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Substrate-dependent, non-hyperbolic kinetics of pig brain prolyl oligopeptidase and its tight binding inhibition by JTP-4819

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Abstract

Prolyl oligopeptidase (POP) is a cytosolic serine protease that hydrolyses small peptides at the carboxyl end of the proline residue. It has raised pharmaceutical interest, since its inhibitors have been shown to have antiamnesic properties. We studied prolyl oligopeptidase kinetics with two 7-amino-4-methylcoumarin derivatives: Z-Gly-Pro-AMC and Suc-Gly-Pro-AMC. Z-Gly-Pro-AMC was found to obey standard Henri–Michaelis–Menten kinetics with a K_m of 30 ± 3 μM, whereas Suc-Gly-Pro-AMC exhibited substrate inhibition kinetics with K_m and K_{is} of 510 ± 150 and 270 ± 90 μM, respectively. Autodock simulations revealed that either the succinyl or the AMC-end of Suc-Gly-Pro-AMC may bind to the S'_1 subsite of the active site. We believe that non-specifically bound Suc-Gly-Pro-AMC allows the simultaneous binding of second substrate molecule to the active site and this leads in substrate inhibition. In addition, we demonstrated that the inhibition type of a well characterized prolyl oligopeptidase inhibitor, JTP-4819, is competitive tight binding with a K_{ic} of 0.045 ± 0.008 nM. We suggest that due to the high concentration of prolyl oligopeptidase in the brain (0.12 nmol/g pig brain), the tight binding nature of the inhibition should be considered when using brain homogenate as the enzyme source in prolyl oligopeptidase inhibition measurements. This is of importance in studying structure–activity relationships of potent prolyl oligopeptidase inhibitors.

Keywords: Prolyl oligopeptidase; Serine protease; Enzyme kinetics; Substrate inhibition; Tight binding inhibition

1. Introduction

Prolyl oligopeptidase (POP) (EC 3.4.21.26), previously called post-proline cleaving enzyme or prolyl endopeptidase, is a large (80 kDa) cytosolic protease that belongs to a new POP family of serine proteases, unrelated to trypsin, subtilisin and carboxypeptidase Y. POP preferentially hydrolyses small peptides at the carboxyl end of a proline residue. Bioactive peptide substrates for POP include substance P, vasopressin, thyroliberin, neurotensin and angiotensins I and II [1,2]. It has been proposed that POP activity could influence learning and memory through its ability to metabolize neuropeptides [3–5].

There are several commercial fluoro- and chromogenic substrates for measuring POP activity and the potency of new POP inhibitors. The hydrolysis kinetics of some naphthylamide and 4-nitroanilide substrate derivatives have been studied [6], but the exact binding mechanism of fluorogenic 7-amino-4-methylcoumarin (AMC) substrates still remains to be clarified. The 3D-structures of POP and its complexes with an inhibitor and a substrate [7,8] indicate that the two widely used fluorogenic substrates, Suc-Gly-Pro-AMC and Z-Gly-Pro-AMC (Fig. 1), may bind via different mechanisms to the active site of the enzyme. In this study, we evaluated the enzyme kinetics of these AMC substrates. Indeed, a difference in their N-terminals resulted in totally distinct hydrolytic kinetics with pig brain homogenate and with purified recombinant pig POP. We also optimized the activity assay by studying the effect of different reaction conditions on substrate hydrolysis. In addition, we discovered that the type of inhibition and inhibition constants of an inhibitor can be determined by measuring IC_{50} values at multiple substrate

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Abbreviations: POP, prolyl oligopeptidase; Suc-Gly-Pro-AMC, *N*-succinyl-glycyl-prolyl-7-amino-4-methylcoumarin; Z-Gly-Pro-AMC, *N*-benzyloxycarbonyl-glycyl-prolyl-7-amino-4-methylcoumarin; AMC, 7-amino-4-methylcoumarin; DMSO, dimethyl sulfoxide.

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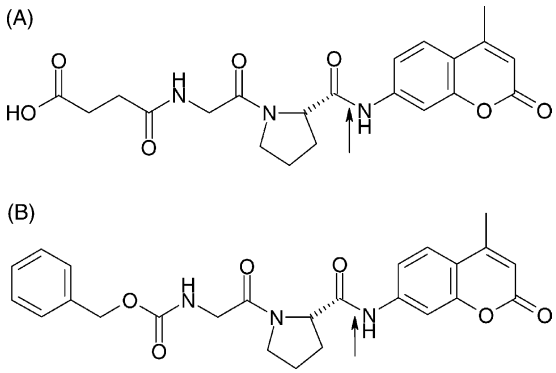

Fig. 1. Chemical structures of two model substrates for POP: Suc-Gly-Pro-AMC (A), and Z-Gly-Pro-AMC (B). POP hydrolyses the amide bond between proline and 7-amino-4-methylcoumarin (AMC) (indicated by arrows).

concentrations, even in the presence of substrate inhibition. Finally, we studied the inhibition kinetics of a well characterized POP inhibitor, JTP-4819, since to our knowledge, its exact inhibition type has not been determined.

2. Materials and methods

2.1. Reagents

N-benzyloxycarbonyl-glycyl-prolyl-7-amino-4-methylcoumarin (Z-Gly-Pro-AMC), *N*-succinyl-glycyl-prolyl-7-amino-4-methylcoumarin (Suc-Gly-Pro-AMC) and 7-amino-4-methylcoumarin were obtained from Bachem AG (Bubendorf, Switzerland). Acetic acid, sodium acetate and dimethyl sulfoxide (DMSO) were purchased from Riedel-de Haën (Seelze, Germany). Na_2HPO_4, KH_2PO_4 and acetonitrile were obtained from Merck (Darmstadt, Germany) and methanol from Labscan Ltd. (Dublin, Ireland).

