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Shusaku Masuda[*] Hironobu Watanabe[†] Masaaki Morioka[‡]

Yukitoshi Fujita[**] Tomiko Ageta[††] Hiroyuki Kodama[‡‡]

[*]Kochi Medical School,

[†]Kochi Medical School,

[‡]Kochi Medical School,

[**]Kochi Medical School,

[††]Kochi Medical School,

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## Abstract

Both prolidase and prolinase from the human prostate were separated into two peaks by TSK DEAE-5PW chromatography. These peaks of prolidase isozymes I and II differed from each other in their responses to preincubation with Mn2+, their substrate specificity, optimal pH, and heat stability. The molecular weights of prolidases I and II were estimated to be 110,000 and 165,000, respectively, by gel filtration. Substrate specificity of prolinase peaks I and II was almost the same, but they differed in optimal pH and heat stability. The molecular weights of prolinases I and II were about 85,000 and 63,000, respectively. These results indicate that two isozymes of prolidase and of prolinase, which differ in various characteristics, are present in the human prostate.

**KEYWORDS:** human prostate, prolidase, prolinase

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# Characteristics of Partially Purified Prolidase and Prolinase from the Human Prostate

SHUSAKU MASUDA, HIRONOBU WATANABE, MASAAKI MORIOKA, YUKITOSHI FUJITA, TOMIKO AGETA[a] AND HIROYUKI KODAMA*[a]

Departments of Urology and [a]Chemistry, Kochi Medical School, Nankoku-shi, Kochi 783, Japan

Both prolidase and prolinase from the human prostate were separated into two peaks by TSK DEAE-5PW chromatography. These peaks of prolidase isozymes I and II differed from each other in their responses to preincubation with $Mn^{2+}$, their substrate specificity, optimal pH, and heat stability. The molecular weights of prolidases I and II were estimated to be 110,000 and 165,000, respectively, by gel filtration. Substrate specificity of prolinase peaks I and II was almost the same, but they differed in optimal pH and heat stability. The molecular weights of prolinases I and II were about 85,000 and 63,000, respectively. These results indicate that two isozymes of prolidase and of prolinase, which differ in various characteristics, are present in the human prostate.

**Key words:** human prostate, prolidase, prolinase

Several types of proline-specific peptidases are present in mammalian organs (1), and these peptidases act together with other endo- and exopeptidases to effect complete hydrolysis of proteins such as collagen. Prolidase (EC 3.4.13.9, amino acyl-L-proline hydrolase) is an iminodipeptidase and is specific for dipeptides with proline residues at the carboxy terminal. The activity of this enzyme has been found in porcine intestinal mucosa (2), horse erythrocytes, porcine kidney (3) and other mammalian organs (4-5), and it has been purified from bovine intestine (6) and human erythrocytes (7). The biological significance of prolidase has been studied in terms of patients with a prolidase deficiency (8-10).

Prolinase (EC 3.4.13.8, L-prolyl-amino acid hydrolase) is another iminodipeptidase and is specific for dipeptides with a proline residue at the amino terminal. Prolinase has been partially purified from human skin fibroblasts and bovine kidney (11, 12) and its activity has also been found in various human and rat tissues (13). However, prolidase and prolinase in the human prostate has not yet been characterized. This report describes the partial purification and characterization of prolidase and prolinase from the human prostate.

## Materials and Methods

***Columns.*** High-performance liquid chromatography columns, TSK gel DEAE-5PW (8 × 75 mm) and TSK G3000SW (8 × 300 mm), were purchased from Tosoh Company, Tokyo, Japan.

***Chemicals.*** Glycylproline (Gly-Pro), alanylproline (Ala-Pro), leucylproline (Leu-Pro), valylproline (Val-Pro), phenylalanylproline (Phe-Pro), methionylproline (Met-Pro), serylproline (Ser-Pro), prolylproline (Pro-Pro), prolylisoleucine (Pro-Ile), prolylleucine (Pro-Leu), prolylvaline (Pro-Val), prolylmethionine (Pro-Met), prolylglycine (Pro-Gly), and prolylalanine (Pro-Ala) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

***Tissues.*** Prostate samples were obtained by suprapubic transvesical prostatectomy from patients with prostatism aged 70-80 years. The samples were examined by pathological examinations and normal portions of the gland were used in this study.

