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Molecular cloning and expression of novel fructosyl peptide oxidases and their application for the measurement of glycated protein

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Abstract

Fructosyl peptide oxidases, enzymes that are active against a model compound of glycated hemoglobin, N^{α}-fructosyl valyl-histidine, were characterized. To identify the primary structure of fructosyl peptide oxidases, we have prepared cDNA libraries from *Eupenicillium terrenum* ATCC18547 and *Coniochaeta* sp. NISL9330. The coding regions, both fungal fructosyl peptide oxidases consisting of 1314-bp, were obtained with degenerated primers based on the amino acid sequences and specific primers by 3′ and 5′ RACE (rapid amplification of cDNA ends). By their sequence similarities and substrate specificities, fructosyl peptide oxidases and their homologs could be categorized into two groups: (A) enzymes that preferably oxidize α-glycated molecules and (B) enzymes that preferably oxidize ε-glycated molecules. We showed that recombinant fructosyl peptide oxidases could be used to detect protease-treated fructosyl-hexapeptide, a glycated peptide that is released from HbA_{1C} by endoproteinase Glu-C, suggesting these enzymes could be useful for the enzymatic measurement of HbA_{1C}.

Keywords: Hemoglobin A_{1C}; Fructosyl peptide oxidase; Fructosyl amino acid oxidase; Amadoriase; Fructosyl-hexapeptide; Enzymatic measurement of HbA_{1C}; *Eupenicillium terrenum*; *Coniochaeta* sp.

Glycation is the non-enzymatic reaction by which Amadori compounds are produced from reducing sugars, such as glucose, and an amine. In vivo, the levels of glycated blood proteins reflect the level of glucose, since blood proteins, such as hemoglobin and albumin, are readily glycated by glucose at their amino groups [1,2]. For clinical diagnosis, the development of a selective and sensitive method for the measurement of glycated hemoglobin (HbA_{1C}) and albumin is of considerable importance. HbA_{1C} is glycated at the N-terminal valine residue of the β-subunit [3], while the glycated sites of albumin are the internal lysine residues of the protein. Therefore, N^{α}-fructosyl valine (Fru-Val) and N^{ε}-fructosyl lysine (εFru-Lys) were applied as model compounds of HbA_{1C} and glycated albumin and an extensive search was carried out to find an enzyme that shows specificity for such fructosyl amino acids.

Fructosyl amino acid oxidase (FAOX), or amadoriase, catalyzes the oxidative deglycation of glycated amino acids to produce the corresponding amino acids, glucosone, and hydrogen peroxide [4]. FAOXs have been isolated from *Corynebacterium* sp., *Agrobacterium* sp., *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. [4–7]. We have shown that the bacterial FAOXs characterized to date are distinct from eukaryotic FAOXs, based on sequence comparisons and substrate specificities [8]. Bacterial FAOXs had sequence similarity with an opine-catabolizing enzyme in *Agrobacterium* and were active against Fru-Val [8,9]. Substrate specificities of eukaryotic FAOXs have also been determined and it was found that εFru-Lys and fructosyl propylamine are good substrates for FAOXs from *Fusarium* sp., *Aspergillus* sp., and *Pichia* sp. [10–12].

To develop enzymatic determination of HbA_{1C}, we have found several proteases that could efficiently liberate fructosyl peptides from HbA_{1C} (our unpublished result). However, the FAOXs characterized previously could not be applied to the enzymatic determination of HbA_{1C}, since they did not have activity toward fructosyl peptides. Therefore, the primary aim of our study was to find a novel fructosyl peptide oxidase (FPOX) that is

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active against fructosyl peptides and that could be applied to the measurement of HbA_{1C}. Previously, we screened a fungal culture collection for FPOX using N^{α}-fructosyl valyl-histidine (Fru-ValHis) as a substrate. We found that the cell extracts of eight genera, *Achaetomiella*, *Achaetomium*, *Chaetomium*, *Coniochaeta*, *Eupenicillium*, *Gelasinospora*, *Microascus*, and *Thielavia*, showed FPOX activity [13]. In the present study, we purified and characterized two FPOXs; one from *Eupenicillium terrenum* ATCC18547 and one from *Coniochaeta* sp. NISL9330. Both enzymes have high activity toward Fru-Val and Fru-ValHis, however they differed in specificity toward εFru-Lys. cDNA coding for FPOX was cloned from the cDNA library of *E. terrenum* and *Coniochaeta* sp. Primary structures were compared to those of known FAOXs in order to elucidate the relationship between the catalytic properties and the protein structure.

Materials and methods

Materials

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt (TOOS) was purchased from Dojindo Laboratories, Japan, and horseradish peroxidase was from Kikkoman, Japan. Fru-ValHis and fructosyl amino acids were prepared as described previously [4].

Methods

Assay of FPOX activity. FPOX activity was measured spectrophotometrically at 37 °C with the peroxidase-coupled reaction system, as described previously [8]. The standard reaction mixtures contained 0.1 M potassium phosphate, pH 8.0, 0.5 mM TOOS, 900 U/L of peroxidase, 0.45 mM of 4-aminoantipyrine, and 20 mM Fru-ValHis in a final volume of 3 ml. One unit of enzyme activity was defined as the amount of enzyme that produced 0.5 μmol of quinoneimine dye per minute at 37 °C.

