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Note

Separation of two erythrocyte prolidase isoforms by fast protein liquid chromatography; application to prolidase deficiency

ISAAC MYARA* and NICOLE MOATTI

*Laboratoire de Biochimie, Hôpital Broussais, 96 Rue Didot, F-75674 Paris Cédex 14 (France)** *and Laboratoire de Biochimie, Faculté des Sciences Pharmaceutiques et Biologiques, Chatenay-Malabry (France)*

and

ALAIN LEMONNIER

Laboratoire de Biochimie, Faculté des Sciences Pharmaceutiques et Biologiques, Chatenay-Malabry (France)

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Prolidase (EC 3.4.13.9) is a dipeptidase which specifically cleaves the X-Pro dipeptides (X = amino acid). Prolidase deficiency is a rare metabolic disorder clinically characterized by dermatological manifestations, especially severe leg ulcerations. Its main biochemical feature is massive urinary elimination of iminodipeptides [1]. When glycylproline (Gly–Pro) was used as a substrate to test the prolidase activity erythrocytes [2–12], leukocytes [2,5], plasma [13] and fibroblasts [4,10], this activity was virtually undetectable. Erythrocytes have also been used to purify human prolidase [14–17] and to study the properties of this enzyme [14,16,18]. With other X-Pro substrates, including Phe–Pro, Ala–Pro, Val–Pro and Leu–Pro, Butterworth and Priestman [19] demonstrated the presence of two prolidase isoforms in fibroblasts, amniotic fluid cells, blood cells, kidney, liver, spleen, pancreas, cortex, heart and skeletal muscle, and separated these isoforms by ion-exchange chromatography. Prolidase I, eluted at the lowest ionic strength, was active with most of the X-Pro dipeptides, whereas prolidase II displayed little activity with Gly–Pro. They also demonstrated, in two prolidase-deficient fibroblast strains, the presence of prolidase II activity and a large deficiency in that of prolidase I.

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Our laboratory confirmed the presence of both prolidases in normal human erythrocytes after batch fixation and DEAE-Sephadex chromatography [16]. However, this method of characterization needed 20 ml of erythrocytes and was time-consuming. We therefore describe here a new method of prolidase isoform separation by fast protein liquid chromatography (FPLC), which requires only 0.5 ml of erythrocytes and takes less than 2 h, including the enzymatic reaction. We applied this method to erythrocytes from three prolidase-deficient patients.

EXPERIMENTAL

Patients

Two of the three prolidase-deficient patients have already been described [13,20,21]. The third was a 13-year-old Moroccan woman who began to develop chronic ulcerations at the age of 10 years. Biological diagnosis was established by routine amino acid chromatography, which revealed massive iminodipeptiduria, and by the absence of plasma [22] and erythrocyte [21] prolidase activity, which was tested using glycylproline as substrate after 24-h preincubation.

As controls, eighteen normal adult subjects with no kidney disturbances were chosen, because slightly reduced erythrocyte prolidase activity has been found in chronic uraemia [23].

Sample preparation

Erythrocytes were obtained from heparinized tubes by low-temperature centrifugation and washed three times with four volumes of ice-cold 0.15 *M* sodium chloride solution. The cells were haemolysed with one volume of ice-cold water, frozen and thawed. A further 30-min centrifugation at 2000 *g* allowed globular stomas to be discarded. The haemolysate was then diluted 1:1 with 0.02 *M* Tris–HCl (pH 8.0) containing 7 m*M* 2-mercaptoethanol (buffer A) and filtered through 0.2-μm filters (Flow-Labs., Les Ulis, France).

FPLC separation

The chromatographic equipment was manufactured by Pharmacia (Uppsala, Sweden). The FPLC system consists of an LLC-500 programmer controlling two P-500 pumps. The sample was introduced via a 0.5-ml loop on to a Mono Q HR 5/5 column. The effluent was monitored by a single-path ultraviolet monitor at 280 nm and 0.5-ml fractions were collected with a Frac-100 fraction collector. Experiments were carried out under the following conditions: buffer A, 0.02 *M* Tris–HCl (pH 8.0) containing 7 m*M* 2-mercaptoethanol; sample, 0.5 ml of erythrocytes diluted 1:1 in buffer A; buffer B, 1 *M* sodium chloride in buffer A; gradient, from 10 to 40% buffer B in 11 min: flow-rate, 1.0 ml/min; fractions, 0.5 ml.

