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D-Aspartyl residue in a peptide can be liberated and metabolized by pig kidney enzymes

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Summary. The presence of an enzyme activity which hydrolyzes glycyl-D-aspartate was found in the homogenates of pig kidney cortex. The activity was inhibited by metal chelating agents and cilastatin, suggesting that the enzyme was a cilastatin-sensitive metallo-peptidase. Of the two hydrolysis products, D-aspartate was found to be less accumulated than glycine. The fate of D-aspartate was, therefore, examined and the amino acid was found to be converted to L-aspartate, L-alanine and pyruvate, in the presence of L-glutamate. Experiments with enzyme inhibitors suggested that the conversion involved D-aspartate oxidase, aspartate aminotransferase and alanine aminotransferase as well as decarboxylation of oxaloacetate produced from D-aspartate. All the results indicate that the enzymes in the pig kidney can liberate the D-aspartyl residue in the peptide and convert it to the compounds readily utilizable. The finding suggests a probable metabolic pathway of the D-aspartate-containing peptide.

Keywords: Amino acids – Hydrolysis of glycyl-D-aspartate – D-Aspartyl residue in peptides – Metallo-enzyme – Conversion of D-aspartate to L-amino acids – D-Aspartate oxidase – Aspartate aminotransferase – Alanine aminotransferase

Introduction

It had been widely thought that mammals contained only L-amino acids (Man and Bada, 1987). However, recent reports have demonstrated that several D-amino acids are present, as free or protein bound form, in mammals including human (Shapira and Chou, 1987; Man and Bada, 1987; Shapira et al., 1988; Fisher et al., 1992; Nagata et al., 1992; Powell et al.,

1992; D'Aniello et al., 1992, 1993; Hashimoto et al., 1993; Konno et al., 1993; Nagasaki, 1994; Fujii et al., 1994; Kera et al., 1995). Among them, D-aspartic acid appears to be one of the most abundant D-amino acids. The physiological origin of the free D-amino acids is still obscure, although several studies (Konno et al., 1990, 1993; Nagata and Akino, 1990; Nagata et al., 1992; Kera et al., 1995) have provided experimental evidence that some are from foods and bacteria under each specified conditions. As for the protein bound D-amino acids, D-aspartic acid has been found in several mammalian endogenous proteins (Bada, 1984; Shapira and Chou, 1987; Man and Bada, 1987; Shapira et al., 1988; Fisher et al., 1992; Powell et al., 1992; Fujii et al., 1994). In addition, it is known that D-amino acids, particularly D-aspartate, are produced from the corresponding L-isomers in dietary proteins during food preparation and processing (Man and Bada, 1987). The D-aspartyl residues in these endogenous or exogenous dietary proteins may serve as a source of free D-aspartate found in mammalian tissues. While proteins containing substantial D-amino acids can be partly broken down by proteolytic enzymes, probably to di- or tripeptides enriched in D-amino acids, there has been no report that D-aspartate is liberated from the peptides in mammals (Man and Bada, 1987).

The mammalian tissues possess D-aspartate oxidase and D-amino acid oxidase, which respectively catalyzes the oxidative deamination of acidic and neutral D-amino acids to the corresponding α-keto acids (Hamilton, 1985). The resulting α-keto acids can undergo decarboxylation or stereospecific transamination yielding the L-enantiomers of the original amino acids (Man and Bada, 1987; Zagon et al., 1994). The involvement of D-amino acid oxidase in utilization of several neutral D-amino acids has been well demonstrated by the studies with mutant mice lacking the enzyme (Konno and Yasumura, 1984, 1992). On the other hand, there is little experimental evidence on D-aspartate utilization in mammals.

This *in vitro* study was carried out to evaluate possibilities whether mammalian tissues would possess any enzyme activity liberating D-aspartic acid in a dipeptide and whether D-aspartate oxidase was involved in further metabolism of D-aspartate.

Materials and methods

Materials

Fresh pig kidneys were obtained from a local slaughter house and the cortex was stored at −45°C until use. Glycyl-D-aspartate was purchased from Sigma (St. Louis, MO, USA). Malate dehydrogenase and lactate dehydrogenase were obtained from Toyobo (Osaka, Japan). Cilastatin (MK0791) (monosodium *Z*-S[6-carboxy-6-{[2,2-dimethyl-(S)-cyclopropyl)carbonyl]amino}-5-hexenyl]-L-cysteine) was a gift from Banyu Pharmaceutical Co. Ltd. (Tokyo, Japan). L-Hydrazinosuccinic acid was synthesized as previously reported (Yamada et al., 1985). A reversed-phase HPLC column (4.6 × 150mm), packed with 4-μm-diameter J'sphere-ODS-M80 was from YMC (Kyoto, Japan). All other chemicals were of analytical purity.

