SHORT COMMUNICATION

The influence of α-aminophosphonic acids on the activity of aminopeptidase from barley seeds—an approach to determine the enzyme specificity

Bartosz Oszywa · Małgorzata Pawełczak · Paweł Kafarski

Abstract Inhibitory potencies of 24 α-aminophosphonic acids against barley seeds (*Hordeum vulgare* L.) metallo-aminopeptidase have been determined to evaluate structural requirements of this enzyme. The enzyme was sensitive mostly to the influence of phosphonic acid analogues of phenylalanine and its homologues, thus showing narrow specificity if compared with porcine aminopeptidases M1 and M17 and with *Plasmodium* aminopeptidase M17.

Keywords α-Aminophosphonic acids · Aminopeptidase · Barley seeds · Inhibitors

Introduction

The aminopeptidases (EC 3.4.11) according to IUBMB classification belong to the class of hydrolase, subclasses of peptidase, and sub-subclass of aminopeptidases. They have capacity to remove individual amino acids from the N-terminus of protein substrates (Lowther and Matthews 2002; Taylor 1993).

The aminopeptidase enzymes are commonly found in biological kingdoms (microorganisms, plants and animals) and can be located in the cytoplasm, or various types of membranes and cell organelles. These enzymes play a number of more or less important and characteristic functions. Together with other peptidases, they steer metabolic pathways, control the metabolism of proteins, and activate other enzymes and hormones necessary for the proper functioning of the body. The aminopeptidase due to the fact that they can recover the amino acids from dietary and endogenous proteins also has a nutritional function.

They are necessary for proper functioning of eukaryotic and prokaryotic cells, but often are also important factors in various pathological states and diseases such as: malaria, cancer, diabetes, HIV, cataracts, angiogenesis, hypertension, systemic lupus (Hooper and Lendeckel 2004; Sanderink et al. 1988; Sanz 2007; Jankiewicz and Bielawski 2003; Pulido-Cejudo et al. 1997; Taylor et al. 1982; Sharma et al. 1996; Inokuma et al. 1999; Umezawa 1980; Pretlow et al. 1994; Mathe 1991).

In plants, the high concentration of aminopeptidases has been found in the seeds, young fast-growing organs, the parts which have been injured or damaged, and in the old, dying plant parts. High levels of these enzymes have been observed in plants grown under harsh conditions, especially under osmotic stress, which is due to water shortage or too high salinity (Bartling and Weiler 1992; Desimone et al. 2000; Thayer et al. 1988; Matsui et al. 2006; Ogiwara et al. 2005; Chien et al. 2002). Therefore, they may be considered as an element of "immunological response" of plants (Fowler et al. 2009; Bae et al. 2013).

Phosphonic acids and their derivatives are acting as inhibitors of aminopeptidases. This results from that these

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compounds imitate the tetrahedral transition state of peptide bond hydrolysis. In these reactions, at least one intermediate, in which the carbon atom participating in the reaction adopts the sp^3 configuration, is formed. Since phosphonic moiety also has tetrahedral structure and is mimicking this state, phosphonates exhibit inhibitory action towards proteolytic enzymes (Giannousis and Bartlett 1987; Dive et al. 2004; Lejczak et al. 1989; Mucha et al. 2008, 2010; Drąg et al. 2005). Thus, they might be used as tools for differentiating aminopeptidases from various sources and to determine the structural requirements for their N-terminal fragment binding.

The results of the studies on the activity of large libraries of fluorogenic substrates tailored to study proteolytic enzymes indicate enzyme preferences toward peptide side chance. In this manner, the sets of data that may be useful for design and synthesis of selective inhibitors are obtained (Drąg et al. 2010; Kasperkiewicz et al. 2012; Poras et al. 2011, 2013; Węglarz-Tomczak et al. 2013; Poręba et al. 2014).

In this paper, we examined the influence of simple α-aminophosphonic acids on activity of novel metalloaminopeptidase isolated from seeds of barley (Oszywa et al. 2013) and compared the results with published data concerning porcine and *Plasmodium* aminopeptidases (Cunningham et al. 2008).

Materials and methods

Plant materials

Fresh seeds of barley were obtained from a local farmer and until use were stored at 4 °C. Aminopeptidases from barley seeds were isolated and purified in accordance with the previous described procedure (Oszywa et al. 2013). The enzyme was purified in six stages. Purification methods: ammonium sulphate precipitation, gel chromatography (Sephadex G-25, Sephacryl HR 300), ion chromatography (DEAE-Sepharose, Macro-Prep Q) and hydrophobic interaction chromatography (Phenyl-Sepharose HP). Purity of the isolated enzyme was determined by SDS-PAGE electrophoresis. The molecular weight of the enzyme calculated based on the gel filtration (Sephacryl HR 300) and SDS-PAGE electrophoresis was 58 kDa.