***Enzyme purification.*** Prolidase and prolinase were partially purified by HPLC at 0-4°C as follows. Prostate samples (0.1 g) were rinsed with 0.9% NaCl, and homogenized in an ice-bath for 10 min with 50 mM Tris-HCl buffer, pH 7.8 (5 ml/0.1 g tissue) using an Ultra-turrax homogenizer. The homogenate was centrifuged at 10,000 × $g$ for 15 min at 4°C, and the resulting supernatant (1 ml) was directly applied to a TSK DEAE-5PW

* To whom correspondence should be addressed.

ion exchange column (8 × 75 mm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0). Elution was performed with 5 ml of a linear gradient of NaCl (0–40 mM) in 50 mM Tris-HCl buffer (pH 7.0), 7.5 ml of 40 mM NaCl in 50 mM Tris-HCl buffer, then with 17.5 ml of linear gradient of NaCl (40–250 mM) in 50 mM Tris-HCl buffer (pH 7.0), collecting 0.5-ml fractions. Fractions containing activities of prolidase and prolinase were combined separately. Combined fractions of prolidase or prolinase were applied to a TSK G3000SW column (8 × 300 mm) (Tosoh Co.) equilibrated with 50 mM phosphate buffer (pH 7.0) and eluted with 50 mM phosphate buffer (pH 7.0) at a flow rate of 0.3 ml/min, collecting 0.3-ml fractions. Fractions containing prolidase or prolinase were combined separately.

***Enzyme assays.*** Prolidase activity was assayed using several iminodipeptides as substrates. The reaction mixture containing 10 $\mu$l of enzyme solution, 80 $\mu$l of 50 mM Tris-HCl (pH 7.4), 10 $\mu$l of 10 mM $MnCl_2$ was preincubated at 37 °C for 10 min. After the addition of 100 $\mu$l of 10 mM substrate to 50 mM Tris-HCl buffer (pH 7.8), the reaction mixture was further incubated at 37 °C for 30 min (prolidase) or 60 min (prolinase). The reaction was stopped with 200 $\mu$l of 10 % TCA. The mixture was then centrifuged at 8,000 × $g$ for 5 min and the amount of proline liberated was determined by spectrophotometry using Chinard's method (14). The assay for prolinase was the same as that for prolidase except that $MnCl_2$ was not contained in the preincubation mixture.

***Molecular weight estimation.*** Estimation of molecular weights of prolidase and prolinase was performed on gel filtration (TSK G3000SW) calibrated by comparison with ferritin (mol wt = 440,000), catalase (mol wt = 232,000), aldolase (mol wt = 158,000), bovine serum albumin (mol wt = 67,000), ovalbumin (mol wt = 43,000), chymotrypsinogen A (mol wt = 25,000) and ribonuclease A (mol wt = 13,700), which were obtained from Pharmacia (Uppsala, Sweden).

***Protein determination.*** Protein concentration was determined by the method of Lowry *et al.* (15), using bovine serum albumin as the standard.

## Results

The separation of prolidase and prolinase was attempted by using ion exchange column chromatography from

