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Identification of active site carboxylic residues in *Trichoderma reesei* endoglucanase Cel12A by site-directed mutagenesis

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Abstract

cDNA of Cel12A (formerly EG III), one of five endoglucanases of *Trichoderma reesei*, was expressed in *Escherichia coli* by using the *tac* promoter of *E. coli*. Transformants of *E. coli* harboring a plasmid pAGmegl3 containing mature form Cel12A cDNA produced Cel12A protein largely as insoluble inclusion bodies in the cytoplasm of the cells. The insoluble fraction was solubilized with urea from which Cel12A was purified by cation chromatography to electrophoretic homogeneity. The purified enzyme was immunologically and enzymologically identical to that of Cel12A purified from *T. reesei*. E116 and E200 of Cel12A of *T. reesei* are completely conserved in family-12 cellulases. In order to investigate the role of these two glutamate residues in the enzymic function of Cel12A, two mutant enzymes were produced at each position, namely E116D/Q and E200D/Q. The specific activity of these mutant enzymes is reduced by more than 98%, revealing the importance of these two residues to the catalytic function of Cel12A. The data demonstrated that E116 and E200 are the nucleophilic and acid-base residues, respectively. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cellulase; Endoglucanase; Catalytic residue; Site-directed mutagenesis; *Trichoderma reesei*

1. Introduction

Plant cell-wall hydrolases, such as cellulases and xylanases, have especially received increasing attention because they convert cellulose and hemicellulose, which are the most abundant carbohydrate produced in the biosphere, into fermentable sugars. Over 1500 sequences of glycosyl hydrolases, including plant cell-wall hydrolases and related proteins, are known to date. They are conveniently classified into a number of families based on amino-acid sequence similarities [1]. With the accumulation of three-dimensional data, the related families have been grouped into clans, since their members share a common fold and the position of their catalytic residues is conserved despite the dissimilarities of their sequences. In addition, it has been found that most glycosyl hydrolases are modular enzymes consisting of discrete catalytic domain and cellulose binding domain [2–6]. Glycosyl hydrolases cleave their target substrates by either a single or double displacement mechanism, resulting in the inversion or the retention of anomeric configuration of the product, respectively [7]. In the active site of these

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enzymes, there are two carboxylic acids residues that participate in catalysis [8]. The two catalytic carboxylates, a general acid and a general base catalysts, for enzymes operating with inversion are normally separated by 9–10 Å, whereas retaining enzymes have two catalytic functions (a nucleophile and a general acid/base) separated by approximately 5.5 Å.

The filamentous fungus *Trichoderma reesei* produces seven cellulases, two cellobiohydrolases (CBH) and five endoglucanases (EG). Cel12A (formerly EG III), which belongs to family 12 of the retaining glycosyl hydrolases, consists of a single polypeptide chain of 218 amino acid residues with a calculated molecular mass of 23,480 Da. The enzyme is a sole cellulase without a cellulose-binding domain and carbohydrates in *T. reesei* [9,10]. In the family-12 cellulases, the structure of the catalytic domain of the *Streptomyces lividans* CelB was recently solved to 1.75 Å resolution by X-ray crystallography by Sulzenbacher et al. [11]. They proposed that Glu-120 and Glu-203 conserved in all the known family-12 sequences are assigned to the catalytic nucleophile and general acid/base, respectively. Zechel et al. [12] suggested that Glu-120 was confirmed to be the nucleophile by trapping the glycosyl-enzyme intermediate with a mechanism-based inactivator, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-cellobioside. These residues correspond to Glu-116 and Glu-200 in *T. reesei* Cel12A. We have cloned Cel12A cDNA and genomic DNA, and their nucleotide sequences were determined. Moreover, the heterologous expression of Cel12A was also performed to secrete it in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and to produce it intracellularly in *Escherichia coli*. Expression of the mature Cel12A in *E. coli* resulted mostly in the formation of enzymatically inactive inclusion bodies [10]. Here, we describe a successful method for recovering the active enzyme from the inclusion body. Furthermore, using the *E. coli* expression system, the potentiality of the Glu residues as two catalytic residues, a nucleophile and an acid/base catalyst, was investigated by site-directed mutagenesis.

