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Amino acid sequence and thermostability of xylanase A from *Schizophyllum commune*

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The amino acid sequence (197 residues) of xylanase A from the fungus, *Schizophyllum commune*, was determined by automated analysis of peptides from proteolytic and acid cleavage. The sequence is similar to two *Trichoderma* xylanases (approximately 56% identical amino acids), but also shows at least 40% identities with xylanases from *Bacillus subtilis*, *B. pumilus* and *B. circulans*. The conserved regions of the enzyme contain only two glutamic acid residues which implicates their possible involvement in catalysis. The disulfide bond in xylanase A is not conserved in this family. In spite of this, the *B. subtilis* xylanase was found to be more thermostable than xylanase A.

Xylanase; Basidiomycete; Homology; *Bacillus*

1. INTRODUCTION

Xylanase-aided bleaching of kraft pulps is currently being practised in several mills in Canada and Scandinavia (for a review see [1]). The enzyme reacts with acidified or neutralised unbleached pulp (brownstock) and allows subsequent savings in bleaching chemicals required to achieve a target brightness [2,3]. The commercially available xylanases differ slightly in their pH and thermal stabilities. Ideally the enzymes should remain stable at temperatures commonly encountered during brownstock storage. With current efforts to conserve water, the brownstock storage temperature is likely to increase beyond the present average of around 50°C. One approach to increased stability is to modify the enzyme structure by protein engineering. In order to understand more fully the structural features which contribute to enzyme stability, and thereby to allow a rational approach to protein engineering, we have determined the structure of a fungal xylanase and compared its thermostability to that of a bacterial xylanase.

The wood-degrading Basidiomycete, *Schizophyllum commune*, produces extracellular xylanases (EC 3.2.1.8) [4–6], which, together with acetyl xylan esterase [7], ferulic acid esterase [8], and xylosidase [9], are capable of complete hydrolysis of xylan, the predominant hemicellulose in deciduous trees and annual plants. One strain of *S. commune* (ATCC 38548) yields 200 U/ml of xylanase when cultured on a wood-meal medium [10], a relatively high productivity which may be of industrial interest. The major xylanase (xylanase A) has been isolated and partially characterized previously [6]. Xylanase A hydrolyses larchwood xylan to xylooligsaccharides, with xylobiose and xylose accumulating as final products. The enzyme active site contains carboxyl groups which are essential for catalytic action [11]. Earlier studies with partial N-terminal amino acid sequence of xylanase A have indicated some similarities to the corresponding sequence of xylanases from *Bacillus subtilis* [12] and *B. pumilus* [13]. We describe here the total amino acid sequence of the enzyme and its striking similarity with xylanases from prokaryotes. We conclude that the unique presence of a disulfide bond in xylanase A does not confer increased thermostability relative to *B. subtilis* xylanase.

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2. MATERIALS AND METHODS

Schizophyllum commune (Delmar) (ATCC 38548) was maintained on malt agar broth. Xylanase A was produced in 10 l liquid spruce wood meal cultures as described previously [10]. Nine-day cultures were centrifuged (7,200 × *g*) and then concentrated (5 ×) with a Millipore ultrafiltration unit (10,000 M_r cut-off membrane). The concentrate was purified by elution from DEAE-Biogel A as described previously [3], followed by further fractionation on Sephacryl S-200 and Mono-Q columns. The *B. subtilis* xylanase was purified from cultures of *Escherichia coli* expressing the gene, as described previously [3].

Xylanase A was reduced and carboxymethylated in 6 M guanidine hydrochloride, 0.05 M Tris-HCl buffer, pH 8.25, by addition of dithiothreitol under nitrogen, followed after 1 h by [^{14}C]iodoacetic acid. Protein was recovered on a Biogel P-6 column. In the absence of guanidinium hydrochloride, no reduction occurred.

The sequence of xylanase A was determined by automated sequence analysis of peptides obtained by digestion of the reduced, alkylated

protein with lysyl- and arginyl-endopeptidases, staphylococcal V8 protease, and hot acetic acid. The large peptides were isolated with TSK SW columns (7.5 × 600 mm × 4) in 6 M guanidine hydrochloride and the smaller peptides were further fractionated with reverse-phase HPLC columns.

