Functional Analysis of Fructosyl-Amino Acid Oxidases in Fungi

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CONTENTS

ABBREVIATIONS

Enzymes

FAOD	Fructosyl-amino acid oxidase
FNK	Fructosamine kinase
FPOD	Fructosyl-peptide oxidase
POX	Pipecolic acid oxidase
SAX	Saccharopine oxidase
SOX	Sarcosine oxidase

Substrates

Fru-Lys	Fructosyl L-lysine
Fru-Val	Fructosyl L-valine
Fru-Z-Lys	N^{ε}-Fructosyl N^{α}-benzyloxycarbonyl-L-lysine

INTRODUCTION

There have been many reports for Maillard reaction *in vivo*, since the finding of glycated hemoglobin (1, 2). Amadori products such as fructosyl-amino acids are formed by nonenzymatic glycation reaction, in which biological amines (e.g., free amino acids, amino acid residues of proteins, and amino groups of nucleic acids) react with reducing sugars, at an early stage of the Maillard reaction. The resultant Amadori compounds undergo repeated dehydration and condensation to form stable compound called advanced glycation end product (AGE) (3–6) (Fig. 0.1). Glycation affects the function of the proteins *in vivo* (2), and protein cross-links with AGE cause the development of diabetic complication and aging (1). Thus, it is important to investigate the intervention against the Maillard reaction (7).

Two types of deglycation enzymes have so far been identified: fructosyl-amino acid oxidases (FAODs) (EC 1.5.3) (8–17) and fructosamine kinases (FNKs) (EC 2.7.1) (18–21).

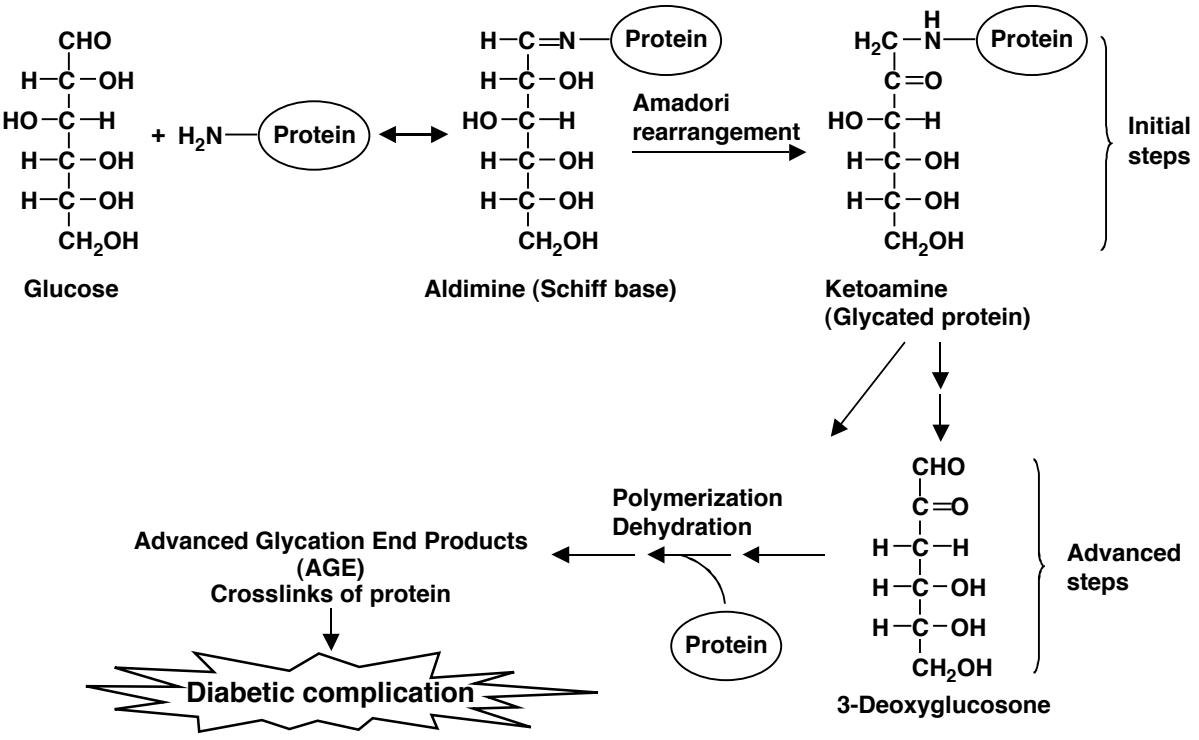

Fig.0.1. Glycation of protein.

Both enzymes act on fructosyl-amino acids but the deglycation mechanisms are quite different. FAOD catalyzes the oxidative deglycation of fructosyl-amino acids, yielding corresponding amino acid, glucosone, and H_2O_2 (Fig. 0.2A) (12, 22). The enzymes were found in different organisms and characterized by the author's group and other research groups. Some of their properties are listed in Table 0.1. Most FAODs show this type of oxidation, but the enzyme found in *Pseudomonas* sp. has a somewhat different reaction mechanism (15) (Fig. 0.2B). This enzyme produces fructosamine from fructosyl-amino acid, indicating that the C-N bonds cleaved are different for these two types of enzyme. FNKs are found in mammals (18, 19) and *Escherichia coli* (20) (Table 0.2) and phosphorylate the third and the sixth carbons, respectively, in the fructosyl moiety of fructosyl-amino acids. The corresponding amino acid and 3-deoxyglucosone are formed from fructosyl-amino acid by the mammalian enzyme (Fig. 0.3A), whereas another hydrolase is needed for deglycation of the phosphorylated compound produced by bacterial FNK (Fig. 0.3B).

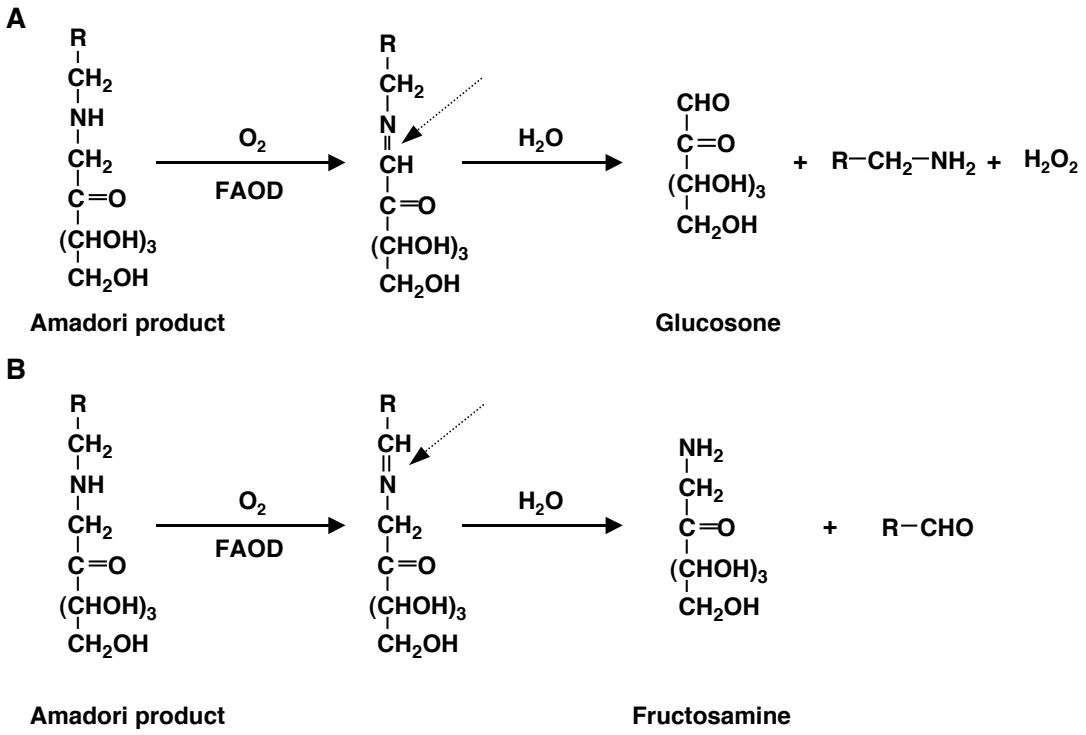

Fig. 0.2. Proposed reaction mechanisms of FAODs. Arrows, C-N bonds to be cleaved. (A) Typical reaction observed with most FAODs; (B) Unusual reaction observed with FAOD from *Pseudomonas* sp.

Table 0.1. FAODs in microorganisms.

Source	Molecular mass (kDa)	Cofactor	K_m (substrate)	V_{max}	References
Aspergillus oryzae (FaoAo1)	49	FAD	0.51 mM (Fru-Z-Lys)	2.7	This study
Aspergillus oryzae (FaoAo2)	98 (dimeric)	FAD	0.22 mM (Fru-Z-Lys)	17.7	This study
			1.38 mM (Fru-Val)	31.2	
Aspergillus fumigatus (Amadoriase I)	51	FAD	3.1 mM (N^{ε}-fructosyl N^{α}-*t*-Boc-lysine)	318	8
			9.75 mM (fructosyl glycine)	36	9
Aspergillus fumigatus (Amadoriase II)	49	FAD	1.6 mM (N^{ε}-fructosyl N^{α}-*t*-Boc-lysine)	330	8
			0.23 mM (fructosyl glycine)	192	9
Aspergillus nidulans	48	FAD	ND*	ND	10
Aspergillus terreus GP1	106 (dimeric)	FAD	0.37 mM (Fru-Z-Lys)	39.7	11
			0.51 mM (N^{α}-Fructosyl N^{ε}-Z-lysine)	67.2	
Fusarium oxysporum S-1F4	50	FAD	0.22 mM (Fru-Z-Lys)	122	12
			1.33 mM (N^{α}-Fructosyl N^{ε}-Z-lysine)	127	
Gibberella fujikuroi AKU3802	52	FAD	0.13 mM (Fru-Z-Lys)	22.9	unpublished
			0.45 mM (N^{α}-Fructosyl N^{ε}-Z-lysine)	38.4	
Penicillium janthinellum AKU3413	49	FAD	0.19 mM (Fru-Z-Lys)	5.15	11
			0.62 mM (N^{α}-Fructosyl N^{ε}-Z-lysine)	149	
Agrobacterium tumefaciens (AgaE-like protein)	85 (dimeric)	FAD	1.64 mM (Fru-Val)	ND	13
			0.31 mM (fructosyl glycine)	ND	
Corynebacterium sp. 2-4-1	88 (dimeric)	FAD	0.74 mM (fructosyl glycine)	527	14
			0.71 mM (fructosyl phenylalanine)	894	
Pseudomonas sp.	106	Cu^{2+}	0.14 mM (ε-fructosyl aminocaproate)	ND	15
Achaetomiella virescens ATCC32393	52	flavin	2.30 mM (N^{α}-fructosyl valylhistidine)	ND	16
(fructosyl-peptide oxidase)			1.69 mM (N^{ε}-fructosyl lysine)	ND	
			1.09 mM (Fru-Val)	ND	
Coniochaeta sp. NISL9330	52	ND*	2.81 mM (N^{α}-fructosyl valylhistidine)	ND	17
(fructosyl-peptide oxidase)			10.6 mM (N^{ε}-fructosyl lysine)	ND	
			0.824 mM (Fru-Val)	ND	
Eupenicillium terrenum ATCC18547	50	ND*	2.76 mM (N^{α}-fructosyl valylhistidine)	ND	17
(fructosyl-peptide oxidase)			0.318 mM (Fru-Val)	ND	

*ND, Not determined.

Table 0.2. Properties of fructosamine kinase.

Source	Molecular mass (kDa)	Carbon atom phospholylated	K_m (substrate)	Reference
Human erythrocyte	35	C3	~1 μM (deoxymorpholinofructose) 13.2 μM (fructosyl lysine) 1.0 mM (fructosyl glycine)	19
Human recombinant	35	C3	~1 μM (deoxymorpholinofructose) 7.2 μM (fructosyl lysine) 2.2 mM (fructosyl glycine)	19
Mouse recombinant	35	C3	~1 μM (deoxymorpholinofructose) 7.4 μM (fructosyl lysine) 1.0 mM (fructosyl glycine)	19
Escherichia coli	28	C6	24 mM (deoxymorpholinofructose) 18 μM (fructosyl lysine) 80 mM (fructosyl glycine)	20

The application of fungal FAODs in the clinical diagnosis of diabetes mellitus have been studied in the author's laboratory (11), since the amounts of glycated proteins such as hemoglobin and albumin in blood reflect the level of blood glucose, and fructosyl-amino acids are model compounds for the glycated proteins. During the studies of fungal FAOD, it was found that FAOD is a peroxisomal enzyme and is widely distributed in filamentous fungi, such as the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Gibberella* (23, 24), while there were only a few reports on yeast and bacterial FAODs (13, 14). Furthermore, FAOD was highly specific for fructosyl-amino acids and did not act toward any amino-acid- and amine-related compounds *in vivo* besides glycated compounds (12). Therefore, it was considered that FAOD plays the important role in fungal cells. However, the definite physiological role of the enzyme is still unknown.

In this thesis, the author examines physiological significance of fungal FAOD. In Chapter I, functional analyses are done on FAOD-homologous genes found in *Schizosaccharomyces pombe* and *Aspergillus oryzae* through comparative study of the genes with the databases from the genome-sequencing projects of the two fungi. Chapter II describes purification and characterization of FAOD isozymes of *A. oryzae*. In Chapter III, the physiological role of FAOD in *A. oryzae* is discussed with the results of expression analysis of the wild-type strain and the gene disruptant.

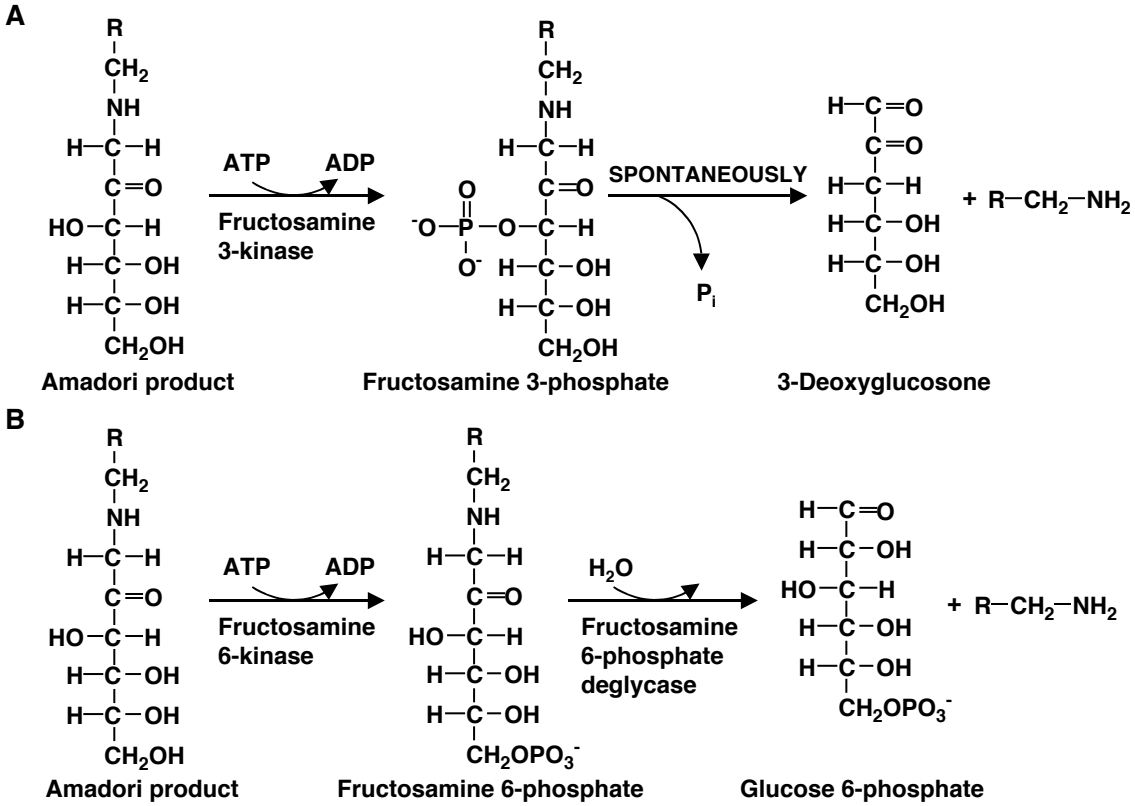

Fig. 0.3. Proposed deglycation pathways of fructosamine by kinase (phosphorylation). (A) Mammalian FNKs; (B) *E. coli* FNK.

CHAPTER I

Fructosyl-Amino Acid Oxidase Homologs in Fungi

It has been known that primary structures of FAODs were partially similar to those of sarcosine oxidases (SOXs) from various organisms in their C-terminal regions. SOX is a flavoprotein which catalyzes the oxidative demethylation of sarcosine (N-methylglycine) to yield formaldehyde, glycine, and H_2O_2. SOX is involved in the metabolism of creatinine and choline (25), and is used in the determination of creatinine and creatine in clinical settings (26). Although both FAOD and SOX cleave the C-N bonds of imino group, there was no relationship between the enzymatic activities of FAOD and SOX.

