Enhancement of Catalytic Activity of Chemically Modified Subtilisin Carlsberg in Benzene by Adjustment of Lyophilization Conditions

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Alteration of the lyophilization conditions significantly enhanced not only the activity of insoluble native subtilisin Carlsberg but also that of solubilized enzyme in benzene. The catalytic activity (k_{cat}/K_m) for the transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol in benzene was enhanced more than 1000 times by the solubilization (100-fold enhancement) and the adjustment of lyophilization conditions (10-fold enhancement). Subtilisin Carlsberg was modified with polyethylene glycol (PEG-Sub) so that it could be solubilized in organic solvents. The effects of pH of the aqueous enzyme solution, of salts, or of a substrate analog in the aqueous solution before the lyophilization process, on the catalytic activity of native subtilisin and PEG-Sub were investigated. The activities of both the native subtilisin and PEG-Sub depended on the pH. The activities of both PEG-Sub and the native subtilisin were enhanced by the presence of salts. The presence of a substrate analog, *N*-Ac–L-Phe, also enhanced the activity of the native subtilisin and PEG-Sub. These results demonstrated that the organic solvent-solubilized subtilisin was affected by the lyophilization conditions.

Recently, many investigations have concentrated their attention on the enhancement of enzyme activity in organic media. These include investigations of optimization of the nature of organic solvent and the water content,[1—3] chemical modification of the enzyme,[4—12] immobilization of the enzyme,[13—15] and extraction of the enzyme with surfactant into organic solvents.[16,17]

It has been demonstrated that the activity of enzymes in organic solvents depends on the conditions of lyophilization. For example, the activity of enzymes lyophilized in organic solvents depends on the pH of the last aqueous solution.[18—20] Khmelnitsky et al.[21] reported that the dramatic enhancement of enzymatic activity in organic solvents was brought about by the presence of KCl in the lyophilized enzyme. In addition, lyophilization of enzymes from aqueous solutions containing inhibitor or substrate analog, followed by their removal, caused significant enhancement of enzyme activity and substrate selectivity.[22—26] The lyophilization of an enzyme usually induces conformational change.[27] However, lyoprotectants, such as sorbitol, sucrose, or polyethylene glycol, significantly enhanced the activity of enzymes in organic media due to the inhibition of conformational change and protection of enzyme molecules from solvent.[28]

Enzymes chemically modified to be soluble in organic solvents are useful as catalysts of organic reactions. However, the effect of lyophilization conditions on the activity of solubilized enzymes has not been investigated. Therefore, in the present work, subtilisin was modified with polyethylene glycol as reviewed by Inada et al.,[29] and the effects of pH, salt, and molecular imprinting on the activity of modified enzyme in benzene were investigated in relation to the conformational change.

Materials and Methods

Materials. Subtilisin Carlsberg (alkaline protease from Bacillus licheniformis, EC 3.4.21.14) was purchased from Sigma Co. (St. Louis, MO), and used without further purification. Benzene (the highest grade) and several kinds of inorganic salts of potassium were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Benzene was dried over molecular sieves for 24 h before use. The substrate *N*-Ac–L-Phe–OEt, and the substrate analog, *N*-Ac–L-Phe, were purchased from Peptide Institute Inc. (Osaka, Japan). 2-Chloro-4,6-bis[ω-methoxy(polyoxyethylene)]-*s*-triazine (activated PEG_2) was purchased from Seikagaku Co. (Tokyo, Japan). Poly(ethylene glycol) (PEG, MW : 4000) was purchased from Sigma Co. (St. Louis, MO).

Modification of Subtilisin with Poly(oxyethylene). To a subtilisin solution (1.5 mg mL^{-1}, 45 mL) in 40 mM sodium tetraborate buffer (pH 9.5), activated PEG_2 (1 g) was added, followed by gentle stirring at 37 °C for 1 h. The reaction mixture was subjected to ultrafiltration using a Centriplus 30 membrane (Amicon, Boston, MA) to remove unreacted activated PEG_2. After the volume of reaction mixture was reduced to 15 mL, the ultrafiltration process was repeated five times with subsequent addition of 170 mL of distilled water. The absence of unreacted PEG_2 was confirmed by infrared spectroscopic data of filtrates. The concentrated solution of subtilisin modified with PEG (PEG-Sub) was lyophilized by a freeze-drier for 48 h at a pressure of 2 Pa and a condenser temperature of −50 °C. Lyophilized protein powder was sealed in vials and stored at −20 °C. The degree of modification of amino

groups in a subtilisin molecule was determined using the trinitrobenzenesulfonate (TNBS) method[30] and elemental analysis. PEG-Sub contains 32% of protein. The hydrolytic activity of the PEG-Sub was determined by monitoring *p*-nitroaniline released from the substrate, Z–Gly–Gly–Leu–*p*NA.

