Biochem. J. (1992) 288, 117–121 (Printed in Great Britain)



# Site-directed mutagenesis at aspartate and glutamate residues of xylanase from *Bacillus pumilus*

Elizabeth P. KO,* Hiroyuki AKATSUKA,* Hideaki MORIYAMA,* Atsuhiko SHINMYO,*‡ Yasuo HATA,† Yukiteru KATSUBE,† Itaru URABE* and Hirosuke OKADA*

*Department of Biotechnology, Faculty of Engineering, Osaka University, and †Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan

To elucidate the reaction mechanism of xylanase, the identification of amino acids essential for its catalysis is of importance. Studies have indicated the possibility that the reaction mechanism of xylanase is similar to that of hen's egg lysozyme, which involves acidic amino acid residues. On the basis of this assumption, together with the three-dimensional structure of *Bacillus pumilus* xylanase and its amino acid sequence similarity to other xylanases of different origins, three acidic amino acids, namely Asp-21, Glu-93 and Glu-182, were selected for site-directed mutagenesis. The Asp residue was altered to either Ser or Glu, and the Glu residues to Ser or Asp. The purified mutant xylanases D21E, D21S, E93D, E93S, E182D and E182S showed single protein bands of about 26 kDa on SDS/PAGE. C.d. spectra of these mutant enzymes show no effect on the secondary structure of xylanase, except that of D21E, which shows a little variation. Furthermore, mutations of Glu-93 and Glu-182 resulted in a drastic decrease in the specific activity of xylanase as compared with mutation of Asp-21. On the basis of these results we propose that Glu-93 and Glu-182 are the best candidates for the essential catalytic residues of xylanase.

## INTRODUCTION

Xylanase (EC 3.2.1.8) is a potentially important enzyme for the use of xylan in agricultural wastes such as rice straw, corn cobs, and hardwood. We have cloned genes for xylan-degrading enzymes, xylanase and xylosidase (EC 3.2.1.37), from *Bacillus pumilus* IPO [1]. The xylanase gene (*xynA*) encodes a pre-xylanase consisting of a signal peptide of 27 amino acid residues at the *N*-terminus and a mature enzyme of 201 amino acid residues [2]. The mature form of xylanase was crystallized [3] and analysed by X-ray crystallography at 0.22 nm (2.2 Å) resolution (Y. Hata, H. Moriyama, A. Shinmyo, H. Okada and Y. Katsube, unpublished work).

To elucidate the reaction mechanism of xylanase, it is important to identify amino acid residues essential for its catalysis. Morosoli *et al.* [4] showed that there is a strong amino-acid-sequence similarity between a certain region of xylanase and the catalytic site of the hen's-egg lysozyme. Recently, Bray & Clarke [5] have reported evidence, from chemical modification, for the involvement of carboxy groups in the catalytic mechanism of xylanase. These studies supported the possibility that the reaction mechanism of xylanase is similar to that of lysozyme. Therefore, on the basis of sequence similarity, three-dimensional structure and site-directed mutagenesis, we identified such essential carboxy groups of xylanase. The results described here show that Glu-93 and Glu-182 are the best candidates for the essential catalytic residues of xylanase.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

*B. pumilus* IPO [6], a donor strain of *xynA*, was used to prepare mature xylanase. *Escherichia coli* KP3998[F$^-$, *hsdS20*($r_B^-m_B^-$) *ara-14 proA2 lacI$^q$ galK2 rpsL29 xyl-5 mtl-1 supE44*, $\lambda^-$] [7] and pKP 1500 [7] were generously given by Dr. T. Miki (Kyushu University). pOXN392R [2] is a hybrid plasmid containing *xynA*.

