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# The effect of chemical modification and additives on the stabilities of lipase from *Aspergillus niger*[1]

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**Abstract.** The lysine residue of *A. niger* lipase was modified with ethyl acetimidate to yield ε-acetamidinated lysine. The modified enzyme showed increased activity toward *p*-nitrophenyllaurate hydrolysis and enhanced stability toward heat and SDS denaturation. The Vm increased 57% and Km increased from 1.43 mM to 2.22 mM after modification. The heat denaturation rate constants for native and modified lipase at 62°C and pH 5.6 were 0.035 and 0.01 $min^{-1}$, respectively. The residual activities for native and modified lipase in 2% SDS for 20 min were 50% and 68%, respectively.

Some carbohydrates, polyhydric alcohols and neutral salts can increase the thermal stability of lipase. The protective effect of 0.1 M additives at 62°C and pH 5.6 followed the order: glucose >sucrose>mannose>$MgCl_2$>$BaCl_2$, sorbitol >glycerol. $ZnCl_2$ significantly destabilized the enzyme. Anions and monovalent cations either had no protective effect or slightly destabilized the enzyme.

The activation energies of thermal denaturation of lipase in the absence, in the presence of 0.1M glucose and in the presence of 0.1 M $MgCl_2$ were 71, 45 and 56 Kcal/mole, respectively. The lipase stability increased as the added glucose concentration increased. However, $MgCl_2$ increased the thermal stability of lipase at low concentration but destabilized the enzyme at high concentration (1.44 M).

**Key words:** *Aspergillus niger*; Carbohydrate; Chemical modification; Lipase; Polyhydric alcohols; Stability.

## Introduction

Lipase {EC 3.1.1.3} has been widely used in fats and oil industry (Posorske, 1984). The enzyme stability is very important for their application in biotechnology. There are various strategies for the stabilization of enzyme activity including enzyme immobilization (Cheng and Shaw, 1980; Cheng and Shaw, 1981; Shaw and Cheng, 1983; Chang and Shaw, 1986; Chang and Shaw, 1987; Shaw *et al.*, 1987.), selective chemical modification (Kutuzova *et al.*, 1984), the addition of neutral salts and ligands (Jenks, 1969; Shaw and Smith, 1976). Recently, we discovered that *Aspergillus niger* lipase efficiently catalyzed hydrolysis and alcoholysis of fully acylated aldose for the preparation of acyl derivatives of 1-hydroxy aldose, which was the useful intermediates for medicines and pesticides (Shaw and Klibanov, 1987; Shaw and Liaw, 1987). In the present work, we have studied the effect of chemical modification and various additives on the stabilities and activities of

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*Aspergillus niger* lipase, which would provide insight into enzyme stabilization mechanism and be useful for lipase application.

## Materials and Methods

### *Materials*

Lipase from *Aspergillus nigers* (AP 6), which had a specific activity of 63.7 Amano units/mg solid, was purchased from Amano Pharm. (Nagoya, Japan). The Amano unit was defined as the amount of lipase to liberate 1 $\mu$eq of acids per min, in a reaction mixture containing 5 ml of olive oil and 4.0 ml of 0.1 M phosphate buffer (pH 6.0) at 37°C. Dialysis membrane (Spectra/por 4,MWCO 12,000-14,000) was from Spectrum Medical Industries, (Los Angeles, CA). Ethyl acetimidate and *p*-nitrophenyllaurate were obtained from Sigma Chemical Co. (St. Louis, MO). All other biochemicals and chemicals were of reagent grade.

### *Enzme Assay*

The lipase activity was assayed by a spectrophotometric method according to Amano technical bulletin for lipase G assay. Amano AP 6 lipase 0.05 ml was added to 0.95 ml of substrate solution (containing 0.85 mg *p*-nitrophenyllaurate and 2.1% Triton X-100 in 1 ml of 52.0 mM acetate buffer, pH 5.6) preincubated at 37°C for 5 min, After 15 min at 37°C, the reaction was terminated by the addition of 2 ml of acetone and the absorbance at 140 nm was measured. For the control reaction, the enzyme solution was added after the addition of acetone. One unit of lipase is defined as the amount of enzyme which can release one micromole of *p*-nitrophenol under the above condition. The enzyme concentrations used were such that kept the enzyme reaction at initial velocity conditions.

