Identification of a glutamine residue essential for catalytic activity of aspergilloglutamic peptidase by site-directed mutagenesis

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Abstract **Aspergilloglutamic peptidase (AGP, formerly called aspergillopepsin II) from *Aspergillus niger* var. *macrosporus* is a unique acid protease recently classified to the peptidase family G1. Our previous study using site-directed mutagenesis on the glutamic and aspartic acid residues of AGP conserved among the G1 family suggested that Glu219 and Asp123 (numbering in the preproform) are important for catalytic activity. However, the Asn mutant of Asp123 retained weak but significant activity and therefore it was unclear whether it is an active site residue. In this study, we performed site-directed mutagenesis on all the other hydrophilic residues including Gln, Asn, Ser, Thr, and Tyr, conserved in this family to screen other residues that might be essential for catalytic function, and found that mutations of only Gln133 resulted in almost complete loss of enzymatic activity without change in the native conformation of the enzyme. Meanwhile, the 3D structure of scytalidoglutamic peptidase, a homologue from *Scytalidium lignicolum*, has been reported, indicating that Glu136 and Gln53 (the counterparts of Glu219 and Gln133 in AGP) form a catalytic dyad. Therefore, the results obtained in this and our previous studies provide with complementary evidence for the definitive conclusion on the catalytic function of the Glu/Gln dyad in glutamic peptidases.**

Keywords: Acid protease; Catalytic residue; Glutamic peptidase; *Aspergillus niger*

1. Introduction

Aspergilloglutamic peptidase (AGP, EC 3.4.23.19), previously called aspergillopepsin II, from *Aspergillus niger* var. *macrosporus* [1,2] is a unique acid protease. It has an optimum pH of 2.0 toward casein [3] and of 1.1 toward hemoglobin [1], suggesting implication of an acidic residue or residues in the catalytic mechanism of the enzyme. This enzyme, however, is not homologous to the aspartic proteases belonging to the peptidases of family A1, which are typical pepsin-type acid proteases, thus being insensitive to their specific inhibitors, such as pepstatin A [4].

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Abbreviations: AGP, aspergilloglutamic peptidase; CD, circular dichroism; SGP, scytalidoglutamic peptidase

The proteins so far known to be homologous to AGP are exclusively of fungal origin; they include scytalidoglutamic peptidase (SGP) from *Scytalidium lignicolum* (formerly scytalidopepsin B) [5], acid peptidases B and C from *Cryphonectria parasitica* (EapB and EapC) [6], and an acid protease from *Sclerotina sclerotiorum* (ACP1) [7]. AGP has a two-chain structure, composed of the light (39 residues) and heavy (173 residues) chains that are non-covalently bound to each other [8], while the other homologous proteases have a one-chain structure. The cDNA structure suggests that AGP is biosynthesized as a preproform of 282 residues including a predicted N-terminal signal peptide of 18 residues [9]. Proteolytic removal of the signal peptide should generate a proenzyme of 264 residues. Under acidic conditions, the proenzyme is auto-processed into the mature form [10].

To identify the catalytic residues of this unique acid protease, we previously performed systematic site-directed mutagenesis on all the Asp and Glu residues conserved among AGP, SGP, EapB, and EapC (Fig. 1). Since the Ala replacement of Glu219 (numbering in the preproform) made the enzyme inactive without changing the circular dichroism (CD) spectrum, we presumed this residue to be a catalytic residue [10]. Indeed, AGP and its homologues are now classified into the peptidase family G1 [11] although they were once classified into the family A4. In addition to Glu219, Asp213 was thought to be important for the catalytic mechanism because Ala mutation of Asp213 resulted in an extensive loss of enzyme activity without changing the CD spectrum [10]. However, it remained questionable whether Asp123 is a catalytic residue since its Asn mutant had a low but significant level of catalytic activity and auto-processing ability. It seemed possible that other residue(s) than Asp and Glu also contribute to the catalytic mechanism. Thus, in this study, we performed site-directed mutagenesis on all the other hydrophilic residues conserved among the family G1 proteases, and found that only Gln133 is indispensable for catalytic activity. Meanwhile, the 3D structure of SGP has been reported [12], indicating that Gln53 and Glu136, the counterparts of Gln133 and Glu219 in AGP, form a catalytic dyad in the active site, although no evidence from site-directed mutagenesis studies has yet been presented. Therefore, the results obtained in this study does provide with complementary evidence for the presumption that the dyad of the Glu and Gln residues are responsible for the catalytic function of glutamic peptidases.