Preparation and incubation of dialyzed homogenates

The frozen pig kidney cortex was minced and homogenized with 5 volumes of 50mM K phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenate was filtered through nylon cloth (100 mesh) to remove fragments of connective fibers. The filtered homogenate was dialyzed twice, for 2hr each time, against 100 volumes of 50mM K phosphate buffer (pH 7.4), in order to remove most of low molecular weight substances.

Incubation of the dialyzed homogenate with glycyl D-aspartate was carried out in a 0.5-ml reaction mixture with or without addition of other reaction components, at 37°C under shaking in the air for a desired period until the reaction was stopped by addition of 9 volumes of cold 8% (v/v) perchloric acid. Incubation of the homogenate with D-aspartate to follow the fate of this amino acid was conducted in a 20-ml reaction mixture in the presence of L-glutamate and FAD under, otherwise, the same conditions as described above, and aliquots were taken out at different times and mixed with 9 volumes of the perchloric acid.

The proteinaceous precipitate formed with perchloric acid was removed by centrifugation and the supernatant was neutralized with KOH, and the precipitated $KClO_4$ was removed. To the supernatant, a neutralized L-cysteic acid solution was added as the internal standard for HPLC analysis at a final concentration of 40μM.

HPLC analysis

The derivatization of amino acids with *o*-phthaldialdehyde and *N*-acetyl-L-cysteine was performed by the method of Aswad (1984). The HPLC analysis was carried out according to our previous study (Kera et al., 1995) with several modifications, using an HPLC system (Shimadzu, Kyoto) equipped with J'sphere-ODS-M80 column. The mobile buffer for assay of acidic amino acids was 50mM Na acetate buffer (pH 5.7) containing 4% (v/v) methanol and that for assay of glycine and L-alanine was 50mM Na acetate buffer (pH 5.9) containing 15% (v/v) methanol. The flow rate was 1.2ml/min. Excitation and emission wavelengths were 320 and 440nm, respectively.

Miscellaneous methods

All measurements of enzyme activities were carried out with dialyzed homogenates, at pH 7.4 and at 37°C, unless otherwise stated. D-Aspartate oxidase activity was measured as previously described (Yamada et al., 1988). Aspartate aminotransferase and alanine aminotransferase activities were assayed by the methods of Schwartz (1971). The concentration of pyruvate in the neutralized perchloric acid extract was estimated with lactate dehydrogenase and NADH, according to Lamprecht and Heinz (1984). Protein concentration was determined according to Lowry et al, (1951).

Results and discussion

Hydrolysis of glycyl-D-aspartate

When a dialyzed pig kidney homogenate was incubated with glycyl-D-aspartate, D-aspartate was liberated time-dependently (Fig. 1). The liberation was not observed when the dipeptide was omitted from the incubation mixture or the homogenate was heated in boiling water for 5min before use, demonstrating the involvement of some enzyme. The simultaneous liberation of glycine, the other hydrolysis product, was also confirmed. However, when the amounts of the two amino acids produced were compared, D-aspartate

was found to be less (68%) than glycine (Fig. 2) The difference was abolished when the incubation was carried out in the presence of *meso*-tartrate, a competitive inhibitor of D-aspartate oxidase (Hamilton, 1985), at a concentration enough for complete inhibition as found in preliminary experiments. The results therefore indicate that a portion of the D-aspartate was further metabolized by the enzyme, resulting in the less accumulation of this amino acid. Another aspect of the results shown in the figure was that *meso*-tartrate decreased the production of both amino acids. This may mean that *meso*-tartrate was inhibitory also to the hydrolytic enzyme, although the inhibition was much weaker than that to the oxidase. The specific activity of the hydro-

