DIPEPTIDE HYDROLYSIS WITHIN INTACT LYSOSOMES *IN VITRO*

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1. Introduction

The enzymes of rat liver lysosomes can digest susceptible proteins to yield amino acids and small peptides, mainly dipeptide [1]. Several studies [2–6] indicate that digestion of endocytosed protein to the level of amino acids takes place *in vitro* within intact rat liver hetero-lysosomes as well as *in vivo* in cultured mouse macrophages. Endocytosis of radioactive labeled proteins resulted in the appearance of radioactivity in compounds identified as the free amino acids iodotyrosine or leucine in the medium. Small amounts of dipeptides could, however, escape detection under the experimental conditions employed. The extent of intralysosomal digestion may depend on both the properties of the lysosomal proteases and dipeptidases and on the permeability of the lysosomal membrane to the intermediate products of protein digestion. Ehrenreich and Cohn [7] and Lloyd [8] studying different systems have suggested that the lysosomal membrane is permeable to dipeptides.

In a previous study [9] it was shown that L-amino acid methyl esters mediate the rupture of rat liver lysosomes while the D-stereoisomers do not affect lysosomal integrity. It was also found that α-L-amino acid esters serve as substrates for neutral lysosomal esterase and transpeptidase activity, while the D-stereoisomers are either not degraded, or degraded at a much slower rate. It was suggested that an intralysosomal enzymic conversion of the esters leads to an intralysosomal accumulation of free amino acids and dipeptides (which being zwitterions are much less permeable than the respective esters). An intralysosomal product accumulation brings about an inward flow of water leading eventually to lysosomal burst.

Based on the observations discussed above it was reasoned that if exogenous dipeptides gain access to the intralysosomal space and if an intralysosomal hydrolysis of the dipeptides does take place, the accumulated products would lead to lysosomal rupture. A neutral dipeptidase activity in rat liver lysosomes has been reported [10].

Eleven dipeptides were tested for their ability to damage lysosomal integrity as reflected in loss of latency with respect to acid phosphatase. Only the dipeptides that were both permeating and hydrolyzable by the lysosomal enzyme composite brought about lysosomal damage when present at a concentration of 0.01 M. Two dipeptides containing D-Ala were resistant to enzymic hydrolysis and caused damage only when present at much higher concentrations. The data suggest that dipeptides, except when negatively charged, can permeate the lysosomal membrane, and are hydrolyzed within the intact organelle.

2. Materials and methods

2.1. *Chemicals*

L-Ala–L-Ala, L-Met–L-Met, Gly–Gly, disodium *p*-nitrophenyl phosphate (PNPP) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), were products of Sigma Chem. Co., St. Louis. D-Ala–D-Ala, D-Ala–L-Ala were kindly supplied by Mr. I. Jacobson of this Department. Gly–L-Met, L-Met–Gly, Gly–L-Glu and L-Met–L-His were products of Mann Research Laboratories, N.Y. and L-Tyr–L-Ala and L-Ser–L-Leu were products of Miles-Yeda Ltd., Israel.

2.2. *Isolation of rat liver lysosomes*

A lysosome rich fraction (in which acid phosphatase specific activity assayed with β-glycerophosphate

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was higher than that of liver homogenate by a factor of 17–23) was obtained by differential centrifugation described previously (Method A) [9].

2.3. *Assay of acid phosphatase activity*

Acid phosphatase activity was assayed in 0.01–0.02 M Na-Hepes buffer (pH 7) using 0.01 M PNPP as substrate. The release of *p*-nitrophenol was followed in a Gilford recording spectrophotometer 2400-S at 420 nm. Three factors determined the conditions chosen for the assay: i) The treatment with the dipeptides was carried out at pH 7, thus medium composition is not changed significantly during the assay. ii) Optimum activity of acid phosphatase on PNPP is at a pH of 5.4 [11]. H^+ concentration lower than pH 7 would lead to very low enzymic activity. iii) The phenol group of *p*-nitrophenol has a pK of 7.15, at a lower pH the fraction of an ionized light absorbing species will substantially decrease.