Recently, the genome-sequencing projects for various microorganisms are now in progress and the author found some 'putative FAOD genes' in these gene databases. Functional analysis of the FAOD-homologous genes would provide helpful information to elucidate the physiological role of FAOD. In this chapter, functional analyses of FAOD homologous genes of two fungi are described.

Section 1 Characterization of fructosyl-amino acid oxidase homologs found in *Schizosaccharomyces pombe* genome

Complete sequencing of *S. pombe* genome (27) revealed two putative FAOD genes, SPBC354.15 and SPAC139.04c of which the gene products show 31.3% and 30.7% identities to FAOD of *Aspergillus terreus* strain GP1 and *Penicillium janthinellum*, respectively. The author designated these genes as *FAP1* and *FAP2*, respectively. Phylogenetic analysis showed that the deduced amino acid sequences from *FAP* genes were similar to those of FAODs rather than those of SOXs. This simple eukaryote is an excellent model for understanding higher eukaryotic system such as cellular process (28), cell-cycle control, chromosome structure (29), and mitosis (30), and could be useful tool for functional analysis of FAOD. In this section, functional analysis of these genes, *FAP1* and *FAP2*, in *S. pombe* is described.

MATERIALS AND METHODS

Materials. Fructosyl-amino acids were kindly provided by Arkray, Inc., Kyoto, Japan. Plasmid vectors, pGEX-6P-1 and pGEX-5X-1, Glutathione-Sepharose 4B, PreScission protease, and HiLoad 16/60 Superdex 200 pg were purchased from Amersham Biosciences K.K., Tokyo, Japan. Bacto Tryptone, Bacto yeast extract, and Bacto malt extract were from Nippon Becton Dickinson Co., Tokyo, Japan. Polypepton® was from Nihon Pharmaceutical Co., Tokyo, Japan. Pronase from *Streptomyces griseus* (EC 3.4.24.4) was from Calbiochem-Novabiochem Co., La Jolla, Calif., USA. Phosphodiesterase from *Crotalus adamanteus* venom (EC 3.1.4.1) was purchased from Wothington Biochemical Corp., Lakewood, N.J.,

USA. Adenylate kinase from *Bacillus strearothermophilus* (EC 2.7.4.3) was purchased from Seikagaku Corp., Tokyo, Japan.

Microorganisms and culture media. *E. coli* strain JM109 (*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17($r_K^-m_K^+$), *relA*1, *supE*44, *Δ*(*lac-proAB*), F'[*traD*36, *proAB*, *lacI* ^{q}, *lacZΔ*M15]), and DH5α (F$^-$, ϕ80d*lacZΔ*M15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17($r_K^-m_K^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1) were cultivated in 2 × YT medium containing 1.6% Bacto Tryptone, 1% Bacto yeast extract, and 0.5% NaCl (pH 7.0) or LB medium containing 1% Bacto Tryptone, 0.5% Bacto yeast extract, and 1% NaCl (pH 7.0). Ampicillin (50 μg/ml) was added to the medium if necessary. *S. pombe* (*279h*$^-$, *ade*$^-$) was cultivated on an MY medium containing 0.3% Bacto yeast extract, 0.3% Bacto malt extract, 0.5% Polypepton®, 1% glucose, and 2% agar (pH 5.5).

Construction of FAOD-overexpression plasmid. cDNA of FAOD from *A. terreus* strain GP1 (FAOD-A) was amplified by PCR. The PCR reaction mixture contained 0.2 ng pFKLT18/μl (31) as a template, 0.2 mM each dNTP, 2 mM $MgCl_2$, 0.5 μM each primer (5'-GGAATTCTTATGCCAGTCACCAAGTCTTC-3' and 5'-AACTGCAGCTATAACTTCG AGATGTCCCT-3' as 5'- and 3'-primer, respectively), and 0.025 unit *Ex Taq* DNA polymerase (Takara Bio, Inc., Otsu, Shiga, Japan)/μl. The PCR reaction was done under the following conditions: 95°C for 5 min; 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min (30 cycles); and finally by 72°C for 3 min using a Thermal Cycler (model Personal TP240; Takara Bio). The PCR product was digested with *Eco*R I and *Pst* I and ligated with pGEX-6P-1. The resultant recombinant plasmid was designated as pGFAOD6P.

Cloning of *FAP* genes of *S. pombe*. A colony of *S. pombe* was picked up from MY plate and suspended in 50 μl of sterilized distilled water. The suspension was incubated at 95°C for 5 min and used as a template for PCR. PCR was carried out using each primer set (for *FAP1*, 5'-CGGATCCATGGTAAAGAATACTAGCGT-3' and 5'-GGAATTCTTAGCTTA

GCTCTTGTCGA-3' as the 5'- and 3'-primer, respectively; for *FAP2*, 5'-CGGGATCCATGTC GAGAACCATTGTCATA-3' and 5'-TCCCCCGGGTCACTTGTCGTAAGTAAACTC-3' as the 5'- and 3'-primer, respectively). These primers were designed by using the nucleotide sequence of *FAPs* (*FAP1*, 1,239 bp; *FAP2*, 1,302 bp) and contained several restriction enzyme sites. PCR was carried out under the following conditions: 95°C for 5 min; 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min (30 cycles); and finally by 72°C for 3 min. The PCR reaction mixture contained an appropriate amount of denatured *S. pombe* cells, 1 μM each primer, 0.2 mM each dNTP, 2 mM $MgCl_2$, and 0.025 unit *Ex Taq* DNA polymerase (Takara Bio)/μl. The PCR products were purified using the QIAquick PCR purification kit (Qiagen K.K., Tokyo, Japan). The purified fragments were digested with restriction enzymes (*Bam*H I and *Eco*R I for *FAP1*, and *Bam*H I and *Sma* I for *FAP2*) and introduced into pGEX-6P-1. These recombinant plasmids were designated as pGPOX6P and pGSP26-2, and used to transform *E. coli* JM109 and DH5α competent cells, respectively, by electroporation using a BTX Electrocell Manipulator (ECM 600; Genetronics, Inc., San Diego, Calif., USA) (32).

Assay of enzyme activity. Oxidase activity was measured at 30°C by the formation of a quinone dye following the absorbance at 505 nm with a Hitachi U-3300 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) or a model 550 Microplate Reader (Nippon Bio-Rad Laboratories, Tokyo, Japan). The reaction mixture contained 33 mM Tris-HCl (pH 8.0), 1.5 mM 4-aminoantipyrine, 2 mM phenol, 2 units horseradish peroxidase (Sigma type IV; Sigma-Aldrich Japan K.K., Tokyo, Japan)/ml, and appropriate amounts of each substrate and enzyme.

Purification of GST fusion proteins. All purification steps were done at 4°C. A total of 5 ml of an overnight culture of *E. coli* transformant was used to inoculate 500 ml of 2 × YT medium containing 50 μg ampicillin/ml. After incubation for 4 h (to an optical

density at 600 nm of 0.30), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium at the final concentration of 0.1 mM. The cells were harvested after cultivation for 26 h by centrifugation at 5,500 × *g* for 10 min, washed with 0.85% KCl, suspended in 25 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3), and then disrupted with a sonicator (model 450 Sonifier; Branson Ultrasonics Corp., Danbury, Conn., USA). Triton X-100 was added to the suspension at the final concentration of 1% and the suspension was incubated on ice for 30 min with gentle agitation. Cell extract was obtained by centrifugation at 6,000 × *g* for 15 min. Two milliliters of the 50% slurry of Glutathione-Sepharose 4B equilibrated with PBS was added to 25 ml of the bacterial sonicate and incubated for 30 min with gentle agitation. The enzyme solution was centrifuged at 500 × *g* for 5 min. The resin was washed 3 times with PBS and once with cleavage buffer (50 mM Tris-HCl [pH 7.0], 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol [DTT]). It was incubated overnight with 20 μl of PreScission protease (40 units) in 980 μl of cleavage buffer to remove GST moiety from the fusion proteins.

Protein determination. The amount of protein was determined by the method of Bradford (33) with a Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin (BSA; Bio-Rad) as a standard. Gel filtration was carried out with HiLoad 16/60 Superdex 200 pg equilibrated with 0.1 M Tris-HCl (pH 8.0) containing 0.1 M NaCl at a flow rate of 1.0 ml/min in an FPLC system (Amersham Biosciences) to determine molecular mass of proteins. Standard proteins were catalase (M_r 240,000), BSA (M_r 68,000), ovalbumin (M_r 45,000), chymotrypsinogen A (M_r 25,000), and cytochrome *c* (M_r 12,500) in a kit from Roche Diagnostics K.K., Tokyo, Japan. The molecular mass of the subunit of the purified protein was determined by SDS-PAGE in a 12.5% gel as described by Laemmli (34) in a Phast System (Amersham Biosciences). Protein was stained with Coomassie Brilliant Blue R-250 (Sigma).

Cofactor analysis. The amount of AMP liberated from the purified protein gave that of FAD. Purified Faps (Fap1, 3.85 nmol; Fap2, 6.83 nmol) in 100 μl of 0.1 M Tris-HCl buffer (pH 8.0) were incubated with 1 mg of pronase at 37°C for 20 h. After inactivation of pronase in boiling water, 0.23 mU phosphodiesterase was added and incubated at 37°C for 30 min. AMP in the reaction mixture was measured by enzymatic method with adenylate kinase (11, 35). To examine cofactor-binding properties, the enzyme was precipitated with trichloroacetic acid at the final concentration of 1%, as follows. The solution was heated in boiling water for 10 min and cooled to room temperature, and centrifuged at 12,000 × *g* for 10 min. The absorption spectrum of the supernatant was measured.

TLC analysis. L-Saccharopine (20 mM) was incubated with 8 μunits Fap2/μl for 20 h at 37°C in 33 mM Tris-HCl buffer (pH 8.0). L-Glutamate and L-lysine at the concentration of 20 mM were used as the standards. TLC was done on precoated plates (silica gel 60 F_{254}, 0.25 mm thick; E. Merck, Darmstadt, Germany). The solvent system was 2-propanol–25% NH_4OH–water (9:1:2). Spots on the plates were visualized by spraying with ninhydrin solution (0.2% ninhydrin and 5% acetic acid in 1-butanol) followed by brief heating at 150°C.

Nucleotide sequence accession numbers. The gene accession numbers used in this study were described as follows: NC_003423 for *FAP1* of *S. pombe*; NC_003424 for *FAP2* of *S. pombe*; Y09020 for FAOD of *A. terreus*; AF035700 for amadoriase I of *Aspergillus fumigatus*; U82830 for amadoriase II of *A. fumigatus*; Y09021 for FAOD of *P. janthinellum* AKU3413; AB035128 for SOX of *Cylindrocarpon didymum*; P40859 for SOX of *Bacillus* sp. B-0618; 2009328A for SOX of *Arthrobacter* sp.; P23342 for SOX of *Bacillus* sp. NS-129; NM_008952 for SOX of *Mus musculus*; and P79371 for SOX of *Oryctolaqus cuniculus*.

RESULTS

Cloning and expression of *FAP1* and *FAP2* genes of *S. pombe*. Phylogenetic analysis (see the next section) showed that the amino acid sequences deduced from *FAP* genes were similar to those of FAODs rather than to those of SOXs and L-pipecolic acid oxidase (POX), showing 27 and 42% identity to that of FAOD of *A. terreus*, respectively. *FAPs* were expressed in *E. coli* as GST fusion proteins, which were purified to apparent homogeneity on SDS-PAGE (Fig. 1.1.1). The molecular masses of Fap1 and Fap2 were estimated to be 46 and 49 kDa, respectively, under the denaturing conditions, corresponding to the molecular masses deduced from the amino acid sequences. The molecular masses of Fap1 and Fap2 were calculated to be about 98 and 56 kDa, respectively, by gel filtration. These results suggested that Fap1 was a homo dimer, whereas Fap2 was a monomeric protein.

Enzyme activities of Fap1 and Fap2. Fap1 and Fap2 had no activity toward N^{ε}-fructosyl N^{α}-benzyloxycarbonyl-L-lysine (Fru-Z-Lys) and fructosyl L-valine (Fru-Val), which were substrates for FAOD. It has been reported that FAODs showed similarity to monomeric SOXs (11). A SOX has high activity toward sarcosine and significant activities toward L-pipecolate and L-proline. The enzyme activities of Faps were measured with these substrates (Table 1.1.1) and it was found that Fap1 oxidized L-pipecolate (20.7 units/mg) and L-proline (6.7% of the activity for L-pipecolate), while the activity toward sarcosine could not be detected. However, Fap2 did not react with any substrates for the enzymes described above. The substrates of FAOD homologs have a common partial structure

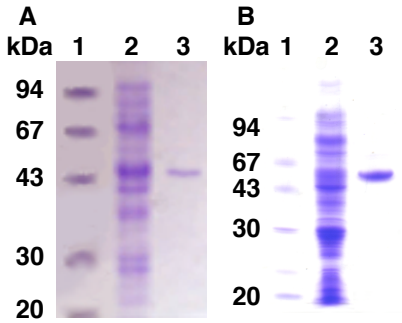

Fig. 1.1.1. SDS-PAGE of fractions obtained during purification of Fap proteins. In panel A, expression and purification of rFap1. In panel B, expression and purification of rFap2. Lane 1, standard proteins; lane 2, cell extract; lane 3, purified recombinant protein.

Table 1.1.1. Relative activities of Faps and other FAOD homologs.

Substrate	*S. pombe*		Monkey	Rabbit	*A. terreus* GP1
	Fap1[1]	Fap2[1]	POX[2]	SOX[3]	FAOD[1]
	(%)	(%)	(%)	(%)	(%)
L-Pipecolate	100	ND	100	30	ND
L-Saccharopine	ND[4]	100	-	-	ND
Sarcosine	ND	ND	10	100	ND
L-Proline	6.7	ND	23	21	ND
Fru-Z-Lys	ND	ND	-	-	100
Fru-Val	ND	ND	-	-	37

[1]This study. The substrates concentrations were 10 mM except for Fru-Z-Lys and Fru-Val, of which the same concentrations were 1.7 mM, respectively.

[2]substrate concentration, 5 mM (36).

[3]substrate concentration, 9.8 mM (37).

[4]ND, not detected.

and the enzymes cleave C-N bonds of the imino group (Fig. 1.1.2). Therefore, we screened many imino compounds that exist *in vivo* as the substrates for Fap2 and L-saccharopine was found to be a suitable substrate for the oxidase activity. FAOD of *A. terreus* did not oxidize any compounds described above except Fru-Z-Lys and Fru-Val (37% of the activity for Fru-Z-Lys) (Table 1.1.1).

The K_m values of Fap1 for L-pipecolate and L-proline were 4.07 and 33.5 mM,

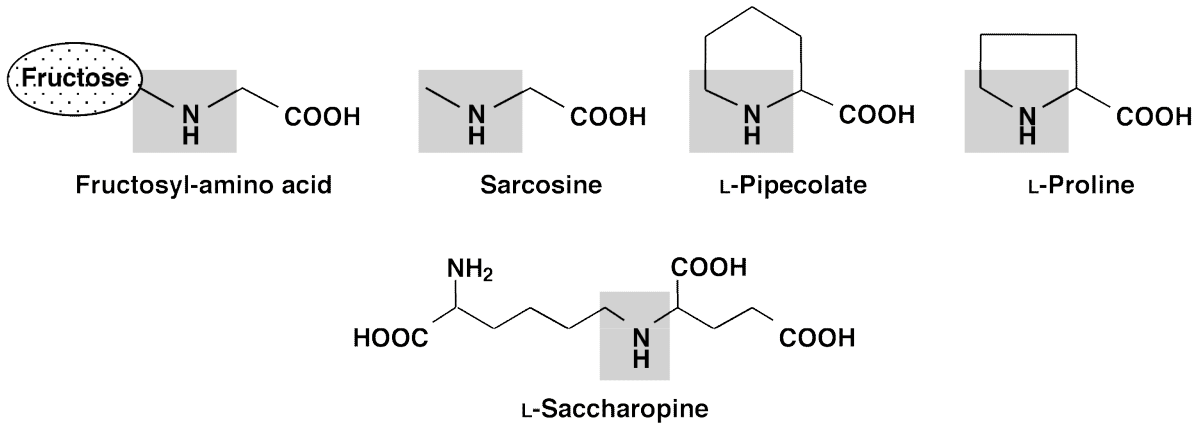

Fig. 1.1.2. Substrates of FAODs and its homologs have similar chemical structures.

respectively, and that of Fap2 for L-saccharopine was 9.02 mM. The V_{max} values of Fap1 for L-pipecolate and L-proline were 46.1 and 9.45 μmol•min^{-1}•mg^{-1}, respectively, and that of Fap2 for L-saccharopine was 6.72 μmol•min^{-1}•mg^{-1}.