Lyophilization of Enzymes. Subtilisin or PEG-Sub was dissolved (5 mg mL^{-1}) in 20 mM aqueous phosphate buffer (K_2HPO_4 and KH_2PO_4) at different pHs and lyophilized for 48 h in an Eyela freeze-drier (Tokyo, Japan).

To investigate the effect of salt present during lyophilization on the catalytic activity of lyophilized enzymes, various amounts of inorganic salts of potassium were added to the aqueous phosphate buffer containing enzymes, which was then freeze dried for 48 h.[20] The concentration of enzyme in benzene solution was determined by using the Coomassie brilliant blue assay[31] after removal of precipitate by centrifugation. The content of KCl in the benzene solution was calculated by weighting after removal of benzene by evaporation, considering the content of enzyme.

To imprint enzymes with substrate analog, *N*-Ac–L-Phe, the native subtilisin and PEG-Sub were lyophilized in the presence of 40 mM *N*-Ac–L-Phe. The substrate analog was added to the aqueous buffer (final pH 8) prior to the addition of enzyme and the subsequent freezing.[22] The imprinted molecules were washed out from the enzyme sample prior to the assay of catalytic activity. The lyophilized powder (1 mg) was placed in 1 mL of 1-propanol, sonicated for 30 s, and shaken at room temperature for 10 min. Then the enzyme was recovered by centrifugation, and the procedure was repeated. After removal of the washing solution, the pellet of enzyme was obtained.

Fourier-Transform Infrared (FT-IR) Spectroscopy. All FT-IR measurements were performed on a PE-2000 FT-IR system (Perkin–Elmer) with a Spectra Tech liquid cell equipped with CaF_2 windows. The lyophilized enzyme was analyzed as a KBr pellet (1 mg of protein powder per 200 mg of KBr). Spectra in water, benzene, and 1-propanol were measured using 15- and 25-μm Teflon® spacers, respectively. A total of 256 scans at 4 cm^{-1} resolution were averaged. Spectra (except those obtained in KBr) were corrected by subtraction of the solvent background, although 1-propanol does not absorb significantly in the amide I region (1700—1600 cm^{-1}).

The spectral data were analyzed according to Griebenow and Klibanov.[32–34] All spectra were resolution-enhanced by Fourier self-deconvolution (FSD). The frequencies of the absorption-band centers found in the second-derivative spectra in the amide I region were used as starting parameters for the Gaussian curve-fitting treatment, which was performed using the software of GRAMS 386 from Galactic Ind. Inc. The contents of secondary structure were calculated from the areas of the individual absorption bands and their proportion of the amide I region.[35] The Gaussian curve-fitting treatment was performed in the amide I region using the resolution-enhanced spectra by FSD. In most instances, the discrepancies between the component frequencies obtained by second derivatization and the Gaussian curve-fitting were less than 4 cm^{-1}. The content of secondary structure was determined from more than three independent spectra. The contents were averaged, and the standard deviations were calculated. The absorptions in the amide I region were assigned in accordance with assignments previously published.[32,33,36–38] The main absorption bands at 1658 and 1666 cm^{-1} were assigned to α-helix, and those at 1693, 1636, and 1625 cm^{-1} to β-sheet.[37] All other absorption bands at 1681, 1672, 1651, 1644, and 1618 cm^{-1} were assigned to β-turn and random-coil structures.

