Effect of Ketonic Organic Solvents on Thermolysin : A Spectrophotometric Approach

M. Nurul Alam[1], Koji TADASA* and Hiroshi KAYAHARA

Department of Bioscience and Biotechnology,
Faculty of Agriculture, Shinshu University
[1]The United Graduate School of Agricultural Science, Gifu University (Shinshu University)

Summary. Ultraviolet spectra of thermolysin on mixing with *n*-alkyl ketones were investigated. Hypochromic effect of UV spectra of enzyme and model compounds was observed. A clear bathochromic shift (red shift) of thermolysin spectrum was also demonstrated. With increase in chain length of ketones, the red shift was found reduced. The hypochromic and bathochromic effects were significantly suppressed when enzyme or model compounds were pre-treated with inhibitor (phosphoramidon). There had been no such effect of ketones upon mixing with substrates. It was speculated that ketones interact with the UV sensitive aromatic amino acid residues at the microenvironment of active site of enzyme.

Keywords : thermolysin, ketone, ultraviolet spectra, hypochromic effect, bathochromic (red) shift

Bacillus thermoproteolyticus thermolysin [EC 3.4.24.4], the best studied member of the thermolysin clan, is a thermostable zinc endopeptidase[1]. It's amino acid sequence[2] and three-dimensional structure[3-5] are known. Thermolysin is constituted by a single polypeptide chain of 316 amino acid residues with a molecular mass of 34.6 kDa.[1] It contains 41 aromatic amino acid residues including 3 tryptophans, 28 tyrosines and 10 phenylalanines.

It is known for a long time that the ultraviolet absorption spectra of protein solutions at wavelengths greater than 250 nm is due chiefly to the aromatic side chains of tyrosine, phenylalanine and tryptophan[6-7]. The enhancement of thermolysin activity in the presence of some neutral salts such as NaCl has been documented[8]. Recently, it has been reported that these neutral salts cause ultraviolet absorption difference spectrum at around 293 nm region, which is the characteristics of the red shift of a tryptophyl residue caused by charge effect. Furthermore, conformational change of the enzyme due to the effect of salt has also been proposed[9-10].

Enzymatic catalysis in organic solvent is of great interest today. In the recent years, we have reported that a small amount of organic solvents, miscible or immiscible in water, greatly affects the activity of thermolysin-catalyzed synthesis of Z-Phe-Phe-OMe[11-12]. In contrast to the well known term "water activity," we have proposed the term "organic activity" to describe this phenomenon. But the realities in the interplay between enzyme (in particular the active site) and organic solvent remains obscure. What makes the enzyme turn into being so potent in the presence of organic solvents? This remains the target of few research groups around the world including ours. In attempts to rationalize the activation or inhibition of thermolysin by organic solvents, we explained these facts by means of kinetic data and proposed models of such phenomena[13-14]. Still, the interaction between enzyme's active site and organic solvent remains uncertain. Until today, there has been no report on the spectrometric study of organic solvent's effect on

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*Corresponding Author ; Fax : (0265) 763928,
e-mail : tadasak@gipmc.shinshu-u.ac.jp

enzyme activity. This paper reports an attempt to rationalize spectroscopically the activation effect of *n*-alkyl ketones on thermolysin.

Materials and Methods

Enzyme Crystalline thermolysin (lot T5CB491) from *Bacillus thermoproteolyticus* (EC 3.4.24.4) with a specific activity of 8960 PU/mg protein was obtained from Daiwa Kasei K. K. (Osaka, Japan) and was used without further purification. Before used, the solution of enzyme was filtered through Millipore membrane filter.

Chemicals Normal-alkyl ketones (acetone, methyl ethyl ketone, methyl propyl ketone, methyl pentyl ketone, methyl hexyl ketone, methyl heptyl ketone and methyl octyl ketone), model amino acid residues (*N*-Ac-Trp-OEt and *N*-Ac-Tyr-OEt) and inhibitor (phosphoramidon) were purchased from Nakalai Tesque, Inc. (Kyoto, Japan) or Wako Pure Industries, Ltd. (Osaka, Japan), Aldrich Chem. Co. (MI, USA) and peptide Institute (Osaka, Japan) respectively. Amino acid derivatives (Z-Phe and Phe-OMe. HCl) were prepared in our laboratory.

Buffer 40 mM Tris-HCl buffer, containing 10 mM $CaCl_2$, pH 7.5 at 40°C was used throughout the study.