2(*S*)-[[2(*S*)-(Hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-*N*-phenylmethyl)-1-pyrrolidinecarboxamide (JTP-4819) was synthesized in our laboratory using a slightly modified synthesis procedure of that described in United States Patent 5536737. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.8–2.3 (8H, m), 3.08 (1H, t, $J = 5.1$ Hz), 3.32 (1H, m), 3.49 (1H, m), 3.62 (1H, m), 3.92 (1H, m), 4.23–4.53 (4H, m), 4.60–4.66 (1H, m), 4.67–4.72 (2H, m), 7.2–7.4 (5H, m). ^{13}C NMR (500 MHz, $CDCl_3$): δ 24.75, 25.44, 28.19, 28.93, 44.74, 45.91, 47.03, 57.86, 61.04, 67.15, 127.29, 127.69, 128.63, 139.34, 156.42, 172.12, 209.18. ESI-MS: $m/z = 360$ $(M+H)^+$. Anal. ($C_{19}H_{25}N_3O_4$) calculated C: 63.49, H: 7.01, N: 11.69; found C: 63.12, H: 7.02, N: 11.58.

2.2. Brain homogenate preparation

Pig brains were obtained from the local slaughterhouse (Atria, Kuopio, Finland). Pigs were about 6 months old, weighing 100–120 kg. Brains, excluding cerebellum, were frozen in liquid nitrogen within half an hour after slaughter and stored at −70° until homogenized in 3 vol. of assay buffer (0.1 M Na–K-phosphate buffer, pH 7.0). The homogenate was centrifuged at 10,000 *g*, 4°, for 20 min. Aliquots of supernatant were frozen and stored at −70°. A total of 1:2 dilutions of the supernatant were used for the enzyme assays.

2.3. Expression and purification of porcine prolyl oligopeptidase

Porcine brain RNA was obtained by the Trizol reagent protocol (Life technologies). POP cDNA was generated by the reverse transcriptase (AMV) reaction using the primer 5′-GATAAAAATCCCCGAGGCAGT-3′. The entire coding region, including the stop codon, was amplified by PCR (*Pfu* DNA polymerase) and *Nco*I/*Hin*dIII sites were introduced at the 3′- and 5′-ends respectively. Primers were: sense, 5′-CCCCAGCCATGGTGTCCTTCC-3′; antisense, 5′-TTAAGCTTATGGAATCC-3′. The *Nco*I/*Hin*dIII digested PCR product was then cloned into the *Nco*I/*Hin*dIII site of pBAD/myc-HisA (Invitrogen) obtaining pBADPigPOP. The recombinant plasmid was used to transform *E. coli* TOP10 competent cells. For expression, a colony of *E. coli* TOP10 harboring pBADPigPOP was grown 12 hr in 50 mL of Luria broth containing ampicillin 50 μg/mL. A total of 30 mL of this culture were used to inoculate 1 L of Luria broth supplemented with ampicillin 50 μg/mL, and grown until an OD_{600} ~0.5, then arabinose was added to 0.02% and the growth continued for additional 4–6 hr. Cells were harvested and the pellet resuspended in 280 mL 10 mM Tris, 100 mM NaCl, 0.5 mM EDTA, pH 8. Cells were treated with lysozyme (0.5 mg/mL) for 30 min at 37°. Sphaeroplasts were centrifuged and the pellets frozen at −70°. Pellet was resuspended in 30 mL 10 mM Tris, pH 8 by sonication (three times for 5 s), and the debris was removed by centrifugation (5 min, 5,000 *g*). Supernatant was then used immediately for POP purification.

POP was purified from cell suspension by following the procedure of Szeltner *et al*. [6]. Practically pure POP was obtained after DEAE and Blue Sepharose chromatography with yield of 68%. The active enzyme concentration was determined by utilizing Morrison equation (Eq. (3)) as described below. Dilutions were made in assay buffer and enzyme concentration of 0.3 nM was used in all experiments.

2.4. Substrate and inhibitor solutions

Suc-Gly-Pro-AMC was dissolved and diluted in assay buffer (0.1 M Na–K-phosphate buffer, pH 7.0). Z-Gly-Pro-AMC was dissolved and diluted in 60% methanol. JTP-4819 was used as a well-established POP inhibitor [5] and a stock solution of 0.1 M was prepared by dissolving the substance in DMSO. Further dilutions were made in assay buffer.

2.5. Enzyme assay

The POP activity assay was modified from the method described by Toide *et al*. [4]. The method was transferred to 48-well plate on the basis of our assay optimization. An enzyme solution (10 μL of 1:2-diluted pig brain homogenate or purified recombinant pig POP) was preincubated with 465 μL of assay buffer for 30 min at 30°. The reaction was initiated by adding 25 μL of substrate and the plates were incubated for 60 min at 30°. The reaction was terminated by the addition of 500 μL of 1 M sodium acetate buffer (pH 4.2). The formation of AMC was measured fluorometrically using a Bio-Tek FL500 fluorescence plate reader. The excitation and emission wavelengths were 360 and 460 nm, respectively. End point measurements were allowed since substrate depletion did not exceed 15% in any experiment and the reaction was linear up to 120 min.

The effect of organic solvents on POP activity was studied in the presence of acetonitrile, DMSO and methanol using Suc-Gly-Pro-AMC as a substrate at a concentration of 200 μM and pig brain homogenate as the enzyme source. Final solvent concentrations in the incubation medium varied between 0.1 and 4%.

The effect of incubation temperature (from 20 to 40°) was measured as described above except for the change in preincubation and incubation temperatures. The pH optimum was determined in the assay buffer at pH values ranging from 5.5 to 8.0. In both experiments, Suc-Gly-Pro-AMC at a concentration of 10 μM was used as the substrate and pig brain homogenate as the enzyme source.

