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Application of aminoacylase I to the enantioselective resolution of α-amino acid esters and amides

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Abstract—Aminoacylase I from *Aspergillus melleus*, a readily available and inexpensive enzyme mainly used in the industrial production of enantiopure L-amino acids from their *N*-acetyl derivatives, is shown to hydrolyze the esters and amides of natural and non-natural amino acids with high enantioselectivity (for the ester hydrolysis, *E* is up to 76, in case of amides *E* >300). The reaction rates of amide and ester hydrolysis are comparable, and in some cases these conversions proceeded even faster than 'traditional' aminoacylase-catalyzed hydrolysis of *N*-acetyl derivatives thus providing new possibilities for the resolution of the corresponding racemates. This novel approach provides an alternative route for the biocatalytic production of optically active amino acids and their derivatives.

1. Introduction

Aminoacylase I (AC, *N*-acyl-L-amino acid amidohydrolase, E.C. 3.5.1.14), is a readily available and inexpensive enzyme with relaxed substrate specificity[1] that is used in the industrial production of enantiopure L-amino acids from their *N*-acyl derivatives.[2,3] In organic and water-organic medium, aminoacylase has been shown to mediate the reverse reaction—the acylation of L-amino acids[4,5]—and also perform enantioselective irreversible acyl transfer from an activated acyl donor to alcohols[6–8] and amines.[9,10] To date, nearly all scientific and industrial applications of the aminoacylase I family of enzymes have exploited the ability of these catalysts to cleave or synthesize the *N*-acyl amino moiety.

Recently, the ability of acylase I to perform enantioselective conversions of carboxylic acid derivatives has been demonstrated, for example, butanolysis and enantioselective hydrolysis of carboxylic acid esters.[11,12] Another example of esterase activity of aminoacylases has been reported,[13] where acylase I was used to hydrolyze *N*-acetyl-D,L-phenylalanine alkyl esters to give L-phenylalanine.

The fact that the activity of aminoacylase is not restricted to *N*-acyl transfer reactions gives new possibilities for the application of these enzymes in enantioselective conversions.

Herein we report on the highly effective and enantioselective hydrolysis of esters and amides of different amino acids catalyzed by aminoacylase I from *Aspergillus melleus*. This approach gives a new alternative route for the biocatalytic production of optically active amino acids and their derivatives, which nowadays are broadly used as physiologically active compounds, chiral auxiliaries or synthons and as intermediates in pharmaceutical, food and agrochemistry.

2. Results and discussion

In spite of the fact that esterase activity of aminoacylase I towards carboxylic and amino acids derivatives has recently been reported,[11] no systematic investigations describing its scope and potential applicability have been performed. Therefore, we have chosen a number of alkyl- and aryl-substituted amino acid esters and amides (see Fig. 1) as potential substrates for aminoacylase I-catalyzed enantioselective hydrolysis.

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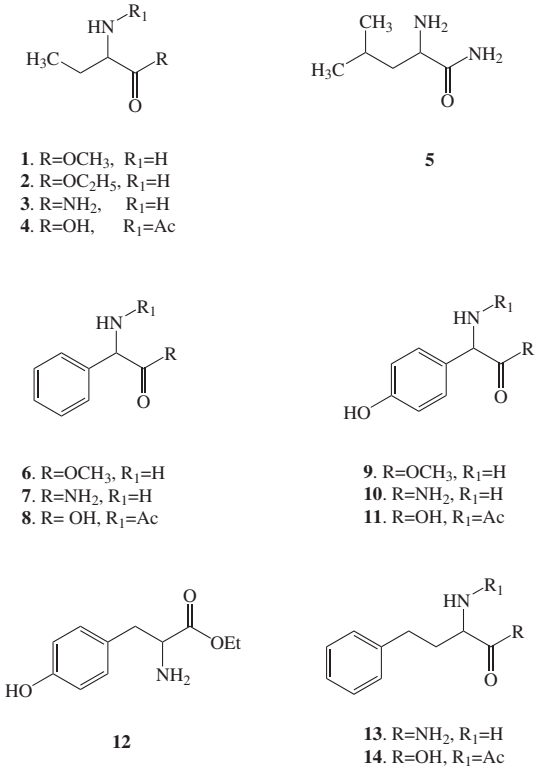

Figure 1. Structures of converted amino acid derivatives.

Hydrolytic reactions were performed in aqueous solutions at pH 7.5 for the amides (which is close to the maximum catalytic activity of aminoacylase I) and pH 6.5 in the case of esters (to suppress the nonenzymatic 'blank' hydrolysis). The results summarized in Tables 1 and 2 show that in both cases the aminoacylase was able to conduct enantioselective hydrolysis of the racemic substrates.