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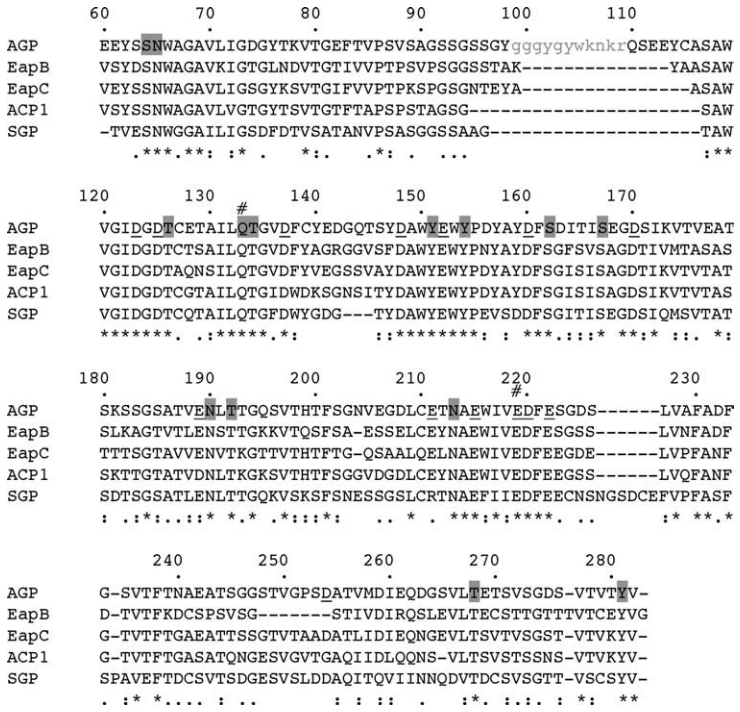

Fig. 1. Amino acid sequence alignment of the peptidases of the family G1. The sequences of the mature forms of AGP [8,9], peptidases B and C from *C. parasitica* (EapB and EapC) [6], the acid peptidase from *S. sclerotiorum* (ACP1) [7], and scytalidoglutamic peptidase from *S. lignicolum* (SGP) [5] are aligned using Clustal W [13]. Residues are numbered according to the preproform of AGP [9]. The small letters in the AGP sequence indicate the intervening sequence that is removed during maturation. The residues whose site-directed mutants were analyzed in the previous study and in this study are underlined and shadowed, respectively. The putative catalytic residues deduced from the present and previous studies are shown with #.

2. Materials and methods

2.1. Materials

The enzymes used for construction of the expression plasmids for AGP mutants were obtained from TAKARA BIO INC. (Ohtsu, Japan). The other reagents (analytical grade) were obtained from Wako Pure Chemicals (Tokyo, Japan).

