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Note

High-performance liquid chromatographic procedure for the determination of serum prolidase activity

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The amino acid sequence of Xaa-Pro-Yaa- (Xaa and Yaa indicate amino acids) is present not only in structural proteins such as collagen [1] and amelogenin [2] but also in biologically active peptides and enzymes [3]. Proline residues in peptides seem to confer a special configuration on the molecule and may help to protect that region against undesired hydrolysis.

During the hydrolysis of these peptides from the N-terminal residue, proline iminopeptidase (EC 3.4.11.5) acts first, then aminopeptidase P (EC 3.4.11.9) splits off the second residue or dipeptidyl peptidase IV (EC 3.4.14.5) removes the dipeptide Xaa-Pro from Xaa-Pro-Yaa- [4,5]. In the latter case, the Xaa-Pro formed should be hydrolysed by prolidase (EC 3.4.13.9), which is a dipeptidase that specifically cleaves this type of dipeptide. Hereditary lack of this enzyme causes iminodipeptiduria, and affected individuals display a number of characteristic features [6–8].

The colorimetric determination of proline by Chinard's method [9] is widely used for measurement of prolidase activity in plasma of patients [10,11]. Earlier we described high-performance liquid chromatographic (HPLC) methods for the determination of dipeptidyl peptidase IV activity [12], aminopeptidase P activity [13] and proline iminopeptidase activity [14]. This paper describes a simple HPLC procedure for the determination of prolidase activity in human serum as a part of our series of studies on proline-specific exopeptidase activities.

EXPERIMENTAL

Chemicals

Gly-L-Pro was obtained from the Peptide Institute (Minoh, Osaka, Japan). Sodium 1-octanesulphonate for ion-pair chromatography was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Other reagents were reagent grade.

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Sample preparation

Serum was separated from the blood of six healthy male persons (20–25 years old). Hemolysed samples were discarded. Sera were stored at −20°C, and enzyme activities were tested within a month after collection.

Selection of some conditions for the determination of serum prolidase activity

Optimization of serum volume, manganese concentration and preactivation time of serum enzyme with manganese was required in order to obtain proper assay conditions. Each of these parameters is described in the main text.

Chromatographic determination of prolidase activity

Prolidase activity was determined chromatographically with Gly-Pro as the substrate as described previously [12]. The assay medium (200 μl), containing 5.0 μmol of Tris–HCl buffer (pH 8.0), 0.4 μmol of manganese(II) chloride and 10 μl of serum was preincubated for 3 h at 37°C to activate the serum enzyme. Then 1.2 μmol of Gly-Pro were added to the assay medium, and incubation was carried out for 30 min at 37°C. The reaction was stopped by the addition of 400 μl of 10% perchloric acid and 200 μl of water. After centrifugation of the assay tubes at 3000 *g* for 10 min, the concentration of the peptide in the clear supernatant solution was determined by HPLC analysis of 10-μl samples. The mean value of three replicates was used for the estimation.

The concentration of unhydrolysed Gly-Pro in the assay mixture was measured at 210 nm by HPLC with a mobile phase of 10.0 m*M* potassium dihydrogenphosphate buffer (pH 2.1) containing 0.5 m*M* 1-octanesulphonate. 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.). The guard column (50 mm × 4.0 mm I.D.) contained Pharmaphase ETH, particle size 30 μm, also from DuPont.

RESULTS AND DISCUSSION

Effect of serum volume

Serum volumes of 10–50 μl were used for the determination of prolidase activity. The Gly-Pro peak, which has a retention time of 4.6 min, gradually decreased with increasing serum volume, though the decrease in Gly-Pro was not linear for serum volumes of more than 20 μl. (Fig. 1A and B). The substrate was not saturated with the volumes of serum used for the measurement.

Effect of manganese concentration

The manganese concentration required for optimal prolidase activity after a 2-h preincubation with the ion at 37°C was determined. Manganese chloride concentrations in the range 1.0–5.0 m*M* were suitable (Fig. 2), as reported previously [10]. Calcium chloride could not be used instead of manganese chloride.

