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A highly enantioselective aminopeptidase from sunflower seed—Kinetic studies, substrate mapping and application to biocatalytic transformations

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ABSTRACT

The sunflower seed (*Helianthus annuus* L.) major aminopeptidase primary specificity has been assessed with a number of specially designed amino acid amides and alkyl esters. Studies with amino acid 4-nitroanilides with straight alkyl side chains suggest the enzyme possesses an S_1 hydrophobic binding site of limited size and effects of substrate sorption are present in both its binding and turnover. The enzyme S_1'-subsite is able to accommodate a number of different leaving groups—primary amide, one- and two-ring condensed aromatic structures, amino acid residues and alkyl ester moieties. The hydrolysis of amide substrates is affected by both their P_1 and the P_1' part. The replacement of the amide leaving group with an ester one tents to minimize the P_1-part effect. The enzyme's high enantioselectivity towards the configuration of the substrate P_1-part has been successfully utilized for resolution of racemic amino acid amides.

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1. Introduction

Aminopeptidases form a broad group of diverse enzymes what concerns their structure and mechanism of action, occurrence and biological role [1–4]. They have been object of numerous studies aiming their basic characterization as well as integration into some practical issues as they effectively catalyze the transformation of a variety of substrate structures [5–8].

The SSMA[1] has been purified and characterized recently [9]. It is an 80 kDa thiol-dependent neutral aminopeptidase. Its mode of interaction with a number of peptide and arylamide substrates has been studied.

The correlation of the kinetic parameters of enzyme action with specific changes in the substrate structure has proved fruitful in mechanistic investigation of a number of enzymes [10–13]. In the present study this method has been utilized in the characterization of the SSMA. The detailed kinetic analysis of the enzyme's primary specificity carried out with several different series of substrates provided an insight into its active site structure and functioning.

Biocatalytic transformation is the method of choice for preparation of a number of enantiomerically pure substances—diols [14], carbonyl compounds [15], aliphatic acid amides [16], chiral drugs [17] and amino acids [18–20]. Racemic resolution of amino acids can be carried out using hydrolytic enzymes in synthetic reactions employing regioselective acyl transfer [21], hydrolytic removal of N-acyl moieties [20] or by hydrolysis of amino acid alkyl esters or amides [18,19,22]. The SSMA has been employed in the latter strategy as it effectively catalyzed the enantioselective conversion of amino acid amides.

2. Results and discussion

A number of amino acid amides and esters were used to map the enzyme primary specificity (Fig. 1). A free amino group and S-configuration of the adjacent carbon atom of the acyl moiety is required for the catalytic process to take place [9].

2.1. Kinetic analysis of the sunflower aminopeptidase S_1-specificity

The kinetic parameters of the enzyme-catalyzed hydrolysis are significantly affected by the nature of the amino acid residue at P_1-position of the substrate (Table 1). The effect of the hydrophobicity of the side chain on the efficacy of catalysis is studied with

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[1] *Abbreviations*: sfl: spectrofluorimetry; sph: spectrophotometry; SSMA: sunflower seed major aminopeptidase.

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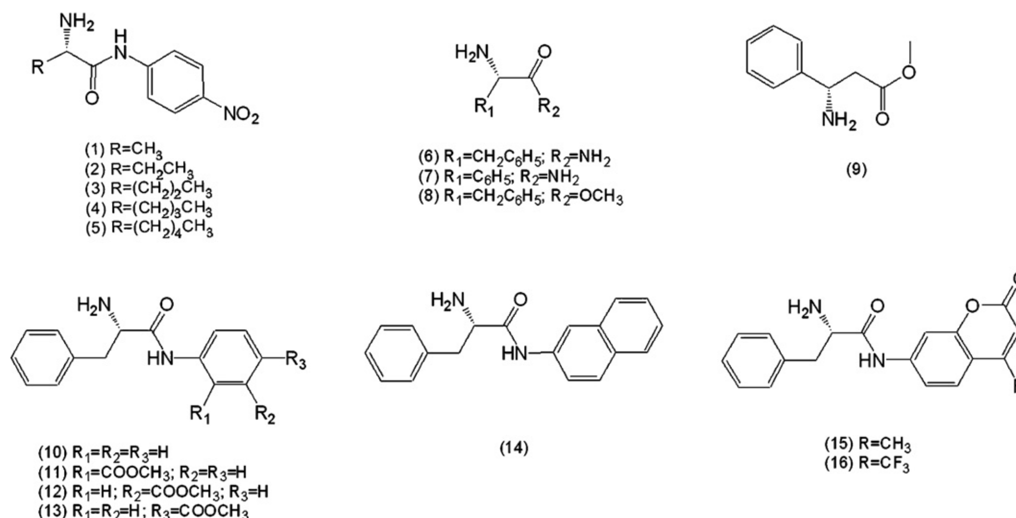

