ELSEVIER

FEBS Letters 339 (1994) 200–204

FEBS 13679

FEBS LETTERS

Acylation of subtilisin with long fatty acyl residues affects its activity and thermostability in aqueous medium

Francisco J. Plou*, Antonio Ballesteros

Departamento de Biocatalisis, Instituto de Catálisis, CSIC, Universidad Autónoma, 28049 Madrid, Spain

Received 27 December 1993

Abstract

Subtilisin Carlsberg has been artificially hydrophobized by acylation with octanoyl or palmitoyl chlorides. Samples with several degrees of substitution were obtained. Hydrophobization facilitates in some cases the binding of synthetic or natural substrates. Furthermore, derivatized subtilisins show improved thermal stability (15-fold at 45°C) in aqueous solution. As a result, octanoyl-subtilisin exhibits enhanced thermostability without losing biological activity.

Key words: Subtilisin Carlsberg; Chemical modification; Enzyme hydrophobicity; In vitro hydrophobization; Enzyme stabilization

1. Introduction

Subtilisins comprise a group of serine endopeptidases (m.wt. ≈ 27,500 Da) that are secreted in large amounts from a wide variety of *Bacillus* species. The large data bank available for subtilisins makes them attractive enzymes to elucidate structure–function relationships [1]. The industrial importance of subtilisins is very high, especially as components of household detergents. In 1988 the world market for industrial enzymes was approximately US $600 million, half of which corresponded to enzymes added to detergents (mainly subtilisins) [2].

One of the most important aims of enzyme engineering is to enhance the thermostability of enzymes. Furthermore, temperature stability of enzymes used in laundry detergents is an important factor in productivity [3]. Protein stabilization has been achieved by several methods (see [3] and references therein), including multipoint immobilization, site-directed mutagenesis, chemical modification, crosslinking or even selection of enzymes from thermophilic organisms.

The hydrophile–lipophile balance (HLB) of an enzyme seems to be essential in most of its properties (recognition of substrates, binding, stability, etc.). In this context, the in vivo posttranslational modification of proteins by covalent attachment of fatty acids and lipids is a widespread phenomenon in nature but not yet completely understood [4–6]. Myristic and palmitic acid are the predominant fatty acid residues linked to proteins. Myristic acid is commonly found attached by an amide bond to an N-terminal glycine; palmitic acid is bound to the protein via an alkali-labile ester linkage, usually a thioester [6]. Several methods for acylation of proteins in vitro have been also described [5,7]. Therapeutical applications of these acyl-proteins and acyl- antibodies are being reported as very promising, since hydrophobization imparts transmembrane properties to water-soluble proteins [8] or makes possible their insertion into the lipid matrix of liposomes [9].

In the present work, and imitating the hydrophobization that takes place in nature for many proteins, we have lowered the HLB of subtilisin Carlsberg – a protein containing 10 amino residues and no cysteines – by chemical acylation of its amino groups (using acyl chlorides of different chain length). Furthermore, the effect of the hydrophobization of the protein on its activity and stability was analyzed.

2. Materials and methods

Subtilisin Carlsberg (protease type VIII from *B licheniformis*), palmitoyl and octanoyl chlorides, sodium cholate, phenylboronic acid, *p*NPB, ATEE, Ac-Tyr-NH_2, *N*-transcinnamoylimidazole, Succ-Ala-Ala-Pro-Phe-*p*NA were all purchased from Sigma. 2,4,6-trinitrobenzenesulfonic acid and PMSF were from Fluka. Casein (Hammarsten) was from Merck. Sephadex G-25 and G-50, PEG 6000 and Dextran 500 were supplied by Pharmacia. All other chemicals used were of the purest grade available.

*Corresponding author. Fax: (34) (1) 5854760.

