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Properties of the prolyl oligopeptidase homologue from *Pyrococcus furiosus*

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Abstract **Prolyl oligopeptidase (POP), the paradigm of a serine peptidase family, hydrolyses peptides, but not proteins. The thermophilic POP from *Pyrococcus furiosus* (*Pfu*) appeared to be an exception, since it hydrolysed large proteins. Here we demonstrate that the *Pfu* POP does not display appreciable activity against azocasein. The autolysis observed earlier was an artefact. We have also found that the pH-rate profile is different from that of the mammalian enzyme and the low pK_a extracted from the curve represents the ionization of the catalytic histidine. We conclude that some oligopeptidases may be true endopeptidases, cleaving at disordered segments of proteins, but with very low efficacy.**
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Keywords: Thermophilic oligopeptidase; Autolysis; Kinetics

1. Introduction

A group of serine peptidases cannot hydrolyse proteins, but readily cleave peptides that are not greater than ~30 amino acids in length. These enzymes are grouped under the prolyl oligopeptidase (POP) family (S9 of clan SC) [1]. The family includes enzymes of different specificities, which are important targets of drug design [2]. The POP has been involved in depression [3] and amnesia [4]. Inhibitors to the enzyme are described as cognitive enhancers, and some entered clinical trials [5]. POP was suggested to be implicated in the regulation of blood pressure [6]. Recently, the enzyme was proposed to use as oral therapeutic agent for celiac sprue because of its unique ability to accelerate the breakdown of proline-rich gluten-derived peptides in the gut lumen [7,8]. The enzyme consists of two domains, a peptidase and a seven-bladed β-propeller, which covers the catalytic triad (Ser554, His680 and Asp641) thereby excluding large proteins from the active site. Oligopeptide substrates can approach the active site between the two domains [9–12].

It was reported earlier that the thermophilic *Pyrococcus furiosus* (*Pfu*) POP was different from the mesophilic porcine POP, since it hydrolysed large proteins; specifically it autolyzed and hydrolysed azocasein [9,13]. This is a surprising observation for an enzyme classified as oligopeptidase (http://merops.sanger.ac.uk/). In our hands, the *Pfu* POP displayed only trace activity against proteins. However, this behaviour can cause a terminology problem, because oligopeptidases are a subgroup of endopeptidases, and therefore any oligopeptidase is logically an endopeptidase. Hence, we introduce two terms for the present discussion: "true endopeptidase" (a peptidase that hydrolyses proteins regardless of size) and "oligopeptidase" (restricted to small substrates).

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Abbreviations: POP, prolyl oligopeptidase; *Pfu*, *Pyrococcus furiosus*; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetatic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Z, benzyloxycarbonyl; Nap, 2-naphthylamine; Nan, 4-nitroanilide

2. Materials and methods

2.1. Enzyme preparation

The gene coding for *Pfu* POP in a pET11d vector [9] was subcloned into a pET3d expression vector using the 5′ *Nco*I and 3′ *Bam*HI restriction sites flanking the coding region of the gene. The recombinant protein was expressed in *Escherichia coli* Rosetta(DE3) strain. Cultures were grown in 300 ml LB containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol in 2000 ml flasks at 37 °C until the OD_{600} reached 0.6. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to 1 mM, and the growth was continued overnight at 37 °C. The bacterial cells were harvested by centrifugation and sonicated on ice in 30 mM Tris–HCl buffer, pH 8.0, containing 10 mM ethylenediaminetetraacetatic acid (EDTA) and 1 mM dithioerythritol. The suspension was centrifuged (18 000 × *g*, 30 min) and the supernatant was incubated at 90 °C for 15 min. After cooling, the precipitated proteins were removed by centrifugation. The supernatant was further purified with ion-exchange chromatography on DEAE and MonoQ columns in 20 mM phosphate buffer, pH 6.5. A linear gradient of 0–0.5 M NaCl was used for the chromatography on the DEAE column. The FPLC chromatography using the MonoQ column was performed with a linear gradient of 10–30% buffer containing 0.5 M NaCl. The degree of purification was monitored with activity measurements using Z-Gly-Pro-Nap substrate, and the homogeneity of the protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). From 1800 ml culture medium 25 mg pure enzyme was obtained, which was stored at −18 °C in the presence of 40% ethylene glycol. Prior to the assays the ethylene glycol was removed by gel filtration on a G-25 Sephadex column.