Preparation of cell extracts and purification of FPOX. E. terrenum ATCC 18547 was grown aerobically at 25 °C for 96 h in 6 L of medium (0.1 g of yeast extract, 0.1 g of malt extract, 0.1 g of KH_2PO_4, and 0.05 g of $MgSO_4$ in 100 ml, pH 7.3). Washed mycelia were suspended in TEG buffer (10 mM Tris–HCl buffer, pH 8.0, containing 1.0 mM EDTA and 1.0% glycerol) and disrupted by a French press. The homogenate was centrifuged at 9000*g* for 30 min to remove intact cells and cell debris. Ammonium sulfate was added to the above supernatant to 40% saturation and then the precipitate formed was pelleted by centrifugation at 15,000*g* for 10 min. The supernatant to which ammonium sulfate was added to 60% saturation was centrifuged at 15,000*g* for 10 min. The resultant precipitate was dissolved in TEG buffer and dialyzed. The dialyzed enzyme solution was applied to an Ultrogel AcA34 (IBF Biotechnics, France) equilibrated with TEG buffer. Gel filtration chromatography was carried out with the same buffer. The active fractions were collected and dialyzed, and then applied to a Q Sepharose FF column (Amersham Bioscience) equilibrated with TEG buffer. The absorbed protein was eluted with a linear NaCl gradient (0–0.5 M). The active fractions were collected and dialyzed by ultrafiltration, and then applied to a Poros R2/M column (PerSeptive Biosystems, Japan) equilibrated with TEG buffer containing 2.0 M ammonium sulfate. The absorbed protein was eluted with a linear gradient of ammonium sulfate (2.0–0 M) in TEG buffer. The specific activity of the purified preparation was 3.6 U/mg. Purification of FPOX from *Coniochaeta* sp. NISL 9330 was carried out as described previously [13].

Protein methods. Protein was measured with a Bio-Rad protein assay kit, using bovine serum albumin as a standard. The molecular mass of each purified enzyme was determined by gel filtration chromatography on a G3000SWXL column (7.8 mm∅ × 30 cm; Tosoh, Japan) equilibrated with 20 mM potassium phosphate, pH 8.0, containing 5% glycerol and 0.15 M NaCl. Glutamate dehydrogenase, lactate dehydrogenase, enolase, myokinase, and cytochrome *c* were used as molecular standards. The molecular masses of the subunit were determined by SDS–PAGE. The purified FPOXs from two fungi were partially digested with trypsin or endoproteinase Lys-C (Roche Diagnostics, Japan), processed with ABI 173A MicroBlotter system (Applied Biosystems, Japan), and then blotted onto a PVDF membrane. The amino acid sequence of the peptide in each spot was determined by Edman's method with a protein sequencer (Applied Biosystems model 476A).

RNA isolation and reverse transcriptase-mediated PCR. Total RNA was isolated from *E. terrenum* and *Coniochaeta* sp. using Isogen (Wako, Japan), essentially according to the manufacturer's instructions. cDNA libraries were synthesized from the total RNA with oligo(dT) primers and Avian-Myeloblastosis virus reverse transcriptase (Takara, Japan). Degenerate PCR oligonucleotides were designed using the peptide sequences generated from the Edman degradation reactions. By using the cDNA library of *Eupenicillium* along with the 5′-degenerated primer (primer 1, Table 3) and 3′-degenerated primer (primer 2), a fragment of *Eupenicillium* FPOX cDNA was obtained by degenerated PCR. Similarly, a fragment of *Coniochaeta* FPOX cDNA was obtained using the cDNA library of *Coniochaeta* with the 5′-degenerated primer (primer 3) and 3′-degenerated primer (primer 4) by degenerated PCR.

3′ and 5′ RACE. Specific primers were synthesized for 3′ and 5′ RACE on the basis of the partial nucleotide sequence of *Eupenicillium* and *Coniochaeta* cDNAs. For 3′ RACE of *Eupenicillium* FPOX cDNA, 3′ RACE primer (primer 5) and oligo(dT)-adapter primer (Takara, Japan) were used. For 5′ RACE of *Eupenicillium* FPOX cDNA, the 5′-Full RACE core set (Takara, Japan) was used with the anchor primer (primer 6), 1st primer pairs (primers 7, 8), and 2nd primer pairs (primers 9, 10). Similarly, 3′ RACE primer (primer 11) and oligo(dT)-adapter primer were used for 3′ RACE of *Coniochaeta* FPOX cDNA. The anchor primer (primer 12), 1st primer pairs (primers 13, 14), and 2nd primer pairs (primers 15, 16) were used for 5′ RACE of *Coniochaeta* FPOX cDNA.

PCR amplification. The *Eupenicillium* and *Coniochaeta* cDNAs were added to the reaction mixture as a template for PCR (2.5 mM $MgCl_2$, 20 pmol of primers, PCR buffer, and 2.5 U of *Taq* polymerase). Amplification consisted of denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s.

