Plasma Prolidase Activity: A Possible Index of Collagen Catabolism in Chronic Liver Disease

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We describe here an easy method of determining prolidase (EC 3.4.13.9) in plasma after preincubation with Mn2+ for 24 h at 37 °C to maximize prolidase activity. The mean activity in 338 patients who were either in hospital or outpatients was 900 U/L ± 520 (2 SD), unrelated to sex or age. In 25 of these 338 samples tested, prolidase activity was between 1500 and 2000 U/L. It exceeded 2000 U/L in eight, all of whom were patients with chronic liver disease. Plasma prolidase activity was normal in cytolytic syndromes such as liver or heart disease. Of the 27 patients with cirrhosis, only five exhibited prolidase activity >2000 U/L. Plasma prolidase activity was uncorrelated with six biochemical indexes to liver function (the aminotransferases, alkaline phosphatase, glutamytransferase, total bilirubin, and serum albumin) or with the degree of cirrhotic fibrosis. We believe that plasma prolidase activity may be high only in the early stage of fibrosis. This hypothesis would be consistent with the data on rat-liver collagenolytic activities during CCL4 administration. Monitoring of plasma prolidase activity might be useful in evaluating fibrotic processes in chronic liver disease in the human.

Additional Keyphrases: fibrosis • cirrhosis • chronic cardiac liver • liver metastasis • phenobarbital induction • liver prolidase activity

Prolidase (EC 3.4.13.9; proline dipeptidase) splits dipeptides with proline or hydroxyproline as their C-terminal amino acid. Because of the high proportion of imino acids in collagen (25% Pro and Hyp), this enzyme plays an important role in its degradation. The existence of prolidase has been known for about 50 years (1), but a deficiency of it was first described only 10 years ago (2, 3). So far, about 20 cases have been reported.

Clinically, deficiency is characterized by dermatological symptoms, especially severe leg ulcers, and different degrees of mental retardation. Massive urinary elimination of iminodipeptides is the predominant biochemical feature.

Prolidase is widely distributed in man and animals (4). This activity is relatively high in kidney, intestinal mucosa, and erythrocytes, and low in liver and plasma, prolidase activity in plasma being about 6% as great as in erythrocytes (5).

We recently re-investigated erythrocyte prolidase activity (5), with colorimetry of proline by Chinard's method (6). This assay of prolidase required preincubation with Mn2+ for 24 h at 37 °C, during which its activity triples. We noted that, for proline assay the reaction cannot be conducted at 100 °C as originally specified but only at 90 °C because of substrate hydrolysis. Several changes in collagen metabolism were recently described in prolidase-deficient cultured skin fibroblasts (7).

The aim of the present work was to investigate the prolidase activity of body fluids in syndromes involving modifications in collagen metabolism. For this purpose, it seemed more convenient to determine prolidase in plasma.

Materials and Methods

Materials

Chinard’s reagent: Mix 600 mL of glacial acetic acid, 400 mL of 6 mol/L orthophosphoric acid (407 mL of orthophosphoric acid 65%, d = 1.7, and 593 mL of water). Dissolve 25 g of ninhydrin in this mixture at 70 °C.

Standard prolidase solution: This is a 650 μmol/L solution in 0.45 mol/L trichloroacetic acid.

Glycyl-L-proline (Sigma Chemical Co., St. Louis, MO 63178): 94 mmol/L in 0.05 mol/L Tris HCl buffer, pH 7.8, containing 1 mmol of MnCl2 per liter.

Population investigated. Plasma prolidase activity was determined in hospitalized subjects or outpatients at the Centre Hospitalier Universitaire, Bicêtre, France. We assayed 338 plasma samples.

Procedures

Sample preparation. Collect venous blood into heparinized tubes (EDTA, fluoride, and sodium citrate are all inhibitors) and separate the plasma by centrifugation at 2300 × g for 15 min. Discard hemolyzed samples because erythrocyte prolidase activity is relatively high. Store plasma samples at −20 °C until assay.

Preincubation. Dilute plasma sixfold with the buffer mixture and preincubate for 24 h at 37 °C.

Enzymatic reaction. Add 100 μL of 94 mmol/L glycyl-L-proline solution to a 100-μL aliquot of the diluted and preincubated plasma (final dipeptide concentration: 47 mmol/L). After incubation for 30 min at 37 °C, stop the reaction by adding 1 mL of 0.45 mol/L trichloroacetic acid and use the 0.5 mL of supernate for prolidase estimation. Add 100 μL of plasma to a control tube after the reaction is stopped.