Fig. 1. SSMA substrates.

the hydrolysis of 4-nitroanilides of amino acids with straight alkyl side chains (compounds 1–5). The dependence of the log k_{cat}/K_m on the Hansch–Fujita hydrophobicity constant, π has a biphasic pattern with two linear portions intersecting at $\pi = 2.0$ (Fig. 2). For substrates with side chains of up to four carbon atoms the efficacy of catalysis increases linearly with extension of the chain. The increment of log k_{cat}/K_m for the side chain containing five carbon atoms is significantly smaller than those for the first four members of the series. That indicates the enzyme possesses an S_1 hydrophobic binding site of limited size. The residue of norleucine is with optimal side chain length (*n*-butyl residue) to fit it and a further enlargement does not improve the efficacy of catalysis. For compounds 1–4 linear correlations of the hydrophobicity constant with each of the kinetic parameters (log k_{cat} and log K_m) have also been obtained indicating the presence of sorption effects in both the substrate binding and the catalytic step of the reaction. Such dependence is better manifested with the effects of substrate binding as seen from the intensity factors of the obtained correlation equations (Fig. 3). This allows the formation of the non-covalent enzyme–substrate complex to be treated in analogy with the transfer of a hydrocarbon residue from water to a non-polar environment which in this case is the binding site of the enzyme active centre.

Table 1
Kinetic parameters of the SSMA-catalyzed hydrolysis of amino acid 4-nitroanilides.

Substrate	π[a]	Kinetic parameters of hydrolysis		
		k_{cat} (s^{-1})	10^4 K_m (M)	10^{-3} k_{cat}/K_m (M^{-1} s^{-1})
1[b]	0.5	64.8	122	5.3
2	1.0	96.3	49	19.7
3	1.5	140	12.5	112
4	2.0	203	3.4	604
5	2.5			684[c]

[a] Data taken from [12].
[b] Data taken from [9].
[c] Specificity constant estimated from pseudo-first order traces at [S] ≪ K_m.

2.2. Effect of the P_1'-moiety

The influence of the leaving group has been studied by the kinetic characterization of a series of substrates with the same acyl part, a residue of phenylalanine (Table 2). The primary amide of phenylalanine is a specific substrate mainly due to its effective binding. The amide of phenylglycine is also hydrolyzed in contrast to the 4-nitroanilide which has been previously found to be ineffective as a substrate [9]. This phenomenon confirms the significance of the leaving group for the catalytic process and suggests the presence

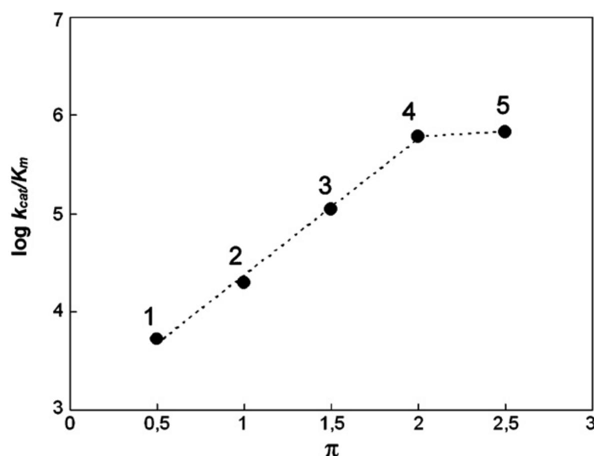

Fig. 2. Dependence of log k_{cat}/K_m on π of the side chain for the SSMA-catalyzed hydrolysis of amino acid 4-nitroanilides. Correlation equation of the function for substrates 1–4: log $k_{cat}/K_m = 1.38\pi + 2.98$ (r^2 0.99).