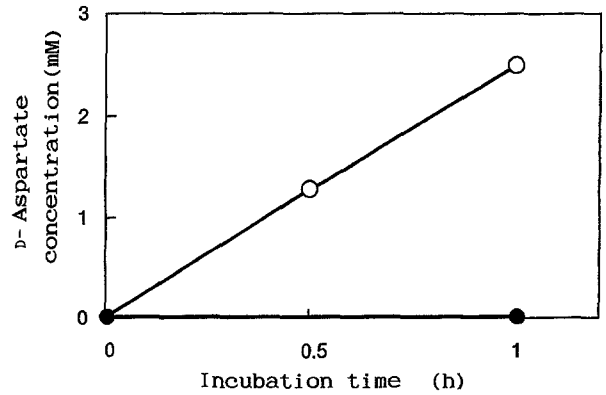

Fig. 1. Liberation of D-aspartate from glycyl-D-aspartate by pig kidney homogenate. The dialyzed homogenate (15–16mg protein/ml) was incubated with glycyl-D-aspartate (10mM) at 37°C without (○) or with (●) 5-min heating in boiling water before use. The results are the means for two separate experiments

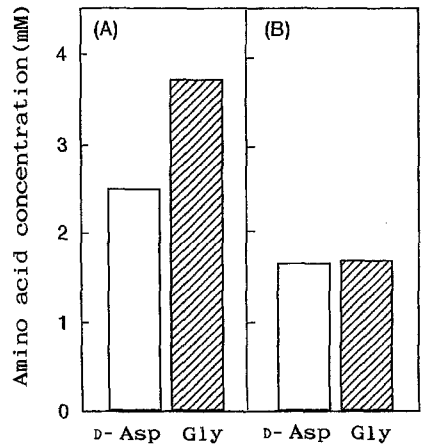

Fig. 2. Effect of *meso*-tartrate on the production of D-aspartate and glycine from glycyl-D-aspartate by pig kidney homogenate. The incubation was carried out at 37°C for 1h, in the absence (**A**) and presence (**B**) of *meso*-tartrate (40mM). The final concentrations of protein and glycyl-D-aspartate were 11–12mg/ml and 10mM, respectively. The results are the means for two separate experiments

Table 1. Effect of inhibitors on hydrolysis of glycyl-D-aspartate by pig kidney homogenate

Inhibitor	Relative activity[1] (%)
None	100
o-Phenanthroline (20mM)[2]	0
EDTA (10mM)[2]	0
p-Chloromercuribenzoate (5mM)[2]	115
N-Ethylmaleimide (5mM)[2]	105
Phenylmethylsulfonyl fluoride (50mM)[2]	118
Cilastatin (5mM)[2]	3

[1]The dialyzed homogenate was preincubated with inhibitors at 37°C for 30min, before addition of glycyl-D-aspartate, and the mixture was then incubated for 1h. The final concentrations of protein and the peptide were 11–12mg/ml and 10mM, respectively. The hydrolytic activity was determined by glycine release for 1h. The results are the means for two separate experiments.
[2]The final concentration is shown in parenthesis.

lytic enzyme in the homogenate was estimated from the glycine release in the absence of *meso*-tartrate to be 4.1nmol/min per mg protein at the substrate concentration of 10mM.

The hydrolytic enzyme was examined with respect to the sensitivity to several enzyme inhibitors (Table 1). Metal chelating agents, EDTA and *o*-phenanthroline, potently inhibited the enzyme activity. No inhibition was observed with thiol reagents, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide and with a serine proteinase inhibitor, phenylmethylsufonyl fluoride. Thus, it is suggested that a metallo-peptidase was involved in the hydrolysis of glycyl-D-aspartate. This may explain the inhibition of the hydrolysis with *meso*-tartrate, described above, since tartrate is known to bind metal ions. Interestingly, the hydrolysis was also inhibited by cilastatin (Table 1), which is known to be a specific inhibitor of a metallo-enzyme, renal dipeptidase (Kahan et al., 1983). The renal peptidase from the pig hydrolyzes glycyl-D-leucine (Campbell et al., 1966) and glycyl-D-phenylalanine (Hooper et al., 1987), and the enzyme from the rat hydolyzes glycyl-D-alanine (Kozak and Tate, 1982). Thus, it appears possible that the enzyme also hydrolyzes glycyl-D-aspartate, although further examinations are required for the identification.