Measurement of Absorption Spectra of Enzyme

A Shimadzu Spectrophotometer PC-2500 was used throughout the investigation. Ten μM thermolysin solutions were prepared in buffer containing different concentrations (percent, v/v) of ketones, filtered, transferred to cells (micro type) and kept for 10 minutes in the slots to reach desired temperature (40°C) Similarly, a blank solution for each was kept in the reference slot and treated as above.

Measurement of Absorption Spectra of Model Compounds *N*-Ac-Trp-OEt or *N*-Ac-Tyr-OEt solutions of 0.1 mM and 0.4 mM respectively were prepared in buffer containing different concentration of solvents, filtered, and then treated as described above to measure the spectra.

Measurement of Absorption Spectra of Inhibitor Thermolysin (10μM) and phosphoramidon (13 μM) were mixed thoroughly with buffer containing different concentrations of solvents, kept for 30 minutes, filtered, and treated as described above to measure the spectra.

Results and Discussion

Effects of n-Alkyl Ketones on the Ultraviolet Spectra of Thermolysin

We have reported that *n*-alkyl ketones greatly affect the thermolysin-catalyzed peptide synthesis of Z-Phe-Phe-OMe.[11] We found that *n*-alkyl ketones from methyl *n*-propyl ketone through methyl *n*-hexyl ketone greatly activate the peptide synthesis whereas dimethyl ketone (acetone) and methyl ethyl ketone inhibit this synthesis at their high concentrations (around

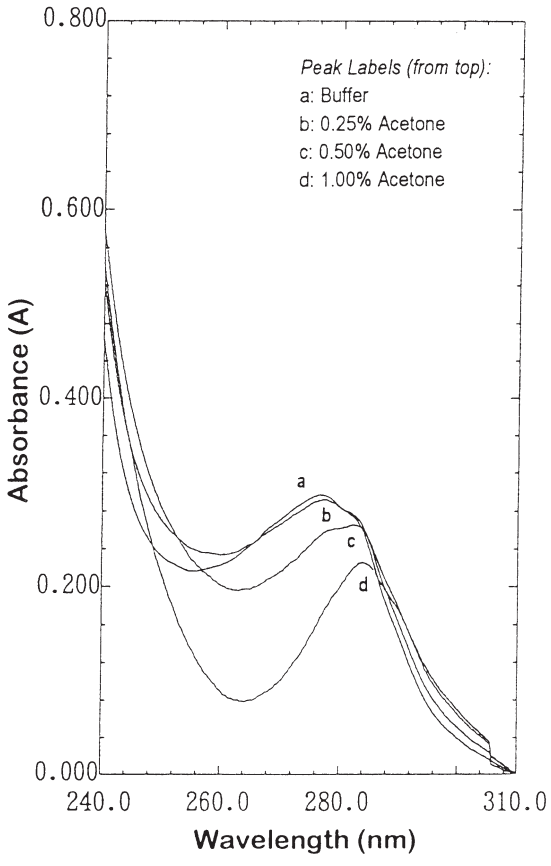

Fig. 1 : Absorption spectra observed on the mixing of thermolysin in buffer with different concentrations (%, v/v) of acetone. The thermolysin concentration was 10 μM in 40 mM Tris-HCl buffer pH 7.5 at 40°C. Each sample was treated equally : same pre-incubation, same slot and same cell in spectral measurement.

Table 1 : Bathochromic red shift at different concentrations of various ketonic solvents. Thermolysin concentration was 10 μM. Other experimental conditions are same as in the Fig. 1.

Solvent	Red shift (nm) in the presence of solvent (% v/v)		
	0.25%	0.50%	1.00%
Acetone	2.35	5.75	7.50
Methyl ethyl ketone	1.85	5.25	6.75
Methyl propyl ketone	1.75	4.85	5.35
Methyl butyl ketone	1.75	3.60	4.35

