Isolation of Acein-2, a novel angiotensin-I-converting enzyme inhibitory peptide derived from a tryptic hydrolysate of human plasma

Kazuya Nakagomi[a,*], Riho Yamada[a], Hidetoshi Ebisu[a], Yutaka Sadakane[a], Toshifumi Akizawa[b], Takenori Tanimura[a]

[a] *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata 570-0101, Japan*

Received 27 December 1999

Edited by Gunnar von Heijne

Abstract We previously described a novel angiotensin-I-converting enzyme (ACE) inhibitory peptide, designated Acein-1, that was isolated from a tryptic hydrolysate of human plasma. We now report a second such inhibitory peptide, Acein-2 obtained from the same hydrolysate. The peptide was purified by gel filtration and cation exchange chromatography followed by reversed-phase gradient and isocratic high performance liquid chromatography. Acein-2 was found to be a tripeptide, Leu-Ile-Tyr, which is thought to correspond to f(518–520) of human α2-macroglobulin. The synthetic tripeptide showed a potent dose-dependent inhibition of ACE, with an IC_{50} value of 0.82 μmol/l. Lineweaver–Burk plots suggested that Acein-2 as well as the previously described Acein-1 are non-competitive inhibitors.

Key words: Acein-2; Acein-1; Angiotensin-I-converting enzyme inhibitory peptide; Tryptic hydrolysate; Human α2-macroglobulin

1. Introduction

Almost all organisms, including animals, plants, and microorganisms, as well as synthetic compounds have been examined in the search for bioactive substances as starting materials for medicines [1–7]. However, we believe that the human body might be the best resource for such bioactive peptides because such substances would be expected to be less toxic to humans. Although numerous substances have been isolated from the human body, there are probably many others, especially degradation products of proteins, that have not yet been identified. To demonstrate this idea, we have isolated Acein-1 [8] and Cabin-1, 2, 3 and 4 [9] as novel bioactive peptides in enzymatic hydrolysates of human plasma. Acein-1, a novel peptide that inhibits angiotensin-I-converting enzyme (ACE, peptidyl dipeptide hydrolase), was isolated from a tryptic hydrolysate of human plasma. The amino acid sequence of Acein-1 (Tyr-Leu-Tyr-Glu-Ile-Ala-Arg) corresponds to f(138–144) of human serum albumin. During the isolation of Acein-1, another active fraction was found in the effluent from a SP-Sephadex C-25 column.

In this paper, we report the isolation of another novel ACE inhibitory peptide, which was derived from a tryptic hydrolysate of human plasma. We also discuss the inhibitory mechanism of Acein-2 based on Lineweaver–Burk plots.

*Corresponding author. Fax: (81)-76-434 5063.
E-mail: nakagomi@ms.toyama-mpu.ac.jp

ACE has classically been associated with the renin-angiotensin system, which regulates peripheral blood pressure. ACE raises blood pressure by converting angiotensin-I, released from angiotensinogen by renin, into the potent vasoconstrictor angiotensin-II. ACE also degrades vasodilative bradykinin in blood vessels and stimulates the release of aldosterone in the adrenal cortex. Consequently, ACE inhibitors may exert an anti-hypertensive effect [10,11].

2. Materials and methods

2.1. Enzymes and other reagents

Freshly frozen human plasma for use in transfusion was used. ACE (EC 3.4.15.1, from rabbit lung) and trypsin (EC 3.4.21.4, from porcine pancreas) were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma Chemical (St. Louis, MO, USA), respectively. Hippuryl-histidyl-leucine (HHL) and bradykinin potentiator B (BPB) were obtained from The Peptide Institute (Osaka). Sephadex G-25 and SP-Sephadex C-25 were from Pharmacia Biotech (Uppsala, Sweden), and the SEP-PAK C18 cartridge was from Waters (Milford, MA, USA). All other reagents were of analytical grade unless otherwise specified.

2.2. Tryptic hydrolysis

Human plasma (10 ml) was diluted with seven volumes of 50 mM Tris–HCl buffer (pH 8.0). Trypsin was added to the plasma solution at an enzyme:substrate ratio of 1:50 and the mixture was hydrolyzed at 37°C for 3 h. The reaction was stopped by heating in boiling water for 10 min, and the hydrolysate was centrifuged for 20 min at 4°C and 3000×*g*. The supernatant was used as a sample.

