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EFFECT OF HYDROPHOBICITY OF ACYL GROUPS ON THE ACTIVITY AND STABILITY OF ACYLATED THERMOLYSIN

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Summary

1. Normal carboxylic acids of different hydrophobicities and similar chain lengths were prepared and used for the modification of amino groups of thermolysin (EC 3.4.24.4). They were 4,7,10,13-tetraoxatetradecanoic acid, 4,7,10-trioxatetradecanoic acid, 4,7-dioxatetradecanoic acid and 4-oxatetradecanoic acid.

2. The modified enzymes were isolated by gel filtration. They had 6—7 acyl groups per molecule. Acylation of amino groups with 4-oxatetradecanoic acid and tetradecanoic acid made the enzyme insoluble.

3. The most hydrophilic enzyme derivative had similar enzyme activity and higher heat resistance than the native enzyme. The most hydrophobic derivative showed lower K_m (50%) and V (40%) values for proteinase activity and lower heat resistance than the former derivative. The trioxa-derivative had intermediate characteristics. The results are discussed with respect to effects on stability and activity of the enzyme.

Introduction

There have been few attempts to change surface properties, especially hydrophobicity, of enzyme molecules. We have prepared a series of normal carboxylic acids having different hydrophobicities and similar chain lengths for the modification of amino groups of enzymes (Fig. 1).

A methylene group increases the hydrophobicity of amino acid side chains by approx. 0.7 kcal/mol and an ether group decreases it by approx. 0.7 kcal/mol [1]. Thus, replacement of an ether group by a methylene group increases hydrophobicity by approx. 1.4 kcal/mol. Therefore, the hydrophobicity of the acyl groups shown in Fig. 1 increases from tetraoxatetradecanoyl to tetradecanoyl with the increasing number of methylene groups replacing the corresponding oxygen atoms.

In this report, the acyl groups were introduced into the free amino groups of

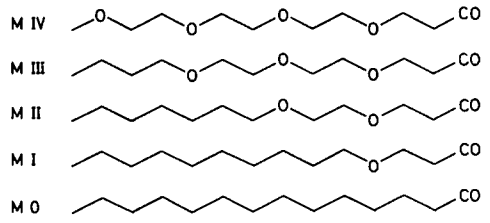

Fig. 1. Structure of the acyl groups. M IV, 4,7,10,13-tetraoxatetradecanoyl; M III, 4,7,10-trioxatetradecanoyl; M II, 4,7-dioxatetradecanoyl; M I, 4-oxatetradecanoyl; M 0, tetradecanoyl.

thermolysin (EC 3.4.24.4), a thermostable neutral protease. The number and position of acylation sites were kept constant as far as possible by acylating almost all free amino groups of the enzyme. Thus, the difference in the activity and stability of the modified enzymes should reflect the difference in the hydrophobicity of the acyl groups.

Materials and Methods

Crystalline thermolysin (*Bacillus thermoproteolyticus* Rokko) was kindly supplied by Daiwa Kasei Co., Osaka. Before use the enzyme was subjected to gel filtration on Sephadex G-50 equilibrated with 20 mM barbital buffer (pH 8.5)/20 mM $CaCl_2$. *N,N*-Dimethylcasein was prepared by the method of Lin [2]. Cbz-Gly-Leu-NH_2 was prepared according to Vaughan [3]. Dioxane was purified by distillation after refluxing with $LiAlH_4$. Ethyl chloroformate was used after distillation. Tetradecanoic acid was purchased from Sigma, St. Louis, Mo., U.S.A. All other chemicals were of reagent grade.

3,6,9-Trioxa-1-decanol. 0.12 mol sodium metal was dissolved in 0.5 mol triethyleneglycol at 80—90°C under N_2 and 0.1 mol dimethyl sulfate was added dropwise with stirring at 50—55°C. The reaction mixture was stirred for 1 h at 80°C. Distillation (three times) under reduced pressure yielded 10.6 g 3,6,9-trioxa-1-decanol.

3,6-Dioxa-1-decanol. 0.1 mol sodium metal was dissolved in 0.5 mol diethyleneglycol at 60—70°C under N_2 and 0.11 mol *n*-butyl bromide was added. The reaction mixture was stirred for 5 h at 70—80°C. Distillation (twice) under reduced pressure yielded 13.4 g 3,6-dioxa-1-decanol.