Conversion of D-aspartate to L-amino acids and pyruvate

Since the results above indicated that D-aspartate liberated from glycyl-D-aspartate was metabolized by D-aspartate oxidase, we attempted to follow the fate of D-aspartate. First, we examined the possibility that oxaloacetate, the oxidation product of D-aspartate would be reaminated to form L-aspartate in the presence of L-glutamate because we found that the organ of the rat and pig

contained a high level of L-glutamate (4–7 μmol/g wet tissue) (Kera et al., 1995 and unpublished results). When the dialyzed pig kidney homogenate was incubated with D-aspartate and L-glutamate, L-aspartate was produced time-dependently (Fig. 3). As shown in Table 2, the production of L-aspartate was not observed without the presence of D-aspartate and L-glutamate, and was inhibited completely with *meso*-tartrate, the inhibitor of D-aspartate oxidase, and significantly with L-hydrazinosuccinate, an inhibitor of aspartate aminotransferase (Yamada et al., 1985), indicating the involvement of these enzymes in the process.

Figure 3 also shows that the concentration of L-aspartate produced was only about a half of the decrease in D-aspartate concentration and L-glutamate concentration. This suggested that a considerable part of the oxaloacetate produced might have remained unchanged or been converted to some other compounds.

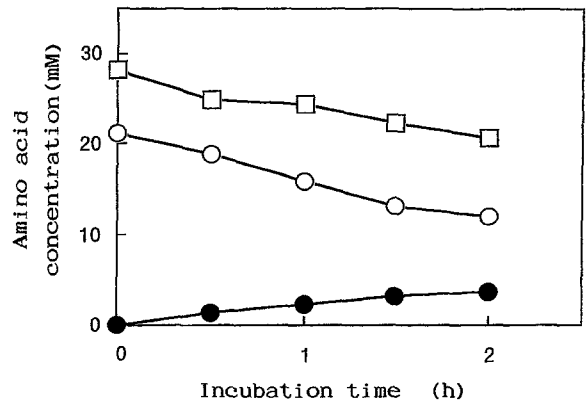

Fig. 3. L-Aspartate production from D-aspartate by pig kidney homogenate. The dialyzed homogenate (15–16 mg/ml) was incubated with D-aspartate (20 mM) and L-glutamate (30 mM) in the presence of FAD (20 μM) at 37°C for 2 h. The results are the means for two separate experiments. (○) D-aspartate; (●) L-aspartate; (□) L-glutamate

Table 2. L-Aspartate production from D-aspartate by pig kidney homogenate under various conditions

Condition	Relative production[1] (%)
Complete	100
− D-Aspartate	0
− L-Glutamate	0
+ *meso*-Tartrate (40 mM)[2]	0
+ L-Hydrazinosuccinate (40 μM)[2]	40

[1] The dialyzed homogenate was incubated at 37°C for 2 h. The "complete" incubation mixture contained the same concentrations of protein, D-aspartate and L-glutamate as in Fig. 3. The results are the means for two separate experiments.
[2] The final concentration is shown in parenthesis.

The previous studies (Still et al., 1949; Jaroszewicz, 1975; Yamada et al., 1988) showed that oxaloacetate, was easily decomposed to pyruvate in tissue homogenates because of its instability. Therefore, we further analyzed for pyruvate and L-alanine which would be formed from pyruvate by transamination involving alanine aminotransferase. Figure 4 shows that the two compounds, of which alanine was the major, increased time-dependently. The total concentration of L-aspartate, L-alanine and pyruvate was found to be approximately equal to the decrease in D-aspartate concentration. Thus the fate of D-aspartate oxidized is mostly explained by the production of the three compounds. The activities of D-aspartate oxidase, aspartate aminotransferase and alanine aminotransferase in the homogenate were found to be 13nmol/min/mg, 600nmol/min/mg and 44nmol/min/mg of protein, respectively. Considering the much lower activity of alanine aminotransferase than that of aspartate aminotransferase, it is noticeable that rather more L-alanine than L-aspartate was accumulated after 2-h incubation. This probably reflects that the concentration of oxaloacetate during the incubation was kept very low because of its instability.