2.3. Sephadex G-25 gel filtration

A sample of the supernatant was concentrated by 5-fold with a vacuum concentrator, and 10 ml of the concentrate was applied directly to a Sephadex G-25 column (30 i.d.×360 mm) equilibrated with 10 mM ammonium acetate (pH 4.0). The same solution was used as the eluent at a flow rate of 3.6 ml/min. Fraction volume was 10 ml. Fraction Nos. 18–22 were found to have activity.

2.4. SP-Sephadex C-25 cation exchange column chromatography

About 50 ml of the combined fractions from the gel filtration was applied to a SP-Sephadex C-25 cation exchange column (30 i.d.×420 mm) equilibrated with 10 mM ammonium acetate (pH 4.0). Materials were eluted with a linear gradient between 250 ml of 10 mM ammonium acetate (pH 4.0) and 250 ml of the same solution containing 500 mM ammonium formate and then 300 ml of the final solution at a flow rate of 3.0 ml/min. Fraction volume was 16 ml. Fraction Nos. 10–14, 34–38 and 42–45 were found to have activity. For each of these three groups of fractions, the fractions were pooled, desalted with a SEP-PAK C18 cartridge and concentrated.

2.5. Reversed-phase gradient high performance liquid chromatography (HPLC)

The desalted samples were purified by HPLC with gradient elution. A GILSON HPLC system was used with an autogradient set (Model 802, 803C, and two Model 302 pumps, GILSON Medical Electronics,

0014-5793/00/$20.00
PII: S0014-5793(00)01163-7

Middleton, WI, USA). Separation was performed at room temperature on a Develosil ODS-5 (Nomura Kagaku, Osaka, Japan) column (4.0 i.d.×150 mm). A linear acetonitrile gradient between 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 80% acetonitrile with 0.1% TFA in water (solvent B) was used as follows: 0% B for 20 min, 0–40% B for 60 min, 40% B for 30 min and 100% B for 10 min. The flow rate was 1.0 ml/min and the effluent was monitored continuously at 215 nm. All peaks were fractionated and evaporated for measuring inhibitory activity.

2.6. Reversed-phase isocratic HPLC

The active fractions were further purified by reversed-phase isocratic HPLC. The HPLC system used was the same as the gradient HPLC system. Separation was performed on a single or a tandem-linked Develosil ODS-5 column (4.0 i.d.×150 mm) at room temperature. 18% Acetonitrile with 0.1% TFA was used as the eluent. The flow rate was 0.5 ml/min and the effluent was monitored continuously at 215 nm. The active fractions were collected and evaporated.

2.7. Inhibitory assay for ACE

The inhibitory activity of ACE was measured spectrophotometrically using HHL as the substrate, by a modification of the method of Cushman and Cheung [12] as described by Maruyama et al. [13]. 5 mM HHL and an appropriate quantity of ACE inhibitor were dissolved in 100 mM sodium borate buffer, pH 8.3, containing 300 mM NaCl, and incubated with 20 mU/ml ACE at 37°C for 30 min. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value. BPB (IC_{50} = 3.3 μM) was used as a positive control for ACE inhibition [14].

2.8. Amino acid sequence analysis and mass spectrometry

The amino acid sequence was determined using Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin amino acid analyzer (Shimadzu PPSQ-21, Kyoto, Japan). A mass analysis of the peptides was carried out using a mass spectrometer (JMS-AX505, JEOL, Tokyo, Japan).

2.9. Preparation of peptides [15]

Peptides were synthesized with the solid phase Fmoc method on a peptide synthesizer (Applied Biosystems 433A, Foster City, CA, USA) and deprotected in the usual manner. Purification was performed by HPLC with a reversed-phase column (Capcell Pak C18, 10 i.d.×250 mm, Shiseido, Tokyo, Japan) employing a linear gradient elution system from H_2O to 50% acetonitrile containing 0.1% TFA for 30 min. The flow rate was set for 3.0 ml/min. Effluents were monitored by UV absorption at 220 nm, and the peaks with 220 nm absorbance were collected and lyophilized.

