original paper

Haematologica 1994; 79:13-8

PROLIDASE DEFICIENCY: BIOCHEMICAL STUDY OF ERYTHROCYTE AND SKIN FIBROBLAST PROLIDASE ACTIVITY IN ITALIAN PATIENTS

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ABSTRACT

Background and methods. **Prolidase deficiency (PD), a rare, autosomally inherited disorder causing iminodipeptiduria is associated with a number of clinical manifestations, the principle feature being chronic skin ulceration. The enzyme prolidase cleaves iminodipeptides containing C-terminal prolyl or hydroxyprolyl residues and is important in the final stages of protein catabolism. We report clinical and biochemical findings in 8 Italian patients with proven prolidase deficiency. There was considerable heterogeneity in age at onset of symptoms (varying from 3-17 years), mental retardation and clinical manifestations (asymptomless to very severe). Prolidase activity was determined in hemolysates of patient erythrocytes and cultured dermal fibroblasts.**

Results. **Prolidase activity was found to be deficient, especially against gly-pro. Erythrocyte and fibroblast enzyme was also separated into two forms, a major isoform (I) and a minor one (II) by fast protein liquid chromatography, and activity against different iminodipeptide substrates was tested. Isoform I activity was markedly reduced in all patients as compared to normal controls, while isoform II activity appeared to be unaltered.**

Conclusions. **We were unable to find any correlation between degree of enzyme activity loss and severity of symptoms.**

Key words: prolidase deficiency, erythrocyte prolidase activity, fibroblast prolidase activity, ulcers

Prolidase deficiency (PD), a rare autosomal recessive phenotype in which deficient prolidase activity leads to massive urinary excretion of imidodipeptides (X-Pro and X-Hyp), and it is associated with a range of clinical manifestations.[1,2] Prolidase (iminodipeptidase E.C. 3.4.13.9), a dimer with a molecular subunit mass of about 54.3 kD is responsible for hydrolyzing dipeptides containing C-terminal prolyl or hydoxyprolyl residues in the final stages of protein catabolism. It is an ubiquitous enzyme whose activity has been documented in erythrocytes, leukocytes, plasma, dermal fibroblasts, the kidney, brain, heart, thymus and uterus. Given the relatively high quantity of iminoacids in collagen and the presence of skin alterations, it is tempting to speculate that this enzyme deficiency results in altered collagen metabolism even though protein catabolism in general is affected. The enzyme probably plays an important role in the re-utilization of proline residues for *de novo* protein synthesis (Figure 1), although the exact correlation between deficient prolidase activity and the pathogenesis of clinical manifestations is still not fully understood.

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Acknowledgements: the authors wish to thank Mr. A. Gallanti and M. Bellaviti for expert technical assistance; they also wish to thank Prof. E. Berardesca, IRCCS Policlinico San Matteo, Pavia; Prof. Fimiani, Dept. Dermatology, Siena University; Dr. G. Pasolini, Spedali Civili, Brescia and Dr. Amato, Dept. Dermatology, Palermo University, Italy, for referring patients. This work was supported by the EC Concerted Action on inheritable Connective Tissue Diseases 1991/1992 and the Biotechnology and Bioinstrumentation Project, CNR, Rome.

Received on September 6, 1993; accepted on November 29, 1993.

Clinical symptoms vary considerably and include dermatological lesions, mental retardation and recurrent infections (otitis media, sinusitis), splenomegaly or hepatosplenomegaly with elevated γ-globulinemia and anemia in some cases. Dermatological lesions (erythematous papular eruptions, telangiectasia with pruritis, dermatitis) may occur all over the body, especially on the lower extremities. The main feature of PD, however, is the chronic recurring skin ulceration, usually confined to the lower limbs. In some cases ulcers are so severe that walking is impossible and even amputation has been required. The age of onset of symptoms varies, but most of them occur before puberty.[2] In some families, sibs of affected individuals have been shown to possess deficient enzyme activity, though they were symptomless at the time of testing.[3-5]

To date only about 40 confirmed cases of PD have been reported; we have had 8 (20%) patients referred to us in the past few years with suspected prolidase deficiency, and were able to confirm this defect in all, including the younger, asymptomatic (at the time of diagnosis) brother of one of them. Although prolidase deficiency is a very rare condition, we think it may be more common than is suspected and some patients may be misdiagnosed.

Here we report the clinical findings from our patients and biochemical data regarding their erythrocyte and skin fibroblast prolidase activity. Prolidase can be separated into two isoforms, a major isoform (I) and a minor one (II). The activity of erythrocyte and fibroblast isoforms I and II towards different substrates was studied. Since our patients were heterogeneous in terms of age at onset, severity of clinical symptoms and mental retardation, we compared findings in an attempt to correlate enzyme activity with severity of symptoms.