Compounds

Aminophosphonic acids were available from earlier studies (Lejczak et al. 1989; Drąg et al. 2005; Cunningham et al. 2008) or were synthesized according to the modified literature procedure (Soroka 1989).

Acetamide (0.2 mol) was dissolved in acetic acid (40 ml) and cooled in an ice bath followed by addition of acetyl chloride (0.1 mol). This resulted in crystallization of unidentified by-product. After 15 min, the appropriate aldehyde was introduced and the mixture was stirred in an ice bath for 30 min. Mixture was allowed to stand for about a day at room temperature. The next day the mixture was cooled again in an ice bath, and then phosphorus trichloride (0.1 mol) was added in portions maintaining ice bath temperature for 30 min. After warming to room temperature, the mixture was heated for 1 h at 70–75 °C to complete the reaction. The volatile components of the reaction mixture were removed on rotary evaporator affording an oily product, which was then hydrolysed by refluxing in 100 ml concentrated hydrochloric acid for 8 h. After evaporation of water solution under reduced pressure, the oily product was dissolved in ethanol and left for a night to allow ammonium chloride and by-products (bisphosphonates) to precipitate (Dziuganowska et al. 2014). After filtration of these by-products, the desired aminophosphonate was crystallized from water/ethanol mixture.

Enzyme assays

Aminopeptidase activity was examined at 37 °C in 50 mM Tris–HCl, pH 7.5 containing NaCl (50 mM) and 2-mercaptoethanol (10 mM). Synthetic substrate L-leucine-p-nitroanilide (solution in DMSO) was added to the assay buffer and the solution was supplemented with the enzyme. The progress of the hydrolysis of L-Leu-pNA was monitored spectrophotometrically (UV–VIS Spectrophotometer Cintra 303) at a wavelength of 405 nm against a control sample lacking enzyme. The measured K_M value was 0.55 mM. The assay mixture, totally 1.15 ml, contained: solution of the synthetic substrate L-Leu-pNA in DMSO (final concentration from 1.5 to 0.2 mM), 50 mM Tris–HCl buffer (pH 7.5), containing 50 mM NaCl, and 10 mM 2-mercaptoethanol, the solution of the potential inhibitor in reaction buffer (concentration of compound dependent on inhibitory activity), and enzyme (0.028 mg of protein). The enzymatic reaction was performed at 37 °C for 15 min.
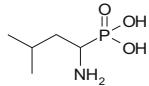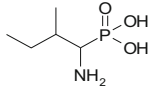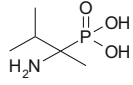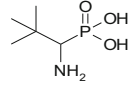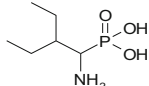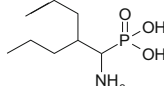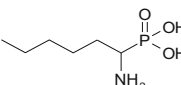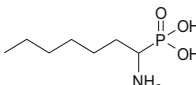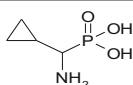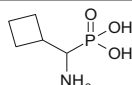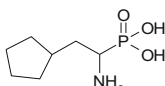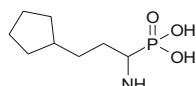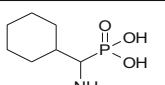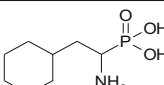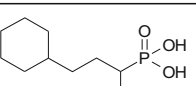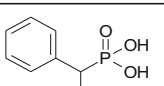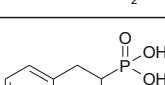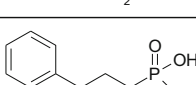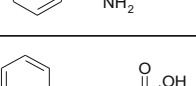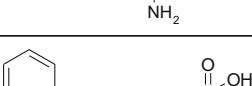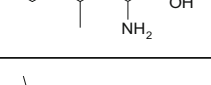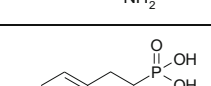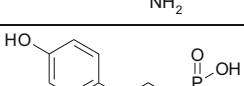
Inhibition constants for the α-aminophosphonic acids toward barley AP were determined basing on Lineweaver–Burk, Dixon, Hanes–Woolf curves and from a half-inhibitory concentration method for the reactions carried out in the presence of inhibitor and lacking an inhibitor. Reaction velocities were determined based on the progression curves (change in the absorbance over time). All reactions were carried out for four substrate concentrations and five concentrations of each inhibitor with each study being repeated three times. For each of

the designated kinetic parameters, V_o, V_{max}, K_M and K_i were calculated relative error. The values given in the Table 1 are the average values of K_i calculated by all these methods. Scatter of results (K_i values) did not exceed 10 % for each of the test inhibitor.

