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Note

Use of L-prolyl-L-leucylglycinamide (MIF-1) for the high-performance liquid chromatographic determination of proline iminopeptidase activity in rat liver

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Proline iminopeptidase (EC 3.4.11.5) is an aminopeptidase that specifically hydrolyses the N-terminal proline residue from peptides, except from the dipeptide Pro-X. The presence of this enzyme activity was found in microorganisms and some animal organs by use of poly-L-proline as substrate [1,2]. However, poly-L-proline cleaving activity in crude extracts can also be due to aminopeptidase P (EC 3.4.11.9) [3]. It is well known that the best substrate for proline iminopeptidase is the tripeptide L-prolyl-L-leucylglycinamide, also known as melanocyte stimulating hormone-release inhibiting factor (MIF-1). This tripeptide has been shown to accumulate and to be metabolized in certain organs.

Earlier we determined proline-specific exopeptidases, including proline iminopeptidase, which was determined with L-prolylglycylglycine as substrate, by a high-performance liquid chromatographic (HPLC) method [4–6]. Because tripeptide aminopeptidase (EC 3.4.11.4) can also cleave Pro-Gly-Gly, in this paper a new method utilizing Pro-Leu-Gly-amide for the determination of proline iminopeptidase activity in rat liver is described, in which identification of L-leucylglycinamide and glycinamide was made after hydrolysis.

EXPERIMENTAL

Chemicals

L-Pro-L-Leu-Gly-amide (peptide content 97%) was obtained from Sigma (St. Louis, MO, U.S.A.). L-Leu-Gly-amide · HBr (peptide content 70%) and Gly-amide · HCl were purchased from Bachem (Bubendorf, Switzerland). All the other reagents were of the highest purity available.

Enzyme preparation

Rat organs were removed from freshly slaughtered animals. About 1 g of

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tissue was homogenized in 9 volumes of 0.25 *M* sucrose–10 m*M* Tris–HCl (pH 7.4)–0.5 m*M* manganese chloride, and the homogenate was then centrifuged for 60 min at 15 000 *g* to obtain the soluble fraction. Protein concentrations were determined by the method of Hartree [7] with bovine serum albumin as standard.

Chromatographic determination of proline iminopeptidase activity

Incubation was carried out in all instances in a standard reaction mixture (500 μl) containing 30.0 μmol of Tris–HCl buffer (pH 8.0), 0.50 μmol of Pro-Leu-Gly-amide, 1.0 μmol of manganese chloride, 20–100 μg of tissue homogenate and water. The temperature was 37°C and the period of incubation was 30 min. The reaction was terminated by addition of 400 μl of 10% perchloric acid followed by centrifugation for 5 min at 10 000 *g* to remove the insoluble material. The concentration of the peptides in the clear medium solution was determined by injection of a 10-μl sample into an HPLC apparatus equipped with an autosampler (Jasco 850-AS with 801-SC). Leu-Gly-amide and Gly-amide, enzymatically formed from the Pro-Leu-Gly-amide in the assay mixture, were chromatographically separated by HPLC with 10.0 m*M* potassium dihydrogenphosphate buffer (pH 2.1) as the mobile phase, and the eluate was monitored for peptides at 210 nm. The column (150 mm × 4.6 mm I.D.) was packed with Zorbax ODS, particle size 5 μm, supplied by DuPont (Wilmington, DE, U.S.A.). A guard column (50 mm × 4.0 mm I.D.) containing Parmaphase ETH of particle size of 30 μm, also supplied by DuPont, was connected to the separation column.

RESULTS AND DISCUSSION

The complete separation of Pro-Leu-Gly-amide, Leu-Gly-amide and Gly-amide was achieved with 10.0 m*M* potassium dihydrogenphosphate buffer (pH 2.1) as the mobile phase. Because it was not possible to avoid the overlapping of Gly-amide and perchloric acid peaks, the amount of Gly-amide present was calculated by subtraction of the perchloric acid value from the total value. The retention times of Pro-Leu-Gly-amide, Leu-Gly-amide and Gly-amide were determined to be 4.5, 2.7 and 1.8 min, respectively. As shown in Fig. 1B, authentic Leu-Gly-amide gave a large peak in the starting area. This peak can probably be attributed to impurities in the peptide product (see *Chemicals*). The activity of soluble proline iminopeptidase was detected in rat liver. Incubation of Pro-Leu-Gly-amide with increasing amounts of liver homogenate (Fig. 1D, E and F) resulted in a gradual decrease in the Pro-Leu-Gly-amide peak (peak 1) with a concomitant increase in the 1.8-min peak (peak 3), corresponding to Gly-amide, and in a small increase in the 2.7-min peak (peak 2), corresponding to Leu-Gly-amide (Fig. 1). The action of several aminopeptidases capable of hydrolysing the Leu–Gly bond of Leu-Gly-amide generated by proline iminopeptidase would result in the non-apparent rapid decrease in Leu-Gly-amide and in the observed increase in Gly-amide in the homogenate sample. The total value for these three