18.6%, v/v) in buffer. Acetone is regarded as water-miscible solvent which is infinitely soluble in water as described in literature. On the contrary, methyl ethyl ketone is soluble upto 18.6% (v/v) in water. Recently, we found (data to be published elsewhere) that acetone, when used in low concentration (around 1.5% v/v) in water, activates the same peptide synthesis and further addition beyond this level causes suppression of the activity. In this article we focused on the possibility of conformational alterations in the microenvironment of active site of enzyme due to the interaction with ketonic organic solvents. Fig. 1 depicts the absorption spectra observed on mixing of thermolysin with buffer containing different concentrations of acetone. Only one sharp peak was observed in each case. One can see that with the increase in the solvent concentration, two types of effects, namely hypochromic (decrease in absorption intensity) and bathochromic/red shift (shift of absorption maximum to the longer wavelength), are clear. The similar profiles were observed when effect of longer *n*-alkyl ketones were examined (data not shown). It was found that the intensities of red shift decrease with the increase in chain length of the solvent added. Table 1 lists the values of bathochromic red shifts caused by acetone and longer ketones. The red shift was calculated by subtracting the wavelengths (nm) of absorption maxima of thermolysin spectra containing different concentrations of ketones from the absorption spectrum of enzyme in buffer without ketone. Similarly, a tendency of 'larger the chain length-weaker the hypochromic effect' was observed.

We checked other group of organic solvents whether they show any red shift like ketones. To this end, we found that other solvents like alcohols and alkyl esters did not show such distinct red shift in the UV spectra of thermolysin (data to be published elsewhere) although absorption intensities were greatly changed.

UV Spectra of Model Compounds

Since spectral change in thermolysin solution was observed upon mixing with solvents, it was

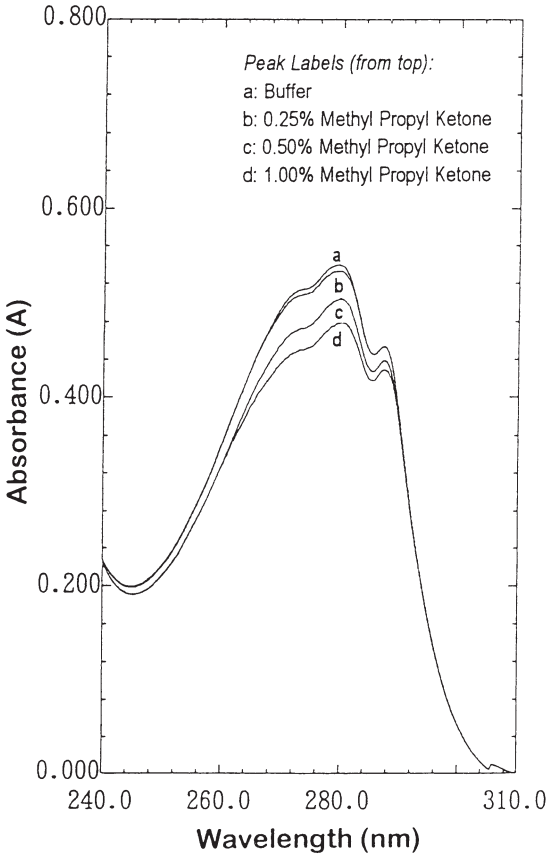

Fig. 2 : Spectra of *N*-Ac-Trp-OEt in buffer with different concentrations of methyl propyl ketone. *N*-Ac-Trp-OEt concentration was 0.1 mM in Tris-HCl buffer pH 7.5 at 40°C. Other experimental conditions are the same as in Fig. 1.

