Influences of the Suspension Method of Thermolysin in Organic Solvents on Enzyme Activity

Keizo TANAKA, Koreyoshi IMAMURA, Takaharu SAKIYAMA,
and Kazuhiro NAKANISHI[†]

*Department of Bioscience and Biotechnology, Faculty of Engineering,
Okayama University, 3-1-1 Tsushima-naka, Okayama 700-8530, Japan*

Thermolysin (EC 3.4.24.4) was suspended in organic solvents containing a small amount of water (aqueous buffer solution) by two different methods, and the catalytic activity as well as the conformational changes in the enzyme were compared. In one method (Method 1), dried powder enzyme was directly suspended in organic solvents containing a small amount of water while in the second method (Method 2), the dried enzyme was first dissolved in the buffer solution and then added to the anhydrous organic solvents containing the same final water content as that used in Method 1. In Method 2, the enzymes showed a catalytic activity 2–10 times as high as that in Method 1. The ratio of the activity obtained in Method 2 to that in Method 1 strongly depended on kinds of organic solvents, and its dependency was reasonably explained by the $E_T(30)$ values, which represent the extent of polarity of an organic solvent. The Fourier transform infrared spectroscopy (FT-IR) analyses indicated that the enzyme conformation in Method 2 was closer to its native one than that in Method 1. The influences of the suspension method on the enzyme activities of the different forms of the enzyme (as donated, dialyzed, and cross-linked) and the courses of the enzyme activities during incubation in Methods 1 and 2 were also investigated

Key words: thermolysin; enzymatic synthesis; organic solvent; cross-linked enzyme crystals; secondary structure

1. Introduction

An enzymatic reaction in an organic solvent has a number of advantages in comparison with that in an aqueous solution such as the shift in the equilibrium of the hydrolytic reaction toward the synthesis and the increase in the solubility of the hydrophobic substrates [1–4].

A number of studies have revealed that the enzymatic reaction rate in organic solvents is affected by various factors such as the kinds of organic solvents, water content, and partition of the substrate/product in aqueous/organic biphasic systems [5]. However, there are still some unknown mechanisms that affect the enzyme activity in organic solvents. Ke and Klibanov [6] and Dai and Klibanov [7] have recently reported that the enzyme activity in organic solvents strongly depends on the enzyme history. Namely, when the enzyme such as α -chymotrypsin, subtilisin, and peroxidase is initially dissolved in an aqueous solution followed by addition to an organic solvent, the reaction rate is higher than that observed when the

enzyme is directly added to the organic solvent containing the same final water content in both cases. They suggested that such a tendency could occur because the preliminary dissolution of the enzyme in an aqueous solution induces renaturation of the enzyme that became denatured due to dehydration during drying whereas the direct suspension into an organic solvent does not.

In this study, we showed that the activity of thermolysin was also different using the suspension method as did the group of Klibanov *et al.* [6,7]. In particular, we investigated the mechanism for the difference in the activity of thermolysin suspended using two suspension methods in different organic solvents. We measured the FT-IR spectra to analyze the conformational changes in the enzyme suspended in the organic solvent, which has not yet been reported in previous studies [6,7]. The influences of the kind of organic solvents, incubation time, the water content of the organic solvent, and the enzyme form (cross-linked and dialyzed forms) on the difference between the enzyme activities obtained by the two suspension methods were also investigated.

2. Materials and Methods

2.1 Materials

Crystalline thermolysin ($1 \times$ crystallized) was donated from Daiwa Kasei K.K. (Osaka, Japan). The enzyme content in the preparation is 60% by weight, and the remaining 40% is mainly calcium acetate [8]. *N*-(Benzyloxycarbonyl)-L-aspartic acid, Z-Asp, and L-phenylalanine methyl ester hydrochloride, PheOMe·HCl, were purchased from the Peptide Institute (Osaka, Japan). L-Phenylalanine methyl ester, PheOMe, was prepared from PheOMe·HCl as previously described [9]. *N*-(Morpholino)ethane sulfonic acid (MES) was purchased from Dojin Co. (Kumamoto, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2 Methods

