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Distribution and Properties of Fructosyl Amino Acid Oxidase in Fungi

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Fructosyl amino acid oxidase, an enzyme that can be used for the determination of glycated proteins in blood samples from diabetic patients, was used to screen cultures in our microorganism culture collection. Fructosyl amino acid oxidase was found only in the strains of four genera of fungi, *Aspergillus*, *Fusarium*, *Gibberella*, and *Penicillium* and exhibited different substrate specificities against fructosyl valine and N^{ε}-fructosyl N^{α}-Z-lysine. A fructosyl valine-specific enzyme from *Penicillium janthinellum* AKU3413 was monomeric (M_r, 49,000), was most active at 35°C and pH 8.0, and had a covalently bound flavin adenine dinucleotide as a prosthetic group.

The Maillard reaction starting from Amadori rearrangement causes the browning of foods and the impairment of their functions during long-term storage (7). In the initial step of the reaction, glucose is attached to the α or ε amino group of amino acids and proteins to form unstable Schiff's base and becomes rearranged to form stable ketoamine products (10). This series of nonenzymatic reactions is called glycation to distinguish it from enzymatic glycosylation. The nonenzymatic reactions also occur in vivo. The glycation of blood proteins, hemoglobin and albumin, has been known to be enhanced in diabetic patients whose blood glucose level is increased (2, 5). The enzymatic determination of glycated amino acids or proteins is expected to be useful for the control of food quality and to monitor blood sugar levels in diabetic patients.

In our previous study (8), N^{ε}-fructosyl N^{α}-Z-lysine (ε-FL), a model glycated protein, was used as a substrate to screen for an enzyme suitable for use in a diagnostic test, because the most probable glycation site in proteins is the ε amino group of the lysine residue. An isolate, *Fusarium oxysporum* S-1F4, was found to produce fructosyl lysine oxidase (FLOD), which was purified and characterized (8). The enzyme catalyzes the following reaction: ε-FL + O_2 → N^{α}-Z-lysine + glucosone + H_2O_2. FLOD has potential for use in the analysis of a variety of glycated proteins. On the other hand, the amount of hemoglobin A_{1c}(HbA_{1c}), the glycated compound of hemoglobin, reflects the blood glucose level in the preceding several months and the high-pressure liquid chromatographic method for the measurement of HbA_{1c} is currently used as a standard routine clinical technique for the diagnosis of diabetes mellitus (4). If an enzymatic determination of HbA_{1c} is established, the diagnostic test would become more convenient. Since the glycation site of HbA_{1c} is the N-terminal valine residue in the β-chain (1, 3, 6), the enzyme that acts on fructosyl valine (FV) is useful for diagnostic analysis. From these viewpoints, we examined the distribution of fructosyl amino acid oxidase (FAOD) in microorganisms using ε-FL and FV as substrates.

Stock cultures in our laboratory (114 strains of actinomyces, 159 strains of bacteria, 43 strains of basidiomyces, 102 strains of fungi, and 193 strains of yeasts) were used for screening FAOD-producing microorganisms with an autoclave-browned medium (GL-AB medium), which contains 20 g of glucose, 10 g of L-lysine · HCl, 1 g of K_2HPO_4, 1 g of NaH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 2 g of yeast extract in 1,000 ml of distilled water (pH 5.5). This has been established as the most convenient medium for FAOD production in our previous paper (9). Organisms were grown in 10 ml of GL-AB medium at 28°C under reciprocal shaking for several days. The cells of bacteria and yeasts were harvested by centrifugation at 8,000 × *g* for 5 min. The fungal mycelia were collected by filtration with filter paper, washed with 0.85% KCl, suspended with 0.1 M Tris-HCl buffer (pH 7.5), and disrupted by a Mini-Beatbeater model 3110BX (Japan Lambda, Co.). The cell extract was obtained by removing cell debris by centrifugation at 8,000 × *g* for 10 min. FAOD activity and protein was determined as described previously (8). FAOD activity was found only in strains belonging to the fungal genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Gibberella* (Table 1). The enzymes from different origins had different activity ratios for the two substrates tested, ε-FL and FV. For instance, the enzymes from *F. oxysporum* S-1F4 and *Gibberella fujikuroi* were more active with ε-FL than with FV. On the other hand, FV was a better substrate for the enzymes from several strains of the genus *Penicillium*. The enzyme was purified from several organisms showing high FLOD activity; as a result, each organism had a single species of the enzyme, indicating that the substrate specificities of enzymes varied greatly in the organisms. These results suggest that it is possible to choose enzymes fitted for the purpose; an enzyme highly active with ε-FL is useful for the determination of proteins that are glycated at an internal lysine residue, while an enzyme specific to FV is more suitable to specific analysis of hemoglobin which is glycated in an N-terminal valine. An enzyme from the latter category was purified from *Penicillium janthinellum* AKU3413 and characterized.