Cloning of PCR products. PCR products were analyzed by electrophoresis on a 1% agarose gel (Takara, Japan). They were cloned into a linearized pT7Blue T-vector (Novagen, UK) and subsequently introduced into *Escherichia coli* JM109 cells. Recombinant plasmids were selected by blue white selection and sequenced using the CEQ 2000XL DNA Analysis System and CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, USA).

Subcloning for FPOX genes. For the amplification of the coding region of the FPOX-E transcript, an upstream primer (primer 17) and a downstream primer (primer 18), corresponding to the amino acid sequences of the N- and C-terminal regions of FPOX-E, were used. PCR was carried out in 50 μl of a reaction mixture containing 50 pmol of each primer, 120 mM Tris–HCl, pH 8.0, 1.0 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 0.2 mM dNTP. Thirty cycles (98 °C for 15 s, 65 °C for 2 s, and 74 °C for 30 s) of PCR were performed using 2.5 U of KOD polymerase (Toyobo, Japan). The amplified fragment was purified by agarose gel electrophoresis and

inserted into the *Sma*I site of pUC19 (Takara, Japan), giving pUC-EFP. In a similar way, *Coniochaeta* FPOX cDNA was inserted into the *Eco*RI site of pKK223-3 (Amersham Pharmacia Biotech) using an upstream primer (primer 19) and a downstream primer (primer 20), giving pKK-CFP. Sequence analysis of the inserted region was carried out by the dideoxy chain termination method.

Expression and purification of recombinant FPOX-E and -C. The plasmid carrying the cDNA of FPOX-E or -C was introduced into *E. coli* JM109 cells and the transformants were cultured in 10 L of LB medium containing 50 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Then, the cells were harvested by centrifugation and FPOX-E or -C was purified by the same procedure used for native FPOXs from fungi cells.

Enzymatic measurement of glycated peptide. N^{α}-fructosyl Val-His-Leu-Thr-Pro-Glu (Fru-hexapeptide; Peptide Institute, Japan) dissolved in water at several concentrations was used as the standard solution. Five microliters of the standard solution and 5 μl of *Aspergillus oryzae* protease (20 mg/ml; Kikkoman, Japan) in 50 mM potassium phosphate buffer (pH 6.5) were incubated at 37 °C for 2 h. After heat inactivation (90 °C, 5 min), 140 μl of reaction mixture was added, incubation was continued at 30 °C for 20 min, and then the absorbance of the solution at 555 nm was measured. The reaction mixture contained 0.1 M potassium phosphate, pH 8.0, 0.5 mM TOOS, 900 U/L peroxidase, 0.45 mM 4-aminoantipyrine, and 0.1 U/ml FPOX-E or -C in a final volume of 150 μl. In addition, bacterial FAOX (Kikkoman, Japan) and fungal FAOX (ketoamine oxidase; Asahi Kasei, Japan) were used for the control experiments.

Results

Purification of FPOXs from E. terrenum and Coniochaeta sp.

FPOX was purified from the cell extract of *E. terrenum* with Fru-ValHis (Table 1). The purified FPOX from *E. terrenum* (FPOX-E) has a specific activity of 3.6 U/mg for the substrate Fru-ValHis. FPOX from *Coniochaeta* sp. (FPOX-C) was also purified from the cell extract by a similar process and its specific activity was 24 U/mg. The molecular mass of the enzyme subunit was estimated by SDS–PAGE to be 50,000 for FPOX-E and 52,000 for FPOX-C. G3000SWXL gel filtration showed that the molecular mass of the protein was 50,000 for FPOX-E and 60,000 for FPOX-C. From these results, the enzymes were considered to be monomeric.

Molecular cloning of FPOX cDNAs from E. terrenum and Coniochaeta sp.

Peptide sequences that were identified from the protease digestion of purified FPOX-E and -C are presented in Table 2. To initiate the cloning of FPOX-E, a PCR approach was taken (see "Materials and methods"), which resulted in the generation of a 924-bp PCR product. Several of the peptides identified from the peptide sequencing were found encoded in the 924-bp product, indicating that a partial cDNA encoding the FPOX-E had been obtained. Based on the partial cDNA for FPOX-E, several primer pairs were designed and used in 3′ and 5′ RACE to obtain sufficient amounts of specific PCR products for sequencing. The sequences of the 3′ and 5′ RACE fragments were connected to the 924-bp product by overlap to deduce the full length of the cDNA for FPOX-E. The sequence was 1314-bp long from the start codon (ATG) to the stop codon (TGA), which encodes a 437 amino acid polypeptide. (Fig. 5). The cDNA clone of FPOX-C was obtained using essentially the same method. The cDNA consisted of 1314-bp, which was of the same length as that of *Eupenicillium* cDNA. The deduced amino acid sequence of FPOX-C showed 76% identity with that of FPOX-E (Fig. 5). The cDNA sequences of FPOX-E and FPOX-C have been submitted to the DDBJ Nucleotide Sequence Database (Accession Nos. AB116146 and AB116147, respectively).

