A unique N-linked glycosylation motif (Asn\textsuperscript{79}-Tyr-Thr) was found in the sequence of type-A feruloyl esterases from Aspergillus spp. To clarify the function of the flap, the role of N-linked oligosaccharides located in the flap region on the biochemical properties of feruloyl esterase (AwFAEA) from Aspergillus awamori expressed in Pichia pastoris was analyzed by removing the N-linked glycosylation recognition site by site-directed mutagenesis. N79 was replaced with A or Q. N-glycosylation-free N79A and N79Q mutant enzymes had lower activity than that of the glycosylated recombinant AwFAEA wild-type enzyme toward /C11-naphthylbutyrate (C4), /C11-naphthylcaprylate (C8), and phenolic acid methyl esters. Kinetic analysis of the mutant enzymes indicated that the lower catalytic efficiency was due to a combination of increased \( K_{m} \) and decreased \( k_{cat} \) for N79A, and to a considerably decreased \( k_{cat} \) for N79Q. N79A and N79Q mutant enzymes also exhibited considerably reduced thermostability relative to the wild-type.

Key words: feruloyl esterase; N-glycosylation; flap; thermostability

Feruloyl esterases (EC 3.1.1.73) hydrolyze ester linkages of ferulic acid in plant cell walls and have been classified into types A, B, C, and D based on protein sequence and substrate specificity.\textsuperscript{1} The classification of carbohydrate esterases (CEs) including feruloyl esterase is also available on the Carbohydrate-Active Enzymes (CAZY) server (http://afmb.cnrs-mrs.fr/CAZY), and CE is defined in 14 families.\textsuperscript{2} Recently, the crystal structure of feruloyl esterases, AnFAEA\textsuperscript{3} and FAE-III\textsuperscript{4} from A. niger, has been solved. Its overall structure is close to that of lipase from Rhizomucor miehei. It has been found that several lipases have an \( \alpha \)-helical loop that acts as a lid covering the active site of the enzyme. An \( \alpha \)-helical loop, designated the flap, was also found in the structure of AnFAEA/FAE-III. Mutational analysis of the flap region in feruloyl esterase from A. niger and A. awamori involved in substrate discrimination has been reported.\textsuperscript{5,6} Two N-acetylglucosamine units linked to N79, which comprises the flap region were visible in the crystal structure of A. niger feruloyl esterase. The oligosaccharide chain is attached to the asparagine residue within the consensus sequence N-X-S/T, where X is any amino acid other than proline,\textsuperscript{7,8} but a consensus sequence followed by a proline residue is unlikely to be glycosylated.\textsuperscript{9} Protein glycosylation in eukaryotic cells is thought to be important for protein folding, transport, and protein stability. The role of N-glycosylation on the activity, thermostability, and secretion of enzymes has been analyzed.\textsuperscript{10–17}

The feruloyl esterase of Aspergillus awamori (AwFAEA) has been purified and characterized,\textsuperscript{18} and the function of the flap relative to substrate discrimination has also been investigated by site-directed mutagenesis.\textsuperscript{6,10} In the present study, to clarify the function of the flap in AwFAEA, we investigated the role of N-linked oligosaccharides located in the flap region on the biochemical properties of recombinant enzyme expressed in Pichia pastoris.

Materials and Methods

Strains and culture conditions. A. awamori NBRC4033 was used as the source of the feruloyl esterase gene, and P. pastoris GS115 (his4) was used as a host for heterologous expression of AwfaeA cDNA. P. pastoris transformants were grown at 30°C in 10 ml
BMGY (1% yeast extract, 2% peptone, 1% [v/v] glycerol, 0.00004% biotin, 1.34% yeast nitrogen base with ammonium sulfate, and 10% [v/v] 1 M potassium phosphate, pH 6.0) in an incubator-shaker to a cell density of OD\textsubscript{660} = 4. Cells were harvested aseptically by centrifugation (3,000 × g, for 10 min at 4 °C). The cells were then resuspended in 100 ml BMMY (BMGY containing 0.5% [v/v] methanol instead of glycerol) in a 500-ml flask to an OD\textsubscript{660} = 1 to start induction. The culture was grown at 30 °C for 4 d in an incubator-shaker (200 rpm), with daily additions of 0.5 ml methanol to maintain induction.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), as described previously.\textsuperscript{6} Base substitutions were introduced by use of the following primers: 5'-CTCGATACTGCTACA-CCCTC-3' and 5'-GAGGGGTAGGAAGAGTAGTATCGAG-3' for the N79A substitution; and 5'-CTCGATACTGCTAGTACACCCCTC-3' and 5'-GAGGGGTAGGAAGAGTAGTATCGAG-3' for the N79Q substitution. All mutations (underlined) were verified by DNA sequencing (results not shown).