Automated gas- and liquid-phase sequence analysis were performed as described previously [14]. Amino acid composition analysis was performed on a Dionex D-500 or Applied Biosystems 420H analyzers. A SCIEX quadrupole mass spectrometer API III with an ion spray interface and a mass range of 0–2400 amu/e [15] was used to analyze a 5% acetic acid solution of the enzyme (0.1–0.2 mg/ml).

Alignments of amino acid sequences and evolutionary relationships of xylanases were determined with Geneworks version 2.2.1. software by Intelligenetics Inc., Mountain View, CA.

3. RESULTS AND DISCUSSION

The sequencing strategy and complete amino acid sequence of xylanase A is shown in Fig. 1. The molecular weight deduced from the sequence was identical (within one unit) to that determined by electrospray mass spectrometry. The cysteine residues (positions 111 and 160) were found to be linked by peptide mapping of proteolytic digests before and after reduction. This is in agreement with earlier experiments [11] which suggested that no free thiols exist in xylanase A.

Gilkes et al. [16] have classified β-1,4-glycanases (mainly cellulases and xylanases) into ten families. Family G is composed of bacterial and fungal xylanases (Fig. 2). An alignment of *S. commune* xylanase A with these sequences, and with *Streptomyces* and *Trichoderma* xylanase sequences determined since the Gilkes classification, indicates extended regions of conserved sequence (Fig. 2). The *S. commune* xylanase is most similar to *Trichoderma harzianum*, *T. reesei*, and *T. viride* xylanases (Fig. 3). The *Bacillus* xylanases are more distantly related and have a lower turnover number on larchwood xylan [27]. Torronen et al. [28] have