The protein concentration in the enzyme preparation was determined with Bio-Rad protein assay kit based on the method of Bradford [9] with bovine serum albumin as the standard.

2.6. Kinetic measurements

The POP activity was measured at several substrate concentrations with both substrates. Measurements were made with both pig brain homogenate and purified recombinant pig POP. The substrate concentrations used were 10–1000 μM for Suc-Gly-Pro-AMC and 2.5–150 μM for Z-Gly-Pro-AMC. Due to its poor solubility, Z-Gly-Pro-AMC had to be dissolved and diluted in 60% methanol. Therefore, when activity was measured with Z-Gly-Pro-AMC, the methanol concentration in the reaction mixture was 3%. The measurements with Suc-Gly-Pro-AMC were made in the absence and presence of 3% methanol to find out whether the solvent has an effect on kinetic parameters. Kinetic measurements were performed with the normal assay, except that the incubation time was shortened to 30 min for Z-Gly-Pro-AMC.

2.7. Calculation of kinetic parameters

POP activity as a function of substrate concentration was fitted with the following two equations utilizing GraphPad Prism® version 3.02 software (GraphPad Software, San Diego, CA, USA):

$$v = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

$$v = \frac{V_{\max}[S]}{K_m + [S] + [S]^2/K_{is}} \quad (2)$$

In Eqs. (1) and (2), v represents the velocity of reaction, V_{max} the limiting rate of the reaction, K_m the substrate concentration which provides half of the limiting rate of the reaction and K_i the inhibition constant for the inactive ternary complex between enzyme and two substrate molecules.

Eq. (1) (Henri–Michaelis–Menten equation) is an expression for steady state enzyme kinetics and normally kinetic measurements fit well to this equation. However, sometimes two substrate molecules can bind to the active site of the same enzyme molecule and form inactive ternary complex. This phenomenon is called substrate inhibition. The additional term in Eq. (2), K_{is}, takes into account the inhibitory interaction of the substrate molecule.

To distinguish between the equations, the kinetic data fits for both substrates were compared with *F*-test. The test calculates a *P*-value that answers the following question: if Eq. (1) is correct, what is the chance that random scatter allowed the Eq. (2) to fit better?

2.8. Determination of tight binding inhibition by JTP-4819 and enzyme concentration in brain homogenate

In tight binding inhibition, the enzyme concentration in the reaction medium is higher or of same magnitude as the inhibition constant of the inhibitor. In this case, when measuring the inhibition kinetics, the depletion of free inhibitor has to be taken into account. The occurrence of tight binding inhibition can be determined by measuring IC_{50} values at several enzyme concentrations. If the IC_{50} value increases linearly as a function of enzyme concentration, the inhibitor is tight binding in the reaction mixture [10].

The IC_{50} value of JTP-4819 was determined with different dilutions of enzyme homogenate using 200 μM Suc-Gly-Pro-AMC as the substrate. The highest concentration of JTP-4819 used in measurements was 10^{-8} M, hence the amount of DMSO in the reaction medium was always below 0.0001%. The IC_{50} values were calculated by a sigmoidal dose–response equation and plotted against enzyme concentration with GraphPad Prism software.

A tight binding inhibitor can be used to determine the active enzyme concentration (E) in the assay by utilizing the Morrison equation [11]:

$$\frac{v_i}{v_0} = 1 - \frac{(E + I + K_i^{app}) - \sqrt{(E + I + K_i^{app})^2 - 4EI}}{2E} \quad (3)$$

where v_0 and v_i are reaction velocities in the absence and presence of the inhibitor (I), respectively, and K_i^{app} is the apparent inhibition constant of the inhibitor.

POP activities were measured with several JTP-4819 concentrations between 0.01 and 1 nM using 200 μM Suc-Gly-Pro-AMC as the substrate. Then, v_i/v_0 was plotted against JTP-4819 concentration and the active enzyme concentration was calculated by using the Morrison equation.

2.9. IC_{50} at multiple substrate concentrations

Inhibition type and inhibition constants of tight binding inhibitors can be measured for an enzyme that follows Henri–Michaelis–Menten kinetics by plotting IC_{50} against substrate concentration [10]. The following equations describe the four possible inhibition types:

$$IC_{50} = K_{ic} + \frac{E}{2} + \frac{K_{ic}}{K_m} S \quad \text{Competitive inhibition} \quad (4)$$

$$IC_{50} = K_{iu} + \frac{E}{2} + \frac{K_{iu} K_m}{S} \quad \text{Uncompetitive inhibition} \quad (5)$$

$$IC_{50} = K_i + \frac{E}{2} \quad \text{Noncompetitive inhibition} \quad (6)$$

$$IC_{50} = \frac{S + K_m}{(K_m/K_{ic}) + (S/K_{iu})} + \frac{E}{2} \quad \text{Mixed inhibition} \quad (7)$$

In Eqs. (4)–(7), K_{ic} represents the competitive inhibition constant, K_{iu} the uncompetitive inhibition constant, K_i the noncompetitive inhibition constant ($K_{ic} = K_{iu}$) and E the enzyme concentration. If IC_{50} increases linearly when plotted against S, the inhibition type is competitive, if it increases linearly when plotted against $1/S$, the type of inhibition is uncompetitive. If IC_{50} remains constant as a function of substrate concentration, the inhibition type is noncompetitive. In mixed inhibition, IC_{50} increases or decreases non-linearly as a function of substrate concentration, depending on the relative values of the inhibition constants of competitive and uncompetitive inhibition types. The values of dissociation constants can be estimated from the intercepts of the IC_{50}-axis. If the enzyme concentration is much lower than the inhibition constant ($E \ll K_{ic}$, K_{iu}, K_i), the term E can be discarded from Eqs. (4)–(7) and the inhibition constant is directly the intercept of the IC_{50}-axis. However, if the inhibitor is tight binding in assay conditions, such that the enzyme concentration in the reaction medium is higher or of same magnitude as the inhibition constant, the intercept of IC_{50}-axis is the inhibition constant + $E/2$.