Abbreviations. *p*NPB, *p*-nitrophenyl butyrate; ATEE, *N*-Acetyl-L-tyrosine ethyl ester; PEG, polyethylene glycol; HLB, hydrophile-lipophile balance; Succ-, Succinyl; *p*NA, *p*-nitroanilide; Ac-Tyr-NH_2, *N*-Acetyl-L-tyrosine amide; PMSF, phenylmethanesulfonyl fluoride.

SSDI 0014-5793(94)00063-2

2.1. Enzyme assays

The hydrolytic activity (esterase/lipase) [10] using *p*-nitrophenyl butyrate as substrate was followed spectrophotometrically at 30°C in a Varian Cary 210 spectrophotometer equipped with magnetic stirring. The assay mixture (3 ml) contained 30–400 μM substrate in 10 mM phosphate buffer (pH 7), including 5% acetone. Initial rates were estimated by measuring the increase in the absorbance at 346 nm – isobestic point of the *p*-nitrophenol/*p*-nitrophenoxide couple – and considering the molar extinction coefficient as 4,800 $M^{-1} \cdot cm^{-1}$ [11].

The esterase activity using ATEE was measured titrimetrically using a pH-stat (Radiometer). The assay mixture was prepared injecting the ester (as a concentrated solution in ethanol) into the aqueous buffer solution. The system contained 6–36 mM substrate in 0.5 mM Tris- HCl buffer (pH 7.8), 0.1 M KCl. Ethanol content was 5% in all assays.

The amidase activity of subtilisin was determined spectrophotometrically (410 nm) at 30°C in 0.1 M phosphate buffer (pH 7), containing 0.1 M Succ-Ala-Ala-Pro-Phe-*p*NA as substrate.

Proteolytic activity was determined using 1% casein as substrate following the protocol described by Laskowsky [12]; incubation time was 20 min.

The Michaelis constant and the k_{cat} of the system were estimated using a non-linear regression adjustment from the SIMFIT package [13]. One unit of activity (U) is the amount of enzyme that hydrolyses 1 μmol of substrate per min under the above conditions.

2.2. Preparation of palmitoyl-subtilisin

Acylation of subtilisin with palmitoyl chloride was carried out according to Torchillin et al. [14], with slight modifications. A reversible inhibitor (Ac-Tyr-NH_2 or phenylboronic acid) of subtilisin was included (at 20 mM) in the reaction medium. The pH was mantained at 8.0 with the aid of a pH-stat using 1 M NaOH. The cholate present (used to solubilize the acylating agent) and the rest of contaminants were removed in a final step by gel filtration in a Sephadex G-25 column. Fractions containing protein were lyophilized and kept at −20°C.

2.3 Preparation of octanoyl-subtilisin

Subtilisin (100 mg) was dissolved in 7.2 ml of 0.1 M phosphate buffer (pH 8), containing 0.1 M NaCl, 20 mM reversible inhibitor and 1% cholate Then, 0.5 ml of 1.8 M octanoyl chloride solution in acetone were added in portions of 0.1 ml. The mixture was kept at 4°C and pH 8.0 under stirring. After the final addition of octanoyl chloride, base was added in a pH-stat until all octanoyl chloride was hydrolyzed, as evidenced by a leveling off of the base consumption curve. The acylated enzyme was then purified by gel filtration (G-25 column).

2.4. Enzyme thermoinactivation

A diluted solution (~2 μM) of subtilisin in 0.1 M phosphate (pH 7) was incubated at the desired temperature. The remaining amidase activity at different times was measured. The half-life ($t_{1/2}$) was calculated from a single exponential adjustment using ENZFITTER program [15].

2.5 Determination of degree of acylation

The content of free lysines before and after acylation was determined using 2,4,6-trinitrobenzenesulfonic acid according to Fields [16]. In order to avoid the effect of the peptides generated by autolysis of subtilisin – that leads to an overestimation of amino groups – samples were irreversibly inhibited with PMSF and chromatographied over Sephadex G-50 (elution with phosphate 20 mM, pH 7) before following Fields' procedure.