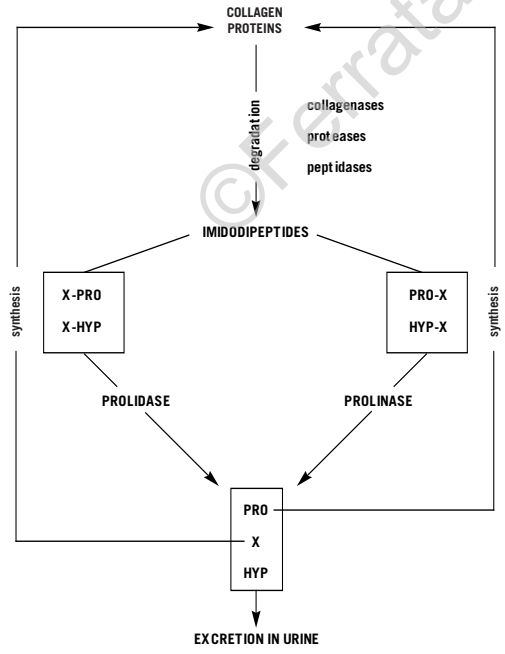

Figure 1. Schematic illustration of protein degradation (collagen). Prolidase is involved in the final stages.

Patients and methods

Clinical cases

Eight patients affected with prolidase deficiency were referred to us. Some have already been described,[5-9] and so the main clinical findings have been listed in table form (Table 1). These findings varied considerably, especially with regard to mental retardation, severity of dermatological lesions and age of onset of clinical symptoms (from 3-17 years). In one patient ulcers appeared on the ankles following a road accident at age 17 and in another[8] ulcers first appeared on the feet at 5 years and then became so severe that by the age of 15 the patient was confined to a wheel chair. His younger brother, now 11, was asymptomatic at the time of the study (3 years ago), but recently small ulcers have started to appear on his feet as well.

Determination of urinary dipeptides

Iminodipeptide levels in the urine were assessed as reported.[9]

Prolidase activity in erythrocytes

Enzyme activity in erythrocytes was determined in hemolysates by quantifying the amount of proline or N-terminal amino acid liberated after hydrolysis of dipeptides containing C-terminal proline.

Briefly, 2 mL fresh, heparinized blood were centrifuged to separate the plasma. The plasma and buffy coat were removed and the erythrocytes were washed in 0.9% (w/v) NaCl, diluted and hemolyzed by freeze-thawing. The hemolysates were dialyzed against 0.05M Tris HCl, pH 7.8, containing 1 mM $MnCl_2$ and then incu-

Table 1. Clinical characteristics of the PD patients studied.

Pat.	*Sex*	*Age at onset*	*Skin manifestations*	*Mental retardation*	*Other clinical features*	*Ref.*
B.A.	F	12	telangiectasia, photosensitivity, leg ulcers	++	recurrent infections, anemia splenomegaly charact. facies[§]	9
B.C	F	3	"	++	"	9
G.D.	M	16	telangiectasia, leg ulcers	+++	anemia	6
C.F.	M	5	pruritis, skin lesions, telangiectasia, foot ulcers	+++	charact. facies[§] recurrent infections	5,7,8
C.M.	M	11	asymptomatic till 8 y, small lesions on feet appeared recently	–	– – –	5,7,8
C.Ma.	M	?	ulcers on feet	+	characteristic facies °	7
P.P.	F	17*	ulcers on feet, pigmented skin telangiectasia photosensitivity	++	characteristic facies °	
P.R.	F	30	leg ulcerations, telangiectasia	++		

*Ulcers first appeared following injury; [§]low hairline, saddle nose;°*bird-like* facies

bated for 15 min at 37°C. The enzyme requires preincubation with Mn^{++} for activation.[10] Aliquots of the hemolysates were incubated with different substrates (gly-pro, ala-pro, phe-pro, val-pro and leu-pro) for 15 min at 37°C. The reaction was stopped by the addition of sulfosalicylic acid, and the supernatant tested for the presence of free proline or N-terminal amino acid. Activity was expressed as µmol proline hydrolyzed per hour per g hemoglobin.

