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Enzymatic resolution of amino acids via ester hydrolysis

Review Article

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Summary. The present review outlines recent examples of enzyme-based resolution procedures for amino acids via the hydrolysis of their esters. The resolutions have been achieved by using proteases (α-chymotrypsin, subtilisin and other microbial proteases, and sulfhydryl proteases of plant origin) and lipases. Relevant work utilizing yeast and other microbial cells is also included.

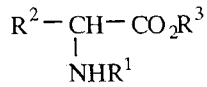
Keywords: Amino acids – Enzymatic resolution – Ester hydrolysis – Proteases – Lipases

Besides some 20 amino acids universally distributed as protein constituents in living organisms, there are hundreds of other amino acids of non-protein origin (Hunt, 1985), which are often referred to as unusual amino acids. Enantiomerically pure unusual amino acids, including those synthetic compounds which have not been found in nature, are useful as building blocks for the synthesis of analogs of biologically active peptides such as toxins, antibiotics, hormones and enzyme inhibitors (Roberts and Vellaccio, 1983). They are also versatile chiral starting materials or chiral catalysts in the synthesis of more complex chiral compounds (Coppola and Schuster, 1987). For the supply of unusual amino acids in quantity, the chemical synthesis of racemic forms followed by their optical resolution is still the preferable way, though a number of methods have been developed for the asymmetric syntheses of amino acids (Williams, 1989; Duthaler, 1994). For the resolution of racemic amino acids, enzymatic methods have often been employed besides chemical methods based on the formation of diastereomeric salts or derivatives (Barrett, 1985; Williams, 1989). The enzymatic methods may be the most advantageous if a number of requirements are met, e.g., the enzyme must be easily available (or inexpensive) and sufficiently stable, the amino acid derivatives to be used as substrates must be readily accessible, the operation must be

easy, the reaction products must be easily separated and purified, and they must have high optical purities. Hydrolytic enzymes have often been used for this purpose. For example, acylase I (aminoacylase; *N*-acylamino acid amidohydrolase; EC 3.5.1.14) has been broadly applied to the resolution of the proteinogenic amino acids and unusual α-amino acids (Greenstein and Winitz, 1961b; Chibata et al., 1976), because it has a wide substrate specificity. Thus, it is the most frequently used enzyme in the chemoenzymatic synthesis of the L ($\equiv$ *S*) forms of amino acids from the racemic *N*-acyl derivatives. An extensive study was conducted of the substrate specificity of porcine kidney acylase and the mold enzyme from *Aspergillus oryzae*, especially for the resolution of unnatural and rarely occurring amino acids (Chenault et al., 1989). However, the most commonly used enzymes such as porcine renal acylase I are not always applicable to the resolution of unusual amino acids. Accordingly, it is worthwhile to develop novel enzymatic resolution methods for them. This review briefly outlines recent examples of enzyme-based resolution procedures for amino acids via the hydrolysis of their esters. These are subdivided into the following major alternative approaches depending on the enzyme used: α-chymotrypsin and subtilisin; lipases; other microbial proteases and sulfhydryl proteases of plant origin; yeast and other microbial cells. The author has already written a review on the enzymatic resolution of racemic fluorine-containing amino acids (Miyazawa, 1995).

1 α-Chymotrypsin and subtilisin

α-Chymotrypsin (EC 3.4.21.1) is one of the most popular enzymes which have been used for the resolution of racemic amino acids through the enantioselective hydrolysis of their esters. This enzyme is a serine protease which catalyzes primarily the hydrolysis of amide bonds adjacent to the carbonyl groups of aromatic amino acid residues. A preparation from bovine pancreas is commercially available inexpensively. The enzyme possesses esterase activity also and catalyzes the hydrolysis of various esters. *N*-Acetyl-DL-amino acid esters were resolved by this method early in the 1950's (Jones and Beck, 1976). The enzyme catalyzes the hydrolysis of L-amino acid ester derivatives, leaving the D-counterparts untouched. *N*-Unprotected esters also have been used as substrates. Thus, ethyl esters of ring-substituted phenylalanines were resolved with α-chymotrypsin at pH 5 (to eliminate the danger of spontaneous hydrolysis of the esters), yielding the L-amino acids and unchanged D-esters in very high enantiomeric excess (Tong et al., 1971). The examples of α-chymotrypsin-catalyzed resolution of amino acids reported before 1980 are compiled in some review articles (Jones and Beck, 1976; Gais, 1995). Here in this review, only those reported afterwards are mentioned. As aromatic amino acids are usually good substrates for α-chymotrypsin, numerous ring-substituted tryptophans and phenylalanines, and amino acids carrying heteroaromatic rings in the side chain have been resolved by this procedure (Scheme 1). In most of these cases, the enantiomeric excess (e.e.) values of the products are not reported, while their



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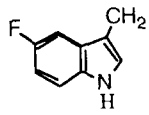
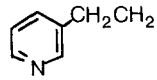
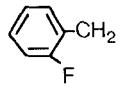
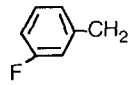
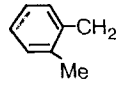
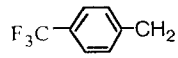
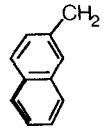
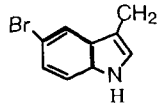
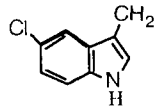
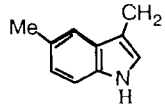
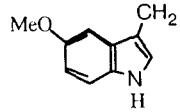
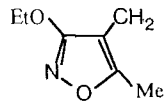
H		Me	5.0	[1]
PhCO		Me	7.8	[2]
H		Et	5.0	[3]
H		Et	5.0	[3]
Ac		Me	7.0	[4]
Ac		Me	7.0	[4]
Ac		Me	7.0	[4]
Ac		Me	7.0	[4]
Ac		Me	7.0	[4]
Ac		Me	7.0	[4]
Ac		Me	7.0	[4]
Ac		Et	7.8	[5]

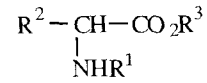
[1] Gerig and Klinkenborg, 1980
[2] Ratcliffe et al., 1985
[3] Sheardy et al., 1986
[4] Porter et al., 1987
[5] Nielsen et al., 1993