Proline measurement. Add 1 mL of glacial acetic acid and 1 mL of Chinard’s reagent to 0.5 mL of supernate. Run a blank and a standard under the same conditions. Instead of supernate, the blank contains 0.5 mL of 0.45 mol/L trichloroacetic acid. After 10 min at 90 °C, measure the absorbance at 515 nm. Adjust the photometer (Gilford 300-N) to 0 with the blank, and calculate the enzyme activities as follows:

For proline determination, we took 0.5 mL from a total volume of 1.2 mL.

\[
\frac{E - C}{S} \times \frac{|S|}{5} \times \frac{12}{5} \times \frac{0.5}{1000} = \text{millimoles of proline formed per} \ 0.1\ \text{mL of plasma, during} \ 30\ \text{min}
\]
where \( E \) is the experimental tube absorbance, \( C \) is the control absorbance, \( S \) is the absorbance of the standard, and [S] is the concentration of the substrate in mmol/L.

If we express the result per litre of sixfold diluted plasma, during one minute, we obtain:

\[
\frac{E - C}{S} \times [S] \times \frac{12}{5} \times \frac{0.5 
1000 \times 6 \times \frac{1}{30} = \text{milli-}
\]

moles of proline formed per litre of plasma per minute at 37 °C and pH 7.8. Simplifying the equation:

\[
\frac{E - C}{S} \times [S] \times 2.4 = \text{mmol \cdot min}^{-1} \cdot \text{L}^{-1}, \text{ or}
\]

\[
\frac{E - C}{S} \times [S] \times 2400 = \mu \text{kat per litre at 37 °C and pH 7.8, or}
\]

\[
\frac{E - C}{S} \times [S] \times 40 = \mu \text{kat per litre at 37 °C and pH 7.8}
\]

**Histological examination.** We chose 13 patients with cirrhosis for this study. Tissue samples were obtained from six of them by needle biopsy and from the other seven by the transcatheter procedure. Tissues were fixed in Bouin’s liquid and embedded in paraffin with 10% pyocolite by routine procedures. Each specimen was step-sectioned at 2 to 3 μm, sampled at six levels. Levels 1, 3, and 5 were stained with hemalum–eosin–safran and levels 2, 4, and 6 by the trichrome, Gordon–Sweet, and Perls techniques. In addition to the diagnosis, the following histological characteristics were examined and graded from 0 (absent) to 3 (very severe): fibrosis of the portal tracts, fibrosis around centrilobular veins, inflammation, steatosis, necrosis, alcoholic hepatitis with liver-cell swelling, Mallory bodies, and neutrophil leukocytes.

The pathologist studied the slides and recorded the findings without prior knowledge of plasma prolidase activity. The mean score for each characteristic was established and then compared with the value for prolidase activity.

**Results**

**Prolidase Activity in Plasma**

**Preincubation.** Plasma prolidase activity increased 10-fold after preincubation with Mn²⁺ for 24 h at 37 °C (Figure 1).

**Prolidase reaction.** After preincubation, prolidase activity was linearly related to time up to at least 4 h (Figure 2).

**Proline measurement.** As measured with a Gilford 300-N spectrophotometer, color intensity was linearly related to proline concentration up to 2 mmol/L (absorbance: 2.300).

**Distribution Frequency of Prolidase in the Population Investigated**

A log-gaussian distribution was obtained (Figure 3). The median value was 800 U/L, the mean value 900 U/L (only for values ≤1500 U/L), and the standard deviation 260 U/L.

The values to be expected for the population investigated were ≤1450 U/L. No sex- or age-related effect was noted (Table 1). Prolidase values were classified as normal (≤1500 U/L), subnormal (1500–2000 U/L), or pathological (≥2000 U/L).

We also classified plasma prolidase activity according to the main disease causing the hospitalization or consultation (Table 2). Of the 338 plasma samples tested, 25 exhibited subnormal values and eight showed pathological values.

**Normal values.** Plasma prolidase activity was normal in samples from patients with bone diseases, myocardial infarction, acute hepatitis, and lithiasis.

**Subnormal values.** Of the 25 patients with subnormal values, nine had cirrhosis, 15 were adults older than 40 years, with no evidence of liver disease, and one was a 22-year-old woman who was taking phenobarbital as an anti-epileptic drug (activity: 1850 U/L). This last case led us to speculate that prolidase might be induced by phenobarbital. We therefore attempted to verify this hypothesis by a daily intraperitoneal injection of phenobarbital (100 mg/kg) into three Wistar male rats for four days. Twenty-four hours
injection, the liver was removed and liver homogenates, microsomes, and cytosol fractions were prepared according to Dieter et al. (8). Those subcellular fractions were assayed for prolidase activity, with and without 24 h of preincubation with Mn2+. We were unable to demonstrate any prolidase induction (Table 3), although bilirubin UDP-glucuronosyl transferase (EC 2.4.1.17) activity tripled. However, it should be emphasized that prolidase is mostly located in the cytosol, and preincubation of the different cellular fractions with Mn2+ is necessary to obtain maximal enzyme activity.

Pathological values. All the eight patients with high prolidase values had chronic liver diseases (one case of hepatic metastasis in the terminal phase of a generalized cancer, two cases of chronic liver disease of cardiac origin, and five of cirrhosis).