### *Acetamidination of Enzyme*

The ε-amino groups of lysine residues in lipase were acetamidinated according to Tuengler and Pfleiderer (1977). Lipase AP 6 (15 g) were dissolved in 60 ml pyrophosphate buffer (0.1 M, pH 8.7) and dialyzed against 10 mM phosphate buffer (pH 8.7) to remove low molecular weight additives. Thirty ml of ethyl acetimidate (150 mg/ml in 0.1 M phosphate buffer, pH 8.7) were mixed with 30 ml of the dialyzed enzyme solutions with stirring for 1.5 h at room temperature. The reactions were stopped by dialysis againt 10 mM phosphate buffer (pH 7.0) at 4°C. After dialysis the pH was adjusted to 5.8. For comparison, control samples were treated by the same procedure except ethyl acetimidate solution was replaced by phosphate buffer alone.

### *Measurement of Protein*

The protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

### *The Effect of Acetamidination and Various Additives on Lipase Stability*

The lipase stabilities were examined by ther mal inactivation kinetics. Prior to use in inactivation studies, lipase was dialyzed against 10 mM potassium phosphate buffer (pH 5.8). Various additives including carbohydrates, neutral salts and polyhydric alcohols were dissolved in the same potassium phosphate buffer. Typically, 1 ml of lipase was mixed with 1 ml of various additive solutions and immersed in a thermostated water bath. After certain period, 50 $\mu$l was removed, mixed with 0.95 ml of *p*-nitrophenyllaurate solution and assayed for the residual enzyme activity (see "Enzyme Assay"). Unheated sample was used as the control. Concentrations, time and temperature are given in the figure legends. First-order rate constants for inactivation were obtained from the slopes of least square fits of logarithm fractional activity remaining versus time.

## Results

### *The Effect of Acetamidination on Lipase Activity and Stability*

With *p*-nitrophenyllaurate as substrate, the acetamidated enzyme showed increased specific activity, 78.81 units/mg, which was 1.54 times higher than the unmodified enzyme, 51.29 units/mg under the assay condition. From Lineweaver-Burk plot (Fig. 1), the Vm and Km for modified enzyme were 28.6 $\mu$mole · min$^{-1}$ · mg$^{-1}$ and 2.22 mM respectively, while those for the unmodified enzyme were 1.82 $\mu$mole · min$^{-1}$ · mg$^{-1}$ and 1.43 mM respectively. Therefore, both Vm and Km were increased as a results of acetamidination of lipase. Both the modified and unmodified enzyme followed first-order kinetics in thermal inactivation experiment (Fig. 2). The thermal inactivation rate constants for unmodified enzyme at 61°C and 63°C were 0.036 min$^{-1}$ and 0.052 min$^{-1}$ respectively, while those for acetamidinated enzyme were 0.01 min$^{-1}$ and 0.035 min$^{-1}$ respectively. Therefore, the modified enzymes were 3.6 times (at 61°C) and 1.5 times (at 63°C) more stable than the unmodified lipase.

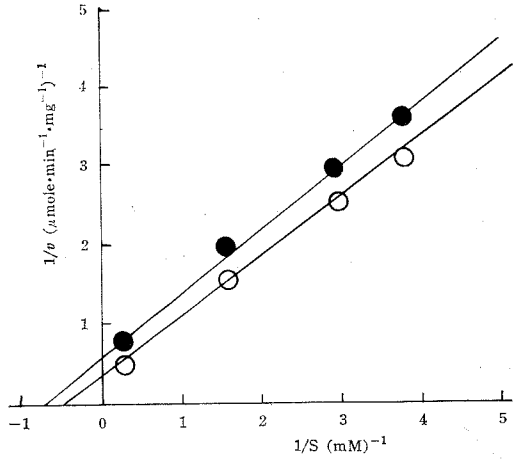



Fig. 1. Lineweaver-Burk plots for the hydrolysis of *p*-nitrophenyllaurate by *Aspergillus niger* lipase. ○-○ modified; ●-● control