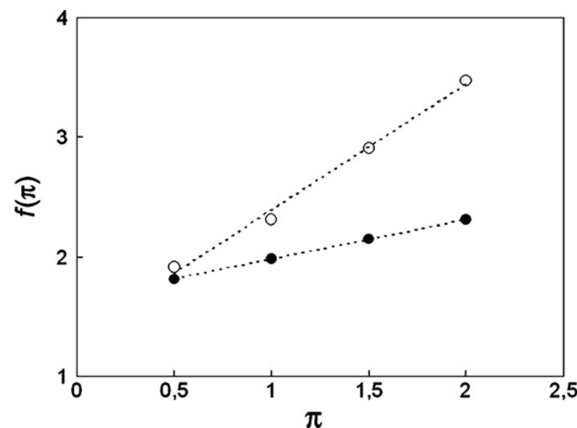

Fig. 3. Dependence of log k_{cat} (●) and log K_m (○) on π of the side chain for the SSMA-catalyzed hydrolysis of amino acid 4-nitroanilides. Correlation equations of the displayed functions: log $k_{cat} = 0.33\pi + 1.65$ (r^2 0.99) and log $K_m = -1.05\pi - 1.33$ (r^2 0.99).

Table 2
Kinetic parameters of the SSMA-catalyzed hydrolysis of S-amino acid amides and esters.

Substrate	Kinetic parameters of hydrolysis		
	k_{cat} (s^{-1})	10^4 K_m (M)	10^{-3} k_{cat}/K_m (M^{-1} s^{-1})
6	242	0.6	4250
7	4.7	1.9	25.1
8	450	1.2	3780
9	458	1.1	4280
10	1030	4.3	2560
11	103	2.8	369
13	1200	3.7	3250
12	184	1.8	1050
14	1290	2.5	5060
			4560[a]
15			3240[a]
16			2100[a]

[a] Specificity constant estimated from pseudo-first order traces at [S] ≪ K_m.

of some steric obstruction in substrate fixation in the case of the arylamide.

With expanding to an aniline ring (compound 10) the specificity of binding decreases in favor of improved catalysis as the turnover number is about six times higher than that for the primary amide. Substitution in the aniline ring (compounds 11–13) significantly affects the parameters of the enzyme-catalyzed hydrolysis. In case of *ortho*- or *metha*-substitution with electron-withdrawing groups the enzyme shows lower K_m values in comparison to the *para*- and non-substituted anilides. The corresponding k_{cat} values are also decreased as the efficacy of catalysis is particularly low with the *ortho*-substituted derivative probably due to a steric hindrance on the reaction centre.

The extension of the arylamine moiety to a two-ring condensed aromatic system in phenylalanine 2-naphthylamide (compound 14) results in a specificity constant that is the highest from all the tested substrates. The two coumaride derivatives (compounds 15 and 16) show decreased efficacy of catalysis. This might be due to unfavorable electronic effects or steric reasons as the oxygen heteroatom allows conformational rotation and the system loses planarity. Phenylalanine 4-trifluoromethyl-7-coumarylamide manifested decreased reactivity in comparison to the corresponding 4-methyl derivative. That is most likely a result of the presence of trifluormethyl residue that can cause both steric hindrance and changes in the electronic structure of the system by its negative inductive effect.

An interesting trend is presented with the enzyme-catalyzed hydrolysis of the methyl esters of phenylalanine (8) and 3-amino-3-phenylpropionic acid (9). Their acyl moieties differ in structure by the place of the primary amino group which significantly affects the hydrolysis of the corresponding 4-nitroanilides. In contrast, the methyl ester leaving group tends to minimize the influence of the substrate acyl part on the catalytic efficacy resulting in almost identical values of the kinetic parameters. Because of its size and structure the methyl ester moiety is probably incapable of establishing the P_1'–S_1' interactions that considerably affect the reactivity of the arylamides.