2.2.1 Enzyme preparations

Three kinds of thermolysin preparations were used in this study, *i.e.*, commercially available crystalline thermolysin without further treatment (abbreviated as powder enzyme), a dialyzed enzyme preparation (dialyzed enzyme), and cross-linked enzyme crystals (CLEC) [8,10]. For preparation of the dialyzed enzyme, the powder enzyme was first dissolved in 25 mM Tris-HCl buffer, pH 7.5, containing 5 M NaBr and 16.6 mM CaCl_2 at -20°C [9]. The enzyme solution was dialyzed against 0.5 mM Tris-HCl buffer, pH 7.5, containing 5 mM CaCl_2 at 4°C overnight and then subjected to freeze-drying after rinsing with 1 mM CaCl_2 solution. The calcium content in the dialyzed enzyme preparation was determined to be around 1/4 that of the powder enzyme using Calcium C-Test Wako (Wako Pure Chemical Industries, Osaka, Japan). CLEC was prepared by a previously described method [8].

2.2.2 Reaction experiments

The thermolysin catalyzed synthesis of *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-AspPheOMe) was carried out at 40°C by the two enzyme suspension methods. In Method 1, 66.5 mg of thermolysin was directly suspended in 16.5 mL of organic solvent containing 0–5.8 mL of 50 mM MES-NaOH buffer, pH 6, containing 5 mM CaCl_2 (abbreviated as MES buffer) and the substrates (80 mM Z-Asp and 80 mM PheOMe). As organic solvents, ethyl alcohol, *n*-propyl alcohol, *n*-butyl alcohol, *n*-amyl alcohol, acetonitrile, *tert*-amyl alcohol, and ethyl acetate were used. The suspension was then sonified at 42 kHz and 180 W for 1 min to disperse the enzyme using an ultrasonic cleaner (Bransononic 2510J, Branson Ultrasonic Corp., Banbury, CT). Immediately after the sonification,

the reaction rate was monitored. In Method 2, 66.5 mg of thermolysin was first dissolved in 0–5.8 mL of MES buffer and then introduced into 16.5–10.7 mL of anhydrous organic solvents containing the substrates (Z-Asp and PheOMe), followed by the sonification using the same method as described above before starting the reaction. Thus, the final water contents in Methods 1 and 2 were 0–35%, in which water is soluble in every organic solvent used in this study. During the reaction, a 0.1-mL portion of the reaction mixture was usually withdrawn every 30 min for analysis using a high-performance liquid chromatograph (HPLC) (LC-6A, Shimadzu Corp., Kyoto, Japan) equipped with an octadecylsilica (ODS) column (STR ODS-H, Shimadzu Corp.) and a UV detector at 254 nm [5,9]. The initial rate was determined from the linear part of the reaction.

2.2.2 Analysis of enzyme structure in organic solvent

The secondary structures of thermolysin in organic solvents were analyzed with an FT-IR spectrometer (Magna 560, Nicolet, Madison, WI) equipped with a horizontal attenuated total reflection (ATR) system (SpectraTech Co., CT). For the FT-IR analyses of the enzymes in the organic solvents, the enzyme concentration was 8 mg/mL in order to obtain a sufficient IR absorbance intensity. The dialyzed enzyme was used to eliminate the IR bands due to calcium acetate contained in the powder enzyme. About 1 mL of the enzyme solution was transferred to a horizontal ATR cell having a Si crystal window, and 128 spectral scans were performed over the range of $1500\text{--}4000\text{ cm}^{-1}$ with a 4-cm^{-1} resolution. The IR spectra of the dialyzed enzyme powders were also measured using a diffuse reflection (DR) technique. For the measurement, the dialyzed enzyme powders were mixed with approximately 100-fold weight of KBr powders and ground into fine powders. The resulting powders were packed in a stainless steel cup and scanned 128 times over the range of $650\text{--}4000\text{ cm}^{-1}$ with a 4-cm^{-1} resolution using a SpectraTech Gemini DR system (SpectraTech Co., CT). The amide I band region in the obtained IR spectra was deconvoluted by using the OMNIC version 4.1a software (Nicolet, Madison, WI). The resolution factor and the half-width of the unresolved bands were set to 2 and 30 cm^{-1} , respectively [11–13]. The overlapping bands in the deconvoluted amide I band region were resolved using the PeakSolve software program (Galactic Ind., Salem, NH).

