SHORT COMMUNICATION

A Sensitive Method for the Assay of Serum Prolyl Endopeptidase

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Summary: A new fluorimetric assay for the determination of prolyl endopeptidase (EC 3.4.21.26) was developed. The synthetic substrate Z-glycyl-prolyl-4-methylcoumarinyl-7-amide (0.2 mmol/l; 5 μl), K-phosphate buffer (100 mmol/l, pH 7.5; 100 μl), and serum (10 μl) are incubated for 120 min at 37 °C. The reaction is stopped with acetic acid (1.5 mol/l; 500 μl) and the released 7-amino-4-methylcoumarin is measured fluorimetrically. The mean value of prolyl endopeptidase catalytic activity concentration in serum for 120 healthy volunteers was 0.455 (SD = 0.092) μmol of 7-amino-4-methylcoumarin released per litre of serum per minute. The proposed procedure is sensitive, robust, and economical.

Introduction

Prolyl endopeptidase (EC 3.4.21.26; post-proline cleaving enzyme) is a proline-specific serine proteinase, first discovered by Walter (1) as an oxytocin-degrading enzyme and found to be widely distributed among different tissues and body fluids (2, 3). It cleaves the peptide bond at the carboxyl side of proline residues in proteins of relatively small molecular mass, which contain the sequence X—Pro—Y, where X is a peptide or protected amino acid and Y is an amide, a peptide, an amino acid, an aromatic amine or alcohol (4). As suggested by Moriyma (5) this enzyme can only cleave small substrates, so it occupies a special position amongst peptidases in its ability to modulate a class of peptides with emerging importance, i.e. the peptide neurotransmitters and neurohormones such as luliberin and substance P. The metabolism of some biologically important peptides has now been reported (6, 7), but there is still no clear evidence for a physiological role for prolyl endopeptidase. The evaluation of prolyl endopeptidase activity in human serum during various pathologies requires a sensitive and accurate method. Our intention here is to optimize and evaluate the reliability of an assay for the determination of serum prolyl endopeptidase activity.

Materials and Methods

Serum samples
Blood was sampled from blood donors, ages 18—65 years. The blood was allowed to clot, centrifuged within 2 h (2000 g, 15 min), and the serum stored at −70 °C. The samples were assayed for enzyme activity within a week of collection.

Reagents
EDTA and dithiothreitol were obtained from Sigma Corp. (St. Louis, USA). N-Benzoxycarbonyl-glycyl-prolyl-4-methylcoumarinyl-7-amide (Z-Gly-Pro-4-methylcoumarinyl-7-amide) and N-benzoxycarbonyl-prolyl-prolinal (Z-Pro-prolinal) were kindly provided to us by Prof. T. Yoshimoto (University of Nagasaki, Nagasaki, Japan). 7-Amino-4-methylcoumarin was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). All the other reagents were of analytical grade and purchased from E. Merck (Darmstadt, Germany).

Materials
Measurements were performed on a Shimadzu RF-5000 fluorimeter obtained from Shimadzu Corp. (Kyoto, Japan). The optical path length was 0.5 cm.

Incubation buffer
The incubation buffer consisted of 100 mmol/l K-phosphate pH 7.5 containing 1 mmol/l NaN₃, 1 mmol/l dithiothreitol and 1 mmol/l EDTA. The pH was adjusted at room temperature and the solution was stored at 4 °C.

Substrate solution
The stock solution of Z-Gly-Pro-4-methylcoumarinyl-7-amide (4.6 mmol/l) was prepared by dissolving the powder in dimethylsulphoxide. This stock stored at 4 °C is stable for at least a month.

Stopping solution
This solution contained 1.5 mol/l acetic acid and stored at 4 °C, is stable for at least two months.
Inhibitor solution
Z-Pro-prolinal (30 mmol/l) was dissolved in the incubation buffer.

Buffers used for the pH dependence determination
The buffer used to determine the pH-optimum consisted of 50 mmol/l TRIS of which the pH was adjusted at room temperature by the addition of acetic acid. The universal buffer used for the pH stability tests contained 0.0286 mol/l citric acid, 0.0286 mol/l KH₂PO₄, 0.0286 mol/l boric acid, and 0.0286 mol/l diethylbarbituric acid. The pH was adjusted at room temperature by the addition of 0.2 mol/l NaOH.

Enzyme assay
The assay was based on the ability of prolyl endopeptidase to cleave Z-Gly-Pro-4-methylcoumarinyl-7-amide. Ten μl of serum were preincubated for 15 min with 100 μl incubation buffer at 37°C, whereupon the reaction was started by the addition of 5 μl Z-Gly-Pro-4-methylcoumarinyl-7-amide stock solution. After 120 min, 500 μl stopping solution was added, and the released 7-amino-4-methylcoumarin was measured fluorimetrically at λₑ 370 nm and λₑ 440 nm. The blank consisted of the incubation buffer and serum to which the stopping solution and the substrate was added after the incubation. The substrate was incubated together with the incubation buffer at 60 °C for 60 min to check for hydrolytic degradation; the increase in fluorescence was negligible.

