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Kinetic properties of human erythrocyte prolidase and optimal conditions for prolidase assay by proline colorimetric determination

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SUMMARY

Prolidase (EC 3.4.13.9) is an iminodipeptidase that catalyses the hydrolysis of C-terminal proline or hydroxyproline containing dipeptides. It is involved in intracellular protein degradation, and apparently contributes to the conservation of iminoacides from endogenous and exogenous protein structures. Analysis of prolidase activity in human biological fluids and tissues has gained attention as a biochemical tool in acute and chronic liver diseases, adenocarcinoma of lungs and as a marker for fetal lung maturation and growth, in addition to long known genetic prolidase deficiency.

In the present study, stability, activation and inhibition kinetics of human erythrocyte prolidase and optimal conditions for human erythrocyte prolidase assay were investigated. Level of proline, product of the enzymatic reaction was measured by Chinard's method.

Human erythrocyte prolidase activity was stable for six months at -10 °C. Preincubation of the enzyme at 37 °C with 1 mM $MnCl_2$ provided appropriate activity.

Preincubation at 45 °C and 55 °C caused enzyme inactivation. After preincubation, prolidase activity was linearly related to incubation time up to at least two hours. Buffer ionic strength between 30-70 mM at pH 8.0 did not cause an obvious difference of prolidase activity. However pH changes between 7.0 and 8.5 caused significant differences with maximal activity at pH 8.0 The Km value for Gly-L-pro dipeptide was approximately 7.0 mM. EDTA, iodo acetamide and some divalent metal ions (Cu^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+}) had inhibitory effect on human erythrocyte prolidase activity.

According to our results, preincubation at 1mM $MnCl_2$ concentration for 2 hours at 37°C and incubation at 30 mM Gly-L-pro dipeptide concentration, 40 mM buffer ionic strength, pH 8.0 for 30 minutes reveals optimal assay conditions for human erythrocyte prolidase. Presence of metal chelating agents and metal ions other than manganese in the reaction media can interfere results, thus should be avoided.

Key Words: Human erythrocyte , prolidase, kinetic properties, inhibition

INTRODUCTION

Prolidase (Peptidase D, Proline Dipeptidase, EC 3.4.13.9) is a highly specific peptidase, the only enzyme known to catalyse hydrolysis of compounds in which the sensitive peptide bond involves the imino nitrogen of proline or hydroxyproline. It is involved in intracellular protein degradation, especially that of collagen that has a high imino acid content (25%), contributes to the recycling of imino acides along with prolinase, dipeptidyl aminopeptidase II and IV and plays an important role in conservation of proline (1). The conformational restrictions imposed by proline motifs in a peptide chain appear to imply important structural or biological functions as can be deduced from their often remarkably high degree of conservation as found in many proteins and peptides, especially cytokines, growth factors, G-protein coupled receptors and neuroactive peptides (2). Thus, the enzyme has a variety of functional role and hereditary prolidase deficiency, the multisystemic disorder is characterized with a wide spectrum of clinical

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manifestations including skin ulcers, mental retardation and susceptibility to infections (3).

Prolidase is widely distributed in human tissues. Its activity is relatively high in kidney, intestinal mucosa, erythrocytes and low in liver and plasma being 6% of erythrocyte activity (4). There are two isoenzymes of prolidase in humans. Prolidase I has a relative molecular mass (Mr) of about 112 000 and its natural substrate is Glycyl-L-proline (Gly-L-pro) whereas prolidase II has a Mr about 185 000 and mainly active towards Met-L-pro substrate. Superiority of the erythrocyte enzyme over plasma enzyme as a possible marker of disease activity could be similarity of prolidase isoenzyme pattern of human erythrocyte (prolidase I: 51% and prolidase II: 49%) and tissues like liver, kidney, duodenum, ileum, jejunum, pancreas, spleen, brain, heart, skin fibroblasts and prostate where only prolidase I activity is present in human plasma. A possible inhibitory effect of albumin is also suggested that makes plasma prolidase assay cumbersome (4,5).

Prolidase is a Mn^{2+} activated enzyme with a 5 to 10 fold increase of the enzyme activity. Optimum pH of human prolidase is reported 7.6-7.8. Some metal ions (Cu^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Hg^{2+} Pb^{2+}, Zn^{2+}) have inhibitory effect on prolidase activity. Iodo acetamid (IAA), p-cloro mercury benzoate (PCMB) are potent inhibitors suggesting that a sulfhydryl group is concerned in the activity of the enzyme. Inhibition with IAA is reversible. Km of the prolidase for Gly-L-pro has been reported between 2,9-30 mM (6-8).