3. Results and Discussion

3.1 Reaction rates of thermolysin suspended by Methods 1 and 2 in various organic solvents

The initial reaction rates of the Z-AspPheOMe syntheses were measured by Methods 1 and 2 in various organic solvents containing 3 wt% water and are listed in Table 1. The reaction rates in Method 2 are much higher than those in Method 1 in all the organic solvents, which is consistent with the tendency observed in previous studies using α -chymotrypsin, subtilisin, and peroxidase [6,7]. In both Methods 1 and 2, the reaction rate in ethyl acetate was the highest among all the tested organic solvents, while the reaction rate in ethyl alcohol was the lowest.

In Fig. 1, the reaction rates in Methods 1 and 2 using the powder enzyme are plotted *versus* the $E_T(30)$ value. The $E_T(30)$ values indicate the extent of the polarity of organic solvents and are cited from the literature [14]. The reaction rates in both Methods 1 and 2 decrease with the increasing $E_T(30)$ value in most organic solvents except for *tert*-amyl alcohol in which the $E_T(30)$ value is 41.9. It can be expected that more hydration water around the enzyme molecules tends to be extracted into the bulk organic solvent with the increasing polarity of organic solvents. Thus, the decrease in the reaction rate with the increasing $E_T(30)$ value is considered to be due to the increase in the amount of hydration water extracted into the organic solvents, demonstrating the importance of the enzyme hydration for the exhibition of the activity in organic solvents as pointed out in the literature [15].

Based on the insight into the importance of the enzyme hydration, the difference in the reaction rates by the suspension methods could be understood as follows. The enzyme may suffer from reversible denaturation due to

Fig. 1 Influences of polarity of organic solvent on initial reaction rate and ratio of initial reaction rate in Method 2 to that in Method 1, $R_{2/1}$. The $E_T(30)$ (kcal/mol) values for organic solvents are 51.9 (ethyl alcohol), 50.7 (*n*-propyl alcohol), 50.2 (*n*-butyl alcohol), 49.1 (*n*-amyl alcohol), 46.0 (acetonitrile), 41.9 (*tert*-amyl alcohol), and 38.1 (ethyl acetate), respectively.

dehydration during drying as found in a number of references [16–19]. However, since the enzyme suspended by Method 2 would fully rehydrate upon dissolving in an aqueous buffer solution before the suspension into organic solvents, the enzyme takes a more native conformation compared to that in Method 1 upon contact with the organic solvents. It is generally known that the native protein has a more hydrophilic surface than the denatured one [20]. Thus, the enzyme in Method 2 would be able to retain more hydrated water in the presence of organic solvents than that in Method 1, yielding a higher activity in Method 2.

The $R_{2/1}$ values defined by the ratio of the reaction rate in Method 2 to that in Method 1 for all the tested organic solvents are also plotted *versus* the $E_T(30)$ value in Fig. 1. The $R_{2/1}$ values for all the organic solvents are greater than 1 and take a maximum at a certain $E_T(30)$ value. The existence of the maximum value indicates that the sensitivities of the reaction rate to the $E_T(30)$ value in Methods 1 and 2 are different, although details are not known at present.

3.2 Enzyme structure in organic solvents

To confirm the above-mentioned explanation for the difference in the reaction rates by the two suspension methods, we investigated the enzyme conformation in an organic solvent. In Fig. 2, the IR spectra for the freeze-dried (curve 1) and aqueous solution samples (curve 2) are shown. The wavenumber at the peak position of the amide I band for the freeze-dried enzyme is higher than that for

Table 1 Initial reaction rates in Methods 1 and 2 at 40°C in organic solvents containing 4 mg/mL enzyme, 3% water (50 mM MES buffer, pH 6), and 200 mM substrates (Z-Asp and PheOMe).