### Modification and subcloning of *xynA*

The following procedures are illustrated in Fig. 1. pOXN392R was partially digested with *Eco*RI, and a 1.5 kbp fragment containing *xynA* was recovered. Both ends of the fragment were blunt-ended with Klenow fragment before it was joined to the *Pst*I linker and digested with *Pst*I and *Hae*III. Plasmid pUC19 [8], on the other hand, was modified by destroying its *Eco*RI and *Hin*dIII sites with the use of restriction enzymes and Klenow fragment. The *Pst*I–*Hae*III fragment obtained as described above was then ligated to the modified pUC19 previously digested with *Pst*I and *Hin*cII, resulting in the plasmid, pUXN1, having unique *Eco*RI and *Hin*dIII sites (Fig. 1*a*). To enhance the expression of xylanase in *E. coli*, a hybrid plasmid, pHIX312, was constructed as follows. The plasmid pUXN1 was digested with *Bst*EII and *Bam*HI, and the *Bst*EII–*Bam*HI fragment containing the main part of *xynA* was recovered. This DNA fragment, together with a synthetic linker (see below), and a modified pKP1500 (without its original *Hin*dIII site) that had been digested with *Eco*RI and *Bam*HI, were ligated to form a hybrid plasmid, pHIX312 (Fig. 1*b*). The synthetic linker is composed of 5′-AATTATGAGAACCATTACGAATAA-TGAAATGG-3 and 3′-TACTCTTGGTAATGCTTATTA-CTTTACCCATTG-5′, which were annealed before use. Plasmid pHIX312 thus prepared contains a modified *xynA* gene in which the signal sequence region of *xynA* was replaced by the initiation codon ATG.

### Site-directed mutagenesis

In mutating Asp-21, Glu-93 and Glu-182, the *Hin*cII–*Eco*RI, *Eco*RI–*Hin*dIII and *Hin*dIII-*Xba*I fragments of pHIX312 respectively were first cloned into M13 RFI to serve as template for mutagenesis brought about by an oligonucleotide-directed '*in vitro*' mutagenesis system (version 2; Amersham Japan, Tokyo, Japan). The mutated DNA fragments were then used to replace

‡ To whom correspondence should be addressed.

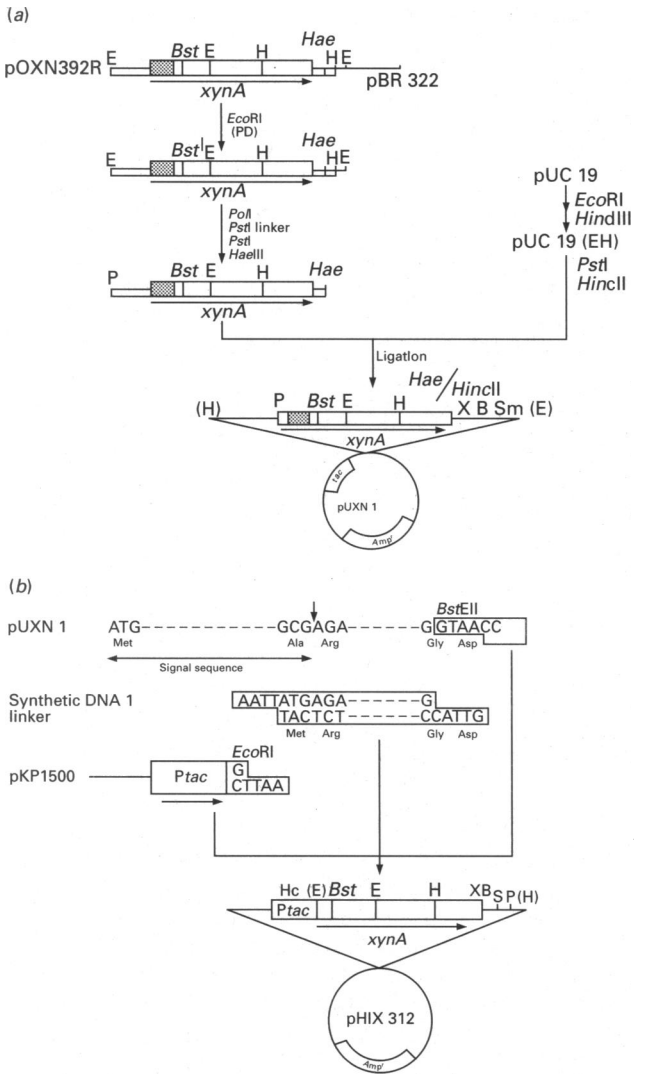


**Fig. 1. Construction of a xylanase high-expression vector (pHIX312)**

Restriction sites are abbreviated as follows: B, *Bam*HI; Bst, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; Hc *Hin*cII; S, *Sal*I; Sm, *Sma*I; P, *Pst*I; and X, *Xba*I. Other abbreviations: PD, partial digestion; (P)*tac*, *tac* promoter; Amp$^r$, ampicillin-resistance-coding region.

their respective original fragments in pHIX312. Mutation of each codon was confirmed by sequencing the mutant fragment by a Toyobo Sequence kit (Toyobo Co., Osaka, Japan).