2.10. Measurement of chymotryptic activity [16]

The chymotrypsin activity was measured by using *N*-benzoyl-Tyr ethyl ester (BTEE) as the substrate. The reaction mixture consisted of 1.5 ml trypsin solution in 80 mM Tris–HCl buffer containing 100 mM $CaCl_2$ (pH 7.8) and 1.5 ml of 1.0 mM BTEE in 50% MeOH. The reaction was started by adding the trypsin solution. The increase in absorbance at 256 nm was measured at precisely 1, 2 and 3 min. The chymotrypsin activity (BTEE U/l) was calculated from the following formula: BTEE (U/l) = 31 100×ΔAbs/min.

3. Results and discussion

With the use of HHL as the substrate, the tryptic hydrolysate of human plasma after a 3 h incubation was found to have significant ACE inhibitory activity. Fractions 18–22 from a Sephadex G-25 column showed ACE inhibition, while the other fractions had no activity. About 50 ml of the combined fractions was subjected to SP-Sephadex C-25 cation exchange chromatography. Some of the SP-Sephadex C-25 cation exchange fractions were found to be active as shown in Fig. 1. Acein-1, which was previously reported [8], was found in fractions 42–45 (striped bars in Fig. 1), while fractions 10–14 and fractions 34–38 (solid bars in Fig. 1) also showed ACE inhibition. These fractions were collected and

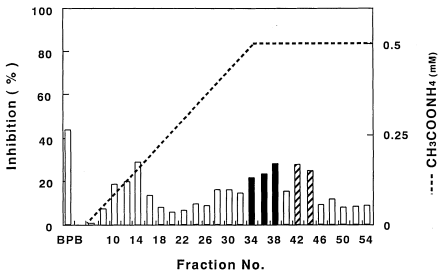

Fig. 1. Fractionation of a tryptic hydrolysate of human plasma on SP-Sephadex C-25. Active fractions eluted from the gel filtration column were applied to a SP-Sephadex C-25 cation exchange column (30 i.d.×420 mm) equilibrated with 10 mM CH_3COONH_4 (pH 4.0). Materials were eluted with a linear gradient formed with 250 ml of 10 mM CH_3COONH_4 (pH 4.0) and 250 ml of 10 mM CH_3COONH_4 (pH 4.0) containing 500 mM $HCOONH_4$, and then 300 ml of the final solution at a flow rate of 3.0 ml/min. Dashed line shows the concentration of $HCOONH_4$ in the eluent. Fractions (16 ml each) were collected and their absorbance at 280 nm was measured. Fractions 34–38 (solid bars) contain Acein-2 and fractions 42–45 (striped bars) contain Acein-1.

concentrated through SEP-PAK C18 cartridges. The sample from fractions 34–38 was purified by reversed-phase HPLC with gradient elution (Fig. 2). The fraction that eluted at around 18% acetonitrile (solid bars in Fig. 2) showed inhibitory activity. This fraction was rechromatographed using isocratic HPLC (18% acetonitrile with 0.1% TFA) and isolated as a single peak. The inhibitory activity in fractions 10–14 was found to decrease after they were desalted and concentrated.

Amino acid sequencing analysis of the purified peptide showed that it was a tripeptide with the sequence of Leu-Ile-Tyr. The mass number of this peptide as determined by mass spectrometry was 408.36 $(M+H)^+$. This value was consistent with the value calculated from the peptide sequence, 408.25. Mass peaks of 295.25 and 182.13 were simultaneously observed, which were consistent with the calculated mass numbers of Ile-Tyr, 295.17 $(M+H)^+$ and Tyr, 182.08 $(M+H)^+$, respectively. Moreover, the purified peptide was identical to the synthetic tripeptide on isocratic HPLC analysis. Consequently, the purified peptide was identified as the tripeptide, Leu-Ile-Tyr, and designated Acein-2.

Acein-2, like Acein-1, was found to originate from human plasma proteins by tryptic digestion, because no activity was observed prior to the digestion. Leu-Ile-Tyr was not a tryptic fragment because the C-terminal amino acid is Tyr, which could not be generated by tryptic digestion. The chymotryptic activity in the trypsin solution was examined, and was determined to be 2 BTEE U/mg protein. Thus, Leu-Ile-Tyr was a product of chymotryptic activity, which contaminated the commercial trypsin.