Solvent	Initial reaction rate (mM/h)	
	Method 1	Method 2
Ethyl alcohol	0.23	0.62
<i>n</i> -Propyl alcohol	0.30	0.64
<i>n</i> -Butyl alcohol	0.46	1.19
<i>n</i> -Amyl alcohol	0.67	2.08
<i>tert</i> -Amyl alcohol	0.41	1.42
Acetonitrile	0.33	3.72
Ethyl acetate	2.61	8.59

Fig. 2 IR spectra (curves 1 and 2) and Fourier self-deconvolved spectra (curves 3 and 4) in the amide I region for enzymes in 50 mM MES-NaOH buffer solution (dashed lines) and dried enzyme powders (solid lines).

the aqueous solution sample. Such a tendency is typical for the protein with a reduced hydration level due to dehydration [21–23].

In Fig. 2, the Fourier self-deconvolved spectra for the freeze-dried (curve 3) and aqueous solution samples (curve 4) are shown, clearly indicating individual secondary structures of the enzyme. The infrared wavenumbers with a half-width, assignments [11–13], and contents of the individual secondary structures are summarized in

Table 2. Table 2 also shows that the amide I band for the enzyme in an aqueous solution consists of three components with a high α -helix content. On the other hand, the spectrum for the freeze-dried enzyme shows another two bands at $1611\text{--}1612\text{ cm}^{-1}$ and 1697 cm^{-1} in addition to the three bands observed for the aqueous solution sample. These bands at $1611\text{--}1612$ and 1697 cm^{-1} are ascribed to the intermolecular β -sheets [11–13]. The formation of the intermolecular β -sheet indicates that the freeze-drying causes aggregation of the enzymes [24,25]. Furthermore, it should be particularly noted that the half width of the band due to the α -helix for the freeze-dried enzyme preparation is much larger than that for the aqueous solution sample. This means that there exists a broad distribution of the C=O stretching frequency in the α -helix of the freeze-dried enzyme preparation, indicating that freeze-drying causes a perturbation of the α -helix of the enzyme in addition to the intermolecular β -sheet formation as described above.

The deconvolved IR spectra of enzymes suspended by Methods 1 and 2 in acetonitrile and ethyl alcohol containing 3% water (MES buffer, pH 6) are shown in Fig. 3. Although we tried to measure the FT-IR spectra in other organic solvents, it was difficult to obtain reliable spectra due to the existence of the peaks arising from the solvent. As shown in Fig. 3, the spectra for the enzyme suspended by Method 2 resemble that for the aqueous-solution sample and are different in tendencies from those for the enzyme suspended by Method 1. The results of the Gaussian curve fittings are summarized in Table 2. The spectra for the enzymes suspended by both Methods 1 and 2 show the bands around 1610 cm^{-1} that indicate the formation of an intermolecular β -sheet. However, the band at 1697 cm^{-1} indicating the intermolecular β -sheet that is observed for the powder enzyme is not detected. The α -

Table 2 Results of Gaussian curve-fitting of Fourier self-deconvolved FT-IR spectra of enzymes in the amide I region.

Solvent	Method	Assignment of resolved bands											
		Unordered			α -Helix			β -Sheet			Intermolecular β -sheet		
		Center (cm^{-1})	Half width (cm^{-1})	Content (%)	Center (cm^{-1})	Half width (cm^{-1})	Content (%)	Center (cm^{-1})	Half width (cm^{-1})	Content (%)	Center (cm^{-1})	Half width (cm^{-1})	Content (%)
50 mM MES-NaOH, pH 6	-	1635	14.7	23.7	1655	15.6	57.4	1680	14.5	19.0	-	-	-
Powder form	-	1639	15.2	19.9	1661	28.6	53.3	1684	18.1	17.0	1612	13.6	4.6
											1697	13.1	5.3
Acetonitrile	1	1637	15.5	25.2	1656	17.6	49.6	1681	19.0	19.6	1611	12.4	5.6
	2	1637	14.2	18.4	1657	16.9	57.0	1681	16.4	19.2	1611	15.2	5.5
Ethyl alcohol	1	1637	14.7	23.7	1656	17.4	48.9	1681	18.8	20.9	1612	12.9	6.7
	2	1637	15.5	20.8	1657	17.2	56.8	1680	20.5	15.9	1612	13.4	6.5