2.2. Construction of expression plasmids for the AGP mutants

The expression plasmids for the AGP mutants were prepared by site-directed mutagenesis of the expression plasmid for the wild-type enzyme pAR-ANA [10]. The oligonucleotide primers used for mutagenesis are given in the order of the forward primer followed by the reverse primer for each mutant as follows. N65A: 5′-GAG GAG TAC AGC TCC GCC TGG GCT GGT GCC-3′; 5′-ACG GCA CCA GCC CAG GCG GAG CTG TAC TCC-3′. T126A: 5′-GAC GGT GAC GCC TGC GAG AC-3′; 5′-TCT CGC AGG CGT CAC CGT CG-3′. Q133A: 5′-GAG ACC GCT ATT CTC GCG ACT GGT GTC GAC-3′; 5′-AAG TCG ACA CCA GTC GCG AGA ATA GCG GTC-3′. Q133E: 5′-CGC TAT TCT CGA GAC TGG TG-3′; 5′-ACA CCA GTC TCG AGA ATA GC-3′. T134A: 5′-ATT CTC CAG GCT GGT GTC GAC-3′; 5′-CGA CAC CAG CCT GGA GAA TAG-3′. Y151F: 5′-GAT GCC TGG TTT GAG TGG TAC-3′; 5′-GTA CCA CTC AAA CCA GGC ATC-3′. Y154F: 5′-TAT GAG TGG TTC CCC GAC TAC-3′; 5′-GTA GTC GGG GAA CCA CTC ATA-3′. S162A: 5′-CTA CGA CTT CGC CGA CAT CAC CAT CTC-3′; 5′-GGT GAT GTC GGC GAA GTC GTA GGC GTA G-3′. S167A: 5′-ATC ACC ATC GCT GAG GGT GAC-3′; 5′-CAC CCT CAG CGA TGG TGA TG-3′. N190A: 5′-AGC GCC ACC GTT GAG GCC CTG ACC ACT GGC-3′; 5′-TGG CCA GTG GTC AGG GCC TCA ACG GTG GCG-3′. T192A: 5′-GAG AAC CTG GCC ACT GGC CAG-3′; 5′-GGC CAG TGG CCA GGT TCT CAA C-3′. N213A: 5′- GAC CTT TGC GAG ACC GCC GCC GAG TGG ATC-3′; 5′- ACG ATC CAC TCG GCG GCG GTC TCG CAA AGG-3′. T268A: 5′-TCC GTC CTC GCC GAG ACC TC-3′; 5′-AGG TCT CGG CGA GGA CGG AG-3′. The first PCR was carried out using pAR-ANA as a template and two sets of primers for each mutant; one is a set of a forward primer for T7-promoter, 5′-TAA TAC GAC TCA CTA TAG GG-3′ (T7p), and the reverse mutant primer, and another is a set of a reverse primer for downstream the cloning site of the T7 expression vector pAR-2113, 5′-AAC TCT CAA GGA TCT TAC CGC-3′ (ARC500), and the forward mutant primer. The second PCR was carried out using the product of the first primer as a template and a set of the primers T7p and ARC500. The amplified DNA was digested with *Nde*I and *Bam*HI and was inserted into the *Nde*I/*Bam*HI site of the expression plasmid. For the Y281F mutant, PCR was performed using the T7p primer and a reverse primer, 5′-GCG CGG ATC CTA TTA GAC GAA GGT GAC AG-3′. The amplified DNA was inserted into the *Nde*I/*Bam*HI site of pAR2113. All the expression plasmids were confirmed by DNA sequencing.

2.3. Expression, refolding, and assay of proteolytic activity

Expression was carried out as described [10]. After the purification of the recombinant protein in a denaturing buffer (8 M urea, 100 mM 2-mercaptoethanol, 50 mM NaCl, 1 mM EDTA, 50 mM sodium phosphate, pH 6.3), the solution was diluted with 10 volumes of a refolding buffer (50 mM sodium acetate, pH 5.25) containing 100 mM 2-mercaptoethanol and left to stand overnight at 4 °C. Then the solution was dialyzed against the refolding buffer twice in the presence of 2-mercaptoethanol and successively twice in the absence of the reductant. Assay of the proteolytic activity in the solution after the dialysis was carried out as described [10].