AwfaeA cDNA expression and enzyme purification. Plasmid pPICZ-αFAE was constructed to clone AwfaeA cDNA into the Clal and XbaI sites of pPICZαC for expression in P. pastoris. pPICZ-αFAE contained the α-factor secretion signal sequence. Hence the region encoding the mature AwFAEA protein was amplified and inserted in-frame downstream of the α-factor secretion signal. AwfaeA cDNA was expressed under the control of the P. pastoris alcohol oxidase 1 (aox1) promoter and terminator, as described above.

The culture was centrifuged at 5,000 × g for 15 min to remove cells and the supernatant was then concentrated by ultrafiltration using a 10,000 Da cut-off filter membrane (Millipore, Tokyo). The concentrate was applied to a DEAE-SPW HPLC anion-exchange column (15 mm × 15 cm; Tosoh, Tokyo). Protein was eluted with stepped increases in NaCl from 0 to 0.45 M in 50 mM sodium acetate buffer (pH 5.0) at a flow rate of 3 ml/min. Fractions containing feruloyl esterase activity were concentrated and characterized further.

Purified proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) on a 10–20% gradient gel (Daiichi Pure Chemicals, Tokyo) using the Laemmli method.\textsuperscript{19}

Enzyme assays. Feruloyl esterase activity was assayed as described elsewhere using α-naphthyl (αNA) esters of butyric (C4) and caprylic (C8) acids.\textsuperscript{20} \(K_m\) and \(V_{max}\) values were determined using GraFit 5.0 software (Erithacus Software, London, UK).

The specific activities of the wild-type and the mutant enzymes toward phenolic acid esters were determined using methyl-esterified substrates (methyl ferulate, sinapate, and caffeate; Apin chemicals, London, UK) at a final concentration of 1 mM in a final volume of 0.5 ml for 15 min in 50 mM sodium acetate buffer (pH 5.0) at 37 °C. Reactions were terminated by the addition of glacial acetic acid (0.2 ml). The amount of phenolic acid released was estimated by reverse phase HPLC using UV detection, and was expressed as the μmol of free phenolic acids/min under the above assay conditions.\textsuperscript{21}

Thermostability of wild-type and mutated feruloyl esterases. Purified recombinant wild-type and mutant feruloyl esterases were incubated for 15 min, 30 min, and 60 min at 50 °C and pH 5.0. After the enzymes were cooled on ice, remaining activity was measured as described by Sundberg et al.\textsuperscript{20} Irreversible thermal inactivation of purified wild-type and mutant feruloyl esterases was also examined by determining \(T_{50}\) values. The \(T_{50}\) value is the temperature at which 50% of initial catalytic activity is lost after treatment. Following incubation at temperatures ranging from 46 to 56 °C for 30 min, enzyme solutions were immediately cooled on ice, and residual activities were measured.

Results and Discussion

Purification of rAwFAEA wild-type and mutant enzymes

Heterologous expression in P. pastoris of recombinant wild-type and mutant A. awamori feruloyl esterases containing the Saccharomyces cerevisiae α-factor secretion signal resulted in active enzymes in the culture broth. The rAwFAEA wild-type and mutant enzymes were purified in a two-step procedure using ultrafiltration and anion-exchange chromatography. SDS–PAGE of the wild-type and N79A and N79Q rAwFAEA enzymes revealed single bands of approximately 37,000, 35,000, and 36,000 relative molecular mass (\(M_r\)) respectively (Fig. 1). A unique N-glycosylation recognition site (Asn\textsuperscript{79}-Tyr-Thr) was found in the
sequence of AwFAEA.6) Purified native AwFAEA migrates in SDS–PAGE gel as a 35,000 M̄ protein, whereas purified native AwFAEA after treatment with endoglycosidase H exhibits an increase in mobility on SDS–PAGE with an M̄ of 34,000,18) suggesting that native AwFAEA possesses N-linked oligosaccharides. The lower mobility of wild-type rAwFAEA expressed in P. pastoris relative to the mobility of the native enzyme from A. awamori suggests that the recombinant enzyme has a greater mannose content. On the other hand, the mobility of the N79A and N79Q mutant enzymes was greater than that of wild-type rAwFAEA, suggesting that the mutant enzymes lack N-glycosylation.