In this study, stability, activation and inhibition kinetics of human erythrocyte prolidase and optimal conditions for human erythrocyte prolidase assay were investigated in order to improve analytical presicion and decrease costs.

MATERIAL AND METHODS

Specimen Collection and Storage: Venous blood obtained from healthy subjects confirmed with clinical and laboratory assessment was collected into heparinized tubes. After centrifugation at 4000 ×g for 3 minutes plasma was discharged. Erythrocytes were washed 3 times with saline (0.85%). 2.5 mM $MnCl_2$ was used as hemolysing reagent. Hemolysate pool stored at -10^{O}C until assay was used.

Erythrocyte Prolidase Assay: The hemolysate was diluted 200 fold with 2.5 mM $MnCl_2$ and preincubated at 37 ^{O}C for 120 minutes. The reaction mixture containing 30 mM Gly-L-Pro substrate, 40 mM Trizma HCl buffer (pH: 8.0) and 100 µl of diluted hemolysate in 1 ml, was incubated at 37 ^{O}C for 30 minutes. After incubation, reaction was stopped by adding 0.5 ml of 20% TCA (tri chloro acetic acid) solution. Supernate was used for determination of proline formed by prolidase by Chinard method (9,10).

One unit of enzyme activity was defined that splits 1 µm Gly-L-Pro per one minute. All reagents were of analytical grade and obtained from Sigma (St Louis, USA) and Merck (Darmstadt, Germany).

Statistical Evaluation: Data is given as raw data. Statistical analysis and scatter diagrams of the results were performed with Microsoft Excel software program.

RESULTS

Impact of Mn^{2+} Concentration in Preincubation Media

Effect of 0, 1, 2.5, 5, 7.5, 10 mM $MnCl_2$ concentrations were investigated. The enzyme did not show any catalytic activity without $MnCl_2$ whereas with 1 and 2.5 mM $MnCl_2$ concentrations higher activity was achieved compared to the higher $MnCl_2$ concentrations. Addition of $MnCl_2$ to incubation media instead of preincubation media caused 1.7 times lower activity compared to the same preincubation concentrations.

Enzyme activity obtained at different $MnCl_2$ concentrations in preincubation media are presented in Figure 1.

Divalent Metal Ions other than Mn^{2+}

Cu^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+} at 2.5 mM concentrations were added to preincubation media instead of $MnCl_2$. None of the divalent metal ions investigated had an activator effect.

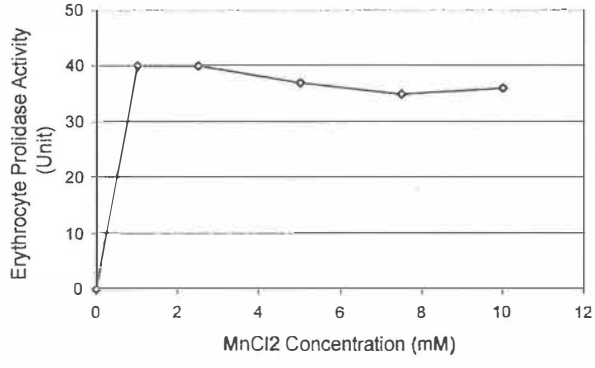

Figure 1. Impact of Mn^{2+} concentration in preincubation media on human erythrocyte prolidase

Table 1. Effect of some divalent metal ions on human erythrocyte prolidase activity

Metal ions (2.5 Mm)	Erythrocyte Prolidase Activity (Unit)
Mn^{2+}	35
Cu^{2+} or Cu^{2+}+ Mn^{2+}	0
Fe^{2+} or Fe^{2+}+ Mn^{2+}	0
Zn^{2+} or Zn^{2+}+ Mn^{2+}	0
Hg^{2+} or Hg^{2+}+ Mn^{2+}	0
Mg^{2+}	0
Mg^{2+}+ Mn^{2+}	35

When added together with 2.5 mM $MnCl_2$, Cu^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+} completely inhibited the enzyme activity whereas Mg^{2+} did not affect results (Table 1).