2.4. CD measurements

The CD spectra were measured at a protein concentration of 0.1 mg/ml in 50 mM sodium acetate (pH 3.5) with a Jasco J-720 spectropolarimeter at room temperature using the water-jacketed quartz cell with a light path of 1 mm. For all measurements, a 1.0-nm bandwidth and a 1.0-s time constant were used, and 32 scans were repeated from 200 to 250 nm at the speed of 50 nm/min with 0.1 nm/point resolution. Spectra were converted to mean residue molecular ellipticity prior to analysis.

3. Results and discussion

3.1. Gln133 is indispensable for enzymatic activity

There are two Ser, four Thr, three Tyr, three Asn, and one Gln residues completely conserved among the hydrophilic residues of the family G1 peptidase AGP, SGP, EapB, EapC, and ACP1 (Fig. 1). In the present study, each of the conserved Ser, Thr, Asn, and Gln residues was replaced with an Ala, and each of the conserved Tyr residues with a Phe. The mutant pro-enzymes were expressed in *Escherichia coli* to form insoluble inclusion bodies. The proteins were extracted under denaturing conditions with 8 M urea at pH 6.2 and refolded by dialysis at pH 5.25 as described [10]. If any of the refolded proteins was potentially active, it is expected to undergo autoprocessing to generate the heavy and light chains. After refolding of the purified mutant proteins, they were dialyzed against a buffer at pH 3.5. Upon SDS–PAGE of the dialyzed samples, the loss of the proform band and appearance of the heavy chain band were observed for each of the Ser, Thr, Tyr, and Asn mutants except N190A (Fig. 2A). All these mutants except N190A were found to be active toward acid-denatured hemoglobin at pH 2 (Fig. 3). Therefore, these residues are not essential for catalytic function. Since the N190A mutant protein aggregated during the refolding process, we could not assay this mutant enzyme. On the other hand, the Ala and Glu mutants of Gln133 showed neither auto-processing (Fig. 2A) nor proteolytic activity toward acid-denatured hemoglobin (Fig. 3) like the Q219A mutant. Therefore, Gln133 is indispensable for the catalytic activity of AGP, while the other conserved hydrophilic residues are not.

3.2. Gln133 is not critical for conformation of AGP

To confirm the correct folding of the mutant enzymes, we examined susceptibility of the recombinant mutants to proteolysis and analyzed their CD spectra. It is known that mature AGP is stable against autolysis, while unfolded AGP is digested by incubation with a small amount of native AGP. Therefore, if the recombinant protein has the correctly folded structure, processing of proAGP into the mature form should occur but no further proteolysis should proceed by incubation with native AGP. Actually, the Q133A and Q133E proform mutants were shown to be processed to the mature form by incubation with a small amount of the native enzyme, but further degradation was not observed (Fig. 2B and C). Therefore, these mutants are considered to have been correctly folded. The CD spectra of the mutant enzymes were compared with that of the wild-type enzyme. As shown in Fig. 4A, both proforms of the mutant Q133A and Q133E showed identical spectra with that of the E219A proform, which is known to show the identical spectrum with the wild-type proform [10]. On the other hand, after incubation with a small amount of native AGP, the spectra of the mutants were changed into that of the mature form (Fig. 4B). These results suggest that both the proforms and the mature forms of the mutant enzymes have the wild-type conformation but lack enzyme activity.