**Thermostability**

Recombinant enzymes whose N-glycosylation sites have been deleted by site-directed mutagenesis have been reported to be significantly less thermostable than their wild-type counterparts.10,11,13) On the other hand, contradictory findings indicating no significant change in thermostability have also been reported12,16) The optimum temperature of the wild-type and both mutant enzymes was about 45 °C. The effect of removing the N-glycosylation recognition site at position 79 of rAwFAEA on thermostability is shown in Fig. 2. The wild-type enzyme retained 70% activity after incubation for 60 min at 50 °C (Fig. 2A), whereas the N79A and N79Q mutant enzymes lost all activity after a 60-min incubation at 50 °C (Fig. 2A).

Removal of the N-glycosylation recognition site at position 79 significantly reduced the T50 value of the mutant enzymes (Fig. 2B and Table 1). The T50 values of the N79A and N79Q mutant enzymes were 48.1 and 47.7 °C, 3.5 and 3.9 °C lower than that of wild-type enzyme respectively (Table 1). In contrast, the wild-type enzyme lost only 10% of its activity at 48 °C (Fig. 2B). These results suggest that either the oligosaccharides linked to rAwFAEA enhance thermostability or refolding of heat-denatured protein, or that the mutation causes structural changes resulting in the observed decrease. Displacement of the lid of lipase exposes the catalytic site, converting the enzyme from the inactive (closed) to the active (open) conformation. It has been reported that the glycan chain attached to Asn79 of AnFAEA contributes to stabilization of the flap in its open conformation.3) The observed decrease in thermostability of the mutant forms of rAwFAEA lacking N79-linked glycans might be due to a decrease in stabilization of the flap.

**Specific activity**

It has been reported the N-glycosylation also influences catalytic properties.11,14,15,17) As shown in Table 2, the specific activity of the N79A and N79Q mutant enzymes decreased significantly toward the butyrate (C4) and caprylate (C8) α-naphthyl esters. While the wild-type rAwFAEA enzyme had a specific activity of 9.0, 16.9, 0.11 units/mg-protein toward methyl ferulate,

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**Fig. 2.** Thermostability of Wild-Type and Mutant rAwFAEA Enzymes.

A. Aliquots of purified wild-type and mutant rAwFAEA enzymes were incubated at 50 °C for different periods of time. After cooling on ice, residual activity was measured at pH 5.0, as described in “Materials and Methods.” B. Aliquots of purified wild-type and mutant rAwFAEA enzymes were incubated for 30 min at different temperatures. After cooling on ice, residual activity was measured at pH 5.0.

**Table 1.** T50 and ΔT50 Values of Wild-Type and Mutant rAwFAEA Enzymes

<table>
<thead>
<tr>
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<th>T50 (°C)</th>
<th>ΔT50 (°C)b</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>51.6 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>N79A</td>
<td>48.1 ± 0.1</td>
<td>−3.5</td>
</tr>
<tr>
<td>N79Q</td>
<td>47.7 ± 0.1</td>
<td>−3.9</td>
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aValues are means of two independent determinations ± standard deviations.
bΔT50 is the change in T50 value for the mutant enzyme relative to the wild type.

**Table 2.** Specific Activity of Wild-Type and Mutant rAwFAEAs toward αNA-Butyrate (C4) and αNA-Caprylate (C8)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Specific activity (units/mg-protein)</th>
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<tbody>
<tr>
<td></td>
<td>αNA-butyrate (C4)</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.59 ± 0.04</td>
</tr>
<tr>
<td>N79A</td>
<td>0.58 ± 0.01</td>
</tr>
<tr>
<td>N79Q</td>
<td>0.19 ± 0.01</td>
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</table>
mutant enzymes exhibited a significant decrease in parameters varied in a mutation-specific manner. Both normal Michaelis-Menten kinetics, while the kinetic shown in Table 3. Both mutant enzymes exhibited the flap in its open conformation.

### Kinetic parameters

Kinetic parameters with respect to αNA-butyrurate are shown in Table 3. Both mutant enzymes exhibited normal Michaelis-Menten kinetics, while the kinetic parameters varied in a mutation-specific manner. Both mutant enzymes exhibited a significant decrease in hydrolysis rate relative to the wild-type enzyme. The $K_m$ value for the N79Q mutant enzyme was close to that of the wild-type enzyme, whereas that for the N79A mutant enzyme had specific activities of 1.5, 9.1, 0.04 units/mg-protein, 0.17-, 0.54-, and 0.36-fold of that of wild-type enzyme toward the same substrates. On the other hand, the optimum pHs of the wild-type, N79A, and N79Q mutant enzymes were similar to that of the native enzyme.

Values are means of two independent determinations ± standard deviations.

### References