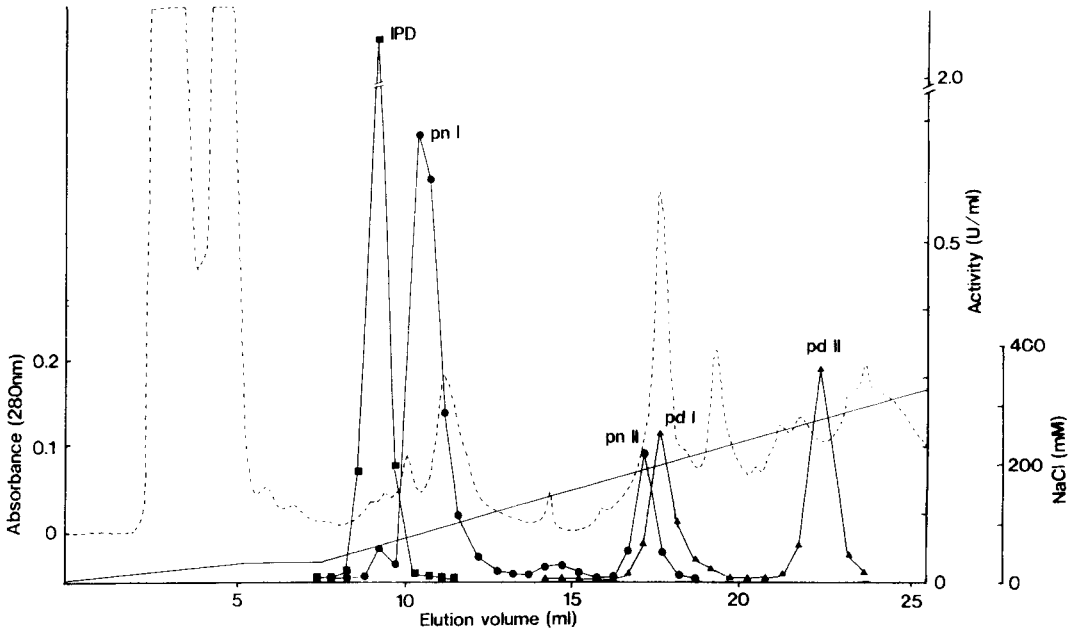


**Fig. 1** TSK DEAE-5PW column chromatography of prolidase and prolinase from the human prostate. Pn I and Pn II: prolinases I and II, Pd I and Pd II: prolidases I and II. Prolidase activity was determined using Met-Pro (▲——▲) and prolinase Pro-Ile (●——●) as substrates. Dashed line, absorbance at 280 nm. IPD; proline iminopeptidase.

**Table 1** The partial purification of prolidase and prolinase from normal portions of prostates from patients with prostatism

| | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| **Prolinase I** | | | | | | |
| Crude homogenate | 1.6 | 9.277 | 3.194 | 0.344 | 100 | 1 |
| TSK DEAE-5PW | 3.6 | 0.367 | 4.196 | 11.433 | 131.400 | 33.235 |
| TSK G3000-5WXL | 0.5 | 0.045 | 1.457 | 32.378 | 45.620 | 94.122 |
| **Prolinase II** | | | | | | |
| Crude homogenate | 1.6 | 9.277 | 0.798 | 0.086 | 100 | 1 |
| TSK DEAE-5PW | 3.9 | 0.803 | 1.054 | 1.313 | 132.100 | 15.267 |
| TSK G3000-5WXL | 0.5 | 0.039 | 0.602 | 7.179 | 75.440 | 83.477 |
| **Prolidase I** | | | | | | |
| Crude homogenate | 1.6 | 9.277 | 1.130 | 0.122 | 100 | 1 |
| TSK DEAE-5PW | 4.0 | 0.824 | 1.042 | 1.265 | 92.210 | 10.369 |
| TSK G3000-5WXL | 0.5 | 0.063 | 0.433 | 6.873 | 38.320 | 56.336 |
| **Prolidase II** | | | | | | |
| Crude homogenate | 1.6 | 9.277 | 1.130 | 0.122 | 100 | 1 |
| TSK DEAE-5PW | 2.8 | 0.223 | 1.171 | 3.375 | 103.600 | 27.664 |
| TSK G3000-5WXL | 0.5 | 0.032 | 0.269 | 8.406 | 23.800 | 68.902 |

Enzyme activites in crude homogenate and enzyme fractions obtained by TSK gel DEAE-5PW or TSK gel G3000-5WXL chromatography. Prolidase I activity was assayed with Ala-Pro; prolidase II was assayed with Met-Pro; and prolinases I and II were assayed with Pro-Leu.

**Table 2** Substrate specificity of prolidases I and II separated from human prostate by high-performance liquid chromatography with an ion-exchange column, TSK gel DEAE-5PW

| | Substrates | | | | | |
|---|---|---|---|---|---|---|
| | Gly-Pro | Ala-Pro | Leu-Pro | Met-Pro | Ser-Pro | Pro-Pro |
| Prolidase I | 0.265 | 1.464 | 0.572 | 0.630 | 1.015 | 0.147 |
| | (18.1) | (100.0) | (39.1) | (43.0) | (69.3) | (13.9) |
| Prolidase II | 0.096 | 0.691 | 0.244 | 2.181 | 0.319 | 0.016 |
| | (4.4) | (31.7) | (11.2) | (100.0) | (14.6) | (0.7) |

The values of prolidase activities from the human prostate against various substrates are given as $\mu$mol/min/mg protein. The numbers in parentheses represent the relative activity (%).