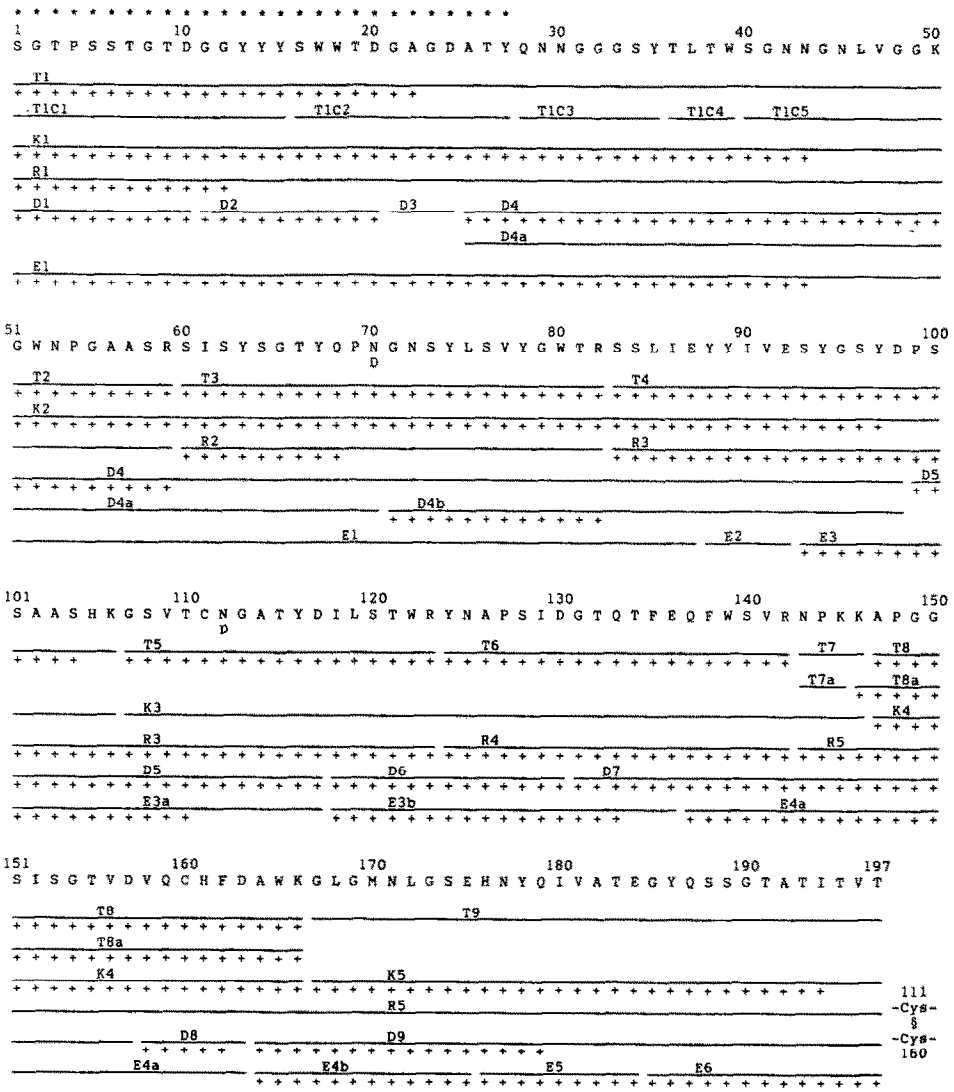

Fig. 1. Amino acid sequence of xylanase A from *S. commune*. The peptides shown were obtained by treatment with trypsin (T), chymotrypsin (C), lys/C enzyme (K) Arg/C enzyme (R), Glu/C enzyme (E), or mild acid hydrolysis (D). + indicates residues identified directly by phenylthiohydantoin amino acid derivatives obtained by automated Edman degradation. The molecular weight calculated from the amino acid sequence was 20,978 Da, and the average mass determined by mass spectrometric analysis was 20,977.

shown a correlation between sequence similarity and pI in the family G xylanases. However, this correlation breaks down with *S. commune* xylanase (pI 4.5 [10]), which is grouped with the alkaline pI *Trichoderma* enzymes.

Of the sequences shown in Fig. 2, only *S. commune* xylanase has a proven disulfide bridge. Generally, such bridges stabilize the protein, due to decreased conformational entropy in the unfolded state [29]. However, when *S. commune* xylanase was compared to the rather similar *B. subtilis* xylanase, the thermal stability was lower (Fig. 4). Evidently other factors, such as electrostatic and hydrophobic interactions [30], play a more important role in establishing thermal stability in the *B. subtilis* xylanase. Reduction and carboxymethylation of denatured xylanase A gave a protein that could not be renatured, as observed in conformational studies of a thermostable xylanase from the fungus, *Humicola lanuginosa* [31]. Other thermostable xylanases, from *Caldocellum saccharolyticum* [32] and *Clostridium thermocellum* [33], are larger enzymes than xylanase A, and are both found in family F of Gilkes classification [16], i.e. they are structurally unrelated to *S. commune* xylanase A.

Carboxylic amino acids are essential for catalytic action of xylanase A [11]. Only two glutamic acids, Glu-87 and Glu-184, are conserved in the sequence alignments shown in Fig. 2. They align with glutamic acid residues which eliminate or reduce enzyme activity when replaced by site-specific mutagenesis in *Bacillus* sequences [27,34]. From previous reports on the functionally similar cellulase enzymes, it appears that either Asp [35] or Glu [36] can serve as catalytic-site residues.

The three-dimensional structures of two bacterial xylanases from *B. pumilus* [34,37] and *B. circulans* (R. Campbell, unpublished data), and one fungal xylanase from *Trichoderma harzianum* (R. Campbell and D.C. Rose, unpublished data) have been determined so far. These xylanases have very similar structures which consist of a single domain containing three β-sheets and one