Flavin studies. Fap1 and Fap2 showed two maxima of absorption at 380 and 454 nm and at 370 and 450 nm, respectively (Fig. 1.1.3). The peaks around 450 nm of the two proteins had a shoulder around 480 nm. Such spectra are typical of flavoproteins. The supernatant after precipitation of these proteins with 1% trichloroacetic acid had no absorbance, whereas the residual pellets were yellow. Measurement of AMP in Faps resulted in 9.01 nmol (Fap1) and 8.55 nmol (Fap2), respectively, indicating that Fap1 and Fap2 contain 1 mol of FAD/1 mol of the subunit as the prosthetic group, respectively. These results suggested that FAD were covalently linked to these protein molecules.

Reaction product of Fap2. To examine the reaction mechanism of Fap2, the reaction products were analyzed by TLC (Fig. 1.1.4). The spot at 0.40 (R_f value) was due to Tris-HCl buffer. Incubation of L-saccharopine with Fap2 gave a spot that was thought to be the reaction product on TLC. The spot was identified as L-glutamate due to its R_f value (0.23).

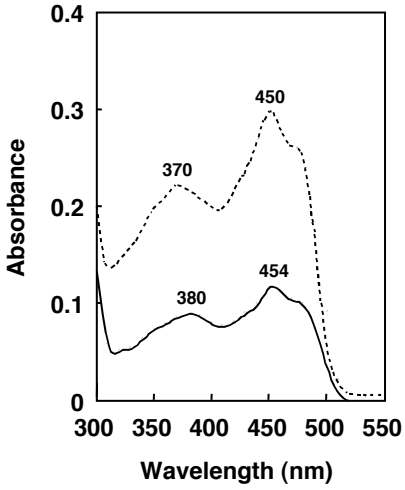

Fig. 1.1.3. Absorption spectra of purified Faps. The concentration of Fap1 and Fap2 were 0.59 and 2.3 mg/ml in the cleavage buffer, respectively. Solid line, Fap1; dotted line, Fap2.

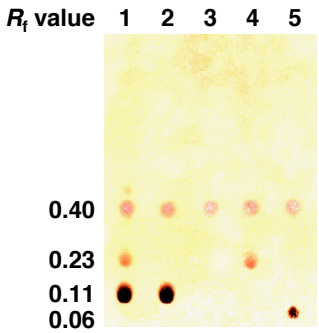

Fig. 1.1.4. TLC analysis of Fap2 enzyme reaction product. Volumes of reaction mixture are all 90 μl. Lane 1, Fap2 (720 μunits) and L-saccharopine (0.5 mg); lane 2, L-saccharopine (0.5 mg); lane 3, Fap2 (720 μunits); lane 4, L-glutamate (0.26 mg); lane 5, L-lysine (0.33 mg).

DISCCUSION

FAODs and their homologs show the high similarity to each other in their C-terminal region (Fig. 1.1.5) (38–43). This region includes a cysteine residue which is conserved in all FAODs and their homologs, and known to be covalently binding sites for FAD in FAODs and SOXs (44, 45). Such cysteine residues are also conserved in Faps, which bind FADs covalently as shown in this study. Most FAODs and their homologs are known to include peroxisome-targeting signal 1 (PTS1) (23, 46). Actually, immuno-electronmicroscopic observation showed that FAODs are localized in peroxisomes in fungi (23). Since Faps did not have PTS1 or other PTSs in their C-terminal and N-terminal regions, respectively, they may not be peroxisomal enzymes.

In mammals, a variety of H_2O_2-producing oxidases including SOX and POX are localized in peroxisomes (36, 37). These enzymes oxidize L-pipecolate, L-proline, and sarcosine (Table 1.1.1). The K_m values of SOX from rabbit kidney suggest that this enzyme prefers L-pipecolate (5.88 mM) rather than sarcosine (66.7 mM) as the substrate *in vivo* (37).

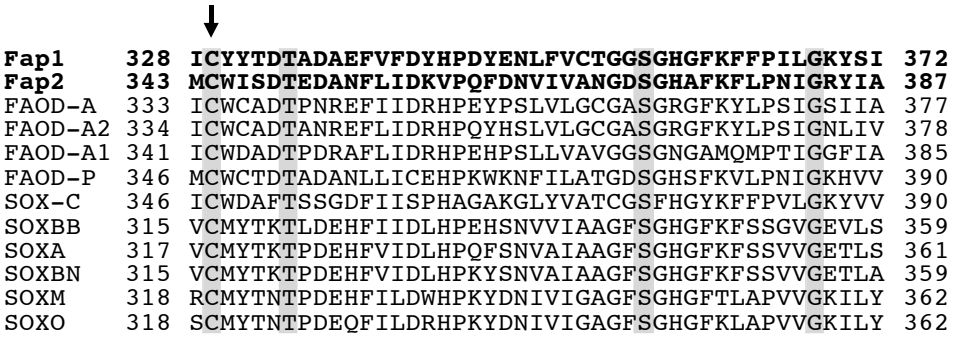

Fig. 1.1.5. Alignment of amino acid sequences of FAODs, and their homologs. Amino acid residues that are identical in all amino acid sequences are shaded, and those indicated by an arrow are covalently binding sites of FAD. Fap1, POX of *S. pombe* (identity to FAOD-A was 27%) (this study); Fap2, SAX of *S. pombe* (42%) (this study); FAOD-A, FAOD of *A. terreus* GP1 (11); FAOD-A2, FAOD of *A. fumigatus* (82%) (38) ; FAOD-A1, FAOD of *A. fumigatus* (51%) (38); FAOD-P, FAOD of *P. janthinellum* AKU3413 (33%) (11); SOX-C, SOX of *C. didymum* M1 (22%) (39); SOXBB, SOX of *Bacillus* sp. B-0618 (16%) (40); SOXA, SOX of *Arthrobacter* sp. (15%) (41); SOXBN, *Bacillus* sp. NS-129 (17%) (42); SOXM, SOX of *M. musculus* (16%) (43); SOXR, SOX of *O. cuniculus* (18%) (37).

The K_m value for L-pipecolate of Fap1 was close to that of POX from monkey liver (3.7 mM) (36), suggesting that L-pipecolate is an *in vivo* substrate of Fap1.

L-Pipecolate and L-saccharopine have been known as the intermediates in L-lysine degradation and biosynthesis pathways in eukaryotes, respectively (47, 48) (Fig. 1.1.6). In eukaryotes, L-lysine is mainly degraded *via* the saccharopine pathway. In this pathway, L-lysine is transformed into L-2-aminoadipic 6-semialdehyde *via* the sequential action of two L-saccharopine dehydrogenase [L-lysine-forming (EC 1.5.1.7) and L-glutamate-forming (EC 1.5.1.10)]. These two reactions are catalyzed by a single bifunctional enzyme in human liver (49, 50). But, in brain, the activity of this bifunctional enzyme is very low and degradation of L-lysine proceeds predominantly *via* L-pipecolate as the intermediate. In this pathway, L-pipecolate is oxidized into Δ^1-piperideine 6-carboxylate by POX, and this intermediate is converted into L-2-aminoadipic 6-semialdehyde by non-enzymatic reaction (49). In *S. pombe*, the two L-saccharopine dehydrogenases are concerned in the L-lysine biosynthesis pathway and L-saccharopine dehydrogenase deficient mutant required L-lysine

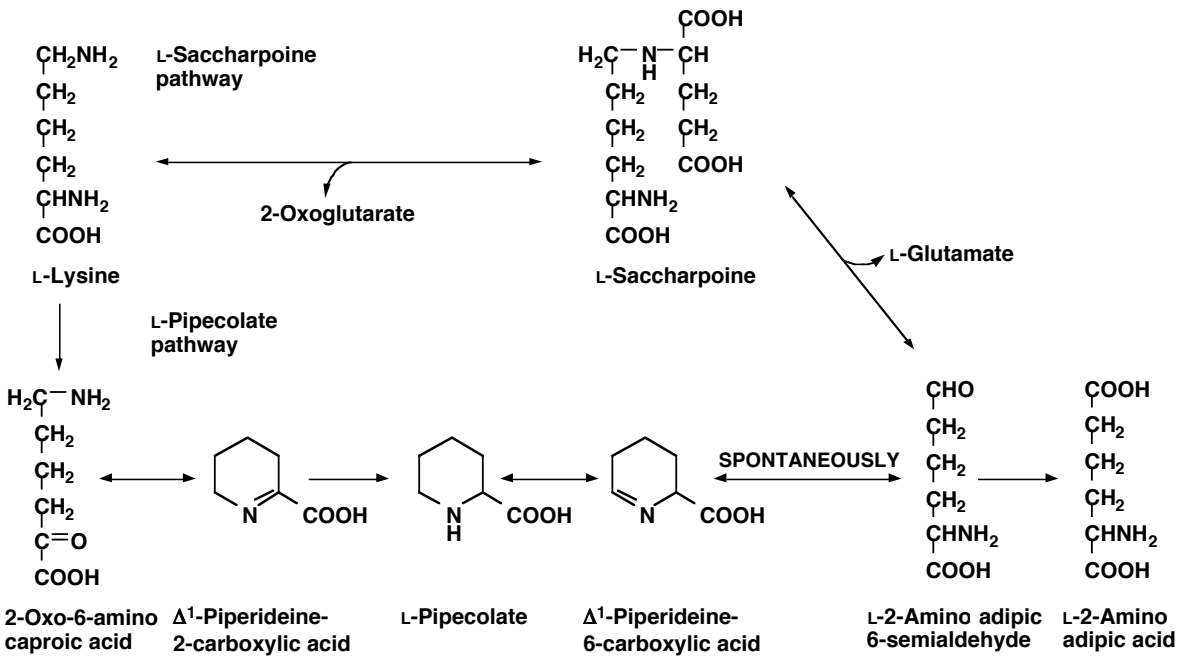

Fig. 1.1.6. Lysine metabolic pathway in eukaryotes.

for growth on the minimum medium (51). In this study, the author found a new enzyme reaction in *S. pombe*, in which Fap2 produced L-glutamate from L-saccharopine by the oxidative reaction (Fig. 1.1.4). This reaction is similar to L-glutamate-forming L-saccharopine dehydrogenase. The reaction mechanism of Fap2 is predicted as follows:

L-Saccharopine + O_2 → L-2-Aminoadipic 6-semialdehyde + L-Glutamate + H_2O_2

Saccharopine oxidase (SAX) was reported in *Rhizoctonia leguminicola* (52). The K_m value for L-saccharopine of Fap2 (9.02 mM) was much higher than that of *Rhizoctonia* SAX (0.128 mM) (52).

Since L-glutamate-forming L-saccharopine dehydrogenase has not been found in *R. leguminicola*, SAX was thought to complement L-saccharopine dehydrogenase reaction in this fungus. It is interesting that *S. pombe* had not only L-glutamate-forming L-saccharopine dehydrogenase (51) but also SAX, and taking account of the K_m values, Fap2 may play an additional role in the L-lysine metabolism when a large amount of L-saccharopine is accumulated.

Section 2 Characterization of fructosyl-amino acid oxidase homologs found in *Aspergillus oryzae* genome

As described in the previous section, the gene products of *FAP* genes did not have FAOD activity. It was known that FAODs are widely distributed in filamentous fungi, such as the genera *Aspergillus*, *Fusarium*, and *Penicillium* (24), but there were only a few reports on yeast and bacterial FAODs (13, 14). In the genome-sequencing projects of filamentous fungi, the whole sequencings have been finished for *Neurospora crassa* and *Magnaporthe grisea*, and the draft sequencings of *Aspergillus nidulans*, *Fusarium graminearum*, and *A. oryzae* have been done. Recently, eleven putative FAOD genes have been found in the draft sequences of *A. oryzae* genome (personal communication from the *A. oryzae* genome sequencing staff). The gene products show 19 to 83% identity to FAOD of *A. terreus* strain GP1, and the author named these genes as *FAO1–11*. FAODs of aspergilli have been reported by several researchers (8, 10, 53) and it may be possible that *A. oryzae* also has FAOD. In this section, characterization of the eleven FAOD homologs was examined.

MATERIALS AND METHODS

Unless otherwise noted, the materials and methods are the same as in section 1.

Materials. The FAOD homolog genes (*FAO1–FAO11*) inserted into pET23-b vector were kindly provided by Gekkeikan Co., Ltd., Kyoto, Japan. pYES2.1/V5-His-TOPO vector was purchased from Invitrogen Corp., Carlsbad, Calif., USA and pESP-2 vector was from Stratagene U. S. A., La Jolla, Calif., USA.

Microorganisms and culture media. *E. coli* strains HMS174(DE3) (F^-, *relA1*, *hsdR* ($r_{k12}^-m_{k12}^+$), RIF^R (DE3)), BL21(DE3) (F^-, *ompT*, $hsdS_B(r_B^-m_B^-)$, *gal*, *dcm*, λ (DE3)), Rosetta(DE3) (F^-, *ompT*, $hsdS_B(r_B^-m_B^-)$, *gal*, *dcm*, *lacY*1 (DE3), pRARE (Cm^R)), and NM522 (*Δhsd*5($r_k^-m_k^+$), *supE*, *thi*-1, *Δ*(*lac-proAB*), F'[*proAB*, lacI^q, *lacZΔ*M15]) were cultivated in 2 × YT medium (pH7.0). *Saccharomyces cerevisiae* strain INV*Sc* (MATa, *his*3Dl, *leu*2, *trp*1-289, *ura*3-52) transformant was cultured at 30°C in SC minimal medium, which is composed of 0.67% yeast nitrogen base (without amino acids and with $(NH_4)_2SO_4$; Becton Dickinson), 2% glucose, 0.01% each adenine and uracil, 0.01% each arginine, cysteine, leucine, lysine, threonine, and tryptophan, and 0.005% each aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine. *S. pombe* strain SP-Q01 (*leu*1-32, h^-) transformant was cultured at 30°C in EMM broth, which is composed of 0.3% potassium hydrogen phthalate, 0.22% Na_2HPO_4, 0.5% NH_4Cl, 2% glucose, 2% volume of salts solution (0.26 M $MgCl_2•6H_2O$, 4.99 mM $CaCl_2•2H_2O$, 0.67 M KCl, and 14.1 mM Na_2SO_4), 0.1% volume of vitamin mixture (4.2 mM pantothenic acid, 81.2 mM nicotinic acid, 55.5 mM *myo*-inositol, and 40.8 mM biotin), and 0.01% volume of mineral solution (80.9 mM boric acid, 23.7 mM $MnSO_4$, 13.9 mM $ZnSO_4•7H_2O$, 7.4 mM $FeCl_3•6H_2O$, 2.47 mM molybdic acid, 6.02 mM KI, 1.6 mM $CuSO_4•5H_2O$, and 47.6 mM citric acid).

Construction of expression plasmids for *FAO4* and *FAO9* genes in *E. coli*. PCR was done with 50 ng of recombinant plasmid/ml which contained *FAO4* gene as a template with 0.2 mM each dNTP, 2 mM $MgCl_2$, 0.02 unit *Ex Taq* DNA polymerase (Takara Bio)/ml, and 0.2 mM each primer; G4Eco5' (5'-GGAATTCATGACATCCTCCAAGTTGACTC-3') and G4Sal3' (5'-AAACGCGTCGACTTAAAGCCGACTCTTATCGCCAATC-3'). G4Eco5' and G4Sal3' primers contained *Eco*R I and *Sal* I sites, respectively. PCR was done by the following protocol: 95°C for 5 min; 94°C for 1 min, 56°C for 1min, 72°C for 2 min (30cycles); 72°C for 10 min. Each PCR product was purified by using Montage PCR

centrifugal filter devices (Nihon Millipore Ltd., Tokyo, Japan) according to the manufacturer's protocol. The purified fragment was digested with *Eco*R I and *Sal* I, electrophoresed in a 1.0% Sea Plaque GTG agarose gel (FMC Bio Products, Rockland, Me., USA) and extracted from the gel by using Wizard SV Gel and PCR Clean-Up System (Promega K.K., Tokyo, Japan). The purified fragment was ligated with pGEX-6P-1 and used to transform *E. coli* HMS174(DE3) competent cells by electroporation (32).

FAO9 gene was amplified by using G9Bam5' (5'-CGGGATCCATGACTGTCGCCAAA TCTTCC-3') and G9Sal3' (5'-AAACGCGTCGACCTACAGCTTCGCAGTATCC-3') as 5'- and 3'-primers, respectively. These primers contained *Bam*H I and *Sal* I sites, respectively, at the 0.2 mM concentration. Fragment obtained was ligated with pGEX-6P-1 under the conditions described above.

Construction of expression plasmids for *FAO4* gene expression in yeasts. PCR was performed using 50 ng recombinant plasmid/ml which retained *FAO4* gene as a template with 0.2 mM each dNTP, 2 mM $MgCl_2$, 0.025 unit *Ex Taq* DNA polymerase/ml, and 0.2 mM each primer; pYES4F (5'-GGAATATGACATCCTCCAAGTTGACTC-3') and G4His3' (5'-AAGCCGACTCTTATCGCCAATC-3') as 5'- and 3'-primers, respectively. PCR was done under the following conditions: 95°C for 5 min; 94°C for 1 min, 57°C for 1 min, 72°C for 2 min (30 cycles); and finally by 72°C for 10 min. PCR product was purified by using Montage PCR centrifugal filter device, ligated with a pYES2.1/V5-His-TOPO vector (Invitrogen), and used to transform *S. cerevisiae* competent cells by lithium acetate method (54).