**Table 3** Substrate specificity of prolinases I and II separated from human prostate by high-performance liquid chromatography with an ion-exchange column, TSK gel DEAE-5PM

| | Pro-Gly | Pro-Ala | Pro-Val | Pro-Ile | Pro-Met | Pro-Pro |
|---|---|---|---|---|---|---|
| Prolinase I | 9.090 | 6.017 | 24.573 | 33.865 | 14.551 | 0.056 |
| | (26.8) | (17.8) | (72.6) | (100.0) | (43.0) | (0.2) |
| Prolinase II | 3.012 | 2.975 | 8.790 | 10.716 | 5.259 | 0.049 |
| | (28.1) | (27.8) | (82.0) | (100.0) | (49.1) | (0.5) |

The values of prolinase activities from the human prostate against various substrates are given as $\mu$mol/min/mg protein. The numbers in parentheses represent the relative, activity (%).

normal portions of the prostate of patients with prostatism. Prolidase and prolinase activities from the prostate were detected by elution with a linear gradient of NaCl (40–250 mM) and each showed two peaks as shown in Fig. 1. These peaks are referred to prolidases I and II and prolinases I and II. The partial purification of prolidases I and II, and prolinases I and II from the prostate using TSK DEAE-5PW and TSK G3000-5WXL is summarized in Table 1.

The results of specific activities of prolidases I and II against six iminodipeptides are shown in Table 2. The activity of prolidase I against six iminodipeptides was in the following order: Ala-Pro > Ser-Pro > Met-Pro > Leu-Pro > Gly-Pro > Pro-Pro. Prolidase II has the

highest activity for Met-Pro among the iminodipeptides tested, but it could not hydrolyze Pro-Pro. The optimal pH for prolidase I was 7.25–7.50, whereas that for prolidase II was 7.50–8.00. The activity of partially purified prolidases I and II from the prostate were compared using Gly-Pro, Ala-Pro and Met-Pro after preincubation with or without 1 mM $Mn^{2+}$ for 0–60 min,