Fig. 3 Fourier self-deconvolved FT-IR spectra in the amide I region for enzymes suspended in (a) acetonitrile and (b) ethyl alcohol containing 3% 50 mM MES-NaOH buffer solution by Methods 1 (curve 1) and 2 (curve 2). Curves 3 mean the spectra for enzymes in 50 mM MES-NaOH buffer solution (pH 6.0).

helix contents of the enzymes in Method 2 are higher than those in Method 1 and nearly the same as those for the aqueous-solution sample, whereas the content of the unordered structure of the enzyme suspended by Method 2 is lower than that by Method 1 and resembles that of the aqueous-solution sample. From these results, the structure of the enzyme suspended by Method 2 is deduced to be in

a more native state than that by Method 1. Denaturation of the enzyme in Method 1 brought about the formation of an intermolecular β -sheet, a decrease in the α -helix content, and an increase in the unordered structure, although the mechanism for the denaturation seems to be different from that by freeze-drying as judged from their IR spectra. These findings support the explanation proposed above, namely, that the enzyme structure is changed by the two suspension methods in organic solvents.

The enzymes formed aggregates in all the organic solvents particularly in Method 1 under the experimental conditions used in this study. Thus, the change in the size of the enzyme aggregate with the suspension method is considered to be another reason for the difference between the reaction rates in Methods 1 and 2. The activity for the enzyme aggregates would depend on their size due to the mass transfer resistance. However, we have preliminarily confirmed that although the enzyme activities obtained for both Methods 1 and 2 increased with the increasing sonification period of the enzyme suspensions, they reached a constant after a 1-min sonification. Thus, the effect of the aggregation on the activity could be ignored under the experimental conditions adopted in this study.

It has been reported that the activity of the enzyme coated by surfactants is quite high even by direct suspension in organic solvents [26]. According to the findings obtained in this study, such a phenomenon may be partly explained by the water adsorbing ability of the surfactant covering the surface of the enzyme molecules. Thus, it may be of crucial importance to coat enzymes with any substances having a suitable ability to maintain sufficient water to show a high activity in organic solvents.

3.3 Course of activity of thermolysin suspended in organic solvent by different methods

The courses of the enzyme activities in Methods 1 and 2 during incubation at 40°C were studied using acetonitrile (Fig. 4). The initial reaction rate of the enzyme that had been incubated for different period was measured. The enzyme activity in Method 2 gradually decreased with time and seems to reach that in Method 1 while the enzyme activity in Method 1 was nearly constant during the incubation as shown in Fig. 4. This may reflect the gradual transition from the native structure to a certain enzyme equilibrium state in the organic solvent, which corresponds to the enzyme in Method 1.

Fig. 4 Time courses of initial reaction rates for thermolysin in Methods 1 (open keys) and 2 (closed keys) in acetonitrile containing 3% MES buffer solution (pH 6).

3.4 Influence of water content in organic solvents on enzyme activity

The initial reaction rates were measured in acetonitrile containing different water contents as shown in Fig. 5. The initial reaction rate in Method 1 increased with the increasing water content in the organic solvent, suggesting that the recovery of the enzyme structure in the organic solvent is promoted by the increase in the water content. In Method 2, with an increasing water content, the reaction rate significantly increased and had a maximum at 8% probably because the amount of water around the enzyme

Fig. 5 Influences of water content of organic solvent (acetonitrile) on initial reaction rate and ratio of initial reaction rate in Method 2 to that in Method 1, $R_{2/1}$.

molecules increased. It then gradually decreased and reached that in Method 1 for a 35% water content.

This decrease in the reaction rate might happen in the following case although the mechanism is unclear at the present time. When the final water content in the organic solvent is low, the enzyme molecules are apt to aggregate on contact with acetonitrile. The aggregated enzyme is considered to strongly maintain water, expressing a high activity. The calcium acetate may gather around the aggregate, which may also contribute to attracting water as reported by Khmeinitzky and Welch [27]. When the water content is high enough, *e.g.*, at 35%, the enzyme molecules would disperse. The dispersed enzyme is considered not to be so strong at attracting water as the aggregate and is probably denatured on contact with the organic solvent.