Results and discussion

Nearly all the studied aminophosphonates appear to inhibit aminopeptidase from barley seeds (Table 1). All of them appeared to be competitive inhibitors (see representative

Table 1 Structures of studied α-aminophosphonic acids and their inhibition constants (K_i) towards aminopeptidase from barley seeds

Cpd.	Structure	Inhibition constant K_i [μM] ± SD	Cpd.	Structure	Inhibition constant K_i [μM] ± SD
1		77±2	2		No inhibition up to 1.12 mM
3		No inhibition up to 2.47 mM	4		No inhibition up to 2.90 mM
5		2736±50	6		186±15
7		1560±60	8		960±40
9		850±40	10		1294±50
11		316±12	12		367±20
13		416±15	14		538±20
15		150±8	16		255±5
17		88±3	18		25±1
19		28±1	20		164±8
21		54±2	22		955±30
23		505±10	24		330±15
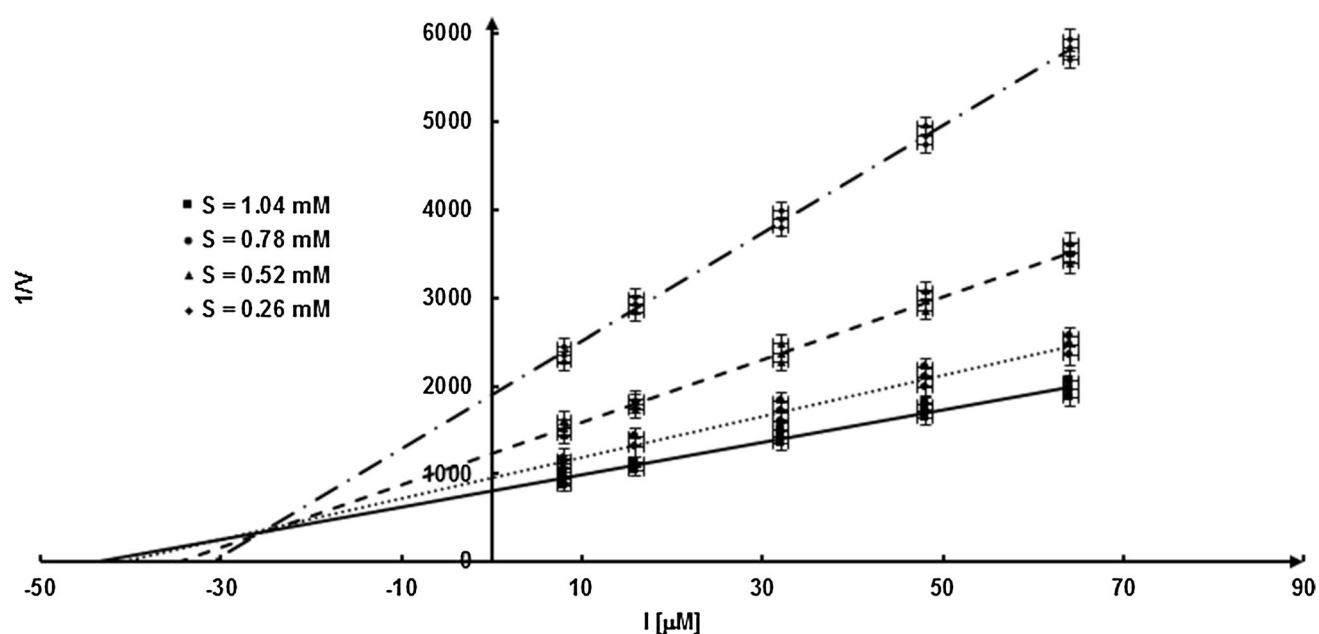
Fig. 1 The type designation inhibition and the inhibition constant K_i by Dixon method for the compound 1(RS)-amino-3-phenylpropanephosphonic acid towards aminopeptidase from barley seeds

Table 2 Comparison of inhibitory activity of α-aminophosphonic acids to mammalian aminopeptidases