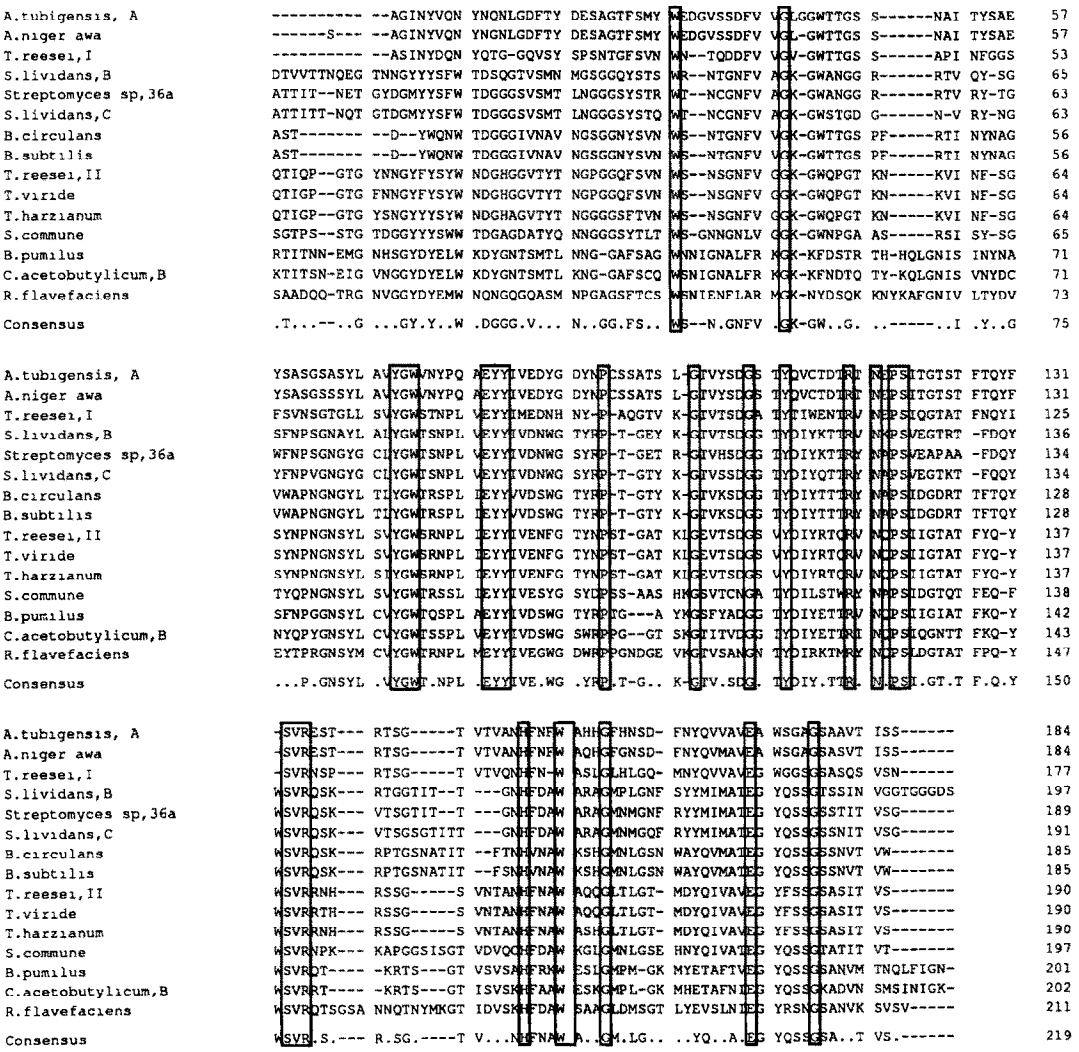

Fig. 2. Alignment of *S. commune* xylanase A with other xylanases from family G. Sequences obtained from *Aspergillus niger* [17], *Aspergillus tubigensis* [18], *Trichoderma reesei* [19], *Streptomyces* sp 36 A [20], *Streptomyces lividans* [21], *Bacillus circulans* [22], *Bacillus subtilis* [12], *Trichoderma viride* [23], *Trichoderma harzianum* [21], *Bacillus pumilus* [13], *Clostridium acetobutylicum* [25], *Ruminococans flavefaciens* [26].

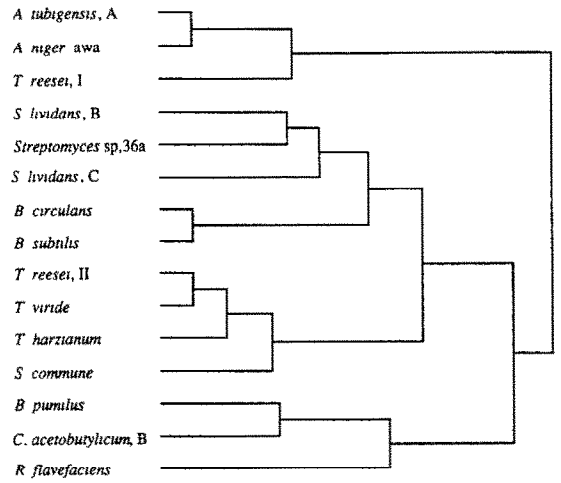

Fig. 3. Possible evolutionary relationships among xylanases from family G, based on the multiple alignments of amino acid sequences. The extended N-terminal sequence of *C. acetobutylicum* xylanase B (residues 1–31) and the C-terminal sequence of *S. lividans* xylanase B (residues 198–293) were omitted.