### Purification of xylanases

*E. coli* KP3998 (pHIX312) cells were grown at 37 °C in Luria–Bertani medium [9] (4 litres) containing ampicillin (50 $\mu$g/ml). After 12–15 h of cultivation, the expression of *xynA* gene in the cells was induced by the addition of isopropyl thiogalactoside (final concn. 0.1 mM) and the culture was further cultivated for 3 h. Cells were harvested and resuspended in 50 mM-potassium phosphate buffer, pH 7.0. DNAase I (Boehringer-Mannheim; 3 units/ml) and RNAase A (Sigma; 0.1 unit/ml) were added to the cell suspension before disrupting the cells with a French pressure cell (Ohtake, Tokyo, Japan). The French-pressed sample was centrifuged (1000 *g*), and the xylanase was purified from the supernatant so obtained by stepwise column chromatography on DEAE-Sephadex, CM-Sephadex and Toyopearl HW60F as described previously [10]. The enzyme from the modified *xynA* was termed 'M-wild xylanase'. Purification of the mutant enzymes was carried out in the same manner as that of the M-wild xylanase. In the case of mutant enzymes, active fractions after column chromatography were detected by Ouchterlony's immuno-double-diffusion test [11], using rabbit antiserum against *B. pumilus* xylanase [1]. The homogeneity of the purified enzymes was checked by SDS/PAGE [12]. The standard marker proteins used were: $\alpha$-lactalbumin (molecular mass 14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase *b* (94 kDa).

### Enzyme assay

Xylanase activity was measured as described previously [10], except that oat spelt xylan (Sigma) was used as a substrate. The rate of the enzyme reaction is expressed as the rate of increase in the amount of reducing sugars equivalent to xylose. The amount of reducing sugars was measured by the Somogyi–Nelson method [13]. Qualitative screening of xylanase activity was done by a slight modification of the Congo Red plate assay [14]. Bacterial colonies were grown on Luria–Bertani medium containing 0.5% xylan in 50 mM-potassium phosphate buffer, pH 6.5, and ampicillin (50 $\mu$g/ml) for 24 h. The colonies were detached from the plates with filter paper before flooding the plates with an aqueous solution of Congo Red (1 mg/ml) for 5 min. The Congo Red solution was then poured off and the plates were washed with 1 M-NaCl for about 15 min until clear zones were revealed. Protein concentrations of xylanase were measured at 280 nm by assuming a molar absorption coefficient of 56 $mM^{-1} \cdot cm^{-1}$. The molar absorption coefficient of xylanase was calculated on the basis of tyrosine and tryptophan content, the molar absorption coefficient at 280 nm for these amino acids being 1260 and 5690 $M^{-1} \cdot cm^{-1}$ respectively.

### C.d. measurements

The c.d. spectra were recorded with a Jasco J-40A automatic recording spectrophotometer equipped with a data processor for c.d. (model DP-501). Each memory unit in the computer stored the c.d. signal for a spectral band of 0.1 nm. Spectra were scanned 32 times at a scan rate of 50 nm/min, using a 0.25 s time constant. The spectra were measured at protein concentrations of 0.8–1.6 mM in 50 mM-potassium phosphate buffer, pH 6.5, by using a cell of 10 mm pathlength at 25 °C.

## RESULTS AND DISCUSSION

### Candidate amino acid residues for site-directed mutagenesis

*B. pumilus* xylanase contains six aspartate and seven glutamate residues, which, except for Glu-165, are located on the $\alpha$-carbon backbone of this enzyme (Fig. 2). On the basis of the three-dimensional structure analysed at 0.22 nm (2.2 Å) resolution (Y. Hata, H. Moriyama, A. Shinmyo, H. Okada and Y. Katsube, unpublished work), this enzyme molecule consists of two structural parts with a cleft region about 3 nm (30 Å) long and 1.5 nm (15 Å) in diameter. The size of the cleft is large enough to accommodate a xylan fibre of about 1.1 nm (11 Å) in diameter [15]. Therefore the candidates for the catalytic residues of this enzyme were narrowed down by presuming that the acidic amino acid residues within the vicinity of the cleft region are most likely to be the best candidates, namely Asp-15, Asp-21, Glu-17, Glu-93, Glu-176 and Glu-182.