A protein database analysis was performed to find the original protein that was hydrolyzed by trypsin (or chymotrypsin) to release Acein-2. Numerous candidates that contained the Leu-Ile-Tyr sequence were found because Acein-2 is only a tripeptide. The most likely protein was human α2-macroglobulin (α2M), which contains two Leu-Ile-Tyr sequences. α2M is one of the major proteins in human plasma, with a concentration of 2–5 mg/ml [17]. α2M is known to be part of the body's defense system that acts as a molecular trap for proteinase molecules [18]. The amino acid sequence of Acein-2 was found to be identical to that of f(518–520) and f(1393–1395) in α2M. We suggest that Acein-2 was derived from

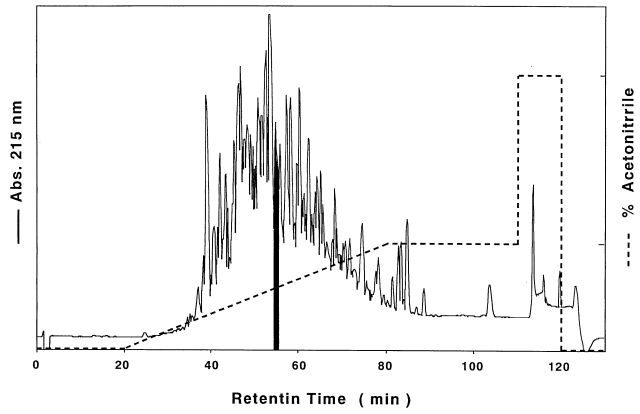

Fig. 2. Reversed-phase gradient HPLC chromatogram. Separation was performed at room temperature on a Develosil ODS-5 column (4.0 i.d.×150 mm). A linear acetonitrile gradient between 0.1% TFA in water and 80% acetonitrile with 0.1% TFA in water is shown as the dashed line. The flow rate was 1.0 ml/min, and the effluent was monitored continuously at 215 nm. The solid bars indicate the fractions with ACE inhibitory activity.

f(518–520) of α2M by chymotryptic cleavage because the preceding residue is Leu. The other fragment f(1393–1395) was probably not the source of Acein-2 because the preceding residue is Val. The other candidates in human plasma for the source of Acein-2 by either tryptic or chymotryptic digestion were human apolipoprotein B-100, human tumor necrosis factor α and the immunoglobulin λ and κ chain V-I regions. The amino acid sequence of Acein-2 was identical to that of f(4505–4507) in human apolipoprotein B-100, and to that of f(57–59) in human tumor necrosis factor α, and both sequences could be digested by chymotryptic activity because the preceding residues are Phe and Tyr, respectively. However, it seems unlikely that Acein-2 is derived from hydrolysates of these proteins because both proteins are minor components in human plasma. Although human immunoglobulin λ and κ chain V-I regions also contain Acein-2 sequences, these sequences occur in variable regions and thus are thought not to be common. As a result, Acein-2 was thought to originate from α2M.

Many bioactive peptides have been isolated from enzymatic hydrolysates of proteins [19–24]. In addition to Acein-1, we have isolated four novel inhibitory peptides from a thermolytic hydrolysate of human plasma. These peptides, designated Cabin-1, 2, 3 and 4, were found to be inhibitors of cathepsin B. Cabin-1 (Leu-Gly-Pro-Val-Thr-Gln-Glu) corresponds to f(64–70) of human apolipoprotein A-I, and Cabin-2 (Val-Leu-Gln-Ser-Ser-Gly-Leu-Tyr-Ser) and Cabin-3 (Val-Val-Ser-Val-Leu-Thr) correspond to f(56–64) and f(185–190) of the human immunoglobulin G γ-chain, while Cabin-4 (Leu-Val-Tyr-Asp-Ala-Tyr) corresponds to f(66–71) of human transferrin [12]. On the other hand, β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), an opioid peptide, was isolated from bovine casein by Brantl et al. [25]. Casoxin D (Tyr-Val-Pro-Phe-Pro-Pro-Phe), a bradykinin agonist, was isolated from a peptic and chymotryptic hydrolysate of human casein [26]. We believe that the human body might be the best resource for such bioactive peptides because such substances would make good starting materials for safer and less toxic medicines. Acein-2, as well as Acein-1 and the Cabin peptides might be good examples for demonstrating this idea.

