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Hydrolysis of Dipeptide Heptyl Esters with Newlase F

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Abstract: Newlase F is a rude enzyme which contains triacylglycerol lipase and acid protease. Hydrolysis of dipeptide heptyl esters with Newlase F was studied in phosphate buffer-organic solvent by HPLC. When the Newlase F's level reached 5 mg/mL under mild condition (pH 7.0, 30°C), the lipase had the highest activity. The reaction was also affected greatly by organic solvents and their concentrations. It is found that protease in Newlase F does not hydrolyze amide bond under this condition (pH 7.0, r.t.).

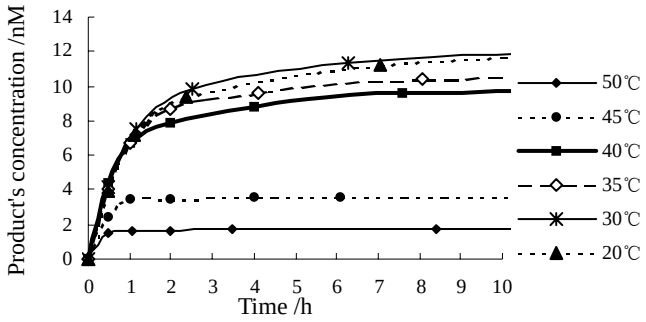
Keywords: Newlase F, dipeptide heptyl esters, HPLC.

Peptides conjugates, such as lipo-, glyco-, phospho- and nucleopeptides[1], are of great importance in the research of signal transmission[2], protein phosphorylation/ dephosphorylation, antibody, and so on. However, the synthesis of these compounds is severely complicated by their pronounced acid and base lability as well as their multifunctionality. Under basic or acidic conditions, β-elimination reactions, anomerization, hydrolysis or rupture of these compounds may occur. The traditional peptide synthesis is inadaptable. However, the enzymatic protecting group techniques can provide a variety of protecting groups which can be removed selectively and orthogonally under the very mild and preferably neutral condition. C-terminal heptyl ester can be selectively demasked through cleavage of heptyl group by lipase from rhizopus niveus (lipase N), with no effect on the N-terminal protecting groups[3]. But lipase N is expensive and not stable enough to use.

Newlase F, which contains lipase from rhizopus niveus and protease, is mainly used in industrial process such as food and protein hydrolysis[4]. Comparing with lipase N, Newlase F is cheaper and more stable. In this paper, Newlase F was used to remove the C-terminal protecting group from dipeptides. The substrates were Boc (t-butyloxycarbonyl) heptyl esters (such as Boc-Ala-Ser-O-Hept) or Z (benzyloxylcarbonyl) protected peptides esters. As these substrates are apolar and not well soluble in phosphate buffer, organic solvents such as acetonitrile or alcohol are needed. In order to study the application of Newlase F in peptide synthesis, the reaction condition and the effect of protease were discussed.

1. Effects of pH and Temperature

Figure 1 Effect of temperature



These reactions were carried out in phosphate buffer containing 20% (volume percent) acetonitrile with various pH and temperature. From **Figure 1** and **Table 1**, it was found that when pH was around 7.0 and temperature was around 30°C, the lipase in Newlase F had the highest catalytic activity. These are slightly different from lipase N[5].

2. Effect of Enzyme Content

In order to find the best enzyme content, the hydrolysis reactions were carried out with various enzyme content in phosphate buffer (pH 7.0) containing 20% (volume percent) acetonitrile at 37°C. As the reaction proceeded, the concentration of substrate decreased and the concentration of product increased (**Figure 1**). After 4 hours, the change of product's concentration was slight. This might due to enzyme deactivity and product inhibition. It was found that the reaction rate increased with the increase of enzyme content. When the enzyme content reached 5.0 mg/mL, the reaction rate and the final concentration of product reached the maximum.

Table 1 Effects of pH and solvent

pH	FCP* (mM)	Solvent	FCP (mM)
2.0	0.00	Isopropanol	20.3
3.0	0.00	Dioxane	16.1
4.0	0.00	Methanol	15.7
5.0	0.00	Acetrone	12.9
6.0	3.81	Ethanol	12.1
7.0	14.90	Benzene	9.8
8.0	6.04	Hexane	6.1
9.0	2.55	Propanol	2.5
10.0	2.24	Heptanol	0.5

* FCP: the final concentration of the product.