Catalytically and structurally important amino acid residues of an enzyme are generally thought to be conserved during the course of evolution. As shown in Fig. 3, the amino acid sequences

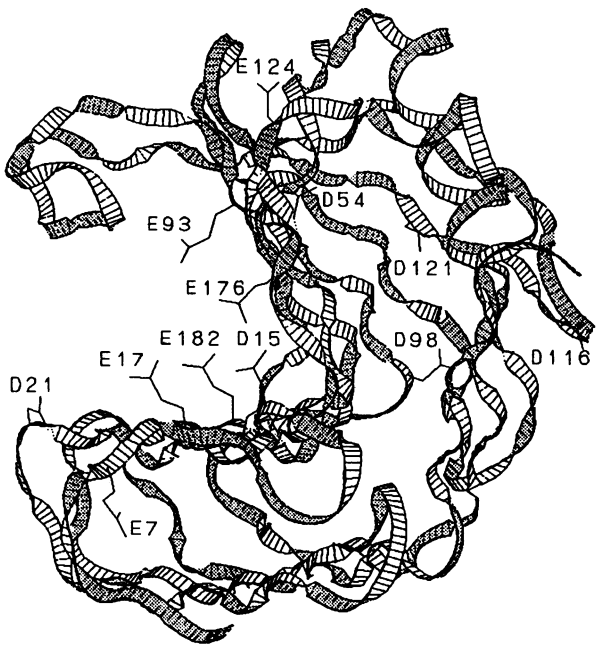


**Fig. 2. Computer graphics of the structure of the *B. pumilus* xylanase molecule**

The protuberance of the side chains of the acidic amino acid residues towards the cleft region of the xylanase is also shown.

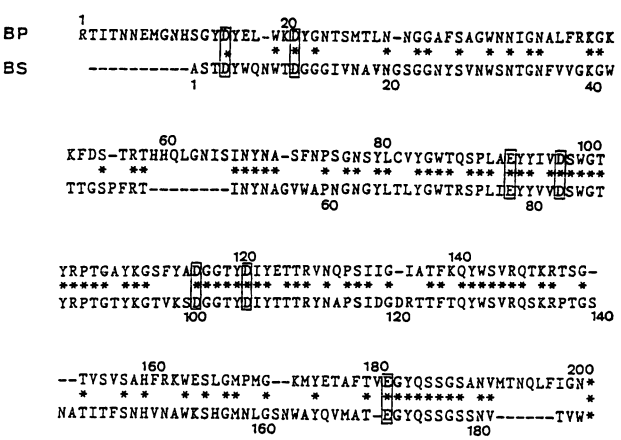


**Fig. 3. Sequence similarity between *B. pumilus* (BP) and *B. subtilus* (BS) xylanases**

Conserved amino acids are shown by asterisks, and conserved aspartate and glutamate residues are boxed.

(mature forms) of *B. pumilus* xylanase [2] and *B. subtilis* xylanase [16] show significant (50%) similarity, suggesting that both enzymes may have evolved from a common ancestral xylanase. Among the deduced candidates for catalytic residues mentioned above, only Asp-15, Asp-21, Glu-93 and Glu-182 are found to be conserved in both sequences (Fig. 3). Furthermore, it was found that all these residues except Asp-15 are also conserved in counterpart xylanases from *Trichoderma hartianum* and *Schizophillum commune* (T. Oku, personal communication). Accordingly, acidic amino acid residues Asp-21, Glu-93 and Glu-183 were the best prospects for site-directed mutagenesis.

As illustrated in Fig. 1, the *xynA* gene was subcloned into a high-expression vector (pKP1500). Beforehand the signal-

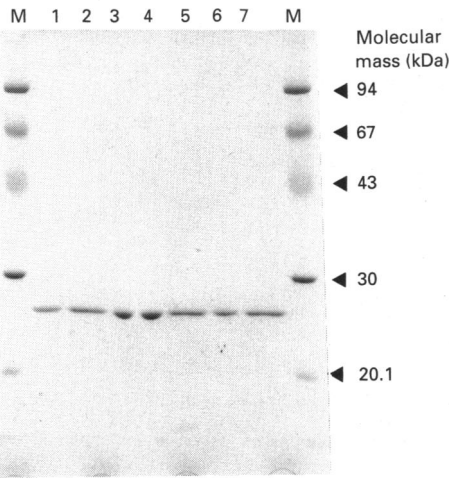


**Fig. 4. SDS/PAGE of mutant xylanases**

Lane 1, E182D; lane 2, E182S; lane 3, E93D; lane 4, E93S; lane 5, D21E; lane 6, D21S; lane 7, M-wild xylanase; lane M, marker proteins.