2. Materials and methods

2.1. Plasmids and strains

E. coli JM109 was used as the host cell for Cel12A expression. Plasmid pAG9-3 [10] was used as an *E. coli* expression vector. Plasmid pT7Blue-T (Novagen) was used for subcloning and sequencing of PCR products. *E. coli* MV1184 and plasmid pKF19k were used for site-directed mutagenesis.

2.2. Modification and subcloning of the cel12A cDNA

The mature Cel12A expression plasmid in *E. coli* was constructed as follows. The DNA fragment comprised of the mature Cel12A-coding region flanked with restriction enzyme sites at both ends was amplified using a PCR method with sequence-specific primers and pT7Blue-egl3 [10] as a template. The primers were 5′-GG*CCATGG*CACAAACCAGCTGTGACCATGGGC-3′ (sense) and 5′-CC*AAGCTT*AGTTGATAGATGCGGTCCAGGA-3′ (antisense) (italicized letters indicate the *Nco*I and *Hin*dIII restriction site, respectively). The PCR fragment was cloned into pT7Blue-T, generating pT7Bluemegl3. Plasmid pT7Bluemegl3 was digested with *Nco*I, blunt-ended and further digested with *Hin*dIII, and a 700 bp fragment was recovered. The fragment was joined between blunt-ended *Eco*RI and *Hin*dIII sites of pAG9-3, resulting in the plasmid pAGmegl3 (Fig. 1). Plasmid pAGmegl3 thus prepared contains a modified Cel12A gene in which the signal sequence region of Cel12A is replaced by the initiation ATG codon and GCA (Ala) codon.

2.3. Preparation of recombinant enzymes

Transformants harbouring the expression plasmid pAGmegl3 were incubated in LB

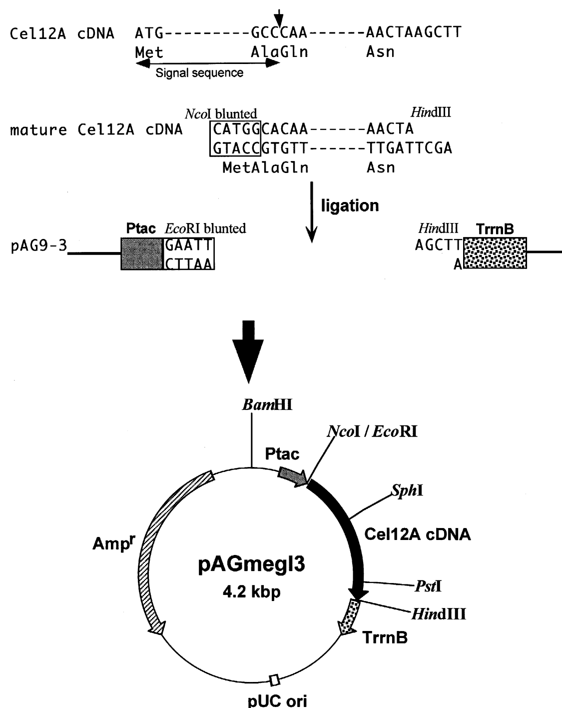

Fig. 1. Construction of Cel12A high-expression vector (pAGmegl3). Ptac and TrrnB indicate *tac* promoter and *rrn* terminator, respectively. Gene for resistance to ampicillin is indicated by Amp^r.