Table 2
Peptide sequences from the protease digestion of FPOX-E and -C

Peptide		Amino acid sequence
FPOX-E	Seq 1	TNVWLESE
	Seq 2	LHQPYGA
	Seq 3	PTDTYP
	Seq 4	NFILA
	Seq 5	LPNIG
	Seq 6	DLAEMPGW
FPOX-C	Seq 1	THAWLDNEDEIL
	Seq 2	QDGGWLAAAKAINAIGQFLK
	Seq 3	DKELFNR
	Seq 4	NFILATGDSGHSF
	Seq 5	HVVELIEGRLPEEMAYQWR
	Seq 6	APPKDLADMPGWKH

Expression of FPOX cDNAs in E. coli

The plasmid carrying FPOX-E or FPOX-C cDNA was introduced into *E. coli* JM109. The cells were disrupted and the extracts were assayed for FPOX activity using Fru-ValHis as a substrate. JM109 [pUC-EFP] and JM109 [pKK-CFP] showed FPOX activity, while

Table 1
Summary of the purification of FPOX from *Eupenicillium terrenum* ATCC 18547

	Total activity (U)	Total protein (mg)	Specific activity (U/ml)	Purification (fold)	Yield (%)
Cell-free extract	0.765	298	0.00257	1	100
Ammonium sulfate (40–60% sat.)	0.780	71.6	0.0109	4.2	100
Ultrogel AcA34	0.643	6.69	0.0961	37	84
Q Sepharose FF	0.507	0.178	2.85	1100	66
POROS PE	0.0612	0.017	3.59	1400	8

Table 3
Primers used in polymerase chain reactions

(1) FPOX-E forward	5′-ACNAAYGTNTGGCTNGARWSNG-3′
(2) FPOX-E reverse	5′-KCCANCCNGGCATYTCNGC-3′,
(3) FPOX-C forward	5′-TGGYTNGAYAAYGARGAYGARAT-3′
(4) FPOX-C reverse	5′-TTRAARTTRTGICCRAARTCICCIGT-3′
(5) FPOX-E 3′ RACE	5′-CATCCCACAGATACCTACCCT-3′
(6) FPOX-E 5′ RACE anchor	5′-TCCCGATGTTTGGCAACAGC-3′
(7) FPOX-E 5′ RACE 1st forward	5′-GTGCCTGTGGTCTATGATGGTG-3′
(8) FPOX-E 5′ RACE 1st reverse	5′-CTCCACGCACCAGCAGCCAAGACAACCTTG-3′
(9) FPOX-E 5′ RACE 2nd forward	5′-ATCAACCGTACGGGGCTGCA-3′
(10) FPOX-E 5′ RACE 2nd reverse	5′-GATCGCATTGATAGCCTTGGC-3′.
(11) FPOX-C 3′ RACE	5′-CCCACAGACACTTATCCAGA-3′
(12) FPOX-C 5′ RACE anchor	5′-ACTCAGCGGCCTCTT-3′,
(13) FPOX-C 5′ RACE 1st forward	5′-AGATGGTACCAAATATTACGCTGACAAG-3′
(14) FPOX-C 5′ RACE 1st reverse	5′-TTTACACCACGTTCTTTCAAGAACTGT-3′
(15) FPOX-C 5′ RACE 2nd forward	5′-AAGGCTTGGGTGTATGCTCATATTCA-3′
(16) FPOX-C 5′ RACE 2nd reverse	5′-TCCTTGTATTTGGTCACGTTGAAGCAA-3′
(17) FPOX-E expression forward	5′-GACATGGCTCATTCGCGTGCAAGC-3′
(18) FPOX-E expression reverse	5′-CAAGAATCACAAATGTGCATCATGC-3′
(19) FPOX-C expression forward	5′-ATGACGTCGAATCGTGCAGATAC-3′
(20) FPOX-C expression reverse	5′-TTACAATTTCGGATCATGTTTCCAT-3′

JM109 [pUC19] and JM109 [pKK223-3] did not, implying that both FPOXs were expressed in active forms in *E. coli*. The FPOX activity in the cell extracts of JM109 [pUC-EFP] and JM109 [pKK-CFP] was 0.01 and 4.7 U/ml, respectively.

Properties of recombinant FPOX-E

The FPOXs from JM109 [pUC-EFP] and JM109 [pKK-CFP] were purified from the cell extract as described in Materials and methods. The purified preparations showed single bands on SDS–PAGE, indicating an apparent homogeneity of the proteins. The purified FPOX-E and FPOX-C had a specific activity of 5.43 and 23.8 U/mg of protein, respectively. The optimum pH for the enzyme activity of FPOX-E and FPOX-C was between 7.5 and 8.0 (Figs. 1A and B). Both enzymes were most active between 30 and 45 °C (data not shown). Incubation at 55 °C for 10 min resulted in complete inactivation of both enzymes (Figs. 2A and B). Enzyme activities were greatly reduced at pH values below 5.5 and above 9.5 (data not shown). In pH and temperature properties, the recombinant proteins were indistinguishable to the native proteins purified from fungi.

