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The use of crude lipase in deprotection of C-terminal protecting groups

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Abstract

A crude lipase, Newlase F, was used to remove C-terminal protecting groups from dipeptide esters. Hydrolysis of dipeptide *n*-heptyl esters with Newlase F was conducted in aqueous media containing acetonitrile. The optimum pH and temperature of lipase in Newlase F were 7.0 and 30 °C, respectively. Low level acetonitrile promoted the hydrolysis of dipeptide *n*-heptyl esters, while high level acetonitrile inhibited the hydrolysis. However, the protease activity in Newlase F was significantly inhibited by acetonitrile. Lipase in Newlase F worked better in a medium containing water-miscible organic solvents than in water-immiscible ones. N-terminal protecting groups were not affected by the protease in the crude enzyme. It was found that the protease in Newlase F did not hydrolyze amide bond with hydrophilic amino acids on either side under these conditions (pH 7.0, room temperature). Newlase F may consequently be used widely in the synthesis of peptide conjugates. The crude enzyme was immobilized on SBA-15 mesoporous molecular sieve. The lipase activity of immobilized preparation was more active on hydrolysis of C-terminal protecting groups and stable than the free enzyme. The immobilization also reduced the protease activity.

Keywords: Newlase F; Dipeptide heptyl esters; Lipase; Protease

1. Introduction

Peptide conjugates, such as phospho-peptides [1], are of great importance in the study of signal transduction [2], protein phosphorylation/dephosphorylation, and so on. However, the chemical synthesis of these compounds was severely complicated owing to their pronounced acid and base lability as well as their multifunctionality. The traditional peptide synthesis will have many side reactions such as β-elimination. However, the enzymatic process can provide a variety of protecting groups, which can be removed selectively and orthogonally under the mild conditions. It was reported that C-terminal protecting heptyl group (Hept) could be hydrolyzed by lipase from *Rhizopus niveus* (Lipase N, Fluka Biochemika) without damaging the N-terminal protecting groups [3]. Therefore, Hept is one of suitable blocking groups for C-terminal protection. But lipase N is expensive and not stable enough to use.

Newlase F, which contains lipase and acid protease from *R. niveus*, has mainly been used in industrial process such as food and protein hydrolysis [4]. Comparing with lipase N, Newlase F is cheaper, more stable and applicable. Hence, Newlase F was selected for removing the C-terminal protecting groups from dipeptide esters. In order to study the application of Newlase F in peptide synthesis, the reaction condition

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and the effect of the protease was discussed in this paper.

2. Materials and methods

2.1. Apparatus

^{1}H NMR spectra were recorded on a Bruker Ac-200p spectrometer at 200 MHz in $CDCl_3$ with chemical shifts referenced to $CDCl_3$ ($\delta_H = 7.24$). Enzymatic hydrolysis was carried out in a water-bath constant temperature oscillator. The amount of substrate and product in the enzymatic reaction were analyzed by HPLC (SHIMADZU LC-9A) equipped with a Zorbax C18 analytic column (4.6 mm i.d. × 25 cm, 5 μm particle size). The UV absorbency was measured spectrophotometrically at room temperature with a Pharmacia Ultraspec 4000 UV-Vis spectrophotometer.

2.2. Materials

p-Nitrophenyl laurate (*p*NPL) was obtained from Fluka Biochemika. CaX (10 X) molecular sieve was purchased from the molecular sieve factory in Shanghai, China. Ala, Ser, $(Boc)_2O$ and Z-Cl were purchased from J&K Chemicals; Boc-Ala, Boc-Ser, Z-Ala, Ala-OMe, Ala-OEt, Ser-OMe were synthesized according to the literature procedure [5]; all other reagents and solvents were analytical grade.

2.3. Synthesis of long-chain alkyl amino acid ester hydrogentoluenesulfonates and protected dipeptides

The synthesis of L-alanine long-chain alkyl ester hydrogentoluenesulfonate Tos·Ala-OR ($R = -(CH_2)_nCH_3$, $n = 0$–9): a mixture of 0.89 g L-Ala (0.01 mol), 2.23 g (0.012 mol) *p*-toluosulfonacid-monohydrate, 0.050 mol long-chain alcohol and 10 ml benzene was refluxed, and then the water was completely removed with a water separator for 6–24 h. After benzene was eliminated by air distillation, long-chain alcohol was removed under high vacuum. The residue was dissolved in ethyl acetate and *n*-hexane was added until no more solid appeared. The mixture was allowed to stand at −20 °C overnight. The solid was separated and recrystallized in ethyl acetate. White solid was obtained with an 80% yield.