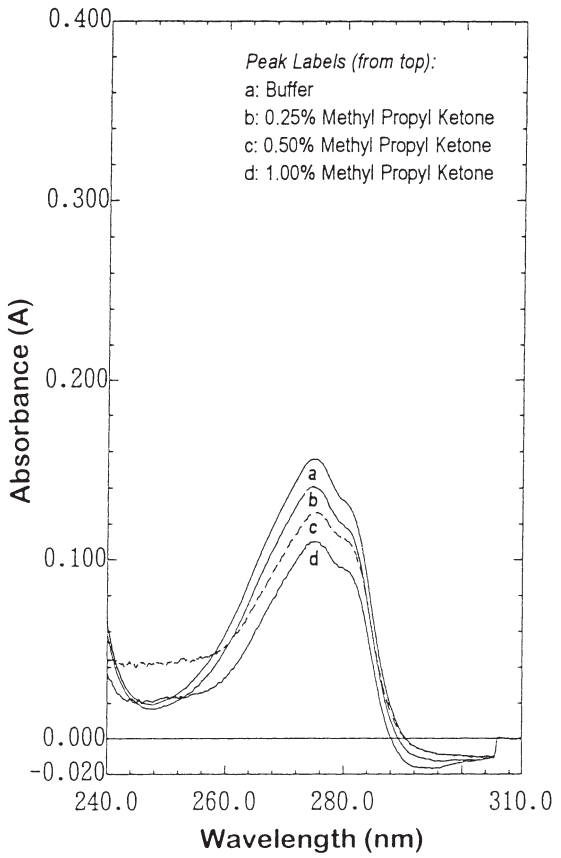

Fig. 3 : Spectra of *N*-Ac-Tyr-OEt in buffer with different concentrations of methyl propyl ketone. *N*-Ac-Tyr-OEt concentration was 0.5 mM in Tris-Hcl buffer pH 7.5 at 40°C. Other experimental conditions are the same as in Fig. 1.

naturally predicted that the aromatic amino acid residues, specially tryptophan or tyrosine, should have undergone similar changes. We have checked this prediction by using *N*-Ac-Trp-OEt and *N*-Ac-Tyr-OEt as model compounds. Free amino acid residues were avoided on the ground of checking similar peptide bonded condition as that in the enzyme. Figs. 2 and 3 depict the spectra of *N*-Ac-Trp-OEt and *N*-Ac-Tyr-OEt respectively in buffer containing different concentrations of methyl propyl ketone. As seen in the Fig. 2, two identical peaks at around 287 nm and 279 nm of tryptophan were observed in the cases of both solvent-free and solvent-containing systems. In the case of tyrosine (Fig. 3), only one sharp peak at 275 nm was observed. For both model compounds, the hypochromic effects were in accordance with those observed upon mixing of ketones with free thermolysin. More clearly, with solvent concentration increased, the hypochromic effects also increased. But the bathochromic red shift, which was very prominent in the case of thermolysin with increasing ketone concentration in buffer, was significantly reduced (less than 1 nm range). This phenomenon seems difficult to understand at the moment. In the strict sense, however, UV sensitive aromatic amino acid residues embedded in thermolysin and the model compounds investigated are in the different states. While these amino acid residues encounter various interactions (viz., electrostatic, hydrophobic force and van der Waals force) in globular or native form in thermolysin, they are not subject to such interactions in their free states (i. e., N-*Ac-Trp-OEt or* *N*-Ac-Tyr-OEt) that we have examined. This could result in such variations in the bathochromic effect.

The relationship between absorption intensity of these model compounds and their carbon length were also investigated for acetone through methyl heptyl ketone and was found to be in good order i.e., longer the carbon length-weaker the absorbance. Phenylalanine was not checked because it's sensitivity to UV light is regarded as the lowest among aromatic amino acid residues in protein. Depending on these results it can be predicted that thermolysin might have undergone some conformational changes (probably the α-helix orientation) due to the effects of ketonic solvents so as to change the sensitivity of it's aromatic amino acid residues to the ultraviolet radiation. These changes, however, were not directly related to the activity of thermolysin in the presence of ketonic-solvents. As mentioned, thermolysin contains 3 tryptophan and 28 tyrosine residues among which Trp 115, Tyr 151 and Tyr 157 are close to active site (Fig. 4) of the enzyme[3,15,16]. Therefore, it seems reasonable to predict that organic solvent molecules interact with these residues and affect the spectra as well as the activity patterns of enzymes.

UV Spectra of Substrate Solutions Mixed with Solvents

To rule out the possibility of interaction of ketones with substrate, Z-Phe & Phe-OMe were