For *S. pombe* transformation, PCR was performed by using 0.2 mM each Nhe-4F (5'-GAATTCGCTAGCATGACATCCTCCAAGTTGACTC-3') and Nhe-4R (5'-GTCGACGC TAGCTTAAAGCCGACTCTTATCGCCAAT-3') as 5'- and 3'-primers, respectively. These primers contained *Nhe* I sites. Other conditions were the same as described above. The

fragment obtained was purified, digested with *Nhe* I, and purified described above, ligated with a pESP-2 vector, and used to transform *S. pombe* competent cells by using Frozen-EZ yeast transformation II kit (Zymo Research, Orange, Calif., USA).

Purification of recombinant proteins. *FAO* gene products were expressed by using pET and pYES vectors, which were His-tagged, and purified from the crude extracts by Mag extractor (Toyobo Co., Ltd., Osaka, Japan) according to manufacturer's instruction. GST fusion proteins were expressed by using pGEX type vectors and purified as described in the previous section. If necessary, the recombinant protein was refolded by using Refolding CA kit (Takara Bio) according to the manufacturer's instruction.

Analytical methods. The method for assay of FAOD activity and the other analytical methods were the same as described in the previous section. To determine the accurate molecular masses, matrix-assisted laser desorption ionization–time-of-flight mass spectra (MALDI–TOF-MS) were measured with a Voyager-DE PRO (Applied Biosystems Japan Ltd., Tokyo, Japan) using (*E*)-α-cyano-4-hydroxycinnamic acid ((2*E*)-2-cyano-3-(4-hydroxy)-2-propenic acid) as a matrix. For analysis of the N-terminal amino acid sequences, the purified FAODs were separated on a gel by SDS-PAGE, blotted electrophoretically onto a polyvinylidene difluoride membrane (PVDF) (Immobilon-P; Millipore) in a Trans-Blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad), and stained with Coomassie Brilliant Blue R-250 (Sigma). N-terminal and internal amino acid sequences were determined by Edman method (55) with a model 476A protein sequencer (Applied Biosystems).

Nucleotide sequence accession numbers. The cDNA sequences of FAODs from *A. oryzae* RIB40 determined in this study (*FAO4* and *FAO9*) have been submitted to the DDBJ under the accession numbers AB180732 (*FAOAo1*) and AB180733 (*FAOAo2*), respectively. The accession numbers for the additional genes used in this section were as follows: AF416568 for *FAOA* of *A. nidulans*; AAD41368 for AgaE-like protein of

Agrobacterium tumeciens; BAB91123 for FAOD of *Corynebacterium* sp. 2-4-1; AB116147 for fructosyl-peptide oxidase (FPOD) of *Coniochaeta* sp. NISL9330; AB116146 for FPOD of *Eupenicillium terrenum*; NP_389049 for SOX of *B. subtilis*; P40875 for SOX of *Corynebacterium* sp.; NP_126465 for SOX of *Pyrococcus abyssi*; Q52671 for SOX of *Rhodobacter capsulatus*; P40854 for SOX of *Streptomyces* sp.; BD171529 for Fao1 of *A. oryzae* RIB40; and NP_057602 for human POX.

RESULTS

Substrate specificities of rFaos. The *FAO* genes in pET23-b vectors were expressed and enzyme activity was assayed. Table 1.2.1 showed specific activities of the cell extracts from *E. coli* transformants containing *FAO* genes. The cell extract from *E. coli* transformant containing *FAO9* gene had FAOD activities toward Fru-Z-Lys and Fru-Val. The cell extracts from *E. coli* transformants having *FAO1*, *-2*, or *-5* gene did not exert FAOD activity. The cell extracts from *E. coli* transformant containing *FAO1* gene had the activity toward L-pipecolate and L-proline, and those for *FAO2* or *FAO5* genes had SOX activity. It was suggested that *FAO1* and *FAO9* encode POX and FAOD, respectively, and both *FAO2* and

Table 1.2.1. Specific activities of the cell extracts from *E. coli* transformants containing an *FAO* gene.

	Specific activity (mU/mg)				
Protein	Fru-Z-Lys	Fru-Val	Sarcosine	L-Pipecolate	L-Proline
rFao1	ND*	ND	ND	910	3.9
rFao2	ND	ND	330	1.9	4.3
rFao5	ND	ND	330	ND	ND
rFao9	470	110	ND	ND	ND

*ND, Not detected.

FAO5 encode SOX. *FAO3*, *-4*, *-6*, *-7*, *-8*, *-10*, and *-11* genes could not be expressed in this system.

Expression of *FAO4* and *FAO9* genes as GST fusion proteins. The phylogenetic analysis of FAOD homologous genes showed that Fao4 was quite similar to amadoriase I showing 78% identity, whereas *FAO4* was not expressed with use of pET vector as described above (Fig. 1.2.1). The other conditions for the expression of *FAO4* gene were also examined. The *FAO4* and *FAO9* genes of *A. oryzae* were expressed in *E. coli* as GST fusion proteins, which were purified to apparent homogeneity on SDS-PAGE (Fig. 1.2.2). The molecular masses of rFao4 and rFao9 were 54 kDa and 46 kDa, respectively, under denaturing conditions. *FAO4* and *FAO9* consisted of 1,338- and 1,311-bp open reading frames, which encode 445 and 436 amino acids, and the molecular masses deduced from the amino acid sequences corresponded to those of the recombinant proteins. Gel-filtration

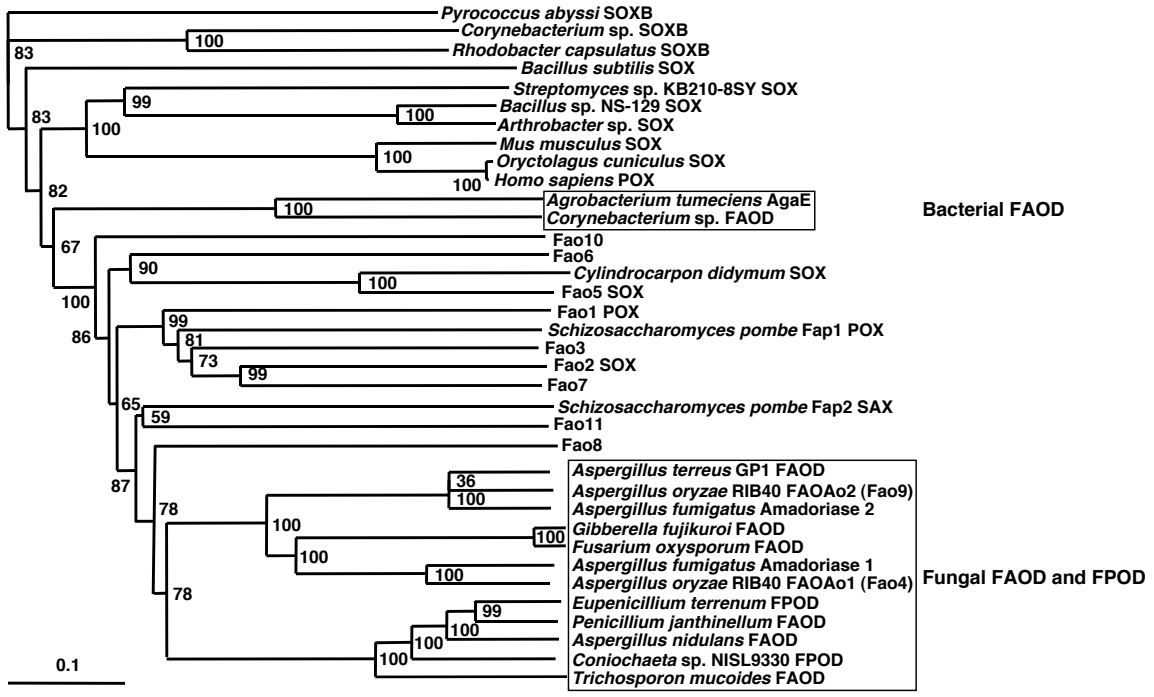

Fig. 1.2.1. Phylogenetic tree of FAODs and their homologs. The amino acid sequences of FAOD and its homologs were retrieved by BLAST program (56) using several databases. The multiple alignment was carried out using Clustal W, version 1.7 (57) and the phylogenetic tree was constructed using neighbor-joining (58) by TreeView, version 1.3 (59). The reliability of cluster formation was analyzed by bootstrap resampling 100 times and the tree was generated with *Pyrococcus abyssi* SOX β-subunit (SOXB) serving as the out-group. The scale bar represents the number of changes per amino acid position.

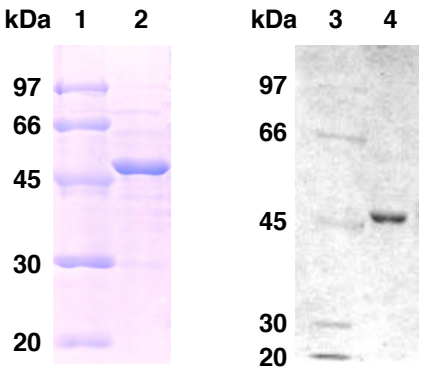

Fig. 1.2.2. SDS-PAGE of purified rFao4p and rFao9p from *E. coli* transformants. Lane 1, 3, standard proteins; lane 2, purified rFao4p; lane 4, purified rFao9p.

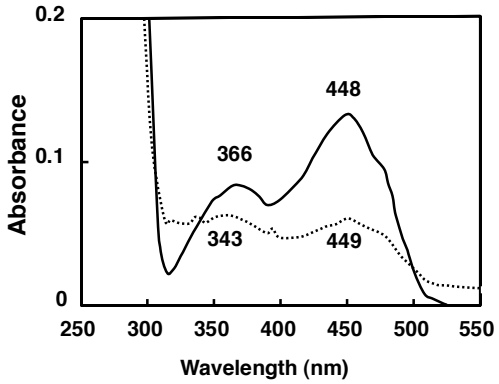

Fig. 1.2.3. Absorption spectra of purified rFao4 and rFao9. The concentration of rFao4 and rFao9 were 0.53 and 0.41 mg/ml in the cleavage buffer, respectively. Solid line, rFao4; dotted line, rFao9.

analysis gave 50 kDa and 100 kDa molecular masses for rFao4 and rFao9, respectively. These results suggested that rFao4 and rFao9 were monomeric and dimeric enzymes, respectively. The absorption spectrum of purified each recombinant protein at pH 8.0 had two maxima and one of them at the longer wavelength had a shoulder around 480 nm (Fig. 1.2.3). After precipitation of these proteins with 1% trichloroacetic acid, each supernatant had no absorption. Residual protein pellets were yellow. It was suggested that flavin was covalently linked to the protein molecules. The K_m values of rFao9 for Fru-Z-Lys and Fru-Val were 0.30 and 1.4 mM, respectively. The V_{max} values of rFao9 for Fru-Z-Lys and Fru-Val were 11.6 and 15.7 μmol•min^{-1}•mg^{-1}, respectively. The recombinant proteins were not active toward opines such as mannopine and saccharopine. The FAOD activity of rFao4 could not be detected with use of various expression systems (Table 1.2.2). Since the molecular mass of rFao4 predicted from the amino acid sequence (50956.78, containing that of covalently bound FAD) was close to that determined by MALDI–TOF-MS analysis (*m/z* 50677.32), protease digestion may not occurred when rFao4 was expressed in *E. coli*. Refolding of the protein with use of any surface-active reagents did not restore FAOD activity (data not shown).

Table 1.2.2. Expression of *FAO4* gene in various host cells.

Vector	Host	Expression	FAOD activity
pET23-b	*E. coli* BL21(DE3)	-	-
	E. coli Rossetta(DE3)	-	-
pGEX-6P-1	*E. coli* JM109	+	-
	E. coli BL21(DE3)	+	-
	E. coli HMS174(DE3)	+	-
	E. coli NM522	-	-
pYES2.1/V5-HIS TOPO	*S. cerevisiae* INV*Sc*	-	-
pESP-2	*S. pombe* SP-Q01	-	-

Expression was confirmed by SDS-PAGE. Fru-Z-Lys was used as a substrate of the enzyme assay.

DISCCUSSION

The results in the previous section provided a new knowledge for characterization of unknown genes, which predicted putative FAOD genes on the databases. The author could predict the function of *FAO* genes in *A. oryzae* from the phylogenetic analysis (Fig. 1.2.1) before the characterization of the corresponding recombinant proteins.

In general, SOX has the activities toward sarcosine, pipecolate, and proline. In combination with the fact that Fap1 of *S. pombe* had the activity for pipecolate, it was suggested that Fao1, -2, -3, -5, -6, -7, and -10 have the oxidase activities toward sarcosine-related compounds described above. Actually, rFao1, -2, and -5 were active toward such compounds. *C. didymum* was known to be the only SOX producing fungus and the fungal SOX was highly specific for sarcosine (60). rFao5 showed the high specificity for sarcosine as expected. Fao11 is close to Fap2 of *S. pombe*, suggesting Fao11 has the activity for saccharopine, although the recombinant protein was not expected in this study.

Fao4 and Fao9 belong to fungal FAOD group in the phylogenetic tree. rFao9 was

active toward fructosyl-amino acids and the substrate specificity was similar to those of aspergilli, corresponding to the phylogenetic distance between these proteins. Although rFao4 had no activity toward substrates for FAOD, various primary structural analyses strongly suggested that *FAO4* gene encoded another FAOD. Furthermore, it is interesting that Fao8 was close to FAOD family rather than SOX family. Fao8 may be another type of FAOD although it is suggested that this protein may not have FAOD activity towards any substrates used in this study from the reason given in the next chapter.

The FAOD cluster in the phylogenetic tree contains fungal fructosyl-peptide oxidases (FPODs), which were active toward fructosyl peptide such as N^{α}-fructosyl valylhistidine. Recently, Hirokawa *et al*. and the author's group reported FPODs (16, 17, 61, 62). Our group proposed a system for enzymatic measurement of glycated albumin in blood using FAODs from fungi such as *A. terreus* GP1 for the first time ever. However, the system is not usable for glycated hemoglobin, in which N-terminal amino acid was valine, since this type of FAOD did not have the activity toward fructosyl peptides containing fructosyl valine residue. For the reason given above, FPOD was screened for from fungi again. FPOD active toward the N-terminal valine can be useful for the measurement of hemoglobin A_{1c} (HbA_{1c}), in which the valine residue at the N-terminal of the β-subunit is glycated.

Figure 1.2.4 shows the alignment of amino acid sequences of FAODs and FPODs containing Fao4 and Fao9 of *A. oryzae*. *FAO4* showed 78% identity with the gene for amadoriase I from *A. fumigatus* and *FAO9* was 83% and 82% identical with the gene for FAOD from *A. terreus* strain GP1 and amadoriase II from *A. fumigatus*, respectively. All amino acid sequences deduced from the FAODs and FPODs genes contained a cysteine residue, to which FAD may be bound covalently (11, 45), and those from the other *FAO* genes also contained the same residue.

Seven of the *FAO* gene products of *A. oryzae* contained peroxisome-targeting signal 1

(PTS1) at their deduced C-terminals (SKL, for *FAO2*, *-3*, *-5*, and *-10*; SRL, for *FAO4*; AKL, for *FAO7* and *-9*) (46). PTS1 is known to be necessary and sufficient for protein sorting to peroxisomes and the consensus sequences for PTS1 are "(S/A/C/N)-(K/H/R/Q/N)-(L/F/Y)". Peroxisome is a subcellular organelle and contains H_2O_2-producing enzymes such as oxidases and H_2O_2-scavending enzymes such as catalases. Thus, it is expected that the levels of the products from these gene are high *in vivo*. The PTS1 sequence of Fao4, SRL, is worthy of notice. Because it was thought that C-terminal SRL is a signal sequence for subcellular sorting of the protein to Woronin body. This subject was described in the last chapter.

Based on the analysis of FAOD homologs in this chapter, these could be categorized as at least three kinds of amine oxidases, which cleave the C-N bonds of the imino group: i) those specific for sarcosine and its related compounds, ii) those specific for saccharopine, and iii) those specific for fructosyl-amino acids.