The catalytic serine (S477A) was eliminated by a two-step polymerase chain reaction (PCR) procedure [14], using the sense/antisense primer pairs (5′-GCATGGGGAAGG**gc**CAACGGaGGCCTCTTGG-3′ and 3′-CGTACCCCTTCC**cgG**TTGCCtCCGGAGAACC-5′, the codon for Ala shown in bold letters). An *Stu*I restriction site was introduced (underlined), which served for the verification of the mutation. The 5′- and 3′-primers for the PCR were 5′-GGTAGCCATGGAAGATCCCTACATA-3′ with an *Nco*I restriction site (underlined) and 3′-CAAGAGTTCTGGGAAAGGATTCCTAGGCGA-5′ with a *Bam*HI restriction site (underlined), respectively. The PCR product was ligated into the pET3d expression vector. The S477A mutation was verified by restriction digestion and the whole construction was sequenced. The inactive S477A variant was purified in the same way as the native enzyme, except that the degree of purification was monitored with SDS–PAGE.

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2.2. Kinetics

The reactions of *Pfu* POP with Z-Gly-Pro-Nap were measured spectrofluorometrically as described for the porcine enzyme [15], except that the cell was closed with a stopper, when the reaction was monitored at high temperature. The buffer for both the standard assays (pH 7.0) and the pH dependence measurements of k_{cat}/K_m at 75 °C contained 30–30 mM acetic acid, MES, HEPES, TAPS and either 0.1 or 0.8 M NaCl. The pH of the buffer was adjusted to the appropriate pH with 1 M HCl or 1 M NaOH at 75 °C. Theoretical curves for the bell-shaped and doubly bell-shaped pH-rate profiles were calculated by non-linear regression analysis as described previously [15], using the GraFit software [16].

The hydrolysis of azocasein with *Pfu* POP was carried out in 100 mM HEPES buffer, pH 8.0. 6–8 vials, each containing 0.10% azocasein and 11 μg/ml *Pfu* POP in 1 ml of reaction mixture, were incubated at 85 °C, and treated with 500 μl of 15% trichloroacetic acid at appropriate times (15–20 min). After incubation for 20 min at 25 °C, the samples were centrifuged and the absorbance values of the supernatants were determined at 410 nm. Alternatively, 500 μl of 1 M NaOH was added to the supernatants, and the absorbance values were determined at 435 nm. The hydrolysis of azocasein with trypsin was measured at 40 °C at an enzyme concentration of 1.5 μg/ml, in the presence of 1 mM $CaCl_2$. Aliquots were taken at appropriate times (2–3 min). The unit of activity was defined previously [13]. The K_i values, the dissociation constants of the enzyme-inhibitor complex, were determined as described previously [17].

2.3. Gel chromatography

A 100 μl of sample was loaded to a Superose 12 column, which was equilibrated with 50 mM phosphate buffer, pH 7.0. The molecular masses were calculated from the calibration curve, using the Sigma Molecular Weight Marker Kit, 12.4–200 kDa.

3. Results and discussion

3.1. Hydrolysis of protein substrates

It has previously been reported that the *Pfu* POP undergoes autolysis and hydrolyses azocasein [13]. As the propeller domain of the enzyme obstructs the access of large molecules to the active site, the hydrolysis of proteins should proceed with a special mechanism. To reveal the peculiarity of protein breakdown, we have undertaken a detailed kinetic investigation.