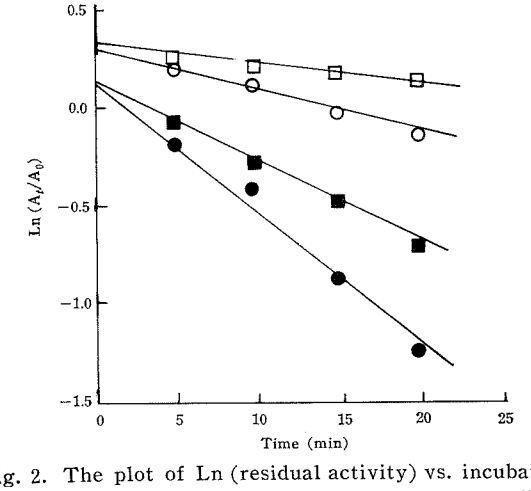



Fig. 2. The plot of Ln (residual activity) vs. incubation time at various temperature for the unmodified and modified lipase. Residual activity of lipase was assayed with *p*-nitrophenyllaurate as substrate. unmodified (●), 61°C; (■), 63°C. modified (○), 61°C; (□), 63°C.

The modified enzyme also showed enhanced stability against detergent denaturation. The residual activites of modified and unmodified enzymes were 68% and 56% respectively after incubation with 1% sodium dodecyl sulfate at pH 5.6 and 25°C for 20 min,

*Effect of Additives on Lipase Stability*

As shown in Table 1, at 0.1 M concentration some carbohydrate, polyhydric alcohols and neutral salts provided significant protection of lipase from thermal inactivation, while some salts made the enzyme more heat labile. For carbohydrates, glucose and sucrose offered the best protective effect, followed by mannose, while galactose showed little effect. For the protective effect of polyhydric alcohols on lipase activity, sorbitol>glycerol> ethylene glycol. For neutral salts effect, divalent cations showed better protective effect, $Mg^{2+}$> $Ca^{2+}$>$Ba^{2+}$, however, $Zn^{2+}$ greatly decreased the thermal stability of lipase. Since $Zn^{2+}$ also greatly inhibited the lipase activity, the decrease of the enzyme stability was attributed to the conformational change induced by $Zn^{2+}$ binding. It was proposed that the stabilizing effect of cations and anions should decrease in the following order (Von Hippel and Schleich, 1969): $(CH_3)_4N^+$>$NH_4^+$ >$K^+$, $Na^+$>$Mg^{2+}$>$Ca^{2+}$>$Ba^{2+}$; $SO_4^{2-}$>$Cl^-$>$Br^-$> $NO_3^-$>$ClO_4^-$>$SCN^-$, with anions and cations being additively effective. This is in accordance with Hofmeister lyotropic series. According to the

**Table 1.** *Effect of various additives on lipase thermostablity*

The lipase samples in the presence of 0.1 M additives in 10 mM phosphate buffer (pH 5.8) were heated at 62°C for 23 min and assyed for residuhl activity. The enzyme activity was assayed in triplicate.

| Additives | | Residual activity (%) |
|---|---|---|
| Control | | 57 |
| Carbohydrate | glucose | 98 |
| (0.1 M) | galactose | 59 |
| | mannose | 89 |
| | sucrose | 96 |
| Polyhydric | ethylene glycol | 58 |
| alcohols | glycerol | 62 |
| (0.1 M) | sorbitol | 66 |
| Neutral salts | $NH_4Cl$ | 57 |
| (0.1 M) | KCl | 50 |
| | $NaCl_2$ | 51 |
| | $CaCl_2$ | 70 |
| | $MgCl_2$ | 75 |
| | $BaCl_2$ | 66 |
| | $ZnCl_2$ | 26 |
| | $(NH_4)_2SO_4$ | 49 |
| | $NaNO_3$ | 44 |
| | NaCl | 51 |
| | $Na_2SO_4$ | 43 |

theory, $(NH_4)_2SO_4$ should be a strong stabilizer of the enzyme. However, in the present experiment with *A. niger* lipase, $(NH_4)_2SO_4$ not only had no protective effect but also slightly decreased the stability of lipase (Table 1). Other neutral salts tested had little effect on lipase stability.