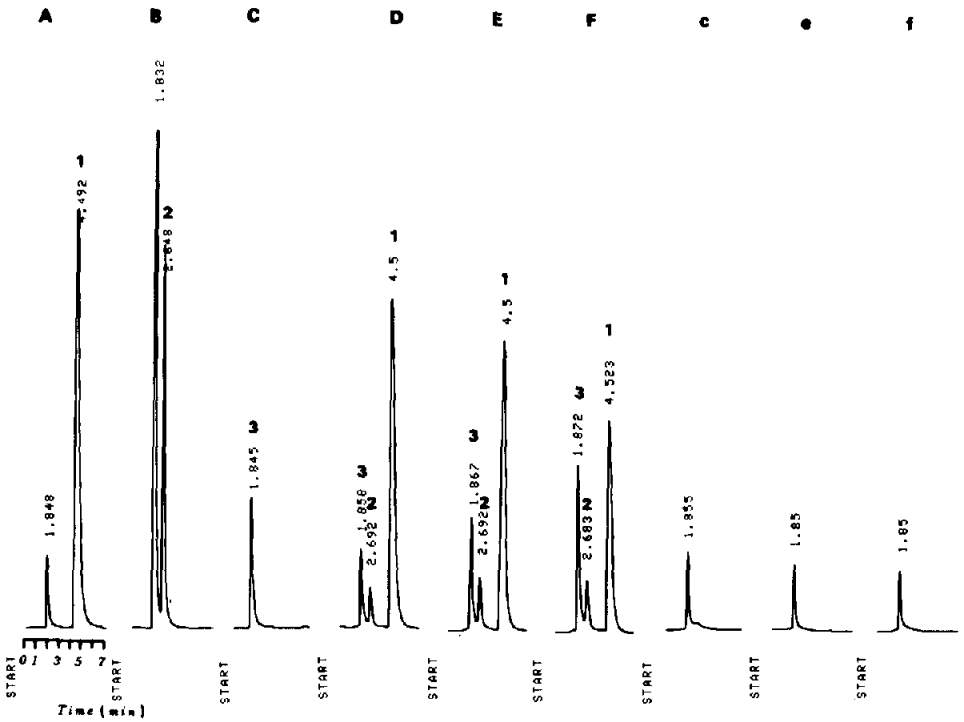

Fig. 1. Typical chromatographic patterns of the authentic peptides and Gly-amide and hydrolysis of Pro-Leu-Gly-amide with different concentrations of rat liver homogenate. (A) Pro-Leu-Gly-amide, 5.6 nmol; retention time, 4.5 min (peak 1). (B) Leu-Gly-amide, 5.6 nmol; retention time, 2.7 min (peak 2). (C) Gly-amide, 5.6 nmol; retention time, 1.8 min (peak 3). (D) Pro-Leu-Gly-amide plus 14.9 μg of rat liver homogenate, (E) plus 29.8 μg of rat liver homogenate and (F) plus 59.5 μg of rat liver homogenate. (c), (e) and (f) are the control of (C) without addition of perchloric acid and controls of (E) and (F) without substrate, respectively. Values on the peaks are retention times (min).

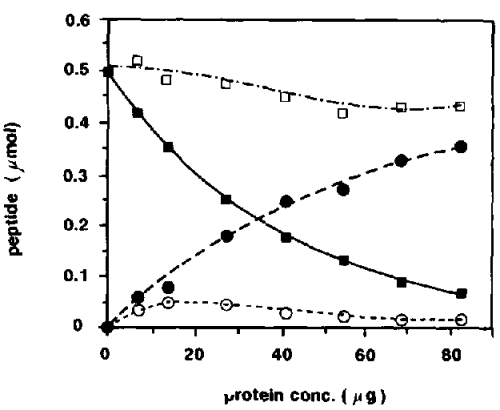

Fig. 2. Dependence of Leu-Gly-amide and Gly-amide formation on rat liver homogenate concentration. (●) Gly-amide was rapidly formed from (■) Pro-Leu-Gly-amide through (○) Leu-Gly-amide by proline iminopeptidase activity. Individual values and (□) the total amount of the three peptides were plotted against the amount of homogenate protein used.

TABLE I

PRO-GLY-GLY AND PRO-LEU-GLY-AMIDE HYDROLYSING ACTIVITY IN DIFFERENT RAT ORGANS

Units, nmol/min · mg protein. Results are means ± S.D. (n = 3).

Organ	Pro-Gly-Gly (A)	Pro-Leu-Gly-amide (B)	A/B
Liver	157 ± 10.5	198 ± 8.2	0.8
Kidney	201 ± 18.6	142 ± 15.3	1.4
Brain	138 ± 12.7	25.0 ± 2.4	5.5
Spleen	123 ± 2.3	14.7 ± 0.3	8.4

peptides and the amount of each detected were plotted against the amount of homogenate protein used (Fig. 2). As can be clearly seen, the Pro-Leu-Gly-amide and Gly-amide concentrations were stoichiometrically related to each other.

In this method, the measurement of the disappearence of the substrate is simpler than measurement of the formation of the products, Leu-Gly-amide and Gly-amide. When we increased the substrate concentration in the assay mixture, however, there was only a slight reduction in peak area. Therefore, the substrate concentration was established as described above. It must also be borne in mind that the protein concentration in an assay mixture should be suitable for the quantitative reduction of the substrate in different tissues examined.

Nordwig and Mayer [8] described a proline iminopeptidase in pig kidney cortex that does not hydrolyse polyproline. Therefore, we examined the distribution of proline iminopeptidase activity in four organs of the rat that were analysed by Sarid *et al.* [2], who used polyproline as substrate, to have a high (liver and kidney) or low (brain and spleen) enzyme content. The data in Table I suggest that the Pro-Gly-Gly hydrolysing activity may be due to tripeptide aminopeptidase [9,10]. The four organs showed nearly the same levels of Pro-Gly-Gly hydrolysis; however, the level of Pro-Leu-Gly-amide hydrolysing activity, which is true proline iminopeptidase activity, was dependent on the organ type and is in agreement with the data of Sarid *et al.* [2]. The previous report [6] on the determination of proline iminopeptidase activity in bovine dental germs using Pro-Gly-Gly as substrate should be re-evaluated with Pro-Leu-Gly-amide as the substrate. In this study, we took advantage of a simple, easy and sensitive technique to achieve the optimum determination of proline iminopeptidase activity. This method can replace the commonly used spectrophotometric method.

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