Optimal temperature
The stability of the enzyme as a function of temperature was tested by preincubating human serum for 60 min at different temperatures, followed by the measurement of the activity at 37 °C. The optimum was determined as above except for the change in temperature, which varied from 23 °C to 48 °C.

pH optimum
The stability of the enzyme with respect to pH was first examined by preincubating the enzyme for 150 min at 4°C at different pH values, after which the reaction was started under standard assay conditions. The pH optimum was determined with the normal assay using TRIS buffers. We investigated the pH dependence at pH values ranging from 6.25 to 9.35.

Calculation of the enzyme activity

\[(F_s - F_b) \times \text{factor} = \text{enzyme activity, expressed in U/l.} \]

\[F_s \text{ and } F_b \text{ are the fluorescence of sample and blank, respectively.} \]

The factor needed for the calculation of activity is determined by making a calibration curve of the fluorescence standard, 7-amino-4-methylcoumarin. The factor is then calculated for each concentration, in a range from \(5 \times 10^{-8} \text{ mol/l to } 5 \times 10^{-2} \text{ mol/l, as follows:} \]

\[\frac{c}{F} \times 0.513 = \text{factor,} \]

where \(c\) is the concentration of 7-amino-4-methylcoumarin in μmol/l, \(F\) is the fluorescence determined at a given \(c\), and 0.513 is the conversion factor needed to obtain U/l. The value 0.513 results from the division of 61.5, the dilution factor of the serum, by 120, the incubation time in min. One unit (U) was defined as the enzyme catalytic activity that releases 1 μmol 7-amino-4-methylcoumarin per minute under these assay conditions.

Inhibition study
50 μl of serum were preincubated together with 25 μl of inhibitor solution at 37 °C for 15 min, after which the remaining prolyl endopeptidase activity was determined by the above method and compared with a serum sample treated in the same way but without inhibitor. The final concentration of Z-Pro-prolinal was 0.87 μmol/l in the reagent solution.

Results
We first examined the linearity of the assay, which is a modified version of the method previously described by Browne & O’Cuinn. The assay was modified for the use of a 10 μl instead of a 100 μl sample. We obtained good correlation with a 20 to 120 min incubation. The line of best fit is given by the equation \(y = 0.02 x + 0.09\) (\(y = \text{amount of 7-amino-4-methylcoumarin released in nmol; } x = \text{time in min.}\) Linear regression analysis of these data yielded an r value of 0.999. As the signal to noise ratio is more favourable for a longer incubation, we suggested 120 min. The detection limit, which we defined as the enzyme activity that still could be determined with a CV smaller than 10% within a single run, was less than 0.1 U/l. The reliability of the assay was tested for two weeks resulting in CV values of 5.9 and 11.1% for within and between runs, respectively. The enzyme is stable between pH 6 and 10, and displays a plateau of optimal activity between 6.3 and 8.1 (fig. 1).
Fig. 2. Histogram of the serum prolyl endopeptidase activity. Mean activity = 0.455 U/l; SD = 0.092 U/l; n = 120.

This broad pH optimum is an advantage for a routine assay, as slight pH changes will not affect the results. The optimal temperature was determined to be 46 °C, which is higher than most prolyl endopeptidases described in the literature (9) (fig. 1b). Nevertheless, the incubations were performed at 37 °C, as this temperature is more representative of the normal physiological conditions in vivo. We also performed the inhibition assay with Z-Pro-prolinal, which is a specific prolyl endopeptidase inhibitor (10). After the preincubation of a serum sample with 0.87 μmol/l of inhibitor, only 0.5% of activity could be detected, which indicates that the proteinase was indeed a post-proline cleaving enzyme. Finally, the mean prolyl endopeptidase activity in serum of 120 healthy volunteers was determined to be 0.455 U/l (SD = 0.092 U/l) (fig. 2).

Discussion

A few methods have been described (11–13) for the determination of prolyl endopeptidase in human serum, but this is the first method that requires only a 10 μl sample. Subsequent clinical trials can therefore also be performed with this small volume. The assay we describe here is accurate, sensitive, robust, and economical, so it can easily be performed as a routine assay for the determination of prolyl endopeptidase in serum. The amount of substrate consumed during our experiments never exceeded 5%. The assay is highly specific for prolyl endopeptidase, because purified dipeptidyl peptidase IV (EC 3.4.14.5) and aminopeptidase P (EC 3.4.11.9), two proline specific aminopeptidases, do not result in any breakdown of the substrate. The possible interference of neutral endopeptidase (EC 3.4.24.11), a metalloprotease which also cleaves between the carboxyl side of proline and an aromatic residue, is prevented by the addition of EDTA to the incubation buffer. Inhibitors of this enzyme are found to have anti-arrhythmic effects in a rat model (14). We examined the effect of a specific prolyl endopeptidase inhibitor, Z-Pro-prolinal, on the serum enzyme and obtained strong inhibition. If later work can prove that the only mode of action of Z-Pro-prolinal is directed towards the active site of prolyl endopeptidase then it may function as an identification of the enzyme. Until there is clear evidence for a physiological role for prolyl endopeptidase, the use of this powerful inhibitor can help to elucidate the function of prolyl endopeptidase in serum. A pathology which results in a statistically significant elevated or lowered prolyl endopeptidase activity in serum has not so far been reported (12, 15, 16). We are now screening a large number of clinically well documented pathological sera, and to complete this search we are in the meantime investigating some tissues which can give rise to serum prolyl endopeptidase.

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References


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