2.6. Partition of subtilisin samples in an aqueous Dextran 500/PEG 6000 biphasic system

The system was prepared as described by Zaslavsky et al. [17]. The composition of the system was 7% (w/w) Dextran 500, 4.4% (w/w) PEG 6000, 0.11 M sodium phosphate buffer (pH 7.4). Enzyme solutions (0.25–0.50 mg/ml) were added to the biphasic system and the phases were allowed to settle for 24 h at 25°C. Enzyme concentration was measured in both phases. The partition coefficient (K) is defined as the ratio of the enzyme concentration in the PEG-rich and the dextran-rich phases. The hydrophobicity of enzyme preparations was expressed as the equivalent number of CH_2 groups (n^{CH_2} [18].

2.7. Other methods

Protein concentration was estimated by the Peterson method [19], suitable for hydrophobic proteins. Active-site titration of subtilisin was performed using *N-trans*-cinnamoylimidazole [20].

3. Results

3.1. Acylation of subtilisin

Several experimental conditions were tested. A reversible inhibitor was included in the reaction medium in order to minimize autolysis at the high pH at which acylation is carried out. Table 1 shows that the presence of an inhibitor increases both the active-enzyme recovery and activity yield.

The degree of acylation can be controlled depending on several parameters, such as the molar ratio acylating agent/protein, the reaction time, the inhibitor used or even the physical state of the enzyme before adding the acylating agent. Acylation degrees ranging from 10– 60% for palmitoylation and 32–57% for modification with capryloyl chloride were obtained. The highest percentage of substitution was in all cases about 60%, which implies introduction of approximately 6 fatty acyl residues into a single enzyme molecule.

It is noteworthy that, after gel filtration, palmitoyl-subtilisin samples showed appreciable naked-eye turbidity, indicating that a good hydrophobization had taken place.

3.2. Evaluation of hydrophobicity

Firstly, the hydrophobization was evaluated measuring the increase in turbidity (absorbance at 400 nm [21]). Fig. 1 depicts the results obtained for subtilisin samples with acyl arms of different length and/or different degree of substitution (turbidity is correlated with titrated enzyme concentration).

Hydrophobicity of acylsubtilisins was also determined studying the behaviour of these biocatalysts in biphasic systems formed by mixtures of polymer solutions. Samples containing different amounts of protein were assayed, and it was observed that enzyme concentration did not affect the partition coefficient. All the samples

Table 1
Active-enzyme recovery (active-site titration with *N*-transcinnamoylimidazole) and activity yield (measured for the hydrolysis of Succ-Ala-Ala-Pro-Phe-*p*NA) after acylation of subtilisin with octanoyl (C_8) and palmitoyl (C_{16}) chlorides, in the presence of two reversible inhibitors

Reversible inhibitor	K_i (mM)	Active-enzyme recovery (%)		Activity yield (%)	
		C_8	C_{16}	C_8	C_{16}
None		55.4	45.2	59.2	35.6
Ac-Tyr-NH_2	120	56.9	53.7	65.5	43.5
Phenylboronic acid	0.23	83.6	60.6	81.0	62.7

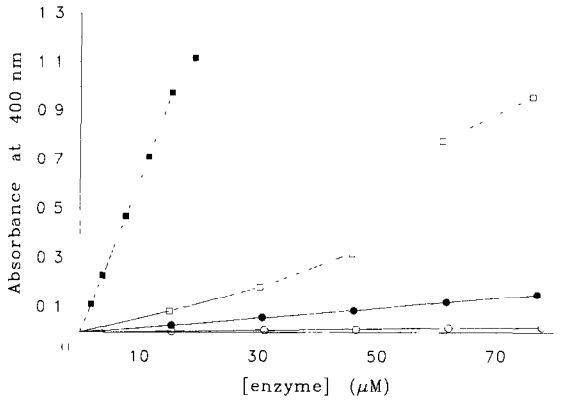

Fig. 1. Turbidity of acyl-subtilisin solutions in water as a function of enzyme concentration (measured by active-site titration). Samples (degrees of acylation in brackets): (○) native; (●) octanoyl-subtilisin (32%); (□) palmitoyl-subtilisin (14%); (■) palmitoyl-subtilisin (39%).

