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Brief technical note

Prolidase deficiency: characteristics of human skin fibroblast prolidase using colorimetric and fluorimetric assays

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Introduction

Prolidase (EC 3.4.13.9) deficiency (McKusick 26413) is an autosomal recessive disease associated with chronic ulcerative dermatitis, mental retardation and imino-dipeptiduria [1]. A single form of prolidase was found in human erythrocytes [2] and an almost total deficiency of the enzyme against the substrate glycyl-proline has been reported [3–9] in the disease. In a preliminary report on cultured human skin fibroblast prolidase [10] we showed that the disease enzyme activity against other substrates was not as reduced. Characteristics of the normal and abnormal enzyme of skin fibroblasts are described that enable diagnosis of the disease to be made using substrates in addition to glycyl-proline.

Materials and methods

Skin fibroblast cultures were obtained from a Turkish male patient (Case 1) [9] and a male Italian patient (Case 2) [4]. The cells were cultured and extracts prepared as previously described [10,11].

Prolidase was assayed using glycyl-proline (gly-pro), phenylalanyl-proline (phe-pro), alanyl-proline (ala-pro), valyl-proline (val-pro), seryl-proline (ser-pro), methionyl-proline (met-pro) obtained from Sigma Ltd. (St. Louis, MO, USA) and glutamyl-proline (glu-pro) obtained from BaChem Ltd. as substrates and followed by estimating liberated proline [10]. Two other possible methods for phe-pro were

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evaluated. Firstly, liberated phenylalanine was estimated spectrophotometrically using a micromethod based upon the nonspecific dipeptidase assay of Shoaf et al [12]. The assay contained 25 μl extract, 50 μl 50 mmol/l barbital–HCl buffer pH 8.0 and 25 μl 80 mmol/l phe-pro and after 30 min incubation at 37 °C the reaction was stopped by heating at 100 °C for 1 min. Reagent (250 μl of 25 mmol/l barbital-HCl buffer pH 8.0 containing 0.27 g/l L-amino acid oxidase, Sigma Ltd. type VI; 20 mg/l horseradish peroxidase, Sigma Ltd. type II; and 0.2 g/l *o*-dianisidine · 2 HCl Sigma Ltd.) was added and after 10 min at 37 °C 250 μl 50% (v/v) sulphuric acid added. The colour was read at 530 nm and compared to a phenylalanine standard curve. Secondly, the method of Matsumoto et al [13] for monoamine oxidase was applied to the fluorimetric estimation of phenylalanine. After heating the assay tubes at 100 °C for 1 min, 400 μl reagent (25 mmol/l barbital–HCl buffer pH 8.0 containing 0.1 g/l L-amino acid oxidase, 10 mg/l horseradish peroxidase, 50 mmol/l EDTA and 0.5 μmol/l homovanillic acid, Sigma Ltd.) was added. After incubation at 37 °C for 10 min, 500 μl 0.5 mol/l NaOH was added and the fluorescence read on a Perkin–Elmer 1000 fluorimeter (excitation 323 nm; emission 426 nm) and related to a phenylalanine standard curve. Addition of EDTA was needed to prevent the precipitation of Mn^{2+} used to activate prolidase. Protein was estimated [14] using bovine serum albumin (Sigma Ltd.) as standard.

The effect of Mn^{2+} on prolidase activity was followed by the addition of increasing amounts of $MnCl_2$ to the assay or by preincubating extract with 1.33 mmol/l Mn^{2+}. Substrate kinetics for enzyme preincubated with 1.33 mmol/l Mn^{2+} for 5 min was calculated by Eadie–Hofstee plots using a Hewlett–Packard HP97 [15] for a number of substrates over the range 1–20 mmol/l. Substrate interaction was also studied for phe-pro by the method of Chrastil and Wilson [16] by following product (phenylalanine) formation with time under limiting substrate level (0.5 mmol/l) for normal and abnormal enzyme at the same protein level. Heat stability of prolidase in the presence (1.33 mmol/l) or absence of Mn^{2+} was investigated by treating extract at 30–60 °C for 5 min or at 48 °C for up to 60 min. For extracts treated without Mn^{2+} an equivalent amount of Mn^{2+} was added with the substrate. The effect of *p*-hydroxymercuribenzoate (PHMB, Sigma Ltd.) on prolidase activity against phe-pro was studied by the addition of 0.01–0.5 mmol/l PHMB before or after treatment with 1.33 mmol/l Mn^{2+}. Based upon these results 0.05 mmol/l PHMB was added to normal and abnormal prolidase following preincubation with 1.33 mmol/l Mn^{2+} and assayed with a number of substrates.