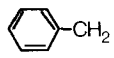
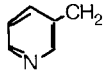
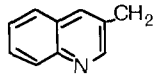
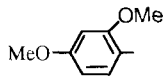
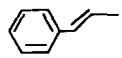
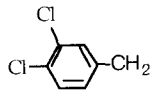
Scheme 1. Amino acid esters resolved by α-chymotrypsin-catalyzed hydrolysis in aqueous solution

optical rotations are given instead. Methyl *N*-acetyl-β-phenylserinates (*erythro* and *threo*), methyl *N*-acetyl-β-(*p*-nitrophenyl)serinate (*erythro*), and methyl *N*-benzoylthreoninates (*erythro* and *threo*) were resolved with high enantioselectivities, yielding the (2*S*)-isomers (Chênevert and Létourneau, 1986; Chênevert et al., 1990). The *threo* form of β-(*p*-nitrophenyl)serinate was not a substrate for this protease.

Amino acid esters bearing aliphatic side chains have also been resolved. A series of ethyl esters of *N*-*t*-butyloxycarbonyl (Boc) derivatized alkenyl-α-amino acids were resolved via hydrolysis by α-chymotrypsin to give the corresponding L-acids (Schricker et al., 1992). Among the hydrolytic enzymes tested, α-chymotrypsin was the most effective, showing broad substrate specificity, high enantioselectivity (86–96% e.e.) and high chemical yields (up to 99%). The resolution of methyl esters of *N*-unprotected α-methylamino acids (α-methyltryptophan, α-methylphenylalanine, and α-methyl-*p*-fluorophenylalanine) was achieved using α-chymotrypsin at pH 5 to give the corresponding L-acids (Anantharamaiah and Roeske, 1982). The hydrolysis rates were slow compared with those of the corresponding simple α-amino acid esters, but sufficiently rapid to be a practical resolution method. The introduction of an α-methyl group into an unacylated amino acid ester lowered the hydrolysis rate much less dramatically than that into an acylated amino acid ester.

Subtilisin Carlsberg (EC 3.4.21.62) is another serine protease which has often been employed as a catalyst for the resolution of amino acids. This protease is isolated from *Bacillus licheniformis* (its source was until recently wrongly refereed to as *Bacillus subtilis*; Jacobs et al., 1985). It is known to display a broader substrate specificity than α-chymotrypsin, though it exhibits an α-chymotrypsin-like preference for aromatic amino acid residues (Morihara, 1974). Although there are significant differences in structure between α-chymotrypsin and subtilisin Carlsberg, the two proteases have a similar catalytic mechanism. Scheme 2 summarizes the examples of amino acids which have been resolved through the enantioselective hydrolysis of their esters using subtilisin Carlsberg. In most cases the methyl or ethyl esters of *N*-protected amino acids were used as substrates, while in some cases *N*-unprotected esters were also employed. The aromatic substrates were usually suspended or slurried before the addition of the protease. The pH range at which hydrolyses were performed was 6.5 to 8.1. The protease preferentially hydrolyzes L-amino acid esters and leaves D-amino esters untouched. Although the e.e. values of the products were not always given explicitly in the literature, the enantioselectivities shown by this protease were good to excellent. In addition to the examples shown in Scheme 2, optically active D-arylglycines were prepared through the enantioselective hydrolysis of their *N*-protected methyl esters using immobilized subtilisin in two phase systems consisting of water and an immiscible organic solvent (methyl isobutyl ketone or methylene chloride) (Schutt et al., 1985). This procedure increases the substrate solubility and reduces the rate of non-enzymatic hydrolysis, improving the yields and optical purities of the products. The proportion of the organic solvent could be 75% by volume without serious damage to the



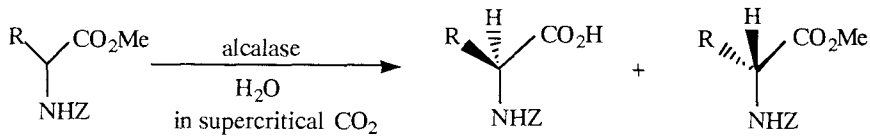
R^1	R^2	R^3	pH	L-Acid		D-Ester		Ref.
				% ee	Yield (%)	% ee	Yield (%)	
Ac		Me	7.5	98	96		98	[1]
PhCO		Me	7.6		91[a]		90	[2]
Ac		Et	6.5–7.0		quant.[a]		99	[3]
Ac		Et		97	95	91	quant.	[4]
Ac		Et		90	89	95	quant.	[4]
Ac		Et		82	quant.	93	95	[4]
Ac		Et	6.5–7.0	96	90		90	[5]

[a] After hydrolysis with HCl.
[1] Roper and Bauer, 1983
[2] Folkers et al., 1984
[3] Acosta et al., 1991
[4] Morgan and Pinhey, 1994
[5] Solladié-Cavallo et al., 1994

Scheme 2. Amino acid esters resolved by subtilisin-catalyzed hydrolysis in aqueous solution

enzyme activity. Even dimethyl sulfoxide was used as a cosolvent in the enantioselective hydrolysis of *N*-acetyl-3-(3-benzo[b]thienyl)alanine ethyl ester (Rao et al., 1987). This shows the robustness of this protease in such a polar solvent, contrary to many other hydrolytic enzymes. Concerning the resolution of aliphatic amino acids using subtilisin Carlsberg, methyl *N*-benzoylthreoninate and methyl *N*-benzoyl-*allo*-threoninate (Chênevert et al., 1990) were resolved with excellent enantioselectivities and methyl *N*-acetyl- 3,3-difluoro-2-aminobutanoate with good enantioselectivity (Ayi et al., 1995).