α-helix. The β-sheets are mostly composed of anti-parallel strands. The active site cleft lies in a deep groove between β-sheets II and III. Two completely conserved glutamic acid residues, which corresponds to Glu-87 and Glu-184 of *S. commune* xylanase A, are found in the active site of the three xylanases. The distance between the two carboxyl groups is about 7 Å [37]. Conversely, none of aspartic acid residues of the xylanases of this family are completely conserved (Fig. 2), and it appears that they are not essential for activity [27,34].

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REFERENCES

[1] Reid, I.D. and Paice, M.G. (1992) in: Industrial Mycology (Leatham, G.F. ed.) pp. 112–126, Chapman-Hall.
[2] Viikari, L., Ranua, M., Kantelinen, A., Sundquist, J. and Linko, M. (1986) Proceedings Third Int. Conf. Biotechnol. Pulp Paper Industry, Stockholm, pp. 67–69.
[3] Paice, M.G., Gurnagul, N., Page, D.H. and Jurasek, L. (1992) Enzyme Microb. Technol. 14, 272–276.
[4] Schmidt, O. and Liese, W., (1980) Holzforschung 34, 67–72.
[5] Varadi, J., Necesany, V. and Kovacs, P. (1971) Drev. Vyskum 16, 147–158.
[6] Paice, M.G., Jurasek, L., Carpenter, M.R. and Smillie, L.B. (1978) Appl. Environ. Microbiol. 36, 802–808.
[7] Biely, P., MacKenzie, C.R. and Schneider, H., (1988) Can. J. Microbiol. 34, 767–772.
[8] Mackenzie, C.R. and Bilous, D., (1988) Appl. Environ. Microbiol. 54, 1170–1173.
[9] Poutanen, K. and Puls, J. (1988) Appl. Microbiol. Biotechnol. 28, 425–432.
[10] Jurasek, L. and Paice, M.G. (1988) Methods Enzymol. 160, 659–662.
[11] Bray, M.R. and Clarke, A.J. (1990) Biochem. J. 270, 91–96.
[12] Paice, M.G., Bourbonnais, R., Desrochers, M., Jurasek, L. and Yaguchi, M. (1986) Arch. Microbiol. 144, 201–206.
[13] Fukusaki, E., Panbangred, W., Shinmyo, A. and Okada, H. (1984) FEBS Lett. 171, 197–201.
[14] Watson, D.C., Yaguchi, M. and Lynn, R. (1990) Biochem. J. 266, 75–81.
[15] Feng, R., Konishi, Y. and Bell, A.W. (1991) J. Am. Soc. Mass Spectrum 2, 387–401.
[16] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. (1991) Microbiol. Rev. 55, 303–315.
[17] Maat, J., Roza, M., Verbakel, J., Stam, H., Santos da Silva, M.J., Egmond, M.R., Hagemans, M.L.D., Gorcom, R.F.M., Hessign, J.G.M., Hondel, C.A.M.J.J. and Rotterdam, C. (1992) in: Xylans and Xylanases (J. Visser et al., eds.) pp. 349–360, Elsevier, Amsterdam.
[18] de Graaf, L.H., van den Broeck, H.C., van Ooijen, A.J.J. and Visser, J. (1992) in: Xylans and Xylanases (J. Visser et al., eds.) pp. 235–246, Elsevier, Amsterdam.
[19] Torronene, A., Mach, R.L., Messner, R., Gonzalez, R., Kalkkinen, N., Harkki, A. and Kubicek, C.P. (1992). Biotechnology 10, 1461–1465.
[20] Nagashima, M., Okumoto, Y. and Okanishi, M. (1989) Trends Actinomycetologia 91–96.