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                    ↓
Fao4      341  ICWDADTVDRAFLIDRHPEYRSLLLAVGGSGNGAMQMPTIGGFIA  385
FAOD-A1   341  ICWDADTPDRAFLIDRHPEHPSLLVAVGGSGNGAMQMPTIGGFIA  385
Fao9      332  VCWCADTANREFIIDRHPEHPSLVLGCGASGRGFKYLPSIGNLIV  376
FAOD-A2   334  ICWCADTANREFLIDRHPQYHSLVLGCGASGRGFKYLPSIGNLIV  378
FAOD-A    333  ICWCADTPNREFIIDRHPEYPSLVLGCGASGRGFKYLPSIGSIIA  377
FAOD-An   346  MCWCTDTADANLLVCEHPRWKGFYLATGDSGHSFKLLPNIGKHVV  390
FAOD-P    346  MCWCTDTADANLLICEHPKWKNFILATGDSGHSFKVLPNIGKHVV  390
FAOD-T    344  MCWCTDTADGALLICEHPRWRNLIIASGDCGHSFKLLPNIGKHIV  388
FAOD-G    340  ICWCADTQDRMFLITYHPRHPSLVIASGDCGTGYKHITSIGKFIS  384
FAOD-F    338  ICWCADTQDRMFLITYHPRHPSLVIASGDCGTGYKHITSIGKFIS  382

FPOD-F1   362  ICWYSDTRDGEWLIDYHPGWKGLFVATGDSGHGFKFLPNLGEKIV  406
FPOD-F2   342  ICWCADTQDRMFLITYHPRHPSLVIASGDCGTGYKHITSIGKFIS  386
FPOD-E    346  MCWCTDTADANLLICEHPKWKNFILATGDSGHSFKLLPNIGKHVV  390
FPOD-C    346  LCWCTDTADAALLMCEHPKWKNFILATGDSGHSFKILPNVGKHVV  390
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Fig. 1.2.4. Alignment of amino acid sequences of FAODs and FPODs. Amino acid residues that are identical in all amino acid sequences are shaded, and those indicated by the arrow are covalently binding sites of FAD. Fao4 and Fao9, FAOD isozymes of *A. oryzae* RIB40 (identities to FAOD-A were 53 and 83%, respectively) (this study); FAOD-A1 and -A2, FAOD isozymes of *A. fumigatus* (51 and 82%, respectively) (38); FAOD-A, FAOD of *A. terreus* GP1 (11); FAOD-An, FAOD of *A. nidulans* (32%) (10); FAOD-P, FAOD of *P. janthinellum* AKU3413 (33%) (11); FAOD-T, FAOD of *Trichosporon mucoides* (31%); FAOD-G, FAOD of *Gibberella fujikuroi* (49%) (unpublished data); FAOD-F, FAOD of *F. oxysporum* (48%) (unpublished data); FPOD-F1 and -F2, FPOD of *F. proliferatum* (23 and 49%, respectively) (61); FPOD-E, FPOD of *Eupenicillum terrenum* (31%) (17); FPOD-C, FPOD of *Coniochaeta* sp. (30%) (17).

Summary

Two FAOD homologs from *S. pombe* were characterized as a POX and SAX, and four of eleven FAOD homologs from *A. oryzae* were characterized as a POX (Fao1), two types of SOXs (Fao2 and -5), and an FAOD (Fao9). Alignment of the primary structures of FAODs strongly suggested that *FAO4* gene encoded another FAOD, although rFao4 had no activity toward the substrates for FAOD. By the analysis of FAOD homologs in this study, these are possibly categorized as at least three kinds of amine oxidases.

CHAPTER II

Purification and Characterization of Fructosyl-Amino Acid Oxidase Isozymes of *Aspergillus oryzae*

In Chapter I, it was shown that *A. oryzae* has two FAOD genes (*FAO4* and *FAO9*). Takahashi *et al*. reported that *A. fumigatus* had at least two FAOD isozymes. Before their examination, our group showed properties of FAOD of *A. terreus* in detail, but, no isozyme was found in *A. terreus*. *A. oryzae* is an important fungus in Japanese fermentation industries (63), and there is a lot of information about genetics and fermentation techniques related to this organism. Therefore, the author used *A. oryzae* strain RIB40 as a model to elucidate the physiological role of FAOD in fungal cells. Chapter II describes purification and characterization of FAOD isozymes of *A. oryzae*.

MATERIALS AND METHODS

Unless otherwise noted, the materials and methods are the same as in previous chapter.

Materials. Fructosyl L-lysine (Fru-Lys) used in cultivation was synthesized according to the method of Hashiba *et al*. (64), and purified with use of Bond Elute PBA column (100 mg; GL Sciences, Inc., Tokyo, Japan). Dried yeast extract-S was from Nihon Pharmaceutical.

Microorganisms and culture media. *E. coli* strain HMS174(DE3) was cultivated in 2 × YT (pH 7.0). A minimum medium MM-1 was used for *A. oryzae* strain RIB40. MM-1 contained 0.3% $NaNO_3$, 0.1% KH_2PO_4, 0.2% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 2% glucose, 0.1%

volume of trace elements solution (0.1% $FeSO_4 \cdot 7H_2O$, 0.88% $ZnSO_4 \cdot 7H_2O$, 0.04% $CuSO_4 \cdot 5H_2O$, 0.01% $Na_2B_4O_7 \cdot 10H_2O$, and 0.005% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$), and 1.5% agar. The pH of this media was adjusted to pH 5.5.

For sporulation of *A. oryzae* strain RIB40, it was cultured in 100 ml of MM-1 medium at 30°C for about 2 weeks. Sporulating mycelia were suspended in 0.8% NaCl containing 0.1% Tween 80 and filtrated through a no. 11G-2 glass filter. The filtrate was centrifuged at 2,200 × *g* for 10 min. The pellet was washed twice with the same solution. For enzyme production, 7.0×10^8 spores were incubated at 30°C for 26 h in 7 liter of autoclave-browned (AB) medium (24) by using a model MDN 10-liter jar fermentor (B.E. Marubishi Co., Tokyo, Japan) with agitation (300 rpm) and aeration (7 liters/min). The AB medium has been used for FAOD induction (10–12, 24). The AB medium is prepared by autoclaving the medium containing glucose and amino acid. The corresponding fructosyl-amino acid, which is the substrate for FAOD, is produced during the autoclaving. The color of the AB medium is brown due to further glycation reaction which may produced AGE-like compounds. The medium, which contained the same constituents as the AB medium but glucose autoclaved separately was added to the medium, did not colored and was used as control medium.

Purification of FAOD isozymes. All purification steps were done at 4°C and column chromatography was done with an FPLC system (Amersham Biosciences). Washed mycelia (520 g as [wet weight]) were suspended in 1.3 liter of 0.1 M Tris-HCl buffer (pH 8.5) containing 2 mM DTT and disrupted in a glass bead mill (Bead-beater model 11079-S; Biospec Products, Bartlesville, Okla, USA). The homogenate was centrifuged at 18,000 × *g* for 75 min to remove unbroken cells and debris as the precipitates. $(NH_4)_2SO_4$ powder was added to the supernatant to give 50% saturation. After gentle stirring for 60 min, the mixture was centrifuged at 18,000 × *g* for 75 min. The supernatant was adjusted to 75% saturation by $(NH_4)_2SO_4$ powder. After another 60 min of stirring and centrifugation under the same

conditions, the precipitate was dissolved in a minimum volume of buffer A (50 mM Tris-HCl buffer (pH 8.5) containing 2 mM DTT). The enzyme solution was then dialyzed against the same buffer for 35 h and centrifuged at 6,500 × *g* for 20 min. The supernatant was applied to a DEAE-Toyopearl column (2.2 by 20 cm; Tosoh Co., Tokyo, Japan) equilibrated with buffer A. The column was washed with 230 ml of the same buffer. The adsorbed protein was eluted with a linear gradient of 0 to 0.1 M NaCl in 450 ml of buffer A. The active fractions were suspended in buffer A containing 40% saturation of $(NH_4)_2SO_4$. The enzyme solution was applied to a Butyl-Toyopearl column (1.6 by 10 cm; Tosoh) equilibrated with the same buffer. After washing the column extensively with the equilibration buffer, elution was done with a linear gradient of 40 to 0% saturation of $(NH_4)_2SO_4$ in 50 ml of buffer A. The enzyme-active fractions were collected and dialyzed with use of a Centricon YM-30 (Millipore), and applied to a HiLoad 16/60 Superdex 200 pg column (1.6 by 60 cm; Amersham Biosciences) equilibrated with 0.1 M Tris-HCl buffer (pH 8.5) containing 0.1 M NaCl and 2 mM DTT. The active fractions were pooled, dialyzed against buffer A, and applied to a Resource Q column (0.64 by 3 cm; Amersham Biosciences). The column was washed with 20 ml of the same buffer. The adsorbed enzyme was eluted with a linear gradient of 0 to 1 M NaCl in 35 ml of buffer A.

Preparation of genomic DNA. Wet mycelia (0.1 g) were ground in liquid N_2 in a mortar. The powdered cells were transferred to a 50-ml conical tube, 8 ml of TE buffer and 8 ml of the lysis buffer composed of 50 mM EDTA and 0.5% SDS were added, and mixed upside down. The mixture was shaken gently at 37°C for 30 min, and centrifuged at 3,000 × *g* and 4°C for 5 min. The supernatant was purified by phenol-chloroform extraction and the precipitate was dissolved in 1.6 ml of TE buffer. Ribonuclease A (50 μg; Nippon Gene Co., Tokyo, Japan) was added to the 400 μl of the DNA solution and the mixture was incubated at 37°C for 30 min. After phenol-chloroform extraction as above, the precipitate

was dissolved in appropriate amount of TE buffer.

Cloning of the partial genes encoding FAOD isozymes. Partial genes encoding FAOD isozymes (*FAO4* and *FAO9*) were amplified by PCR with *A. oryzae* strain RIB40 genome as a template using an FAOD-specific primer (degenerate primer) set; 5'-GGBTTYTTCWTSGARCCNRAYGA-3' and 5'-GTRTCVGYRYMCCAGCAVAT-3' as the 5'- and 3'-primer, respectively. The FAOD-specific primer set was designed from the consensus sequences for FAODs (see the legend for Fig. 2.4). PCR was done under the following conditions: 94°C for 1 min; 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min (35 cycles); and finally by 72°C for 3 min. Purified PCR fragment was ligated with a pCR2.1-TOPO vector by using TOPO TA cloning kit (Invitrogen). The ligated product was used to transform *E. coli* strain JM109 competent cells by electroporation with a BTX Electro Cell Manipulator ECM 600 (Genetronics) (32).

RESULTS

Purification and characterization of FAOD isozymes from *A. oryzae* strain RIB40. Three FAOD-active fractions were found on the DEAE-Toyopearl chromatogram. One of these enzyme activities was observed in a washing fraction (FAOD-Ao1), and the other fractions were eluted with NaCl (FAOD-Ao2a and -Ao2b) (Fig. 2.1). These fractions were further separately purified. The results of the purifications were shown in Table 2.1. Each enzyme showed a single band on SDS-PAGE (Fig. 2.2). The molecular mass of FAOD-Ao1 was estimated to be 49 kDa, and those of FAOD-Ao2a and -Ao2b were estimated to be 48 kDa. Superdex 200 gel filtration showed that the molecular mass of FAOD-Ao1 was 39 kDa, and those of FAOD-Ao2a and -Ao2b were 98 kDa, suggesting that FAOD-Ao1 was

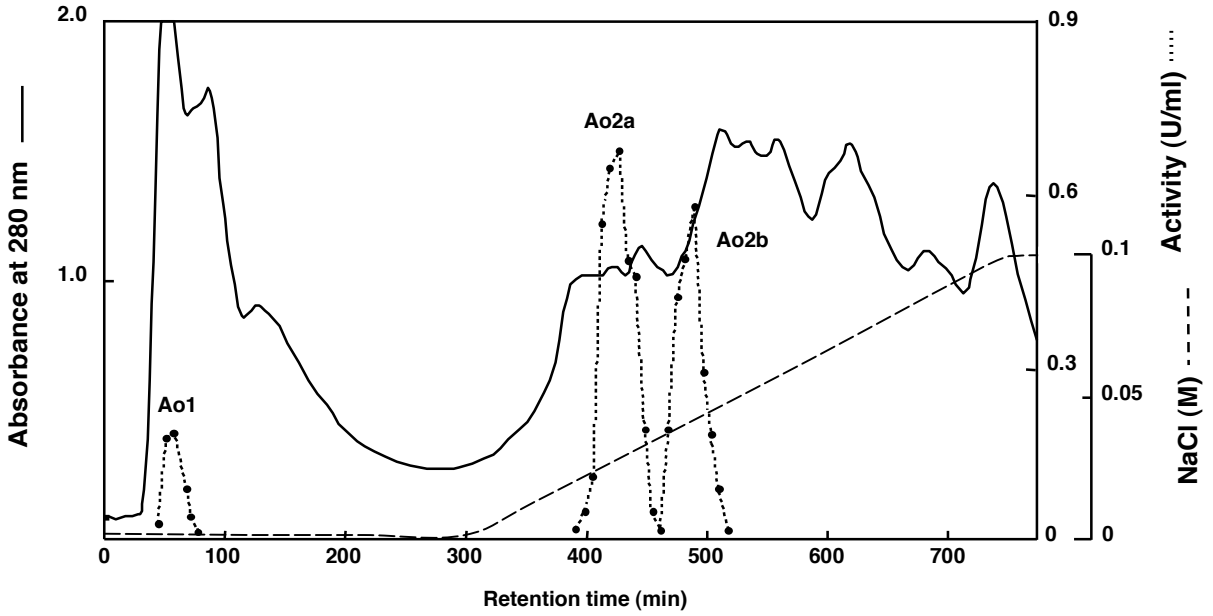

Fig. 2.1. Elution profile of FAOD isozymes in *A. oryzae* strain RIB40 from DEAE-Toyopearl column.

monomeric and the others were dimeric enzymes. FAOD-Ao1 and -Ao2a showed two maxima of absorption at 385 and 447 nm and at 363 and 445 nm, respectively. One of the peaks of each protein around 450 nm had a shoulder around 480 nm. Such spectra are typical of flavoproteins. The supernatant after precipitation of these proteins with 1% trichloroacetic acid had no absorbance, whereas the residual pellets were yellow. Each flavin was identified as covalently bound FAD by the enzymatic method with adenylate kinase (11). N-terminal amino acid sequences of FAOD-Ao2a and -Ao2b were identical, but FAOD-Ao1 had a different N terminus from the other isozymes (Table 2.2). Furthermore, N-terminal amino acid sequences of FAOD-Ao1 and -Ao2a corresponded to those of the gene products of *FAO4* and *FAO9* (Chapter I, Section 2), respectively. Thus, since FAOD-Ao2a and -Ao2b were thought to be derived from the same origin, FAOD-Ao1 and -Ao2a were used for further examination.

The isozymes (FAOD-Ao1 and -Ao2a) were active toward Fru-Z-Lys, but FAOD-Ao1 was not active toward Fru-Val. L-Pipecolate, sarcosine, and L-proline were the substrates for sarcosine-related enzymes but were not the substrate for FAOD isozymes in *A. oryzae*.

Table 2.1. Purification of FAOD isozymes from *A. oryzae* strain RIB40.

Enzyme	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg•protein)	Purification (fold)	Yield (%)
	Cell extract	294	6410	0.0459	1	100
	Ammonium sulfate (50-75% satn.)	243	2270	0.107	2.33	83
FAOD-Ao1	DEAE-Toyopearl	5.56	47.6	0.117	2.49	1.9
	Butyl-Toyopearl	3.85	6.47	0.595	13.0	1.3
	Superdex 200	1.46	0.59	2.47	53.8	0.5
	Resource Q	1.34	0.53	2.52	54.9	0.5
FAOD-Ao2a	DEAE-Toyopearl	41.9	21.2	1.98	43.1	14
	Butyl-Toyopearl	24.5	2.53	9.68	211	8.3
	Superdex 200	10.1	0.566	17.8	388	3.4
	Resource Q	2.78	0.0913	30.4	662	0.9
FAOD-Ao2b	DEAE-Toyopearl	14.5	16.2	0.895	19.5	4.9
	Butyl-Toyopearl	7.4	1.38	5.36	117	2.5
	Superdex 200	2.78	0.272	10.2	222	0.9
	Resource Q	0.947	0.0481	19.7	429	0.3

Furthermore, the isozymes were not active toward opines, such as mannopine and saccharopine. The K_m values of FAOD-Ao1 and -Ao2a for Fru-Z-Lys were 0.51 and 0.22 mM, respectively, and that of FAOD-Ao2a for Fru-Val was 1.38 mM. The V_{max} values of FAOD-Ao1 and -Ao2a for Fru-Z-Lys were 2.7 and 17.7 μmol•min^{-1}•mg^{-1}, respectively, and that of FAOD-Ao2a for Fru-Val was 31.2 μmol•min^{-1}•mg^{-1}.

The optimal pH values for the enzyme reaction of FAOD-Ao1 and -Ao2a were at 6.6 and 8.2, respectively (Fig. 2.3). FAOD-Ao2a did not have enzymes activity at pH 6.0, whereas FAOD-Ao1 was 85% active at this pH. Both isozymes were inactivated gradually when incubated at above 30°C in Tris-HCl buffer (pH 8.0) and completely lost the activity when incubated at 60°C for 10 min (data not shown).

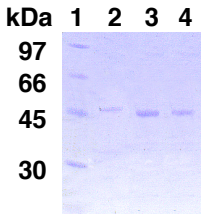

Fig. 2.2. SDS-PAGE of purified FAOD isozymes observed in the anion exchange column chromatography. Lane 1, standard proteins; lane 2, FAOD-Ao1; lane 3, FAOD-Ao2a; lane 4, FAOD-Ao2b. Proteins were stained with Coomassie Brilliant Blue R-250 (Sigma).