The crude subtilisin "alcalase" has also been employed for the resolution of amino acids. This enzyme preparation is a mixture of proteases, the major component being subtilisin Carlsberg. It is inexpensive and has proven to be



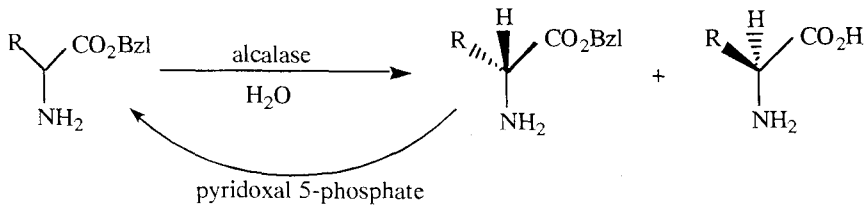
R	% ee (L-acid)	% ee (D-ester)
Et	>99	92
n-Pr	99	88
n-Bu	99	45
n-Hexyl	96	30
$(CH_2)_3CO_2Me$	82	72

Scheme 3

very stable and robust. The methyl esters of racemic phenylalanine and tyrosine were resolved with alcalase at pH 6–8 to give the corresponding L-amino acids and unreacted D-esters both in >90% e.e. (Chen et al., 1986). Benzyl DL-alaninate was also enantioselectivity hydrolyzed. Alcalase was found to be very stable in organic solvents and useable as a catalyst for the resolution of *N*-benzyloxycarbonyl (Z) protected amino acid methyl esters (Chen et al., 1991). Hydrolyses were performed in *t*-butanol containing 5% (v/v) phosphate buffer (pH 8.2). This procedure was favorable for the resolution of hydrophobic amino acid derivatives which were insoluble in aqueous solutions. The corresponding L-acids and unreacted D-esters were obtained in high yields and high enantioselectivities. This enzyme preparation was applied also to the enantioselective hydrolyses in supercritical carbon dioxide containing 0.5% phosphate buffer (pH 8.2) (Chen et al., 1994a). Amino acids bearing aliphatic side chains were chosen as target compounds for resolution (Scheme 3). The small amount of water in the reaction solution was found to be enough for the resolution purpose. A procedure was developed for the complete conversion of a racemic amino acid into the L-enantiomer by the alcalase-catalyzed resolution of the amino acid ester in 2-methyl-2-propanol/water (19:1, v/v) coupled with in situ racemization of the unhydrolyzed D-enantiomer mediated by pyridoxal 5-phosphate (Scheme 4) (Chen et al., 1994b). The benzyl, *n*-butyl and *n*-propyl esters of amino acids were used as substrates, because they were stable during the racemization process contrary to the methyl ester. Resolutions of the methyl esters of 2-amino-3-(2,2′-bipyridin-4-yl)propanoic acid and 2-amino-3-(2,2′-bipyridin-5-yl)propanoic acid (Imperiali et al., 1993) and the methyl ester of *N*-acetyl-(*Z*)-2-amino-4-methylthio-3-butenoic acid (Alks et al., 1992) were also achieved with alcalase.

2 Lipases

Lipases (triacylglycerol lipase; triacylglycerol acylhydrolase; EC 3.1.1.3) are a group of enzymes which have been used most frequently as practical catalysts



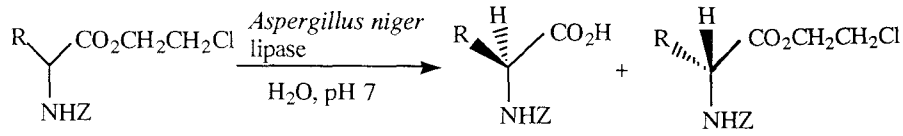
R	L-Acid	
	% ee	Yield (%)
$PhCH_2$	98	92
4-OH-$PhCH_2$	97	95
n-Pr	91	87
n-Bu	90	87
i-Bu	93	87

Scheme 4

for organic syntheses, because they are easily available from variety of sources, inexpensive, and stable, and they require no added cofactors. They are originally hydrolases which catalyze the hydrolysis of triacylglycerols into fatty acids and glycerol. In contrast to esterases, lipases show little activity with substrates soluble in aqueous solutions. They increase their activity at lipid-water interface (Desnuelle, 1972; Alberghina et al., 1991). Lipases from porcine pancreas and various microorganisms are commercially available. A variety of racemic carboxylic acids as well as alcohols have been resolved via the lipase-catalyzed hydrolysis of their esters (Wong and Whitesides, 1994). We reported the first attempt to resolve non-protein amino acids through the microbial lipase-catalyzed hydrolysis of their *N*-protected esters (Miyazawa et al., 1988). The easily removable Z group was chosen for the *N*-protection of amino acids. Thus, the 2-chloroethyl esters of several *N*-Z-α-amino acids, including some proteinogenic amino acids, were treated with lipases from *Aspergillus niger*, *Pseudomonas cepacia*, and *Candida rugosa** at pH 7 (maintained constant) and 25°C. Of the microbial lipases examined, *A. niger* lipase showed the superior broad substrate specificity and enantioselectivity (Scheme 5), the latter being judged by the *E* values** (18–95) (Chen et al., 1982). The L-enantiomers were preferentially hydrolyzed to give *N*-Z-L-amino acids, while the D-counterparts remained unchanged. The methyl

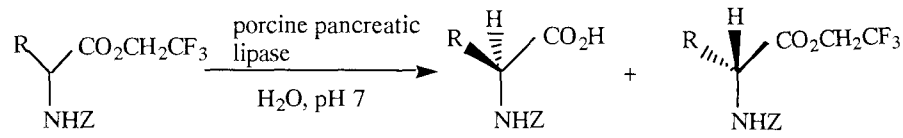
* *Candida rugosa* was previously classified as *Candida cylindracea*. The microbe from which *Pseudomonas cepacia* lipase (Amano) is prepared was previously classified as *Pseudomonas fluorescens* (Kazlauskas et al., 1991).

** The parameter *E* (enantiomeric ratio) is commonly used in characterizing the enantioselectivity of a reaction. Generally speaking, *E* values below 15 are unacceptable for practical purposes. They can be regarded as moderate to good from 15 to 30, and above this value they are excellent (Faber, 1995).



