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Studies on the enhancement of enantioselectivity in the microbial protease-catalyzed hydrolysis of *N*-free non-protein amino acid esters

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Abstract

We have recently reported a marked enhancement of enantioselectivity by switching the conventional methyl ester to esters with a longer alkyl chain such as the isobutyl ester in the *Aspergillus oryzae* protease-catalyzed enantioselective hydrolysis of *N*-unprotected amino acid esters. The present study reveals that the enantioselectivity enhancement is also attained by changing the pH of the reaction mixture, the optimal pH in terms of enantioselectivity being around 6.2. Kinetic measurements indicate that the most significant factor responsible for the observed enantioselectivity enhancement is the change of the K_m value for the L-series.

Keywords: *Aspergillus oryzae* protease; Enantioselective hydrolysis; Isobutyl ester; Enantioselectivity enhancement; pH effect; Enzyme kinetics

1. Introduction

Homochiral non-protein amino acids are useful building blocks for the synthesis of analogs of biologically active peptides [1] and versatile chiral starting materials or chiral auxiliaries for other synthetic purposes [2]. We investigated previously the resolution of non-protein amino acids via the enantioselective hydrolysis of the methyl esters of their *N*-benzyloxycarbonyl (Z) derivatives using two microbial proteases from *Aspergillus oryzae* and *Bacillus subtilis* [3,4], the latter protease generally yielding better results than the former one. In some cases, however, the hydrolysis rates were rather slow, which makes the method less practical. Accordingly, we examined amino acid esters bearing a free α-amino group as substrates which have better solubilities than the corresponding *N*-protected derivatives in the expectation of an enhancement of the hydrolysis rate. When an *N*-free amino acid ester was substituted for the corresponding *N*-protected amino acid ester, however, the enantioselectivity was deteriorated strikingly with the conventional methyl ester. We have recently reported a marked enhancement of enantioselectivity by modifying the ester moiety in the *A. oryzae* protease-catalyzed enantioselective hydrolysis of *N*-unprotected amino acid esters [5]. Thus, the above-mentioned difficulty was overcome by employing esters bearing a longer alkyl chain such as the isobutyl ester. Utilizing this ester, amino acids carrying an aromatic side chain were resolved with excellent enantioselectivities. With amino acids bearing an aliphatic side chain also, good results in terms of the hydrolysis rate and enantioselectivity were obtained by employing such an ester as the isobutyl ester. Moreover, the enantioselectivity proved to be enhanced further by conducting the reaction at low temperature.

In the present study, the effect of pH on the enantioselectivity of the ester hydrolysis was investigated using *A. oryzae* protease. Furthermore, in order to gain an insight into the marked enhancement of enantioselectivity caused by the use of esters with a longer alkyl chain, the kinetic parameters were determined for the *A. oryza*e protease-catalyzed hydrolyses.

2. Experimental

2.1. General

^{1}H NMR spectra were obtained at 300 MHz on a Varian Unity 300 spectrometer using DMSO-d_6 as a solvent with TMS as an internal standard. Mp's were determined on a Yamato MP-21 apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-4 digital polarimeter. All organic solvents

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were distilled following standard protocols. L- and D-Norvaline (2-aminopentanoic acid, Nva) were purchased from Aldrich. The liquid chromatograph employed was a Shimadzu LC-10AS instrument, equipped with a Rheodyne 7725i sample injector and a Shimadzu SPD-10A variable wavelength UV monitor. A Shimadzu C-R6A data processor was used for data acquisition and processing.

2.2. Materials

2.2.1. Preparation of esters of DL-, L- and D-Nva

The preparation of the methyl or isobutyl ester of DL-Nva employed in this study has been described elsewhere [5]. L-Nva isobutyl ester hydrochloride was prepared by modifying the procedure for preparing amino acid methyl esters proposed by Brenner and Huber [6]. Thionyl chloride (8 ml) was added dropwise below 0 °C under stirring to isobutyl alcohol (2-methyl-1-propanol; 50 ml) which had been pre-cooled to −10 °C. After 15 min the L-Nva (2.51 g) was added and the reaction mixture was stirred at 40 °C for 90 h. The mixture was evaporated in vacuo, and the residue was recrystallized from ethyl acetate–ether to yield white needles; 4.49 g (99%); mp 90–91.5 °C; $[\alpha]_D^{25}$ + 9.6° (*c* 1.0, MeOH); >99.9% enantiomeric excess (e.e.) by chiral HPLC on a Sumichiral OA-5000 column (4.6 mm i.d. × 150 mm; Sumika Chemical Analysis Service, Japan) under the following conditions [7]: mobile phase, 3 mM copper(II) sulfate in water–2-propanol (95:5, v/v); flow rate, 1.0 ml min^{-1}; column temperature, 30 °C and detection, UV at 254 nm. ^{1}H NMR (DMSO-d_6) δ 0.86 (3H, t, J = 7.2 Hz), 0.89 (6H, d, J = 6.6 Hz), 1.20–1.45 (2H, m), 1.76 (2H, q-like, J = ca. 7.5 Hz), 1.83–1.96 (1H, m), 3.88–4.00 (3H, m), 8.50 (3H, s). D-Nva isobutyl ester hydrochloride was likewise prepared: mp 90–91.5 °C; $[\alpha]_D^{25}$ − 9.7° (*c* 1.0, MeOH); >99.9% e.e. by HPLC.