medium supplemented with ampicillin (50 μg/ml) at 37°C for 16 h, and the cultures were inoculated in 2 × YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) with ampicillin at a ratio of 1:25. After 2 h of incubation, expression of recombinant Cel12A was induced with isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 mM. After further 6 h of cultivation, the cells were harvested, homogenized by sonication and centrifuged to obtain precipitates. The sonicated pellets were washed twice with 50 ml of STE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl), twice with 5 ml of 10% Triton X-100 and once with 50 ml of H_2O. The final pellet gave an inclusion body containing recombinant Cel12A. The inclusion body was dissolved in 50 ml of a denaturing buffer (10 mM acetate buffer, pH 4.8, containing 8 M urea and 1 mM dithiothreitol (DTT)) with stirring at 4°C for 16 h. After centrifugation, the supernatant was diluted with 950 ml of a renaturing buffer (10 mM acetate buffer, pH 4.8, containing 1 mM DTT) and stirred. The solution was kept at 4°C for 16 h, and the enzyme activity was assayed. After centrifugation at 6000 × *g* for 10 min, the supernatant was applied to an SP-Sepharose FF (Pharmacia) column (2.5 × 11 cm) previously equilibrated with 10 mM acetate buffer, pH 4.8. The column was washed with the same buffer and the protein was eluted with a linear gradient of the buffer containing 0 to 0.5 M NaCl. The active fractions were pooled, and concentrated and desalted by ultrafiltration. The purity of recombinant Cel12A was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed under reducing conditions [13].

2.4. Site-directed mutagenesis

In mutating Glu-116 and Glu-200, the *Xba*I–*Hin*dIII fragment of pT7Bluemegl3 was first cloned into pKF19k to serve as a template for mutagenesis, generating plasmid pKFmegl3. Site-directed mutagenesis was performed by PCR mutagenesis strategy using Mutan-Super Express Km Kit (Takara Shuzo, Kyoto, Japan) according to the supplier's instructions. The primers used were shown in Table 1. PCR was performed in a final volume of 50 μl with 10 ng of the template DNA, pKFmegl3, 5 pmol of each primer and 0.4 mM dNTPs. Each of the 30 amplification cycles consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 4 min. After

Table 1
Oligonucleotide primers used for site-directed mutagenesis
All the oligonucleotide primers were 5′-phosphorylated. Nucleotides that do not pair with a nucleotide in the gene for Cel12A are italicized.

Mutation	Sequence (from 5′ to 3′)
Glu 116 → Gln (E116Q)	GGGAGACTAC*C*AACTCATGA
Glu 116 → Asp (E116D)	GGGAGACTAC*GA**T*CTCATGA
Glu 200 → Gln (E200Q)	TTTGGTACC*C*AGCCCTTCAC
Glu 200 → Asp (E200D)	TTTGGTACCGA*T*CCCTTCAC

ethanol precipitation, the amplified DNA was transformed with *E. coli* MV1184 and plated on LB medium supplemented with kanamycin (50 μg/ml). The mutated DNA fragments obtained as an *Sph*I–*Pst*I fragment were used to replace their corresponding original fragment in pAGmegl3 (Fig. 1) and the sequences of inserts were confirmed by nucleotide sequencing with BcaBEST Dideoxy Sequencing Kit (Takara).

2.5. Circular dichroism

Circular dichroism (CD) spectra of the wild-type (wt) Cel12A and its mutants were obtained in 10 mM acetate buffer (pH 4.8) using a CD spectrophotometer (model J-600: Jasco, Tokyo, Japan) at 15°C. The protein concentration of each enzyme was 0.2 mg/ml. All data were averaged from six acquisitions at a scan rate of 10 nm/min between 255 and 200 nm.