R	% Convn.	% ee (L-acid)	*E*
Et	40	96	95
n-Pr	27	86	18
n-Bu	32	85	18
n-Pentyl	44	95	88
n-Hexyl	31	94	49
Allyl	34	86	21
$PhCH_2$	32	94	50
4-Thiazolylmethyl	37	94	56

Scheme 5

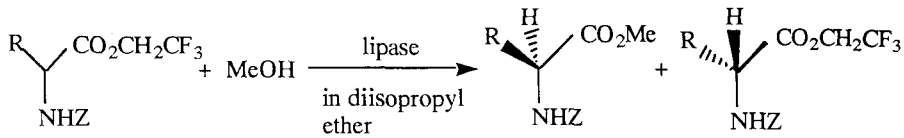


R	% Convn.	% ee (L-acid)	*E*
Et	40	97	130
n-Pr	39	98	190
n-Bu	38	92	42
i-Pentyl	40	95	75
Allyl	38	93	49
Ph	44	97	150
2-F-$PhCH_2$	40	97	130
3-F-$PhCH_2$	40	95	75
4-F-$PhCH_2$	40	90	35
4-Cl-$PhCH_2$	28	94	46
4-Thiazolylmethyl	44	89	36

Scheme 6

esters of the *N*-Z-amino acids were not hydrolyzed with all the microbial lipases tested.

We also found that porcine pancreatic lipase could be used for the same purpose as above (Miyazawa et al., 1989). Thus, the 2,2,2-trifluoroethyl esters of *N*-Z-amino acids were hydrolyzed at pH 7 with this mammalian lipase to yield the L-acids (Scheme 6). For some amino acids, this lipase was superior to the above-mentioned microbial lipases in both the hydrolysis rate and enantioselectivity. The enantioselectivity depends largely on the length of the side chain of the amino acid. For the amino acids with aliphatic side chains, high enantioselectivities ($E > 20$) were obtained with alkyl groups of 2 to 5



R	Lipase[a]	% Convn.	% ee (methyl ester)	*E*
Et	PCL	38	92	42
n-Pr	PCL	38	91	37
n-Bu	PCL	38	89	30
n-Pentyl	PCL	35	87	23
n-Hexyl	PCL	36	90	31
$PhCH_2$	RJL	38	84	19
3-F-$PhCH_2$	RJL	38	73	9.9
4-Thiazolylmethyl	RJL	38	76	12

[a] *PCL, Pseudomonas cepacia* lipase; *RJL, Rhizopus javanicus* lipase.

Scheme 7

carbon atoms. This is also the case with aromatic amino acids, if an aromatic ring is supposed to correspond to a straight chain of 4 carbon atoms. The successful resolution of amino acid derivatives using porcine pancreatic lipase is worthy to note, because low enantioselectivities had been reported in the hydrolysis of other α-substituted carboxylic acid esters (Fülling and Sih, 1987; Dernoncour and Azerad, 1987).

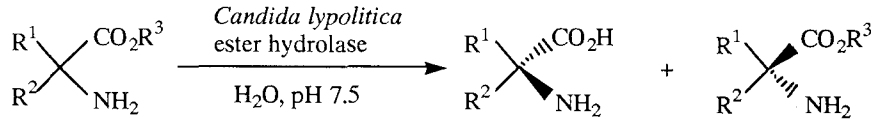
The lipase-catalyzed enantioselective ester hydrolysis was applied to the resolution of pipecolic acid (2-piperidinecarboxylic acid) (Ng-Youn-Chen et al., 1994). Crude lipase from *A. niger* was identified as the most enantioselective catalyst for the hydrolysis of racemic methyl pipecolate: E = 20 in favor of the L-enantiomer at pH 7. The addition of an *N*-acetyl group decreased the enantioselectivity. Partial purification of *A. niger* lipase by fractional precipitation with ammonium sulfate (25–45% saturation) increased the enantioselectivity ($E > 100$). Further purification of the enzyme confirmed that the lipase, and not an impurity, was responsible for the enantioselective hydrolysis of pipecolic acid esters.

The resolution of non-protein amino acids using lipases can also be achieved via the transesterification of esters of their *N*-Z-derivatives with achiral alcohols in anhydrous organic solvents (Miyazawa et al., 1992a). Thus, the 2,2,2-trifluoroethyl esters of *N*-Z-amino acids were reacted with methanol in diisopropyl ether using lipases from *P. cepacia* and *Rhizopus javanicus*. The esters of amino acids carrying aliphatic side chains gave the L-amino acid methyl esters generally with high enantioselectivities in the presence of *P. cepacia* lipase (Scheme 7). The enantioselectivities observed with aromatic amino acids were lower, *R. javanicus* lipase showing better enantiodiscrimination than *P. cepacia* lipase. The lipase-catalyzed enantioselective alcoholysis was applied to the resolution of aziridinecarboxylic acid derivatives (Martres et al., 1994). The 2,2,2-trifluoroethyl ester of *N*-isopropylaziridinecarboxylic acid was reacted with *n*-butanol in hexane at

40°C in the presence of *C. rugosa* lipase. The e.e. values of the newly formed butyl ester and the remaining substrate at 73% conversion (after 5 h) were 68% and 91%, respectively ($E = 32$). However, the absolute configuration of the optically active products obtained was not determined. Porcine pancreatic lipase was used for the transesterification between *N*-unprotected DL-phenylalanine methyl ester and 1-octanol in benzene, affording the L-octyl ester in 95% e.e. (70% yield) and the D-methyl ester in 90% e.e. (80% yield) (Xie et al., 1995).

Candida lipolytica ester hydrolase, a minor component in commercial preparations of *C. lipolytica* lipase, was found to be highly effective in resolving a range of tertiary α-substituted esters including α,α-disubstituted α-amino acids (Yee et al., 1992; Kallwass et al., 1994). A crude commercial lipase preparation was used for the hydrolysis of esters of α,α-disubstituted α-amino acids at pH 7.5 (Scheme 8). The (*S*)-enantiomers were preferentially hydrolyzed and the unchanged (*R*)-esters with high e.e. were recovered. The purification and characterization of *C. lipolytica* ester hydrolase were achieved and its activity was compared with those of structurally related serine carboxypeptidases in resolving racemic α-tertiary ester substrates. A series of α,α-disubstituted α-amino acids were resolved through enantioselective ester hydrolysis catalyzed by *Humicola* amino esterase, a minor component from crude *H. langinosa* lipase (Liu et al., 1995). This enzyme accepts only amino esters with a free amino group as substrates. Amino acid ethyl esters were treated with partially purified *Humicola* amino esterase solution at pH 7.5. Various substrates including aliphatic and aromatic amino esters were resolved into optically active esters and acids with good enantioselectivity (Scheme 9). The varying enantioselectivity of this enzyme toward different amino esters should be noted. Most of the amino esters were resolved into (*R*)-amino acids and (*S*)-amino esters, contrary to most of the enzymatic resolutions reported in the literature.