The IC_{50} values of JTP-4819 on POP were measured at Suc-Gly-Pro-AMC concentrations of 20, 100, 200, 400 and 600 μM as described above.

2.10. Molecular modeling

The modeling of POP-enzyme and substrates (Z-Gly-Pro-AMC Suc-Gly-Pro-AMC) was carried out using Sybyl 6.8 software (Tripos Inc., St. Louis, MO, USA) running on SGI Octane2 workstation. The crystal structure of POP [7] was taken directly from the PDB. Water molecules were removed, essential hydrogens added and Kollman united-atom charges loaded. The structures of substrates were created using the crystal structure of Z-Pro-Prolinal [7] as a starting point and further modified with the standard Sybyl tools. The resulting structures were minimized with MMFF94s force field [12] as implemented in Sybyl and charges (MNDO scaled ESP charges) (Quantum Chemistry Program Exchange, Indiana University, Bloomington, IN, USA) were calculated. All resulting structures (POP and substrates) were used in molecular docking experiments with Autodock 3.0 [13] software, using 100 GA docking runs method for each substrate.

3. Results and discussion

3.1. Reaction conditions

Since POP activity is known to be very sensitive to the effects of organic solvents [14,15] we wanted first to find the least interfering solvent in order to optimize our assay method (Fig. 2A). At 4%, DMSO decreased the enzyme activity by 80%, acetonitrile by 40% but methanol only by 25%. Due to its low inhibitory activity, methanol was chosen as the solvent for Z-Gly-Pro-AMC and it was used in the enzyme assay at a 3% concentration which decreased POP activity less than 20%. DMSO was used as the solvent for JTP-4819, but the final DMSO concentration was very low (below 0.0001%) in the incubation medium and this did not have any effect on the POP activity.

The optimum pH for the POP activity using substrate Suc-Gly-Pro-AMC was 6.3 but the activity was fairly constant between pH values 6 and 7 (Fig. 2B). The activity decreases rapidly at higher pH values: at pH 8 the velocity of reaction was only 10% of maximum. The pH optima for purified rat brain and human lymphocyte POP have been reported to be between 5.8 and 6.5 [16] and between 6 and 7 [17], respectively.

The temperature optimum for POP was 37° (Fig. 2C). However, we decided to perform our enzyme assay at 30°, since variation between measurements seemed to increase at higher temperatures. Also Toide *et al.* [4] have used 30° as the incubation temperature. Purified bovine brain POP has been reported to have a temperature optimum of 40° [18].

3.2. Enzyme kinetics

Interestingly, formation of AMC in the hydrolysis of Suc-Gly-Pro-AMC and Z-Gly-Pro-AMC showed very different kinetics with pig POP (Fig. 3). First, the catalysis mechanisms of substrate hydrolysis differed. Suc-Gly-Pro-AMC did not follow the standard Henri–Michaelis–Menten equation (Eq. (1)), but the data was better described by the

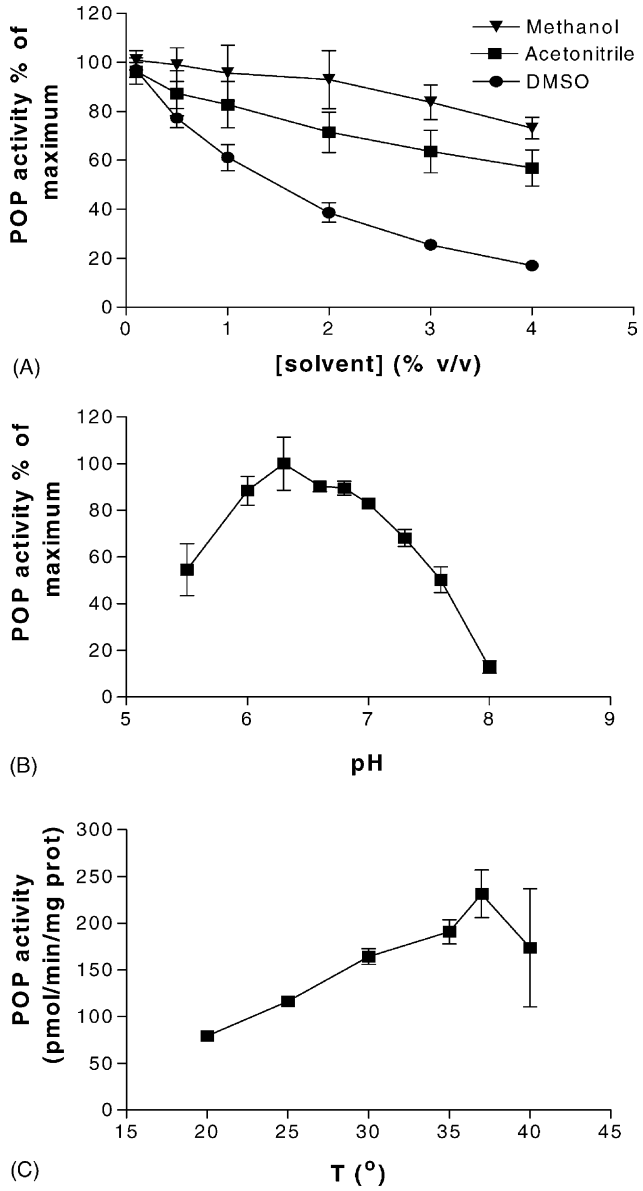

Fig. 2. Effect of organic solvents (A), pH (B), and temperature (C) on POP activity of pig brain homogenate. Measurements were made with Suc-Gly-Pro-AMC and pH in measurements for panels A and C was 7.0. Data are expressed as the mean ± SEM of three independent measurements.