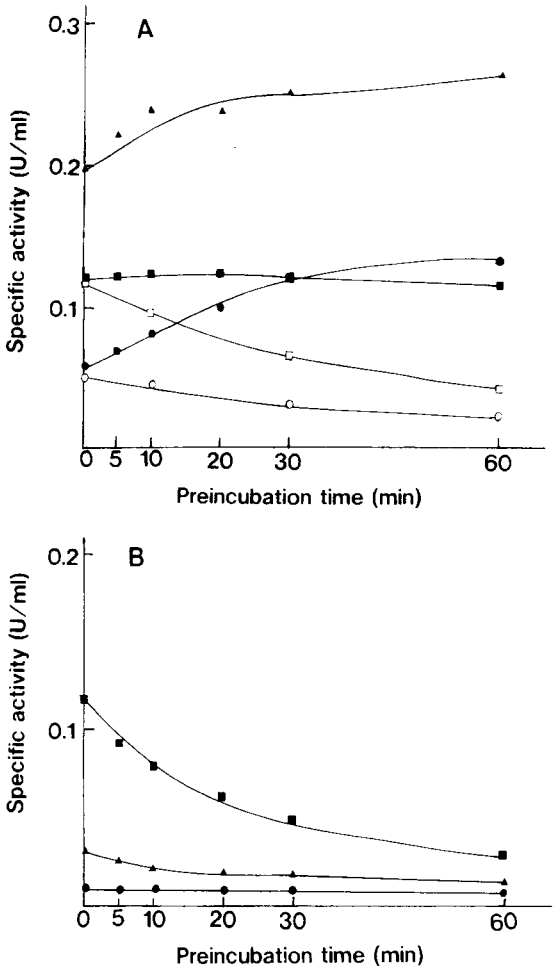


**Fig. 2** The effect of preincubation with or without $Mn^{2+}$ on prolidase I (**A**) and II (**B**) activities isolated from the human prostate. Preincubation was performed at 37°C with or without 1 mM $Mn^{2+}$ for the time shown, and then the enzyme reaction was performed for 30 min with the substrates.
**A**: Gly-Pro ($Mn^{2+}$: +, ●—●; −, ○—○); Ala-Pro ($Mn^{2+}$: +, ■—■; −, □—□); Ser-Pro ($Mn^{2+}$: +, ▲—▲).
**B**: Gly-Pro ($Mn^{2+}$: +, ●—●); Ser-Pro ($Mn^{2+}$: +, ▲—▲); Met-Pro ($Mn^{2+}$: +, ■—■).

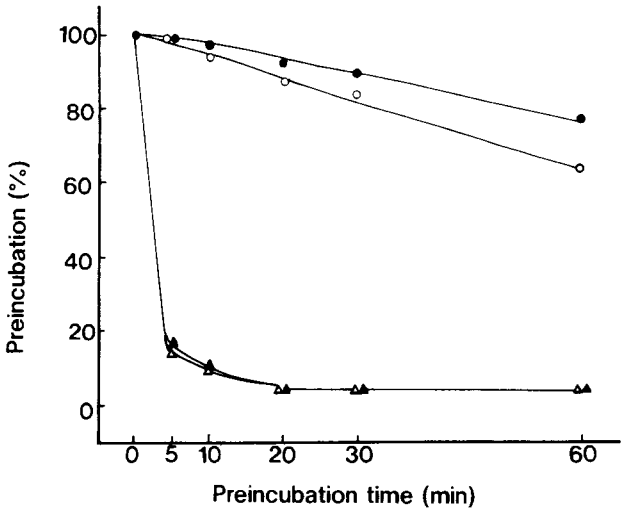


**Fig. 3** The effect of preincubation with or without $Mn^{2+}$ on prolinase I and II activites isolated from the human prostate. Reaction was performed as that in Fig. 2 using Pro-Leu as substrate for 60 min. Prolinase I ($Mn^{2+}$: +, ●—●; −, ○—○); Prolinase II ($Mn^{2+}$: +, ▲—▲; −, △—△).

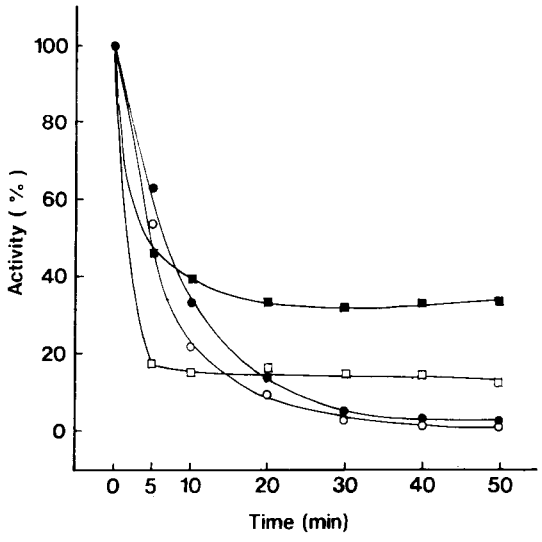


**Fig. 4** The effect of heat treatment 50°C on prolidase I and prolinase I activities. The activities of prolidase I and prolinase I from the human prostate were assayed with Ala-Pro or Pro-Leu, respectively, in the presence or absence of 1 mM $Mn^{2+}$. Ala-Pro ($Mn^{2+}$, +, ■—■; −, □—□); Pro-Leu ($Mn^{2+}$, +, ●—●; −, ○—○).