The ACE inhibitory activity of synthetic Acein-2 was found to be dose-dependent with an IC_{50} value of 0.82 μmol/l (Fig. 3). This value was greater than the IC_{50} value of Acein-1 (16 μmol/l).

Miyoshi et al. reported that some tripeptides isolated from a hydrolysate of α-zein, a maize endosperm protein, act as ACE inhibitors [27]. These peptides, Leu-Arg-Pro, Leu-Ser-Pro, Leu-Gln-Pro and Leu-Ala-Tyr, had IC_{50} values of 0.27, 1.7, 1.9 and 3.9 μmol/l, respectively. They also found that Leu-Arg-Pro had a hypotensive activity, as a 30 mg/kg intravenous injection decreased the blood pressure of spontaneously hypertensive rats by 15 mm Hg. Matsumura et al. isolated four ACE inhibitory peptides from an autolysate of bonito bowels [28]. These peptides, Leu-Arg-Pro, Ile-Arg-Pro, Val-Arg-Pro and Ile-Lys-Pro, had IC_{50} values of 1.0, 1.8, 2.2 and 2.5 μmol/l, respectively. It should be noted that peptides derived from food proteins such as α-zein have been shown to have significant physiological effects and have been used in an attempt to prevent hypertension [20,27]. It is unknown whether Acein-2 has a hypotensive activity in human or rat. Further experiments are needed to answer this question.

To clarify the inhibition mechanism kinetically, Lineweaver–Burk plots were determined for Acein-2 [29]. These plots showed that it is a non-competitive inhibitor, with an intercept on the 1/[S] axis as shown in Fig. 4. These plots suggested that Acein-2 could not bind to the catalytic site of ACE and that it could not be hydrolyzed by ACE. Acein-1 was also a non-competitive inhibitor. Acein-2 might be able to enter the catalytic site, but it did not bind due to the non-competitive

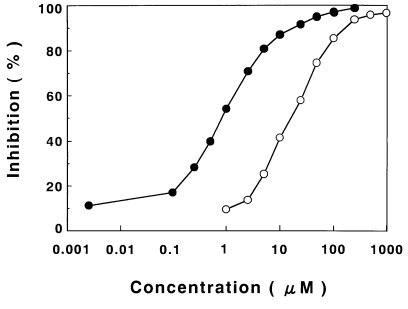

Fig. 3. Dose-dependent inhibition of ACE by Acein-2 (●) and Acein-1 (○). Each point represents the mean value of three experiments.

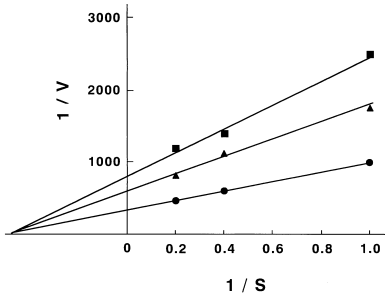

Fig. 4. Lineweaver–Burk plots of the inhibition of ACE by Acein-2. Each point represents the mean value of three experiments. ACE activities were measured in the absence (●) or in the presence of 0.5 μmol/l (▲) or 1.0 μmol/l (■) of Acein-2.

inhibition. These findings suggest that another explanation is needed for the kinetic mechanism of Acein-2.

The present results suggest that Acein-2, as well as Acein-1, could be used as a starting material for anti-hypertensive drugs against ACE. It is not yet known whether Acein-2 is released mainly from α2M by endogenous chymotryptic hydrolysis in the human body to inhibit ACE as part of the physiological regulation.

Acknowledgements: The authors would like to thank Dr. S. Maruyama, National Institute of Bioscience and Human-Technology, for helpful discussions. We also wish to thank Mr. J. Raymond for reading the manuscript and making a number of helpful comments.