substrate inhibition equation (Eq. (2); F-test: $P < 0.0001$ with purified recombinant pig POP and pig brain homogenate). In contrast, Z-Gly-Pro-AMC obeyed standard Henri–Michaelis–Menten kinetics (F-test: $P = 0.20$ and 0.16 for purified pig POP and pig brain homogenate, respectively). Second, kinetic parameters showed that K_m value for Z-Gly-Pro-AMC was one order of magnitude lower than for Suc-Gly-Pro-AMC (Table 1) and that K_{cat} for Suc-Gly-Pro-AMC was three times higher than for Z-Gly-Pro-AMC. Purified pig POP and pig brain homogenate showed similar behavior, indicating that other proteins in the brain homogenate did not interfere with the reaction. Addition of methanol in the reaction medium did not change the inhibitory behavior of Suc-Gly-Pro-AMC,

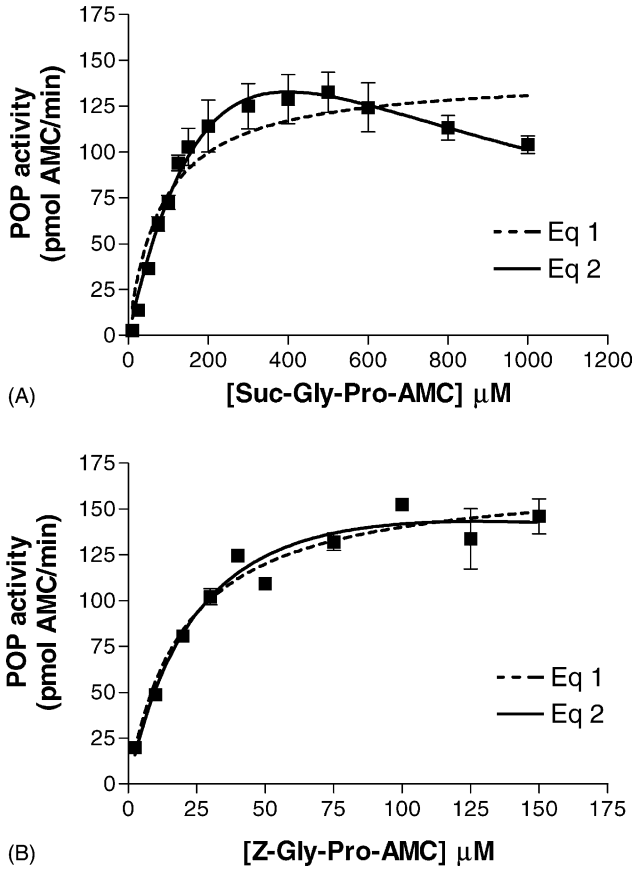

Fig. 3. Dependence of purified recombinant pig POP activity on substrate concentration at pH 7.0. In panel A, POP activity is measured with Suc-Gly-Pro-AMC (substrate inhibition in the reaction mechanism). In panel B, POP activity is measured using Z-Gly-Pro-AMC as a substrate. Eq. (1) describes standard steady state kinetics and Eq. (2) defines substrate inhibition kinetics. Values are shown as the mean ± SEM of three determinations. Enzyme concentration of 0.30 nM was used with both substrates.

Table 1
Kinetic parameters for prolyl oligopeptidase

	Z-Gly-Pro-AMC, 3% methanol	Suc-Gly-Pro-AMC	
		No methanol	3% methanol
Pig brain homogenate			
K_{cat} (s^{-1})	27 ± 1	73 ± 17	62 ± 29
K_m (μM)	30 ± 3	510 ± 150	620 ± 360
K_{is} (μM)	–	270 ± 90	360 ± 240
Purified pig POP			
K_{cat} (s^{-1})	19 ± 1	48 ± 19	60 ± 48
K_m (μM)	20 ± 3	460 ± 230	780 ± 730
K_{is} (μM)	–	350 ± 200	240 ± 250

Parameters were calculated using Eq. (1) for substrate Z-Gly-Pro-AMC and Eq. (2) for Suc-Gly-Pro-AMC. Equations were compared using F-test and better equation was used in calculations. The reaction mixture contained 3% methanol with Z-Gly-Pro-AMC, measurements with Suc-Gly-Pro-AMC were made in the absence and presence of methanol. The enzyme concentrations used in measurements were 0.32 and 0.30 nM for pig brain homogenate and purified recombinant pig POP, respectively. Values represent the mean ± SEM from 2 to 4 independent measurements. K_{is} values for Z-Gly-Pro-AMC is not shown because the substrate did not show substrate inhibition.

so the difference between two substrates did not result from the organic solvent used with Z-Gly-Pro-AMC (Table 1).

The difference in K_{cat} values was only theoretical, since substrate inhibition prevented any increase of velocity above 300 μM of Suc-Gly-Pro-AMC. Hence, the highest measured reaction rate with Z-Gly-Pro-AMC was higher than with Suc-Gly-Pro-AMC. The K_m value for hydrolysis of Z-Gly-Pro-AMC by rat brain POP has been reported to be 40 μM [3], which is close to our own result (30 and 20 μM for brain homogenate and purified pig POP, respectively). The K_m for chromogenic Suc-Gly-Pro-Nan, has been reported to be 350 μM, but this substrate did not exhibit substrate inhibition [6]. Substrate inhibition has not been reported earlier for POP, but this kind of inhibition has been observed with oligopeptidase B (EC 3.4.21.83), which is a serine protease structurally and mechanistically closely related to POP [19].

