splitting activity was strongly lowered It resulted in considerable diminution of the ratios activity on chromogenic substrate/activity on L-leucine amide, falling to 1 88 and 2 8% of their initial values, respectively, for L-leucyl β-naphthylamide and L-leucyl *p*-nitroanilide It should agree with the hypothesis that activity toward chromogenic substrates in crude homogenate should be mostly due to enzymes different from L-leucine aminopeptidase However, purified enzyme still retaining a very weak but not negative activity toward L-leucyl β-napthylamide and L-leucyl *p*-nitroanilide as regards L-leucine amide splitting activity (ratios 0 00051 and 0 00025, respectively) it does not actually seem possible to completely discard the hypothesis that it should be related to a secondary insignificant activity of L-leucyl aminopeptidase

Tamura et al [5] after treatment of rat liver homogenate with bromelain separated five peaks of aminopeptidase activity by DEAE-cellulose column chromatography One of the enzymes showed a specificity toward L-leucine amide while others showed different substrate specificities toward both L-leucine amide and L-leucyl β-naphthylamide Because no full purification was attempted by Tamura, and the swine liver enzyme has only limited activity at pH 7 5, it is not possible to compare these results with our experiments The leucine aminopeptidase from swine liver purified here is apparently unrelated to the amino acid naphthylamidase partially purified from human liver by Smith et al [9], the latter failing to promote any hydrolysis of L-leucine amide and L-leucyl glycine

For swine liver, hog kidney and eye-lens enzymes with L-leucine amide substrate, Mg^{2+} and Mn^{2+} strongly enhanced activity, whereas Zn^{2+} and Co^{2+} either were devoid of any effect or produced inhibition Once again these results contrast with those obtained with microbial aminopeptidases, which are activated by Zn^{2+} and Co^{2+} bivalent cations but not by Mn^{2+} and Mg^{2+}

Swine liver, swine kidney and bovine eye-lens L-leucine aminopeptidase had about the same behaviour in the presence of following inhibitors EDTA and citrate that are effective chelating agents for bivalent cations, strongly inhibit enzymatic activity Competitive inhibitors like aliphatic alcohols inhibit liver and kidney enzymes SH group-specific reagents do not have always the same action *p*-chloromercuribenzoate clearly inhibits swine liver L-leucine aminopeptidase This is in favour of the involvement of a thiol group in binding the metal ion However, monoiodoacetic acid does not inhibit either liver or kidney swine enzymes These apparent contradictions would disappear with the hypothesis that SH group is not inside but quite near the active site, and that *p*-chloromercuribenzoate inhibition is produced by steric hindrance

The molecular weight established for swine liver L-leucine aminopeptidase (M_r 268 000 ± 50 000) is comparable to the value obtained by Smith and Hill [2] (M_r 300 000) for swine kidney L-leucine aminopeptidase in ultracentrifugation (S_{20w} = 12 65) and by Hanson et al [4] (M_r 326 000 ± 20 000) for bovine eye-lens L-leucine aminopeptidase The molecular weight differences between these three enzymes need not be retained as significant because of the lack of precision of the analytical procedures used

Himmelhoch [17] in the L-leucine aminopeptidase of swine kidney found a Zn^{2+} to protein ratio between 4 and 6 gatom/300 000 g protein, the partial

replacement of the 'native' Zn^{2+} by Mn^{2+}, resulting in activation of the enzyme Hanson et al [18] have found, that crystalline enzyme from the bovine lens contain 8–12 gatom Zn^{2+}/326 000 g protein Our results for swine liver L-leucine aminopeptidase (11 4 gatom Zn^{2+} and 2 8 gatom Mg^{2+}) are in the same order of magnitude as the preceding results The relatively poor precision of metal determinations (blanks must be substracted) may explain the variations

The swine liver L-leucine aminopeptidase optimum pH was established as 10, near to the results obtained by Smith and Hill [2] for swine kidney and by Hanson et al [4] for eye-lens enzymes It contrasts with the microbial classified enzymes whose optimum pH is between 7 and 8

Swine liver L-leucine aminopeptidase easily hydrolyzes L-leucine amide but also L-leucyl-glycine dipeptide and L-leucyl-glycyl-glycine tripeptide The K_m for L-leucine amide substrate was established by us as 1 54 10^{-2} M at 37°C, pH 10, in the presence of 1 5 mM Mg^{2+} and as 2 85 10^{-2} M without any addition of Mg^{2+} We should mention that Hanson et al [4] found 3 125 10^{-2} at pH 9 and 40°C in the presence of 2 mM Mn^{2+} for crystalline eye-lens enzyme, and that Bryce and Rabin [19] found a 5 21 10^{-3} M K_m value at 25°C, pH 8 4, for swine kidney L-leucine aminopeptidase in the presence of 5 mM Mg^{2+} and 15 7 10^{-3} M K_m value in the presence of 2 mM Mn^{2+}

In conclusion, swine liver L-leucine aminopeptidase should be included in the 'true' cytoplasmic L-leucine aminopeptidase group It is a Zn^{2+}-containing metallo-enzyme and is activated by Mg^{2+} and Mn^{2+} L-Leucine amide is the most convenient substrate at pH 10, because it is not hydrolyzed by microbial aminopeptidase Swine liver enzyme splits only very slightly 'chromogenic' substrates like L-leucyl β-naphthylamide and L-leucyl *p*-nitroanilide Thus "L A.P Tests" and other kits distributed by manufacturers using these chromogenic substrates to determine a human serum 'so called' L-leucine aminopeptidase in liver diseases, do not show a good sensitivity regarding L-leucine amide substrate to measure true leucine aminopeptidase issued from liver cytolysis, but should mainly detect other aminopeptidases

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