Boc-protected dipeptide esters and Z-Ala-Ala-OR ($R = -(CH_2)_nCH_3$, $n = 0$–9) were synthesized by a mixed anhydride method (according to the literature procedure) [5]. Boc-Ala-Ala, Boc-Ala-Ser and Z-Ala-Ala were prepared by hydrolyzing Boc-Ala-Ala-OMe, Boc-Ala-Ser-OMe, and Z-Ala-Ala-OMe, respectively, under alkaline conditions.

2.4. The assay of lipase activity and protease activity of Newlase F

Newlase F (NW09504) was donated by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan).

2.4.1. Lipase activity assay in microemulsion system

Titrimetric assay of lipase activity was carried out in microemulsion system (according to the literature procedure) [6]. One unit of lipase activity (LU) was defined as the amount of enzyme that released 1 μmol of fatty acid from olive oil/min at 37 °C. Lipase activity of Newlase F was 17 000 LU/g.

2.4.2. Lipase activity assay in aqueous media

Lipase activity in Newlase F was assessed using *p*NPL as substrate spectrophotometrically at 37 °C (according to the literature procedure) [7].

2.4.3. Protease activity assay

Protease activity in Newlase F was determined following the method proposed by Charney and Tomarelli [8]. One protease unit (PU) was defined as the amount of protease that liberated 1 μg tyrosine from casein/min under these conditions. The protease activity of Newlase F was 2800 and 3000 PU/g at pH 7.0 and 3.3, respectively.

2.5. Immobilization of Newlase F

SBA-15 mesoporous molecular sieve was a gift from Prof. Xiao (Department of Chemistry, Jilin University). SBA-15 mesoporous molecular sieve (50 mg) was suspended in 1 ml of 2 mg/ml Newlase F solution for 1 h at 37 °C with stirring. The supernatant was separated from the solid materials by

centrifugation. The lipase activities of the supernatant and the immobilized lipase were assayed by *p*NPL method.

2.6. Enzymatic hydrolysis of protected dipeptide alkyl esters

The general procedure of enzymatic hydrolysis of protected dipeptide alkyl esters: to a mixture of 50 μl of 0.060 M Boc-Gly or phenyl acetic acid (PhAc) (used as internal standard for HPLC analysis), 100 μl of 0.20 M phosphate buffer (pH 7.0) and 50 μl of 10.0 mg/ml Newlase F solution, was added 50 μl of 0.133 M dipeptide esters acetonitrile solution. The mixture was stirred at 37 °C for a certain period of time. The reaction was stopped by adding 400 μl acetonitrile. The solvent was vaporized at 80 °C for 1 h and 200 μl eluant was then added.

2.7. HPLC condition

For the reaction mixtures containing Boc-protected peptide esters: eluant, 25% acetonitrile and 75% 0.10 M phosphate buffer (pH 3.3); flow rate, 0.6 ml/min; detected wavelength, 210 nm; internal standard, Boc-Gly.

For the reaction mixtures containing Z-protected peptide esters: eluant, 40% acetonitrile and 60% 0.10 M phosphate buffer (pH 3.3); flow rate, 0.6 ml/min; detected wavelength, 254 nm; internal standard, PhAc [9].

3. Results and discussion

3.1. Optimum pH and temperature of the lipase in Newlase F

To find the optimum pH and temperature of the lipase in Newlase F, the hydrolysis was carried out with Boc-Ala-Ser-OHept as substrate. When pH was about 7.0 and temperature was about 30 °C (Fig. 1 and Table 1), the lipase in Newlase F had the highest activity [10]. These are slightly different from Lipase N, which showed a pH optimum of pH 6.0–6.5 and a temperature optimum of 35 °C [11]. These may be due to the different substrates in hydrolytic reaction and the existence of acid protease in Newlase F. Since lipase from *R. niveus* has a catalytic center which

Table 1
Effect of pH

pH	Final content of the product (mM)
2.0	0.00
3.0	0.00
4.0	0.00
5.0	0.00
6.0	3.81
7.0	14.90
8.0	6.04
9.0	2.55
10.0	2.24

Reaction conditions: 0.04 M phosphate buffer (30 °C) containing 20% CH_3CN; substrate, 26.6 mM Boc-Ala-Ser-OHept; internal standard, 12.0 mM Boc-Gly; Newlase F level, 2.0 mg/ml; final product level, 14.9 mM (at pH 7.0, 24 h).