The experimental conditions in assaying POP activity may induce artifacts that mimic substrate inhibition. The decrease in fluorescence at high substrate concentrations could arise from the so called inner filter effect, i.e., a substrate absorbs light at the emission wavelength of hydrolyzed substrate and therefore decreases the measured fluorescence intensity. In our method, the inner filter effect would occur if Suc-Gly-Pro-AMC could absorb light at 460 nm. We excluded that effect by diluting the samples 1/10 after the reaction and remeasuring the fluorescence intensities but this did not have any effect on the kinetic parameters (data not shown). Therefore Suc-Gly-Pro-AMC is suggested to be a new inhibitory substrate among substrates for serine protease family [19].

Interestingly, a minor difference in the N-terminal end of the two substrates results in totally distinct kinetics (Fig. 1). The succinyl group differs at least in two major ways from the benzyloxycarbonyl group. First, a negative charge of succinate residue at pH 7 and an additional hydrogen bond accepting carbonyl oxygen. A hydrogen bond could be formed between the carbonyl oxygen of succinyl group and a hydrogen donor residue of POP, e.g., arginine 643. The bond would correspond to a reported hydrogen bond P4 between synthetic octapeptide and enzyme [8]. Nonetheless, it is unlikely that the substrate inhibition would be due to this bond because an additional hydrogen bond should make substrate binding even more specific than with Z-Gly-Pro-AMC but, on the contrary, the K_m values are indicative of an opposite effect.

We studied the binding of these two substrates by automated molecular docking runs utilizing 3D-structure of POP [7] and Autodock [13] software. These docking experiments yielded 100 individual solutions for both of the substrates. Almost all docked substrates were found within the actual binding site as identified by the crystal structure [7]. However, there was some variation between individual solutions. Energetically, the most favorable dockings are shown in Fig. 4. As a clear trend, Suc-Gly-Pro-AMC had two principal ways to be anchored in the active site, AMC- (orange structure in Fig. 4) or succinyl end (magenta structure in Fig. 4) at the S'_1 site, whereas Z-Gly-Pro-AMC has only one way in which it can bind, AMC-end at the S'_1 site [20] (blue structure in Fig. 4). Predicted free energy of binding for Suc-Gly-Pro-AMC was near −7 kcal/mol (for both orientations) and corresponding value for Z-Gly-Pro-AMC was −10 kcal/mol. These values are in accordance with the K_m values of these substrates.

However, this non-productive binding of Suc-Gly-Pro-AMC can not explain the observed substrate inhibition as such, since non-productive inhibition is described by following equation:

$$v = \frac{V_{max}[S]}{K_m + K_m[S]/K_i + [S]} \quad (8)$$

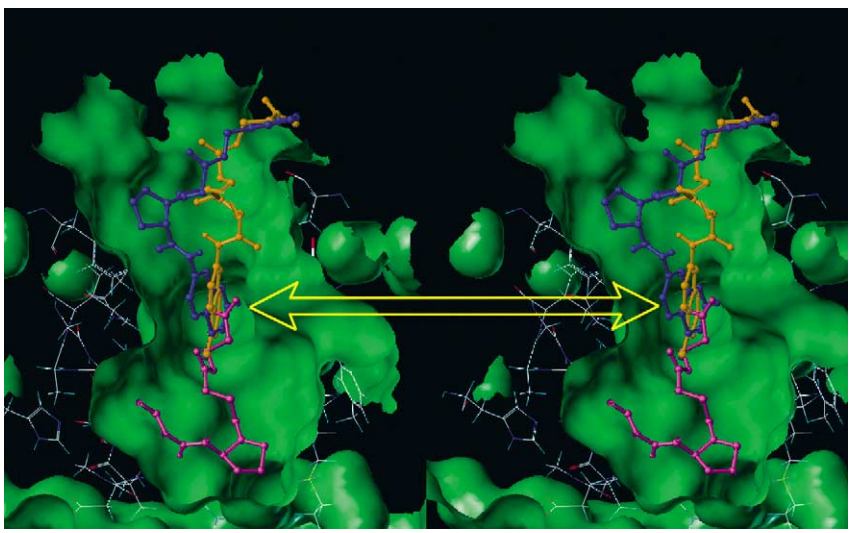

Fig. 4. Dockings for Suc-Gly-Pro-AMC (orange and magenta) and Z-Gly-Pro-AMC (blue) within POP. The green surface corresponds to the Connolly surface and yellow arrows indicate the S'_1 site of the enzyme.

This form is the same as in the equation for competitive inhibition with the inhibitor replaced for substrate. Henri–Michaelis–Menten kinetics is followed and existence of non-productive binding can not be observed from kinetic measurements. [21]. Hence, our data with Z-Gly-Pro-AMC did not follow this equation. In substrate inhibition, second substrate molecule binds to the active site, forming inactive complex. In the case of Suc-Gly-Pro-AMC, it is possible that non-specifically (succinyl end at the S'_1 site) bound substrate allows the simultaneous binding of second, inhibitory substrate molecule to the enzyme active site. Similarly, productively bound Z-Gly-Pro-AMC molecule might prevent the binding of another substrate molecule to the active site.

3.3. Determination of tight binding inhibition of JTP-4819 and enzyme concentration in pig brain homogenate

JTP-4819 was chosen as a model inhibitor for POP, since its neurochemical and behavioral properties have been widely described [22]. However, as far as we are aware, its exact inhibition kinetics has not been determined. The IC_{50} value of JTP-4819 has been reported to be 0.83 nM in rat brain homogenate [4]. This IC_{50} value is so low that we wanted to test whether the enzyme concentration in reaction medium is of the same magnitude suggesting that tight binding inhibition has to be taken into account. Tight binding inhibition of another potent POP inhibitor, Z-Pro-Prolinal, has been studied earlier [23]. However, in that study purified human POP was used as the enzyme source and the results do not describe the enzyme concentration in widely used brain homogenates.