Determination of prolidase activity

Prolidase activity was determined immediately after FPLC separation. For this purpose, 0.1 ml of 60 m*M* phenylalanylproline (Sigma, St. Louis, MO, U.S.A.), prepared in buffer A supplemented with 3 m*M* manganese(II) chloride, was added to 0.2 ml of the chromatographic eluate and incubated for 30 min at 37°C. The reaction was stopped with 1 ml of glacial acetic acid. The proline released was measured after adding 1 ml of Chinard's reagent (600 ml of glacial acetic acid, 400 ml of 6 *M* orthophosphoric acid and 25 g of ninhydrin) and heating the preparation for 10 min at 100°C. The absorbance was read at 515 nm. All absorbances above 5% of the maximum absorbance were considered when calculating the activity of isoforms I and II:

$$\text{Prolidase I (\%)} = \frac{\text{absorbance of peak I}}{\text{absorbance of peak I} + \text{peak II}}$$

$$\text{Prolidase II (\%)} = \frac{\text{absorbance of peak II}}{\text{absorbance of peak I} + \text{peak II}}$$

Sodium measurement

The sodium concentration in the chromatographic eluates was determined with a flame photometer (Corning 455, Ciba Corning Diagnostic, Le Vésinet, France).

RESULTS AND DISCUSSION

Conditions of prolidase activity

2-Mercaptoethanol was essential for preserving prolidase II activity during FPLC separation. A concentration of 7 m*M* (0.5 ml/l) in all the buffers was sufficient. In addition, it was necessary to work with freshly prepared filtered solutions.

Manganese was essential to reveal prolidase II activity during the enzymatic reaction. Without manganese, this activity was virtually undetectable. At a final manganese concentration between 0.5 and 2.0 m*M*, the ratio of prolidase I to prolidase II activity was constant. At higher concentrations, the activity of prolidase I increased proportionally more than that of prolidase II. We arbitrarily chose a manganese concentration of 1 m*M*.

It was not possible to use the Gly–Pro dipeptide as substrate, because prolidase II displayed very little activity with it. Prolidase II was very active with Met–Pro [16], but this dipeptide is very expensive and its preservation was poor. We therefore chose the Phe–Pro dipeptide already used by others [19], and selected 20 m*M* as the final substrate concentration. It was not necessary to increase this concentration because the increase in activity observed was