3.5 Enzyme activity of dialyzed enzyme and CLEC

In Fig. 6(a), the initial reaction rates for CLEC and the

Fig. 6 Initial reaction rates (a) and ratio of initial reaction rate in Method 2 to that in Method 1, $R_{2/1}$, (b) for different forms of enzymes.

dialyzed enzymes suspended by Methods 1 and 2 are shown. The reaction rates of the dialyzed enzymes and CLEC are lower than those for the powder enzyme in both Methods 1 and 2. This may occur probably because the dialysis and cross-linking of the enzymes lower the amount of hydration water for the enzyme molecules responsible for maintenance of the enzyme structure. The calcium acetate contained in the powder enzyme preparation, which would help the enzyme to maintain water molecules around its surface as described above, was removed from the enzyme by the dialysis; cross-linking of the enzyme by glutaraldehyde having a hydrophobic backbone may lower the hydrophilicity of the surface of an enzyme crystal to reduce the amount of hydrated water.

The $R_{2/1}$ values for the dialyzed enzyme and CLEC are shown in Fig. 6(b). For the dialyzed powder enzyme and CLEC, the $E_T(30)$ value showing the highest $R_{2/1}$ value shifted to a lower value than that for the powder enzyme although the reason is unknown now.

4. Conclusion

It was demonstrated that the activity of thermolysin (EC 3.4.24.4) in organic solvents containing a small amount of water strongly depended on the suspension methods. When the dried enzyme was dissolved in an aqueous buffer solution before suspension into an organic solvent (Method 1), the enzyme activity was much higher than that for the direct suspension into the organic solvent (Method 2) even though the final water content in the organic solvents was equivalent. This tendency agreed well with those previously observed in the studies using α -chymotrypsin, subtilisin, and peroxidase [6,7]. The ratio of the reaction rates for the two suspension methods showed a maximum at a certain polarity value of the organic solvent. FT-IR analyses indicated that the enzyme structure for Method 2 takes more a native one in Method 1. These results suggest that the difference in the enzyme activity by the suspension methods would be due to the extent of renaturation of the enzymes denatured during drying. The difference in the enzyme activity by the suspension methods became smaller with time and the increasing water content of the organic solvent.

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References

- 1) A.M. Klibanov; Enzymes that work in organic solvents. *Chemtech.*, **16**, 354-359 (1986).
- 2) C.-H. Wong; Enzymes in synthesis. *Chemtracts-Organic Chemistry*, **3**, 91-111 (1990).
- 3) C. Laane, S. Boeren, K. Vos; On optimizing organic solvents in multi-liquid-phase biocatalysis. *Trends Biotechnol.*, **3**, 251-252 (1985).
- 4) K. Nakanishi, R. Matsuno; *Synthetic Peptides in Biotechnology - Advances in Biotechnological Process* vol. 10, 173-202, A. Mizrahi ed., Alan R. Liss, Inc., New York (1988).
- 5) M. Miyanaga, M. Ohmori, K. Imamura, T. Sakiyama, K. Nakanishi; Kinetics and equilibrium for thermolysin-catalyzed syntheses of dipeptide precursors in aqueous/organic biphasic system. *J. Biosci. Bioeng.*, **90**, 43-51 (2000).
- 6) T. Ke, A.M. Klibanov; On enzymatic activity in organic solvents as a function of enzyme history. *Biotechnol. Bioeng.*, **57**, 746-750 (1998).
- 7) L. Dai, A.M. Klibanov; Striking activation of oxidative enzymes suspended in nonaqueous media. *Proc. Natl. Acad. Sci. USA*, **96**, 9475-9478 (1999).
- 8) M. Miyanaga, M. Ohmori, K. Imamura, T. Sakiyama, K. Nakanishi; Stability of immobilized thermolysin in organic solvents. *J. Biosci. Bioeng.*, **87**, 463-472 (1999).
- 9) K. Nakanishi, T. Kamikubo, R. Matsuno; Continuous synthesis of N-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester with immobilized thermolysin in an organic solvent. *Bio/technology*, **3**, 459-464 (1985).
- 10) A.L. Margolin; Novel crystalline catalysts. *Trends Biotechnol.*, **14**, 223-230 (1996).
- 11) D.M. Byler, H. Susi; Examination of the secondary structure on proteins by deconvolved FTIR spectra. *Biopolymers*, **25**, 469-487 (1986).
- 12) K. Griebenow, A.M. Klibanov; On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. *J. Am. Chem. Soc.*, **118**, 11695-11700 (1996).
- 13) K. Griebenow, A.M. Klibanov; Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin Carlsberg in organic solvents? *Biotechnol. Bioeng.*, **53**, 351-362 (1997).
- 14) R. Christian; Empirical parameters of solvent polarity as