Indeed, JTP-4819 was found to be a tight binding inhibitor of POP, since its IC_{50} value increases linearly as a function of enzyme concentration (Fig. 5A). The intersection of *y*-axis, 0.14 ± 0.02 nM, gives a good approximation of the IC_{50} value in non-tight binding conditions ($E \ll K_i$). JTP-4819 was used to determine the active enzyme concentration by utilizing the Morrison equation (Fig. 5B). The enzyme concentration was calculated to be 0.32 ± 0.01 nM in the reaction medium, thus the concentration of POP in 10,000 *g* supernatant of pig brain homogenate was 31.5 ± 0.8 nM. This concentration is so high that tight binding inhibition would exist even with inhibitors with moderate affinity, if the homogenate is not diluted extensively. Since POP has a molecular mass of 80 kDa and the protein concentration in 10,000 *g* supernatant of pig brain homogenate was 8.6 mg/mL, it can be calculated that POP accounts for about 0.029 % of cytosolic protein present in pig brain homogenate. This corresponds to 9.9 μg (0.12 nmol) of POP per gram of wet weight of pig brain tissue.

3.4. Determination of inhibition type of JTP-4819 by measuring IC_{50} values at multiple substrate concentrations

IC_{50} values of JTP-4819 on pig brain POP are plotted against substrate (Suc-Gly-Pro-AMC) concentration in Fig. 6. The IC_{50} increased linearly as a function of substrate concentration, indicating a pure competitive type of inhibition. The linear regression of observed points intercepts the *y*-axis at 0.20 ± 0.01 nM corresponding to $K_{ic} + E/2$.

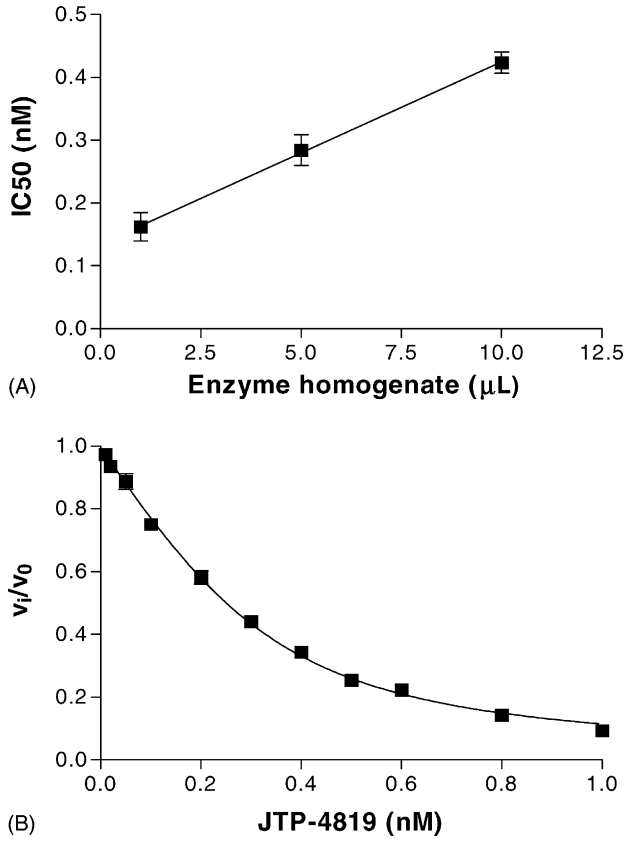

Fig. 5. JTP-4819 as a tight binding inhibitor of POP. The effect of pig brain homogenate concentration on IC_{50} values of JTP-4819 on POP (A). Increasing IC_{50} as a function of enzyme concentration indicates tight binding inhibition. Effect of various concentrations of JTP-4819 on POP activity (B). Data were fit to Eq. (3) and the curve was generated by using the best least-squares fit values of the parameters, $E = 0.32$ nM and $K_i = 0.09$ nM. Values of each panel show the mean ± SEM of three determinations. pH was 7.0.

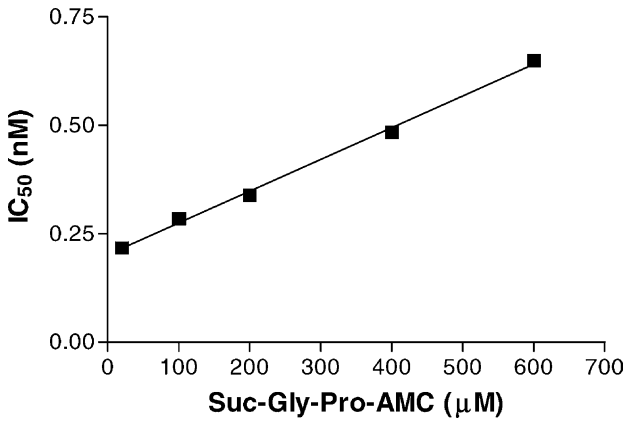

Fig. 6. Effect of substrate concentration on IC_{50} values of JTP-4819 on POP. Linearly increasing IC_{50} value as a function of substrate concentration is characteristic of a competitive inhibition. pH was 7.0.

Hence, JTP-4819 is a tight binding competitive inhibitor of POP with a K_{ic}-value of 0.045 ± 0.008 nM. This value is much lower than the enzyme concentration in the reaction mixture (0.32 ± 0.01 nM). Thus, misinterpretation of the inhibitory potency is obtained if the presence of the tight binding inhibition is ignored. In fact, if $K_i \ll E$ in the reaction medium, the result of IC_{50} determination would always be $E/2$ regardless of the true IC_{50} value.

Obviously, the tight binding type of inhibition could be avoided by diluting the enzyme so that $E \ll K_i$. However, usually the sensitivity of the method does not allow the activity measurements at picomolar levels of enzyme. In addition, despite the extensive dilution, one cannot rule out the tight binding inhibition unless the IC_{50} values are measured with different homogenate concentrations or unless the active enzyme concentration is defined.