2.6. Enzyme assay

Endoglucanase activity was determined by measuring the enzymatic release of reducing sugars from carboxymethyl cellulose (CMC). Standard assay mixture (total volume, 0.5 ml) contained 0.4 ml of 1.25 % CMC in 50 mM acetate buffer, pH 5.5, and 0.1 ml of appropriately diluted enzyme solution. After incubation at 50°C for 15 min, the amount of reducing sugar was determined by the Somogyi–Nelson method described earlier [14], using glucose as a standard. All of the assays were done in triplicate. One unit of the activity was defined as the amount of enzyme that liberates reducing sugars equivalent of 1.0 μmol of D-glucose from the substrate per minute. For determinations of K_m and k_{cat}, various concentrations (0.04–1.25%) of CMC in 50 mM acetate buffer, pH 5.5, were used. The optimum pH and temperature for endoglucanase activity of Cel12A were determined as described previously [15], using 50 mM citrate/phosphate buffer (pH 3–8) for optimum pH. The pH stability was examined by preincubating the enzyme in the absence of substrate at 50°C for 30 min in 25 mM citrate/phosphate buffer (pH 3–8) before determining the endoglucanase activities. For the determination of thermostability, the enzyme was preincubated in 50 mM acetate buffer (pH 5.5) at various temperatures (30–70°C) for 30 min.

2.7. Other methods

SDS-PAGE and subsequent Western blotting were performed as described before [10]. Sequencing of Cel12A protein transferred from an SDS-polyacrylamide gel to PVDF membrane was carried out by automated N-terminal Edman degradation using a PPSQ-21 protein sequencer (Shimadzu, Kyoto, Japan).

3. Results and discussion

3.1. Production of recombinant Cel12A in E. coli

The expression vector for the mature form of Cel12A, pAGmegl3, was constructed by inserting Cel12A cDNA into the multicloning site of pAG9-3 as shown in Fig. 1. ATG (Met) codon and GCA (Ala) codon containing a *Nco*I site were inserted to immediate upstream from the Gln17 codon of Cel12A cDNA by using a synthetic oligonucleotide linker to delete the signal sequence and introduce a translation initiation codon. By this construction, it was expected that the protein with the extra Met–Ala amino acids added at its N-terminus could be expressed in *E. coli*. pAGmegl3 was introduced into several *lacI*^{q} derivatives of *E. coli* K-12, and the transformants were cultivated in 2 × TY medium containing 50 μg/ml of ampicillin for IPTG induction. Cultured cells were disrupted by sonication, and the suspensions were centrifuged to obtain a supernatant and a pellet. The highest CMCase activity in the supernatant of 58 mU/ml was observed using *E. coli* JM109 as a host (data not shown). However, it

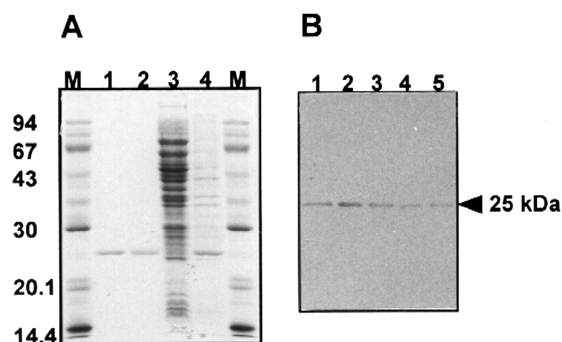

Fig. 2. SDS-PAGE (A) and immunoblot analysis (B) of Cel12A produced in *E. coli* JM109. (A) Each sample was put on an SDS-PAGE gel (15%), and the gel was stained with Coomassie Brilliant Blue R-250. Lane 1, native Cel12A purified from *T. reesei* culture supernatant; lane 2, recombinant Cel12A purified from *E. coli*; lane 3, soluble fraction of the *E. coli*-transformed cell extract; lane 4, insoluble fraction. (B) The protein was transferred onto PVDF membrane and the rabbit anti Cel12A antiserum was used as primary antibody. Lane 1, soluble fraction; lanes 2,3, and 4, insoluble fraction solubilized by 10% SDS; lane 5, insoluble fraction solubilized by 8M urea; volume ratio per culture broth was 1:1/5:1/10:1/20:1/10.

