Pergamon

Tetrahedron: Asymmetry, Vol. 8, No. 3, pp. 367–370, 1997

Printed in Great Britain
PII: S0957-4166(97)00002-5
0957-4166/97 $17.00 + 0.00

Resolution of non-protein amino acids via microbial protease-catalyzed ester hydrolysis: marked enhancement of enantioselectivity by the use of esters with longer alkyl chains and at low temperature

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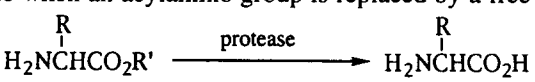
Abstract: In the microbial protease-catalyzed hydrolysis of amino acid esters with the free α-amino group, the enantioselectivity can be enhanced greatly by employing esters with longer alkyl chains such as the isobutyl ester instead of the conventional methyl ester and by conducting the reaction at low temperature. © 1997 Elsevier Science Ltd. All rights reserved.

Microbial proteases from a variety of sources are commercially available now. Although they are inexpensive, stable and easy to handle (*e.g.*, they require no added cofactors), their use as synthetic catalysts has been limited. We report here the utilization of microbial proteases from *Aspergillus oryzae* and *Bacillus subtilis*[1,2] for the resolution of non-protein amino acids through the enantioselective hydrolysis of their *N*-unprotected esters. Homochiral non-protein amino acids are useful building blocks for the synthesis of analogs of biologically active peptides and versatile chiral starting materials or chiral auxiliaries for other synthetic purposes. During the course of this investigation, we have found that the enantioselectivity can be significantly improved by choosing an appropriate ester grouping and reaction temperature. This information forms the subject of the present communication.

Initially, the methyl esters of several halogenated phenylalanines **1** (R=X-PhCH$_2$) were subjected to hydrolysis with *A. oryzae* protease (protease A[3]) at pH 7 and 30°C. After the desired degree of conversion (*ca.* 40%), the liberated amino acid **2** was isolated and its enantiomeric excess (ee) was determined by chiral HPLC analysis (on Sumichiral OA-5000). Although the hydrolysis of these methyl esters with the free α-amino group proceeded more smoothly than that of the corresponding *N*-protected esters,[2] the enantioselectivities deteriorated strikingly, as judged from the values of enantiomeric ratio, *E*[4] (entries 1, 8 and 10, Table 1).[5] Accordingly, the influence of ester groups was examined next using 4-fluorophenylalanine **1** (R=4-F-PhCH$_2$) as a model amino acid. It was gratifying to find that the use of the *n*-butyl (entry 3) or isobutyl ester (entry 4) resulted in no retardation of the hydrolysis rate and yet with a marked enhancement of enantioselectivity. Thus, the resolution of other halogenated phenylalanines was examined by employing their isobutyl esters[6] as substrates, and sufficiently high enantioselectivities were achieved in all the cases (entries 7, 9 and 11–14).[7] These results[8] imply that the role of the ester moiety becomes relatively important in the substrate recognition by the protease when an acylamino group is replaced by a free amino group.



1 **2**

Ring-substituted phenylalanines have been resolved by the action of α-chymotrypsin on their *N*-free ethyl esters.[9] We observed that 4-fluorophenylalanine methyl ester was hydrolyzed at pH 5.0 using this mammalian protease with an excellent enantioselectivity (94% ee(P) at 47% convn., *E*=100),

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Table 1. *Aspergillus oryzae* protease-catalyzed hydrolysis of aromatic amino acid esters **1**[a]

Entry	R	R'	% convn.	Time/min	% ee(P)[b]	E[c]
1	4-F-PhCH$_2$	Me	48	79	47	4.2
2	4-F-PhCH$_2$	*i*-Pr	35	230	67	7.1
3	4-F-PhCH$_2$	*n*-Bu	34	24	99	300
4	4-F-PhCH$_2$	*i*-Bu	47	25	97	170
5	4-F-PhCH$_2$	Bzl	39	5	91	37
6	3-F-PhCH$_2$	Me	23	30	84	15
7	3-F-PhCH$_2$	*i*-Bu	33	30	98	150
8	2-F-PhCH$_2$	Me	24	45	7.5	1.2
9	2-F-PhCH$_2$	*i*-Bu	44	55	92	50
10	4-Cl-PhCH$_2$	Me	43	59	46	3.7
11	4-Cl-PhCH$_2$	*i*-Bu	46	30	98	340
12	2-Cl-PhCH$_2$	*i*-Bu	38	20	99	720
13	4-Br-PhCH$_2$	*i*-Bu	52	25	91	120
14	PhCH$_2$	*i*-Bu	31	24	>99	>300