manifested preference for the most hydrophobic phase (the PEG-rich one), this preference being higher when subtilisin was acylated (Table 2). In this way, the equivalent number of CH_2 groups (n^{CH_2} increases more than 2-fold when octanoyl- (34% acylation) or palmitoyl-subtilisin (14%) are compared with the native enzyme. However, palmitoyl-subtilisin with a high degree of substitution (39%) deposits on the interphase of the system, yielding erratic K values.

3.3. Kinetic parameters

The kinetic constants for the hydrolysis of different substrates, natural or synthetic, were evaluated. Table 3 summarizes the results encountered for the hydrolysis of several ester or amide substrates. Kinetic constants for the hydrolysis of the two esters assayed, ATEE and *p*NPB, do not show a significant variation for acylated subtilisins compared with the wild-type enzyme. However, the binding of the long amide substrate Succ-Ala-Ala-Pro-Phe-*p*NA seems to be favoured when subtilisin is acylated (i.e. a slight decrease in K_m is observed). Nevertheless, k_{cat} values indicate that catalysis is not improved despite the better binding.

Results found in the hydrolysis of casein are presented in Fig. 2. The octanoyl-subtilisin exhibits higher hydrolysis rates than the native enzyme, whereas the subtilisin derivatized with palmitoyl chloride hydrolizes casein with a low velocity, possibly due to steric hindrance.

Table 2
Relative hydrophobicity of the modified preparations of subtilisin, measuring the partition coefficient of enzyme between PEG 6000 and Dextran 500

Protein	n^{CH_2}
Bovine serum albumin	6.5
Subtilisin Carlsberg	7 3
Palmitoyl-subtilisin (14%)	17.5
Octanoyl-subtilisin (32%)	18.1

Bovine serum albumin (Sigma) is presented as a standard. Degrees of substitution are in brackets.

3.4. Stability experiments

Stability of acyl-subtilisins in aqueous medium (pH 7) at 45°C and 65°C was studied. Low enzyme concentrations were used in order to minimize autolysis and/or aggregation processes [22]. The acyl-subtilisins with the highest percentages of modification were compared with the native enzyme. Table 4 summarizes the thermoinactivation constants, k_{in}, and half-lives, $t_{1/2}$, of the preparations. Hydrophobization of subtilisin with acyl (C_8 or C_{16}) chlorides gives rise to preparations whose thermostability is enhanced. This stabilizing effect (ratio of the rate constant of thermoinactivation of the native and the modified enzymes) is about 15-fold at 45°C and about 2.5-fold at 65°C.

4. Discussion

Recent papers have shown that varying the hydrophilic-lipophilic character of an enzyme through covalent modification of their amino groups can critically alter their activity and stability in aqueous and organic media. Thus, Mozhaev et al. [22] hydrophilizated α- chymotrypsin by reaction with anhydrides of aromatic carboxylic acids: the modified protein was extremely stable against irreversible thermal inactivation. And Ampon et al. [23], after reductive alkylation of lipase (with aldehydes), found that the enzyme had altered enzymatic activities and was more stable. However, the rules that govern the new properties of the modified enzymes are still not clear.