Transesterification Activity. Transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester (APEE) (1—100 mM) with 1-propanol (0.5 M) was investigated in benzene in the presence of the native subtilisin and PEG-Sub (1 mg mL^{-1}). The reaction mixture (1 mL) was placed in a screw-cap vial (4-mL capacity) and shaken in an incubator shaker at 25 °C and 170 rpm. Samples (1 μL) were periodically taken out for analysis. Gas chromatography (GC) was used to determine the formation of *N*-acetyl-L-phenylalanine propyl ester (APPE). The GC conditions were as follows: a TC-7 capillary column (GL Sciences Ind., Osaka, Japan); the injector and flame ionization detection (FID) temperature 280 °C; gaseous helium for carrier gas. The rate of transesterification reaction in the absence of enzyme was negligible as compared with that in the presence of enzyme under the same conditions. The percentage of catalytically active subtilisin molecules in the enzyme preparation was determined by titration with *N-trans*-cinnamoylimidazole according to the method of Zaks and Klibanov.[19] The percentage of catalytically active subtilisin in the native subtilisin and PEG-Sub in benzene were 28 and 25%, respectively. The kinetic parameters were calculated from Lineweaver–Burk plots using the active enzyme concentration.

Results and Discussion

Synthesis and Hydrolytic Activity of Poly(oxyethylene)-Modified Subtilisin (PEG-Sub). Fifty-four percent of amino groups on the surface of the subtilisin molecule were found to have been modified with polyethylene glycol. This result indicates that in the PEG-Sub a subtilisin molecule carries about 10 chains of poly(oxyethylene), considering the PEG_2 has two chains. Bovara et al.[39] prepared subtilisin coupled to 2, 4, or 5 polyethylene glycol chains. Subtilisin Carlsberg, carrying 6 polyethylene glycol chains, is commercially available from Sigma Co., Ltd. Measurement of the hydrolytic activity in aqueous buffer using Z–Gly–Gly–Leu–*p*NA as a substrate showed that the hydrolytic activity of PEG-Sub was 70% of the activity of native subtilisin under the same conditions.

Effect of pH on Enzymatic Activity. The native subtilisin or PEG-Sub was dissolved in an aqueous solution of different pH and lyophilized, then the transesterification activity in benzene was investigated (Fig. 1). The catalytic activity was profoundly affected by the pH of the aqueous solution from which subtilisin was lyophilized. Subtilisin showed the 'pH memory' which has been reported previously.[20] PEG-Sub was more active than subtilisin and also showed the pH memory. In addition, because the poly(oxyethylene) chain enhances the solubility of the enzyme in benzene, the rate of the transesterification reaction was significantly enhanced by the modification with polyethylene glycol. PEG-Sub lyophilized from acidic solutions retained higher activity than native subtilisin. This result may be due to the fact that 54% of the amino groups on the surface of the subtilisin molecule were modified with polyethylene glycol and the net charge of enzyme was thereby changed.

Effect of Salt on Enzymatic Activity. To investigate the effect of salt on enzymatic activity, the native subtilisin and PEG-Sub were lyophilized in the presence of several kinds of inorganic salts of potassium (Fig. 2). The transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester

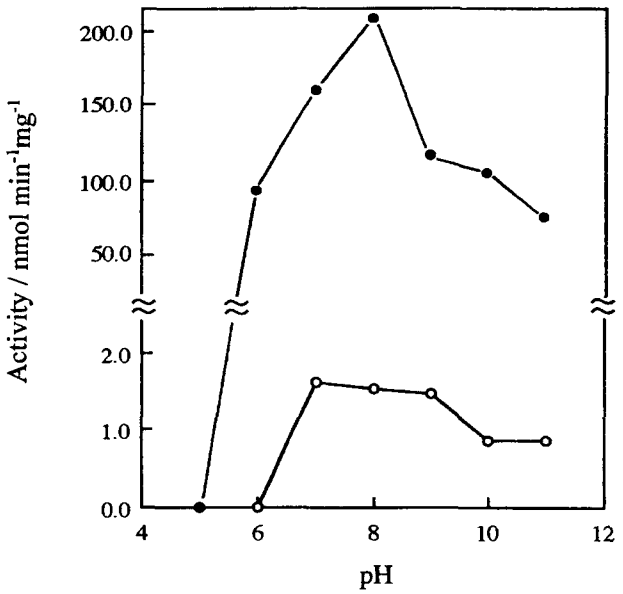

Fig. 1. Transesterification activity in benzene of the native subtilisin (open circles) and PEG-Sub (closed circles) lyophilized at different pHs.