The reaction of *Pfu* POP with azocasein was measured under the same conditions (buffer, pH, temperature, enzyme and substrate concentrations) as described earlier [13], except that we used pure *Pfu* POP rather than partially purified enzyme, and the slope of absorbance change vs. time was calculated from 6 to 8 points, rather than from two points, where the linearity of the curve is not apparent. We have also examined the inactive S477A variant, which is devoid of the catalytic serine, in a parallel experiment and found that the activities of the wild type enzyme and the S477A variant were 0.015 and 0.00026 U/mg protein, respectively, whereas the previously reported activity for the wild type enzyme was 16.4 U/mg protein [13]. This value corresponds to a normal activity, comparable to that we found with trypsin (13.0 U/mg), whereas the activity of the inactive S477 variant was similar to the very low spontaneous hydrolysis of azocasein. We have also determined the activities at alkaline pH (see Section 2), because under these conditions a greater and real absorbance maximum develops at 435 nm [18]. However, the large difference between the earlier and the present data was independent of the methods employed.

These results indicated that the *Pfu* POP displayed rather low activity against the protein substrate. There may be two explanations for the conflicting results. (i) In the previous study partially purified enzyme was used [13], which might contain some inadvertent proteolytic enzyme(s). (ii) The azocasein preparation could have hydrolysable oligopeptides as impurities.

Besides azocasein hydrolysis, we have examined the possible breakdown of the thermophilic acylaminoacyl peptidase from *Aeropyrum pernix* K1. Samples from the reaction mixtures were withdrawn at two-hour and overnight incubations at 85 °C and analyzed on SDS–PAGE. There was no difference between the proteins incubated with or without *Pfu* POP. Other proteins, like bovine serum albumin, denatured at the high temperature and were useless for this test.

Occurrence of autolysis leading to complete protein breakdown during the incubation of the *Pfu* POP was inferred from SDS–PAGE, which demonstrated that the protein band disappeared after incubation for 24 h at 95 °C in the presence of 10% glycerol, while those treated at 4, 37, and 65 °C did not change [13]. We could confirm this finding using SigmaUltra, >99% (GC) glycerol (G 6279), but some spreading of the band was noticed at 60 °C. However, taking samples at intermediate times, we observed that the protein bands tended to blur after 1–2 h of incubation at 85 °C, spreading both upward and downward (Fig. 1A, lanes 1–5). At 95 °C the spreading was more extensive so that the overnight sample band of the enzyme became practically invisible (Fig. 1B, lane 2). In addition, we arrived at the same results also with the inactive S477A variant, which was evidently unable to autolyze (not shown). These results clearly indicated that the protein failed to break down during incubation at the catalytically optimum temperature.

Addition of trichloroacetic acid to the samples incubated at 85 °C with glycerol, which apparently disappeared from the SDS–PAGE (Fig. 1A, lane 5), gave similar amount of precipitate as the non-incubated sample (Fig. 1A, lane 1), confirming that the protein did not break down in the heated mixtures. Provided that the enzyme does not autolyze, the protein samples should elute from the size exclusion chromatography column as a single peak. The native protein eluted from a Superose 12 FPLC column at 61 kDa instead of the expected 71 kDa (Table 1), which indicated that the protein was slightly retarded on the column. This has previously been observed with and without 150 mM KCl [13]. Interestingly, the porcine POP is also eluted at a molecular mass lower than expected [10]. The protein incubated overnight 85 °C in the presence of 10% glycerol emerged as a single peak at 110 kDa (Fig. 2 and Table 1), demonstrating that the *Pfu* POP was not broken down to small peptides, but its structure changed to some extent as a result of the reaction with some inadvertent compound(s) in glycerol. The peak of the incubated sample is broader (peak width at half height, w_h = 1.25 min) compared with the control (w_h = 0.83 min) though the effect is not as great as it appears from the SDS–PAGE. Of course the two different methods do not necessarily provide similar results.