2.4. *Analysis of enzyme-reaction products by high voltage electrophoresis*

Electrophoresis was carried out in a model LT-36 electrophoresis tank (Savant Instruments). 25 μl aliquots of reaction mixtures (see legends to figs. 1 and 2) were applied to Whatman No. 3 MM paper. Pyridine acetate buffer of pH 3.5 (glacial acetic acid–pyridine–water (10:1:89, by volume)) was used. The electrophoresis (40 min) was run at a gradient of 50 V/cm.

Table 1
Effect of dipeptides on the latency of acid phosphatase and its release to the medium.

Dipeptide	Available acid phosphatase activity (% of total activity)	Acid phosphatase activity detected in the medium (% of available activity)
–	19 ± 2	42
Gly–Gly	19 ± 2	47
Ala–Ala	70 ± 3	42
Met–Met	30 ± 7	75
Met–His	78 ± 5	70
Gly–Met	28 ± 3	–
Met–Gly	66 ± 6	85
Gly–Glu	17 ± 2	50
Tyr–Ala	60 ± 5	80
Ser–Leu	53 ± 2	90

All the amino acids in the dipeptides having optic isomery were of the L-configuration. The preincubation mixtures consisted of 0.01 M of the indicated dipeptide, 0.01 M Na-Hepes (pH 7), 0.25 M sucrose and a lysosome rich fraction (0.8 mg protein per ml). After a preincubation period of 30 min at 25°C the lysosomal suspensions were kept in an ice bath until assayed. For assay the samples were diluted 1:1 with a solution containing 0.02 M PNPP, 0.25 M sucrose, 0.02 M Na-Hepes. Available activity was recorded for 6 min after which time the reaction mixture was made 0.1% in Triton X-100, and the total activity recorded for an additional period of 6 min. A parallel set of preincubation mixtures was cooled and centrifuged at 4°C for 30 min in a Sorvall RC2-B centrifuge at 12 300 *g*. Acid phosphatase activity in the supernatant was assayed as described above.

Table 2
Stereospecificity and concentration dependence of lysosomal rupture by alanine dipeptides.

Dipeptide	Concentration of added dipeptide (M)	Available acid phosphatase activity (% of total activity) 0 min	15 min	30 min	60 min
–	–	15	17	17	18
L-Ala–L-Ala	0.01	–	42	62	68
D-Ala–L-Ala	0.01	–	11	16	15
D-Ala–D-Ala	0.01	–	15	19	19
L-Ala–L-Ala	0.2	13	–	62	78
D-Ala–L-Ala	0.2	–	–	83	94
D-Ala–D-Ala	0.2	19	–	52	64

Preincubation mixtures consisted of i) 0.01 M alanine dipeptide, 0.25 M sucrose, 0.02 M Na-Hepes containing 1 mg/ml of lysosomal protein and ii) 0.2 M alanine dipeptide, 0.05 M sucrose, 0.02 M Na-Hepes pH 7 and containing 4 mg/ml of lysosomal protein. Acid phosphatase activity was determined on samples withdrawn from the suspension (each containing 40 μg of lysosomal protein) and diluted into an ice cold 0.25 M sucrose solution, 0.02 M in Na-Hepes (total volume 0.8 ml). Prior to assay, the diluted samples were supplemented with 0.2 ml of 0.05 M PNPP, 0.25 M in sucrose. The release of *p*-nitrophenol was followed as described in Methods.

After ninhydrin staining the color was fixed a $Cu(NO_3)_2$ solution.

2.5. *Protein determination*

Protein was determined by the method of Lowry et al. [12] with bovine serum albumin as standard.