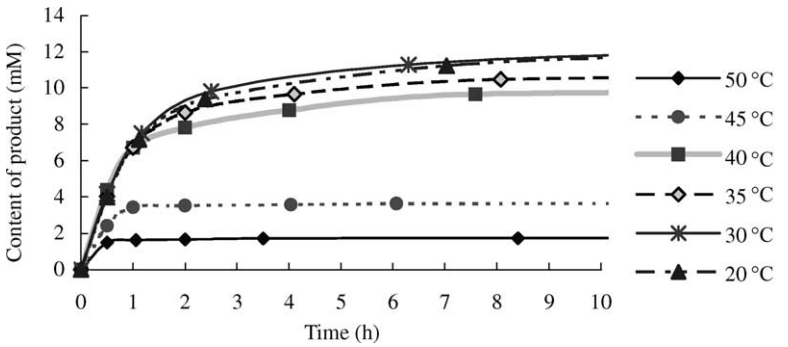

Fig. 1. Effect of temperature. Reaction conditions: 0.04 M phosphate buffer (pH 7.0) containing 20% CH_3CN; substrate, 26.6 mM Boc-Ala-Ser-OHept; internal standard, 12.0 mM Boc-Gly; Newlase F level, 2.0 mg/ml; final product level, 14.9 mM (at 30 °C, 24 h).

contains a triad of three amino acids (Ser, Asp, His) [12], high acidic or basic condition cannot make the catalytic center work. On the other hand, the acid protease in Newlase F worked better in acidic condition [13]. Therefore, it was possible to inhibit the acid protease in the hydrolysis of C-terminal protecting group by changing reaction conditions.

3.2. The effect of acetonitrile concentration

The effect of acetonitrile in different concentration on the activity of Newlase F was listed in Table 2. The final content of the product Boc-Ala-Ser-OH and the initial reaction rate rose with the amount of acetonitrile increased when the level of acetonitrile was low. The final content of the product reached the maximum at 10% acetonitrile. Meanwhile, the lipase had the highest initial reaction rate at 20% acetonitrile. Since Boc-Ala-Ser-OHept is not dissolved in aqueous medium, high concentration of acetonitrile can make more substrates dissolve in organic solvent and more product transfer to aqueous solution. In addition, it can make lipase lose its activity easily. So, the level of acetonitrile with the highest initial rate was different from that having the maximum product. When the level of acetonitrile was higher than 20%, the lipase activity in Newlase F decreased obviously with increasing the amount of acetonitrile. When the content of acetonitrile reached 50%, only a slight lipase activity was detected. So, high level acetonitrile can be used as an inhibitor to terminate the reaction.

Table 2
Effect of acetonitrile concentration

Acetonitrile content (vol.%)	Initial reaction rate (mmol/l h)	The final content of the product (mmol/l)
20[a] (control)	0.0083	0.10
0	4.35	4.07
5	7.00	20.95
10	8.70	24.05
15	9.35	14.57
20	10.85	9.92
33	0.88	2.09
50	0.0031	0.11

Reaction conditions: 0.04 M phosphate buffer (pH 7.0, 37 °C); substrate, 26.6 mM Boc-Ala-Ser-OHept; internal standard, 12.0 mM Boc-Gly; Newlase F level, 2.0 mg/ml; final product level, 24.05 mM (at 10% CH_3CN, 24 h).

[a] Control experiment: in the same reaction condition but without Newlase F.

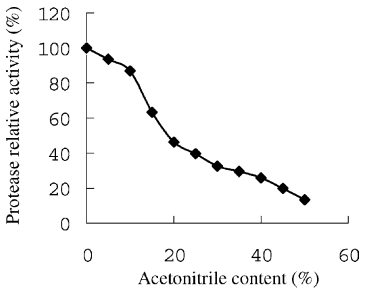

Fig. 2. Effect of acetonitrile content on protease activity. Reaction conditions: 0.04 M phosphate buffer (pH 7.0, 30 °C) with different level CH_3CN; substrate, 5.5 mg/ml casein [8]; Newlase F level, 2.0 mg/ml.

Casein-hydrolysis activity in Newlase F also decreased significantly with increasing the amount of acetonitrile (Fig. 2). At 20% acetonitrile, the protease activity was only 45% of the activity without acetonitrile, and little protease activity was detected at 50%. So, the use of acetonitrile can improve the selectivity of the hydrolysis and increase the reaction rate of lipase.