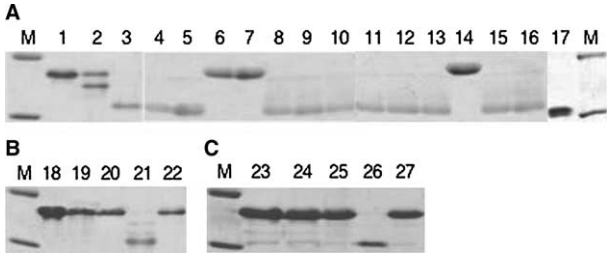

Fig. 2. SDS–PAGE of the recombinant AGP mutants after refolding in vitro and subsequent incubation at pH 3.5. (A) Lanes M, molecular size marker. The upper and lower bands are ovalbumin (45 kDa) and carbonic anhydrase (31 kDa), respectively. Lane 1, AGP denatured in the presence of 8 M urea and 100 mM 2-mercaptoethanol. Lane 2, after dilution of the denatured sample in a refolding buffer (50 mM sodium acetate, pH 5.5). Lane 3, after successive dialysis against the refolding buffer and an activation buffer (50 mM sodium acetate, pH 3.5). Lanes 4–17, the AGP mutants after the refolding and activation process: lane 4, N65A; lane 5, T126A; lane 6, Q133E; lane 7, Q133A; lane 8, T134A; lane 9, Y151F; lane 10, Y154F; lane 11, S162A; lane 12, S167A; lane 13, T192A; lane 14, E219A; lane 15, T268A; 16, Y281F; and lane 17, N213A. (B,C) Processing by native AGP of the Q133E and Q133A mutants, respectively. Lanes 18 and 23, the AGP mutants denatured in the presence of 8 M urea and 100 mM 2-mercaptoethanol. Lanes 19 and 24, after dilution of the denatured proteins in the refolding buffer. Lanes 20 and 25, after successive dialysis against the refolding buffer and the activation buffer. Lanes 21, 22, 26 and 27, the refolded proteins after incubation at pH 3.5 and 37 °C for 4 h in the presence (lanes 21 and 26) and absence (lanes 22 and 27) of a small amount of native AGP. The light chain band is not shown.

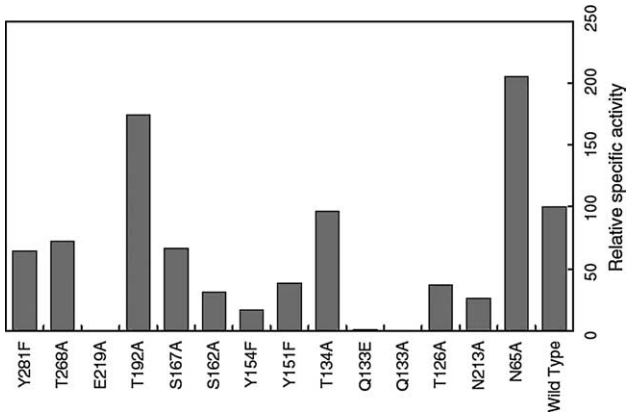

Fig. 3. Relative specific activities of wild-type AGP and the mutant enzymes toward acid-denatured hemoglobin. After refolding, the proteolytic activities of the sample solutions toward acid-denatured hemoglobin at pH 2.0 were assayed [10]. The activity of the wild-type enzyme is taken as 100%.

3.3. Glu219 and Gln133 are the catalytic residues

Very recently, the 3D-structure of SGP has been reported [12] indicating that Glu136 and Gln53 form a catalytic dyad. Glu136 and Gln53 in SGP correspond to Glu219 and Gln133 in AGP, respectively, which were demonstrated to be important for catalytic activity by site-directed mutagenesis in our previous [10] and present studies, respectively. In this study, we could not assay the N190A mutant because of aggregation during refolding process. Asn107 of SGP, which is the counterpart of Asn190 of AGP, is located at the C-terminal end of the β-strand 10 that is away from the active site [12]. Therefore, Asn190 is not thought to participate in the catalytic mechanism although it can be important for folding of AGP.

The 3D structure of AGP has also been determined recently, which is very similar to that of SGP [Sasaki, H. et al., unpublished data]; Glu219 and Gln133 in AGP thus constituting the same dyad with the Glu136 and Gln53 in SGP. Therefore, the dyad of Glu and Gln is considered to be essential for the catalytic function of the glutamic peptidases. On the other