Methyl esters were prepared at ordinary temperature according to the literature [6]. L-Nva methyl ester hydrochloride: mp 103–105 °C (EtOH–ether); $[\alpha]_D^{25}$ + 21.7° (*c* 1.0, MeOH); >99.9% e.e. by HPLC. ^{1}H NMR (DMSO-d_6) δ 0.85 (3H, t, J = 7.2 Hz), 1.20–1.45 (2H, m), 1.74 (2H, q-like, J = ca. 7.4 Hz), 3.72 (3H, s), 3.96 (1H, t, J = 6.3 Hz), 8.59 (3H, s). D-Nva methyl ester hydrochloride: mp 104–105 °C; $[\alpha]_D^{25}$ −21.5° (*c* 1.0, MeOH); >99.9% e.e. by HPLC.

2.2.2. Enzymes

Protease from *A. oryzae* (protease A) was supplied by Amano Pharmaceutical Co. (Japan) and had a specific activity of 10 U/mg solid (pH 7.0) (one Amano unit is defined as the enzyme quantity which hydrolyses casein to produce color equivalent to 400 mg of L-Tyr per 60 min at 37 °C, according to the supplier). This enzyme preparation was used "straight from the bottle".

2.3. pH effect on the A. oryzae protease-catalyzed hydrolysis of DL-Nva isobutyl ester

DL-Nva isobutyl ester hydrochloride (84 mg; 0.4 mmol) was dissolved in 2.5 ml of a buffer solution with a certain pH, i.e., 0.1 M phosphate buffer (pH 7) or McIlvaine buffer (pH 6.5, 6.2, 6.0, 5.8, 5.5 or 5.0) (made with 0.1 M citric acid and 0.2 M dibasic sodium phosphate). The pH was adjusted to the desired value with 0.5 M NaOH. On the other hand, a protease preparation (20 mg) was added to 1 ml of the same buffer, mixed up and centrifuged, and 0.5 ml of the supernatant was added to the above substrate solution. The resulting mixture was stirred at 30 °C. The pH was maintained constant with 0.1 M NaOH using a pH-stat autotitration system (Hiranuma Autotitrator COMTITE-550, Japan). The progress of the reaction was followed by the consumption of the alkali. After the desired degree of conversion, the reaction mixture was treated as usual to separate the liberated amino acid, the e.e. value of which was estimated by chiral HPLC as described before [5]. The results are shown in Table 1.

Table 1
pH effect on the *A. oryzae* protease-catalyzed hydrolysis of the isobutyl ester of DL-Nva[a]

pH	% Convn.	Time (min)	% e.e.$_P$[b]	*E*
7.0	40	77	92	45
6.5	40	240	93	52
6.2	40	630	95	75
6.0	40	770	95	75
5.8	40	24.5 h	95	75
5.5	30	22.5 h	93	41
5.0	20	27.5 h	94	41

[a] Reactions were conducted as described in Section 2 using 0.4 mmol of DL-Nva isobutyl ester hydrochloride at 30 °C.
[b] Enantiomeric excess of the liberated amino acid.

2.4. Kinetic measurements

The kinetic studies were conducted by measuring the initial rates of hydrolysis of L- and D-Nva methyl ester (hydrochloride) and L- and D-Nva isobutyl ester (hydrochloride). In a typical experiment, the substrate stock solution (240 mM) was prepared by dissolving each substrate in 0.1 M NaCl. On the other hand, the enzyme stock solution (33.3 mg ml^{-1}) was prepared by dissolving *A. oryzae* protease in 1 mM HCl. A certain amount of the substrate solution (0.5–4.0 ml), 50 μl of the enzyme solution and suitable amount of 0.1 M NaCl (necessary for making up the total volume of 20 ml) were stirred at 25 °C. A stream of nitrogen was blown over the reaction mixture to prevent CO_2-absorption. The liberated acid was titrated with 0.01 M NaOH back to the initial pH using the pH-stat autotitration system. The initial rate of the reaction was measured by following the consumption of the alkali. Some 16 data points were usually collected in a run. Initial concentration of the substrate ester was changed from 6 to 48 mM. The Lineweaver–Burk ($1/V_0$ against $1/[S]_0$) plot was used for calculating V_{max} and K_m, assuming Micheelis–Menten kinetics [8]. The V_{max} and K_m values thus obtained by the least-squares method are shown in Table 2.