sequence region of the *xynA* gene from *B. pumilus* was first replaced with initiation codon to avoid heterogeneity of the *N*-terminus of xylanase produced by *E. coli* cells. In addition, the hybrid plasma, pHIX312, was designed to have a unique *Eco*RI and *Hin*dIII sites in the xylanase gene (234 bp and 498 bp from the initiation codon respectively). This construction facilitates the handling of the 5′, central and 3′ regions of the structural gene as a cassette mode for site-directed mutagenesis.

The xylanase encoded by this modified *xynA* was termed 'M-wild xylanase'. The *N*-terminal sequence of the M-wild xylanase, purified as described in the Materials and methods section, was confirmed by sequential Edman degradation (Applied Biosystems model 470A instrument) to have a methionine residue before the mature sequence. The specific activity of the M-wild enzyme is the same as that of the wild-type enzyme produced by *B. pumilus*, indicating that the presence of the extra methionine residue does not affect the catalytic activity of this enzyme.

### Site-directed mutagenesis

Alterations of Asp-21, Glu-93 and Glu-182 were carried out by site-directed mutagenesis in which the maintenance of the net charge or hydrophilicity of the protein molecule was taken into consideration. The alterations are as follows: Asp (GAT) → Glu (GAA) or Ser (TCT), Glu (GAA) → Asp (GAT) or Ser (TCA); the mutant proteins thus purified from the transformants harbouring the hybrid plasmid containing the mutated genes are designated as D21E, D21S, E93D, E93S, E182D and E182S. Xylanase activity of the transformants was screened qualitatively by colony assay and by the Somogyi-Nelson [13] reaction test of the crude enzyme extracts as described in the Materials and methods section. The results obtained after subjecting *E. coli* (pHIX312) to the said qualitative tests served as the positive control. Haloes (clearing zones) of uniform sizes were produced by transformants of D21E and D21S, whereas none were observed with that of E93D and E93S. Moreover, the haloes produced by the transformants of E182D and E182S are of different sizes. The reason for this heterogeneity is yet unclear, though a smaller phenomenon was reported for a site-directed mutant of lysozyme [17]. This unusual phenomenon requires further investigation. In the case of the qualitative Somogyi–Nelson test, resulting colours of the reaction mixture containing the mutant enzymes ranged from faint blue to greenish, in

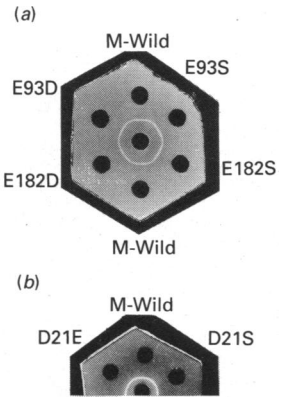


**Fig. 5. Double-immunodiffusion test**

Antiserum against *B. pumilus* xylanase was placed in the centre well.

**Table 1. Estimated secondary structures of mutant xylanases**

| Xylanase | Distribution of secondary structure (%) | | |
|---|---|---|---|
| | α-Helix | β-Sheet | β-Turn |
| M-wild | 0 | 0.58 | 0.21 |
| D21E | 0.07 | 0.40 | 0.22 |
| D21S | 0 | 0.51 | 0.18 |
| E93D | 0 | 0.56 | 0.24 |
| E93S | 0 | 0.51 | 0.12 |
| E182D | 0 | 0.48 | 0.22 |
| E182S | 0 | 0.61 | 0.16 |

contrast with that containing the M-wild xylanase, which is of a dark-blue colour.

After selecting the transformants from the initial screening, DNA sequencing of the entire structural gene harboured in these transformants was performed to ensure that no other simultaneous mutation aside from the targeted site had occurred during the manipulation of the gene. For E182D and E182S, colonies showing a negligible halo were selected. The mutant xylanases were purified as described in the Materials and methods section, and the homogeneity of the mutant enzymes checked by SDS/PAGE (Fig. 4). These results show that all the enzymes migrate as a single protein band with the same molecular size (about 26 kDa). Furthermore, these enzymes show a fused single precipitin line with rabbit antiserum against *B. pumilus* xylanase by the double-immunodiffusion test (Fig. 5), thereby confirming the identity of the mutant enzymes.