Table 1. Aminoacylase I-catalyzed enantioselective hydrolysis of amino acid esters

Compound	Time (h)	Preferred configuration	Conversion (%)	Ee_s (%)	E
1	3.2	L	53	92	33
2	4.0	L	52	97	76
2[a]	6.0	L	49	90	90
9	5.0	L	56	88	15
6	5.0	L	48	50	5
12	5.5	L	51	82	21

Reaction conditions: 10 mM of starting racemic compound, pH 6.5, 25 °C, 50 U of aminoacylase I.
[a] For immobilized aminoacylase I.

Among the ester substrates, the highest enantioselectivity ($E = 76$) was observed for the short alkyl-substituted compound **2**, while for the aryl-substituted amino acid esters the enantioselectivity varied from poor ($E = 5$, compound **6**) to moderate ($E = 21$, compound **12**). It is worth noting that the enantioselectivity increased significantly (more than two times) on switching from the methyl to the ethyl ester of amino butyric acid **1** and **2**.

Table 2. Aminoacylase I-catalyzed enantioselective hydrolysis of amino acid amides

Compound	Time (h)	Preferred configuration	Conversion (%)	Ee_s (%)	E
3	1.0	L	49.2	96.3	>300
5	0.5	L	50.2	97.8	320
5[a]	1.0	L	49.3	95.7	>300
7	1.5	L	50.6	>99	>300
10	1.0	L	50.1	>99	>300
13	2.5	L	48.5	92.0	240

Reaction conditions: 10 mM of starting racemic compound, pH 7.5, 25 °C, 50 U of aminoacylase I.
[a] For immobilized aminoacylase I.

In contrast with the ester substrates, all the tested amino acid amides showed excellent enantioselectivity (E >240). From Table 2 it can be seen that both alkyl- and aryl-substituted amino acid amides were hydrolyzed with extremely high enantioselectivity. In some cases (compounds **3**, **7** and **10**) it was very hard to detect even traces of D-enantiomeric hydrolysis (E >300). This is illustrated in Figure 2 for the example of aminoacylase-catalyzed hydrolysis of compound **10**. It is clear that over the course of the reaction only the L-form of the amide was converted to the corresponding L-amino acid, whereas the hydrolysis of the D-form was negligible. Similar results were obtained with substrates **3**, **7** and **13**.

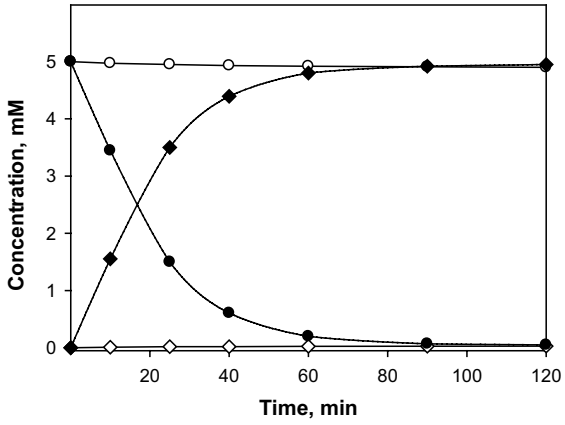

Figure 2. Reaction curves for aminoacylase I-catalyzed hydrolysis of compound **8**. ○ D-HPGA, ● L-HPGA, ◇ D-HPG, ◆ L-HPG. Reaction conditions: pH 7.5, 25 °C, 50 U of aminoacylase I.

We also performed the enantioselective hydrolysis of compounds **2** and **5** using a commercially available immobilized preparation of aminoacylase I. As can be seen from Tables 1 and 2 that the reactions were catalyzed by the immobilized enzyme proceeded as well as with the native enzyme (in both cases, enantioselectivity was slightly higher compared to the native enzyme-catalyzed hydrolysis).

As noted above, the aminoacylase I-catalyzed enantioselective hydrolysis of amino acid esters and amides can be an alternative route for the biocatalytic production of optically active amino acids and their derivatives. As a

result, it was interesting to compare these new reactions with 'traditional' aminoacylase I-catalyzed cleavage of *N*-acetyl amino acid derivatives. For this purpose, we synthesized several *N*-acetylated amino acid derivatives and compared the reaction rates and enantioselectivities of their enzymatic hydrolysis with the corresponding parameters for the hydrolysis of the carboxylic derivatives (amides) of the same amino acids. As can be seen from Table 3, the highest enantioselectivity was observed for the aminoacylase I-catalyzed hydrolysis of amino acid amides in all cases. Furthermore, in some cases the conversion of these substrates proceeded faster than the hydrolysis of the corresponding *N*-acetylated compounds (compare compounds **7** and **8** and **10** and **11**), thus demonstrating the potential of the method for the production of enantiomerically pure amino acids and their derivatives.