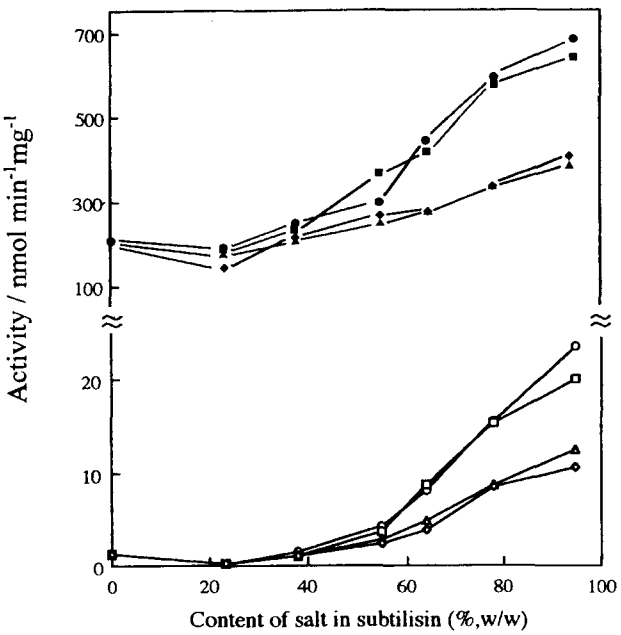

Fig. 2. The effect of salt on the enzymatic activity of the native subtilisin (open) and PEG-Sub (closed): KCl (circles), KNO_3 (squares), KOAc (triangles), and KF (diamonds).

with 1-propanol in benzene was measured. Khmelnitsky et al.[21] reported that the catalytic activity of native subtilisin dramatically increased with increasing content of added salt in hexane. Similarly, in the present study, the addition of KCl or KNO_3 markedly increased the reaction rate in benzene. However, the rate enhancement was much less than that reported previously. The reason is considered to be the difference of organic solvent. In the present study, benzene was used to dissolve PEG-Sub.

Instead of native subtilisin, PEG-Sub was lyophilized in the presence of salts and its catalytic activity in benzene suspension was measured. Although the lyophilized powder was not soluble in benzene, the activity of PEG-Sub was higher than that of native subtilisin and was affected by the addition of salts (Fig. 2). The addition of KCl or KNO_3 markedly increased the reaction rate of PEG-Sub.

The kinetic parameters for the transesterification reaction by the native subtilisin and PEG-Sub lyophilized from an aqueous suspension containing high content of KCl or PEG were determined (Table 1). The catalytic activity (k_{cat}/K_m) was markedly enhanced by lyophilization in the presence of KCl. The increase of enzymatic activities by the addition of KCl during lyophilization of the native subtilisin and PEG-Sub appears to be the result of increasing k_{cat} rather than decreasing K_m. The presence of uncoupled PEG enhanced the catalytic activity 11-fold, but KCl was even more effective than uncoupled PEG. No significant synergistic effect by PEG and KCl was observed. The covalent coupling of PEG enhanced the catalytic activity by 100-fold and the effect was further enhanced by KCl (final enhancement over 1000-fold).

The mechanism of salt-induced activation of insoluble enzymes in benzene has been explained in two different ways: The salt may protect the enzyme from direct inactivation by the organic solvent (salt matrix effect), or the salt may help to maintain the enzyme's native structure during lyophilization (lyoprotectant effect). Khmelnitsky et al.[21] proposed that a salt matrix protected the enzyme from solvent-induced inactivation. Alternatively, Griebenow and Klibanov[33] proposed the lyoprotectant effect because the α-helix content of the native subtilisin lyophilized from an 89% aqueous KCl solution is much higher than that of subtilisin lyophilized from a KCl-free solution. We found that KCl prevented

Table 1. The Effect of Additives in Aqueous Enzyme Solution on the Catalytic Parameters of the Lyophilized Native Subtilisin and PEG-Sub in Benzene