2.3. Enantioselective hydrolysis of amino acid amides

In tests with R- and S-isomers of a number of amides (alaninamide, leucinamide, phenylalaninamide, phenylglycinamide) and arylamides (4-nitroanilides of leucine and phenylalanine [9]) the SSMA displayed high stereoselectivity towards the configuration of the acyl part as only the S-isomers were hydrolyzed. This implied the possible utilization of the enzyme for resolution of racemic amino acid derivatives. Two types of amino acid derivatives are usually employed in such reactions—primary amides and alkyl esters [19]. Amino acid methyl esters are relatively easy to prepare in almost quantitative yields but their susceptibility to non-enzymatic hydrolysis in alkaline medium lowers the enantiomeric purity of the final products. Thus amino acid amides were chosen for the biocatalytic transformation. In experiments with the racemic amides of phenylalanine and phenylglycine complete enantiomeric resolution was achieved as the S-enantiomer was fully converted and the R-enantiomer was intact (Table 3). The higher stability of the amino acid amides allowed the reaction to be carried out at higher temperatures (45 °C) and optimal for the enzyme action pH value (7.5) thus decreasing the process time. Constant rates of substrate conversion until completion of the reaction were observed. They were assured by the substrate concentrations used that were significantly higher than the respective K_m values. The constant rates also suggest the enzyme was stable over the course of reaction and no product inhibition was present. Separation of the enzyme from the reaction mixture upon completion of the reaction was achieved by ultrafiltration on a 30 kDa polysulfone membrane. In this way the enzyme could be continuously reused for another batches of racemic substrate or alternatively employed in a continuous-flow membrane reactor.

3. Conclusion

The ability of the sunflower aminopeptidase to accommodate a variety of substrates in its active centre and to catalyze their hydrolysis shows its flexibility towards the disposed substrate structure. The data for the enzyme S_1 specificity indicates the presence of a hydrophobic site that provides for binding the substrate P_1 amino acid side chain. The linear free energy relationships established for its functioning suggest the presence of sorption effects in both substrate binding and catalysis. The enzyme S_1' subsite functioning appears to be far more complex as a great variety of leaving groups can be accommodated in it. Except peptides which are the natural substrates of the enzyme a number of one- and two-ring aromatic moieties are tolerated as P_1' parts including ones containing substituents and heterocycles. Significant differences in the kinetic parameters of enzyme-catalyzed hydrolysis of substrates with the same acyl part can be caused by altering the leaving group. The replacement of the amide leaving group with an ester one tents to minimize the P_1-part effect.

The SSMA has been successfully employed in resolution of racemic mixtures of amino acid amides. Its catalytic efficiency and high enantioselectivity toward the configuration of the substrate P_1-part ensured the facile preparation of the desired amino acids in enantiomerically pure form.

Table 3
SSMA-catalyzed enantioselective hydrolysis of amino acid amides.

Substrate	Enzyme concentration (nM)	Preferred configuration	Conversion (%)	*ee* %[a]	*E*
(RS)-Phenylalaninamide	1	S	49.5	>99	>320
(RS)-Phenylglycinamide	100	S	49.2	>99	>320

Reaction conditions: 25 mM substrate in 38 mM K–Na phosphate buffer, pH 7.5, 45 °C, reaction time 4 h.
[a] *ee* % for the S-isomer.

4. Experimental

4.1. Materials and equipment

The sunflower seed major aminopeptidase was purified as previously described [9] and stored in 50% (v/v) glycerol at −20 °C. (S)-2-Aminopentanoic acid, (R)- and (S)-phenylglycine, (S)-phenylalanine methyl ester hydrochloride, (S)-phenylalanine 2-naphthylamide and (S)-phenylalanine 4-methyl-7-coumarylamide were from Sigma; (S)-2-aminoheptanoic acid, (S)-2-aminohexanoic acid, (R)- and (S)-phenylalanine, 1-fluoro-2,4-dinitrophenyl-5-(S)-alaninamide and phosphorous pentachloride were from Fluka; (S)-3-amino-3-phenylpropionic acid was from Reanal; the amides of (R)- and (S)-alanine, leucine, phenylalanine and phenylglycine were from Bachem. All other reagents were of analytical grade. NMR spectra were recorded on a Bruker Avance DRX 250 spectrometer and infrared spectra—on a Bruker IFS 113 V spectrometer. UV–vis absorption and fluorescent spectra and kinetic analysis were performed on a Shimadzu UV-3000 spectrophotometer and PerkinElmer LS-5 luminometer respectively. HPLC analysis was carried out on an Agilent 1100 Series Binary LC-system equipped with a LiChrospher 100 RP-8, 5 μm column (4.6 mm × 250 mm) termostated at 20 °C.