Table 2 Effect of CH_3CN concentration

CH_3CN (%)	IRR* (mmol/L·h)	FCP (mmol/L)
20 (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

* IRR: initial reaction rate

3. Effect of Acetonitrile Concentration

The effects of acetonitrile at various concentrations on the activity of Newlase F were listed in **Table 2**. The initial reaction rate rose with the increase of the amount of acetonitrile up to 20% of acetonitrile. When the level of acetonitrile was higher than 20%, the lipase's activity in Newlase F decreased greatly. At the level of 10% acetonitrile, the final concentration of the product reached the maximum. When the content of acetonitrile reached 50%, no lipase's activity was detected (no hydrolysis reaction was detected). So high level of acetonitrile can be used as an inhibiting reagent to terminate the reaction.

4. Effect of Solvents

Generally lipases catalyze only on the interface between the aqueous solution and organic phase[6]. It is important to find that in which solvent lipase catalyzes the reaction best. From **Table 1**, it was found that lipase worked well in branched alcohol medium, such as isopropanol, but not in long-chain alcohol medium. Since Boc protected peptide esters with long-chain alkyl group do not dissolve in the phosphate buffer, an interface is formed when organic solvent solutions containing peptide esters are added to the buffer. Water-miscible organic solvents can promote the forming of the interface, while water-immiscible ones can't. So the reaction works best in water-miscible organic solvents.

Figure 2 The possible cleavage sites of lipase and protease and their products

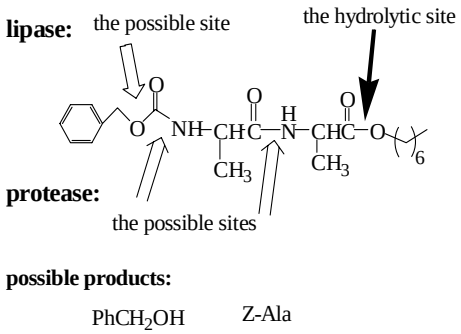
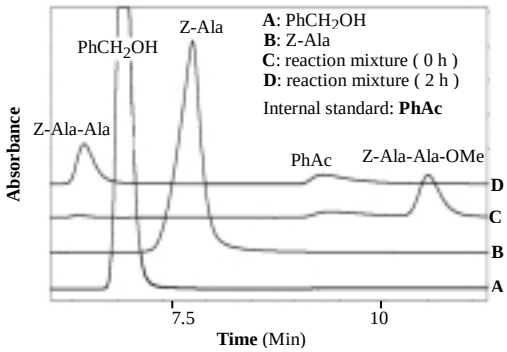


Figure 3 Effect on the N-terminal protecting group and amide bond



5. Effect on N-Terminal Protecting Groups

Benzyl alcohol would be produced if Newlase F could hydrolyze the N-terminal protecting groups (**Figure 2**). But from the HPLC analysis as shown in **Figure 3**, the corresponding peak to $PhCH_2OH$ was not detected in the hydrolysis of Z-Ala-Ala-OR. The N-terminal protecting group was not affected for Boc protected dipeptide heptyl esters, too. So it can be used as an enzymatic deprotecting group technique which is

orthogonal to N terminal protecting groups.

6. Effect of Protease in Newlase F

Newlase F is a rude mixed enzyme. There is a risk of hydrolyzing the peptide bond by the protease in Newlase F. This may limit its application. From **Figure 3**, the corresponding peak of Z-Ala, which is the product by protease, was not detected during the hydrolysis of Z-Ala-Ala-OR (R= $-(CH_2)_nCH_3$, n=0~9), similar to Boc-protecting dipeptide heptyl esters. It means that the protease in Newlase F does not cleave the amide bond of dipeptides under this condition. So protease in Newlase F did not cause any side reaction and produce the corresponding by-products[7].

Conclusion

The reaction conditions, such as pH, temperature, enzyme content and organic solvents, were optimized in this paper. In this reaction condition, Newlase F neither hydrolyzed the N-terminal protecting group nor cleaved the amide bond in dipeptides. So Newlase F may be used as an enzyme to remove C-terminal protecting group in peptide synthesis and be applied widely in organic synthesis.

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