AMINOPEPTIDASE *PC* FROM KAMCHATKA CRAB *PARALITHODES CAMTSCHATICA* HEPATOPANCREAS: SUBSTRATE SPECIFICITY AND INHIBITOR STUDIES

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Aminopeptidase *PC* from Kamchatka crab *Paralithodes Camtschatica* has mixed substrate specificity. It effectively splits off the N-terminal hydrophobic as well as charged amino acids residues.

The K_M values for the hydrolysis of alkaline amino acids *p*-nitroanilides is a one fold lower than those for the hydrolysis of hydrophobic amino acids *p*-nitroanilides. There is a strong correlation between K_M and k_{cat} values and the amino acid residue structure for the hydrolysis of hydrophobic amino acids *p*-nitroanilides. The enzyme is a one fold more effective at the hydrolysis of the alkaline amino acids *p*-nitroanilides. The binding efficiency and the hydrolysis rate for Arg-pNA and Lys-pNA are virtually the same. The enzyme has the highest specificity towards these substrates, therefore it can be considered as an arginine aminopeptidase.

The binding mode of bestatin by aminopeptidase *PC* is close to noncompetitive when the enzyme is saturated with the Arg-pNA substrate. The K_I value was found to be 0.18 mM.

Aminopeptidase from Kamchatka crab *Paralithodes Camtschatica* (aminopeptidase *PC*) is a protease contained in the medicine proteolitic complex Moricrase.

Homogeneous aminopeptidase *PC* was isolated in the previous work [10] with 67% yield and purification degree 237 from the hepatopancreas of the Kamchatka crab *Paralithodes camtshatica* by ion-exchange chromatography on DEAE-Sepharose, hydrophobic chromatography Phenyl-Sepharose and gel-filtration on Sephadex G-150. The enzyme is a homodimer with a molecular weight 220 kDa (110x2). Its pI is 4.1. It hydrolyzes Leu-pNA optimally at pH 6.0; the optimal temperature is 36–40 °C. In the presence of Ca^{2+} the enzyme is stable at pH range 5.5–8.0. Aminopeptidase PC is activated by Ca^{2+}, Mg^{2+} and Fe^{2+}; it is completely inhibited by EDTA, *o*-phenantroline, *p*-chloromercurilbenzoate and bestatin. The enzyme contains 4 Zn^{2+} ions per molecule and therefore it is a metalloaminopeptidase. The binding mode of bestatin, the specific metalloaminopeptidase inhibitor—transition state analog, and the corresponding K_I value were not determined in that work.

It was shown that the aminopeptidase *PC* effectively splits off N-terminal residues Arg and Lys as well as Leu, Phe and Met. The residues of D-amino acids cannot be splitted off. Thus, the enzyme has a mixed substrate specificity, which is atypical for the metalloaminopeptidases of higher animals.

The aims of the present work are: the determination of the kinetic parameters of the amino acids *p*-nitroanilides hydrolysis reaction; the aminopeptidase *PC* substrate specificity study; the determination of the correlation between K_M and k_{cat} values and the N-terminal amino acid residue structure; the determination of the inhibitor binding mode and the K_I for the inhibition of aminopeptidase *PC* by bestatin, the specific metalloaminopeptidase inhibitor.

Materials and Methods

The homogenous aminopeptidase *PC* isolated from the proteolitic complex Moricrase [10] was used in the present work.

Buffer solutions: 50 mM MES-NaOH containing 2.5 mM $CaCl_2$, pH 6.0.

The substrate specificity of aminopeptidase *PC* was studied by measuring the hydrolysis rate of the amino acids *p*-nitroanilides (Serva, Germany). The substrate solutions (0.025–0.8 mg/ml) in 50 mM MES buffer were used to determine the dependence of the enzyme activity from the substrate concentration. The aminopeptidase *PC* concentration was 0.35 mg/ml. The K_M and k_{cat} values were determined by the Lineweaver–Burk linearization of the obtained data.