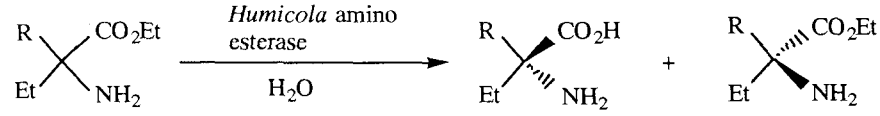
The lipase-catalyzed enantioselective hydrolysis of 5(4*H*)-oxazolone derivatives in aqueous media in combination with in situ racemization of the less reactive enantiomer was reexamined (Scheme 10), though the principle had been well-established many years ago (De Jersey and Zerner, 1969; De Jersey,



R^1	R^2	R^3	% Convn.	% ee ((*R*)-ester)
3-Indolylmethyl	Me	*n*-Bu	60	95
$PhCH_2$	CHF_2	Me	68	97
$3,4\text{-}(OMe)_2\text{-}PhCH_2$	Me	Et	50	>99
$PhCH_2$	Me	Et	50	>99

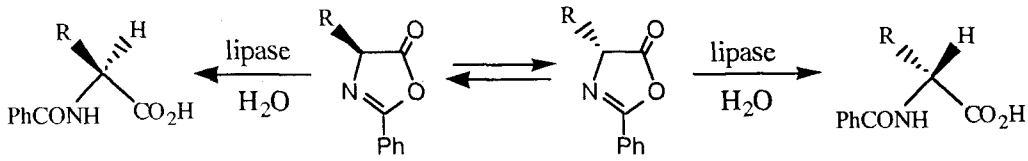
Scheme 8

1970). Thus, porcine pancreatic lipase hydrolyzed 4-substituted 2-phenyloxazolin-5-ones enantioselectively to give *N*-benzoyl-L-amino acids, while *A. niger* lipase exhibited an opposite stereochemical preference and converted the same racemic substrates into the D-amino acid derivatives (Gu et al., 1992). The best substrate was the 4-benzyl derivative, affording *N*-benzoyl-L-phenylalanine in >99% e.e. and the D-counterpart in >99% e.e., respectively. The lipase-catalyzed enantioselective alcoholysis of 5(4*H*)-oxazolone derivatives in organic media with in situ racemization was also investigated (Bevinakatti et al., 1990). *Mucor miehei* lipase catalyzed the ring-opening of 2-phenyl-4-methyloxazolin-5-one with *n*-butanol in diisopropyl ether to give *n*-butyl *N*-benzoyl-L-alaninate (57% e.e. at 45% conversion). The solvent effect on the enantioselectivity of this reaction was also reported (Bevinakatti et al., 1992). A detailed investigation on the methanolysis of 5(4*H*)-oxazolone derivatives was carried out using *P. cepacia* lipase in *t*-butyl methyl ether (Crich et al., 1993). Optically active *N*-benzoyl-L-amino acid methyl esters were obtained in 66–95% e.e. (Scheme 11). The enantioselectivity appeared to improve as the C-4 substituent increased in size. The methanolysis products were then subjected to a second enzyme-catalyzed enantioselective hydrolysis to improve the enantioselectivity: proteases from Amano (prozyme 6 and protease N) catalyzed the hydrolysis of the L-esters at pH 6.8, yielding *N*-benzoyl-L-amino acids of high enantiomeric purity. Enantiomeric alcoholysis of the 5(4*H*)-oxazolone derivatives in organic solvents was applied to the resolution of *t*-leucine (3,3-dimethyl-2-aminobutanoic acid). Treatment of 2-phenyl-4-*t*-butyloxazolin-5-

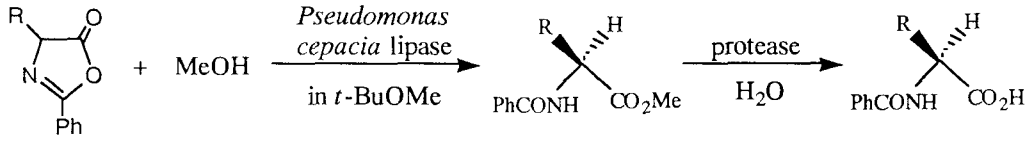


R	% Convn.	% ee (acid)	% ee (ester)	*E*
n-Nonyl	50	91 (*R*)	–	58
n-Hexyl	50	90 (*R*)	99 (*S*)	58
$PhCH_2$	50	85 (*R*)	–	35
n-Bu	46	92 (*R*)	–	58
Allyl	42	94 (*R*)	66 (*S*)	66
Me	72	36 (*S*)	100 (*R*)	20

Scheme 9



Scheme 10



R	L-Methyl ester	
	% ee	Yield (%)
i-Bu	78	82
$MeS(CH_2)_2$	71	56
2-Naphthylmethyl	75	90
4-Me-$PhCH_2$	66	86
$PhCH_2$	69	93
$Ph(CH_2)_3$	84	91

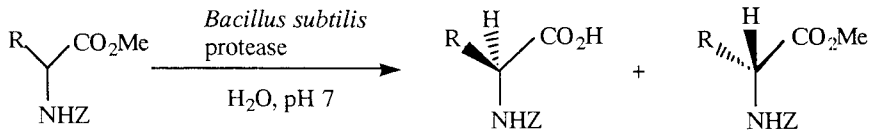
Scheme 11

one with *M. miehei* lipase (Lipozyme) in toluene containing *n*-butanol and a catalytic amount of triethylamine resulted in a 94% yield of *N*-benzoyl-L-*t*-leucine *n*-butyl ester with 99.5% e.e. Omission of triethylamine resulted in a significant decrease in both the yield and the e.e. of the product (Turner et al., 1995).