Cloning of partial *FAOAo1* and *FAOAo2* genes. To clone the whole gene, the 230-bp fragment was amplified by PCR from *A. oryzae*

Table 2.2. N-terminal amino acid sequences of purified FAOD isozymes.

Isozyme	N-terminal amino acid sequence
FAOD-Ao1	TSSKLTPTSSILIVGAGT
FAOD-Ao2a	TVAK···SSSILIIGAGT
FAOD-Ao2b	TVAK···SSSILIIGA

strain RIB40 genome by using FAOD-specific primer set (Fig. 2.4). Only two different sequences were obtained from this fragment and were highly similar to the partial nucleotide sequences of the genes encoding various FAODs (*FAO*s). Furthermore, these corresponded just to the partial nucleotide sequences of *FAO4* and *FAO9*, respectively (data not shown). The N-terminal and internal amino acid sequences determined from the purified isozymes with use of peptide sequencer were found in the deduced amino acid sequences. Thus, it was confirmed that the two genes (*FAOAo1* and *FAOAo2*) encode FAOD-Ao1 and -Ao2a, and corresponded to *FAO4* and *FAO9* (Chapter I, Section 2), respectively.

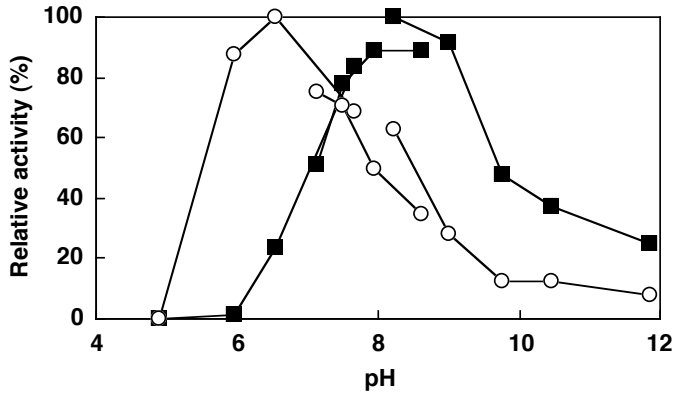

Fig. 2.3. Effect of pH on the activities of FAOD-Ao1 and -Ao2a. The enzyme activities were measured using the following buffers: McIlvaine (pH 4.97 to 7.85), Tris-HCl (pH 7.00 to 8.50), and glycine-NaOH (pH 8.30 to 12.0). Fru-Z-Lys (1.7 mM) was used as a substrate. The circles represent the activity of FAOD-Ao1 and the squares represent the activity of FAOD-Ao2a.

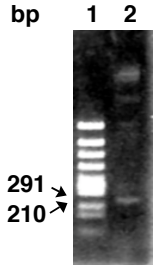

Fig. 2.4. Amplification of partial *FAO* genes with an FAOD-specific primer set. The FAOD-specific primer set was designed from the alignment of fungal FAODs reported previously. The nucleotide positions of these FAODs for 5'- and 3'-primer designs were as follows: FAOD of *A. terreus* at positions 784 to 806 and 997 to 1,016; amadoriase I of *A. fumigatus* at positions 797 to 819 and 1,016 to 1,035; amadoriase II of *A. fumigatus* at positions 787 to 809 and 1,000 to 1,019; FOD of *F. oxysporum* at positions 796 to 818 and 1,012 to 1,031. Lane 1, ϕX174-*Hinc* II digest; lane 2, PCR product.

DISCUSSION

Properties of rFao9 were well consistent with those of FAOD-Ao2a and -Ao2b, and it was confirmed that rFao4 corresponded to FAOD-Ao1 by various analyses, although the activity of rFao4 was not detected. Thus, it should be concluded that there are two types of FAOD in *A. oryzae*.

Before the author obtained the information about *A. oryzae* genome draft sequence, attempted whole gene cloning of *FAOAo1* and *FAOAo2*, and partial gene cloning of *FAOAo1* and *FAOAo2* were done with use of an FAOD-specific primer set in order to clone the FAOD genes. PCR with this primer set can amplify FAOD gene fragment from *F. proliferatum* (61). The fact that only two different fragments were amplified from *A. oryzae* genome demonstrates that this primer set is useful tool for cloning of FAOD genes from fungi.

Takahashi *et al*. also reported that there are four FAOD-active fractions in *A. fumigatus* (8, 38). Three of them give almost the same enzymatic properties and N-terminal sequences, suggesting that this results from partial proteolytic degradation during isolation. The two FAOD isozymes in *A. oryzae* were different in substrate specificities for α- and ε-glycated compounds. The glycation sites of Fru-Z-Lys and Fru-Val are ε- and α-amino groups, respectively (12). It is noteworthy that FAOD-Ao1 was not active toward Fru-Val. This is the same for amadoriase isozymes in *A. fumigatus*; one of the isozymes, amadoriase I, prefers N^{ε}-fructosyl N^{α}-*t*-Boc-lysine (ε-glycated) to N^{α}-fructosyl N^{ε}-acetyllysine (α-glycated), while the other, amadoriase II, is active toward both compounds to the same extent (8). In general, the amino groups in the side chains of Lys and Arg residues in proteins are susceptible to glycation (Fig. 2.5) (2). Thus, the author suggests that Fru-Lys and fructosyl arginine in nature are derived from the glycated proteins. Actually, Yoshida *et al*. detected Fru-Lys and fructosyl arginine in fungal cells cultivated for a long period in the minimum medium not

containing any amino acids (N. Yoshida *et al.*, submitted for publication). The author thinks that FAOD-Ao2 is used for utilization of exogenous fructosyl-amino acids, whereas FAOD-Ao1 is used for deglycation of endogenously produced fructosyl-amino acid residues in their own cells. This is also considered in the next chapter.

Corynebacterium sp. strain 2-4-1 is known to be the FAOD producer in prokaryotes (14, 65). *Corynebacterium* FAOD showed high similarity to AgaE-like protein of *Agrobacterium* sp. (13). During infection, *Agrobacterium* sp. transfers a fragment of oncogenic DNA into the nuclei of plant cells, and then infected plant cells produce large amounts of opines such as octopine, lysopine, and mannopine. AgaE-like protein is concerned with the opine metabolism and has the oxidase activities toward opines such as mannopine as the substrates (13). AgaE-like protein has FAOD activity. The AgaE-like protein and FAOD of *Corynebacterium* sp. had similar properties such as molecular weight, coenzyme binding, and substrate specificity. Therefore, the origin of prokaryotic FAOD

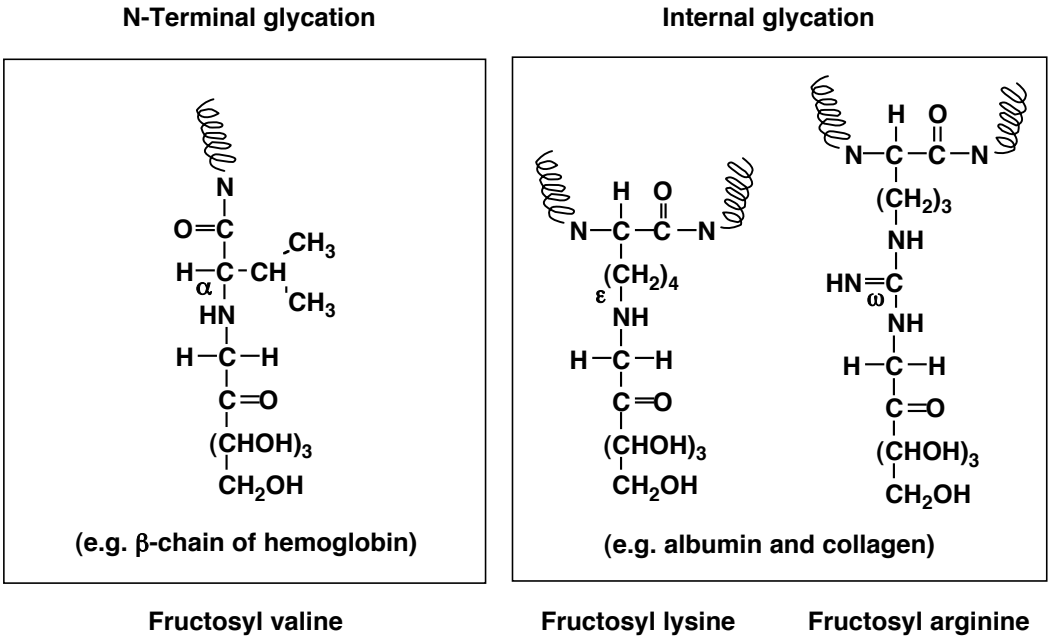

Fig. 2.5. Possible glycation sites of proteins.

gene is thought to be the result of the horizontal propagation of *Agrobacterium* gene. However, FAOD isozymes from *A. oryzae* were not active toward any opines used, indicating that physiological role of eukaryotic FAOD is different from that of prokaryotic FAOD.

Summary

Three active fractions of fructosyl-amino acid oxidase (FAOD-Ao1, -Ao2a, and -Ao2b) were isolated from the cells of *A. oryzae* strain RIB40. N-terminal and internal amino acid sequences of FAOD-Ao2a corresponded to those of FAOD-Ao2b, suggesting that these two isozymes were derived from the same protein. FAOD-Ao1 and -Ao2 were different in the substrate specificity and the subunit assembly; FAOD-Ao2 was active toward Fru-Z-Lys and Fru-Val, whereas FAOD-Ao1 was not active toward Fru-Val. N-terminal and internal amino acid sequences determined for the purified isozymes were also found in the deduced amino acid sequences of Fao4 and Fao9, respectively.

CHAPTER III

Possible Functional Characterization of Fructosyl-Amino Acid Oxidases in *Aspergillus oryzae*

In the preceding chapters, the author found two FAOD isozymes in *A. oryzae* and their difference in the substrates specificities and cellular localization (peroxisome or Woronin body), which suggested different physiological roles between the two isozymes.

Jeong *et al*. reported that the disruption of *faoA* gene encoding FAOD in *A. nidulans* did not affect its phenotype (e.g., growth and physiological appearance) (10). They suggested that the putative role of FAOD in *A. nidulans* could be the provision of a carbon and/or a nitrogen source necessary for sexual development since the *faoA* gene expression was induced by fructosyl amines and depended on the expression of *veA* gene (a sexual development regulator gene) (10). However, these authors did not use the medium containing a fructosyl-amino acid as the sole carbon and/or nitrogen sources. FAOD activity in cell extract of *A. nidulans* was low, and the biochemical and physicochemical characterizations of the enzyme have not been examined. In Section 1, disruption of *FAO9* gene of *A. oryzae* was done to investigate role of FAOD. In Section 2, FAOD genes in *A. oryzae* are examined by expression analysis.

Section 1 Disruption of fructosyl-amino acid oxidase gene in *Aspergillus oryzae* genome

The gene disruption of fungi has been carried out using host-vector systems with auxotrophic mutants (66). Recently, transformation system of filamentous fungi have been developed by using aureobasidin or pyrithiamine as dominant selectable markers (67, 68). As shown in Chapter II, Fao9 was well characterized and highly active toward fructosyl-amino acids. In this section, *FAO9* gene disruptant is constructed by using pyrithiamine as a dominant selectable marker. *A. oryzae* strain niaD-2 (*ΔniaD*) (nitrate reductase-deficient mutant) is used as the wild-type strain as the basis of constructing double mutant for FAOD isozymes.

MATERIALS AND METHODS

Unless otherwise noted, all experiments were done as already described.

Microorganisms and culture media. A minimum medium MM-2 was used for *A. oryzae* strains niaD-2 (*ΔniaD*) (nitrate reductase-deficient mutant). MM-2 used 0.3% $NaNO_2$ as a sole nitrogen source in MM-1 (described in Chapter II). The pH of this media was adjusted to pH 5.5.

For sporulation, *A. oryzae* strain niaD-2 was cultured in 100 ml of MM-2 medium at 30°C for about 2 weeks, and treated as describe in Chapter II.

For the analysis of utilization of carbon and nitrogen sources, *A. oryzae* strains were cultivated at 30°C in a medium composed of 0.1% KH_2PO_4, 0.2% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, a 0.1% volume of the trace elements solution used in Chapter II, 1% carbon source, 0.3%

nitrogen source, and 1.5% agar (pH 5.5). Spores (10^3) of each strain were spotted on the center of a plate and incubated at 30°C.

Construction of *FAO9* gene disruption cassette. *FAO9* gene disruption cassette was constructed by replacing the middle region of the gene with the pyrithiamine resistance gene (*ptrA*) from *A. oryzae*. The pPTR I plasmid (Takara Bio) was used for amplification of *ptrA* gene. Disruption cassette was constructed by three-step PCR method (Fig. 3.1.1) (69, 70).

Amplification of *ptrA* fragment (fragment 1) was done with 50 ng pPTR I plasmid as a template/ml with the primers 5'-GGGGTGACGATGAGCCGCTC-3' and 5'-GGGCAATTGA TTACGGGATCCC-3'. PCR was done under the following conditions: 95°C for 5 min; 94°C for 1 min, 61°C for 1 min, and 72°C for 3 min (30 cycles); and finally by 72°C for 10 min. Amplification of the 5'-flanking region of *FAO9* (fragment 2; containing a 5' part of *FAO9* structural gene) was performed using 50 ng *A. oryzae* strain niaD-2 genomic DNA/ml with

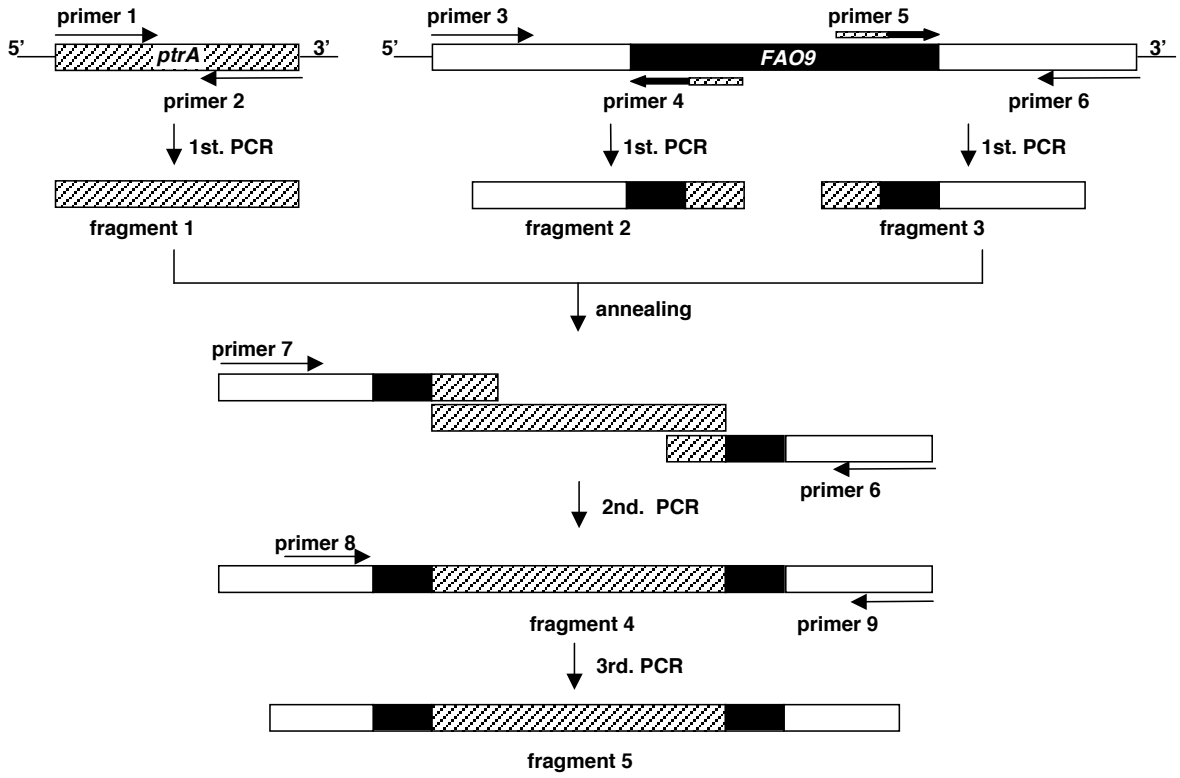

Fig. 3.1.1. Construction of *FAO9* gene disruption cassette. The middle region of *FAO9* gene from *A. oryzae* strain niaD-2 was replaced with *ptrA* by using three-step PCR. Hatched box, *ptrA* gene; solid box, *FAO9* gene; open box, adjacent region of *FAO9* gene.

primers 5'-GGATAGTTAGCAAGACGACATGGTG-3' and 5'-GAGCGGCTCATCGTCACC CCTGACTCTTCCTTGGGTGCCTG-3' under the following conditions: 95°C for 5 min; 94°C for 1 min, 60°C for 1 min, and 72°C for 5 min (30 cycles); and finally by 72°C for 10 min. The 3'-flanking region of *FAO9* (fragment 3; containing a 3' part of *FAO9* structural gene) was amplified as described above with the primers 5'-ATGGGATCCCGTAATCAATTG CCCAGTTTGCCCTTTGAGAAGACCC-3' and 5'-TTGACACCATAGAGATGCCTCGTAG -3'. (The underlined regions shown above correspond to 5'- and 3'-flanking region of the *ptrA* gene, respectively.)