The above results clearly point to the presence of some impurities in glycerol. Therefore, we have examined two other batches of glycerol. With the Sigma glycerol G 5516 (>99%, for molecular biology) we reproduced the previous results obtained with the SigmaUltra glycerol (Fig. 1B, lane 3 vs lane 2). On the other hand, using REANAL glycerol (analytical reagent grade, Budapest, Hungary) the enzyme gave a normal band with a minor cleavage on SDS–PAGE after incubation at 95 °C for 20 h (Fig. 1B, lane 4). In accord with the gel pattern, the activities of the samples incubated with the REANAL

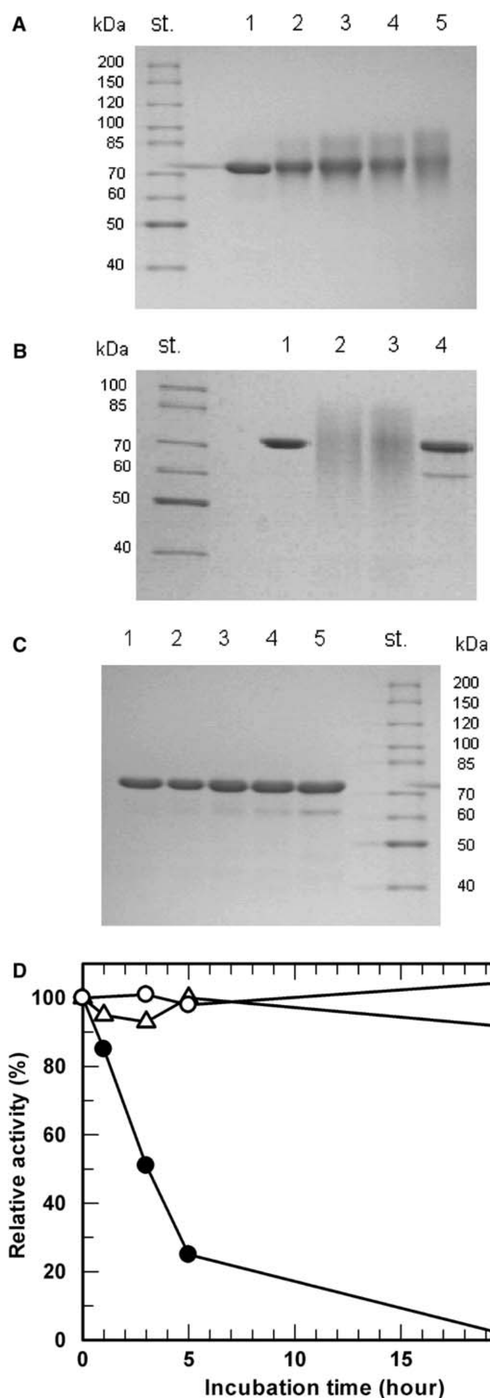

Fig. 1. (A) SDS–PAGE of *Pfu* POP incubated at 85 °C for appropriate times in the presence of 10% SigmaUltra glycerol. The protein concentration was 0.45 mg/ml. Lanes 1–5 stand for samples incubated for 0, 1, 3, 5 and 21 h, respectively. (B) SDS–PAGE of *Pfu* POP incubated at a concentration of 0.54 mg/ml, at 95 °C for 21 h. Lane 1 is the sample without heat treatment. Lanes 2–4 represent the proteins incubated in the presence of 25% SigmaUltra, Sigma >99% and REANAL glycerols, respectively. (C) SDS–PAGE of *Pfu* POP incubated at 85 °C for appropriate times. The protein was incubated at a concentration of 0.45 mg/ml in the absence of glycerol. Lanes 1–5 stand for 0, 1, 3, 5 and 21 h of incubation, respectively. Marking 'st.' indicates molecular mass standards, shown to the left or right of the figure. (D) Relative activities of *Pfu* POP after heating. The incubation and activity measurements were carried out in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. Conditions: (●) as in (A), (△) as in (C), and (○) as in (B), lane 4.

Table 1
M_w for Pfu POP determined by size exclusion chromatography

Conditions[a]	t_R (min)[b]	M_w (kDa)[c]
25 °C no glycerol	23.53	61.0
60 °C no glycerol	23.53	61.0
85 °C no glycerol	23.47	62.2
25 °C 10% glycerol	23.53	61.0
60 °C 10% glycerol	22.91	75.3
85 °C 10% glycerol	21.80	109.8

[a]In 50 mM phosphate, pH 7.0, after an overnight incubation using SigmaUltra glycerol.
[b]Retention time on Superose 12 column in 50 mM phosphate, pH 7.0, error ±0.02 min.
[c]Calculated from the calibration curve using Sigma Molecular Weight Marker Kit, 12.4–200 kDa.

