Analysis of a Catalytic Acidic Pair in the Active Center of Cellulase from Aspergillus aculeatus

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Four acidic amino acid residues, Asp97, Asp101, Glu118, and Glu202, were located in the cleft from the X-ray crystallographic analysis of FI-CMCase, endo-1,4-β-glucanase (EC: 3.2.1.4) of Aspergillus aculeatus No. F-50. To identify the catalytic residues of the FI-CMCase, these residues were mutated to Glu or Ser from Asp97 and Asp101, and to Asp or Ser from Glu118 and Glu202 by site-directed mutagenesis, and totally 8 single mutant enzymes expressed in Escherichia coli were prepared: D97E, D97S, D101E, D101S, E118D, E118S, E202D, and E202S. Mutant enzymes E118S and E202S were not shown to have any detectable activity. Kinetic parameters of other mutant enzymes were measured after purification. The Km of mutant enzymes were not much different from that of wild type FI-CMCase, while the Vmax of mutant enzymes D97E, D97S, D101E, D101S, E118D, E118S, and D202E were much decreased to 1/50, 1/20, 1/4000, 1/2000, 1/800, and 1/1600 of the wild type FI-CMCase, respectively. From these results we concluded that Glu118 and Glu202 were most probable candidates for a catalytic pair of acidic amino acids in FI-CMCase.

Key words: site-directed mutagenesis; active site; cellulase; family 12

FI-CMCase is the most abundant cellulase of the 9 cellulases produced by Aspergillus aculeatus No. F-50.1) The purification and properties have been reported.1) The cloning of the cDNA2) and the genomic DNA2) encoding FI-CMCase were described and the expression in Escherichia coli4) and Saccharomyces cerevisiae5) of the cDNA was also reported. The effects of the 5′-upstream region of the structural gene on the expression were studied.7) Also the crystal structure of FI-CMCase was resolved to 2.2 Å.5,6) FI-CMCase can be classified on the basis of amino acid sequence as a glycosyl hydrodase of family 12,10) which includes small molecular weight endoglucanases consisting of only a catalytic domain.

The catalytic center in many glycosidases is believed to be composed of a pair of facing acidic amino acid residues located in the cleft.9) From the crystal structure of FI-CMCase, 2 acidic amino acid pairs in the cleft can be considered as candidates: Asp97 on β-10 and Asp101 on β-11, and Glu118 on β-12 and Glu202 on β-18 as shown in Fig. 1. The distances between α-carbon of Asp97 and Asp101, and that of Glu118 and Glu202 in the cleft are around 9 Å and 7 Å, respectively.9) For this work, 8 mutants of FI-CMCase cDNA were constructed by site-directed mutagenesis: Asp97 and Asp101 → Glu or Ser; Glu118 and Glu202 → Asp or Ser. Mutant FI-CMCase cDNAs were expressed in E. coli harboring an expression vector, and mutant enzymes were purified to homogeneity, and the kinetic parameters were measured. From the data we conclude that Glu118 and Glu202 are an acidic amino acid pair of the catalytic center.

Materials and Methods

Bacterial strains and plasmids. Escherichia coli JM10911) was used as a host strain for plasmid construction and for mutant enzyme production. M13 mp1911) was used in subcloning of the FI-CMCase gene for site-directed mutagenesis. pHEM069 was used as an E. coli expression vector for the wild type FI-CMCase and its mutant enzymes.

Media and culture conditions. E. coli JM109 was grown at 37°C in LB medium (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) or 2 × TY medium (1.6% bactotryptone, 1% yeast extract, 1% NaCl, pH 7.5). E. coli transformants were selected on LB-Amp plates (50 μg/ml ampicillin) containing 1.5% agar. CMC plates (LB medium containing 10 mg/ml carboxymethyl cellulose (CM-cellulose), 50 μg/ml ampicillin, and 1.5% agar) were used for cellulase activity detection by halo formation.

Site-directed mutagenesis and expression of mutant

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enzyme. The 830 bp EcoRI-PstI fragment containing the FI-CMCase coding region of cDNA from pHEM06 was inserted into the same site of M13 mp19 RF. Single-stranded DNA isolated from E. coli was used for site-directed mutagenesis, which was done by the method of Kunkel[2] with a Muta-Genie in vitro mutagenesis kit (Bio-Rad Japan, Tokyo). Alterations of codon for the four acidic amino acid residues by using oligonucleotides as follows: Asp97 (GAC) to Glu (GAA) for D97E and to Ser (TCC) for D97S; Asp101 (GAT) to Glu (GAA) for D101E and to Ser (TCT) for D101S; Glu101 (GAG) to Asp (GAT) for E118D and to Ser (TCC) for E118S; Glu202 (GAG) to Asp (GAT) for E202D and to Ser (TCC) for E202S. The nucleotide sequences of mutated genes were confirmed by sequencing fragments with a Thermo Sequencing Kit (Amersham). The EcoRI-PstI DNA fragments containing each mutant FI-CMCase gene were used to replace the same DNA fragment of pHEM06. E. coli JM109 was transformed with each of the expression plasmids. The transformants were cultivated in 2 x TY medium containing ampicillin at 37°C, and harvested bacterial cell was used for enzyme purification.