Content of lyophilized powder	K_m	k_{cat}	k_{cat}/K_m
wt%	mM	s^{-1}	$M^{-1}\,s^{-1}$
100% subtilisn	61 ± 4	0.11 ± 0.01	1.8 ± 0.1
62.5% subtilisin+37.5% PEG	35 ± 5	0.70 ± 0.09	20.0 ± 0.5
5% subtilisin+95% KCl	30 ± 3	3.3 ± 0.1	110 ± 7
5% subtilisin+2% PEG+93% KCl	30 ± 6	3.6 ± 0.4	121 ± 12
100% PEG-Sub	35 ± 4	12.3 ± 0.3	351 ± 11
5% PEG-Sub+95% KCl	19 ± 2	40.2 ± 0.9	2120 ± 42

a decrease of α-helix content of the native subtilisin during lyophilization (Table 2). PEG also had the same effect on the conformation of native subtilisin. However, KCl was more effective in enhancing the catalytic activity of subtilisin than PEG. Therefore, we concluded that the protective effect of KCl on insoluble enzymes was due to both the salt matrix effect and the lyoprotectant effect.

However, the present investigation indicated that KCl was also effective on PEG-Sub. When the reaction suspension was separated into soluble and precipitate parts, 98% of PEG-Sub and 5% of KCl were found in benzene and the high activity was observed in the benzene solution. The precipitate was almost totally salt and the catalytic activity was owing to the soluble enzyme. Therefore, it is difficult to explain the salt effect on soluble enzyme by the above-mentioned salt matrix effect. Table 2 shows that there was no significant difference between the conformation of PEG-Sub in the absence and in the presence of KCl. It is known that subtilisin catalyzes the transesterification reaction through a charged and highly polar transition state, and that a polar environ-

Table 2. The Effect of Additives in Aqueous Enzyme Solution on the Content of Secondary Structure of the Lyophilized Native Subtilisin and PEG-Sub

Content of lyophilized powder	State	Content (%) of secondary structure		
wt%		α-Helix	β-Sheet	Disordered
100% subtilisin[a)]	In water	34 ± 0	19 ± 0	47 ± 0
100% subtilisin	Powder	28 ± 2	22 ± 4	50 ± 3
100% subtilisin	In benzene	29 ± 3	26 ± 3	45 ± 4
62.5% subtilisin+37.5% PEG	In benzene	33 ± 3	23 ± 2	44 ± 3
5% subtilisin+95% KCl	In benzene	34 ± 2	22 ± 2	44 ± 3
5% subtilisin+2% PEG+93% KCl	In benzene	34 ± 3	23 ± 3	43 ± 3
100% PEG-Sub	In water	34 ± 2	23 ± 2	43 ± 3
100% PEG-Sub	Powder	33 ± 3	22 ± 3	46 ± 3
100% PEG-Sub	In benzene	33 ± 1	24 ± 1	43 ± 2
5% PEG-Sub+95% KCl	In benzene	34 ± 2	23 ± 2	43 ± 3

a) The result of Xu et al. (1997).[40]

Table 3. The Catalytic Parameters for Transesterification Reaction Catalyzed by the Native Subtilisin, PEG-Sub, Imprinted[a)] Subtilisin, Imprinted[a)] Subtilisin Containing PEG, and Imprinted[a)] PEG-Sub

Content of lyophilized powder	K_m	k_{cat}	k_{cat}/K_m
wt%	mM	s^{-1}	$M^{-1} s^{-1}$
100% native subtilisin	61 ± 4	0.11 ± 0.01	1.8 ± 0.1
100% imprinted subtilisin	49 ± 5	6.3 ± 0.2	129 ± 7
62.5% imprinted subtilisin+37.5% PEG	51 ± 6	9.4 ± 0.6	184 ± 9
100% PEG-Sub as prepared	35 ± 4	12.3 ± 0.3	351 ± 11
100% imprinted PEG-Sub	27 ± 5	31.3 ± 0.7	1160 ± 13

a) The concentration of imprinting molecule, *N*-Ac–L-Phe, was 40 mM.