Results and discussion

Assays for prolidase

Myara et al [8] reported a method using Chinard's ninhydrin reagent optimised for the assay of prolidase. With this method gly-pro was rapidly hydrolysed, such that at 100 °C an absorbance of 1.0 was reached after 20 min necessitating the use of a lowered temperature which reduced assay sensitivity. The present method was based on the ninhydrin method of Mayer and Nordwig [17] and at 100 °C the absorbance was only 0.09 after 20 min enabling 100 °C to be used. The development

of the proline colour and hydrolysis of gly-pro to give the substrate blank were critically dependent upon the temperature. Using heating blocks in which there was a difference of only a degree between blocks gave unreliable readings. This problem was overcome by ensuring a uniform temperature in a boiling waterbath by continuous stirring. All the other substrates were more stable and gave a lower blank than gly-pro. Glycine was found to increase the proline standard reading and its inclusion in the method, as recommended by Mayer and Nordwig [17], eliminated any tendency for the glycine liberated by the enzyme from gly-pro to increase the reading.

The L-amino acid oxidase colorimetric method gave 3.8 times the colour for a given phenylalanine standard (10 nmol = absorbance 0.285) compared to an equivalent proline standard in the Mayer and Nordwig [17] method and a slightly better enzyme reading to blank ratio. The method was linear up to at least an absorbance of 1.0 equivalent to 35 nmol phenylalanine. The L-amino acid oxidase fluorimetric method gave a reading of 900 fluorescent units for 10 nmol phenylalanine with the fluorimeter standardised to 200 U with 1 mg/l quinine and an enzyme reading to blank ratio twice as high as the proline method. Hence, both L-amino acid oxidase methods gave larger readings for a given enzyme level than the proline method and were suited for the rapid analysis of a large number of samples as generated by kinetic and chromatographic studies.

An optimum of pH 8.0 was obtained for normal and abnormal fibroblast prolidase using barbital–HCl buffer. The three enzyme assays were linear with time for at least 60 min and with protein up to at least 40 μg/assay. A range of 5–15 μg protein/assay was used for the L-amino acid oxidase methods and 15–40 μg protein/assay for the proline method. The three methods gave similar prolidase activities for a number of fibroblast cultures.

Effects of manganese

Control prolidase was activated by the addition of Mn^{2+} with the substrate (2.5 mmol/l Mn^{2+} optimal gly-pro; 1 mmol/l Mn^{2+} optimal other substrates), except for glu-pro which did not require Mn^{2+}. Preincubation of the enzyme with Mn^{2+} at 37 °C further increased control prolidase activity, with the same levels of Mn^{2+} being optimal (Fig. 1). Preincubation for 60 min for gly-pro and 5 min for the other substrates (phe-pro and leu-pro shown) were required for optimal activation. The increase in activity with preincubation at 37 °C did not occur at 48 °C. The abnormal enzyme was activated by the addition of Mn^{2+} with the substrate, but the optimal Mn^{2+} for Case 1 prolidase activity against phe-pro was about 0.05 mmol/l Mn^{2+} and Case 2 showed about 90% of maximum activity at this level (Fig. 2). Abnormal prolidase against phe-pro was always reduced by preincubation irrespective of the level of Mn^{2+}. However, both cases still required 60 min preincubation for gly-pro, but maximum prolidase activity only required 0.05 mmol/l Mn^{2+}. Previous studies have used different conditions for Mn^{2+} ranging from no Mn^{2+} [5], 10 mmol/l [4] or 16 mmol/l [6] with no preincubation to 1 mmol/l preincubated for 2 h [3,7] or 24 h [8] or 10 mmol/l for 1 h at 37 °C [3,6]. Based upon the present data these variable Mn^{2+} conditions would partly explain the very variable prolidase

levels reported [3–9]. In addition, Mn^{2+} conditions optimised for control prolidase are not necessarily those needed by the abnormal enzyme.