*Effect of Glucose and $MgCl_2$ on Lipase Inactivation Kinetics*

The effects of carbohydrate and neutral salt on lipase were apparently different. As shown in Figs. 3 and 4, the lipase stability increased as glucose concentration increased, while $MgCl_2$ increased the lipase stability at lower concentration but decreased the lipase stability at higher concentration. The first-order thermal inactivation rate constants for lipase in the presence of 0, 0.16, 0.04 and 1.44 M glucose concentration at 62°C and pH 7.0 (potassium phosphate buffer) were 0.023, 0.014, 0.01, 0.002 $min^{-1}$, respectively while those in the presence of 0.10, 0.04, and 1.44 M $MgCl_2$ concentration under the same condition were 0.018, 0.022 and 0.026 $min^{-1}$, respectively. It is interesting to see that lipase in the presence of 0.19 M or 0.64 M $MgCl_2$ concentration exhibited higher activity under short time (10 min) than unheated control (Fig. 4). An intermediate enzyme form with higher activity might be involved in the thermal inactivation process (Henley and Sadana, 1985).

The inactivation rate constants ($k$) of lipase at different temperatures were analyzed by Arrhenius plot (Fig. 5). From $\ln k = \ln A - (Ea/R)\ 1/T$, where A, R and T represent constant, gas constant and absolute temperature respectively, the activation energy Ea can be calculated from the slope. From Fig. 5, it was clear that both $MgCl_2$ and glucose at 0.1 M showed high temperature

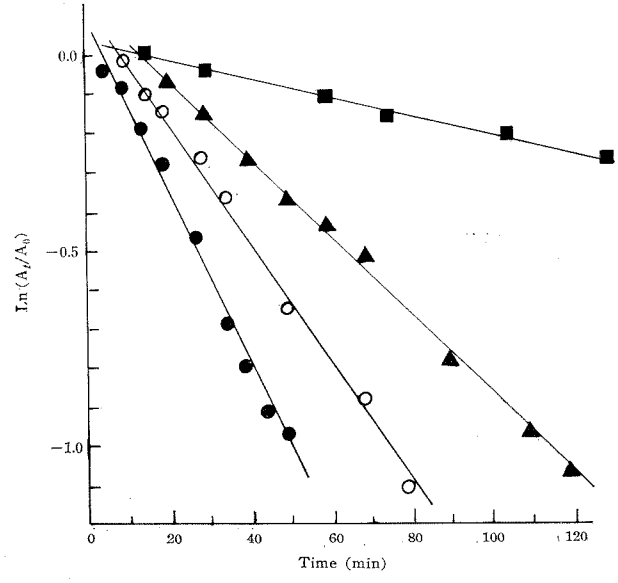



Fig. 3. Effect of glucose concentration on lipase thermal stability. The lipase samples in the pressence of varying glucose concentration in 10 mM phosphate buffer (pH 5.8) were heated at 62°C for various time intervals and assayed for residual activites. ●-● 0 M; ○-○ 0.16 M; ▲-▲ 0.64 M, ■-■ 1.44 M.