Table 3. Comparison of the enantioselectivity and the reaction rates for the aminoacylase I-catalyzed hydrolysis of *N*-acetylated amino acids and the corresponding amino acid amides

Compound	Initial rate, mM min^{-1}	Preferred configuration	*E*
3	0.33	L	>300
4	0.41	L	>300
7	0.35	L	>300
8	0.04	L	96
10	0.21	L	>300
11	0.03	L	110
13	0.09	L	240
14	0.62	L	70

Reaction conditions: 10 mM of starting racemic substrate, pH 7.5, 25 °C, 50 U of aminoacylase I.

3. Conclusions

Aminoacylase I from *A. melleus* was shown to catalyze the hydrolysis of the esters and amides of natural and non-natural amino acids with high enantioselectivity. The reaction rates of these conversions are comparable, and in some cases much higher than those for the 'traditional' aminoacylase-catalyzed hydrolysis of *N*-acetyl derivatives of the corresponding compounds.

4. Experimental

4.1. Materials

Acylase I from *A. melleus*, 1.3 U mg^{-1}, and immobilized (on Eupergit C) acylase I from *Aspergillus*, 63 U g^{-1} (moist material) were from Fluka. The activity of aminoacylase was measured using the standard hydrolytic assay of *N*-acetyl L-methionine.[9] One unit (U) would liberate 1 μmol of L-methionine per min. D-(−)- and L-(+)-2-Aminobutyric acid, *N*-acetyl-L-methionine, D-(−)- and L-(+)-leucine, D-(−)- and L-(+)-homophenylalanine, D-(−)- and L-(+)-tyrosine and L-tyrosine ethyl ester hydrochloride were purchased from Acros, Belgium. D-(−)- and L-(+)-leucine amide, D-(−)- and L-(+)-homophenylalanine ethyl ester, D-(−)- and L-(+)-phenylglycine amide, D-(−)- and L-(+)-*p*-hydroxy-phenylglycine methyl ester hydrochloride and D,L-tyrosine ethyl ester were purchased from Fluka.

D-(−)- and L-(+)-Aminobutyric acid ethyl and methyl esters, D-(−)- and L-(+)-aminobutyric acid amide and D-(−)- and L-(+)-homophenylalanine amide were synthesized in the Laboratory of Biocatalysis and Organic Chemistry, TUDelft, Netherlands, D-(−)- and L-(+)-phenylglycine, D-(−)- and L-(+)-phenylglycine methyl ester and D-(−)- and L-(+)-*p*-hydroxy-phenylglycine and D-(−)- and L-(+)-*p*-hydroxy-phenylglycine amide were purchased from DSM Fine Chemicals, The Netherlands. The organic solvents and buffer compounds were commercial products of analytical or HPLC grade.

4.2. Analysis and equipment

The progress of the reactions was monitored by reversed phase HPLC chromatography using a Chrompack 4.6×50 mm 5×Nucleosil C-18 column, with detection on a Waters 486 tunable absorbance detector with Waters Millenium[32] software. The hydrolysis of *N*-acetyl-L-methionine was monitored by using an acetonitrile–aqueous 50 mM phosphate buffer pH 3.0 (15:85, v/v) as an eluent at flow rate 0.5 mL min^{-1} and detection at 210 nm. The mobile phase for monitoring the hydrolytic reactions of corresponding amides, esters and their *N*-acetylated derivatives was prepared by adjusting a 0.68 g L^{-1} solution of KH_2PO_4 in acetonitrile–water (30:70, v/v) containing 0.68 g L^{-1} sodium dodecylsulfate to pH 3.0 with phosphoric acid. The flow rate was 0.5 mL min^{-1}; detection at 210 nm. The retention times of the products corresponded well to those of the chemically prepared samples.

Reaction curves and enantiomeric composition of the reaction mixtures starting from the racemic compounds were analyzed by chiral HPLC using a 150×4 mm Crownpak CR (+)-column, with detection on a Waters 486 tunable absorbance detector at 210 nm with Waters Millenium[32] software. The enantiomeric ratio (*E*) of the enantioselective conversions was calculated using sets of experimental points of ee_sor ee_p versus ξ [where ee_s (or ee_p) is the enantiomeric excess, and ξ is the conversion of the initial substrate], as described earlier.[10] The eluents used and retention times found are compiled in Table 4.