The specific inhibitor bestatin (Serva, Germany) was used for the enzyme inhibition studies. To the enzyme buffer solutions, the precalculated amount of 1 mM bestatin solution was added for the inhibitor concentrations to be 50–400 μM. The solutions were preincubated for 1 h at 20 °C, the substrate solutions were added afterwards. The effective K_M and k_{cat} values for different concentrations of bestatin were determined by the Lineweaver–Burk linearization. The K_I value was determined by the method described in Ref. [13].

Results and Discussion

The summary of the kinetic parameters determination is shown in Table 1. The study revealed that the K_M values

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Table 1

Aminopeptidase *PC* substrate specificity

Substrate	K_M, mmol/l	k_{cat}, s^{-1}	k_{cat}/K_M, $\text{l}\cdot\text{mmol}^{-1}\cdot\text{s}^{-1}$
Leu-pNA	2.27	2.05	0.91
Ala-pNA	2.20	2.20	1.01
Phe-pNA	2.11	1.25	0.59
Val-pNA	1.26	0.39	0.31
Lys-pNA	0.14	1.10	7.91
Arg-pNA	0.12	0.88	7.44

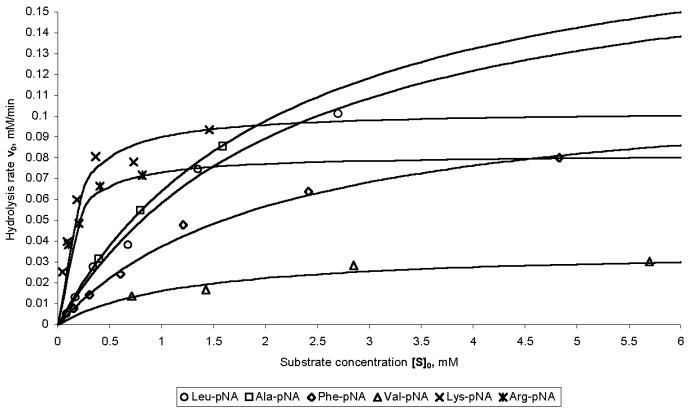

Fig. 1. The correlation between the reaction rate and the substrate concentration. The normal lines are the approximations built by the determined K_M and k_{cat} values.

for the hydrolysis of hydrophobic amino acids *p*-nitroanilides are a one fold higher than those for the hydrolysis of the alkaline amino acids *p*-nitroanilides. The enzyme is slowly saturated with the increasing concentrations of hydrophobic substrate, as shown in Fig. 1. The enzyme saturation is much faster in case of the alkaline amino acids *p*-nitroanilides.

The side chain structure of hydrophobic amino acids has little impact on the corresponding K_M value. This is not the case only for Val-pNA: the corresponding K_M value is almost twice lower compared to the reference K_M value for Leu-pNA. The hydrolysis efficiency is even more low for Val-pNA: the k_{cat} value found for this substrate is five times lower than the referential value for Leu-pNA. It seems that the branching at the β-carbon of the hydrophobic amino acid residue at P_1' worsens the substrate binding and the catalytic activity of the enzyme. Thus it can be hypothesized that the enzyme has rather small hydrophobic binding site at S_1'. It's also interesting to note the lowering of the hydrolysis efficiency in case of Phe-pNA. The K_M value is almost the same with the reference for the hydrolysis of this substrate.

The presence of branching at the γ-carbon of the hydrophobic amino acid residue doesn't seem to affect the substrate binding and the enzyme catalytic activity. The K_M and k_{cat} values are almost the same for the hydrolysis of Leu-pNA and Ala-pNA. Thus It can be supposed that the hydrophobic amino acid residue binds at S_1' only by its β-carbon atom, while the γ-carbon atom doesn't play role in substrate binding.

Comparing the hydrolysis reactions of alkaline amino acids *p*-nitroanilides, it can be noted that the binding as well as hydrolysis efficiency are virtually the same for Lys-pNA and Arg-pNA. The enzyme is eight time more efficient at the splitting off the Arg and Lys residues than hydrophobic amino acids residues, as seen in Table 1.