3-Oxa-1-decanol. 0.17 mol sodium metal in 0.8 mol ethyleneglycol stirred with 0.14 mol *n*-heptyl bromide for 6 h at 60—70°C. Distilation (twice) under reduced pressure yielded 3-oxa-1-decanol.

4,7,10,13-Tetraoxatetradecanoic acid. 0.12 mol 3,6,9-trioxa-1-decanol and 0.23 mol 3-propiolactone were heated together at 100°C for 24 h. 120 ml 6 M KOH was added and the solution was kept at 31°C for 24 h. The reaction mixture was acidified with HCl and extracted with dichloromethane. The dichloromethane solutions were dried over anhydrous Na_2SO_4 and evaporated to remove the solvent. Distillation (twice) under reduced pressure yielded 4,7,10,13-tetraoxatetradecanoic acid.

4,7,10-Trioxatetradecanoic acid. This was similarly prepared from 0.05 mol 3,6-dioxa-1-decanol and 0.1 mol 3-propiolactone.

4,7-Dioxatetradecanoic acid. A small amount of sodium metal was dissolved in 19 mmol 3-oxa-1-decanol and 22 mmol methyl acrylate was added to the solution at 5°C with stirring. After 3 h the reaction mixture was acidified with 3 M HCl and extracted with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to remove the solvent. Distillation under reduced pressure yielded methyl 4,7-dioxatetradecanoate. 2.3 g KOH in 3 ml water was mixed with 10 ml methanol and 4.1 mmol methyl-4,7-dioxatetradecanoate. After 2 h at room temperature, the reaction mixture was acidified with 3 M HCl, evaporated, and extracted with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to remove the solvent. Distillation under reduced pressure yielded 4,7-dioxatetradecanoic acid.

4-Oxatetradecanoic acid. 10 mmol 1-decanol and 50 mmol 3-propiolactone were heated at 100°C for 2 days. Distillation under reduced pressure yielded 4-oxatetradecanoic acid.

Acylation of enzyme. Thermolysin was acylated with mixed carboxylic acid anhydrides of the analogues of tetradecanoic acid described above. A solution of 0.1 mmol tetradecanoic acid analogue and 0.1 mol triethylamine in 0.2 ml dioxane was cooled to 10°C, and 0.08 mmol ethylchloroformate in 0.2 ml dioxane was added. After 30 min at this temperature, the solution was diluted with dioxane to the required concetration. Acylation of thermolysin was carried out at 40°C. The reaction mixture contained 8 ml enzyme solution (70—80 μM) in 20 mM barbital buffer (pH 8.5)/20 mM $CaCl_2$ and 4 ml mixed carboxylic acid anhydride (9—16 mM) in dioxane. After 1 h, 1 ml 1.3 M NH_2OH (pH 8.5) was added to the solution to remove *O*-acyl groups on tyrosyl residues of the enzyme [4]. After 1 h at 40°C, the precipitate formed was centrifuged off and the clear solution was passed through Sephadex G-25 equilibrated with 10 mM barbital buffer (pH 7.5)/20 mM $CaCl_2$ at 4°C to separate modified thermolysins from the reaction mixture. The enzyme concentration was calculated from the absorbance at 280 nm, based on a molar absorption coefficient of 52400 $M^{-1} \cdot cm^{-1}$ [5].

The number of free amino groups was determined using 2,4,6-trinitrobenzenesulphonic acid according to Fields [6]. The reaction mixture contained 0.5 ml enzyme solution in 10 mM barbital buffer (pH 7.5)/20 mM $CaCl_2$ and 2 ml 0.4 M sodium borate/14 mM 1,10-phenanthroline/1 mM $CaCl_2$/5% CH_3OH. The reaction was initiated by adding 1 ml 0.05% trinitrobenzenesulphonic acid/5 mM Na_2SO_3 and allowed to proceed at 40°C for 2 h. Absorbance was measured at 420 nm. The molar absorption coefficients of the trinitrophenylated α- and ϵ-amino groups were assumed to be 22000 and 19000 $M^{-1} \cdot cm^{-1}$, respectively [6]. Approx. 7.5 of the 12 amino groups [7] on thermolysin reacted with trinitrobenzenesulphonate, assuming that the N-terminal α-amino group was most reactive [6]. The number of amino groups acylated on a modified thermolysin was estimated from the decrease in the number of free amino groups.