### C.d. spectra of the enzymes

Since the conformation of a protein necessary for its biological function is determined by its amino acid sequence [18], the effects of the mutations mentioned above on the secondary structure of xylanase were also investigated by c.d. As Fig. 6 shows, the c.d. spectra of the M-wild xylanase and the mutant enzymes in the region 210–260 nm at pH 6.5 and 25 °C display profiles and well-resolved troughs at 220 nm, except that of D21E, which is at 223 nm. In addition, although the depth of the trough at 220 nm of the mutant enzymes varies from that of the M-wild xylanase, the shape of their spectra are similar, except that of D21E. The composition of the secondary structures of the mutant proteins were estimated on the basis of the method described by Provencher & Glockner [19] with a set of 15 reference proteins [20], and the results are shown in Table 1. The values for the M-wild enzyme coincide well with those obtained from the three-dimensional structure of the wild-type enzyme (Y. Hata, H. Moriyama, A. Shinmyo, H. Okada and Y. Katsube, unpublished work). The contents of α-helix, β-turns and β-sheets of the mutant xylanases do not vary much from that of the M-wild xylanase, except in the case of D21E, where a very slight increase in the α-helix content was obtained. It is noteworthy that the depth of the trough at 220 nm is sensitive to these mutations without changing the β-sheet content. This may be due to the very low α-helix content of this enzyme, because the trough due to β-sheets is much smaller than that due to α-helices.

### Activity of the mutant xylanases

The specific activities and kinetic constants of the M-wild and the mutant xylanases are listed in Table 2. These results show that the mutations of Glu-93 to Asp or Ser and Glu-182 to Asp or Ser lead to very significant decrease in the activity of xylanase, especially with the activities of E93S, E182D and E182S, which fall below the limit of detection. These results indicate that alterations in the amino acid residues at positions 93 and 182,

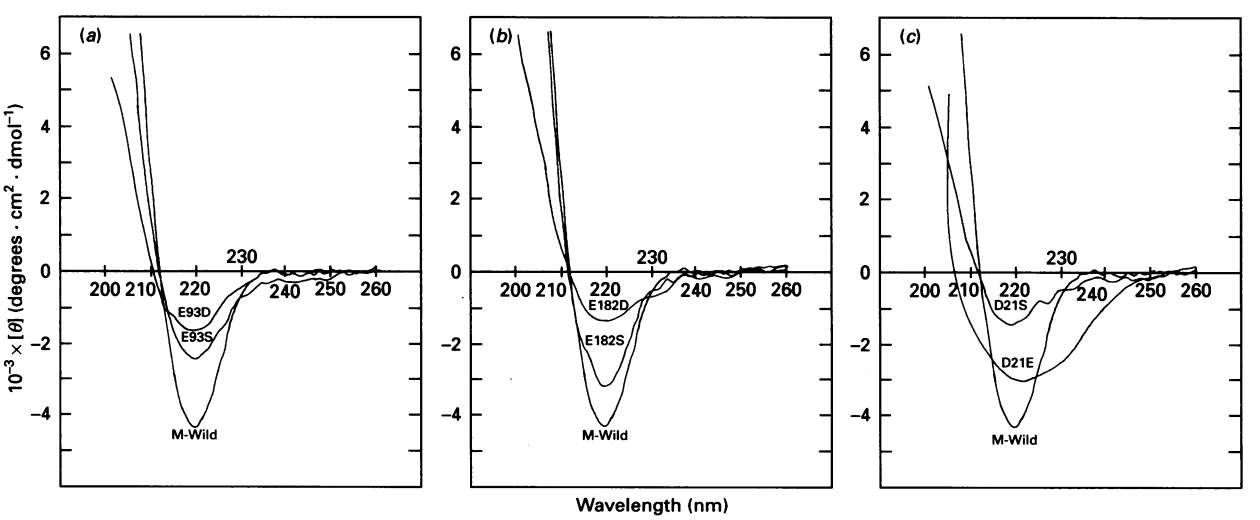


**Fig. 6. C.d. spectra of mutant xylanases**

The c.d. spectra were measured as described in the Materials and methods section. The data were expressed in terms of mean residue ellipticity ($\theta$). (*a*) E93D, E93S and M-wild xylanase; (*b*) E182D, E182S and M-wild xylanase; (*c*) D21E, D21S and M-wild xylanase.