Substrate specificities of recombinant FPOXs

To investigate the substrate specificity of FPOXs, the enzyme assay was performed with varying concentrations of substrate. A typical Michaelis–Menten curve was obtained either when FPOX-E activity was measured with Fru-ValHis and Fru-Val (Fig. 3A) or when FPOX-C activity was measured with Fru-ValHis, Fru-Val, and εFru-Lys (Fig. 3B). Apparent K_m values of

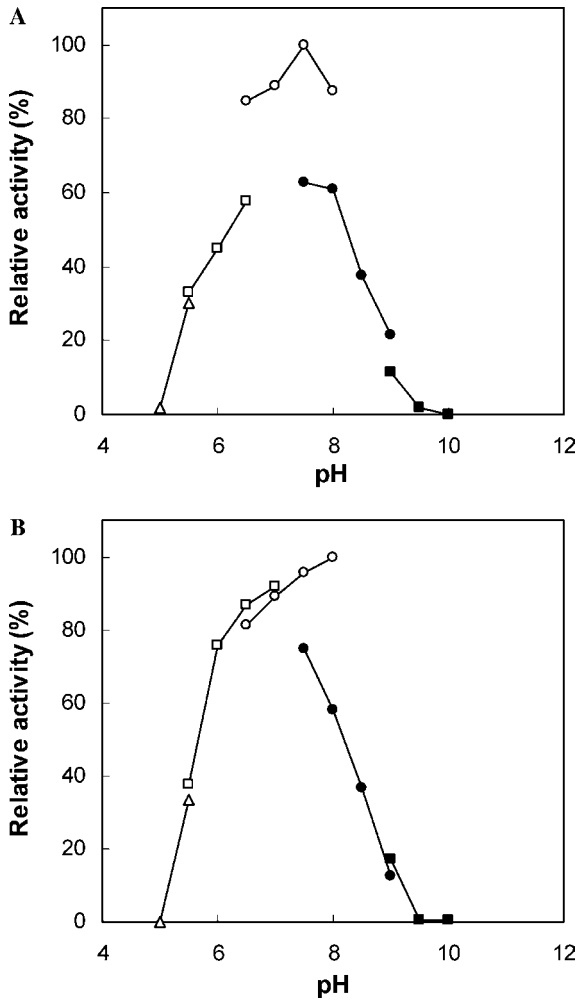

Fig. 1. Effects of pH on the activities of FPOX-E (A) and FPOX-C (B). Enzyme activities were measured in a standard reaction mixture in various buffers at 100 mM. Buffers: (△) acetate; (□) Mes–NaOH; (○) phosphate; (●) Tris–HCl; and (■) Ches–NaOH.

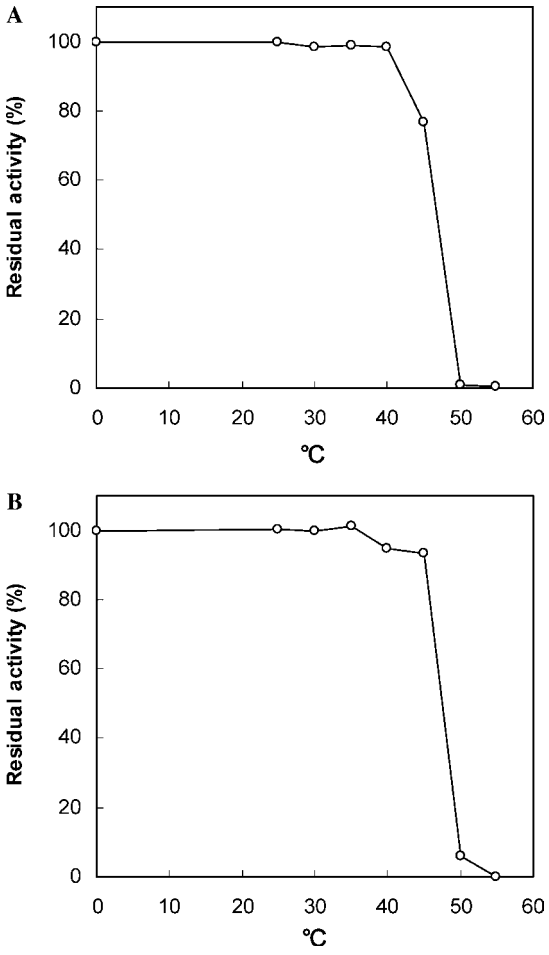

Fig. 2. Thermostability of FPOX-E (A) and FPOX-C (B). The activity remaining was measured after the purified enzyme was incubated in 100 mM potassium phosphate buffer (pH 8.0) at various temperatures for 10 min.