Proteinase and peptidase activity were assayed with *N,N*-dimethylcasein and Cbz-Gly-Leu-NH_2 as substrate, respectively. Standard assay conditions were: 0.5% *N,N*-dimethylcasein or 10 mM Cbz-Gly-Leu-NH_2, 5 mM barbital, and 1 mM $CaCl_2$ (pH 7.5, 40°C). When Cbz-Gly-Leu-NH_2 was used as substrate, 5% CH_3OH was added to the reaction mixture irrespective of the substrate concen-

tration. 0.2 ml of the reaction mixture were withdrawn by pipette at 1-min intervals and added to 2 ml 0.4 M sodium borate/10 mM EDTA to stop the reaction, and the amount of liberated α-amino groups was determined using trinitrobenzenesulphonate.

4,7,10,13-Tetraoxatetradecanoyl thermolysin, derivative 4; 4,7,10-trioxatetradecanoyl thermolysin, derivative 3; 4,7-dioxatetradecanoyl thermolysin, derivative 2; 4-monooxatetradecanoyl thermolysin, derivative 1.

Results

Amino groups on thermolysin were acylated with mixed carboxylic acid anhydrides of the tetradecanoic acid analogues. Tyrosyl residues of the enzyme could also be acylated with the reagents and *N,O*-acyl-enzymes had much lower proteinase activity than corresponding *N*-acyl-enzymes. Similar results were reported by Ohta et al. [8] using acetyl-thermolysin. In this report, *O*-acyl groups were deacylated with hydroxylamine and the effect of *N*-acylation was investigated.

The number of free amino groups on thermolysin was approx. 7.5, and 6—7 amino groups were acylated with 4,7,10,13-tetraoxatetradecanoic acid, 4,7,10-trioxatetradecanoic acid, or 4,7-dioxatetradecanoic acid as shown in Tabel I. However, complete acylation of free amino groups with 4-oxatetradecanoic acid or tetradecanoic acid was unsuccessful because the acylated enzymes were insoluble. Therefore, the characteristics of the three acyl-enzymes, derivatives 2, 3 and 4, were mainly investigated.

Table I shows the activity of the acyl-enzymes. The proteinase activity of derivative 4, the most hydrophilic acyl-enzyme, was equal to that of the native thermolysin, and decreased with increasing hydrophobicity of the acyl groups. The proteinase activities of derivative 1 and tetradecanoyl-thermolysin with 2 or 3 acyl groups per enzyme molecule, were about 40% of that of the native enzyme.

Peptidase activities of derivatives 2 and 3 were a little higher than those of native enzyme of derivative 4. The peptidase activity was assayed in the presence of 5% CH_3OH, and the addition of methanol might cancel the effect of different hydrophobicities of the acyl groups. But the proteinase activity in the

TABLE I

ENZYMATIC ACTIVITY OF NATIVE AND ACYL-THERMOLYSIN DERIVATIVES

Proteinase and peptidase activities were measured at 40°C with 0.5% *N,N*-dimethylcasein and 10 mM Cbz-Gly-Leu-NH_2, respectively.

Enzyme	No. of $-NH_2$ acylated	Proteinase activity		Peptidase activity	
		(mol/mol per s)	(%)	(mol/mol per s)	(%)
Thermolysin	0	95	100	13	100
Derivative 4	6.8	94	99	12	92
Derivative 3	7.0	76	80	16	123
Derivative 2	6.4	38	40	15	115

TABLE II

MICHAELIS PARAMETERS OF NATIVE AND ACYL-THERMOLYSIN DERIVATIVES FOR THE HYDROLYSIS OF *N,N*-DIMETHYLCASEIN AND Cbz-Gly-Leu-NH_2

Activity was assayed at 40°C in 5 mM barbital buffer (pH 7.5)/1 mM $CaCl_2$ with six substrate concentrations ranging from 0.2 to 2.0 mg/ml of *N,N*-dimethylcasein or from 2 to 20 mM of Cbz-Gly-Leu-NH_2.

Enzyme	*N,N*-dimethylcasein		Cbz-Gly-Leu-NH_2
	K_m (mg/ml)	V (mol/mol per s)	V/K_m (1/M per s)
Thermolysin	0.9	108	$1.45 \cdot 10^3$
Derivative 4	1.3	114	$1.38 \cdot 10^3$
Derivative 3	1.4	92	$1.85 \cdot 10^3$
Derivative 2	0.7	45	$1.77 \cdot 10^3$

presence of 5% methanol was similar to that given in Table I. Therefore, the increase in hydrophobicity of the acyl groups decreased only proteinase activity of acyl-thermolysin.