3. Results

Lysosomal suspensions incubated for 30 min at 25°C with 0.01 M of certain dipeptides lose, to a varying degree, their latency with respect to acid phosphatase activity, concomitant with the appearance of the latter activity in the medium. The data presented in table 1 show that Ala–Ala, Met–His, Met–Gly, Tyr–Ala and Ser–Leu are potent lysosomal labilizers, i.e. available enzyme activity in the lysosomal suspensions increased from 19% to 50–80% of the total activity. Met–Met and Gly–Met were of a markedly smaller potency in damaging lysosomal integrity, while Gly–Gly and Gly–Glu did not affect acid phosphatase latency under

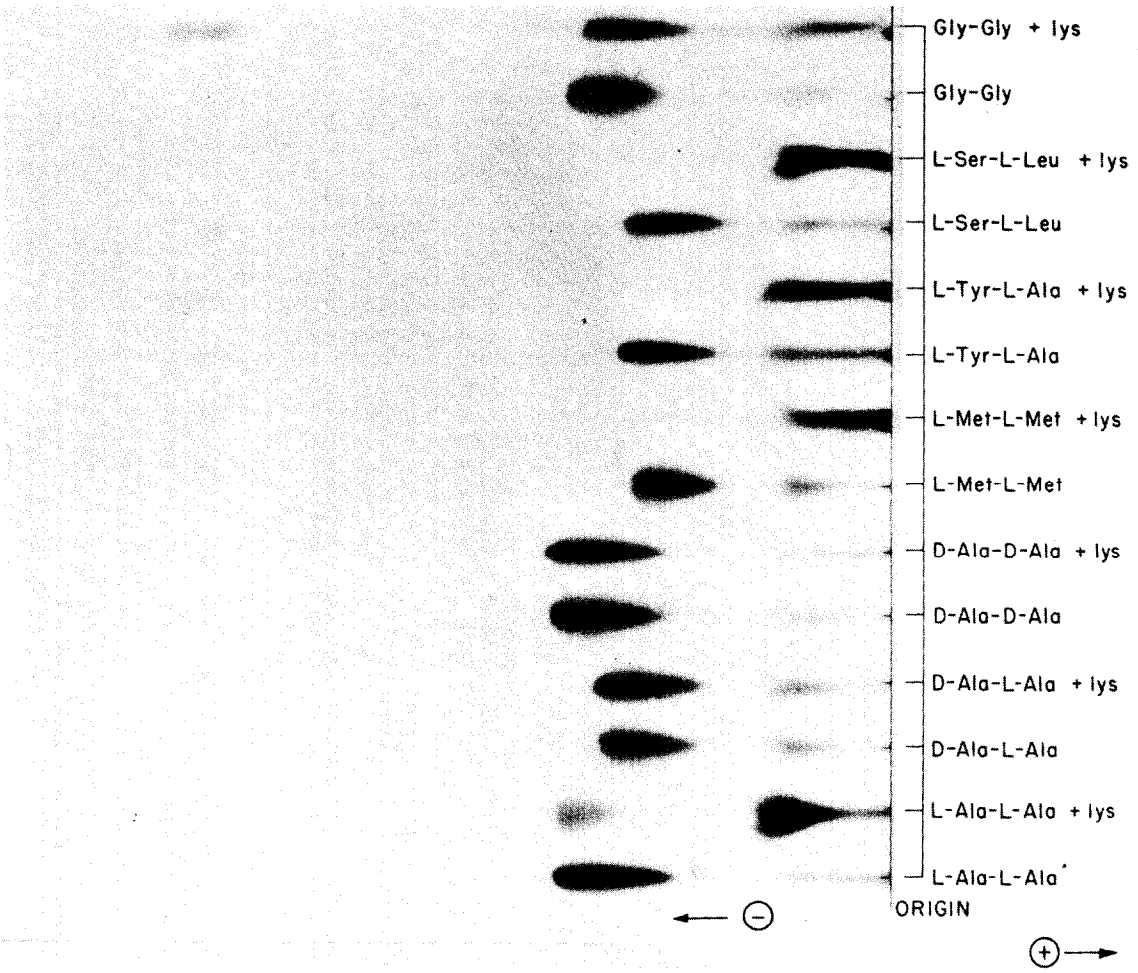

Fig. 1. Dipeptides as substrates for lysosomal enzymes. The specified dipeptides at a concentration of 0.02 M (0.02 M in Na-phosphate buffer pH 7.0) were incubated with (denoted by + lys) or without a lysosome rich fraction (0.25 mg protein/ml) for 2 hr at 37°C. Aliquots of 25 μl were then