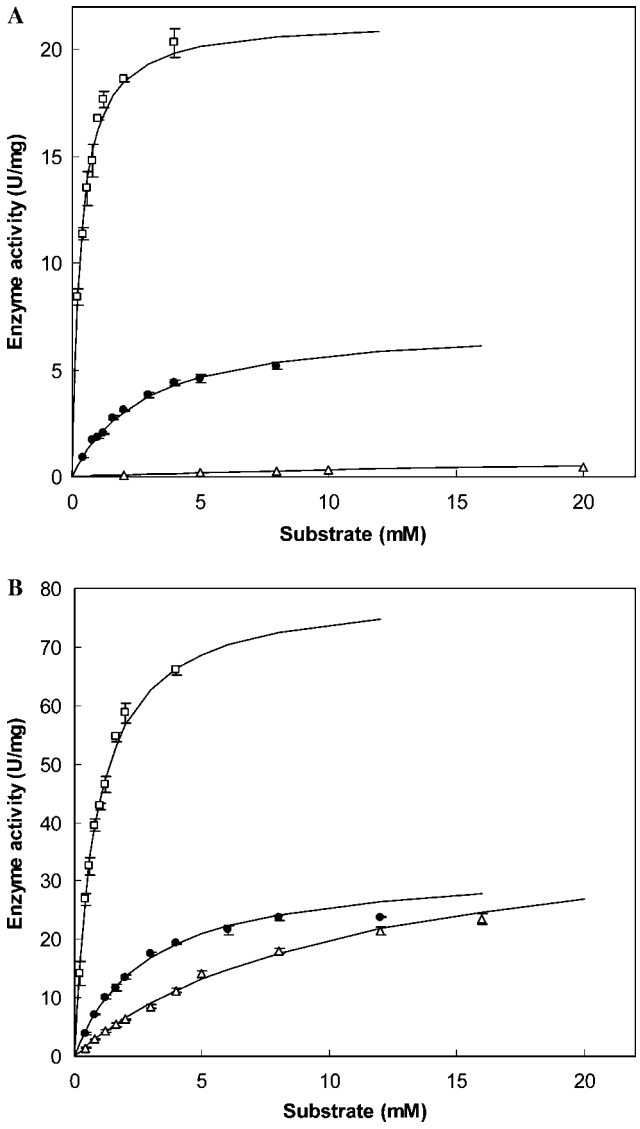

Fig. 3. Michaelis–Menten plots of FPOX-E (A) and FPOX-C (B) for glycated molecules. The plots were obtained by the standard reaction of FPOXs with different concentrations of N^{α}-fructosyl valine (□), N^{α}-fructosyl valyl-histidine (●), and N^{ε}-fructosyl lysine (△).

FPOX-E were 2.76 mM for Fru-ValHis and 0.318 mM for Fru-Val (Table 4), indicating high specificity toward Fru-Val. FPOX-C was also active toward εFru-Lys, although FPOX-E had quite low activity against εFru-Lys (Figs. 3A and B). Apparent K_m values of FPOX-C were 2.81 mM for Fru-ValHis, 0.824 mM for Fru-Val, and 10.6 mM for εFru-Lys, indicating also high specificity toward Fru-Val (Table 4).

Enzymatic measurement of glycated hexapeptide

HbA_{1C} is measured with the β-N-terminal hexapeptide (Fru-hexapeptide), which is released by enzymatic cleavage of the intact glycated hemoglobin molecule with endoproteinase Glu-C, using HPLC-ESI/MS or HPLC-CE [14]. For the enzymatic determination of Fru-hexapeptide, protease-treated Fru-hexapeptide was used as substrate for the FPOX reaction. As shown in Fig. 4, a linear relationship existed between the concentrations of protease-treated Fru-hexapeptide and the absorbance for FPOX reactions. A number of proteases were tested in the reaction, and the *Aspergillus* protease was found to be one of the most effective for liberating substrate for FPOXs from Fru-hexapeptide. Since no absorbance was detected in the reaction of bacterial FAOX and fungal FAOX, FPOX activity toward fructosyl peptide was suggested to be indispensable for the enzymatic measurement of glycated hexapeptide.

Sequence similarity of FPOXs and FAOXs

A sequence alignment of FPOXs with existing FAOXs and other related proteins is shown in Fig. 5. The N-terminal AMP-binding motif, GxGxxG, and FAD attachment residue, Cys342 [15], were conserved in all proteins. Among six FPOX homologs, FAOD-P and

Table 4
Properties of N^{α}-fructosyl valine oxidases and N^{ε}-fructosyl lysine oxidases

	Molecular mass		Specific activity (U/mg)					Michaelis constant (mM)				
	Gel filtration	SDS–PAGE	α-Glycated molecule		ε-Glycated molecule			α-Glycated molecule			ε-Glycated molecule	
			Fru-Val	Fru-ValHis	αFru-εZlys	εFru-αZLys	εFru-Lys	Fru-Val	Fru-ValHis	αFru-εZlys	εFru-αZLys	εFru-Lys
Fru-Val oxidase												
FPOX-C	60	52	66.0	23.8			23.4	0.824	2.81			10.6
FPOX-E	50	50	20.6	5.43			0.42	0.318	2.76			
FAOX-P [6]	39	49	18.6		18.6	4.18				0.62	0.19	
εFru-Lys oxidase												
FAOX-A [6]	106	51	9.62		9.62	32.0				0.51	0.37	
FLOD S-1F4 [7]	45	50	<0.01		<0.1		48.9			1.33	0.22	0.22
FLOD IFO9972 [17]	48	47	3				30				0.194	

Z-, benzyloxycarbonyl-.