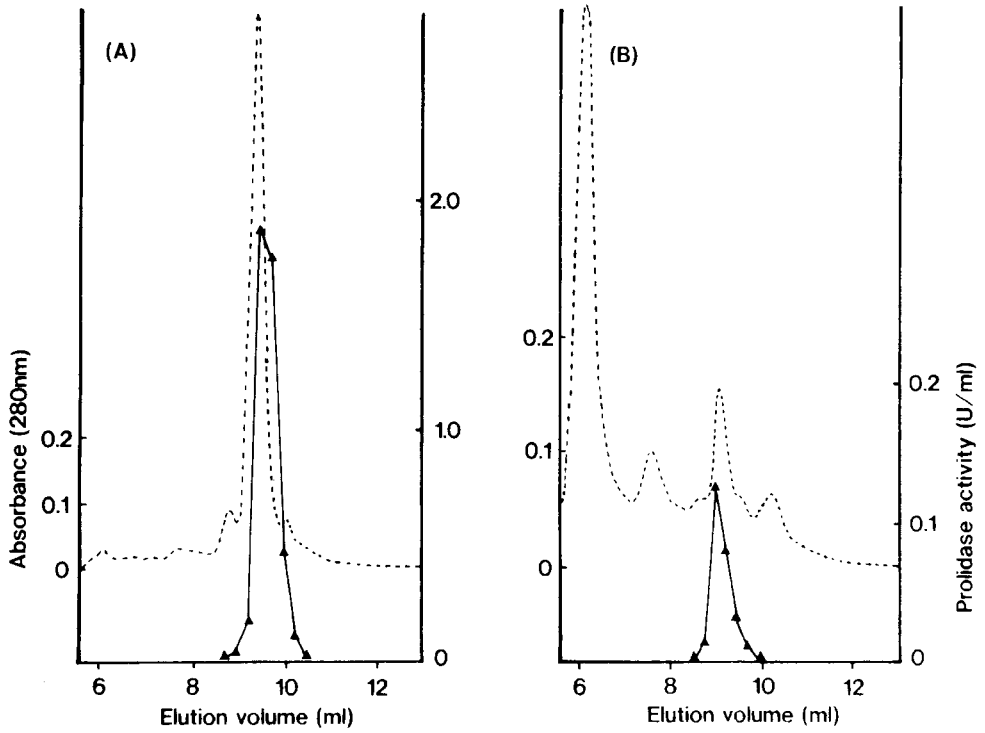


**Fig. 5** TSK gel G3000-5WXL chromatography of prolidase I (Pd I) (**A**) and prolidase II (Pd II) (**B**) obtained by TSK gel DEAE-5PW. Both prolidase activities were measured using Ala-Pro (Pd I) and Met-Pro (Pd II) as substrates. Dashed lines, absorbance at 280 nm.

as shown in Fig. 2A, B. The activity of prolidase I against the substrates tested progressively decreased after preincubation without $Mn^{2+}$ for 0–60 min, but the reduction of activity of prolidase I was prevented by the presence of 1 mM $MnCl_2$ (Fig. 2A). The activity of prolidase II was very labile, whether or not $Mn^{2+}$ was present (Fig. 2B). The activity of prolidase I was very thermostable as compared with prolidase II.

The results of specific activity of prolinases I and II against six iminodipeptides are shown in Table 3. The activity of prolinases I and II against six iminodipeptides was in the following order: Pro-Ile > Pro-Val > Pro-Met > Pro-Gly > Pro-Ala > Pro-Pro. The optimal pH of prolinase I was 7.75–8.25 and that of prolinase II was 7.50–8.00. The activity of partially purified prolinase I against Pro-Leu did not change after preincubation with or without $Mn^{2+}$, whereas the activity of prolinase II was very labile whether or not $Mn^{2+}$ was present (Fig. 3). Therefore, the activities of prolidase I and prolinase I were estimated against Ala-Pro or Pro-Leu, respectively, after heating with or without 1 mM $Mn^{2+}$ for 0–50 min at 50 °C (Fig. 4). The activity of prolidase I against Ala-Pro in the absence of $Mn^{2+}$ was reduced to about 18 %, after 10 min at 50 °C. The reduction of prolidase I activity against Ala-Pro at 50 °C was partially prevented (40 %) by the presence of 1 mM $Mn^{2+}$, whereas the activity of prolinase I was progressively decreased regardless of the presence of $Mn^{2+}$, and the activity was almost completely lost after 30 min at 50 °C.

The elution profiles of prolidases I and II from a gel filtration column TSK gel G3000SW (Fig. 5) indicate that prolidase II is larger than that of prolidase I, and apparent molecular weights of prolidases I and II were estimated to be about 95,000 and 165,000, respectively. Likewise, the molecular weights of prolinases I and II were estimated to be about 85,000 and 63,000, respectively.