4.2. Substrate synthesis

Compounds 1–5 and 10–13 were synthesized by acylation of the appropriate arylamine with the mixed anhydride of the corresponding amino acid by a modification of the method of Sliede et al. [23]. The powdered amino acid (2 mmol) and phosphorous pentachloride (2 mmol) were mixed in 10 ml dry dichloromethane on ice–water bath at continuous stirring. The mixture was agitated for another 3 h and evaporated to dryness under reduced pressure. The residue was dried for 1 h over potassium hydroxide *in vacuo* and the arylamine (2 mmol) dissolved in 5 ml dry tetrahydrofuran was added. The mixture was carefully heated to boiling, left for 1 h at room temperature and taken up to dryness *in vacuo*. The solid was mixed with 20 ml of water, shaken for 30 min and filtered. The filtrate was washed with 3 × 5 ml ethylacetate and then the pH was raised to 8–9 with saturated sodium hydrogencarbonate. The product was extracted with 3 × 5 ml ethylacetate, the extract was washed with brine, dried with anhydrous sodium sulfate and evaporated to dryness *in vacuo* resulting in white to yellowish crystalline product. Compound 10 crystallized from the water phase after raising pH and was collected by filtration on Buchner funnel, washed with ice-cold water and dried over potassium hydroxide *in vacuo*.

(S)-Phenylalanine 4-trifluoromethyl-7-coumarylamide (16) was synthesized as described for the corresponding 4-methyl-7-coumarylamide [24]. The product was purified by preparative thin-layer chromatography on Silica gel 60 in chloroform–methanol.

(S)-3-Amino-3-phenylpropionic acid methyl ester hydrochloride (9) was prepared by Fischer esterification. (S)-3-Amino-3-phenylpropionic acid (6 mmol) was mixed with 10 ml methanol and concentrated sulfuric acid (2 ml) was added dropwise at continuous stirring on ice–water bath over 1 h. The mixture was left to warm to room temperature and was then refluxed for 6 h. After cooling 3.9 g of anhydrous sodium carbonate were added in small portions, the mixture was shaken for a few minutes and the crystal mass was filtered off and rinsed with methanol. The filtrate was combined with the washings and taken up to dryness *in vacuo*. The solid was partitioned between 10 ml chloroform and 10 ml saturated sodium hydrogencarbonate. The organic phase was washed with brine, dried with anhydrous sodium sulfate and taken up to dryness. The solid was dissolved in 2 ml 1 M hydrochloric acid and evaporated. The residue was recrystallized from ethylacetate–methanol to yield the product as white needles.

The homogeneity of the obtained products was checked by thin-layer chromatography on Silica gel 60 in the following systems: carbon tetrachloride–chloroform–methanol 5:5:1 and carbon tetrachloride–chloroform–ethylacetate–methanol 5:5:0.5:0.5 for the arylamides; chloroform–methanol 5:1 and chloroform–methanol–ammonia 10:0.5:0.5 for the ester. The structures of the synthesized substances were confirmed by their electronic, infrared and NMR-spectra.

4.3. Substrate characterization

4.3.1. (S)-2-Aminobutyric acid 4-nitroanilide (**2**)

Yield 78%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 313 (12,600); IR (KBr) 1672, 1342 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 10.02 (s, 1H), 8.22 (d, 2H, *J* = 9.0 Hz), 7.78 (d, 2H, *J* = 9.0 Hz), 3.45–3.50 (m, 1H), 1.94–2.10 (m, 1H), 1.62–1.75 (m, 2H), 1.04 (t, 3H, *J* = 7.4 Hz).

4.3.2. (S)-2-Aminopentanoic acid 4-nitroanilide (**3**)

Yield 55%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 313 (12,200); IR (KBr) 1695, 1341 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 10.03 (s, 1H), 8.22 (d, 2H, *J* = 9.0 Hz), 7.78 (d, 2H, *J* = 9.0 Hz), 3.49–3.55 (m, 1H), 1.89–2.04 (m, 1H), 1.36–1.66 (m, 4H), 0.98 (t, 3H, *J* = 7.0 Hz).