Purification of wild-type and mutant FI-CMCases. The 15-h grown E. coli JM109 cells carrying each expression vector from 10 l of culture broth were harvested by centrifugation, and resuspended in 100 mM sodium acetate buffer (pH 5.5) containing 20 mM EDTA, and the cells were disrupted with a French pressure. A crude enzyme solution was obtained by centrifuging the disrupted cells and then streptomycin (10 mg/ml) was added to it. After 1 h on ice, the precipitates were removed by centrifugation. From the supernatant obtained, the enzyme was purified by gel filtration with Sephadex G-200 and G-75 as described previously. As the activity of the mutant enzyme was very low, the cellulase protein was detected by an immunological method.

Measurement of the activity. Cellulase activity of the enzyme was measured by using CM-cellulose as a substrate as described previously. The reaction mixture containing 5 mg/ml CM-cellulose and the enzyme in 1 ml of 50 mM acetate buffer (pH 5.0) was incubated at 37°C for 10 min. The amount of reducing sugar released was measured by the Somogyi method. One unit of the enzyme was defined as the activity producing 1 μmole of reducing sugar as a glucose equivalent per minute.

Circular dichroism (CD) measurements. CD spectra were recorded with a Jasco J-40A automatic recording spectrophotometer with a data processor. Each memory unit in the computer stored the CD signal for a spectral band of 0.1 nm. Spectra were scanned 10 times at a scan rate of 50 nm/min, using 0.25 s as the time constant. The spectra were measured at protein concentrations of 1 μg (24 μg/ml equivalent) in 50 mM acetate buffer, pH 5.5, by using a cell of 10-mm path length at 25°C. The protein concentration of the FI-CMCase was used as a molecular extinction coefficient.

Results

Candidates for a pair of acidic amino acid residues in the active center

The four acidic amino acids of FI-CMCase, Asp97, Asp101, Glu118, and Glu202, in the conserved region in family 12 of glycosyl hydrolases are shown in Fig. 2. Glu118 and Glu202 are strictly conserved in all enzymes, but Asp97 is conserved in the enzymes of Aspergillus sp. and Asp101 is also well conserved in this family except for the enzymes of Thermotoga sp.. These four amino acids are located within the cleft built up by two sheets of antiparallel β-strands (β-2, 3, 6, 10, and 18, and β-7, 17, 11, 12, 15, 13, 16, and 14) of the FI-CMCase molecule as shown in Fig. 1. Asp97 and Glu202 are located on the 10th and 18th β-strand of one side of the β-sheet, but Asp101 and Glu118 are located on the 11th and 12th of the opposite side of β-sheet. A pair of acidic amino acid residues are most likely for the active center of catalysis. Therefore, Asp97, Asp101, Glu118, and Glu202 were targeted for identification as an active center and treated to site-directed mutagenesis.

Purification of wild-type FI-CMCase and its mutants

It is expected that mutant enzymes of FI-CMCase have very little or no activity, because the mutations are situated near or the active center and the activity would be seriously injured. So, we compared the protein elution profile to that in the purification of the wild type FI-CMCase. In the purification of mutant enzymes, enzyme fractions after column chromatography were detected by rabbit anti-FI-CMCase antiserum. Purified mutant enzymes were run on SDS-PAGE. They showed a single protein band with the molecular weight of 24,000, the same as wild type FI-CMCase (data not shown).

Double-immunodiffusion assay

Double-immunodiffusion assay was done with purified mutant enzymes and rabbit antiserum against FI-CMCase from A. aculeatus. Each purified mutant enzyme showed a single precipitin line with the antiserum which was fused with that of wild type FI-CMCase (data not shown), thereby confirming mutated enzymes have the same immunological properties as wild type FI-CMCase, that is, the surface structures of mutated enzymes are not changed much.

CD spectra of the enzymes

Since the conformation of a protein necessary for its biological function is governed by its amino acid sequence, the effects of the mutations mentioned above on the secondary structure of FI-CMCase were also investigated by the CD spectrum analysis. As shown in Fig. 3, the CD spectra of the wild type FI-CMCase and mutant enzymes in the region 205–260 nm at pH 5.5 and 25°C display profiles and are well-resolved through at 217 nm. In addition, the shapes of the spectra of wild type FI-CMCase and mutant enzymes are very similar.
Activity of the mutant enzymes