could be judged that the active soluble form was still expressed in little amount. SDS-PAGE analysis of the pellet also showed that the transformant of *E. coli* JM109 produced the largest amount of Cel12A protein as an inclusion body (data not shown). The Cel12A synthesized in *E. coli* JM109 was the major protein in the insoluble pellet, with the same apparent molecular mass as that of the native Cel12A purified from a *T. reesei* culture supernatant, as shown in SDS-PAGE in Fig. 2A (lane 4). Immunoblot analysis after SDS-PAGE of the soluble and insoluble fractions showed that the amount of Cel12A protein expressed as an insoluble form was about 10-fold over that of the soluble form (Fig. 2B).

3.2. Renaturation and purification of recombinant Cel12A

Since recombinant Cel12A was produced in *E. coli* mainly in the insoluble fraction, we developed a protocol that allowed the recovery of significant amounts of active Cel12A. The inclusion body was solubilized with 8 M urea, and the supernatant was analysed by SDS-PAGE, indicating that about 80% of the insoluble Cel12A could be solubilized (data not shown). A preliminary experiment demonstrated that more than 90% of the activity of Cel12A in the solution was maintained at a urea concentration below 0.5 M at 4°C. After denaturation, therefore, the supernatant was diluted 20-fold with the renaturation buffer to 0.4 M urea concentration. The renaturated protein solution was applied to SP-Sepharose column chromatography, by which most of the contaminant proteins were removed. In this way, active recombinant Cel12A was purified with the yield of 36 μg of the protein and 468 mU of the activity per ml of culture, which corresponds to eightfold activity of the soluble fraction. The specific activity of recombinant Cel12A (13.1 U/mg) was almost the same as that of the native Cel12A (12.7 U/mg). The recombinant enzyme exhibited quite the same enzymological properties as those of the native one, such as the specific activity, pH and temperature characteristics (Table 2), and also the CD spectrum (data not shown). The N-terminal amino acid sequence identified by an automated protein sequencer was Ala-Gln-Thr-Ser-(Xaa)-Asp-Gln-Trp-Ala-Thr, which agreed with the constructed Cel12A cDNA ex-

Table 2
Properties of the Cel12As from *T. reesei* and *E. coli*

	T. reesei	*E. coli*
Molecular mass (kDa)	25	25
Specific activity (U/mg)	12.7	13.1
Optimum pH	5.5	5.5
Optimum temperature (°C)	55	55
pH stability	5.0–5.5	5.0–5.5
Temperature stability	up to 45°C	up to 45°C
N-terminal amino acid sequence	pyrGlu–Thr–Ser–	Ala–Gln–Thr–Ser–

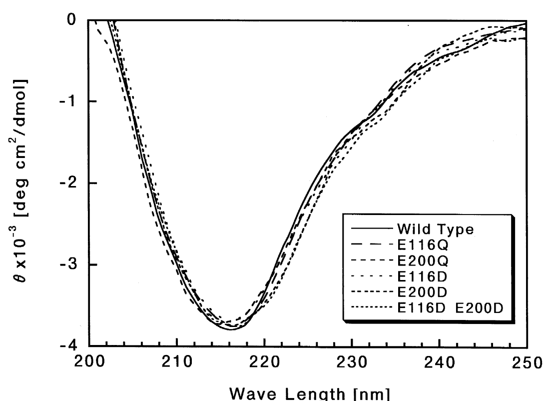

Fig. 3. Circular dichroism spectra of wild-type and mutant forms of Cel12A.

cept for no detection of the first methionine residue, which is assumed to be deleted with endogeneous methionine aminopeptidase in *E. coli*. The fifth amino acid residue, (Xaa), indicated as an unidentified amino acid may be a cystein residue in view of the nucleotide sequence. These results indicate that the presence of the extra alanine residue does not affect the catalytic activity of this enzyme. Furthermore, this *E. coli* expression system appears to be suited to the study of the catalytic function of Cel12A.