Substrate specificity

Skin fibroblast prolidase activated by preincubation for 5 min at 37 °C with 1.33 mmol/l Mn^{2+} showed a tight range of values for all the eight substrates (20

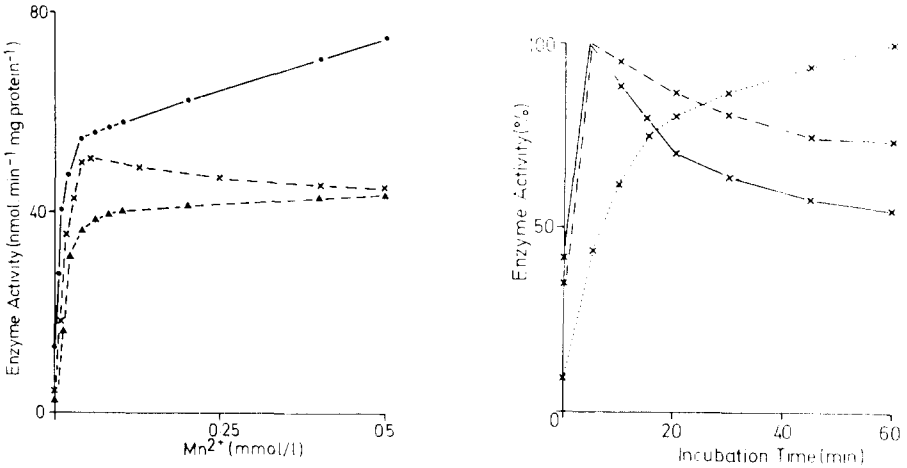

Fig. 1. Effect of preincubation with 1.33 mmol/l Mn^{2+} at 37 °C on control prolidase activity against gly-pro (...), phe-pro (–) and leu-pro (- - -).

Fig. 2. Effect of preincubation with different Mn^{2+} levels for 5 min at 37 °C on control (●), Case 1 (×) and Case 2 (▲) prolidase activity against phe-pro.

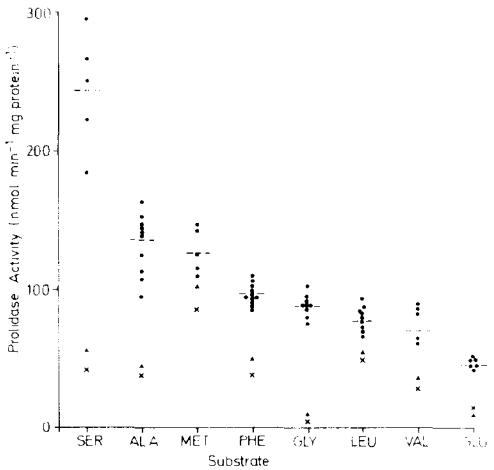

Fig. 3. Activity of control (●), Case 1 (×) and Case 2(▲) skin fibroblast prolidase (- - -: mean activity) following preincubation with 1.33 mmol/l Mn^{2+} for 5 min at 37 °C against 20 mmol/l substrate (x-pro; x = amino acid shown).

mmol/l) used (Fig. 3). The highest specific activity was against ser-pro, whilst the lowest was against glu-pro, the only substrate not requiring Mn^{2+}. Activity against gly-pro, when fully activated by 60 min preincubation with 2.5 mmol/l Mn^{2+}, would be increased about 2.5 times to a mean specific activity of 220 nmol · min^{-1} · mg^{-1} protein. The reported mean specific activity (nmol · min^{-1} · mg^{-1} protein) of skin fibroblast prolidase ranged from 7.2 [5], 20 [4], 50 [8] to 154 [9], all less than the present level, and, in part, reflects the variable Mn^{2+} conditions used. Abnormal prolidase activity against gly-pro has consistently been reported [3–10] to be absent or very low and in the present study activity in the two cases was reduced to 3–6% depending upon the Mn^{2+} conditions. The reduction in activity against the other substrates was not as marked ranging from 27 to 80% (Fig. 3). The enzyme deficiency against substrates with a polar amino acid (gly, ser, glu) was greater than against those with a non-polar amino acid (ala, val, phe, leu, met).