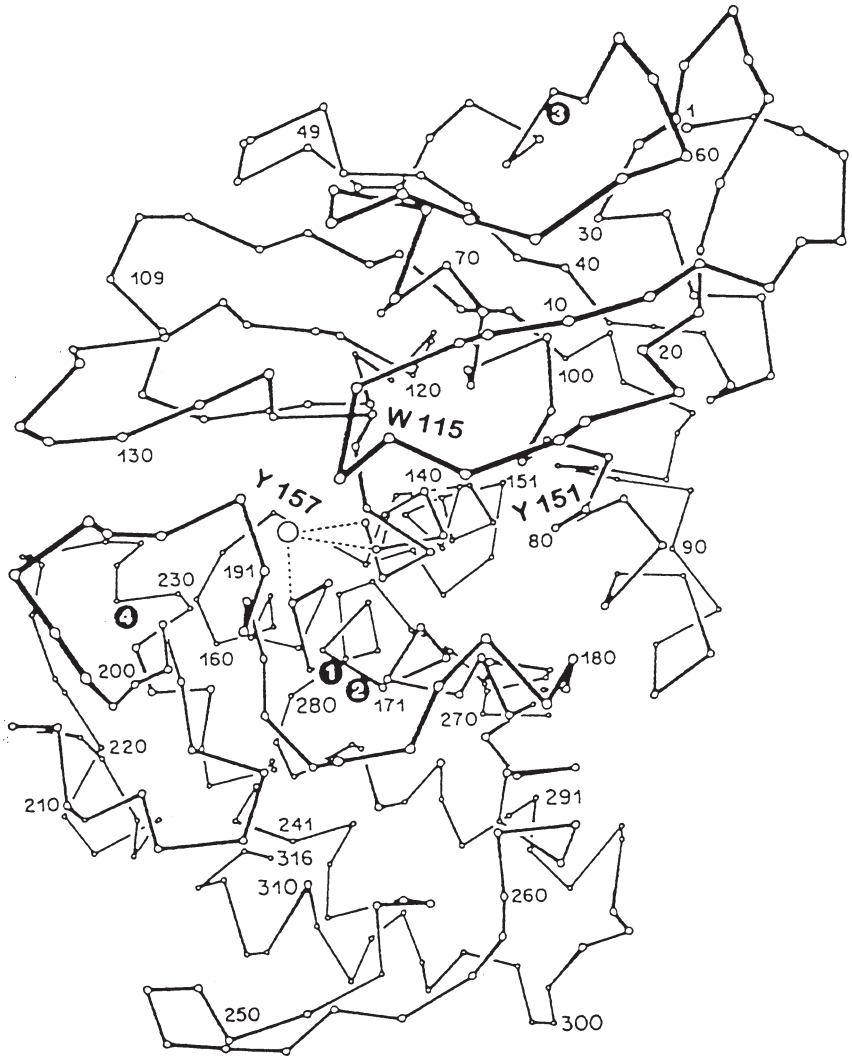

Fig. 4 : Perspective drawing illustrating the backbone conformation of the thermolysin molecule showing the zinc and calcium ions represented by open and closed circles respectively. The tryptophan and tyrosine residues are represented by one letter symbols and their respective positions in thermolysin (Adapted from Colman, P. M., Jansonius, J. N., And Matthews, B. M., *J. Mol. Biol.*,**70**, 701 (1972).

mixed with different concentrations of ketones and spectra were taken. Results are shown in the Fig. 5. One broad peak at around 250 nm and a sharp peak at 211 nm region were observed. There was neither significant hypochromic nor bathochromic effect upon mixing of acetone with these substrates that we have used for model reaction to investigate organic solvent effects on thermolysin. Therefore, the prediction of interaction of solvent with enzyme molecule, not with substrate, seems to be correct.

Spectra of Phosphoramidon Treated Thermolysin Upon Mixing With Ketones

Some potent competitive inhibitors of thermolysin are known and their interaction with the enzyme has been studied extensively[17–21]. Fig. 6 illustrates schematically the proposed mode of binding of phosphoramidon and zincov to the active site of thermolysin[17–21]. We selected phosphoramidon as inhibitor in this study. The concentration of inhibitor used here was decided by carrying out an experiment which showed no synthetic or hydrolytic activity of enzyme. Therefore, it can be predicted that the active site of thermolysin was covered and turned inactive. It was found that the hypochromic effects become

weaker, but not totally lost, upon mixing with phosphoramidon prior to the addition of ketonic solvents. It was speculated that the interaction of solvent with the active site aromatic residues of thermolysin is predominant though not exclusive. Direct evidence of such interaction seems difficult at the moment and awaits future research.