4.3.3. (S)-2-Aminohexanoic acid 4-nitroanilide (**4**)

Yield 48%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 313 (13,400); IR (KBr) 1692, 1338 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 10.06 (s, 1H), 8.22 (d, 2H, *J* = 9.0 Hz), 7.78 (d, 2H, *J* = 9.0 Hz), 3.47–3.57 (m, 1H), 1.94–2.08 (m, 1H), 1.21–1.61 (m, 6H), 0.96 (t, 3H, *J* = 7.0 Hz).

4.3.4. (S)-2-Aminoheptanoic acid 4-nitroanilide (**5**)

Yield 47%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 313 (14,700); IR (KBr) 1695, 1342 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 10.03 (s, 1H), 8.22 (d, 2H, *J* = 9.0 Hz), 7.78 (d, 2H, *J* = 9.0 Hz), 3.46–3.54 (m, 1H), 1.90–2.04 (m, 1H), 1.83–1.88 (m, 2H), 1.16–1.62 (m, 6H), 0.90 (t, 3H, *J* = 6.5 Hz).

4.3.5. (S)-3-Amino-3-phenylpropionic acid methyl ester hydrochloride (**9**)

Yield 68%. IR (KBr): 1748 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6) δ (ppm): 7.22–7.38 (m, 5H), 4.24 (dd, 1H, *J* = 7.3 Hz, *J* = 6.0 Hz), 3.65 (s, 3H), 3.05–3.24 (m, 2H).

4.3.6. (S)-Phenylalanine anilide (**10**)

Yield 30%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 245 (10,800); IR (KBr) 1667 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6) δ (ppm): 7.59 (d, 2H, *J* = 9.8 Hz), 7.15–7.34 (m, 7H), 6.96–7.07 (m, 1H), 3.58 (dd, 1H, *J* = 7.8 Hz, *J* = 5.5 Hz), 3.01 (dd, 1H, *J* = 13.3 Hz, *J* = 5.3 Hz), 2.73 (dd, 1H, *J* = 13.5 Hz, *J* = 8.0 Hz).

4.3.7. (S)-Methyl 2-(phenylalaninamido)benzoate (**11**)

Yield 38%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 252 (9200), 305 (3800); IR (KBr) 1705, 1685 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 12.11 (s, 1H), 8.86 (dd, 1H, *J* = 8.0 Hz, *J* = 1.0 Hz), 8.08 (dd, 1H, *J* = 8.0 Hz, *J* = 1.5 Hz), 7.60 (t, 1H, *J* = 8.0 Hz), 7.28–7.39 (m, 5H), 7.15 (t, 1H, *J* = 8.0 Hz), 3.96 (s, 3H), 3.83 (dd, 1H, *J* = 9.5 Hz, *J* = 4.0 Hz), 3.40 (dd, 1H, *J* = 14.0 Hz, *J* = 4.0 Hz), 2.84 (dd, 1H, *J* = 14.0 Hz, *J* = 9.5 Hz).

4.3.8. (S)-Methyl 3-(phenylalaninamido)benzoate (**12**)

Yield 40%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 242 (10,400), 292 (1400); IR (KBr) 1704, 1686 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 9.57 (s, 1H), 8.09 (t, 1H, *J* = 2.0 Hz), 7.97–8.01 (m, 1H), 7.78 (dt, 1H, *J* = 8.0 Hz, *J* = 1.3 Hz), 7.42 (t, 1H, *J* = 8.0 Hz), 7.24–7.38 (m, 5H), 3.92 (s, 3H), 3.76 (dd, 1H, *J* = 9.5 Hz, *J* = 4.0 Hz), 3.38 (dd, 1H, *J* = 14.0 Hz, *J* = 4.0 Hz), 2.81 (dd, 1H, *J* = 14.0 Hz, *J* = 9.5 Hz).

*4.3.9. (S)-Methyl 4-(phenylalaninamido)benzoate (**13**)*

Yield 46%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 278 (17,800); IR (KBr): 1702, 1683 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 9.73 (s, 1H), 8.06 (d, 2H, J = 9.0 Hz), 7.72 (d, 2H, J = 9.0 Hz), 7.26–7.41 (m, 5H), 3.94 (s, 3H), 3.79 (dd, 1H, J = 9.5 Hz, J = 4.0 Hz), 3.42 (dd, 1H, J = 14.0 Hz, J = 4.0 Hz), 2.82 (dd, 1H, J = 14.0 Hz, J = 9.5 Hz).