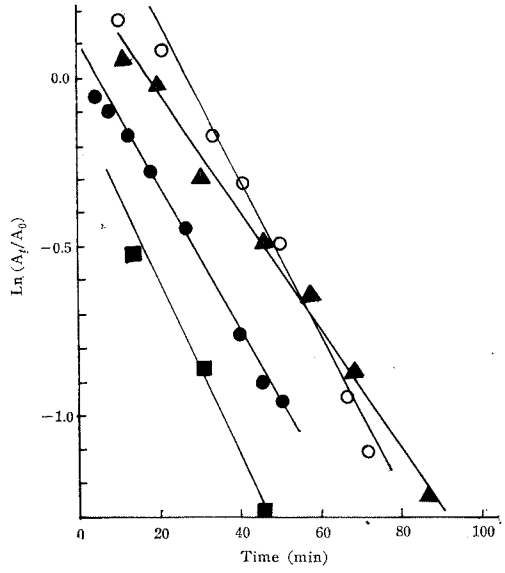



Fig. 4. Effect of $MgCl_2$ concentration on lipase thermal stability. The lipase samples in the presence of varying $MgCl_2$ concentration in 10 mM phosphate buffer (pH 5.8) were heated at 62°C for various time intervals and assayed for residual activities. ●-● 0 M; ○-○ 0.64 M ■-■ 1.44 M.

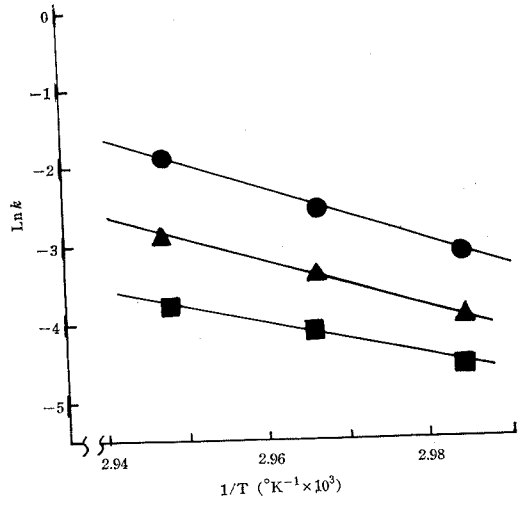


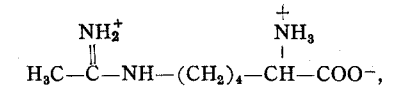

Fig. 5. Arrhenius plots of Ln $k$ vs. reciprocal of temperature. $k$ is the thermal denaturation rate constant at various tempeatures.

stabilization effect, i.e., the stabilization can be observed only above the isokinetic temperature and is greater as the temperature is higher (Kutuz *et al.*, 1984). The activation energies of thermal denaturation of lipase in the absence, in the presence of 0.1 M glucose and in the presence of 0.1 M $MgCl_2$ were 71, 45 and 56 Kcal/mole, respectively.

## Disscusion

The development of enzyme engineering for industrial applications required the improvement of enzyme stability such that enzyme can be used over prolonged period of time. In the present work, we found that actamidination of lipase and the addition of carbohydrate effectively increassed the lipase stability, while divalent cations and polyhydric alcohols were less effective (Fig. 2 and Table 1). On the contrary, some neutral salts such as $ZnCl_2$ greatly reduced the lipase stability (Table 1).

Acetamidination of proteins is a selective and mild modification method for ε-amino groups of lysine residues in proteins without altering the charge. The acetamidinated lysine,



has an arginine-like structure with a similar *pk* value (*pk*=12.5) (Means and Feeney, 1971; Tuengler and Pfleiderer, 1977). Since lysine is not involved in lipase active site, the acetamidination presumably modifies the lysine residues located on enzyme surface. Generally chemical modification of enzymes leads to reduced enzme activity. It has been reported that acetamidinated lactate dehydrogenase reduce 20% enzyme activity as compared to the native enzyme but greatly increased stability toward heat, alkaline denaturation and tryptic digestion (Tuengler and Pfleiderer, 1977). In the present work, the acetamidination also significantly increased the enzyme stabillity (Fig. 2) and suprisingly, increased the enzyme activity as well (Fig. 1). It is possible that modification of lipase induces conformational change which increases the catalytic

efficiency and thermostability. It has also been proposed that increase of the water layer around the enzyme or ionic effects due to the change of ε-amino groups of lysine to arginine-like groups would lead to the enzyme stabilization. (Tuengler and Pfleiderer, 1977). The thermostable enzyme from thermophilic microorganism differs from the thermolabile one by a higher arginine/lysine ratio (Kutuzova *et al.*, 1984).