Preincubation Temperature

37°C, 45°C and 55°C preincubation temperatures were investigated. Preincubation at 55°C for two hours resulted in complete inactivation, and at 45°C in 17% residual activity compared to 37°C (Figure 2).

Table 2. Inhibitor effect of EDTA on human erythrocyte prolidase activity (preincubation mixture containing 2.5 mM $MnCl_2$).

EDTA concentration (Mm)	Addition time	Enzyme Activity	
		Unit	%
0	-	36,1	100
2,5	preincubation	14,0	38,9
5,0	preincubation	0	0
2,5	incubation	37,0	100
5,0	incubation	37,0	100

Incubation Time

After preincubation with 2.5 mM $MnCl_2$ at 37°C for 120 minutes, human erythrocyte prolidase activity was linear at least up to 120 minutes (Figure 3).

Ionic Strength and pH

Buffer ionic strength between 30-70 mM at pH 8.0 did not cause an obvious difference of prolidase activity. However pH changes between 7.0 and 8.5 caused significant differences with maximal activity at pH 8.0 (Figure 4).

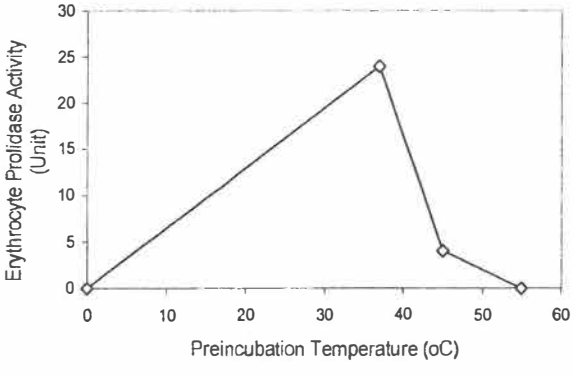

Figure 2. Prolidase activity after two hours preincubation at 37°C, 45°C and 55°C

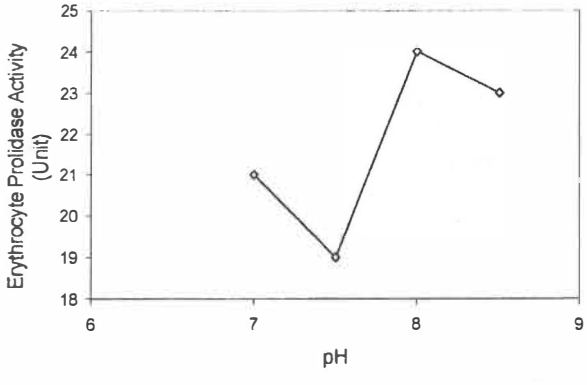

Figure 4. pH dependent behavior of human erythrocyte prolidase

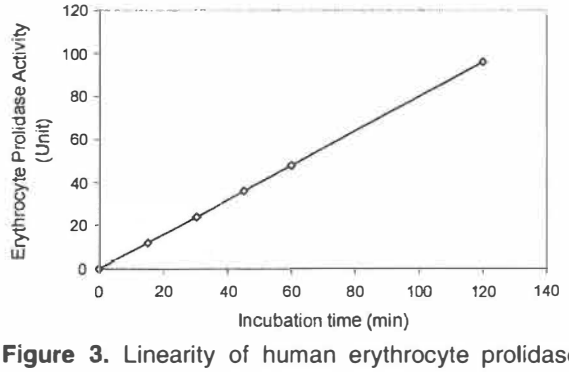

Figure 3. Linearity of human erythrocyte prolidase activity up to 120 min.

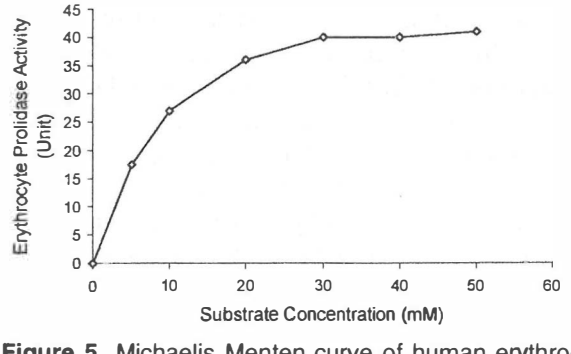

Figure 5. Michaelis Menten curve of human erythrocyte prolidase at 37°C, pH 8.0 against Gly-L-pro

Table 3. Inhibitor effect of IAA on human erythrocyte prolidase activity (preincubation mixture containing 2.5 mM $MnCl_2$).