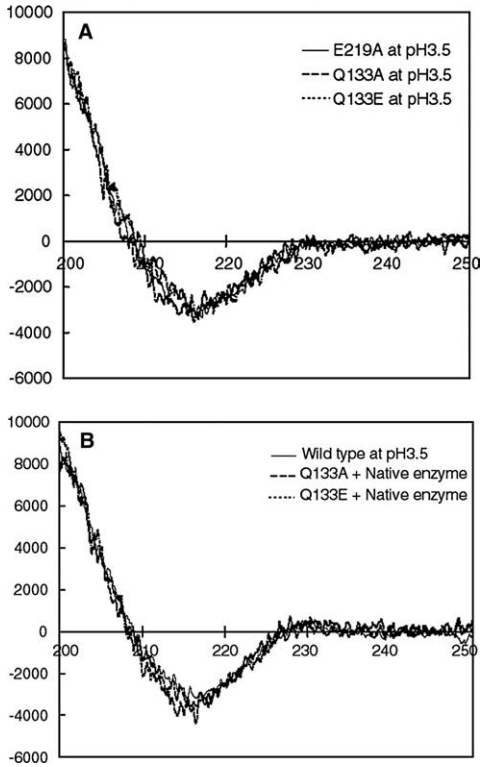

Fig. 4. CD spectra of the Q133A and Q133E mutants at pH 3.5. (A) Spectra of the recombinant proforms of the Q133A and Q133E mutants. The spectrum of the E219A mutant, which has an essentially the same spectrum with the wild-type proenzyme [10], is shown for comparison. (B) Spectra after maturation of the recombinant mutants by incubation with a small amount of native AGP. The spectrum of the wild-type enzyme is shown for comparison. The CD spectra were measured at a protein concentration of 0.1 mg/ml in 50 mM sodium acetate (pH 3.5) with a Jasco J-720 spectropolarimeter at room temperature using the water-jacketed quartz cell with a light path of 1 mm.

hand, Asp123 in AGP, whose mutations abolished or reduced the enzyme activity, and Asn190, whose mutation resulted in aggregation, have been shown to be well apart from the catalytic site like the corresponding Asp43 and Asn190, respectively in SGP.

3.4. The double mutant Q133E/E219Q is inactive

To investigate whether the positions of the Glu and Gln residues of the catalytic dyad can be functionally exchangeable, the double mutant E219Q/Q133E was prepared. As in the cases of the mutants Q133E and E219Q, the Q133E/E219Q double mutant showed no auto-processing nor maturation by incubation with a small amount of native AGP, hence no activity toward acid-denatured hemoglobin (Fig. 2). The CD spectra of the mutant, however, were indistinguishable from those of the wild-type protein both in pro and mature forms (data not shown). These results suggest that the residues at positions 133 and 219 have different roles in the catalytic mechanism, and thus unexchangeable.

4. Conclusion

The previous [10] and present studies using site-directed mutagenesis have shown that, among all the conserved hydrophilic residues in the peptidase family G1, Glu219, Gln133, and Asp123 are important for the catalytic function of AGP [10]. On the other hand, the X-ray crystallographic studies on SGP [12] have revealed that Gln53 and Glu136 (Gln133 and Glu219 in AGP) constitute a catalytic dyad, whereas Asp43 (Asp123 in AGP) is distant from the active site, leading to the conclusion that Gln53 and Glu136 in SGP are the catalytic residues. These results are also consistent with those obtained by X-ray crystallographic studies on AGP [Sasaki, H. et al., unpublished]. Since the assignment of the catalytic residues in SGP as well as in AGP was based only on the crystal structure, it is important to obtain further evidence to support it. Combination of our results from site-directed mutagenesis on AGP with the structural analysis on SGP [12], leads us to a more definite conclusion that the glutamic peptidases of the family G1 have a novel type of catalytic dyad composed of a Glu and a Gln residue. Fujinaga et al. [12] have proposed a plausible hydrolytic mechanism in which the Glu acts as a general base and the Gln provides with electrophilic assistance and oxyanion stabilization. However, further studies seem to be required to get a definite conclusion on the mechanism. Studies are now in progress along this line with AGP.

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