[a] An amino acid ester hydrochloride (0.2 mmol) was dissolved in 2.5 ml of 0.1 M phosphate buffer (pH 7). The pH was adjusted to 7.0 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 shaken at 30 °C. The reaction was monitored by HPLC on a Nucleosil 5C$_8$ column. [b] Enantiomeric excess of the liberated amino acid **2**. [c] Enantiomeric ratio.[4]

but not enantiospecifically.[10] Moreover, the enantioselectivities observed with the methyl esters of other halogenated phenylalanines were rather low (3-F derivative, E=23; 2-F derivative, E=1.3; 4-Cl derivative, E=1.0). In contrast, replacing the methyl ester with the *n*-butyl (E=200) or isobutyl ester (E=280) led to a marked enhancement of enantioselectivity, as illustrated in the case of 4-fluorophenylalanine.

The influence of the ester grouping was also explored with *N*-unprotected amino acids bearing aliphatic side-chains. When a series of esters of norvaline **1** (R=*n*-Pr) and norleucine **1** (R=*n*-Bu) were subjected to hydrolysis with *A. oryzae* protease, the enantioselectivity was increased progressively with the length of the ester alkyl chain (entries 1–5 and entries 8–11, Table 2). In these cases also, good results in terms of the hydrolysis rate and enantioselectivity were obtained using isobutyl esters. The enantioselectivity seems to fall again when the ester alkyl chain becomes longer (entry 7). Thus, the isobutyl esters of other aliphatic amino acids were hydrolyzed to show rather high enantioselectivities (entries 13 and 15–17), which, however, were lower than those observed with the aromatic amino acid derivatives. We found that conducting the hydrolysis at a low temperature (5°C) was quite effective in improving the enantioselectivity, as illustrated by the cases of norvaline (entry 6), norleucine (entry 12) and 2-aminobutanoic acid **1** (R=Et) (entry 14), though much longer times were necessary to achieve reasonable degrees of conversion.[11]

The enhancement of enantioselectivity by modifying the alcohol moiety of the substrate ester seems to be rather general, and this possibility should be considered when the enantioselectivity is inadequate with the methyl or ethyl ester usually used in the ester hydrolysis. The kinetic aspect must be investigated for a full understanding of this phenomenon.

Table 2. *Aspergillus oryzae* protease-catalyzed hydrolysis of aliphatic amino acid esters **1**[a]

Entry	R	R'	% convn.	Time/min	% ee(P)[b]	E[c]
1	*n*-Pr	Me	40	170	58	5.5
2	*n*-Pr	Et	40	280	72	10
3	*n*-Pr	*n*-Pr	40	250	75	12
4	*n*-Pr	*n*-Bu	40	150	88	30
5	*n*-Pr	*i*-Bu	40	77	92	45
6[d]	*n*-Pr	*i*-Bu	40	400	97	130
7	*n*-Pr	*n*-Pentyl	40	190	77	13
8	*n*-Bu	Me	40	160	53	4.6
9	*n*-Bu	Et	39	200	67	7.8
10	*n*-Bu	*n*-Pr	39	170	86	24
11	*n*-Bu	*i*-Bu	40	140	95	76
12[d]	*n*-Bu	*i*-Bu	41	19.7 (h)	98	220
13	Et	*i*-Bu	40	190	78	14
14[d]	Et	*i*-Bu	40	22.3 (h)	95	78
15	*i*-Bu	*i*-Bu	40	320	94	66
16	*n*-Pentyl	*i*-Bu[e]	33	280	89	27
17	*i*-Pentyl	*i*-Bu[e]	33	14.5 (h)	77	11

[a] The hydrolysis was conducted as described above except that 0.4 mmol of a substrate ester hydrochloride (unless otherwise noted) was used, the pH was maintained at 7 by automatic titration with 0.1 M NaOH, and stirring was employed instead of shaking. The progress of the reaction was followed by the consumption of the alkali. The reaction temperature was 30 °C unless otherwise noted. [b] Enantiomeric excess of the liberated amino acid **2**. [c] Enantiomeric ratio.[4] [d] At 5 °C. [e] Tosylate.

Acknowledgements

We are grateful to Amano Pharmaceutical Co. for the generous gift of proteases used in this study. This work was financially supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (to T. M.).

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(Received in Japan 2 December 1996)