- linear free-energy relationships. *Angew. Chem. Int. Engl.*, **18**, 98-110 (1979).
- 15) J. Partridge, P.R. Dennison, B.D. Moore, P.J. Halling; Activity and mobility of subtilisin in low water organic media: hydration is more important than solvent dielectric. *Biochim. Biophys. Acta*, **1386**, 79-89 (1998)
- 16) J. Geigert; Overview of the stability and handling of recombinant protein drugs. *J. Parenter. Sci. Tech.*, **43**, 220-224 (1989).
- 17) M.C. Manning, K. Patel, R.T. Borchardt; Stability of protein pharmaceuticals. *Pharm. Res.*, **6**, 903-918 (1989).
- 18) M.J. Pikal; Freeze-drying of proteins. Process, formulation and stability. In *Formulation and delivery of proteins and peptides*. Cleland, J. L. and Langer, R., eds., ACS Symposium Series, **567**, 120-133 (1994).
- 19) K. Geibenow, A.M. Klibanov; Lyophilization-induced reversible changes in the secondary structure of proteins. *Proc. Natl. Acad. Sci. USA*, **92**, 10969-10976 (1995).
- 20) J.C. Kendrew; The three-dimensional structure of a protein molecule. *Sci. Am.*, **205**, 96-110 (1961).
- 21) G.A. Careri, A. Giansanti, E. Gratton, Samprogetti; Lysozyme film hydration events: An IR and gravimetric study. *Biopolymer*, **18**, 1187-1203 (1979).
- 22) P.L. Poole, J.L. Finney; Sequential hydration of dry proteins: A direct difference IR investigation of sequence homologs lysozyme and α -lactoalbumin. *Biopolymer*, **23**, 1647-1666 (1984).
- 23) J.F. Carpenter, J.H. Crowe; An infrared spectroscopic study of interactions of carbohydrates with dried proteins. *Biochemistry*, **28**, 3916-3922 (1989).
- 24) S.D. Allison, A.D. Dong, J.F. Carpenter; Countering effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying, and rehydration. *Biophys. J.*, **71**, 2022-2032 (1996).
- 25) A. Dong, H. Ping, W.S. Caughey; Protein secondary structures in water from second derivative amide I infrared spectra. *Biochemistry*, **29**, 3303-3308 (1998).
- 26) S. Okazaki, N. Kamiya, M. Goto; Application of novel preparation method for surfactant-protease complexes catalytically active in organic media. *Biotechnol. Prog.*, **13**, 551-556 (1997)
- 27) Y. Khmeinitzky, S.H. Welch; Salts dramatically enhance activity of enzymes suspended in organic solvents. *J. Am. Chem. Soc.*, **116**, 2647-2648 (1994).

*** 和文要約 ***

サーモライシンの有機溶媒への懸濁方法が酵素活性に及ぼす影響

田中恵三, 今村維克, 崎山高明, 中西一弘

岡山大学工学部生物機能工学科

有機溶媒中における酵素反応に関してこれまで多くの研究が行われており、有機溶媒中の水分濃度が酵素活性に大きく影響することが明らかになっている。さらに、近年、有機溶媒の含水率が同じでも、予め一定量の水溶液を加えた含水有機溶媒に酵素を懸濁した場合と酵素を予め水溶液に溶解したうえで有機溶媒に懸濁した場合で酵素活性が著しく異なるという現象が報告されている[6,7]。