3 Other microbial proteases and sulfhydryl proteases of plant origin

Microbial proteases from a variety of sources are commercially available now. Although they are inexpensive, stable and easy to handle, their use as synthetic catalysts has been limited. We reported the use of two kinds of microbial proteases, from *Aspergillus oryzae* (Amano A) and from *Bacillus subtilis* (Amano N), for the resolution of non-protein amino acids through the enantioselective hydrolysis of their *N*-protected esters (Miyazawa et al., 1992b). The methyl esters of racemic *N*-Z-α-amino acids were found to be substrates of these microbial proteases. The hydrolyses were conducted at pH 7 in the presence of 20% (v/v) *N*,*N*-dimethylformamide (DMF). Contrary to the lipase-catalyzed hydrolysis, the amino acid ester substrates must be dissolved by the addition of the organic cosolvent. In general, *B. subtilis* protease gave better results than *A. oryzae* protease. Using the former protease, the amino acids bearing aliphatic side chains were resolved with good to excellent enantioselectivities and reasonable hydrolysis rates (Scheme 12). Halogenated phenylalanines and phenylalanine homologs were also resolved generally with high enantioselectivities, though the hydrolysis rates were not always reasonably fast. In all the cases, the L-enantiomers were preferentially hydrolyzed to give Z-L-amino acids.

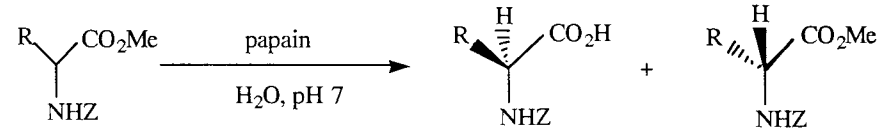
Sulfhydryl proteases have been isolated from a variety of sources and those of plant origin are most easily available: papain (EC 3.4.22.2) from the papaya (*Carica papaya*) latex, ficin (EC 3.4.22.3) from the fig (*Ficus carica*)



R	% Convn.	% ee (L-acid)	*E*
Et	40	95	75
n-Pr	40	91	39
n-Bu	40	>99	>400
n-Pentyl	40	98	200
n-Hexyl	38	99	370
n-Octyl	39	98	190
n-Decyl	40	98	200
4-F-$PhCH_2$	40	85	22
4-Cl-$PhCH_2$	40	94	62
4-Br-$PhCH_2$	40	90	35
$Ph(CH_2)_3$	40	>99	>400

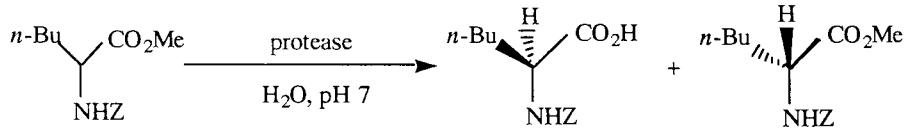
Scheme 12

latex, and bromelain (EC 3.4.22.4) from the pineapple (*Ananas sativus*) stem. Of these sulfhydryl proteases, only papain had been employed for synthetic purposes: e.g., the enzymatic peptide synthesis (Kullmann, 1987) and the resolution of racemic amino acids via the amide bond formation with aniline, phenylhydrazine, etc (Greenstein and Winitz, 1961a). We found that the methyl esters of a number of *N*-Z-amino acids were good substrates for the papain-catalyzed hydrolysis (Miyazawa et al., 1994). The hydrolyses were carried out at pH 7 in the presence of 20% DMF as an organic cosolvent. The esters of amino acids carrying aliphatic side chains were hydrolyzed in high enantioselectivities to give *N*-Z-L-amino acids (Scheme 13). On the other hand, the enantioselectivities observed with aromatic amino acid derivatives were generally lower. The results obtained using other sulfhydryl proteases, ficin and bromelain, had a close resemblance to those obtained using papain in both the enantioselectivity and hydrolysis rate. Papain was also used for the enantioselective hydrolysis of *N*-protected racemic 2-furylglycine methyl ester (Drueckhammer et al., 1988). From *N*-benzoyl-DL-furylglycine methyl ester, *N*-benzoyl-L-furylglycine with 97% e.e. and *N*-benzoyl-D-furylglycine methyl ester with 97% e.e. were obtained at 50% conversion. A much lower e.e. was obtained when methanol was used as a cosolvent instead of DMF. *N*-Boc-allylglycine ethyl ester was also hydrolyzed by papain to give the *N*-protected L-amino acid with 96% e.e. (86% yield) (Schricker et al., 1992). Bromelain was found to hydrolyze *N*-acetyl-*erythro*-β-phenylserine methyl ester with very high enantioselectivity to give the (2*R*,3*R*)-acid ($\geq$98% e.e., 68% yield) and the (2*S*,3*S*)-ester ($\geq$98% e.e., 96% yield) (Chênevert et al., 1990). On the other hand, the enantioselectivity toward the *threo* isomer was very low. These compounds were not substrates for papain and ficin. It is worthwhile to note that the stereochemical preference exhibited by bromelain in this case is a notable exception to the usual *S*-stereopreference of proteases.



R	% Convn.	% ee (L-acid)	*E*
Et	40	99	400
n-Pr	41	95	78
n-Bu	41	>99	>400
i-Pentyl	40	>99	>400
n-Heptyl	40	98	200
Allyl	43	95	84
$Ph(CH_2)_2$	45	96	120
$Ph(CH_2)_3$	42	92	48

Scheme 13

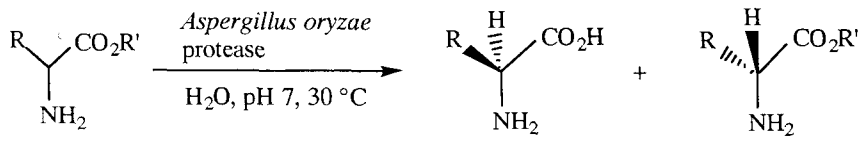


Protease	% Convn.	% ee (L-acid)	*E*
Papain	41	>99	>400
Ficin	40	96	95
ex *Aspergillus oryzae* (Amano A)	40	98	200
ex *Aspergillus melleus* (Amano P)	40	>99	>400
ex *Aspergillus sojae* (Sigma Type XIX)	41	94	64
ex *Bacillus subtilis* (Amano N)	40	>99	>400
ex *Bacillus licheniformis* (Sigma Type VIII)	39	>99	>380
ex *Rhizopus niveus* (Amano Newlase F)	39	>99	>380
α-Chymotrypsin	40	98	200

Scheme 14

We examined the effect of different proteases on enantiodiscrimination by using *N*-Z-norleucine (2-aminohexanoic acid) methyl ester as a substrate (Miyazawa et al., 1993). The hydrolysis was conducted at pH 7 at 35°C in the presence of 20% DMF (Scheme 14). The sulfhydryl proteases of plant origin and most of the microbial proteases examined exhibited excellent enantioselectivities with the substrate, though some proteases showed rather low hydrolysis rates. On the other hand, when *N*-Z-*p*-fluorophenylalanine methyl ester was used as a substrate, the enantioselectivities were generally lower and the hydrolysis rates were reduced significantly.