3.3. Effect of organic solvents

Generally, lipases catalyze only the hydrolysis on the interface between the aqueous solution and organic phase [14]. In lipase from *R. niveus* [12], an α-helix which acts as a lid is in a position where it shelters the catalytic triad. On the interface, the lid moves to expose the catalytic center and allows to bind the substrates in it. It is important to find that in which solvent lipase catalyzes the hydrolysis best. From Table 3, it was found that lipase worked well in branched alcohol medium, such as *t*-butanol and isopropanol. Adding water-miscible organic solvents, such as dioxane and methanol, to the reaction mixture can improve catalytic activities greatly. Moreover, the effect of water-immiscible ones such as hexane was less. These are contrary to the usual lipase-catalyzed reaction: addition of water-immiscible organic solvents to form a biphasic medium will improve the catalytic activity [14]. Since Boc-protected peptide esters with long-chain alkyl were not dissolved well in aqueous medium, an interface was formed when

Table 3
Effect of solvents

Solvent	Product concentration (mM)
t-Butanol	20.7
i-Propanol	20.3
Methanol	15.71
Ethanol	12.11
Propanol	2.55
Tetrahydro-furan	1.86
Dioxane	16.1
Acetone	12.9
Benzene	9.83
Butanone	8.18
Hexane	6.13
Ethyl acetate	5.86

Reaction conditions: 0.04 M phosphate buffer (pH 7.0, 37 °C) containing 20% (v/v) organic solvent; substrate, 26.6 mM Boc-Ala-Ser-OHept; internal standard, 12.0 mM Boc-Gly; Newlase F level, 2.0 mg/ml.

organic solvent solutions containing Boc-protected peptide esters were added to the media. These can explain why the reaction worked better in water-miscible organic solvents than in water-immiscible ones. When the substrate was added, the solutions become turbid. So, adding organic solvents can favor the hydrolysis to proceed. This was in agreement with the fact [15] that the hydrolysis with lipase N was often carried out in water-miscible organic solvents, such as acetone.

3.4. Effect of C-terminal protecting groups

For Z-Ala-Ala-OR (R = $-(CH_2)_nCH_3$, n = 0–9), Z-Ala-Ala was found in all the hydrolysis reactions. As shown in Table 4, it was found that the hydrolysis was slow and incomplete for the long-chain C-terminal protecting group (n = 7–9). In addition, the rate for hydrolyzing C-terminal protecting group of Z-Ala-Ala-OR (R = $-(CH_2)_nCH_3$, n = 0–6) was fast. However, for Boc-protected dipeptides, the favor C-terminal protecting groups are heptyl or 2-bromoethyl group [3,15]. For the existence of highly hydrophobic Z-protecting group, Z-Ala-Ala-OR was not dissolved in aqueous media. So, the dipeptide esters were assembled easily in the interface and the hydrolysis rate was improved greatly.

Table 4
Relative product content in the hydrolysis of Z-Ala-Ala-O$(CH_2)_nCH_3$

n	Relative product content (%)
0	94.64
1	97.55
2	100.00[a]
3	93.89
4	61.08
5	56.63
6	55.74
7	16.69
8	15.74
9	4.28

Reaction conditions: 0.04 M phosphate buffer (pH 7.0, 37 °C) containing 20% CH_3CN; substrate, 20.0 mM Z-Ala-Ala-O$(CH_2)_nCH_3$; internal standard, 10.0 mM PhAc; Newlase F level, 2.0 mg/ml; final product level, 19.0 mM (at n = 2, 24 h).

[a] 100%: 19.0 mM (at n = 2, 24 h).

3.5. Effect of acid protease in Newlase F

Since Newlase F was a crude enzyme from *R. niveus*, there was a risk that the protease in Newlase F might lead to the hydrolysis of the peptide bond, which may limit its application. The N-terminal protecting groups and the amide bond may be affected by Newlase F. Benzyl alcohol or Z-Ala would be produced if protease hydrolyzes the N-terminal protecting group or amide bond in Z-protected dipeptide esters (Fig. 3). But Fig. 4 indicated that the corresponding peaks of Z-Ala and $PhCH_2OH$ were not detected during the hydrolysis of Z-Ala-Ala-OR (R = $-(CH_2)_nCH_3$, n = 0–9). For Boc-protected dipeptide ester, such as Boc-Ala-Ala-OHept, Boc-Ala-Ser-OHept and

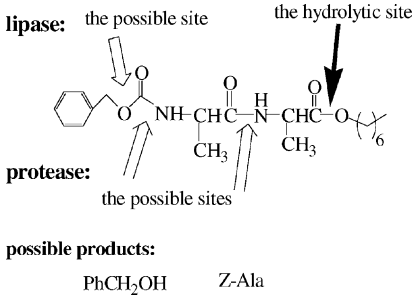

Fig. 3. Possible cleavage sites of Newlase F and their products.