本研究ではまず、酵素としてサーモライシン (EC 3.4.24.4) を用いて、有機溶媒への懸濁方法による酵素活性が変化することを検証した。種々の有機溶媒 (エチルアルコール, *n*-プロピルアルコール, *n*-ブチルアルコール, *n*-アミルアルコール, アセトニトリル, *tert*-アミルアルコール, 酢酸エチル) を用いて二つの異なる酵素の懸濁方法 (方法 1, 2) に対する酵素反応速度を調べた。方法 1 では予め基質 (80 mM Z-Asp, 80 mM PheOMe) と 50 mM の MES 緩衝液を添加した有機溶媒に酵素 (4.0 mg/mL) を懸濁することで酵素反応を開始した。方法 2 では酵素を 50 mM の MES 緩衝液に溶解したのち、基質を含む有機溶媒に懸濁することで反応を開始した。このとき、溶媒の含水率は方法 1, 2 とも 3 % に設定した。酵素反応速度はサーモライシンによるジペプチド (*N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester) の合成初速度より評価した。その結果、いずれの有機溶媒においても、方法 2 の場合の方が方法 1 の場合よりも高い反応速度を示すことが分かった。また、有機溶媒の種類によって反応速度が著しく変化することが分かった。

有機溶媒の極性と酵素反応速度の関係について検討するため、方法 1, 2 における酵素反応速度を溶媒の極性を表す $E_T(30)$ に対してプロットした。その結果、有機溶媒の極性が増すにつれて反応速度が低下することが分かった。これは有機溶媒の極性が高くなるほど、酵素の触媒活性に不可欠な水和水が有機溶媒に抽出されやすくなるためと考えられる。また、本知見は有機溶媒中の酵素反応における酵素表面の水和水の重要性を示唆している。この水和水の重要性を加味すると、懸濁方法による酵素反応速度の変化は次のように説明することができる。まず、懸濁前の酵素は乾燥操作により構造変化を生じている。しかし、方法 2 では予め水溶液に溶解するた

め酵素は十分再水和することができ、有機溶媒と接触する段階において酵素の構造は方法 1 の場合に比べてよりネイティブなものとなっているものと考えられる。一般的に未変性状態のタンパク質は変性状態のものより親水的な表面を有することが知られている。そのため、方法 2 の場合には酵素が有機溶媒中でも水和水を高度に保持することができ、その結果、方法 1 よりも高い酵素活性を示すものと考えられる。また、方法 1, 2 の反応速度の比は中程度の極性 ($E_T(30)=46.0$) で最大を示すことが分かった。

つぎに、酵素の有機溶媒への懸濁方法が酵素の構造に及ぼす影響を調べた。FT-IR により各懸濁方法を用いた場合の酵素の IR スペクトルを測定し、得られたスペクトルをフーリエセルフデコンボリューション、ガウシアンカーブフィッティング処理することによって各二次構造の含有率を算出した。その結果、方法 1 では unordered に起因するピークが水溶液中の結果に比べ大きくなっているのに対し、方法 2 のスペクトルは水溶液中のスペクトルと良好に一致することが分かった。この結果は、有機溶媒への懸濁方法により酵素の構造が変化することを表しており、上述の懸濁方法による酵素反応速度の差異に対する考察を強く支持するものである。

本研究では懸濁後の保存時間が酵素活性に及ぼす影響についても検討を加えた。酵素を方法 1 あるいは 2 で有機溶媒に懸濁し、40 °C で 0–12 h 保持したのち、基質を添加することで反応を開始した。有機溶媒としてはアセトニトリルを用いた。その結果、方法 1 の場合は保存時間によらずほぼ一定な反応速度が見られたのに対し、方法 2 では時間とともに緩やかに反応速度が低下していく傾向が見られた。さらに有機溶媒 (アセトニトリル) 中の含水率が酵素活性に及ぼす影響を調べた結果、方法 1 の場合は含水率の上昇とともに酵素反応速度は上昇した。一方、方法 2 の場合は含水率が 8 wt% のときに反応速度が最大となり、さらに含水率が増すと反応速度が低下するという傾向が見られた。また、酵素として透析処理したもの (透析酵素)、酵素の結晶をグルタルアルデヒドで架橋したもの (CLEC) を用いて各種有機溶媒中における酵素反応速度を調べた。その結果、透析および架橋処理により酵素反応速度の有機溶媒の極性に対する依存性が変化することが示唆された。