For unspecific effect of additives, it has been proposed that compounds having a strong interaction with the enzyme but low interaction with water will favor enzyme denaturation due to the stabilization effect on unfolded structure. On the contrary, compounds which interact strongly with water but weakly with the enzyme will enhance enzyme stability by reducing the amount of free water (water activity) (Monsan and Combes, 1983). Therefore, carbohydrate, polyhydric alcohols and neutral salts which have water-activity depressive effect would in general have protective effect on enzyme. It is clear that carbohydrate and polyhydric alcohols in general have protective effect on lipase (Table 1). The protective effect followed the order: glucose >sucrose >mannose >sorbitol >glycerol >galac tose. Since glycerol has a slightly greater water-activity depressing effect than sorbitol but has less protective effect on lipase, the protective effect of polyhydric alcohol is not simply related to the modification of water activity of the medium. Recently, it becomes clear that lipase can accept alcohols, polyhydric alcohols, mono-and disaccharide as substrates in alcoholysis of ester or ester formation reaction (Seino *et al*, 1984; Shaw and Klibanov, 1987; Shaw and Liaw, 1987. Therefore, the protective effect of carbohydrates and polyhydric alcohols probably involves both nonspecific water activity depressive effect and specific ligand binding effect.

For the nonspecific effect of neutral salts, it has been proposed that the stabilization effect of neutral salts is due to the salting out effect of hydrophobic residues from the surface into the interior of enzyme molecule which would compress the enzyme conformation and leads to the higher thermal stability of enzyme (Klibanov, 1983). The stabilization effects of cations and anions should correlate with Hofmeister lyotropic series, According to the theory $(NH_4)_2SO_4$ should be a strong stabilizer. However, in the present experiment only some divalent cations $Mg^{2+}$, $Ca^{2+}$ and $Ba^{2+}$ provided some protective effect. The anions and monovalent cations tested either had no protective effect or made the lipase more heat labile. $ZnCl_2$ (0.1 M) greatly destabilized the lipase (Table 1). $MgCl_2$ increased the lipase stability at lower concentration but destabilized the lipase at higher concentration (Fig 4). These results suggest that the effects of salts on lipase stability are largely due to specific binding of cations to the lipase molecule inducing conformations which are either more heat stable or labile.

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# 化學修飾與添加物對黑麴菌脂肪酶穩定性之影響

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黑麴菌脂肪酶之離胺酸可以 ethyl acetimidate 修飾。此修飾後之酵素對 p-nitrophenylaurate 基質之水解活性增加而且對於熱與 SDS 變性作用之穩定性提高。修飾酵素之 Vm 增加 57%，而 Km 從 1.43 mM 增大爲 2.22 mM。未修飾與修飾脂肪酶在 62°C 與 pH 5.6 下之熱變性常數各爲 0.035 與 0.01 $min^{-1}$ 於 1% SDS 中 20 分鐘後之殘餘活性，未修飾與修飾酵素各爲 56%與 68%。

有一些醣類、多羥基醇類與中性鹽類可以增强脂肪酶之熱穩定性。在 62°C 與 pH 5.6 下，0.1 M 之下列各物質對脂肪酶之保護作用依序遞減，葡萄糖>蔗糖>甘露糖>$MgCl_2$>$BaCl_2$～山梨糖醇>甘油。$ZnCl_2$ 反而使酵素不穩定，陰離子與單價陽離子對酵素穩定性沒有什麼影響。

脂肪酶單獨存在、添加 0.1 M 葡萄糖與添加 0.1 M $MgCl_2$ 之熱變性活化能各爲 71，45 與 56 Kcal/mole，脂肪酶之穩定性隨葡萄糖濃度之增加而上升。然而 $MgCl_2$ 在低濃度下增加酵素穩定性而在高濃度下反而降低酵素之熱穩定性。