4.3. Chemical acylation of racemic amino acids

N-Acetylated compounds **6**, **9** and **12** were prepared by acylation of the corresponding amino acids with acetic anhydride in an alkaline aqueous solution.[14] Thus, 15 mmol of acetic anhydride was added dropwise to the aqueous solution, containing 10 mmol of the corresponding compound at pH 11 at 4 °C. The reaction mixture was stirred for about 30 min and then the pH value adjusted to 1.5. The precipitated product was filtered, washed with acidic ice-cold water and dried. The yields were in the range of 80–85% (w/w).

Table 4. Chiral HPLC for compounds **1**–**14**

Compound	Column	Mobile phase	Flow rate (mL min^{-1})	Retention time (min)	
				D	L
1	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.3, 20 °C	0.6	5.0	12.9
2	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.3, 20 °C	0.6	9.5	25.3
3	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.3, 20 °C	0.6	2.5	3.7
4[a]	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.0, 20 °C	0.6	2.1	3.2
5	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.3, 20 °C	0.6	5.6	9.7
6	Crownpak CR(+)	Aqueous $HClO_4$ pH 2.0, 20 °C	0.6	14.3	24.9
7	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.0, 20 °C	0.6	5.6	26.1
8[a]	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.5, 20 °C	0.6	9.1	33.7
9	Crownpak CR(+)	Aqueous $HClO_4$ pH 2.0, 20 °C	0.6	8.4	19.7
10	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.0, 20 °C	0.6	3.8	21.8
11[a]	Crownpak CR(+)	Aqueous $HClO_4$ pH 1.5, 20 °C	0.6	5.8	28.4
12	Crownpak CR(+)	Aqueous $HClO_4$ pH 2.0, 20 °C	0.6	18.5	35.7
13	Crownpak CR(+)	MeOH–aqueous $HClO_4$ pH 1.5 (10:90, v/v), 20 °C	0.6	19.5	37.0
14[a]	Crownpak CR(+)	MeOH–aqueous $HClO_4$ pH 2.0 (10:90, v/v), 20 °C	0.6	41.2	87.5

[a] In these cases, the reactions were followed by monitoring the free D- and L-amino acids, accumulated over the course of enzymatic hydrolysis.

^{1}H NMR (300 MHz, DMSO-d_6) of **4**: δ (ppm) 0.95 (t, 3H, CH_2CH_3), 1.78 (m, 2H, CH_2CH_3), 1.93 (s, 3H, $COCH_3$), 4.1 (d, 1H, $CHCO_2H$), 8.43 (d, 1H, NH), 11.9 (s, 1H, CO_2H).

^{1}H NMR (300 MHz, DMSO-d_6) of **8**: δ (ppm) 1.92 (s, 3H, $COCH_3$), 5.4 (d, 1H, $CHCO_2H$), 7.38 (m, 5H, aromatic protons), 8.5 (d, 1H, NH), 12.6 (s, 1H, CO_2H).^{1}H NMR (300 MHz, DMSO-d_6) of **11**: δ (ppm) 1.89 (s, 3H, $COCH_3$), 5.35 (d, 1H, $CHCO_2H$), 6.57 (s, 1H, PhOH), 7.11–7.44 (dd, 4H, C_6H_4(OH)), 8.54 (d, 1H, NH), 12.7 (s, 1H, CO_2H).

^{1}H NMR (300 MHz, DMSO-d_6) of **14**: δ (ppm) 1.89 (s, 3H, $COCH_3$), 1.97 (m, 2H, CH_2CH_2), 2.62 (m, 2H, $PhCH_2$), 4.15 (q, 1H, $CHCO_2H$), 7.26 (m, 5H, aromatic protons), 8.1 (d, 1H, NH), 12.38 (s, 1H, CO_2H).

4.4. Enzymatic hydrolytic reactions

Enzymatic reactions were carried out in a 10 mL closed reaction vessel at the appropriate temperature and pH values under permanent stirring. The reactants were dissolved in 50 mM Tris buffer with reactions initiated by adding 50 U of aminoacylase I. Further details are given in the notes to the tables. Nonenzymatic (blank) reactions were performed under the same conditions but without adding enzyme. Samples were withdrawn over the course of the reaction, centrifuged and subjected to HPLC analysis as described above.

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