The effect of substrate concentration on the activity of the acyl-enzymes was investigated. The hydrolysis of *N,N*-dimethylcasein and Cbz-Gly-Leu-NH_2 followed Michaelis-Menten kinetics. The values of the Michaelis constant (K_m) and maximum velocity (V) for proteinase activity were calculated from Lineweaver-Burk plots. For peptidase activity, the value of V/K_m was calculated from Hofstee plots because of the high K_m values (approx. 50 mM) for Cbz-Gly-Leu-NH_2. These values are summarized in Table II. As for proteinase activity, derivatives 3 and 4 had larger K_m values and derivative 2 a smaller K_m value

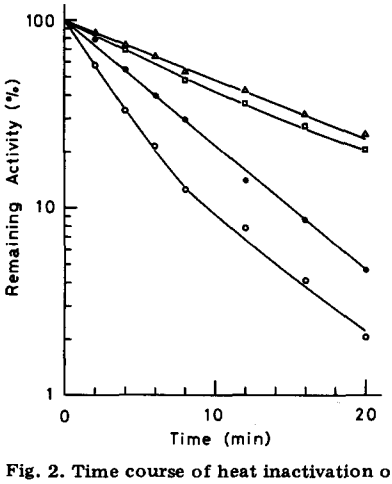

Fig. 2. Time course of heat inactivation of native and acyl-thermolysin derivates. Enzymes (approx. 3 μM) in 10 mM barbital buffer (pH 7.5) containing 10 mM $CaCl_2$ were heated at 90°C. At the time indicated, the enzyme solution was cooled and activity was assayed with *N,N*-dimethylcasein as described under Materials and Methods. ●, Thermolysin; △, derivative 4; □, derivative 3; ○, derivative 2.

than the native enzyme; the V values of acyl-thermolysins decreased with increase in the hydrophobicity of the acyl group. As for peptidase activity, derivatives 2 and 3 had higher values of V/K_m than the vative or derivative 4.

The effect of acylation on the heat resistance of the enzyme was tested at 90°C. Fig. 2 shows the time course of heat inactivation of the enzymes. The heat inactivation rate constants calculated from the linear parts were 0.16, 0.072, 0.084 and 0.26 min^{-1} for the native enzyme and derivatives 4, 3 and 2, respectively. Derivatives 3 and 4 had higher, and derivative 2 lower, heat resistance than the native enzyme. The order of heat resistance was the same at 80°C. These results show that hydrophilic modification increases and hydrophobic modification decreases heat resistance of thermolysin.

Discussion

Titani et al. [7] showed that thermolysin has 12 amino groups per molecule and we found that approx. 7.5 amino groups reacted with trinitrobenzenesulphonic acid, in contrast with the results of Oshima et al. [9]. This discrepancy is probably due to the effect of autolysis of thermolysin. We used the enzyme preparation immediately after gel filtration and added 1,10-phenanthroline and $CaCl_2$ to the reaction mixture to minimize the effect of autolysis.

Derivatives 2, 3 and 4 had 6—7 acyl groups per molecule. As 6 lysyl groups participate in salt bridge formation [10], the remaining 6 amino groups (Ile-1, Lys-45, Lys-182, Lys-239, Lys-307 and Lys-316) should be acylated in these acyl-thermolysin derivatives. These acyl groups have similar chain lengths and different hydrophobicities. Therefore, the differences in enzymatic property between these acyl-enzymes are due mainly to the differences in the hydrophobicity of the enzyme surface. Derivative 3 had intermediate enzymatic property, but the difference between derivatives 4 and 3 was smaller than that between derivatives 3 and 2. There may be a critical value of hydrophobicity of the enzyme surface for the enzymatic property. Derivative 1 and tetradecanoylthermolysin, with more than four acyl groups, were insoluble. The surface hydrophilicity of a soluble enzyme like thermolysin is thought to have an important effect not only on its solubility but also on its activity and stability. Kapmeyer et al. [11] reported a hydrophobically-modified lactate dehydrogenase, which contained 38 ϵ-(N-2,4-dinitrophenyl)aminocaproamidino groups per tetramer. The modified lactate dehydrogenase had poor solubility, lower activity (42%), and lower thermostability. These results accord well with ours. On the other hand, hydrophilic modification enhanced heat resistance without changing enzymatic activity. This finding will be useful for increasing the heat resistance of a soluble enzyme. These changes in enzymatic property may be due to conformational change by these modifications. Further investigation will be needed concerning the relationship between activity, stability and structure of the enzyme.

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