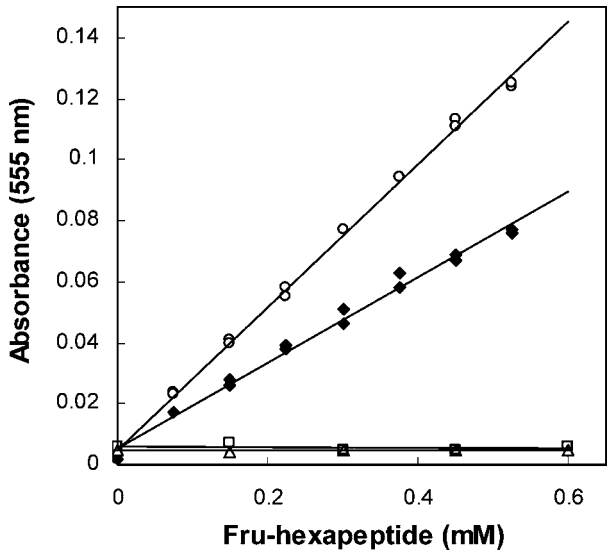

Fig. 4. Enzymatic measurements of Fru-hexapeptide with FPOXs. Fru-hexapeptide solutions at various concentrations were used for the protease-coupled FPOX activity. Symbols: (○) FPOX-C; (◆) FPOX-E; (△) bacterial FAOX; and (□) fungal FAOX.

FaoA showed striking similarity (70–85%) with FPOXs. On the other hand, FAOD-A, Amadoriase II, Amadoriase I, and FLOD showed low levels of similarity to FPOXs (30–40%), and their sequences were very similar to each other (≧50%). From these results, FPOXs and their homologs could be divided into two groups, the upper and lower groups in Fig. 5. This is reasonable given the substrate specificity of the enzymes, because the enzymes in the upper group (including FPOXs) had a similarly high level of activity against α-glycated molecules (e.g., Fru-Val), while those of the lower group had high levels of specificity toward ε-glycated molecules (e.g., εFru-Lys) (Table 4).

Discussion

Recently we have screened for a novel fructosyl peptide oxidase, an enzyme that could be used for the measurement of glycated hemoglobin levels in diabetic subjects with hyperglycemia [13]. Fructosyl peptide oxidases have been found in strains of eight genera: *Achaetomiella*, *Achaetomium*, *Chaetomium*, *Coniochaeta*, *Eupenicillium*, *Gelasinospora*, *Microascus*, and *Thielavia*. By using a purified FPOX from *Achaetomiella virescens*, we have shown that Fru-ValHis was consumed and the same molar amount of valyl-histidine was produced by the FPOX reaction [13]. The properties of the FPOXs characterized in this study are summarized in Table 4. FPOX-E and -C showed higher specificity toward Fru-Val than Fru-ValHis, however the activity toward Fru-ValHis is novel and particularly applicable to the enzymatic measurement of HbA_{1C}