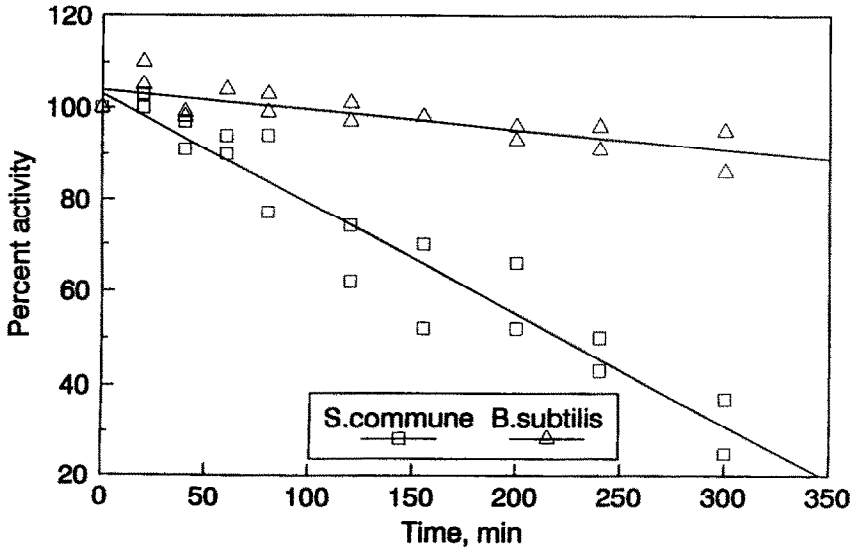

Fig. 4. Stabilities of *S. commune* and *B. subtilis* xylanase enzymes at 50°C.

[21] Shareck, F., Roy, C., Yaguchi, M., Morosoli, R. and Kluepfel, D. (1991) Gene 107, 75–82.
[22] Yang, R.C.A., Mackenzie, C.R. and Narang, R.A. (1988) Nucleic Acids Res. 16, 7187.
[23] Yaguchi, M., Roy, C., Ujie, M., Watson, D.C. and Wakarchuck, W. (1992) in: Xylans and Xylanases. (J. Visser et al., eds.) pp. 149–154, Elsevier, Amsterdam.
[24] Yaguchi, M., Roy, C., Watson, D.C., Rollin, F., Tan, L.U.L., Senior, D.J. and Saddler, J.N. (1992) in: Xylans and Xylanases (J. Visser et al., eds.) pp. 435–438, Elsevier, Amsterdam.
[25] Zappe, H., Jones, W.A. and Woods, D.R. (1990) Nucleic Acids Res. 18, 2179.
[26] Zhang, J. and Flint, H.J. (1992) Mol. Microbiol. 6, 1013–1024.
[27] Wakarchuk, W., Methot, N., Lanthier, P., Sung, W., Seligy, V., Yaguchi, M., To, R., Campbell, R. and Rose, D. (1992) in: Xylans and Xylanases (J. Visser et al., eds.) pp. 439–442, Elsevier, Amsterdam.
[28] Torronen, A., Kubicek, C.P. and Henrissat, B. (1993). FEBS Lett. 321, 135–139.
[29] Creighton, T.E. (1988) Bioessays 8, 57–63.
[30] Mrabet, M.J. Van den Broeck, A., Van den Brande, I., Stanssesns, P., Laroche, Y., Lambeir, A.M., Matthijsseno, G., Jenkino, J., Chiadmi, M., van Thilbeurgh, H., Rey, F., Janin, J., Quax, J., Larsters, I., de Maeyer, M. and Wodak, S.J. (1992) Biochemistry 31, 2239–2253.
[31] Tatu, U., Murthy, S.K. and Vithayathil, P.J. (1992) J. Protein Chem. 9, 641–649.
[32] Luthi, E., Love, D.R., McAnulty, J., Wallace, C., Caughey, P.A., Saul, D. and Bergquist, P.L. (1990) Appl. Environ. Microbiol. 56, 1017–1024.
[33] Grepinet, O., Chebrou, M.C. and Beguin, P. (1988) J. Bacteriol. 170, 4582–4588.
[34] Ko, E.P., Akatsuka, H., Moriyama, H., Shinmyo, A., Hata, Y., Katsube, Y., Uraba, J. and Okada, H. (1992) Biochem. J. 228, 117–121.
[35] Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C. and Jones, T.A. (1990) Science 249, 380–386.
[36] Mitsuishi, Y., Nitisinprasert, S., Saloheimo, M., Biese, I., Reinikainen, T., Claeyssens, M., Keranen, S., Knowles, J.K.C. and Teeri, T.-T. (1990) FEBS Lett. 275, 135–138.
[37] Katsube, Y., Hata, Y., Yamaguchi, H., Mariyama, H., Shinmyo, A. and Okada, H. (1990) in: Protein Engineering: Protein Design in Basic Research, Medicine and Industry (M. Ikehara, ed.) pp. 91–96, Japan Scientific Societies Press.