IAA (Mm)	Erythrocyte Prolidase Activity			
	Preincubation		Incubation	
	Unit	%	Unit	%
0	30,0	100	30,0	100
10	22,3	74,1	26,6	88,5
20	15,2	50,6	26,1	86,9
30	11,8	39,2	25,6	85,3
40	6,6	22,0	23,9	79,7
50	3,4	11,3	21,4	71,4

Michaelis Menten Curve

Michaelis Menten curve is presented in Figure 5. Km value of the enzyme for Gly-L-pro was approximately 7.0 mM.

Impact of EDTA, IAA and Glutathione

Effect of ethylene diamine tetra acetic acid (EDTA) at 2.5 and 5.0 mM concentrations in incubation and preincubation media was investigated (preincubation media containing 2.5 mM $MnCl_2$). An inhibitory effect of EDTA was observed only in the preincubation media, in a concentration dependent manner (Table 2).

Effect of iodo acetamide (IAA) at 10-50 mM concentrations (10 mM intervals) was investigated (preincubation media containing 2.5 mM $MnCl_2$). A concentration dependent inhibitory effect of IAA for human erythrocyte prolidase was observed which was more prominent in preincubation media (Table 3).

Glutathione itself did not show an activator effect at 10-50 mM concentrations (10 mM intervals) on prolidase activity, instead had inhibitor effect.

DISCUSSION

Prolidase has been known for over sixty years as a key enzyme involved in imino acid metabolism, and as a diagnostic tool for genetic deficiency of prolidase. However, its value as an indicator of impaired collagen metabolism in liver diseases was discovered after 1980s (9,10). Its relation to immune system, insulin-like growth factor I, cell growth and regulation of prolidase expression by beta 1 integrin receptors suggesting a possible role of prolidase in tumourogenesis and metastasis was reported in 1990s (11-13). Elevated prolidase activity of lung adenocarcinomas confined to G_3-poorly differentiated cases compared to lung parenchyma had been reported (14,15). Prolidase activity is depressed in human breast cancer MCF-7 cells which is proposed as a result of disturbances in signaling mediated by beta 1 integrin-collagen interaction (16). We have recently observed elevated erythrocyte prolidase activity in a group of patients with lung adenocarcinoma that supports alteration of prolidase activity in malign neoplasms (17,18). Prolidase activity in amniotic fluid was suggested as a marker for fetal lung maturation and growth as well (19). Thus, analysis of prolidase activity in human biological fluids and tissues has gained clinical utility as a biochemical marker.

Although kinetic properties of purified or partially purified prolidase has been exclusively investigated before, behavior of the enzyme may not be the same in analytical medium. Establishment of kinetic properties of the enzyme in analytical conditions may help to improve method, decrease costs and time-consuming.

Activation of prolidase I by preincubation of manganese has long been used. Myara et al. proposed optimization of erythrocyte prolidase activity using 1mM $MnCl_2$ for 24 hours at 37°C (20). Times can be reduced in the presence of higher manganese concentrations (e.g. 25 mM $MnCl_2$ for 45 min) or by using higher temperatures (1mM $MnCl_2$ for 1 hour at 50°C) (8). However even at 45 °C a remarkably high inactivation was observed in our study and higher manganese concentrations up to 10 mM caused negligible inhibition. A relatively shorter preincubation time, 120 minutes in our study, revealed comparable results. In addition, human erythrocyte prolidase consists of the two isoforms and prolidase II activity completely disappears after prolonged preincubation (8,20).

Manganese activation seems to be irreversible, as judged by the stability of the enzyme action against EDTA after preincubation. However the stability was partial in case of IAA suggesting that a sulfidryl group and manganous ion to be present within the prolidase active site and related to each other. Inhibitor effect of divalent metal ions can be attributable to competition of these ions to manganous ions but magnesium ions did not show a similar effect. This discrepancy remains to be elucidated (6-8, 20).

According to our results preincubation at 1mM $MnCl_2$ concentration for 2 hours at 37°C and incubation at 30 mM Gly-pro dipeptide

concentration, 40 mM buffer ionic strength, for 30 minutes reveals optimal assay conditions for human erythrocyte prolidase. Presence of metal chelating agents and metal ions other than manganese in the reaction media can interfere results, thus should be avoided.

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