Papain-catalyzed esterification of *N*-Boc protected amino acids in a biphasic system (using methylene chloride as the organic phase) was found to be another method of resolution of amino acids using this protease. For example, esterification of *N*-Boc-DL-aspartic acid with benzyl alcohol gave



R	R′	% Convn.	% ee (L-acid)	*E*
4-F-PhCH$_2$	Me	48	47	4.2
4-F-PhCH$_2$	*i*-Bu	47	97	170
3-F-PhCH$_2$	*i*-Bu	33	98	150
2-F-PhCH$_2$	*i*-Bu	44	92	50
4-Cl-PhCH$_2$	*i*-Bu	46	98	340
4-Br-PhCH$_2$	*i*-Bu	52	91	120
n-Pr	Me	40	58	5.5
n-Pr	*i*-Bu	40	92	45
n-Pr (5°C)	*i*-Bu	40	97	130
n-Bu	*i*-Bu	40	95	76
i-Bu	*i*-Bu	40	94	66

Scheme 15

only the L-α-monoester, leaving the D-amino acid untouched (Cantacuzène et al., 1987). This procedure was applied to the resolution of vinylglycine (2-amino-3-butenoic acid) (Hallinan et al., 1994). Esterification of the *N*-Boc derivative with ethanol gave the ethyl ester of the L-derivative in 80% yield. Dichloromethane was employed as the organic phase. To obtain the D-enantiomer in optically pure form, a second incubation was carried out to remove the remaining L-isomer.

We examined also the microbial protease-catalyzed hydrolysis of esters of *N*-unprotected amino acids (Miyazawa et al., 1997a). Initially, the methyl esters of several halogenated phenylalanines were subjected to hydrolysis with *A. oryzae* protease at pH 7 and 30°C. Although the hydrolysis of these methyl esters with a free α-amino group proceeded more smoothly than that of the corresponding *N*-protected esters, the enantioselectivities deteriorated. Accordingly, the influence of ester groups was examined next using *p*-fluorophenylalanine as a model amino acid. The use of the *n*-butyl or isobutyl ester 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 as substrates, and sufficiently high enantioselectivities were achieved in all the cases (Scheme 15). Similarly a marked enhancement of enantioselectivity by replacing the methyl ester with the isobutyl ester was generally achieved in the hydrolysis mediated by another microbial protease from *B. subtilis*. These results imply that the role of the ester moiety becomes relatively important in the substrate recognition by the proteases when an acylamino group is replaced by a free amino group. 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 (2-aminopentanoic acid) and norleucine were subjected to hydrolysis with *A. oryzae* protease, the enantioselectivity was

increased progressively with the length of the ester alkyl chain. 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. Thus, the isobutyl esters of other aliphatic amino acids were hydrolyzed to show rather high enantioselectivities, 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, though much longer times were necessary to achieve reasonable degrees of conversion.

Little has been investigated on the effect of temperature on the stereoselectivity of enzymatic transformations, though temperature is an easily controllable parameter in the experimental conditions. When $-RT\ln E$ obtained from the E value at each temperature was plotted against the absolute temperature T, straight lines were obtained for the *A. oryzae* protease-catalyzed hydrolysis of the isobutyl esters of 2-aminobutanoic acid and norvaline (Miyazawa et al., 1997b) (Fig. 1). The existence of a linear relationship between $-RT\ln E$ and T indicates that the temperature effect on the enantioselectivity of this enzymatic hydrolysis is governed by the following equation as in the ordinary chemical reactions: $-RT\ln E = \Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$, where $\Delta\Delta G^{\ddagger}$, $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ represent the difference in the activation free energy, activation enthalpy and activation entropy, respectively, between the enantiomers (Phillips, 1992; Phillips, 1996). This means also that the enzyme retains its active conformation in the temperature range examined. As the majority of enzymes used for biotransformations are not thermally stable, reactions at higher temperatures are largely restricted, but lowering temperature seems to have less operational limits. In cases in which

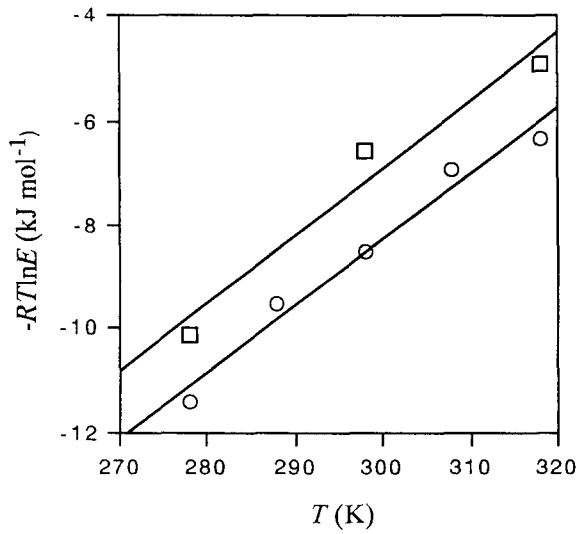


Fig. 1. Influence of temperature (T) on the difference in the activation free energy ($\Delta\Delta G^{\ddagger} = -RT\ln E$) between the enantiomers for the *Aspergillus oryzae* protease-catalyzed hydrolysis of the isobutyl esters of 2-aminobutanoic acid (square) and norvaline (circle)

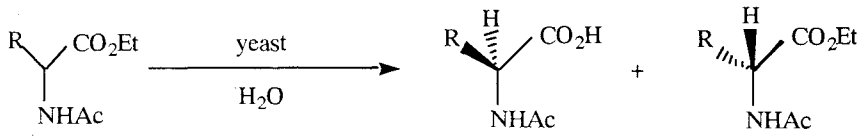
the "racemic temperature", at which no enantiomeric discrimination occurs, exists above ordinary temperatures (this must be often the case), there is the possibility of improving the enantioselectivity by conducting the reaction at lower temperature.