Substrate kinetics

The response of normal and abnormal prolidase (Case 2), following preincubation with 1.33 mmol/l Mn^{2+} for 5 min at 37 °C, to a number of substrates was analysed by Eadie–Hofstee plots [15] and the results for corrected Km and Vmax given in Table I. The estimate of data variation [SD(E)rad; 15] for all substrates was less than 0.07 indicating the results to be acceptable. The normal enzyme exhibited biphasic kinetics against phe-pro, whilst the abnormal enzyme showed only a single line. The abnormal enzyme had a lowered affinity for all the substrates with the smallest change being for glu-pro and the largest for ser-pro, phe-pro and leu-pro. The maximum activity of the abnormal enzyme, calculated from the Eadie–Hofstee plots was increased for met-pro, unchanged for phe-pro and leu-pro, somewhat reduced for ser-pro and val-pro and most reduced for ala-pro, glu-pro and gly-pro. Owing to the altered Km values the deficiency in abnormal prolidase activity would be more marked the lower the substrate level used. The substrate (gly-pro) level used in previous work has varied from 1 [4], 3.7 [3,7], 7 [5], 10 [6] to 47 mmol/l [8] which

TABLE I

Substrate kinetics of control and Case 2 prolidase

Substrate	Km		Vmax	
	Control	Case	Control	Case
Ser-pro	3.8	36.2	279	142
Ala-pro	2.3	8.1	221	62
Met-pro	4.5	14.4	147	210
Phe-pro	2.8, 6.8	24.9	136	147
Gly-pro	2.8	13.7	150	20
Leu-pro	1.3	10.4	80	72
Val-pro	0.9	3.3	68	40
Glu-pro	4.4	6.1	33	6

1–20 mmol/l substrate analysed by Eadie–Hofstee plots [15]

would differentially affect the level of control and abnormal prolidase. As with Mn^{2+} conditions, the level of substrate optimised for the assay of control prolidase would not be optimal for the measurement of the abnormal enzyme.

Analysis of product formation using phe-pro as substrate by the method of Chrastil and Wilson [16] indicated the abnormal enzyme had an unchanged n (sterical structure constant) value of over 0.9 and $p\infty$ (maximum product formation) value of 0.35 mmol/l, but the k (rate constant related to diffusion coefficient) was reduced to 0.0023 as compared to 0.0086 $l \cdot g^{-1} \cdot s^{-1}$ of the normal enzyme. The data indicated no major alteration in enzyme structure or absorption affinity for the substrate, but faulty activation or presence of inactive enzyme.

Heat stability

Both normal and abnormal prolidase activity against phe-pro were thermolabile above 45 °C with only about 10% residual activity at 55 °C after 5 min (Fig. 4A). The addition of Mn^{2+} increased the heat stability of normal, but decreased that of the abnormal enzyme. Following the loss of activity at 48 °C with time showed (Fig. 4B) that 70–90% of control enzyme activity (example shown typical of six tested) was lost after 60 min without Mn^{2+}, but that with 1.33 mmol/l Mn^{2+} only about 25% was lost. The two cases (Case 2 shown) showed a similar pattern of loss without Mn^{2+} as control enzyme, but in the presence of 1.33 mmol/l Mn^{2+} the abnormal enzyme was initially even more labile. These results indicate that normal prolidase consists of two forms, one labile and one stable in the presence of Mn^{2+} and that for the abnormal enzyme the stable form is largely absent.

Effect of p-hydroxymercuribenzoate (PHMB)

The effect of PHMB on prolidase activity was tested, as PHMB binds to swine intestinal prolidase [18] and sulphydryl reagents inhibit swine kidney prolidase [19]. The normal and abnormal prolidase activity against phe-pro was strongly inhibited by treatment with PHMB prior to incubation with Mn^{2+}. However, preincubation with 1.33 mmol/l Mn^{2+} protected the normal enzyme, but not the abnormal enzyme