The activity of prolidase in the supernatants of prostate homogenate and erythrocyte lysates from normal

**Table 4** The substrate specificity of prolidase in the supernatants of homogenates from human prostate and erythrocytes

| Substrate | Enzyme source | |
|---|---|---|
| | Prostate | Erythrocytes |
| Gly-Pro | 0.312 ± 0.026 (45.3) | 4.254 ± 0.53 (100) |
| Ala-Pro | 0.688 ± 0.045 (100) | 3.183 ± 0.36 (74.8) |
| Val-Pro | 0.373 ± 0.034 (54.2) | 0.960 ± 0.17 (22.6) |
| Leu-Pro | 0.383 ± 0.032 (55.7) | 1.416 ± 0.18 (33.3) |
| Met-Pro | 0.562 ± 0.061 (81.7) | 2.573 ± 0.27 (60.5) |
| Ser-Pro | 0.669 ± 0.067 (97.2) | 3.456 ± 0.26 (81.2) |
| Phe-Pro | 0.349 ± 0.041 (50.7) | 1.392 ± 0.19 (32.7) |
| Pro-Pro | 0.198 ± 0.026 (28.8) | 0.828 ± 0.12 (19.5) |

Values of prolidase activity against various substrates in the supernatants of homogenates from human prostate and erythrocytes (mean ± SD: n = 5). Numbers in parentheses represent the relative activity of prolidase against substrate.

individuals was assayed using Gly-Pro, Ala-Pro, Val-Pro, Leu-Pro, Met-Pro, Ser-Pro, Phe-Pro and Pro-Pro as substrates after preincubation with 1 mM $Mn^{2+}$. As shown in Table 4, Gly-Pro was the most reactive substrate for erythrocyte prolidase, which hydrolysed eight iminodipeptides in the following order: Gly-Pro > Ser-Pro > Ala-Pro > Met-Pro > Leu-Pro > Phe-Pro > Val-Pro > Pro-Pro. On the other hand, Ala-Pro was the most reactive substrate for prostate prolidase, which hydrolysed eight iminodipeptides in the following order: Ala-Pro > Ser-Pro > Met-Pro > Leu-Pro > Val-Pro > Phe-Pro > Gly-Pro > Pro-Pro.

## Discussion

The several properties and separations of prolidase and prolinase from normal portions of the prostates of patients with prostatism were studied. The present study on the separation of prolidase from prostate specimens agreed well with previous papers on human erythrocytes (16, 17) and cultured skin fibroblasts (18). The activity of prolidase I from erythrocytes was the highest for Gly-Pro as reported in previous papers (6, 10), but that obtained from prostate tissue was the highest for Ala-Pro. The activity of prolidase II from prostate was the highest for Met-Pro, which was almost the same as that of prolidase II from erythrocytes. The activity of prolidase I against Met-Pro from erythrocytes (16) and fibroblasts (19) was higher than that of prolidase II, but the activity of prolidase I against Met-Pro from the prostate was lower than that of prolidase II (Fig. 1). These results indicate that the ratio of prolidase I and II activities is tissue-specific. The response for divalent cation ($Mn^{2+}$) and the heat stability of prolidases I and II from the prostate were very similar to those of prolidases I and II from erythrocytes (16). The molecular weight of prolidase I from the prostate was the same as that of the prolidase purified from erythrocytes by Endo *et al.* (7). The molecular weight of prolidase II from prostate was the same as that of prolidase II from erythrocytes reported by Ohhashi *et al.* (16). These results suggest that prolidase I from erythrocytes and prolidase I from prostate might not be the same enzyme, but prolidase II in each tissue is the same enzyme.

Our method for the separation of prolinases I and II from prostate gave a better result (Fig. 1) than the procedure used by Butterworth and Priestman (11). The relative activities against various substrates of prolinases I and II from the prostate were similar, and were the same as those reported in previous papers (10, 11). The activity of prolinase I was very thermostable compared with prolinase II. The present study indicates that prolinase from the prostate also exists in two forms. At present, no patients with prolinase deficiency have yet been found, and this might be due to the presence of two forms of prolinase in human tissues. If such a patient is found, it will be necessary to determine activities of two prolinase forms separately as done in the case of prolidase deficiency (16-19).

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