The FL-CMCase from the E. coli, termed wild type FL-CMCase, has an extra methionine residue at an amino-terminus of the mature enzyme due to deleting the signal peptide as a translation initiation codon. The specific activity of this enzyme was the same as that of the FL-CMCase produced by A. aculeatus, indicating that the presence of an extra methionine residue does not affect the activity of this enzyme. The specific activities of the wild type and mutant enzymes are listed in Table 1. Mutant enzymes D97E and D97S still have considerable specific activities. The mutant enzymes of D101E, D101S, E118D, and E202D had very low specific activities (less than 0.3% of the wild type FL-CMCase). The kinetic parameters of the wild type and mutant enzymes except for E118S and E202S were measured over the CM-cellulose concentration range 1.0 to 10 g/l. The $K_m$ and $V_{max}$ of the wild type and mutant enzymes are also listed in Table 1. All of the $K_m$ of mutant enzymes were similar to that of the wild type FL-CMCase. However the $V_{max}$ of the mutant enzymes were drastically decreased to less than 1/800 of the wild type enzyme, especially in the mutant enzymes of Asp101, Glu118, and Glu202. These results suggested that the affinities toward the substrate of the mutant enzymes were not affected but the catalytic rates were greatly affected, indicating that those amino acid residues are located in the catalytic center but not in the substrate binding site.

### Discussion

In general, an acidic amino acid pair in the active site of glycosidases is involved in the reaction mechanism. One carboxyl group acts as a proton donor to protonate the glucosidic oxygen and the other carboxyl group acts...
Fig. 2. Alignment of the Amino Acid Sequences Around the Putative Active Site in Family 12 of Glycolysyl Hydrolases.

Amino acid residues identical to those of FI-CMCase are indicated by a white letter in black boxes. Hyphens indicate gap. Acidic amino acid residues within the cleft of FI-CMCase are marked by asterisks. AA FICMC, Aspergillus ficuum FI-CMCase; AK CMC1, A. kawachii CMCase-1 (accession No. S55931); AN ENGA, A. niger endoglucanase A (accession No. A1224451); AO CELA, A. oryzae CelA (accession No. D83731); AA ENG, A. aculeatus xylolucan-specific endo-β-glucanase (accession No. AF043595); TR EGIII, Trichoderma reesei EGIII (accession No. AB003694); EC CELS, Erwinia carotovora CelS (accession No. JU0328; EC CELB, E. carotovora CelB (accession No. AF025769); RM CELA, Rhodothermus marinus CelA (accession No. U72637); SH CEL2, Streptomyces halstedii CelA2 (accession No. U51222); SL CELB, S. lividans CelB (accession No. U04629); SR EGLS, S. rochei EglS (accession No. X73953); SV CELS1, S. viridogriseus CelS1 (accession No. AF130408); TM CELA, Thermodiplodica maritima CelA (accession No. Z69341); TM CELB, Th. maritima CelB (accession No. Z69341); TN CELA, Th. neapolitana CelA (accession No. U93354); TN CELB, Th. neapolitana CelB (accession No. U93354).

as a base to promote the formation of a hydroxyl ion, which attacks a new anomic carbon. They support the general hypothesis that the enzymatic hydrolyses of carbohydrates is involved in Taka-amylase and various cellulases.

The three-dimensional structure of FI-CMCase is very close to Bacillus pumilus xylanase which belongs to family 11 of glycosyl hydrolases. The two catalytic Glu residues (Glu93 and Glu182) in the xylanase have been identified from mutation analysis. Torrønen et al. also predicted that Glu118 and Glu202 for FI-CMCase would be essential for catalysis from the significant relationship between the two families by hydrophobic cluster analysis. Based on the data of the three-dimensional structure of FI-CMCase and the conserved acidic amino acid
residues among family 12 cellulases, Glu118 and Glu202 are supposed to be a most likely candidates for a pair of acidic amino acids in the catalytic residues. In addition Asp97 and Asp101 are also located within the cleft of the FI-CMCase molecule. Therefore these four amino acid residues in FI-CMCase were replaced by other acidic amino acid or serine by site-directed mutagenesis to identify the catalytic residues. The molecular weight, the immunological properties, and the CD spectra of the purified mutant enzymes were quite similar to that of the wild type enzyme. The activities of mutant enzymes E118S and E202S were not detected because they were less than 10^{-4} of the wild type FI-CMCase, indicating the acidic amino acids at position 118 and 202 were essential for the catalysis. Both Asp97 mutations to Glu (D97E) and Ser (D97S) still kept considerable activities, indicating that Asp97 is not very important for the catalysis and not involved in the catalysis, but both mutation of Asp101 to Glu (D101E) and Ser (D101S) drastically decreased the activity. The decreases of specific activities in mutant enzymes D101E and D101S were not much different, suggesting that carboxyl group of Asp101 may not be directory required for the catalysis but was an important role in the catalytic reaction of the enzyme. While mutant enzymes E118D and E202D had a very low level of activity as same as that of the Asp101 mutants, but mutant enzymes E118S and E202S did not have a detectable activity, whose differences were higher than 10^{-4}. This results suggest that carboxyl groups of Glu118 and Glu202 are very important, or indispensable for the reaction. Thus, we conclude that Glu118 and Glu202 are the most likely candidates for participating in the catalytic mechanism of FI-CMCase.

References