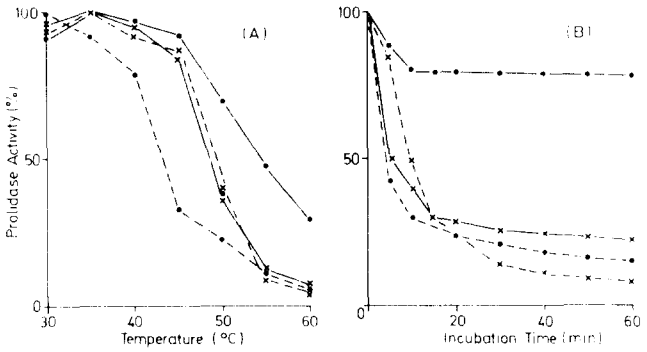

Fig. 4. Heat stability of control (–) cand Case 2 (- - -) prolidase activity against phe-pro without (×) or with (●) 1.33 mmol/l Mn^{2+}. A. Effect of 5 min at 30–60 °C. B. Effect of preincubation at 48 °C for up to 60 min.

against PHMB inhibition (Fig. 5). Addition of PHMB and Mn^{2+} simultaneously gave an intermediate result for control prolidase with 0.05 mmol/l PHMB plus 1.33 mmol/l Mn^{2+} inhibiting 30% compared to < 5% when PHMB was added after the Mn^{2+}. The abnormal enzyme (Cases 1 and 2) was inhibited > 95% under both these conditions. A level of 0.05 mmol/l PHMB was, therefore, chosen to analyse the effect of PHMB on the normal and abnormal enzyme for a number of substrates (Table II). The addition of Mn^{2+} prior to PHMB protected normal prolidase activity for all the substrates, except for a small reduction with gly-pro and a moderate reduction with met-pro. However, the abnormal enzyme was markedly inhibited by PHMB for all the substrates, except for a moderate reduction of the small residual activity against gly-pro. Inhibition of swine kidney prolidase activity by iodoacetamide has previously been noted [19] to be prevented by Mn^{2+}. Since PHMB and iodoacetamide react with SH groups, it seems likely that Mn^{2+} activates prolidase by binding to SH groups.

TABLE II

Relative activity (%) of prolidase against different substrates following PHMB treatment

Substrate	Controls			Cases	
	1	2	3	1	2
Ser-pro	100	97	100	14	14
Ala-pro	92	98	100	0	4
Met-pro	45	53	56	4	3
Phe-pro	113	108	100	6	3
Gly-pro	76	81	80	58	58
Leu-pro	117	93	75	2	4
Val-pro	131	126	120	4	3

Prolidase activated by preincubation with 1.33 mmol/l Mn^{2+} for 5 min at 37 °C and PHMB added with the substrate to give a concentration of 0.05 mmol/l.

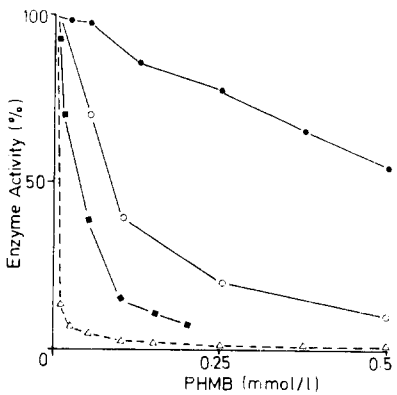

Fig. 5. Effect of PHMB on control (–) and abnormal (- - -) prolidase activity against phe-pro. ■ = PHMB added before Mn^{2+}; ○ = PHMB added with Mn^{2+}; ● = PHMB added after Mn^{2+}; △ = PHMB added before or after Mn^{2+}.

Diagnostic properties of abnormal prolidase

The present data showed that the following properties can distinguish control and abnormal prolidase: (1) Mn^{2+}: control activated but abnormal inactivated by preincubation with high level of Mn^{2+}; (2) substrates: abnormal enzyme reduced specific activities and increased Km values compared to control; (3) heat stability: control more stable, but abnormal enzyme more labile to heat treatment in presence of Mn^{2+} and (4) PHMB: inhibition of control prevented, but abnormal enzyme not protected by Mn^{2+}. Any one of these properties could be used to detect the abnormal prolidase in cultured skin fibroblasts. The results could be explained by either a change in the properties of a single form of prolidase or the presence of two forms of prolidase at least one of which is altered. Although only a single form of prolidase was found in erythrocytes using gly-pro as substrate [2], the present results indicate that a second form of the enzyme would not be able to hydrolyse gly-pro efficiently and would require the use of another substrate for detection. Which of the possibilities is correct will be resolved by the use of chromatographic procedures and detection of prolidase with a number of substrates.

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