Enzyme activity in skin fibroblasts

Total prolidase activity was determined in skin fibroblasts cultivated *in vitro*.[11] The skin fibroblasts were grown in 125 cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a 5% CO_2 humidified atmosphere at 37°C. At confluence, media were removed and the cell layers washed with PBS, scraped into a small volume of 0.05M Tris-HCl, pH 7.8, and homogenized. After centrifugation the cell extract was incubated in the presence of 1 mM $MnCl_2$ for 15 min at 37°C, and then incubated for 60 min with different substrates (final concentration 80 mM).

The quantity of proline liberated was assayed using a colorimetric method,[12] and activity was expressed as nmoles proline hydrolyzed per minute per mg protein.

Separation of the two prolidase isoforms

Erythrocyte and fibroblast prolidases were separated into two isoforms (I and II) using *fast protein liquid chromatography* (FPLC).

Hemolysates were diluted 1:1 (v/v) with 0.02M Tris-HCl, pH 8.0, containing 7 mM mercaptoethanol added to prevent deactivation of isoform II.[13] FPLC separation was performed on a Mono Q HR 5/5 column (Pharmacia) and elution was in Tris buffer as above, utilizing a gradient from 0.1M-0.4M NaCl; 0.5 mL hemolysate was injected and, following elution, prolidase activity was assayed on fractions after incubation in the presence of substrate. The amount of

Table 2. Urinary excretion of glycylproline.

Patient	Age	Sex	Gly-Pro (mg/24h/m^2)
BA	37 y	F	470.0
BC	21 y	F	320.2
GD	31 y	M	1171.4
CF	13 y	M	835.7
CM	18 y	M	681.3
PP	23 y	F	ND
PR	40 y	F	676.8
CMa	55 y	M	616.5

Controls: glycyl proline not normally present in the urine.
N.D. not determined.

proline liberated during hydrolysis was assayed.[12]

Results

The initial diagnosis was confirmed in all our patients by findings of massive urinary excretion of gly-pro (Table 2) and deficient prolidase activity.

Total erythrocyte prolidase activity from patients and controls was tested against different C-terminal proline-containing dipeptides. Activity was greatest against gly-pro in control erythrocyte hemolysates (in the order gly-pro>ala-pro>phe-pro>o>val-pro>leu-pro). In contrast, activity against a gly-pro substrate was undetectable or extremely reduced (< 7%) in all patient hemolysates and activity towards other substrates was slightly reduced. The order of enzyme activity was different from that of controls and was val-pro>ala-pro>phe-pro>leu-pro>gly-pro in 4/5 patients tested, and ala-pro>val-pro>phe-pro>leu-pro>gly-pro in the fifth.

In some cases skin biopsies were available to start fibroblast cultures so that total prolidase activity could be assayed. In contrast to erythrocyte prolidase, fibroblast enzyme activity was highest against the ala-pro substrate, in the order ala-pro>gly-pro>phe-pro. Patient fibroblast prolidase showed drastically reduced activity towards substrate gly-pro, as did the erythrocyte enzyme.

The two forms of the enzyme derived from erythrocytes could be separated by FPLC as described by other authors.[11, 13] Results for the erythrocyte isoforms are shown in Figure 2.

In control erythrocytes the activity of isoform I was always higher than that of isoform II for