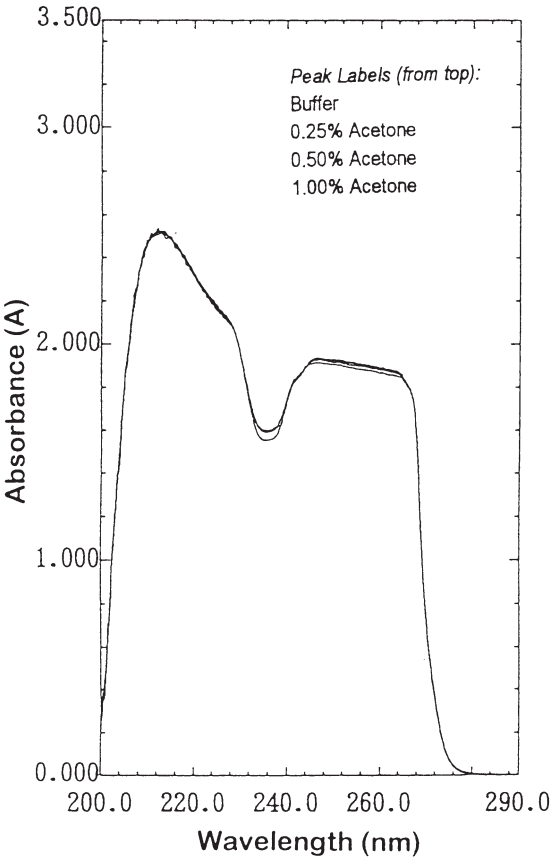

Fig. 5 : UV spectra of Z-Phe and Phe-OMe mixed with different concentrations of acetone. Z-Phe and Phe-OMe concentrations were 10 mM and 20 mM respectively in Tris-HCl buffer pH 7.5 at 40°C. For further details, see text.

Conclusion

We demonstrated that ketonic organic solvents induce clear bathochromic red shift and hypochromic effects in the UV spectra of thermolysin. The red shift of spectra of tryptophyl residues in thermolysin due to their interaction with neutral salts has been justified by the charge effect[9-10]. Since the red shift is prominent with ketones but not with alcohols and esters, the interaction between UV sensitive residues (especially tryptophan) with keto group is of particular importance. But this can not be discussed straightforward. Moreover, the bathochromic (red) shift in the UV spectra is caused by the greater mobilization of π electron of chromophore. Our data demonstrate that the red shift becomes weaker in the cases of longer ketones. Therefore, it can be speculated that with the chain length increased, the hydrophobic interaction between the carbon chain of ketones and the arylic π electrons in the same chromophore may become stronger and the π electrons may become less mobilized. As a result, the intensities of red shift become weaker which is evident in the data presented in this article. It was also predicted that ketonic solvents induce the conformational change in thermolysin structure. This change might be very minute to be detected conventionally, but enough to affect the activity profiles of thermolysin which necessitates further study.

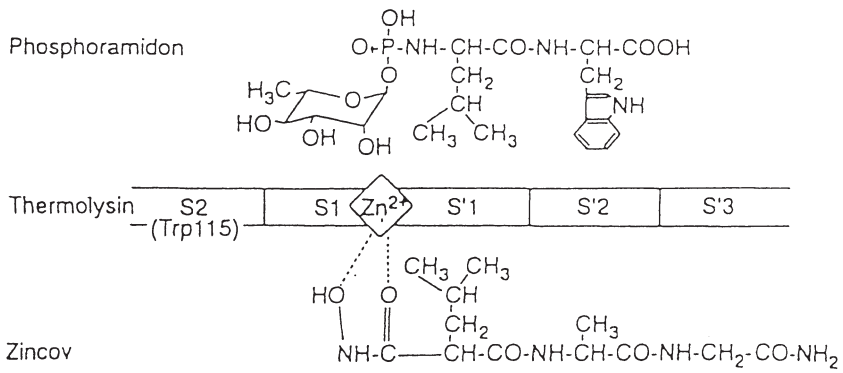

Fig. 6 : Schematic diagram showing the proposed interaction of phosphoramidon and zincov at the active site of thermolysin (Adapted from Inouye et al. ; Ref. 10).

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サーモリシンに及ぼすケトン性有機溶媒の影響：紫外スペクトル測定分析

M．ヌラル アラム[1]・只左　弘治・茅原　紘

信州大学農学部応用生命科学科

[1]岐阜大学大学院連合農学研究科（信州大学）

要　約

サーモリシン，サーモリシン活性中心に位置する二種のUV感受性芳香族アミノ酸に相当するアミノ酸の合成モデル化合物，及び，サーモリシン特異的阻害剤とサーモリシンの複合体に関するUVスペクトルを測定，分析した。それらの系にn-アルキルケトンを加えた場合，サーモリシンとモデル化合物では淡色効果が見られた。また，すべてのケトン添加サーモリシン系では明白な深色効果が認められた。深色効果の程度はケトンの炭素鎖長と共に減少した。両効果に由来するサーモリシン活性中心の構造的変化を推論した。

キーワード：サーモリシン，ケトン，紫外スペクトル，淡色効果，深色移動