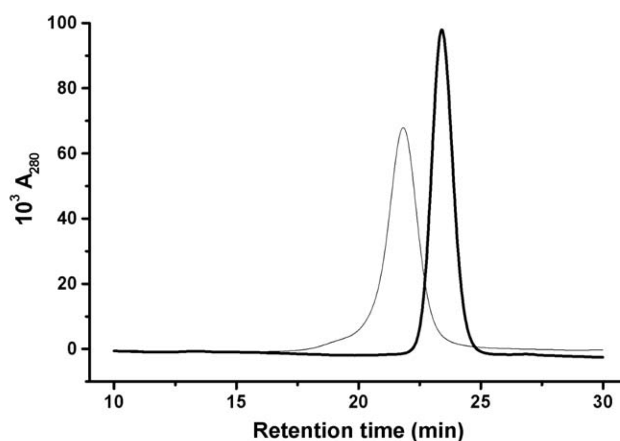

Fig. 2. Size exclusion chromatography of *Pfu* POP. The enzyme is shown with (thin line) and without (thick line) an overnight incubation in the presence of 10% SigmaUltra glycerol at 85 °C.

glycerol were practically identical (Fig. 1D) with that of the non-incubated control, whereas those incubated with the Sigma preparations lost practically all activities (remaining activities 0.1% and 0.3%). The decrease of activity paralleled with the spreading of the protein band in the SDS–PAGE. After 1, 3, 5 and 21 h of incubation with the SigmaUltra glycerol the activities were ~85%, 50%, 25% and 0%, respectively (Fig. 1D).

The different effects of the different batches of glycerol correspond to their spectra (Fig. 3). For the REANAL glycerol the increase in absorbance after an overnight heating at 85 °C was much lower than those of the Sigma preparations. Addition of DTT to the incubation mixture protected the enzyme activity to some extent (3% vs. 0.1% of activity remained), suggesting that the active agent in glycerol could be an aldehyde or ketone derivative.

When the active enzyme was overnight incubated at 85 °C in the absence of glycerol, we observed a minor cleavage product, using SDS–PAGE (Fig. 1C, lanes 1–5). However, during the first 4–5 h of incubation there was practically no breakdown. The extremely slow reaction, similarly to the azocasein hydrolysis, does not match the value of the usual enzymatic reaction rate. Using size exclusion chromatography, the partially autolyzed sample displayed a single peak practically at the same place, where the native enzyme eluted (Table 1). This indicated that the enzyme retained the main features of its structure, even though an accessible loop was in part cleaved. This was confirmed by the intact catalytic activity of the enzyme.

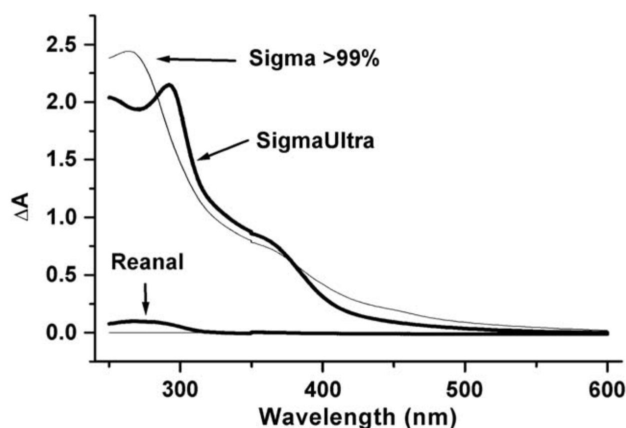

Fig. 3. Increase in absorbances of various batches of glycerol during heating. Difference spectra of glycerol samples diluted with equal volumes of 50 mM phosphate buffer, 1 mM EDTA, pH 7.0, obtained from recording before and after incubation at 85 °C for 24 h.