Fusion PCR reaction was done by using 133 ng each of the fragments 1 to 3/ml as templates with primer 5'-ACAGCCACGACGATAACCTTTCCAC-3' and the 3'-primer used before for the amplification of 3'-flanking region of *FAO9* under the following conditions: 95°C for 5 min; 94°C for 1 min, 60°C for 30 s, and 68°C for 8 min, (30 cycles); and finally by 68°C for 10 min. PCR products were size-fractionated by electrophoresis, and the expected band (fragment 4) was extracted from the gel.

The nested PCR was done by using 145 ng fragment 4/ml as a template with primers 5'-GCAAGATTTGGTAACCTACGGGAGT-3' and 5'-AGGATGGAGAAGTTTTCCGAGC-3' under the following conditions: 95°C for 5 min; 94°C for 1 min, 68°C for 7 min (30 cycles); and finally by 68°C for 10 min.

The final PCR product, fragment 5, was ligated with a pCR2.1-TOPO vector and used to transform *E. coli* BL21(DE3) competent cells by electroporation (32). The fragment 5 was reamplified in the same way and used to transform *A. oryzae* strain niaD-2 cells.

Disruption of *FAO9* gene. *A. oryzae* strain niaD-2 mycelia (150 mg/ml) were incubated at 30°C for 3 h with protoplasting solution containing 20 mg of Yatalase (Takara Bio)/ml, 5 mg of cellulase (Onozuka R-10; Yakult Pharmaceutical Industry, Tokyo, Japan)/ml, 0.8 M NaCl, 10 mM DTT, and 10 mM sodium phosphate buffer (pH 6.0). After

filtration through an 11G-2 glass filter, the protoplasts were washed with 0.8 M NaCl by centrifugation at 700 × *g* for 5 min. The pellet was suspended in solution I composed of 0.8 M NaCl and 10 mM $CaCl_2$ in 10 mM Tris-HCl (pH 8.0) to a final concentration of 2×10^8 cells/ml, and 0.2 volumes of solution II containing 40% (wt/vol) PEG 4000 (Wako Pure Chemical Industries, Osaka, Japan), 50 mM $CaCl_2$, and 50 mM Tris-HCl (pH 8.0) was then added to the mixture. The 0.2-ml portion of this suspension and 20 μg of the *FAO9* disruption cassette were mixed on ice for 30 min. One milliliter of the solution II was added to the mixture, followed by incubation and incubated at room temperature for 15 min. After the addition of 8.5 ml of solution I, the mixture was centrifuged at 700 × *g* for 5 min. The pellet was suspended in 0.2 ml of solution I, and a small portion of the suspension was spread on an MM-2 medium containing 0.8 M NaCl and 0.1 mg pyrithiamine/ml. After incubation at 30°C for 3 days, each colony was picked up and culture was repeated three more times.

Southern blot analysis. A partial *FAO9* open reading frame (the nucleotide position 1 to 513) was amplified by PCR and used as a probe. The PCR fragment was labeled by DIG-High Prime (Roche Diagnostics). The genomic DNA of *A. oryzae* strain niaD-2 transformants (7.5 μg) were digested with *Bam*H I and fragments were separated by electrophoresis. The fragments were transferred onto a Hybond-N+ membrane (Amersham Biosciences) with 0.4 N NaOH. The membrane was heated in an oven at 120°C for 30 min. The membrane was transferred to prehybridization solution (DIG Easy Hyb; Roche Diagnostics) and preincubated at 50°C for 1 h. Digoxigenin-labeled probe was added to the prehybridization solution to the final concentration of 50 ng/ml, and hybridization was done overnight at 50°C. The membrane was washed twice by incubating at room temperature in 0.1% SDS in 2 × SSC (0.3 M NaCl and 30 mM trisodium citrate dihydrate [pH 7.0]) for 10 min and washed twice by incubating at 68°C in 0.1% SDS in 0.1 × SSC for 15 min. Detection was done by using DIG-High Prime DNA labeling and detection starter kit II.

RESULTS

Disruption of *FAO9* gene. A gene disruption cassette containing *ptrA* gene (5.5 kb) was constructed by three-step PCR. The cassette was used to transform protoplasts of *A. oryzae* and the pyrithiamine-resistant transformants were obtained (Fig. 3.1.2). The disruption was confirmed by Southern blot analysis. As shown in Fig. 3.1.2B, the disruptant gave a 2.8-kb band with *Bam*H I-digested genome as the template. A 4.0-kb band was observed in the wild-type strain, suggesting there was no insertion of *ptrA*. This result showed that a single copy of disruption cassette was integrated into the *FAO9* locus of the wild-type strain.

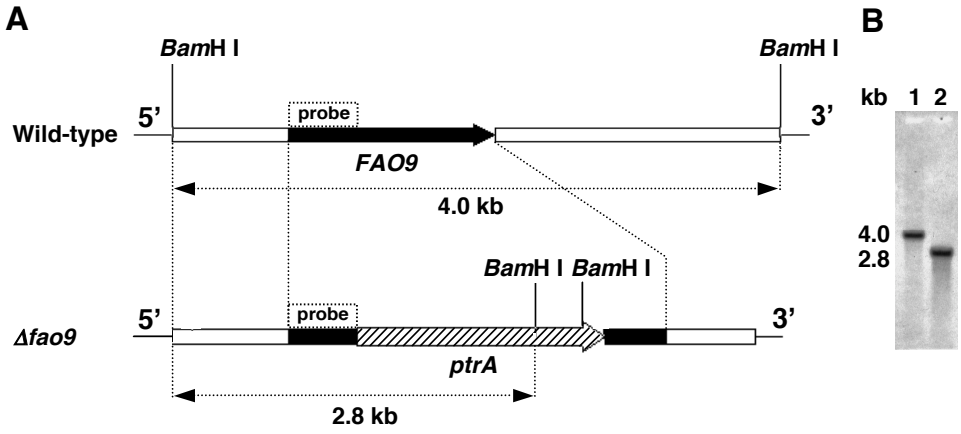

Fig. 3.1.2. Physical map (A) and Southern blot analysis (B) of the *FAO9* loci in the wild-type and disruptant strains. In panel A, solid boxes show *FAO9* gene containing its 5'- and 3'-flanking regions. Upper map represents the physical map of *FAO9* locus in the wild-type strain and the lower map represents the result of homologous recombination of the gene disruption cassette. Hatched arrow shows *ptrA* gene. In panel B, genomic DNA was digested with *Bam*H I and separated by electrophoresis on a 1% agarose gel. A part of *FAO9* gene (513 bp; shown in panel A) was used as a probe. Lane 1, the wild-type strain; lane 2, strain *Δfao9*.

Effects of carbon and nitrogen sources on the growth of wild-type and *Δfao9* strains. Table 3.1.1 and Fig. 3.1.3 show the growth of the wild-type and *Δfao9* strains on various solid media. The wild-type strain niaD-2 (*ΔniaD*) grew on the medium containing Fru-Val or Fru-Lys as the sole carbon and/or nitrogen sources to the same level as found with the medium containing glucose and $NaNO_2$ as the sole carbon and nitrogen sources, respectively. However, strain *Δfao9* did not grow on Fru-Val as the sole carbon and/or nitrogen sources in agreement with the fact that the gene product of *FAO9* was active toward Fru-Lys and Fru-Val. Strain *Δfao9* grew on the medium containing Fru-Lys as the sole carbon and nitrogen sources, however, slower than the wild-type strain. The wild-type strain grew on the minimum medium containing glucose and Fru-Val to the same level as found with the medium containing glucose and nitrite as the sole carbon and nitrogen sources,

Table 3.1.1. Growth of the wild-type and *Δfao9* strains of *A. oryzae* on various solid media.

Source		Growth	
C	N	Wild type	*Δfao9*
Fru-Val	Fru-Val	+++	-
Fru-Lys	Fru-Lys	+++	++
Fru-Val	$NaNO_2$	-	-
Fru-Val	$(NH_4)_2SO_4$	+++	-
Glc*	Fru-Val	+++	±
Glc	$NaNO_2$	+++	+++
Glc	$(NH_4)_2SO_4$	+	+
Glc	$NaNO_2$, $(NH_4)_2SO_4$	-	-
Glc	Fru-Val, $NaNO_2$	-	-
Glc	$NaNO_3$	-	-

*Glc, Glucose.

The wild-type niaD-2 and *Δfao9* strains of *A. oryzae* were cultivated at 30°C in the media for assimilation test containing various carbon and nitrogen sources. Growth (as measured by the mycelial diameter): +++, >5 cm; ++, 3 to 5 cm; +, <3 cm; -, no growth; ±, the same growth level was observed on the medium without nitrogen source.

respectively, indicating that catabolite repression did not affect the expression of *FAO9*. The maximum growths were observed after 11–12 days in all the cases described above. The wild-type strain and disruptant did not grow on the medium containing glucose, nitrite, and ammonium, and on the medium containing nitrite and Fru-Val.

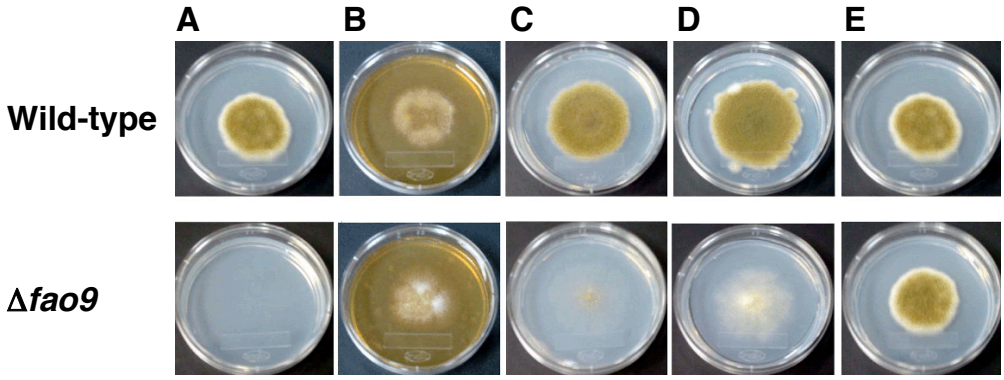

Fig. 3.1.3. Growth of the wild-type and *Δfao9* strains of *A. oryzae* on various solid media. The photographs are in accordance with Table 3.1.1. (A) Fru-Val was used as carbon and nitrogen sources; (B) Fru-Lys was used as carbon and nitrogen sources; (C) Fru-Val and $(NH_4)_2SO_4$ were used as the carbon and nitrogen sources, respectively; (D) Glc and Fru-Val were used as the carbon and nitrogen sources, respectively; (E) Glc and $NaNO_2$ were used as the carbon and nitrogen sources, respectively.

DISCUSSION

In the nitrogen catabolism of fungi, ammonium and glutamine are known as primary nitrogen sources (71). Figure 3.1.4 shows the nitrogen metabolic pathway in *A. oryzae* illustrated with regard to the nitrogen metabolisms of other filamentous fungi (72–74). Ammonium and glutamine repress expression of the enzymes involved in utilization of secondary nitrogen sources such as nitrate, methylamine, and other amino acids. This reaction is called ammonium repression. Furthermore, it was reported that growth of

A. nidulans is inhibited on the medium containing ammonium and nitrite, suggesting that ammonium repression occurs on the nitrite metabolism and the remaining nitrite exhibits cytotoxicity in *A. nidulans* (75). The author found that such an ammonium repression on the nitrite metabolism also occurs in *A. oryzae* and Fru-Val may repress the expression of nitrite reductase. The wild-type strain could grow normally on the medium containing Fru-Val and ammonium, indicating that ammonium repression did not affect at least the expression of *FAO9* gene.

Slower growth of *Δfao9* strain on Fru-Lys indicates that the gene products of *FAO4* in *Δfao9* strain was involved in the degradation of Fru-Lys. It is consistent with the substrate specificity of the two FAOD isozymes in *A. oryzae*.

In this study, by using a fungi imperfecti *A. oryzae*, the author showed that *FAO9* gene disruptant had different phenotype from the wild-type strain and *FAO9* was greatly engaged in the utilization of fructosyl-amino acid from the medium.

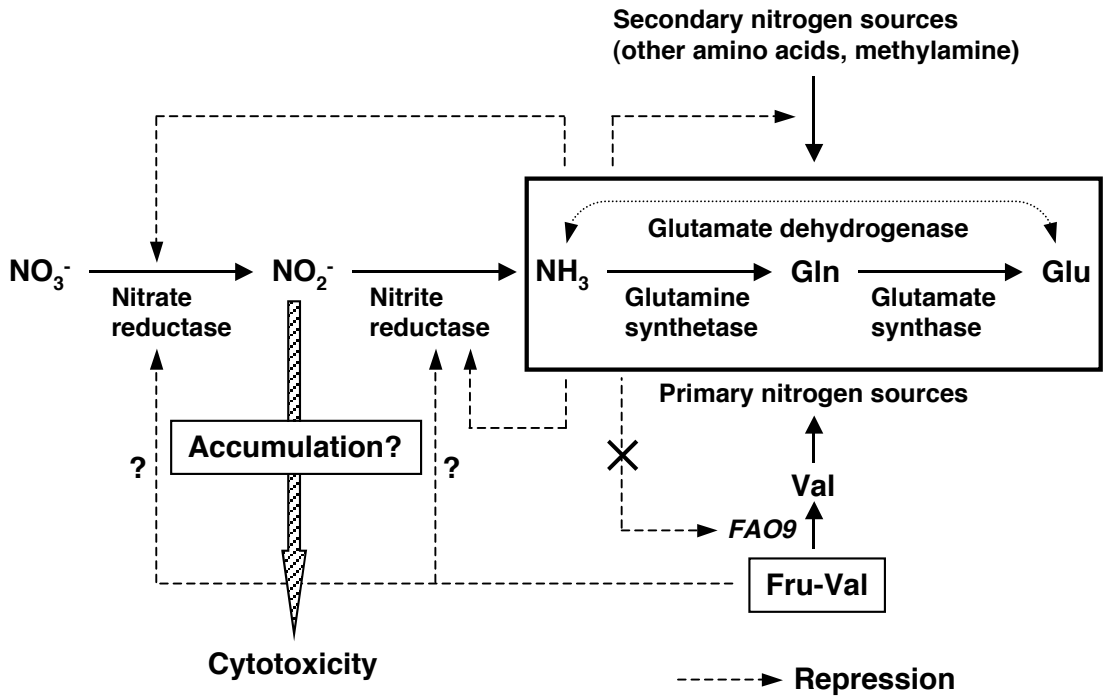

Fig. 3.1.4. Postulated regulation by fructosyl-amino acid in nitrogen metabolism of *A. oryzae*.

Section 2 Expression analysis of fructosyl-amino acid oxidase genes in *Aspergillus oryzae*

Enzymes involved in the metabolism of nitrogen sources have been commonly found in small amounts during growth of microorganisms on ammonium (71). This phenomenon is called ammonium repression. Especially, nitrate reductase is very sensitive to ammonium repression (71). In the previous section, the author predicted that ammonium repression dose not affect the expression of *FAO9* gene and that Fru-Val represses the expression of the enzymes concerning nitrate metabolism. In this section, the author deals with expression of the genes concerning nitrate metabolism and the two FAOD genes of *A. oryzae* strain RIB40, and discusses the FAOD gene expression in nitrogen metabolism in *A. oryzae*. The author attempts to hypothesize the physiological roles of FAOD isozymes in *A. oryzae* from the results of this series of study in Chapters I–III.

MATERIALS AND METHODS

Unless otherwise noted, all experiments were done as already described.

Induction of *FAO* genes on solid media. Spores (4.0×10^7) of *A. oryzae* strain RIB40 were used to inoculate 400 ml of MM-1 and incubated at 30°C for 36 h. Mycelia were collected on a filter paper (weighing 0.04 g), washed with 0.85% NaCl, and used to inoculate agar plates containing various nitrogen sources. After 1.5 h and 3.0 h of incubation at 30°C, the mycelia were collected, washed with 0.85% NaCl, and used as the source of total RNA, which was analyzed by Northern blotting.

Induction of *FAO* genes in AB medium. Spores (10^7 spores) of *A. oryzae* strain

niaD-2 were incubated at 30°C for 21–26 h in 100 ml of autoclave-browned (AB) medium (described in Chapter II) containing 2% glucose and 1% amino acid to induce *FAO* gene expression. Glucose was separately sterilized to make the non-browned media, which were used as the control. The pH of these media was adjusted to pH 5.5. The mycelia were collected and washed with 0.85% NaCl, and total RNA was prepared and analyzed as before.