Patients with cirrhosis. Of the 338 patients, 27 were cirrhotic. Of these, 13 had a normal prolidase value, nine had a subnormal value, and five had a pathological value.

Liver samples from 13 patients were histologically examined (two from subjects with pathological values, five with subnormal values, and six with normal values). Although the number of patients in this series was admittedly not sufficient for statistically significant calculations, we could see no correlation between histological characteristics and plasma prolidase activity, in particular for fibrosis.

Correlation of Prolidase Activity with Other Biochemical Variables

Blood tests. There was no correlation between plasma prolidase activity and results of the following routine laboratory tests: aspartate or alanine aminotransferase, glutamyltransferase, alkaline phosphatase, total bilirubin, and serum albumin (Table 4).

Study of ascites fluid. Ascites fluid was checked for prolidase activity. No correlation was found between prolidase activity in plasma and ascites fluid. There was, however, a correlation between the prolidase and protein concentration in ascites fluid (Figure 4).

Discussion

Prolidase activity is easy to measure in plasma, and no sample dilution is required to accommodate measurement of the usual pathological values. However, preincubation with Mn2+ for 24 h at 37 °C is essential for maximum activity, as is also true for erythrocytes (5), cultured skin fibroblasts, tissue homogenates, ascites fluid, and human erythrocyte purified enzyme (unpublished data). Values of 900 ± 520 U/L (2 SD) were found in the population investigated. Values exceeding 2000 U/L were only observed in patients with chronic liver diseases. In bone diseases, high prolidase activity was never detected. We had no cases of Paget's disease in whom to evaluate this activity. We found that plasma prolidase activities were normal in our cases of bone metastasis and primary bone cancers, and in cytolytic syndromes (liver or heart diseases). We saw no correlation between prolidase activity and results of some routine laboratory tests of liver function. Nor was plasma prolidase activity correlated with liver histology in cirrhotic patients. In ascites fluid, protein concentrations and prolidase activity were correlated, suggesting that, in exudates, prolidase production may parallel that of other proteins.

Some authors have suggested that collagen synthesis or degradation (or both) may alter during chronic liver diseases (9, 10). Such diseases are often characterized by fibrosis, which may be defined as an increase of connective tissue deposits (11). Collagen is the best known of all connective tissue components, and many parameters relating to collagen have been proposed for the assessment of hepatic fibrosis. In cirrhotic liver, a four- to sevenfold increase was found in the amount of collagen (9). Normal liver contains both type I and type III collagens as well as small amounts of several basement-membrane collagen. The ratio of type I to type III has been determined by
different authors in both normal and cirrhotic liver, but with marked discrepancies (12–14). Collagen synthesis can be investigated in primary-cell liver cultures (15–17) as well as in tissue slices, by using the in vitro incorporation into collagen of radiolabeled amino acids. Increased incorporation of radioactive proline into collagen was reported with fibrotic liver specimens (18, 19). In addition, many workers have measured, in liver and blood samples from man and animals, the activity of the following enzymes that are involved in collagen formation: prolyl hydroxylase (20–22), lysyl hydroxylase (24, 25), glucosyl and galactosyl transferases (24, 25), and lysyl oxidase (33). Although collagen synthesis has been widely explored in relation to the pathogenesis of hepatic fibrosis, only a few experiments have been devoted to collagen catabolism. In human liver, collagenase activity was shown to be high in cirrhosis, but low in alcoholic hepatitis (10). After CCl₄ administration to rats, liver collagenolytic activity rose in early stages of fibrosis and declined in advanced fibrosis (34, 35). Using immunofluorescent antibodies against collagenase, Montfort and Perez-Tamayo (36) showed that collagenase disappeared in the late stage of CCl₄-induced liver cirrhosis. These data led us to speculate that hepatic collagenolytic activity might be related to the different steps in the development of fibrosis. As collagenase activity cannot be tested in plasma because of the presence of anticolagenases, we proposed to follow up collagen catabolism in chronic liver disease by plasma prolidase assay. However, we found no correlation between plasma prolidase activity and the histological degree of fibrosis. In the early stage of fibrosis, plasma prolidase activity might be high and might subsequently drop in advanced fibrosis. This hypothesis would corroborate the modifications of collagenolytic activity observed in rats after CCl₄ administration. Therefore, plasma prolidase activity may be similar in early and advanced fibrosis. No correlation was observed between plasma prolidase activity and other biochemical measures of hepatic function. Thus this activity does not seem to well reflect chronic liver dysfunction. Some cirrhotic patients showed a normal plasma prolidase activity, even though hepatic function was severely disturbed. Some other cirrhotic patients showed a high prolidase activity with subnormal results for hepatic tests.

We propose to study this enzyme activity in liver fibrosis as a reflection of a modification of liver collagen catabolism. We are planning a particular series of assays concerning plasma prolidase activity in the same patient at different stages of chronic hepatitis, and in the rat after CCl₄ intoxication.

References

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