3.3. Site-directed mutagenesis and characterization of mutants

Two glutamic acids, Glu-116 and Glu-200 of *T. reesei* Cel12A are conserved in family-12 cellulases and presumed to act as the nucleophile and the acid/base in the double displacement mechanism. To confirm this, we investigated the effect of replacement of Glu-116 and Glu-200 with Asp or Gln separately. Site-directed mutagenesis was performed by the PCR mutagenesis method described in Section 2.4. A double mutant having two aspartates at these putative catalytic residues was also prepared. All mutants were expressed in *E. coli* as inclusion bodies, which were solubilized and purified according to the same procedure as that for the wt Cel12A. Based on an SDS-PAGE analysis, the mutant enzymes were obtained with a similar yield to that of the wt enzyme, and the molecular mass of each mutant was estimated to be the same as that of the wt Cel12A (data not shown). Comparison of CD spectra of the mutants with that of the wt enzyme in the far-UV regions did not reveal any significant difference, indicating that the mutations did not modify the fold of the enzyme (Fig. 3).

3.4. Kinetic studies

Specific activities and kinetic parameters, K_m and k_{cat}, of the wt Cel12A and the mutants were determined with CMC as a substrate. These measurements were made at pH 5.5, at which the wt Cel12A had the highest activity (Table 2). Specific activities and the parameters determined by the Lineweever–Bark plots are summarized in Table 3. The specific activities of the Asp mutants, E116D and E200D, were 1.7% and 0.4% of that of the wt Cel12A, respectively, while the double mutant E116D:E200D displayed little activity. The k_{cat} values of the mutant enzymes E116D and E200D were 50-

Table 3
Specific activities and kinetic parameters of wild-type and mutant enzymes with CMC as a substrate

Enzyme	Specific activity		K_m (g/l)	k_{cat} (s^{-1})	k_{cat}/K_m (l/g · s)
	(U/mg)	(%)			
Wild type	13.1	(100)	2.00	5.50	2.8
E116D	0.214	(1.7)	4.17	0.106	0.025
E200D	0.0483	(0.40)	0.500	0.0265	0.053
E116D:E200D	1.82×10^{-3}	(0.015)	–	–	–
E116Q	1.61×10^{-3}	(0.013)	–	–	–
E200Q	2.39×10^{-3}	(0.019)	–	–	–

and 200-fold lower than that of the wt enzyme, respectively, which corresponded to the decreases in specific activity. On the other hand, both of the Gln mutants, E116Q and E200Q, had the activity reduced almost at all, namely to 0.01–0.02% of that of the wt enzyme, strongly suggesting that these glutamic acids play an important role in the catalysis of hydrolysis. The K_m value of the E116D mutant (4.2 g/l) was twofold higher than that of the wt Cel12A (2.0 g/l), while that of the E200D mutant (0.5 g/l) was reduced by fourfold compared with that of the wt Cel12A. Furthermore, the catalytic efficiencies (k_{cat}/K_m) of the mutant enzyme E116D was more than two orders of magnitude lower than that of the wt enzyme. Reduction of catalytic efficiency was less pronounced when E200 was replaced by D. It is observed in other retaining glycosidases [16–20] that the general acid/base residue less contributes to catalysis relative to the nucleophile. Although further detailed kinetic analysis, or investigation of azide-effects or pH effects on the reaction by the mutant enzymes will be necessary to clarify the function of the active residues, it is plausible that Glu-116 and Glu-200 act as the nucleophile and the general acid/base, respectively, in the catalysis of hydrolysis. This is consistent with the prediction of Sulzenbacher et al. [11] by X-ray analysis of the *S. lividans* CelB and with the nucleophile identification of it by Zechel et al. [12].