Cheng and Prusoff [24] have discussed the relationship between inhibition constant and IC_{50} and Cha [10] has extended their equations to accommodate tight-binding inhibitors. Cha [10] also noted that inhibition type and inhibition constants could be determined by plotting IC_{50} against substrate concentration. Here we show that this method can be applied also for substrates that exhibit substrate inhibition. This method proved to be very convenient in the case of substrate inhibition, but it also has some advantages for substrates that follow standard kinetics. First, usually inhibition type is determined by measuring apparent K_m and V_{max} at several inhibitor concentrations and these parameters cannot be measured reliably in the presence of substrate inhibition. Second, high substrate concentrations are often needed to measure these parameters which may result in solubility problems.

The measurements of IC_{50} values are widely used to compare the potency of inhibitors in drug development. However, it should be borne in mind that the IC_{50} values are valid for comparison of inhibitors only when determined under identical conditions. Determination of IC_{50} values at several substrate concentrations would readily provide the K_i value of new inhibitors.

In conclusion, we optimized the method for assaying POP activity and found that the two widely used substrates for POP bind via different mechanisms to the enzyme active site. This was observed both with pig brain homogenate and purified recombinant pig POP. We also studied the inhibition kinetics of a well characterized POP inhibitor, JTP-4819 and our results indicate that JTP-4819 is a competitive tight binding inhibitor of POP with a K_{ic}-value of 0.045 ± 0.008 nM. The tight binding inhibitor allowed us to measure the concentration of POP protein in the pig brain homogenate and we found the concentration to be such a high (0.12 nmol/g pig brain), that classical methods for measuring IC_{50} values give erroneous results for potent POP inhibitors. These findings are of importance for structure–activity relationship studies when developing new POP inhibitors.

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References

[1] Cunningham DF, O'Connor B. Proline specific peptidases. Biochim Biophys Acta 1997;1343:160–86.
[2] Rennex D, Hemmings B, Hofsteenge J, Stone S. cDNA cloning of porcine brain prolyl endopeptidase and identification of the active-site seryl residue. Biochemistry 1991;30:2195–203.
[3] Atack J, Suman-Chaunan N, Dawson G, Kulakowski J. In vitro and in vivo inhibition of prolyl oligopeptidase. Eur J Pharmacol 1991;205:157–63.
[4] Toide K, Iwamoto Y, Fujiwara T, Abe HJ. JTP-4819: a novel prolyl endopeptidase inhibitor with potential as a cognitive enhancer. Pharmacol Exp Ther 1995;274:1370–8.
[5] Toide K, Shinoda M, Fujiwara T, Iwamoto Y. Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on spatial memory and central cholinergic neurons in aged rats. Pharmacol Biochem Behav 1997;56:427–34.
[6] Szeltner Z, Renner V, Polgár L. Substrate- and pH-dependent contribution of oxyanion binding site to the catalysis of prolyl oligopeptidase, a paradigm of the serine oligopeptidase family. Protein Sci 2000;9:353–60.
[7] Fülöp V, Böcskei Z, Polgár L. Prolyl oligopeptidase: an unusual β-propeller domain regulates proteolysis. Cell 1998;94:161–70.
[8] Fülöp V, Szeltner Z, Renner V, Polgár L. Structures of prolyl oligopeptidase substrate/inhibitor complexes. J Biol Chem 2001;276:1262–6.
[9] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[10] Cha S. Tight-binding inhibitors. I. Kinetic behavior. Biochem Pharmacol 1975;24:2177–85.
[11] Copeland RA. Enzymes. In: A practical introduction to structure, mechanism and data analysis. New York: Wiley, 2000.
[12] Halgren TA. MMFF VI. MMFF94s option for energy minimization studies. J Comp Chem 1999;20:720–9.
[13] Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. Automated docking using Lamarckian genetic algorithm and an empirical binding free energy function. J Comp Chem 1998;19:1639–62.
[14] Polgár L. Prolyl oligopeptidases. Methods Enzymol 1994;244:188–200.
[15] Polgár L. pH-dependent Mechanism in the catalysis of prolyl oligopeptidase from pig muscle. Eur J Biochem 1991;197:441–7.
[16] Kato T, Nakano T, Kojima K, Nagatsu T, Sakakibara S. Changes in prolyl endopeptidase during maturation of rat brain and hydrolysis of substance P by the purified enzyme. J Neurochem 1980;35:527–35.
[17] Goossens F, De Meester I, Vanhoof G, Hendriks D, Vriend G, Scarpé S. A sensitive method for the assay of serum prolyl endopeptidase. Eur J Clin Chem Clin Biochem 1992;30:235–8.
[18] Yoshimoto T, Oyama H, Koriyama N, Tsuru D. Prolyl endopeptidase from bovine testis: purification, characterization and comparison with the enzymes from other tissues. Chem Pharm Bull 1988;36:1456–62.
[19] Polgár L. Oligopeptidase B: a new type of serine peptidase with a unique substrate-dependent temperature sensitivity. Biochemistry 1999;38:15548–55.

[20] Sybyl Mol2-files are available on request (antti.poso@uku.fi).
[21] Cornish-Bowden A. Fundamentals of enzyme kinetics. London: Portland Press Ltd, 1995.
[22] Toide K, Shinoda M, Miyazaki A. A novel prolyl endopeptidase inhibitor, JTP-4819. Its behavioral and neurochemical properties for the treatment of Alzheimer's disease. Rev Neurosci 1998;9:17–29.
[23] Bakker AV, Jung S, Spencer RW, Vinick FJ, Faraci WS. Slow tight-binding inhibition of prolyl oligopeptidase by benzyloxycarbonyl-prolyl-prolinal. Biochem J 1990;271:559–62.
[24] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–108.