References

[1] Anonymous (1989) In vitro screening of traditional medicines for anti-HIV activity: memorandum from a WHO meeting, Bull. WHO. 67.
[2] Ichikawa, K., Kinoshita, T. and Sankawa, U. (1989) Chem. Pharm. Bull. 37, 345–348.
[3] Wagner, H. (1988) Planta Med. 55, 235–241.
[4] Omura, S. (1992) General Screening of Enzyme Inhibitors, Springer-Verlag, New York.
[5] Umezawa, H. (1972) Enzyme Inhibitors of Microbial Origin, University of Tokyo Press, Tokyo.
[6] MacAllan, D., Sohal, J., Walker, C., Hill, D. and Moore, M. (1997) J. Recept. Signal Transduction Res. 17, 521–529.
[7] DesJarlais, R.L., Seibel, G.L., Kuntz, I.D., Furth, P.S., Alvarez, J.C., Ortiz de Montellano, P.R., DeCamp, D.L., Babe, L.M. and Craik, C.S. (1990) Proc. Natl. Acad. Sci. USA 87, 6644–6648.
[8] Nakagomi, K., Fujimura, A., Ebisu, H., Sakai, T., Sadakane, Y., Fujii, N. and Tanimura, T. (1998) FEBS Lett. 438, 255–257.
[9] Nakagomi, K., Fujimura, A., Maeda, H., Sadakane, Y., Fujii, N. and Tanimura, T. (1999) Biochem J. (submitted).
[10] Cheung, H.S., Wang, F.L., Ondetti, M.A., Sabo, E.F. and Cushman, D.W. (1980) J. Biol. Chem. 255, 401–407.
[11] Cushman, D.W., Cheug, H.S., Sabo, E.F. and Ondetti, M.A. (1977) Biochemistry 16, 5484–5491.
[12] Cushman, D.W. and Cheung, H.S. (1971) Biochem. Pharmacol. 20, 1637–1648.
[13] Maruyama, S. and Suzuki, H. (1982) Agric. Biol. Chem. 46, 1393–1394.
[14] Kato, H. and Suzuki, T. (1971) Biochemistry 10, 972–980.
[15] Akizawa, T., Osaki, M., Matsukawa, M., Hamai, M., Itoh, M., Seiki, M. and Yoshioka, M. (1996) Peptide Chem. 1995, 305–308.
[16] Rick, W. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.U., Ed.), 2nd edn., Vol. 1, pp. 1006–1012, Academic Press, New York.
[17] Barrett, A.J. (1981) in: Methods in Enzymology (Lorand, L., Ed.), Vol. 80, pp. 737–754, Academic Press, New York.
[18] Salvesen, G. and Enghild, J.J. (1993) in: Methods in Enzymology (Lorand, L. and Mann, K.G., Eds.), Vol. 223, pp. 121–141, Academic Press, New York.
[19] Yamamoto, N. (1997) Biopolymers 43, 129–134.
[20] Chiba, H. and Yoshikawa, M. (1991) Kagaku to Seibutsu 29, 454–458.
[21] Meisel, H. (1993) in: Food Proteins-Structure Functionality (Schwenke, K.D. and Mothes, R., Eds.), pp. 67–75, Weinheim, New York.
[22] Maruyama, S., Nakagomi, K., Tomizuka, N. and Suzuki, H. (1985) Agric. Biol. Chem. 49, 1405–1409.
[23] Mullally, M.M., Meisel, H. and FitzGerald, R.J. (1997) FEBS Lett. 402, 99–101.
[24] Mullally, M.M., Meisel, H. and FitzGerald, R.J. (1996) Biol. Chem. Hoppe-Seyler 377, 259–260.
[25] Brantl, V., Teschmacher, H., Henschen, A. and Lottspeich, F. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1211–1216.
[26] Kato, M., Fujiwara, Y., Okamoto, A., Yoshikawa, M., Chiba, H. and Udaka, S. (1995) Biosci. Biotech. Biochem. 59, 2056–2059.
[27] Miyoshi, S., Ishikawa, T., Kaneko, F., Tanaka, H. and Maruyama, S. (1991) Agric. Biol. Chem. 55, 1313–1318.
[28] Matsunuma, N., Fujii, M., Takeda, Y., Sugita, K. and Shimizu, T. (1993) Biosci. Biotech. Biochem. 57, 695–697.
[29] Michal, G. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.U., Ed.), 2nd edn., Vol. 1, pp. 144–156, Academic Press, New York.