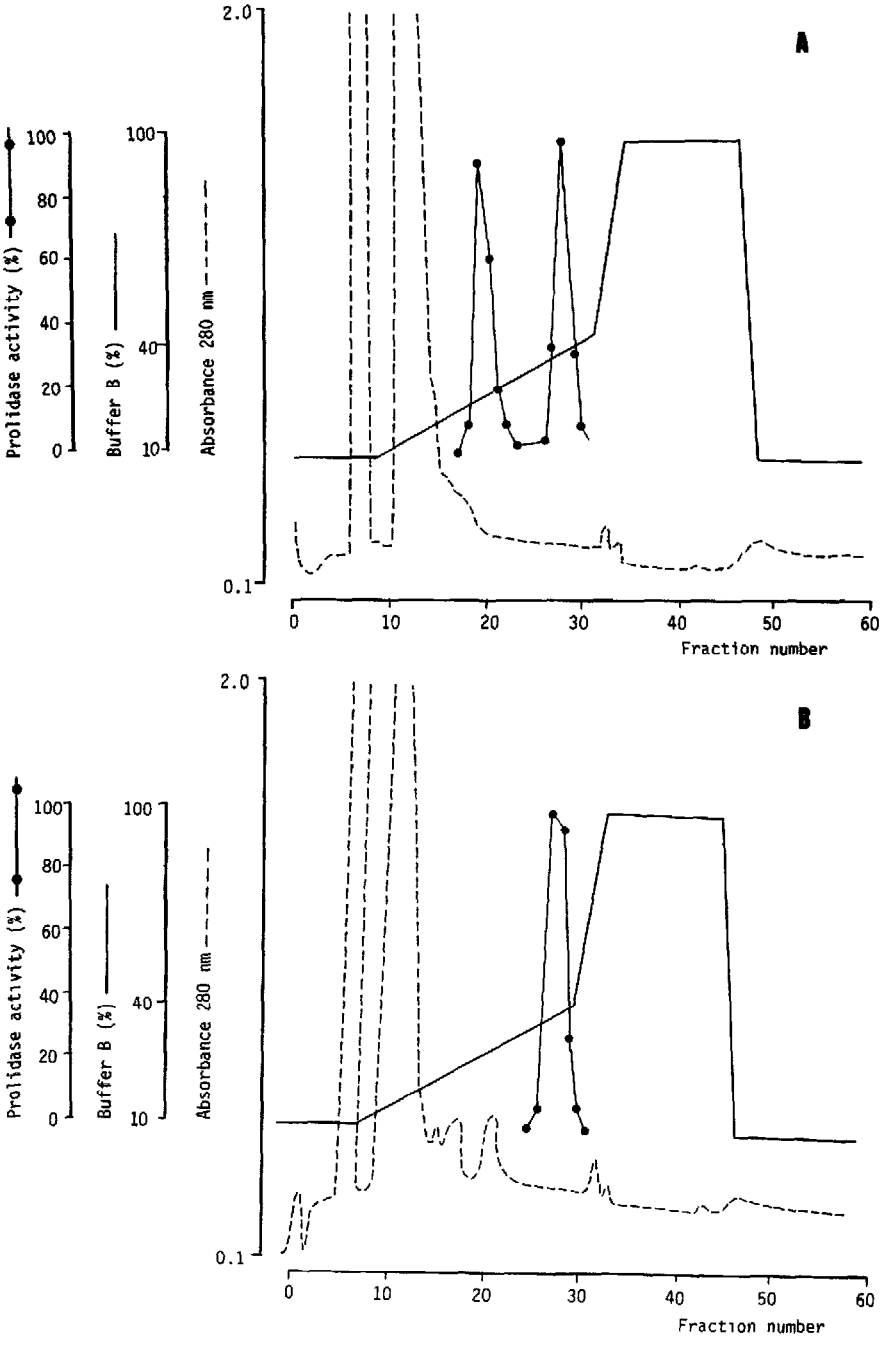

Fig. 1. FPLC separation of the erythrocyte prolidases on a Mono Q HR 5/5 column. (A) Normal erythrocytes; (B) prolidase-deficient erythrocytes.

slight, and there was an increase in substrate hydrolysis during spectrophotometric proline determination [21].

The incubation time was an important parameter; the longer it lasted, the larger was the proportion of prolidase I activity. We chose a time of 30 min, which allowed good sensitivity.

FPLC separation

Excellent separation of the two prolidase activities was obtained for the erythrocytes from normal subjects using the methodology described (Fig. 1A). Prolidase I activity was eluted with a sodium chloride concentration of 181 ± 6.7 m*M* (mean $\pm$ S.D., $n = 18$) and prolidase II activity with a concentration of 283 ± 15.4 m*M*. In the eighteen normal subjects, the proportion of prolidase I activity was $51 \pm 4.5\%$. The last fraction of prolidase I activity and the first of prolidase II activity were separated by three or four vials. This methodology can therefore be incorporated in a scheme for purifying prolidase II, which has so far not been isolated.

Application of the method to prolidase deficiency

Prolidase I activity was not detectable in the erythrocytes of the three prolidase-deficient patients (Fig. 1B). This activity was also undetectable in other cells such as leukocytes and cultured skin fibroblasts. This confirms the previous results of Butterworth and Priestman [19] for fibroblasts. As Endo et al. [24] recently showed after isolation of prolidase I and immunoblotting, this lack of activity is due to the absence of enzyme protein.

CONCLUSION

The proposed method for the separation by FPLC of erythrocyte prolidase I and prolidase II was simple and fast. In prolidase-deficient erythrocytes, the absence of prolidase I was shown in less than 2 h. This method can be applied to other prolidase-deficient cells such as cultured skin fibroblasts and leukocytes, and can be incorporated in a scheme for purifying prolidase from both normal and prolidase-deficient tissues.

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