It can be concluded that the *Pfu* POP is a stable enzyme, which does not break down during incubation at the catalytically optimum temperature and retains its activity during heating overnight at 85 °C without the addition of any protective agent. The use of glycerol as additive may lead to artefacts. The spectral analysis applied here offers a useful test for probing the competence of the reagent in future studies.

3.2. Kinetics with small substrates

Because of the incongruous data with the protein substrates in the present and previous studies, we have undertaken the kinetic analysis of small peptide reactions. The pH-rate profiles for the *Pfu* POP published in the previous report are not exactly consistent with our expectation based on an earlier finding. Specifically, it was demonstrated that the doubly bell character of the porcine POP substantially changed after replacing a cysteine residue near the active site (Cys255) by a threonine, serine or alanine residue. In these cases the major, alkaline enzyme form virtually disappeared, but the acidic shoulder maintained the catalytic activity [14]. The *Pfu* POP does not contain the corresponding cysteine residue; therefore, it can be anticipated that the pH-rate profiles will differ from that of the porcine enzyme resembling more that of the Cys255 variant.

The pH dependence of the specificity rate constant (k_{cat}/K_m) for the *Pfu* POP was determined with Z-Gly-Pro-Nap. Fig. 4A does not support the similarity between the pH-rate profiles of the thermophilic and the mesophilic enzymes. As for the *Pfu* POP, we have obtained an approximately bell-shaped profile. The $k_{cat}/K_m(\text{limit})_1$ and $k_{cat}/K_m(\text{limit})_2$ apparent in the porcine POP (thin line in Fig. 4A) refer to the limiting values of the low- and high-pH forms of the enzyme, respectively. Only the low-pH form is apparent in the C255T variant of the mesophilic POP (dotted line in Fig. 4A). It appears that the $k_{cat}/K_m(\text{limit})_2$ decreased with respect to the $k_{cat}/K_m(\text{limit})_1$ in the case of the *Pfu* POP, and the similarity of the two limiting values led to the broad maximum of the pH dependence curve. Indeed, the points also fit to a doubly bell curve (not shown for clarity). We have measured the pH-rate profiles several times using different enzyme preparations, different substrates (Z-Gly-Pro-Nap, Z-Gly-Pro-Nan), different temperatures (60 and 75 °C), and different ionic strength (0.1 and 0.8 M salt