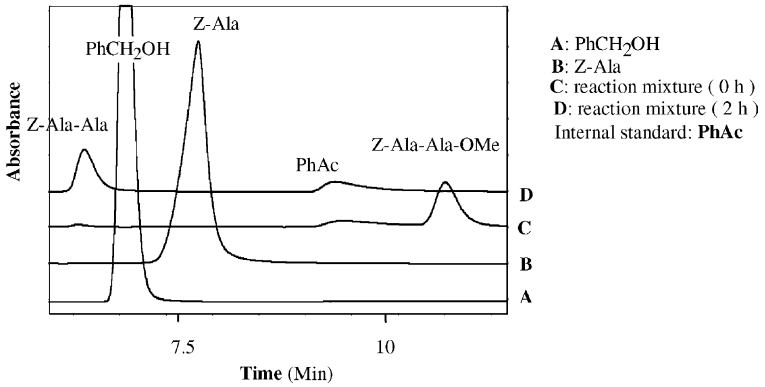

Fig. 4. HPLC analysis of reaction mixture obtained by hydrolysis of Z-Ala-Ala-OMe with Newlase F. Reaction mixture C (0 h) contained 20.0 mM Z-Ala-Ala-OMe, 10.0 mM PhAc and 2.0 mg/ml Newlase F. Reaction mixture D (2 h) is C after incubating in 0.04 M phosphate buffer (pH 7.0, 30 °C) with 20% CH_3CN for 2 h.

Boc-Ser-Ala-OHept, the results were similar. It meant that the protease in Newlase F did not cleave the amide bond of dipeptides containing hydrophilic amino acids on either side under this condition. Firstly, the acid protease showed substrate specificity for peptide bonds located between large hydrophobic residues [16]. Secondly, the activity of acid protease might be weak in neutral condition [13,16]. For dipeptides containing hydrophilic residues, the protease in Newlase F did not cause any side reaction. So, Newlase F can be used for enzymatic deprotection in peptide synthesis.

3.6. Immobilization of crude lipase on SBA-15 mesoporous molecular sieve

Immobilization of enzymes on solid supports can improve the stability greatly. The pores of SBA-15 mesoporous molecular sieve are well defined, with narrow distributions of pore diameter (75 Å). The internal surfaces of these pores, which can achieve 800–1000 m^2/g, are lined with silanol hydroxyls. These possess potential suitable for the physical adsorption of large molecules, such as through hydrogen bonding. For example, mesoporous SBA-15 molecular sieve have been used for immobilizing trypsin [17].

The crude enzyme was immobilized on SBA-15 mesoporous molecular sieve. From the UV spectra of the enzyme solution before and after adsorption, it was found that some of the crude enzyme was adsorbed by the SBA-15 molecular sieve. The lipase activities of the supernatant and the immobilized enzyme were assayed by *p*NPL method. It was found that the lipase activity of the supernatant was only 10% of the crude enzyme, while the lipase activity of the immobilized enzyme was 50–300% higher than the crude enzyme. In addition, the protease activity of the supernatant reached 80% of the crude enzyme, and the protease activity of the immobilized enzyme was 40–50%. But for the immobilized enzyme by the CaX molecular sieve, the lipase activity and protease activity of the absorbed crude enzyme was very low. Therefore, the internal surfaces of these pores in SBA-15 mesoporous molecular sieve increased the activity of crude lipase greatly. From the above discussion, the lipase in Newlase F may be absorbed easily in the internal surfaces of these pores. And the silanol hydroxyls can change the environment of lipase and improve the activity. So, the lipase activity of immobilized enzyme by SBA-15 mesoporous molecular sieve was shown to be more active and stable than free crude enzyme, while the protease activity was reduced.

4. Conclusion

The reaction conditions, such as pH, temperature and organic solvents, were optimized in this paper.

Newlase F may be used to remove C-terminal protecting groups which are orthogonal to N-terminal protecting group in peptide synthesis. In these reaction conditions, Newlase F did not hydrolyze the N-terminal protecting group and the protease did not cleave the amide bond with hydrophilic amino acids on either side in dipeptides. The lipase activity of immobilized enzyme by SBA-15 mesoporous molecular sieve was shown to be more active and stable than free crude enzyme, while the protease activity was reduced. So, Newlase F may be used as a lipase to remove C-terminal protecting group in peptide synthesis and be applied widely in organic synthesis.

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