Due to the drastic reaction conditions, the previous studies on chemical modification of proteases reported low recoveries of active-enzyme and activity, especially

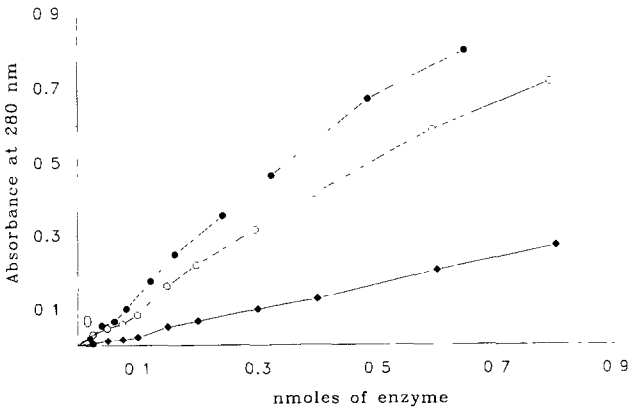

Fig. 2. Determination of proteolytic activity of acyl-subtilisins using 1% casein as substrate at 30°C. Incubation time: 20 min. (○) native; (●) octanoyl-subtilisin, (32%); (◆) palmitoyl-subtilisin (14%).

Table 3
Kinetic constants for the hydrolysis of several substrates catalyzed by native and acylated subtilisins

Enzyme preparation	Acylation degree (%)	*p*NPB		ATEE		Succ-Ala-Ala-Pro-Phe-*p*NA	
		K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
Native		0.086 ± 0.01	2.7 ± 0.1	44 ± 13	790 ± 150	0.32 ± 0.07	830 ± 100
Octanoyl-	32	0.063 ± 0.005	2.4 ± 0.1	87 ± 10	1,370 ± 150	0.21 ± 0.03	620 ± 50
Palmitoyl-	14	0.099 ± 0.009	2.1 ± 0.1	61 ± 10	820 ± 100	0.15 ± 0.02	90 ± 4

when high degrees of modification were achieved. Therefore, a few works [7,22,24] report the use of a reversible inhibitor (or a substrate) in order to prevent the modification of active site residues, minimize autolysis and, as a consequence, improve process yield [25]. Table 1 clearly shows how the presence of an inhibitor with good affinity for subtilisin (K_i equal to 0.23 mM) increases the recovery of active enzyme and activity; thus, phenylboronic acid exhibits some properties that make it a suitable compound in this kind of processes: non-toxic at millimolar concentrations, great dependence of K_i vs. pH that permits to control the magnitude of the binding, and reasonable price. In absence of inhibitors, activity yields reported in similar acylations are commonly under 50% [14,26].

Hydrophobicity of the enzyme surface has been correlated in some reports with turbidity of its aqueous solutions [7]. In the present work, the optical density at 400 nm increases considerably with both the number of residues attached to the biocatalyst and the length of the fatty chain (Fig. 1). Alternatively, the determination of hydrophobicity by measuring the partition of the protein in biphasic systems (Ficoll 400/Dextran 70 [22] or Phenyl Sepharose/$(NH_4)_2SO_4$ [26]) is often more sensitive but is time-consuming. Concerning our samples, the observed differences in n^{CH_2} (Table 2) are doubtless indicative of the differences in their superficial hydrophobicity.

The better binding in the hydrolysis of Succ-Ala-Ala-Pro-Phe-*p*NA when acyl-subtilisins were assayed can be explained considering that a change in the microenvironment of the enzyme site increases the interaction of the substrate with the protein. The specificity constant (k_{cat}/K_m) for the octanoyl-modified enzyme is even higher than the corresponding value for the native protein (Table 5). Results found in the hydrolysis of casein correlate quite well with those obtained for Succ-Ala-Ala-Pro-Phe-*p*NA hydrolysis. The results obtained in the present work confirm the convenience of using substrates with different size and different scissile bonds when testing kinetic properties of modified enzymes. Results using only short, synthetic substrates, as is commonly done for proteases (e.g. in site-directed mutagenesis), can be misleading.