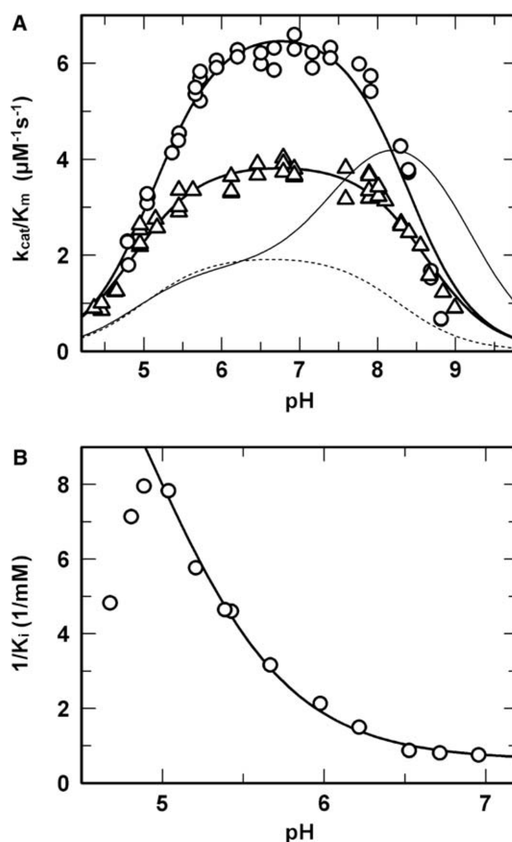

Fig. 4. (A) The pH dependence of *Pfu* POP with Z-Gly-Pro-Nap. The reactions were measured in the presence of 0.1 (△) and 0.8 M NaCl (○). The parameters for the bell-shaped curves are: (△), $k_{cat}/K_m(\text{limit}) = 3.91 \pm 0.07\ M^{-1}\ s^{-1}$, $pK_1 = 4.85 \pm 0.04$, $pK_2 = 8.62 \pm 0.04$, (○), $k_{cat}/K_m(\text{limit}) = 6.76 \pm 0.14\ M^{-1}\ s^{-1}$, $pK_1 = 5.12 \pm 0.04$, $pK_2 = 8.39 \pm 0.04$. For the porcine POP the data were fitted to a doubly bell-shaped curve illustrated with a thin line [22]. The C255T enzyme variant displayed a bell-shaped curve, shown with a dotted line [14]. (B) Titration of *Pfu* POP with Z-Gly-Pro-OH. The points above pH 5 were fitted to a simple sigmoid curve.

concentration, including the condition employed in the previous publication [9]) and always obtained the approximately simple bell-shaped profile shown in Fig. 4A. We cannot offer an explanation for the difference in the k_{cat}/K_m values, which was lower for the native enzyme ($0.145 \pm 0.008\ \mu M^{-1}\ s^{-1}$ for Z-Gly-Pro-Nan) in the previous report [9] than in the present study (2.35 ± 0.06 and $3.91 \pm 0.07\ \mu M^{-1}\ s^{-1}$ for Z-Gly-Pro-Nan and Z-Gly-Pro-Nap, respectively).

The $pK_1 = 4.85 \pm 0.04$ extracted from the pH-rate profile agrees well with the pK_a of 4.91 ± 0.11 obtained from the titration of the catalytic histidine with the product-like Z-Gly-Pro-OH inhibitor (Fig. 4B) with the method used with the porcine enzyme [17]. This supports that the pK_1 refers to the ionization of the catalytic histidine. Taking into account the $d(pK_a)/dT = -0.020$ for imidazole, this value for the *Pfu* POP measured at 75 °C is consistent with the pK_a of 6.2 found for the porcine POP at 25 °C.

These data are at variance with the results obtained in the previous work [9], which demonstrated a doubly bell pH dependence with three pK_a values: $pK_1 = 4.69$, $pK_2 = 7.49$, and $pK_3 = 8.70$. The authors assigned the pK_2 for the catalytic His and pK_1 for a carboxyl group from either an aspartic or a glutamic acid. If pK_2 reflected the His, there would not be an

appreciable activity at pH 5, contrary to the finding. It is unlikely that a carboxylate ion far from the active site could substitute the His as a general base catalyst. The catalytic His must operate even at pH 5, but the dissociation of a carboxyl group may disturb the pK_a value of the catalytic histidine, resulting in two apparent constants, pK_1 and pK_2, as also observed with the porcine enzyme.

3.3. Conclusion

It has been a stimulating question whether the serine oligopeptidases are capable of hydrolyzing proteins. Usually they cannot split large molecules because a propeller domain covers their active site. However, several papers indicated that some serine oligopeptidases hydrolyse large proteins. For example, fibroblast activation protein has gelatinase and collagenase activities [19], oligopeptidase B cleaves at a restricted site of histone proteins [20], POP digests itself [9,13] or degrades a splice variant protein of p40-phox [21]. In all of these cases the hydrolytic reactions required unusually long times, at least an overnight reaction, thus specific rate constants were not determined. We have also observed a trace activity with the wild type *Pfu* POP. The hydrolysis of large substrates may only occur with low probability, when the two domains of serine oligopeptidases assume a wide opening, which allows a flexible protein segment to approach the active site. Accordingly, these oligopeptidases can be considered as true endopeptidases, but with trace activities.

Because of the high specificity of POP for proline residue, there has been a great interest in using this enzyme for analysis of the primary structure of proteins as an alternative to trypsin, the mostly employed enzyme. It appeared that the *Pfu* POP could meet this requirement, but the trace activity of the enzyme is at variance with the expectations. Such trace activities may not be of importance in any practical system, and can therefore be ignored for practical purposes, although admitted for theoretical purposes.

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