Northern blot analysis. Total RNA (10 μg) was separated on a formaldehyde/agarose gel, and transferred onto a Hybond-N+ membrane. The membrane was treated with 0.05 N NaOH solution, baked at 120°C for 30 min, and UV light was irradiated from a trans illuminator for 3 min to fix the RNA. Probes used in this study are listed in Table 3.2.1. The probes were labeled by using PCR with the PCR DIG probe synthesis kit (Roche Diagnostics), PCR conditions as follows: 95°C for 3 min; 94°C for 1 min, 62°C (for *FAO4* and *FAO9* probes), 55°C (for *niaD* probe), or 60°C (for β-actin probe) for 1 min, 72°C for 1 min (30 cycles); and finally by 72°C for 5 min. Hybridization and detection were done as described in the previous section for Southern blot analysis.

Nucleotide sequence accession numbers. The accession number of the gene *niaD* of *A. oryzae*, newly used in this section, was D49701.

Table 3.2.1. Primers used for probe synthesis.

Target gene	Name	Expected size (bp)	Sequence (5'→3')
FAO4	RIBNo45'-3	514	5'-primer: ATACCATTCTTTCCGCAGGGGC
	RIBNo43'-3		3'-primer: AGAGCATCCGCTATGAACCCAC
FAO9	RIBNo95'-2	500	5'-primer: CGCTGTGCTGAAAGGAAACTTC
	RIBNo93'-2		3'-primer: GGGTCTTCTCAAAGGGCAAACTC
niaD	AoniaDp5'	505	5'-primer: TGAAACCAGCCAGAAAGGGAC
	AoniaDp3'		3'-primer: CGGACCACAAATCAGGACCATAC
β-actin	RIBActin5'	467	5'-primer: GTTTTCGAGACTTTCAACG
	RIBActin3'		3'-primer: GTGGTTACGTGGATTCCCC

RESULTS

Expression analysis of *FAO* genes in various solid media. Figure 3.2.1 shows the effects of glucose and ammonium on the expression of *FAO4* and *FAO9* genes. The transcription of both *FAO* genes was observed at 1.5 h after the non-induced mycelia were transferred on the inducing media. The transcriptional levels of *FAO4* and *FAO9* were constant regardless of adding glucose and/or ammonium sulfate in the inducing media.

In order to examine whether the growth inhibitions in the media containing nitrite were the results of repression of nitrite reductase (*niiA*) and nitrite toxicity as described in the previous section, a part of the *niiA* gene was cloned and used as the probe for Northern blot. Although dot blot analysis with this probe was done on the total RNAs from various mycelial samples, remarkable spot was not detected. Thus, the probe could not be used for the expression analysis. Instead of *niiA* gene, expression of nitrate reductase gene (*niaD*) in *A. oryzae* strain RIB40 was analyzed (Fig. 3.2.2). Expression of *niaD* gene was observed when the medium containing glucose and nitrate was used, but was not observed when medium containing Fru-Val and nitrate was used. Although the expression of *niaD* gene was

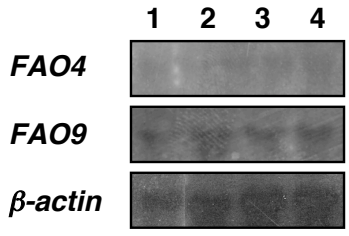

Fig. 3.2.1. Carbon catabolite and ammonium repressions on *FAO* genes expression. Northern blot analysis of *FAO* genes in *A. oryzae* strain RIB40 was carried by using total RNA extracted from the mycelial samples induced on the various media. Lane 1, 1% Fru-Val and 0.3% $(NH_4)_2SO_4$ were used as the carbon and nitrogen sources, respectively; lane 2, 1% glucose, 1% Fru-Val, and 0.3% $(NH_4)_2SO_4$ were used as the carbon and nitrogen sources; lane 3, 1% glucose, 0.3% Fru-Val, and 0.3% $(NH_4)_2SO_4$ were used as the carbon and nitrogen sources; lane 4, 1% glucose, 0.3% Fru-Val, and 0.3% $NaNO_3$ were used as the carbon and nitrogen sources. The extractions were done at 1.5 h after inductions.

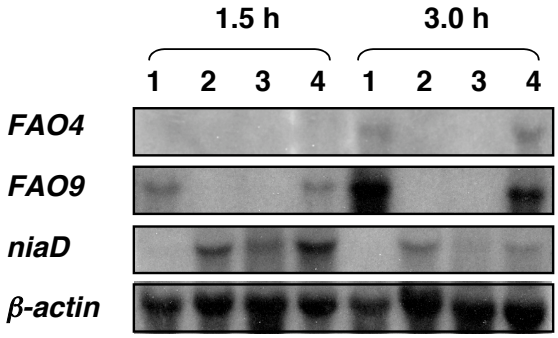

Fig. 3.2.2. Transcriptional levels of *FAO* and *niaD* genes in *A. oryzae* strain RIB40 with the various nitrogen compounds. Northern blot analysis of *FAO* and *niaD* genes in *A. oryzae* strain RIB40 was carried by using total RNA extracted from the mycelial samples induced in the various media. Lane 1, 1% Fru-Val and 0.3% $NaNO_3$ were used as the carbon and nitrogen sources, respectively; lane 2, 1% glucose and 0.3% $NaNO_3$ were used as the carbon and nitrogen sources, respectively; lane 3, 1% glucose, 0.3% $(NH_4)_2SO_4$, and 0.3% $NaNO_3$ were used as the carbon and nitrogen sources; lane 4, 1% glucose, 0.3% Fru-Val, and 0.3% $NaNO_3$ were used as the carbon and nitrogen sources. The extractions were carried out at 1.5 h and 3.0 h after inductions.

observed at 1.5 h when the medium containing glucose, ammonium, and nitrate was used, the band was diminished in another 1.5 h of incubation. Expression of *niaD* gene was slightly observed after 3.0 h of culture in the medium containing glucose, Fru-Val, and nitrate.

Induction of *FAO* genes in AB media. Transcriptional level of FAOD genes were examined by using various AB media (containing lysine, valine, arginine, or asparagine). As shown in Fig. 3.2.3, the *FAO9* transcriptional level was much higher in mycelia induced in the AB medium containing valine or lysine than the non-browned medium. When arginine and asparagine were used with glucose in AB media, *FAO9* gene expression was hardly seen. The expression level for *FAO4* gene was slightly higher in mycelia induced in the medium containing lysine or valine than in those with non-browned medium, and these levels were much lower than those for *FAO9*.

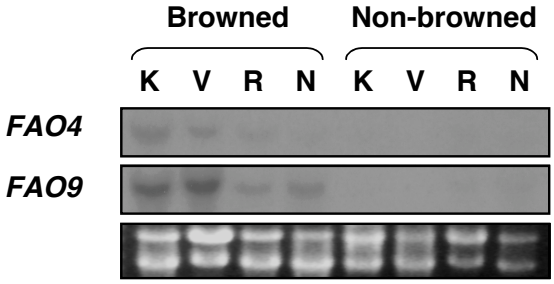

Fig. 3.2.3. Induction of *FAO* genes in autoclave-browned medium. Northern blot analysis of *FAO* genes in *A. oryzae* strain niaD-2 was carried by using total RNA extracted from mycelial samples cultured in autoclave-browned media containing 2% glucose and 1% various amino acid indicated in the figure.

DISCUSSION

Ammonium repression is found in the nitrogen metabolisms in *A. nidulans* and *N. crassa*. In this study, transcriptional analysis of *niaD* gene revealed that ammonium repression also occurs in the expression of *niaD* in *A. oryzae*, and Fru-Val repressed the *niaD* expression. AreA is known to be the major nitrogen regulatory protein which activates transcription of many structural genes encoding enzymes for secondary nitrogen source catabolism under nitrogen-limiting conditions (71–73). For example, the transcription of nitrate reductase needs the pathway-specific transcriptional factor NirA, and the AreA. The mechanism of ammonium repression on *niaD* gene expression of *A. nidulans* is accounted for as follows: ammonium ion in growth medium represses the expression of AreA. Since NirA needs to bind the AreA for the transcription of *niaD*, *A. nidulans* is not able to utilize nitrate in the medium with ammonium ion, in which *areA* expression is repressed. From the finding described above, the author assumes that Fru-Val dose not affect the expression of *niaD* directly but that of *areA*. Although the analysis of *niiA* expression was unsuccessful in this

study, it was suggested that *niiA* was also repressed by Fru-Val or ammonium, and consequently this fungus could not grow because of toxicity of nitrite in the medium as shown in the previous section.

It was revealed that catabolite and ammonium repression did not affect the expression of *FAO* genes by Northern blot analysis, in agreement with the growth test described in the previous section. Inducing effects of fructosyl-amino acids were remarkable on *FAO9* but less on *FAO4* in any case (Figs. 3.2.1–3). These results suggested the function of *FAO9* in utilization of fructosyl-amino acids as nitrogen sources, and really significant amount of fructosyl-amino acids is found in nature.

Maillard himself suggested that the Maillard reaction occurs in nature (76) and there have been many reports for involvement of this reaction in the formation of humic substances in soil (77). However, there has not been any report for the existence of fructosyl-amino acid in soil. Extremely acidic or alkaline treatments have been used for separation of soil humic substances, however, fructosyl-amino acids are unstable under such conditions. There may be more Amadori compounds including fructosyl-amino acids in soil than expected, and extraction method should be improved.

Low inducibility of *FAO4* may be referable to localization in certain organelle and substrate specificity of the gene product. As shown in Chapter I, it is suggested that Fao4 is localized in Woronin body. Woronin body is a peroxisome-derived dense-core vesicle that is specific to several genera of ascomycotina and fungi imperfecti. Recently, *HEX1* gene, which encodes the major protein of Woronin body, was isolated from *N. crassa* (78). *A. oryzae HEX1* ortholog (*HEXA*) was also reported (79). It is known that, in these fungi, Woronin body seals septal pores in response to cellular damage (78, 80, 81), and also that fungal mycelia lacking *HEX1* function were unable to survive nitrogen starvation *in vitro* (82). C-terminal sequence of *HEX1* is tripeptide SRL and Hex1 protein localizes in Woronin bodies,

when the *HEX1* gene is expressed (78). Minami *et al*. showed that recombinant EGFP, in which SRL was introduced in the C-terminal, was localized with Hex1 protein in *A*. *oryzae* (83). These results suggest that C-terminal SRL is a signal for Woronin body and, until now, the SRL in PTS1 consensus sequences is the only signal sequence found for subcellular sorting of the protein to Woronin body. The PTS1 sequence in Fao4 is 'SRL'. So, it is expected that Fao4 and Fao9 are localized in peroxisomes (Fig. 3.2.4A), but, under the condition for Woronin body formation, Fao4 is sorted into Woronin bodies (Fig. 3.2.4B). Fao4 was specific for Fru-Lys and biological Fru-Lys was strongly suggested to come from glycated proteins, or, in other words, from damaged proteins. Taking all together, the author builds up a hypothesis as follows: when the glycated proteins in cell membrane or septum are increased, the fluidity of the membrane is lost, causing some pores. Woronin body containing Fao4 is sorted to the cell membrane or septum to seal the pores and to deglycate the proteins. Thus, the author predicts that Fao4 acts toward endogenous fructosyl-amino acids, and that Fao9 is for use of exogenous fructosyl-amino acids.

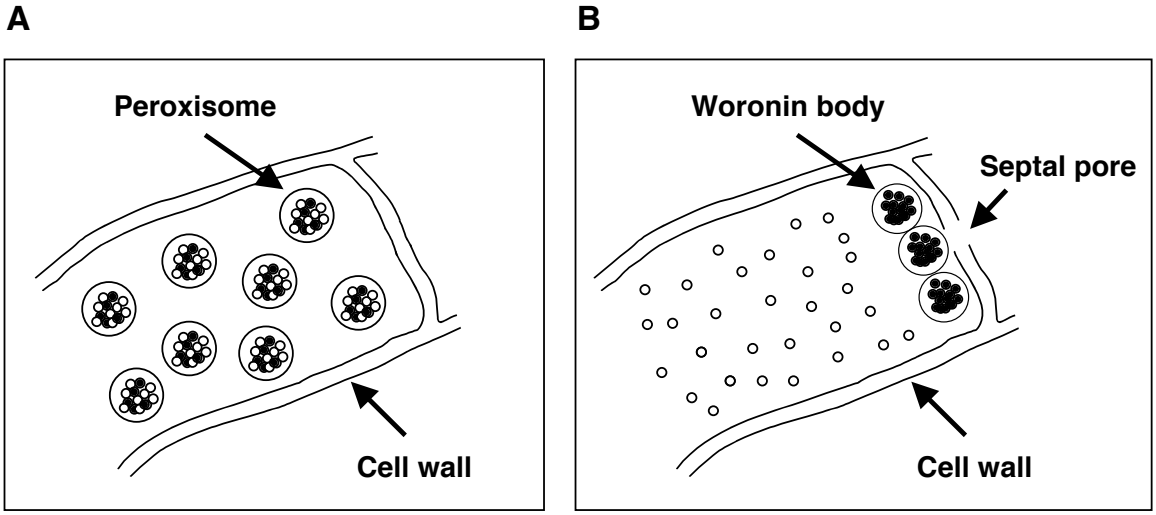

Fig. 3.2.4. Putative cellular localization of FAOD isozymes. A part of the fungal cells is shown. The small black and white particles represent Fao4 and Fao9 proteins, respectively. In panel A, under conditions for peroxisome formation. In panel B, under conditions for Woronin body formation.

Summary

Strain *Δfao9* did not grow on Fru-Val as the sole carbon and/or nitrogen sources. Carbon and nitrogen catabolite repressions did not affect the growth of *A. oryzae*. Northern analysis revealed that Fru-Val repressed the *niaD* expression, and catabolite and ammonium repressions did not affect the expression of *FAO* genes. The transcriptional level of *FAO9* was higher than that of *FAO4* in any case.

From these results, combined with the previous results, the author predicts that Fao4 acts toward endogenous fructosyl-amino acids, and that Fao9 is for use of exogenous fructosyl-amino acids.

CONCLUSION

FAOD homolog genes found in genomes of *S. pombe* and *A. oryzae* were examined. Two homologs from *S. pombe* were characterized as a POX and a SAX, and four of eleven homologs from *A. oryzae* were characterized as a POX (Fao1), two types of SOXs (Fao2 and 5), and an FAOD (Fao9). Alignment of the primary structures of FAOD strongly suggested that *FAO4* gene encodes another FAOD, although rFao4 had no activity toward substrates for FAOD. The deduced amino acid sequences of Fao4 and Fao9 revealed that different PTS1 sequences in their C-terminal. It was expected that Fao4 and Fao9 are localized in peroxisomes, but, under some conditions, Fao4 is sorted to Woronin bodies, suggesting different role of these two isozymes *in vivo*.

By the analysis of FAOD homologs in this study, they were thought to be categorized as at least three kinds of amine oxidases, which cleave the C-N bonds of the imino group: i) those enzymes specific for sarcosine and its related compounds; ii) those specific for saccharopine; and iii) those specific for fructosyl-amino acids.

FAOD-Ao1, -Ao2a, and -Ao2b, as the active fractions of FAOD, were isolated from *A. oryzae* strain RIB40. N-terminal and internal amino acid sequences for FAOD-Ao2a corresponded for those of FAOD-Ao2b, suggesting that these two isozymes were derived from the same protein. The genes encoding the FAOD isozymes, *FAOAo1* and *FAOAo2*, corresponded to *FAO4* and *FAO9*, respectively. Thus, it was concluded that there are two types of FAOD in *A. oryzae*. The two FAOD isozymes were different in the substrate specificity for α- and ε-glycated compounds. It is noteworthy that FAOD-Ao1 was not active toward Fru-Val. Similar results were known with amadoriase isozymes in *A. fumigatus*; one of the isozymes, amadoriase I, preferred ε-glycated to α-glycated compounds, whereas another, amadoriase II, was active toward the both compounds to the

same extent (8). Since Fru-Lys in nature are thought to be derived from the glycated proteins, the author predicted *FAO4* is used for deglycation of endogenously produced fructosyl-amino acid residues in fungal cells. In addition, FAOD isozymes from *A. oryzae* were not active toward any opines tested, indicating that physiological role of eukaryotic FAOD is different from that of prokaryotic FAOD.

The author attempted disruption of *FAO9* gene of *A. oryzae* strain niaD-2 (*ΔniaD*) and expression analysis of two FAOD genes to elucidate the physiological role of FAOD. Strain *Δfao9* did not grow on Fru-Val as the sole carbon and/or nitrogen sources. Carbon and nitrogen catabolite repressions did not affect the growth of *A. oryzae*. Northern analysis revealed that Fru-Val repressed the *niaD* expression, and catabolite and ammonium repression did not affect the expression of *FAO* genes.

The author thinks that lower inducibility of *FAO4* than *FAO9* by fructosyl-amino acids is referable to its localization in an organelle and the substrate specificity of the gene product and that Fao4 in *A. oryzae* is concerned with recycle of amino acids from fructosyl-amino acids in the cell. From the results in these studies, the author hypothesized that Fao4 acts toward endogenous fructosyl-amino acids, and that Fao9 is engaged in the utilization of exogenous fructosyl-amino acids.

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Shin-ichi Akazawa

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