Cpd.	Stereomer	AP barley K_i (µM)	LAP porcine K_i (µM)[b]	APN porcine K_i (µM)[b]
1.	1 R	77	0.23[a]	53[a]
2.	RR:RS, 1:1	NI	–	–
3.	1RS	NI	798	NI
4.	1RS	NI	–	–
5.	1R	2,736	243	138
6.	1RS	186	33	NI
7.	1RS	1,560	1[a]	26.5[a]
8.	1RS	960	–	–
9.	1RS	850	84.5	178
10.	1RS	1,294	22.7	77.4
11.	1RS	316	6.92	47.7
12.	1RS	367	6.35	18.5
13.	1R	416	7.89	161
14.	1RS	538	0.75	54.7
15.	1RS	150	0.21	37.1
16.	1RS	255	–	–
17.	1RS	88	–	–
18.	1RS	25	0.14	15.9
19.	1RS	28	0.26	36.9
20.	1RS	164	0.33	3.69
21.	1RS	54	NI	168
22.	1RS	955	1.37	NI
23.	1RS	505	0.33	170
24.	1RS	330	0.12	23.7

NI no inhibit enzyme activity

[a] Lejczak et al. (1989)

[b] Drąg et al. (2005)

Fig. 1) and, in opposition to porcine aminopeptidase M17, slow-binding kinetics has not been observed. Analogues of aliphatic amino acids appeared to be weak or negligible inhibitors of the enzyme with phosphonic acid analogue of leucine (compound **1**) being moderately active. Compounds bearing phenyl ring appeared to be far more active, with phosphonic acid analogue of homophenylalanine (compound **18**) acting as the most effective one. Its homologue, compound **19**, bearing an additional methyl at carbon atom in position 3 also exhibited a significant activity against this enzyme. A similar model of activity was observed for the porcine aminopeptidases (Table 2) and aminopeptidase from *Plasmodium falciparum* (value of IC_{50} for compound **18** is 22 μM; Cunningham et al. 2008). This, alongside with significant activity of compound **21**, indicates that there is a spacious binding site able to accommodate hydrophobic N-terminal fragments of inhibitors. The activity of this particular compound is somewhat surprising since it appeared to be far less active towards porcine enzymes. This, alongside with significantly weaker activity of compound **20** and these carrying alkyl substituents, indicates that the part of the barley enzyme binding hydrophobic fragment of aminophosphonate inhibitors differs from those observed in the case of porcine and *Plasmodium* enzymes. This is also seen from introduction of hydrophilic moiety into phenyl ring (compounds **22**, **23** and **24**), which resulted in significant decrease in affinities of such compounds towards aminopeptidase from barley seeds, whereas these compounds exhibited quite pronounced action towards porcine and *Plasmodium* enzymes.

Thus, comparison of the patterns of inhibitory activities of barley enzyme with two porcine aminopeptidases M17 (LAP, EC 3.4.11.1) and M1 (APN, EC 3.4.11.2) (Lejczak et al. 1989; Drąg et al. 2005) indicates that aminopeptidase from barley seeds is quite different from the two bovine enzymes being nearly exclusively sensitive to the action of phosphonic acid analogues of phenylalanine (compounds **17–20**) (Table 2). Aminopeptidase M17 prefers both aliphatic and aromatic hydrophobic aminophosphonates (compounds **18–20** and **1**, **5** and **13**; with analogues of leucine and homoalanine being of choice), whereas aminopeptidase M1 is preferably inhibited by aminophosphonates containing additional hydrophilic group in side chain, with analogue of homotyrosine (compound **24**) being the most active. Although *Plasmodium falciparum* aminopeptidase M17 (EC 3.4.11.1) prefers aminophosphonates bearing hydrophobic aromatic moiety in their side chain (Cunningham et al. 2008), the pattern of activity against this enzyme is also quite different because it is not sensitive to compounds **10–13**. Thus, our study indicates that the use of libraries of simple inhibitors could be a useful tool in determining specificities of aminopeptidases and their fingerprints.

Conclusions

α-Aminophosphonic acids appeared to be moderate or weak inhibitors of newly isolated aminopeptidase from barley seeds. Its pattern of activity is significantly different from those found for structurally related aminopeptidases M17 from *Plasmodium* and bovine lens, as well as porcine M1 aminopeptidase. Although the plant enzyme is also metallopeptidase phosphonic acid, analogues of amino acids appeared to be significantly weaker inhibitors of the enzyme.

Author contribution statement Bartosz Oszywa: protocol execution, graphics, data collection, statistical analysis, partial writing of the paper. Małgorzata Pawełczak: conceptualization hypothesis, design of the research, publication search, mentorship and consultation, partial writing of the paper, paper revision prior to submission. Paweł Kafarski: synthesis compounds, data interpretation conclusions, writing the paper, mentorship and consultation, grant and funding acquisition. All the authors read corrected and approved the manuscript in its final form.

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