spotted on a 3 MM Whatman paper and subjected to high voltage electrophoresis (see Methods). The controls contain the same quantity of lysosomal protein that has been preincubated for 2 hr at 37°C, and added to the dipeptide prior to spotting on paper.

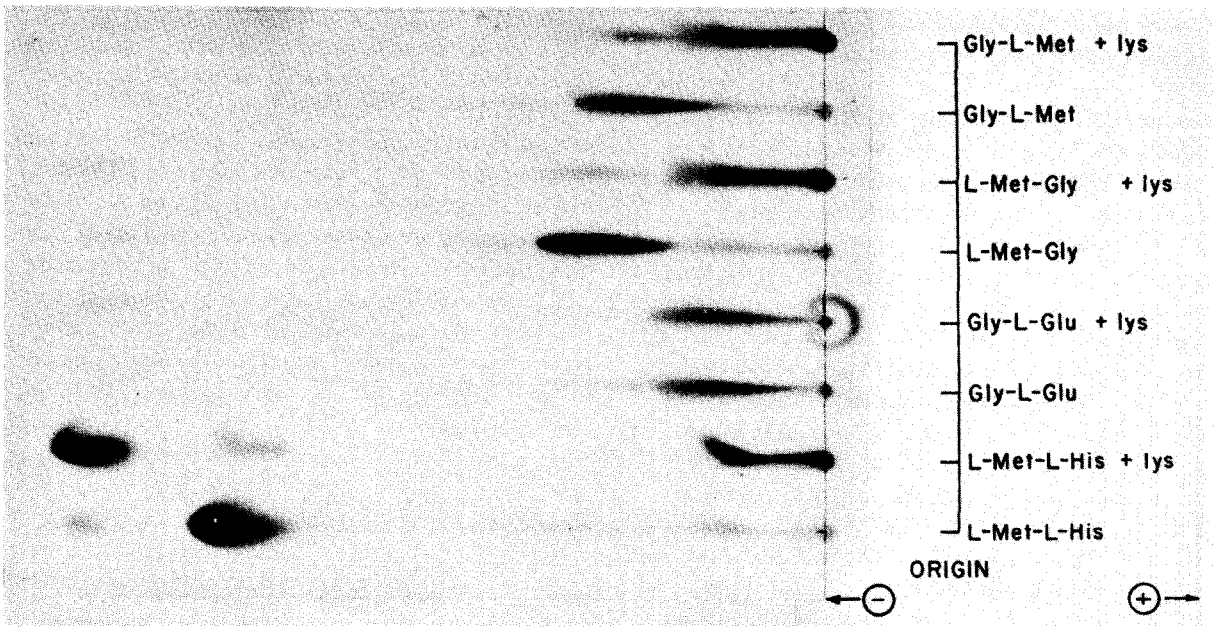

Fig. 2. Dipeptides as substrates for lysosomal enzymes. Details as in the legend to fig. 1.

the experimental conditions employed. Of the available activity in the control, about 42% resides in the supernatant of a 30 min centrifugation at 12 300 *g*. The dipeptides which were found to increase the available activity (except for Ala–Ala) were also effective in releasing that activity to the medium. Preincubation with Met–His and Met–Gly led to a release of up to 56% of the total activity to the supernatant. Three cycles of freeze–thawing in the presence and in the absence of these dipeptides led to an 80% and 53% release of acid phosphatase to the medium, respectively.