Thermitase, an alkaline serine protease from *Thermoactinomyces vulgaris*, was used for the enantioselective hydrolysis of *N*-Boc-amino acid methyl esters (Łankiewicz et al., 1989). The following amino acids were resolved in aqueous DMF (25–30%) at 55°C: *p*-chlorophenylalanine, 2,4-dichlorophenylalanine, 3,4-dichlorophenylalanine, β-(2-naphthyl)alanine, and *S*-benzylcysteine.

Pronase, a proteolytic complex of several enzymes from *Streptomyces griseus*, was reported to have good activity and high enantioselectivity toward the L-antipode in the hydrolysis of amino acid methyl esters. This enzyme preparation is commercially available and often used for complete hydrolysis of peptides and proteins (Nomoto et al., 1960). The methyl esters of tryptophan, phenylalanine, leucine and valine were enantioselectively hydrolyzed (Yamskov et al., 1981) and this procedure was later applied to the methyl esters of non-protein amino acids: β-(1-naphthyl)alanine, β-(2-naphthyl)alanine, phenylglycine, 2-amino-4-phenylbutanoic acid, β-(2-thienyl)alanine, 2-aminohexanoic acid, 2-aminopentanoic acid, and 2-aminobutanoic acid (Pugniere et al., 1994). The L- and D-enantiomers were obtained in high enantioselectivities and good yields under reaction conditions which were optimized for each amino acid.

4 Yeast and other microbial cells

The hydrolytic properties of baker's yeast (*Saccharomyces cerevisiae*) were employed for the resolution of *N*-acetylamino acid esters (Glänzer et al., 1987). The active enzyme involved was thought to be an unspecific protease, from the close analogy of its hydrolytic behavior with that of α-chymotrypsin. The carboxylic ester moiety was hydrolyzed and the amide group remained unchanged in all the cases examined (Scheme 16). In general, anaerobic conditions were employed for the fermentation, providing a sufficient hydrolysis rate to accomplish a conversion greater than 50% within 48 h. Only the L-derivatives were cleaved while the D-enantiomers remained untouched and could be recovered. The corresponding *N*-acetyl-L-amino acids were not isolated. The substrates bearing unbranched alkyl or arylalkyl substituents were hydrolyzed in excellent enantiomeric excess. While the α-ester was preferentially hydrolyzed the ω-ester moiety remained unchanged, yielding the ω-half esters of *N*-acetyl-L-aspartic and L-glutamic acid. This microorganism was applied to the resolution of the three positional (*o*-, *m*- and *p*-) isomers of fluorophenylalanine (Csuk and Glänzer, 1988). Racemic *N*-acetylfluorophenylalnine ethyl esters were hydrolyzed at pH 7.5 using lyophilized yeast. The pH optimum for lyophilized yeast for this reaction was found to be 7.5, while fermenting yeast itself showed a pH optimum between 3 and 4. The reaction stopped after 6–8 h at 52–53% conversion. No difference was observed in the hydrolysis rates of the three isomeric substrates. The



R	D-Ester	
	% ee	Yield (%)
Me	100	47
Et	>96	48
i-Bu	92	48
$PhCH_2$	>97	38
CH_2CO_2Et	86	49
$(CH_2)_2CO_2Et$	89	46

Scheme 16

unhydrolyzed *N*-acetylamino acid esters were obtained in good yields. Their enantiomeric purities were determined to be >96%.

Yeast cells cross-linked with glutaraldehyde were used for the enantioselective hydrolysis of methyl esters of racemic *N*-acetyl-α-amino acids (phenylglycine, phenylalanine and alanine) in reverse micelles of bis(2-ethylhexyl)sulfosuccinate sodium salt in chloroform-isooctane (1:9, v/v) (Fadnavis et al., 1989). The liberated L-acid due to yeast-mediated hydrolysis was periodically removed from the micellar medium, minimizing the product inhibition. The *N*-acetyl-L-amino acids and the remaining *N*-acetyl-D-amino acid esters were obtained in 98 ± 2% e.e. and 40–60% yield. The hydrolysis proceeded more efficiently in reverse micelles than in water, eliminating the solubility limitation of the reactant.

The hydrolytic potentialities of cells of *Sulfolobus solfataricus*, a thermoacidophilic archaeobacterium, trapped in sodium alginate was preliminarily reported (Trincone et al., 1990). By this approach the methyl esters of racemic α-amino acids (phenylalanine, methionine, serine, valine and alanine) were hydrolyzed; at 50% conversion, the remaining substrate was shown to be the D-enantiomer with high optical purity. However, *S. solfataricus* alginate beads were able to hydrolyze also the D-enantiomer, reaching 100% conversion in each case within 24 h at 40°C.

5 Final remarks

Enzymatic resolution of DL-amino acids continues to be the method of choice for many workers, particularly because it, in principle, allows the recovery of both enantiomers. As shown in this review, enantioselective ester hydrolysis has been exploited for the enzyme-based resolution of a wide variety of amino acids since the pioneering work using α-chymotrypsin early in the 1950's. Applications of enzymes have been expanding considerably for the purpose: not only proteases but also lipases have been employed with success. The resolution of non-protein amino acids has been attracting particular interest.

In some reports conventional applications are described with novel details, while in others new enzymes or new procedures are proposed. Thus, the enzymatic approaches via ester hydrolysis have proved to be complementary to the more extensively studied resolution through deacylation of *N*-acylated amino acids using such hydrolytic enzymes as acylase I. We hope that this review might help our readers in finding some suggestions for choosing the most appropriate procedure for the resolution of amino acids of interest. We also hope that the range of both enzymes and amino acids will be further widen in the future studies.

Acknowledgments

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