Since the aminopeptidase *PC* reveals the highest specificity towards the alkaline amino acids *p*-nitroanilides, it should be considered as an arginine aminopeptidase. However, the enzyme has the mixed substrate specificity in general, which is atypical for metalloaminopeptidases (see Table 2), e. g. aminopeptidase B [2, 5–9]. For example, the aminopeptidase *PC* k_{cat}/K_M value found for the hydrolysis of Ala-pNA is almost a one fold higher than that for aminopeptidase B by the hydrolysis of this substrate analog.

Bestatin, the specific inhibitor of metalloaminopeptidases—transition state analog [3, 13] was used to study the inhibition of aminopeptidase *PC*. The substrate used was Arg-pNA as one of the most specific substrates for the aminopeptidase *PC*. In the perliminary experiment it was established that the enzyme is inhibited by 17% and 71% in the presence of bestatin in concentrations of 50 μM and 500 μM respectively. This was shown by the hydrolysis of 0.1 mM Arg-pNA with the one-hour preincubation of the enzyme with bestatin.

The binding mode of bestatin is close to noncompetitive in the presence of the substrate in high concentrations (0.4 mM and above), as seen in Fig. 2. For most known metalloaminopeptidases, the bestatin binding mode is competitive. The typical example is the bovine lens leucine aminopeptidase [12]. However, the noncompetitive binding mode of bestatin was recently described [4, 11] for the porcine liver arginine aminopeptidase (also known as aminopeptidase B). It was supposed that the substrate and bestatin bind differently to this enzyme. Namely, the substrate binds at the standard S_1 and S_1' sites while bestatin binds at S_1' and S_2'. This explains the noncompetitive inhibition type of this enzyme. The unusual noncompetitive binding mode of bestatin to the aminopeptidase *PC* lets

Table 2

The comparison of different aminopeptidases substrate specificity (relative units)

Aminopeptidase *PC*		Leucine aminopeptidase [1]		Aminopeptidase B [8, 9]	
Leu-pNA	12.2	Leu-N_2H_3	= 100	Leu-βNA	0
Ala-pNA	13.5			Ala-βNA	3.4
Phe-pNA	8.0	Phe-N_2H_3	17.9		
Val-pNA	4.1			Val-βNA	0
Lys-pNA	106			Lys-βNA	50.4
Arg-pNA	= 100	Arg-βNA	= 100		

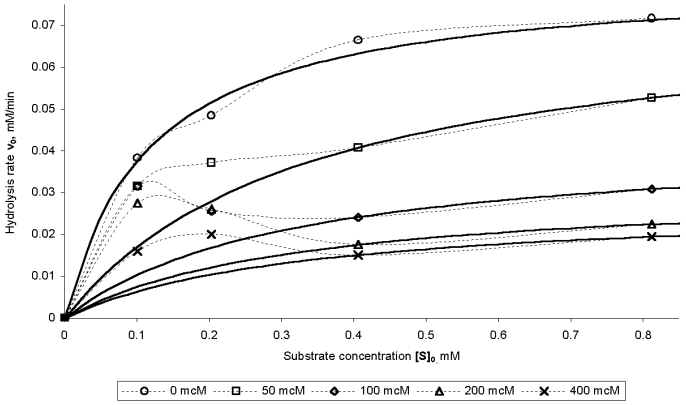

Fig. 2. The correlation between the hydrolysis rate of Arg-pNA and its concentration in the presence of bestatin in different concentrations. The dotted lines are the experimental data, the normal lines are the approximations built by the determined effective K_M and k_{cat} values.

to suppose that the aminopeptidase PC may belong to this subtype of arginine aminopeptidases. The K_I value found for aminopeptidase *PC* is 0.18 mM, which is three fold higher than that described for aminopeptidase B (0.16 μM) [4].

According to the data obtained, it can be supposed that the aminopeptidase *PC* has the active center structure similar to that of aminopeptidase B. At the same time the enzyme has mixed substrate specificity not typical for this class of enzymes. The existence of two binding sites at S_1', one for charged and the other for hydrophobic amino acids residues can be proposed to explain the mixed substrate specificity. Supposedly, the hydrophobic binding site is rather small, and the β-carbon of the amino acid residue plays vital role in the substrate binding.

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