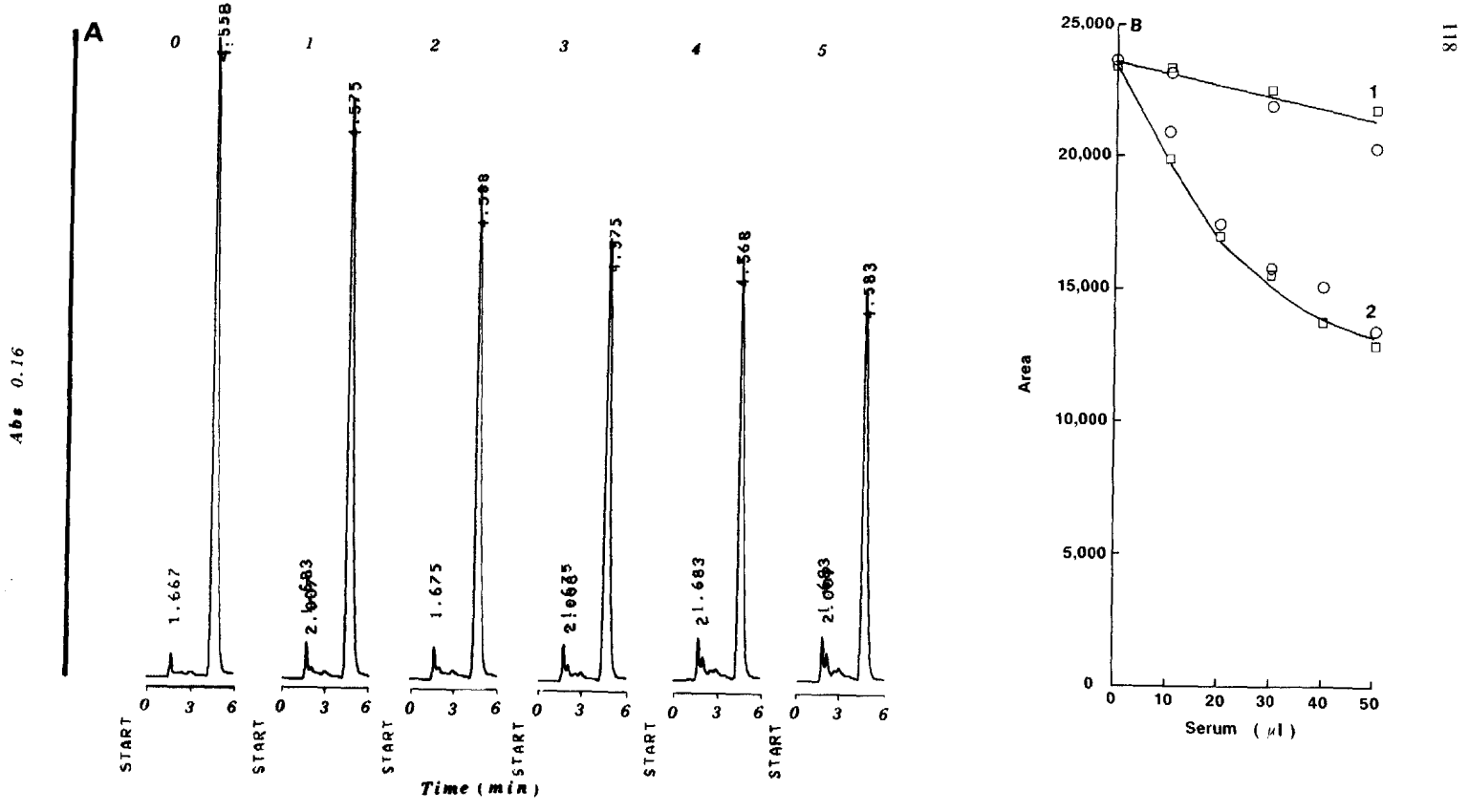

Fig. 1. (A) Typical chromatographic patterns of the hydrolysis of Gly-Pro with different volumes of human serum: 0, 0 μl; 1, 10 μl; 2, 20 μl; 3, 30 μl; 4, 40 μl; 5, 50 μl. Gly-Pro concentration without serum indicates 15.0 nmol. Mobile phase, 10.0 m*M* potassium dihydrogenphosphate containing 0.5 m*M* 1-octanesulphonate (pH 2.1). (B) Dependence of decrease in Gly-Pro concentration (expressed by area) on human serum volume. 1, Without preincubation; 2, with preincubation (3 h). Plots are indicated for two samples in each case.