Concerning thermal stability, this parameter can be influenced by many factors (temperature, pH, ionic strength, enzyme concentration, etc.) that need to be carefully controlled. At least two interpretations can be postulated for the stabilizing effect observed: first, attachment of acyl arms to enzyme can increase resistance to autolysis as a consequence of steric hindrance; second, the substitution –by uncharged (acyl) groups– in the protein surface of some unfavourably positively charged (repulsive) groups that promote unfolding can enhance thermostability. In this context, hydrophilization of protein surface area usually leads to enhanced stability [22,27]; on the other hand, hydrophobization of the protein has led both to an increase [28,29] or a decrease [22,30] in thermostability. It seems likely that there is an optimum ratio of surface hydrophilicity/hydrophobicity and that this ratio is different for each enzyme. So each enzyme needs to be tackled differently and it may be difficult to lay down general strategies for stabilization [30]

For the industrial application of subtilisins and similar enzymes, primarily in laundry detergents, it is attractive to obtain derivatives with increased thermal stability and yet maintaining high activity. Thus hydrophobization via acylation (in particular with octanoyl chloride) can be considered an alternative to site-directed mutagenesis to get samples with enhanced thermal stability without losing biological activity. In conclusion, the study of hydrophobic modifications of native enzymes can be

Table 4
Thermoinactivation constants of native and acylated subtilisins at pH 7

Subtilisin	Acylation degree (%)	k_{in} (s^{-1})		$t_{1/2}$ (h)	
		45°C	65°C	45°C	65°C
Native		$8.0 \pm 0.5 \cdot 10^{-5}$	$1.3 \pm 0.1 \cdot 10^{-4}$	2.4	1.4
Octanoyl-	57	$6.0 \pm 1.0 \cdot 10^{-6}$	$6.7 \pm 0.6 \cdot 10^{-5}$	30.4	2.9
Palmitoyl-	60	$5.2 \pm 0.8 \cdot 10^{-6}$	$4.4 \pm 0.6 \cdot 10^{-5}$	37.2	4.4

Table 5
Specificity constants for the hydrolysis of several substrates catalyzed by native and acylated subtilisins

Subtilisin	Acylation degree (%)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)		
		*p*NPB	ATEE	Succ-Ala-Ala-Pro-Phe-*p*NA
Native		$3.1 \cdot 10^4$	$1.8 \cdot 10^4$	$2.6 \cdot 10^6$
Octanoyl-	32	$3.8\ 10^4$	$1.6 \cdot 10^4$	$3.0 \cdot 10^6$
Palmitoyl-	14	$2.1 \cdot 10^4$	$1.3 \cdot 10^4$	$6.0 \cdot 10^5$

very interesting in both fundamental and applied enzymology.

Acknowledgments. We are grateful to Drs. C. Otero and M.L. Rúa for their assistance in the early stages of this work, and to Prof. W.G. Bardsley (University of Manchester) for providing us with a copy of his SIMFIT programme. This work has been supported by the EEC (BRIDGE project No. BIOT-CT90-0176) and the Spanish CICYT (project No. BIO91-0861-CE).