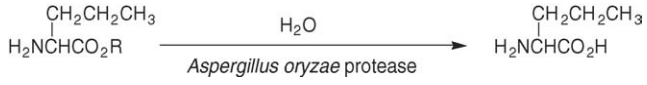
3. Results and discussion

We have recently reported a marked enhancement of enantioselectivity by modifying the ester moiety in the *A. oryzae* protease-catalyzed enantioselective hydrolysis of *N*-unprotected

Table 2
Kinetic parameters for the *A. oryzae* protease-catalyzed hydrolysis of Nva esters[a]

Ester	V_{max} (mM s^{-1} mg(protease)$^{-1}$)	K_m (mM)
L-Nva-OMe	$(1.3 \pm 0.1) \times 10^{-4}$	$(1.4 \pm 0.1) \times 10^{2}$
D-Nva-OMe	$(2.7 \pm 0.7) \times 10^{-5}$	$(2.7 \pm 0.4) \times 10^{2}$
L-Nva-OBu^{i}	$(1.5 \pm 0.3) \times 10^{-4}$	$(1.4 \pm 0.3) \times 10$
D-Nva-OBu^{i}	$(4.0 \pm 0.5) \times 10^{-5}$	$(3.9 \pm 0.4) \times 10^{2}$

[a] Reactions were conducted in 0.1 M NaCl (pH 6.2) at 25 °C as described in Section 2.



Scheme 1. *A. oryzae* protease-catalyzed hydrolysis of Nva esters (R = Me or Bu^{i}).

amino acid esters [5]. With the conventional methyl ester, the enantioselectivity was deteriorated strikingly when an *N*-free amino acid ester was substituted for the corresponding *N*-protected amino acid ester [3,4]. This difficulty was overcome by employing esters bearing a longer alkyl chain, such as the isobutyl ester. Utilizing this ester, amino acids carrying an aromatic side chain were resolved with excellent enantioselectivities. With amino acids bearing an aliphatic side chain also, good results in terms of the hydrolysis rate and enantioselectivity were obtained by employing such an ester as the isobutyl ester. In all the cases examined, the preferential hydrolysis of the L-enantiomers was confirmed by comparison with authentic samples prepared from the optically active amino acids on HPLC or suggested from the regularity of elution order of the enantiomers on HPLC [7].

Of potential factors affecting the enantioselectivity as well as the activity, optimization of pH must be extremely important in optimizing enzymatic reactions. Accordingly, we examined the effect of pH on the *A. oryzae* protease-catalyzed ester hydrolysis employing the isobutyl ester of Nva as a substrate (Scheme 1). Table 1 shows the changes of the hydrolysis rate and enantioselectivity at varying pH values (7.0–5.0). Lowering the pH from 7 resulted in a marked decrease in the hydrolysis rate, which was extremely retarded below pH 6. Concerning the effect on enantioselectivity, a rough bell-shaped correlation was observed between the enantioselectivity of the hydrolysis and the pH of the reaction mixture. The highest value of E [9] was obtained around pH 6.2. Accordingly, the following experiments were conducted at this pH value.

In order to gain an insight into the marked enhancement of enantioselectivity caused by the use of esters with longer alkyl chains such as the isobutyl ester, the kinetic parameters were determined for the *A. oryza*e protease-catalyzed hydrolyses. The methyl or isobutyl ester of enantiomerically pure L- or D-Nva were subjected to hydrolysis at pH 6.2. The pH of the reaction mixture was kept constant by automatic titration with 0.01 M NaOH during the hydrolysis. Its progress was monitored by the consumption of the alkali to obtain the initial rate (V_0). The concentration of the substrate ester $[S]_0$ was systematically changed, and the Lineweaver–Burk ($1/V_0$ against $1/[S]_0$) plot afforded a straight line [8]. The V_{max} and K_m values were obtained by the least-squares method. The results are shown in Table 2. The V_{max} value for the L-methyl ester was a few times larger than that for the D-counterpart, and the K_m value for the latter ester was only about twice as large as that for the former. This explains the L-stereopreference with a rather low enantioselectivity observed when the methyl ester was employed as the substrate. When the substrate was changed from the methyl ester to the isobutyl ester, the K_m value for the L-series was reduced to ca. 1/10, while the corresponding V_{max} value was almost unaltered. By contrast, only a small change was observed with the K_m value as well as with the V_{max} value for the D-series. Thus, the most significant factor responsible for the observed enhancement of enantioselectivity is the change of the K_m value for the L-series. These results indicate that the role of the ester moiety becomes relatively important in the substrate recognition by the protease when an acylamino group such as the *N*-Z-amino group is replaced by a free amino group: the ES-complex derived from the L-isobutyl ester became one order of magnitude more stable than that from the L-methyl ester. On the contrary, the rate of acyl-enzyme formation, to which the observed V_{max} value must be related, was not largely affected by the difference in the ester alkyl groups, as understood easily by comparing their electronic nature.

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