Production of L-aspartate and L-alanine from glycyl-D-aspartate

In order to confirm that D-aspartate liberated from glycyl-D-aspartate itself would be converted to L-aspartate and L-alanine, the dialyzed homogenate was incubated with the dipeptide and L-glutamate. As shown in Fig. 5, L-aspartate and L-alanine were, in fact, produced time-dependently, although the concentrations of the amino acids were low in comparison with those shown in Fig. 4. This smaller production is not unreasonable, since the average concentration of D-aspartate during the experiment was much lower than that in the experiment for Fig. 4 as seen from the figures. In this connection, it should be pointed out that the apparent *Km* value for D-aspartate of pig

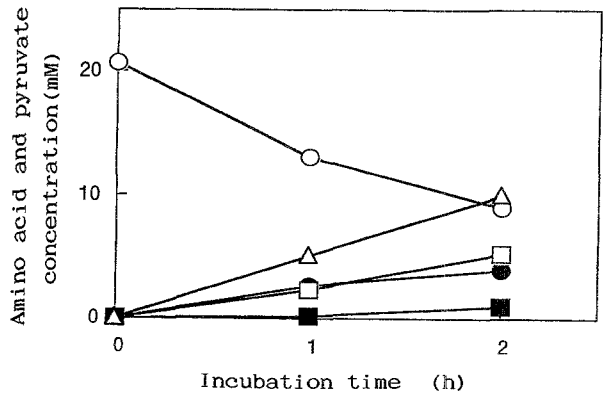

Fig. 4. Production of L-aspartate, L-alanine and pyruvate from D-aspartate by pig kidney homogenate. The incubation condition was the same as in Fig. 3. The results are the means for two separate experiments. To avoid complexity, L-glutamate was omitted from the figure. (○) D-aspartate; (●) L-aspartate; (□) L-alanine; (■) pyruvate; (△) L-aspartate + L-alanine + pyruvate

kidney D-aspartate oxidase is approximately 5mM (De Marco and Crifo, 1967).

All the results in the present study provide evidence that pig kidney enzymes *in vitro* hydrolyze glycyl-D-aspartate, deaminate the liberated D-aspartate oxidatively, and reaminate the resulting oxaloacetate and its decarboxylation product, pyruvate, to L-aspartate and L-alanine respectively, in the presence of L-glutamate. The evidence suggests a possibility that D-aspartate in the peptide is metabolized *in vivo* via a similar pathway to yield the L-amino acids and α-keto acids which are readily utilizable. It requires further studies to determine if the pathway in fact works in pig kidneys, since *in vivo* metabolism depends on many factors such as localization of enzymes.

There had been no report on mammalian enzyme activity which liberates D-aspartate from peptides containing the amino acid, although peptidyl-D-amino acid hydrolase in cephalopods was shown to hydrolyze a D-aspartate-containing peptide (D'Aniello and Strazzullo, 1984). In mammals, liberation of D-aspartic acid residues in the proteins containing the amino acid appeared unlikely to occur, since D-amino-acid containing peptides were not hydrolyzed by the enzymes involved with protein digestion, by which the proteins were partly broken down to yield D-amino-acid rich peptides (Man and Bada, 1987). However, the present finding of the novel pig kidney enzyme activity which hydrolyzes glycyl-D-aspartate leaves little doubt that D-aspartate in the proteins can be liberated, at least in case that the same peptide is released by proteolysis of the proteins. The finding has also opened a possibility that many other small peptides containing D-aspartate may be hydrolyzed by mammalian enzymes, and therefore, D-aspartate in proteins may be mostly liberated after partial degradation of the proteins by usual proteases and peptidases. Studies are in progress to test this possibility, in addition to identify the enzyme hydrolyzing glycyl-D-aspartate.

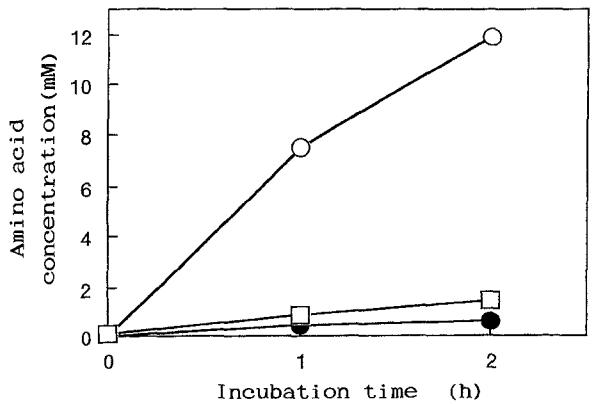

Fig. 5. Production of L-aspartate and L-alanine from glycyl-D-aspartate by pig kidney homogenate. The dialyzed homogenate (15–16mg/mg) was incubated with glycyl-D-aspartate (20mM) and L-glutamate (30mM) in the presence of FAD (5μM) at 37°C for 2h. The concentration of L-glutamate was not determined in this incubation. The results are the means for two separate experiments. (○) D-aspartate; (●) L-aspartate; (□) L-alanine

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