*4.3.10. (S)-Phenylalanine 4-trifluoromethyl-7-coumarylamide (**16**)*

Yield 22%. UV (CH_3OH) λ_{max}, nm (ε, M^{-1} cm^{-1}): 235 (6800), 336 (10,100); ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 9.87 (s, 1H), 7.90 (s, 1H), 7.67 (d, 1H, J = 9.0 Hz), 7.49 (d, 2H, J = 9.0 Hz), 7.24–7.39 (m, 5H), 6.70 (s, 1H), 3.79 (dd, 1H, J = 9.5 Hz, J = 4.0 Hz), 3.37 (dd, 1H, J = 14.0 Hz, J = 4.0 Hz), 2.84 (dd, 1H, J = 14.0 Hz, J = 9.5 Hz).

4.4. Enzyme kinetic experiments

The kinetic experiments with amino acid arylamides were carried out in 50 mM Tris/HCl buffer, pH 7.5 containing 10% (v/v) *N*,*N*-dimethylformamide at 20 °C. The increase of the liberated arylamine was followed continuously by spectrophotometric or spectrofluorimetric assay. The methods used for the different arylamines were as follows: *4-nitroaniline* (*sph*, 410 nm, ε = 9300 M^{-1} cm^{-1}); *aniline* (*sph*, 295 nm, ε = 580 M^{-1} cm^{-1}); *methyl 2-aminobenzoate* (*sph*, 350 nm, ε = 2100 M^{-1} cm^{-1}); *methyl 3-aminobenzoate* (*sph*, 320 nm, ε = 1920 M^{-1} cm^{-1}); *methyl 4-aminobenzoate* (*sph*, 320 nm, ε = 1600 M^{-1} cm^{-1}); *2-naphthylamine* (*sph*, 340 nm, ε = 1650 M^{-1} cm^{-1}; *sfl*, λ_{ex} = 340 nm, λ_{em} = 410 nm); *7-amino-4-methylcoumarine* (*sfl*, λ_{ex} = 360 nm, λ_{em} = 440 nm); *7-amino-4-(trifluoromethyl)coumarine* (*sfl*, λ_{ex} = 400 nm, λ_{em} = 505 nm).

Kinetic experiments with amino acid methyl esters and amides were carried out in 38 mM potassium–sodium phosphate buffer, pH 7.5 at 20 °C. The reactions were followed by HPLC (isocratic elution with 30% CH_3CN in 65 mM KH_2PO_4, pH 4.5, UV-detection at 210 nm) up to 10% depletion of the substrate.

The initial rates of hydrolysis were determined in triplicate for seven substrate concentrations varying from 0.5 to 5 K_m. Kinetic parameters V_{max} and K_m were derived from nonlinear regression data analysis of the dependence of the initial rates on the substrate concentration using Enzfitter software [25]. For compounds 5, 10, 11, 12 and 20 the specificity constants k_{cat}/K_m were obtained from evaluation of the complete reaction curves at $[S] \ll K_m$.

Standard deviations were less than 10% of the corresponding mean values in all cases. The enzyme concentration was calculated from the concentration of the total protein assuming it was fully active thus the obtained k_{cat} values were minimal estimates.

4.5. Enantioselective hydrolysis of amino acid amides

The racemic amino acid amide (25 mM) in 38 mM potassium–sodium phosphate buffer, pH 7.5 was incubated at 45 °C with the purified SSMA. The substrate hydrolysis was followed by HPLC (isocratic elution with 30% (v/v) CH_3CN in 65 mM KH_2PO_4, pH 4.5, UV-detection at 210 nm). The enantiomeric purity of the produced amino acid was assessed by derivatization with 1-fluoro-2,4-dinitrophenyl-5-(S)-alanine amide (Marfey's reagent) [26] and HPLC—analysis of the resulting diastereomers (isocratic elution with 30% (v/v) CH_3CN in 0.2% triethanolamine-*ortho*-phosphoric acid, pH 3.0, UV-detection at 210 nm). The enantiomeric ratio (E) and enantiomeric excess (*ee*) were determined as described by Chen et al. [27].

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