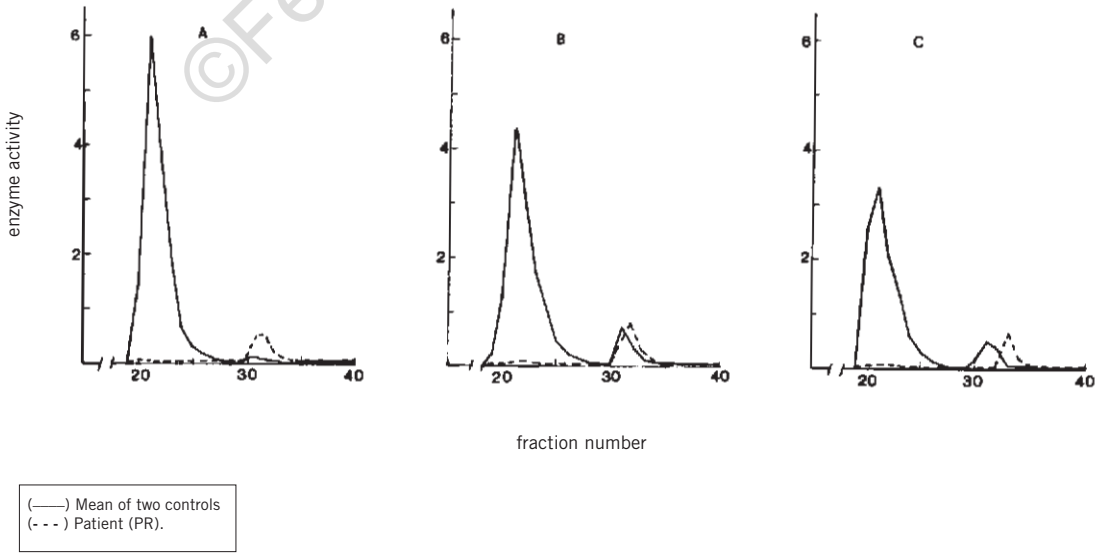

Figure 2. FPLC separation of the two erythrocyte prolidase isoforms. Enzyme activity towards substrates gly-pro (A), ala-pro (B) and phe-pro (C) was evaluated as mmoles proline hydrolyzed/h/mL. Values were normalized according to Hb concentration.

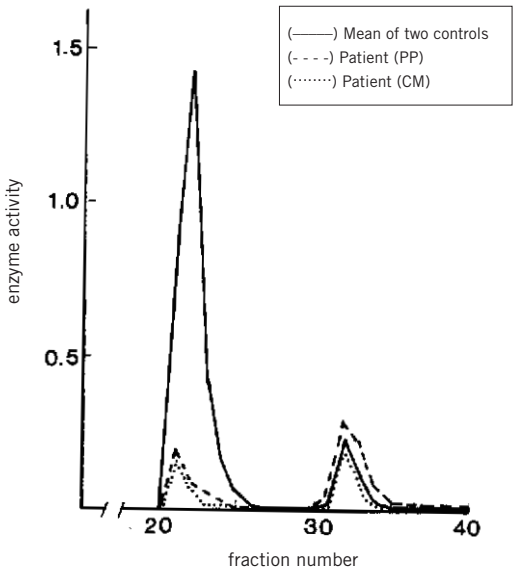

Figure 3. Chromatographic separation using FPLC of the two prolidase isoforms obtained from cultured skin fibroblasts. Enzyme activity towards ala-pro is expressed as nmoles proline hydrolyzed /min/mg protein.

all substrates considered, especially towards gly-pro. In the case of the patients, however, isoform I activity was markedly reduced, while isoform II activity remained unaltered.

We used similar conditions to separate fibroblast prolidase isoforms. Again, as in controls, isoform I activity was greater than that of isoform II. Isoform I activity was considerably reduced in patient cells (< 5% normal activity), while isoform II activity remained unchanged (Figure 3).

Discussion

Clinical findings are extremely heterogeneous in prolidase deficiency. The clinical phenotypes of our patients were extremely varied: near-normal intelligence to severe mental retardation, variable age of onset of symptoms and different expression of dermatological manifestations. The reason for this heterogeneity is still not clear, and even more puzzling is the clinical variability which exists even between sibs. One of our patients developed lesions at age 5 and from 11 years on presented an extremely severe clinical picture, while his younger brother was asymptomatic at age 8 and has only recently developed small ulcers.

Biochemical results were similar in all cases: prolidase activity in hemolysates prepared from patient red cells was markedly reduced against gly-pro (< 10% normal levels), although activity was detected against val-pro, leu-pro, met-pro, ala-pro and phe-pro in agreement with other reports.[2] Most enzyme activity in controls was associated with isoform I, which shows the most affinity for gly-pro. Since erythrocyte hemolysate isoform I activity against gly-pro was virtually undetectable in patient cells, the residual activity towards other X-pro substrates observed in hemolysates is probably due to prolidase II activity. This is in agreement with other reports.[14, 15]

The higher affinity of prolidase I for gly-pro with respect to other X-pro substrates is still not understood. It has been suggested that defective recycling of proline residues in particular is related to clinical phenotype. Since gly-pro is a frequently recurring sequence in collagen, this dipeptide is heavily excreted in the urine of PD patients, and since the skin of patients is most affected, much attention has been focused on how defective prolidase activity affects collagen metabolism. However, PD is probably not specifically related to defective collagen metabolism. In fact, many neuropeptides (i.e. MSH, MIF, thyrotropin-releasing hormone) contain proline, so brain tissue prolidase plays a role in neuropeptide turnover.[16] Altered enzyme activity could also contribute to the pathogenesis of mental retardation. Studies have shown that normal human fibroblasts are able to recycle proline from glycylpro, whereas PD patient cells are not.[17] This lends support to the idea that the pathogenesis of the PD phenotype is possibly correlated with defective proline recycling.

We were unable to correlate enzyme activity with clinical severity in our patients; previous attempts to do this were also unsuccessful.[14] It would appear that there are other unknown factors secondary to the defect in prolidase which are involved in the pathogenesis of this disease.

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