References

[1] Wells, J.A., Powers, D.B., Bott, R.R., Katz, B.A., Ultsch, M.H., Kossiakoff, A.A., Power, S.D., Adams, R.M., Heyneker, H.H., Cunningham, B.C., Miller, J.V., Graycar T.P. and Estell, D.A. (1987) Protein Eng., 279–287.
[2] Arbige, M.V. and Pitcher, W.H. (1989) Trends Biotechnol. 7, 330–335.
[3] Shami, E.Y., Rothstein, A. and Ramjeesingh, M. (1989) Trends Biotechnol. 7, 186–190.
[4] Schultz, A.M., Henderson, L.E. and Oroszlan, S. (1988) Annu. Rev. Cell Biol. 4, 611–647.
[5] Kabanov, A.V., Levashov, A.V. and Alakhov, V.Y. (1989) Protein Eng. 3, 39–42.
[6] McIlhinney, R.A.J. (1990) Trends Biochem. Sci. 15, 387–391.
[7] Martins, M.B.F., Jorge, J.C.S. and Cruz, M.E.M. (1990) Biochimie 72, 671–675.
[8] Kabanov, A.V., Ovcharenko, A.V., Melik-Hubarov, N.S., Bannikov, A.I., Alakhov, V.Yu, Kiselev, V.I., Sveshnikov, P.G., Kiselev, O.I., Levashov, A.V. and Severin, E.S. (1989) FEBS Lett. 250, 238–240.
[9] Martins, M.B.F., Gonzalves, A.P.V., Jorge, J.C. and Cruz, M.E.M. (1990) Eur. J. Pharmacol. 183, 401.
[10] Plou, F.J., Nuero, O.M., Alfonso, C., Reyes, F. and Ballesteros, A. (1993) Applied Biochem. (submitted). Biotechnol.
[11] Fletcher, P.D.I, Robinson, B.H., Freedman, R.B. and Oldfield, C. (1985) J. Chem. Soc. Faraday Trans. 81, 2667–2679.
[12] Laskowski, M. (1955) Methods Enzymol. 2, 32–34.
[13] SIMFIT, 'A package for simulation, curve-fitting and statistical analysis in the life sciences' (1993) by Bardsley, G.W., Manchester University, UK.
[14] Torchilin, V.P., Omelyanenko, V.G., Klibanov, A.L., Mikhailov, A.I., Goldanskii, V.I. and Smirnov, V.N. (1980) Biochim. Biophys. Acta 602, 511–521.
[15] ENZFITTER. 'A non-linear regression data analysis program' (1987) by Leatherbarrow, R.J., Elsevier-Biosoft.
[16] Fields, R. (1971) Biochem. J. 124, 581–590.
[17] Zaslavsky, B.Y., Mestechkina, N.M. and Rogozhin, S.V. (1983) J. Chromatogr. 260, 329–336.
[18] Zaslavsky, B.Y., Mestechkina, N.M., Miheeva, L.M. and Rogozhin, S.V. (1983) J. Chromatogr. 253, 49–59
[19] Peterson, G.L. (1977) Anal. Biochem., 83, 346–356
[20] Bender, M.L., Begue-Canton, M.L., Blakeley, R.L., Brubacher, L.J., Feder, J., Gunter, C.R., Kezdy, F.J., Kilheffer, J.V., Marshall, T.H., Miller, C.G., Roeske, R.W. and Stoops, J.K (1966) J. Am. Chem. Soc. 88, 5890–5913.
[21] John, R.A. (1992) in: Enzyme Assays (Eisenthal, R. and Danson, M.J. Eds.), pp. 82–83, Oxford University Press, Oxford.
[22] Mozhaev, V.V., Siksnis, V.A., Melik-Nubarov, N.S , Galkantaite, N.Z., Denis, G.J., Butkus, E.P., Zaslavsky, B.Y., Mestechkina, N.M and Martinek, K. (1988) Eur. J. Biochem. 173, 147–154.
[23] Ampon, K., Salleh, A.B., Salam, F., Wan Yunus, W.M.Z., Razak, C.N.A. and Basri, M. (1991) Enzyme Microb. Technol. 13, 597–601.
[24] Goldmacher, V.S. (1983) Biochem. Pharmacol. 32, 1207–1210.
[25] Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, Holden-Day Inc., San Francisco.
[26] Vazquez-Duhalt, R., Fedorak, P.M. and Westlake, D.W.S. (1992) Enzyme Microb Technol. 14, 837–841.
[27] Mozhaev, N.S., Melik-Nubarov, N.S., Siksins, V. and Martinek, K. (1990) Biocatalysis 3, 189–196.
[28] Schmid, R.D. (1979) in: Advances in Biochemical Engineering (Ghose, T.K., Fietcher, A. and Blackebrough, N., Eds.) Vol. 12, pp. 41–117, Springer, New York.
[29] Shatsky, M.A., Ho, C.H. and Wang, J.H.C. (1973) Biochim. Biophys. Acta 303, 298–307.
[30] Tyagi, R. and Gupta, M.N. (1993) in: Thermostability of Enzymes (Gupta, M.N Ed.) pp. 146–160, Springer, Berlin.