**Table 2. Xylanase activity of mutant enzymes**

| Xylanase | Specific activity* (relative to M-wild) | $K_m$† (mg/ml) | $V_{max.}$† (μmol/min per mg) |
|---|---|---|---|
| M-wild | 1 | 6.3 | 383 |
| D21E | 0.03 | 22.7 | 86.0 |
| D21S | 0.18 | 18.6 | 175 |
| E93D | $6.6 \times 10^{-4}$ | 16.7 | 0.4 |
| E93S | $< 4.0 \times 10^{-5}$ | – | – |
| E182D | $< 8.8 \times 10^{-5}$ | – | – |
| E182S | $< 7.4 \times 10^{-5}$ | – | – |

* Specific activity of M-wild xylanase was 284 units/mg.
† Kinetic constants were measured over the xylan concentration range 0.4–2.0%.

which are both located within the cleft region, can cause a drastic diminution in the catalytic activity of the xylanase. On the other hand, the loss of activity due to the mutation of Asp-21 to Glu or Ser is not as large as that found with the other mutations. The decrease in the activity of the xylanase due to the mutation of Asp-21 to Glu may be due to the small change in the secondary structure (Table 1). Though these results clearly show that Glu-93 and Glu-182 are the best candidates for the essential catalytic residues of this enzyme, further studies on site-directed mutagenesis of these residues and X-ray crystallographic analysis of substrate-bound xylanase are necessary if we are to be provided with more conclusive evidence for the participation of these residues in the catalytic mechanism of xylanase.

We are grateful to Dr. Katsuhide Yutani for his helpful advice on c.d. spectra of mutant xylanases. This work was supported in part by a grant-in-aid (04203118) from the Ministry of Education, Science and Culture, Japan.

Received 9 March 1992/11 May 1992; accepted 27 May 1992

## REFERENCES

1. Panbangred, W., Kondo, T., Negoro, S., Shinmyo, A. & Okada, H. (1983) Mol. Gen. Genet. **192**, 335–341
2. Fukusaki, E., Panbangred, W., Shinmyo, A. & Okada, H. (1984) FEBS Lett. **171**, 197–201
3. Moriyama, H., Hata, Y., Yamaguchi, H., Shinmyo, A., Sato, M., Tanaka, N., Okada, H. & Katsube, Y. (1987) J. Mol. Biol. **193**, 237–238
4. Morosoli, R., Roy, C. & Yaguchi, M. (1986) Biochim. Biophys. Acta **870**, 473–478
5. Bray, M. R. & Clarke, A. J. (1990) Biochem. J. **270**, 91–96
6. Panbangred, W., Shinmyo, A., Kinoshita, S. & Okada, H. (1982) Agric. Biol. Chem. **47**, 957–963
7. Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T. & Horiuchi, T. (1987) Protein Eng. 1, 327–332
8. Yanisch-Perron, C., Vieria, J. & Messing, J. (1985) Gene **33**, 103–119
9. Miller, J. (1972) in Experiments in Molecular Genetics, pp. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
10. Okada, H. & Shinmyo, A. (1988) Methods Enzymol. **160**, 632–637
11. Ouchterlony, O. (1949) Acta Pathol. Microbiol. Scand. **26**, 507–517
12. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
13. Somogyi, M. (1952) J. Biol. Chem. **195**, 19–23
14. Teather, R. M. & Wood, P. J. (1982) Appl. Env. Microbiol. **43**, 777–780
15. Chanzy, M., Dube, M. & Marchessault, R. H. (1979) Polymer **20**, 1037–1039
16. Paice, M. G., Bourbonnais, R., Desrochers, M., Jurasek, L. & Yaguchi, M. (1986) Arch. Microbiol. **144**, 201–206
17. Malcolm, B. A., Rosenberg, S., Corey, M. J., Allen, J. S., Baetselier, A. & Kirsch, J. F. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 133–137
18. Anfinsen, C. B. & Scheraga, H. A. (1975) Adv. Protein Chem. **29**, 205–300
19. Provencher, S. W. & Glockner, J. (1981) Biochemistry **20**, 33–37
20. Chang, C. T., Wu, C.-S. C. & Yang, J. T. (1978) Anal. Biochem. **91**, 13–31