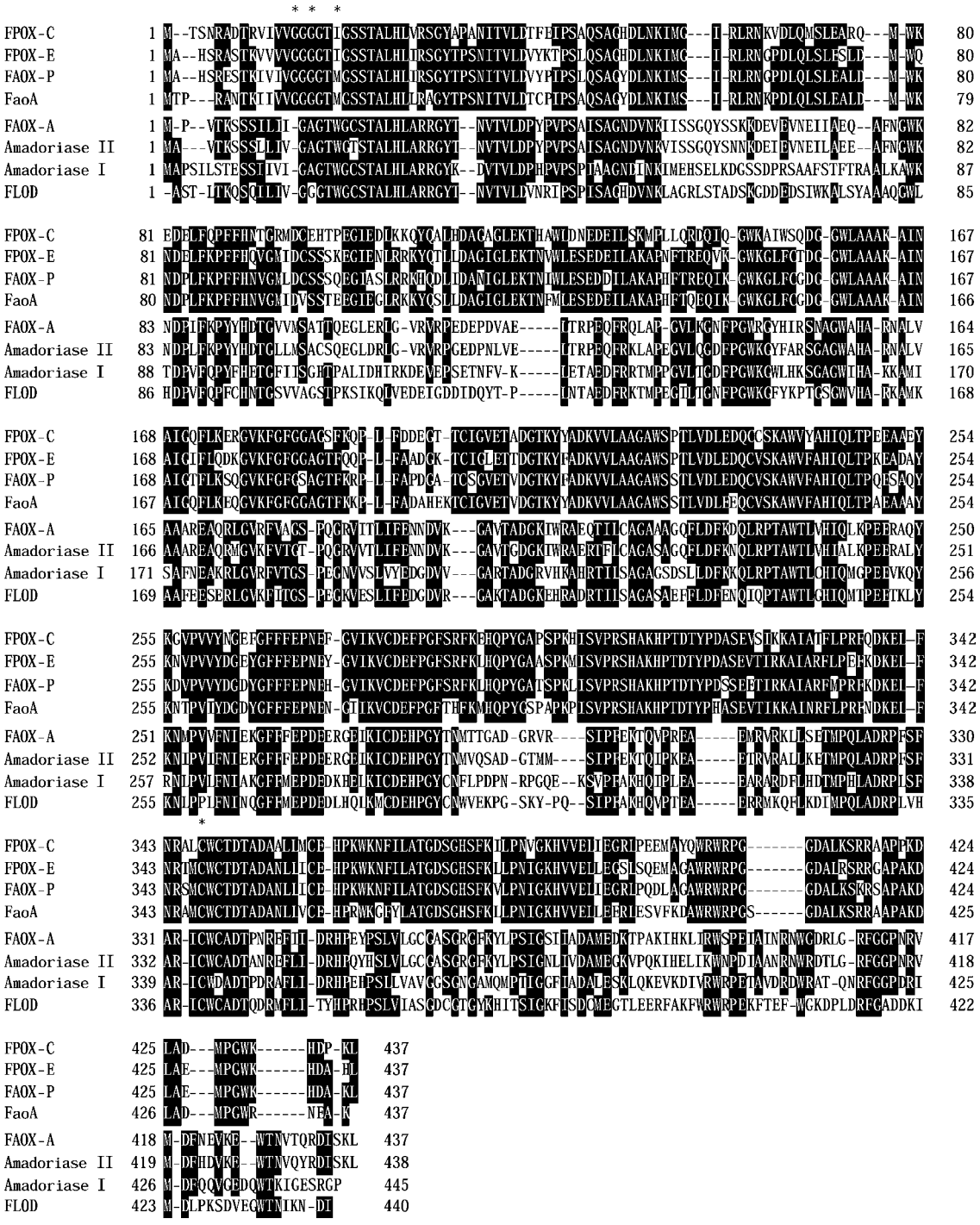

Fig. 5. Multiple sequence alignment of deduced amino acid sequences of FPOX-C, FPOX-E, and their homologs. Amino acid residues conserved in at least three of the four sequences in each group (upper, Fru-Val oxidases; lower, εFru-Lys oxidases) are shaded. Gaps (indicated by dashes) were appropriately introduced for optimal alignment. The residues of the AMP-binding motif and FAD attachment site of Cys are marked by asterisks. FPOX-C, FPOX from *Coniochaeta* sp.; FPOX-E, FPOX from *E. terrenum*; FAOX-P, FAOX from *Penicillium janthinellum* [6]; FaoA, FAOX from *Aspergillus nidulans* [16]; FAOX-A, FAOX from *Aspergillus terreus* [6]; Amadoriase II, I, Amadoriase from *Aspergillus fumigatus* [11,15]; and FLOD, N^{ε}-fructosyl lysine oxidases from *Fusarium oxysporum* IFO 9972 [17].

(discussed below). It is interesting that no FPOX activity was observed in FAOXs in spite of high sequence similarity toward FPOXs (Fig. 5). This difference in substrate recognition is thought to be determined by the substrate binding site of the enzymes. To reveal this mechanism of substrate recognition, cassette mutagenesis of FPOXs and FAOD-P or FaoA would provide important information.

FPOX-E and -C showed higher activity and lower K_m values toward Fru-Val than εFru-Lys (Table 4). And FAOX-P also showed that V_{max}/K_m for αFru-εZLys was much higher than that for εFru-αZLys [6], indicating that these three enzymes had higher specificity toward α-glycated molecules than ε-glycated molecules. In contrast, FAOX-A, FLOD S-1F4, and FLOD IFO9972 showed lower activity and/or higher K_m values toward α-glycated molecules (such as Fru-Val and αFru-εZLys) than ε-glycated molecules (such as εFru-Lys and εFru-αZLys), indicating high specificity toward ε-glycated molecules. Therefore, we categorized FPOXs and their homologs into two groups: (A) enzymes that preferably oxidize α-glycated molecules and (B) enzymes that preferably oxidize ε-glycated molecules (formerly described as fructosyl lysine oxidases [7]) (Table 4). A comparison of amino acid sequences revealed that these two groups (A and B) corresponded to the two groups that show high sequence similarity (Fig. 5). Therefore, they could be described as Fru-Val oxidases and εFru-Lys oxidases, and our novel FPOXs belonging to the former group. This relationship between sequence similarities and substrate specificities seemed reasonable, however further studies are needed to confirm this speculation.

FPOXs could be a useful diagnostic tool for determining the amount of HbA_{1C}. We are currently developing an enzymatic assay system for HbA_{1C} using FPOXs, since there are several proteases that are able to generate fructosyl peptides quite efficiently. In the present study, we have cloned, purified, and characterized two FPOXs; one from *Eupenicillium* and one from *Coniochaeta*. Both enzymes have strong activity toward Fru-Val and Fru-ValHis, however they differed in specificity toward ε-glycated molecules; FPOX-C acted on εFru-Lys, while FPOX-E showed slight activity toward εFru-Lys. Since Fru-hexapeptide is reported to be liberated from HbA_{1C} by Glu-C endoproteinase [14], we examined the enzymatic measurement of Fru-hexapeptide using FPOXs. As shown in Fig. 4, a linear relationship was obtained between the concentration of protease-treated Fru-hexapeptide and the absorbance for the FPOX reaction. The absorbance was not detected when we used bacterial FAOX and fungal FAOX (neither enzyme showed activity toward fructosyl peptide), indicating that not Fru-Val but Fru-ValHis was released by the protease treatment.

In this study, it was suggested that both FPOX-E and -C were available for assaying Fru-hexapeptide. However, FPOX-E could be a better candidate for the assay of HbA_{1C}, because internal Lys residues of proteins were glycated when the glucose level was high, and so εFru-Lys could become a major contaminant of the assay system. As FPOX-E acts mainly against Fru-ValHis even in the presence of εFru-Lys, it should provide for a precise assay of HbA_{1C}. Study is in progress to establish a rapid and simple enzymatic assay system for HbA_{1C} using FPOX-E.

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