Table 4. The Content of Secondary Structure of Native Subtilisin, Imprinted Subtilisin, Imprinted Subtilisin Containing PEG, PEG-Sub, and Imprinted PEG-Sub

Content of lyophilized powder	State	Content of secondary structure (%)		
wt%		α-Helix	β-Sheet	Disordered
100% native subtilisin[a)]	In water	34 ± 0	19 ± 0	47 ± 0
100% native subtilisin (lyophilized)	Powder	28 ± 2	22 ± 4	50 ± 3
100% imprinted subtilisin	In 1-propanol	33 ± 1	29 ± 2	38 ± 3
100% imprinted subtilisin	In benzene	33 ± 1	28 ± 2	39 ± 2
62.5% imprinted subtilisin+37.5% PEG	In benzene	35 ± 3	27 ± 3	38 ± 3
100% PEG-Sub	In water	33 ± 2	22 ± 2	45 ± 2
100% PEG-Sub (lyophilized)	Powder	33 ± 3	22 ± 3	45 ± 3
100% imprinted PEG-Sub	In 1-propanol	34 ± 2	22 ± 3	44 ± 3
100% imprinted PEG-Sub	In benzene	34 ± 1	23 ± 3	43 ± 3

a) The result of Xu et al. (1997).[40]

ment stabilizes the transition state. Therefore, the increased enzymatic activity of PEG-Sub by KCl may be explained by the formation of a polar environment by salts adsorbed on soluble PEG-Sub to favor the polar transition state of the enzymatic reaction, in addition to the lyoprotectant effect.

Effect of Substrate Analog on Enzymatic Activity. Transesterification activities of the native subtilisin and PEG-Sub lyophilized in the presence of *N*-Ac–L-Phe were investigated. Ståhl et al.[23] and Braco et al.[26] reported that the substrate analog molecule bound to the active site of enzymes induced a molecular imprinting effect. Table 3 shows that the enzymatic activity of the native subtilisin imprinted with *N*-Ac–L-Phe was 70 times higher than that of the nonimprinted counterpart. The effect was stronger than that of PEG or KCl. The presence of both analog and PEG further enhanced the activity. The increase of enzymatic activity of the imprinted subtilisin is a result of significantly increased k_{cat} plus slightly decreased K_m.

The activity of PEG-Sub was also enhanced by the presence of *N*-Ac–L-Phe during lyophilization. The increased enzymatic activity comes primarily from increasing k_{cat} and secondarily from decreased K_m. This result was similar to that of the native subtilisin. The relative k_{cat}/K_m values in the presence of *N*-Ac–L-Phe versus in the absence of *N*-Ac–L-Phe for the native subtilisin and PEG-Sub were 102 and 3.3, respectively.

Table 4 shows α-helix and β-sheet contents of the enzymes as determined from the amide I spectral region of FT-IR. The decrease of α-helix and β-sheet contents of the native subtilisin and PEG-Sub during lyophilization was not found in the presence of *N*-Ac–L-Phe. Griebenow and Klibanov[33] reported that the secondary structure of imprinted subtilisin resembled that of the native enzyme in aqueous solution much more closely than did that of the nonimprinted enzyme powder. The same phenomenon was observed with PEG-Sub.

Despite the exciting potential of enzyme imprinting, its generality and molecular mechanisms have remained matters of speculation. Dabulis and Klibanov[28] reported that the effects of the ligands and of lyoprotectants were nonadditive, and suggested the same mechanism of action. It is difficult to explain the molecular mechanism of activity enhancement by the substrate analog. However, the present study clearly indicated that the ligand effect can be exerted on organic solvent-soluble enzymes.

The present investigation demonstrated that the high catalytic activity of organic solvent-soluble enzyme can be further enhanced by appropriate conditions in the lyophilization, similar to that of insoluble native enzyme.

References

1 A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, **263**, 8017 (1988).

2 K. Ryu and J. S. Dordick, *Biochemistry*, **31**, 2588 (1992).

3 A. M. Klibanov, *Trends Biotechnol.*, **15**, 97 (1997).

4 A. Matsushima, M. Okada, and Y. Inada, *FEBS Lett.*, **178**, 275 (1985).

5 K. Takahashi, A. Ajima, T. Yoshimoto, M. Okada, A. Matsushima, Y. Tamaura, and Y. Inada, *J. Org. Chem.*, **50**, 3414 (1985).