It was reported that the removal of the catalytic nucleophile of the retaining β-glucosidase and β-1,3-1,4-glucanase yields an enzyme with the regio- and stereo-specific glycosylation of α-glycosyl fluoride with various glycoside acceptors [21,22]. From this viewpoint, further research will be conducted to synthesize new β-1,4-glycans using the mutant E116Q prepared in this study.

References

[1] B. Henrissat, Biochem. Soc. Trans. 26 (1998) 153.
[2] L.M.A. Ferreira, T.M. Wood, G. Williamson, C. Faulds, G.P. Hazlewood, G.W. Black, H.J. Gilbert, J.H. Clarke, Biochem. J. 294 (1993) 349.
[3] S.J. Millward-Sadler, K. Davidson, G.P. Hazlewood, G.W. Black, H.J. Gilbert, J.H. Clarke, Biochem. J. 312 (1995) 39.
[4] H.J. Gilbert, J. Hall, G.P. Hazlewood, L.M.A. Ferreira, Mol. Microbiol. 4 (1990) 759.
[5] L.E. Kellett, D.M. Poole, L.M.A. Ferreira, A.J. Durrant, G.P. Hazlewood, H.J. Gilbert, Biochem. J. 272 (1990) 369.
[6] P. Tomme, R.A.J. Warren, N.R. Gilkes, Adv. Microbiol. Physiol. 37 (1995) 1.
[7] S.G. Withers, in: S.B. Petersen, B. Svensson, S. Pedersen (Eds.), Progress in Biotechnology, Vol. 10, Elsevier, Amsterdam, p. 97.
[8] J.D. McCarter, S.G. Withers, Curr. Opin. Struct. Biol. 4 (1994) 885.
[9] Y. Morikawa, A. Takahashi, K. Yano, M. Yamasaki, H. Okada, in: K. Shimada, S. Hashino, K. Ohmiya, K. Sakka, Y. Kobayashi, S. Karita (Eds.), Genetics, Biochemistry and Ecology of Lignocellulose Degradation, Uni Publisher, Tokyo, p. 458.
[10] H. Okada, K. Tada, T. Sekiya, K. Yokoyama, A. Takahashi, H. Tohda, H. Kumagai, Y. Morikawa, Appl. Environ. Microbiol. 64 (1998) 555.
[11] G. Sulzenbacher, F. Shareck, R. Morosoli, C. Dupont, G.J. Davies, Biochemistry 36 (1997) 16032.
[12] D.L. Zechel, H.E. Shouming, C. Dupont, S.G. Withers, Biochem. J. 336 (1998) 139.
[13] U.K. Laemmli, Nature 227 (1970) 680.
[14] Y. Morikawa, T. Ohashi, O. Mantani, H. Okada, Appl. Microbiol. Biotechnol. 44 (1995) 106.
[15] H. Okada, T. Sekiya, K. Yokoyama, H. Tohda, H. Kumagai, Y. Morikawa, Appl. Microbiol. Biotechnol. 49 (1998) 301.
[16] S.L. Lawson, W.W. Wakarchuk, S.G. Withers, Biochemistry 36 (1997) 2257.
[17] S.L. Lawson, W.W. Wakarchuk, S.G. Withers, Biochemistry 35 (1996) 10110.
[18] A.M. MacLeod, D. Tull, K. Rupitz, R.A.J. Warren, S.G. Withers, Biochemistry 35 (1996) 13165.
[19] Q. Wang, D. Trimbur, R. Graham, R.A.J. Warren, S.G. Withers, Biochemistry 34 (1995) 14554.
[20] Q. Wang, R.W. Graham, D. Trimbur, R.A.J Warren, S.G. Withers, J. Am. Chem. Soc. 116 (1994) 11594.
[21] C. Malet, A. Planas, FEBS Lett. 40 (1998) 208.
[22] L.F. Mackenjee, Q. Wang, R.A.J. Warren, S.G. Withers, J. Am. Chem. Soc. 120 (1998) 5583.