The stereospecificity as well as the concentration dependence of lysosomal rupture mediated by dipeptides was tested with alanine dipeptides. The data presented in table 2 show that of the three alanine dipeptides tested at a concentration of 0.01 M, only L-Ala–L-Ala was effective in damaging lysosomal latency. The availability of acid phosphatase depends on the time of preincubation with the dipeptide. The stereospecificity of the effect is lost upon increasing the concentration of the dipeptides in the incubation mixture to 0.2 M (table 2).

The ability of the tested dipeptides to serve as substrates for lysosomal dipeptidase activity was also studied. Results of these studies are given in figs. 1 and 2. It is observed that only two out of eleven dipeptides were resistant to hydrolysis by freeze–thawed lysosomal suspensions, namely D-Ala–L-Ala and D-Ala–D-Ala. Under the experimental conditions employed Met–His, Met–Met, Tyr–Ala, Ser–Leu, Met–Gly were almost totally hydrolyzed, while Gly–Gly, Gly–Glu and Gly–Met were hydrolyzed only to a limited extent. The hydrolysis of Gly–Gly yields in addition to Gly, an unidentified spot of a cationic nature.

4. Discussion

At a concentration of 0.01 M various dipeptides have been shown to affect the latency of lysosomal acid phosphatase while others had no effect on this phenomenon. There is a remarkable correlation between the capacity of a dipeptide to labilize lysosomes and its hydrolysis by a lysosomal enzyme composite. D-Ala–L-Ala and D-Ala–D-Ala were neither damaging lysosomal latency nor were they hydrolyzed after a 2 hr incubation period at 37°C (fig. 1). Gly–Glu has been shown to be hydrolyzed by the enzyme composite and its ineffectivity is probably indicative of an inability to penetrate the lysosomal membrane. It

was shown [13] that anions such as gluconate, lactate and glucuronate at 0.25 M provide good osmotic protection to lysosomes, and it was implied that lysosomes are impermeable to negatively charged species.

Further evidence for the correlation between lysosomal damage effects, hydrolyzability of the dipeptides and membrane permeability is obtained from the study of the stereospecificity of the labilization process and its concentration dependence (table 2). Loss of stereospecificity upon increasing the concentration of the dipeptide from 0.01 M to 0.2 M suggests that all of the three alanine dipeptides permeate the lysosomal membrane. The penetrating dipeptides probably interfere with the osmotic balance and drive an inward flow of water leading to lysosomal burst. Using the same criterion Gly–Gly was shown [8] to permeate lysosomal membranes. Lysosomal damage upon incubation in 0.01 M of a dipeptide is therefore best explained by an intralysosomal dipeptide hydrolysis. A non hydrolyzable dipeptide that permeates the membrane will reach an equilibrium distribution between the extra and intralysosomal space. At this low extralysosomal concentration it does not lead to significant osmotic inbalance or inward water flow. An intralysosomal dipeptide hydrolysis leads, however, to a constant inward flow of substrate, and to the intralysosomal accumulation of free amino acids. Rat liver lysosomal membranes are considerably less permeable to free amino acids than to dipeptides [8]. The imbalance between the inward flow of dipeptide and outward flow of its products is most probably the cause for lysosomal rupture.

Ehrenreich and Cohn [7] tested a series of small peptides (mol. wt. 160–294) for ability to induce vacuolization in cultured mouse macrophages. Only two compounds were effective, D-Ala–D-Ala–D-Ala and D-Glu–D-Glu, while 5 dipeptides (mol. wt. 160–190) that were equally resistant to enzymic hydrolysis (each containing D-amino acids) were not effective. The conclusion of these authors was that the latter could pass the lysosomal membrane while the two effective ones could not and were therefore concentrating within the lysosomes. Ehrenreich and Cohn have put forward a concept of a barrier to molecules of a molecular weight greater than 200, or of a molal volume greater than about 160 ml/mole. Three of the dipeptides that have been shown in the present study to permeate rat liver lysosomes are of molecular weights and molal volumes considerably higher than these suggested to lead to impermeability in the mouse macrophage system (mol. wt. Tyr–Ala, 252; Met–His, 286; Met–Met, 280).

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