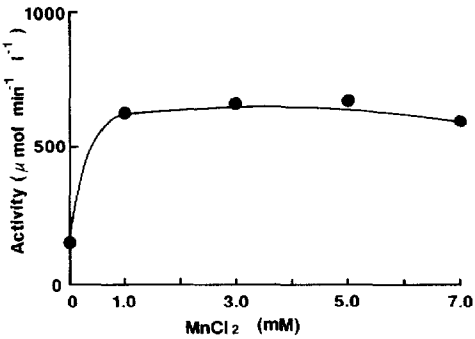

Fig. 2. Effect of Mn^{2+} concentration on human serum prolidase activity. Assay mixtures, containing 10 μl of serum, were preincubated for 2 h at 37°C.

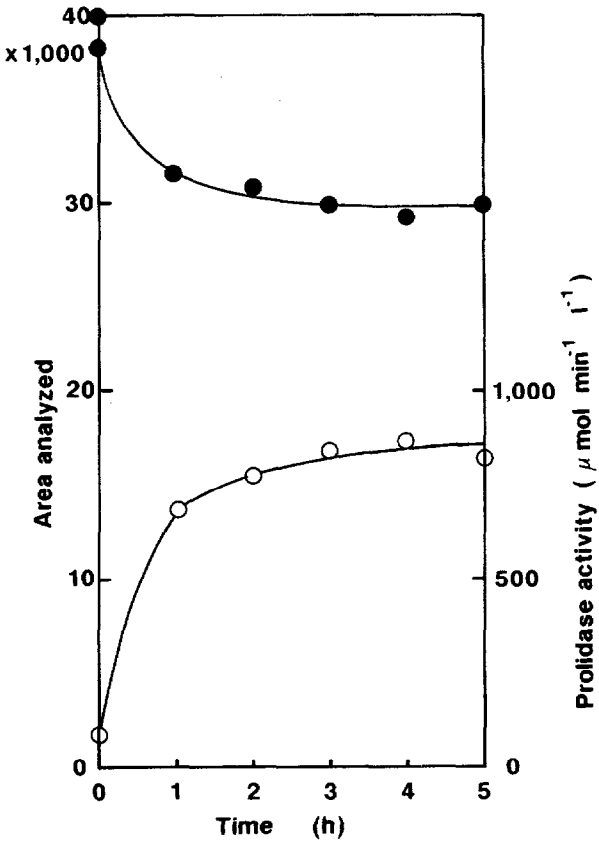

Fig. 3. Effect of preincubation time with Mn^{2+} on prolidase activity. The Mn^{2+} concentration in the assay mixture was 3.0 m*M*.

TABLE I

PROLIDASE ACTIVITY IN HUMAN SERUM

Preincubation was at 37°C for 3 h, with 3.0 m*M* Mn^{2+}. Serum volume, 10.0 μl.

Sample	Activity (μmol min^{-1} l^{-1})
1	784
2	666
3	638
4	783
5	726
6	777
Mean ± S.D.	729 ± 64.0

Effect of preincubation time

In order to obtain the highest serum prolidase activity, we examined the effect of preincubation time with a 3.0 m*M* concentration of manganese. As shown in Fig. 3, a preincubation time of 3–4 h yielded the highest activity. In contrast, Myara *et al.* [10] reported that a 24-h preincubation at 37°C was required for the highest activity. We did not carry out a 24-h preincubation, as such a long time does not seem to be required for activation of the enzyme.

Prolidase activity in human serum

The serum prolidase activities in six healthy young adults (20–25 years old) are shown in Table I. The mean value was calculated to be 729 ± 64.0 μmol min^{-1} (l of serum)$^{-1}$. In plasma, the usual values reported for adult controls were 0.825 ± 0.335 mmol min^{-1} l^{-1} [10] and 900 ± 260 μmol min^{-1} l^{-1} [15].

Prolidase activity in serum samples of the present study was a little lower than the activity reported previously. One reason might be that the substrate was not saturated, since the K_M value of human erythrocyte prolidase was estimated to be fairly high [16]. The relationship between the remaining concentration of the Gly-Pro was not proportional to the increasing amount of serum volume in the assay mixture. When we increased the substrate concentration in the assay mixture to ten times the K_M value of prolidase, however, there was only a slight reduction in peak area.

Studies of prolidase have been extended to cDNA cloning and primary structure determination [17], to plasma activity levels in alcoholic liver disease [18] and to patientes with prolidase deficiency [11,19,20].

The present simple HPLC assay method should be applicable not only to serum but also to other tissue preparations, especially for routine determination of a small number of samples. If an autosampler is used then the method is not restricted to a small number of samples.

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