6 W. Tsuzuki, Y. Okahata, O. Katayama, and T. Suzuki, *J. Chem. Soc., Perkin Trans. 1*, **1991**, 1245.

7 Y. Okahata and K. Ijiro, *Bull. Chem. Soc. Jpn.*, **65**, 2411 (1992).

8 Y. Okahata, Y. Fujimoto, and K. Ijiro, *J. Org. Chem.*, **60**, 2244 (1995).

9 M. Goto, N. Kamiya, M. Miyata, and F. Nakashio, *Biotechnol. Progr.*, **10**, 263 (1994).

10 Y. Ito, Y., H. Fujii, and Y. Imanishi, *Biotechnol. Progr.*, **10**, 398 (1994).

11 Y. Ito, N. Sugimura, O. H. Kwon, and Y. Imanishi, *Nature Biotechnol.*, **17**, 73 (1999).

12 Y. Ito, *J. Biomater. Sci., Polym. Ed.*, **10**, 1237 (2000).

13 P. Aldercreutz, *Eur. J. Biochem.*, **199**, 609 (1991).

14 H. Kise, A. Hayakawa, and H. Noritomi, *J. Biotechnol.*, **14**, 239 (1990).

15 M. Norin, J. Boutelje, E. Holmberg, and K. Hult, *Appl. Microbiol. Biotechnol.*, **28**, 527 (1988).

16 V. M. Paradkar and J. S. Dordick, *Biotechnol. Bioeng.*, **43**, 529 (1994).

17 P. P. Wangikar, P. C. Michels, D. S. Clark, and J. S. Dordick, *J. Am. Chem. Soc.*, **119**, 70 (1997).

18 A. Zaks and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 3192 (1985).

19 A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, **263**, 3194 (1988).

20 Z. Yang, D. Zacherl, and A. J. Russell, *J. Am. Chem. Soc.*, **115**, 12251 (1993).

21 Y. L. Khmelnitsky, S. H. Welch, D. S. Clark, and J. S. Dordick, *J. Am. Chem. Soc.*, **116**, 2647 (1994).

22 A. J. Russell and A. M. Klibanov, *J. Biol. Chem.*, **263**, 11624 (1988).

23 M. Ståhl, U. Jeppsson-Wistrand, M. Månsson, and K. Mosbach, *J. Am. Chem. Soc.*, **113**, 9366 (1991).

24 J. O. Rich and J. S. Dordick, *J. Am. Chem. Soc.*, **119**, 3245 (1997).

25 K. Dabulis and A. M. Klibanov, *Biotechnol. Bioeng.*, **39**, 176 (1992).

26 L. Braco, K. Dabulis, and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 274 (1990).

27 P. Mishra, K. Griebenow, and A. M. Klibanov, *Biotechnol. Bioeng.*, **52**, 609 (1996).

28 K. Dabulis and A. M. Klibanov, *Biotechnol. Bioeng.*, **41**, 566 (1993).

29 Y. Inada, M. Furukawa, H. Sasaki, Y. Kodera, M. Hiroto, H. Nishimura, and A. Matsushima, *Trends Biotechnol.*, **13**, 86 (1995).

30 A. F. S. A. Habeeb, *Anal. Biochem.*, **14**, 328 (1966).

31 M. N. Bradford, *Anal. Chem.*, **72**, 248 (1976).

32 K. Griebenow and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 10969 (1995).

33 K. Griebenow and A. M. Klibanov, *Biotechnol. Bioeng.*, **53**, 351 (1997).

34 K. Griebenow and A. M. Klibanov, *J. Am. Chem. Soc.*, **118**, 11698 (1997).

35 D. M. Byler and H. Susi, *Biopolymers*, **25**, 469 (1986).

36 M. Jackson and H. H. Mantsch, *Biochim. Biophys. Acta*, **1078**, 231 (1991).

37 U. Görne-Tschelnokow, D. Naumann, C. Weise, and F.

Hucho, *Eur. J. Biochem.*, **213**, 1235 (1993).
38 A. Dong, S. J. Prestrelski, S. D. Allison, and J. F. Carpenter, *J. Pharm. Sci.*, **84**, 415 (1995).
39 R. Bovara, G. Carrea, A. M. Gioacchini, S. Riva, and F. Secundo, *Biotechnol. Bioeng.*, **54**, 50 (1997).
40 K. Xu, K. Griebenow, and A. M. Klibanov, *Biotechol. Bioeng.*, **56**, 485 (1997).