All procedures in the enzyme purification were performed at 0 to 4°C. An appropriate concentration of potassium phosphate buffer, pH 8.5, containing 0.1 mM dithiothreitol (KPD buffer) was used throughout the purification procedure unless otherwise stated. *P. janthinellum* AKU3413 was cultivated at 28°C for 36 h with 10 liters of GL-AB medium in a 15-liter jar fermentor. Washed mycelia (425 g [wet weight]) were suspended in 0.1 M KPD buffer and disrupted by a Dyno-Mill (Willy A. Bachofen Manufacturing Engineers, Basel, Switzerland). The cell extract was obtained as a supernatant after centrifugation at 9,000 × *g* for 30 min. Ammonium sulfate was added to 40% saturation to the cell extract, and then the precipitate formed was discarded with centrifugation at 15,000

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TABLE 1. Distribution of FAODs in fungi

Strain[a]	FAOD activity (U/ml) with substrate:		Ratio[b]
	ε-FL	FV	
Aspergillus candidus AKU3310	0.289	0.063	4.6
Aspergillus flavus AKU3368	0.324	0.054	6.0
Aspergillus flavus IAM2007	0.080	0.280	0.3
Aspergillus flavus IAM2119	0.165	0.053	3.1
Aspergillus oryzae IFO4242	0.054	0.012	4.5
Aspergillus oryzae IFO5710	0.161	0.027	6.0
Aspergillus oryzae IAM2603	0.181	0.025	7.2
Aspergillus oryzae IAM2609	0.107	0.278	0.4
Aspergillus terreus IFO6365	0.367	0.044	8.3
Fusarium oxysporum f. sp. *lini* IFO5880	0.678	0.229	3.0
Fusarium oxysporum f. sp. *batatas* IFO4468	0.159	0.026	6.1
Fusarium oxysporum f. sp. *niveum* IFO4471	0.114	0.031	3.7
Fusarium oxysporum f. sp. *cucumerium* IFO6384	0.065	0.018	3.6
Fusarium oxysporum f. sp. *melongenae* IFO7706	0.151	0.023	6.6
Fusarium oxysporum f. sp. *apii* IFO9964	0.083	0.014	5.9
Fusarium oxysporum f. sp. *pini* IFO9971	0.053	0.010	5.3
Fusarium oxysporum f. sp. *fragariae* IFO31180	0.402	0.098	4.1
Fusarium oxysporum S-1F4	0.462	0.018	25.7
Gibberella fujikuroi IFO6356	0.307	0.010	30.7
Gibberella fujikuroi IFO6605	0.059	0.009	6.6
Penicillium oxalicum IFO5748	0.037	0.203	0.2
Penicillium javanicum IFO4639	0.056	0.239	0.2
Penicillium notatum IFO4640	0.086	0.040	2.2
Penicillium chrysogenum IFO4897	0.040	0.245	0.2
Penicillium janthinellum AKU3413	0.075	0.434	0.2
Penicillium cyaneum IFO5337	0.136	0.484	0.3

[a] AKU, Faculty of Agriculture, Kyoto University, Japan; IAM, Institute of Applied Microbiology, University of Tokyo; IFO, Institute for Fermentation, Osaka, Japan.
[b] The ratio of FAOD activity with ε-FL as the substrate to FAOD activity with FV as the substrate.

$\times g$ for 10 min. The supernatant to which ammonium sulfate was added to 75% saturation was centrifuged at 15,000 $\times g$ for 10 min. The resultant precipitate was dissolved in 50 mM KPD buffer and dialyzed overnight against about 50 volumes of the same buffer at 4°C. The dialyzed solution was applied to a DEAE-Sephacel column (5.1 [inner diameter] by 24 cm) equilibrated with 50 mM KPD buffer. The enzyme activity was found in the washed fractions with the same buffer. The ammonium sulfate precipitate (55% saturation) was dissolved in 50 mM KPD buffer containing 25% saturation of ammonium sulfate, and then the solution was loaded on a phenyl-Sepharose HR10/10 column equilibrated with 50 mM KPD buffer containing 25% saturation of ammonium sulfate. For the first 5 min, the column was run isocratically with the equilibration buffer and then a linear gradient of up to 50 mM KPD buffer for 40 min (flow rate, 2 ml/min) was run. The active fractions were combined and precipitated with 75% saturation of ammonium sulfate. The precipitate was dissolved with 0.1 M KPD buffer containing 0.1 M NaCl and chromatographed on a Superdex 200 HR16/60 column. The procedures are summarized in Table 2.

The purified enzyme showed an apparent homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had a specific activity of 18.6 U/mg of protein. The relative molecular mass was estimated to be 39,000 by Superdex 200 gel filtration and 49,000 by SDS-PAGE, indicating that the enzyme is monomeric. The purified enzyme had an adsorption spectrum with two absorption peaks at 366 and 448 nm, characteristic of flavin compounds (Fig. 1). The absorption maximum at 448 nm disappeared when the enzyme was incubated with FV as the substrate. Identification and quantitation of this flavin were carried out by the enzymatic method with adenylate kinase (Sigma Co., St. Louis, Mo.) (8). As a result, 2.57 nmol of AMP was liberated from 2.49 nmol of enzyme protein by phosphodiesterase treatment. Therefore, the enzyme had 1 mol of flavin adenine dinucleotide (FAD) as a prosthetic group per mole. Trichloroacetic acid treatment did not liberate the chromophore and yellow pigment that migrated with the enzyme protein by SDS-PAGE. These results suggest that the chromophore (FAD) binds covalently to the protein.

The optimum temperature and pH of the enzyme were 25°C

TABLE 2. Purification of FAOD from *P. janthinellum* AKU3413

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg · protein)	Purification (fold)	Yield (%)
Cell extract	317	3,260	0.0971	1	100
Ammonium sulfate (40–75% saturation)	294	904	0.351	4	93
DEAE-Sephacel	196	171	1.15	12	62
Ammonium sulfate (0–55% saturation)	133	24.7	5.39	56	42
Phenyl-Sepharose 6FF	49.8	3.98	12.5	129	16
Superdex 200	12.8	0.692	18.6	192	4.0

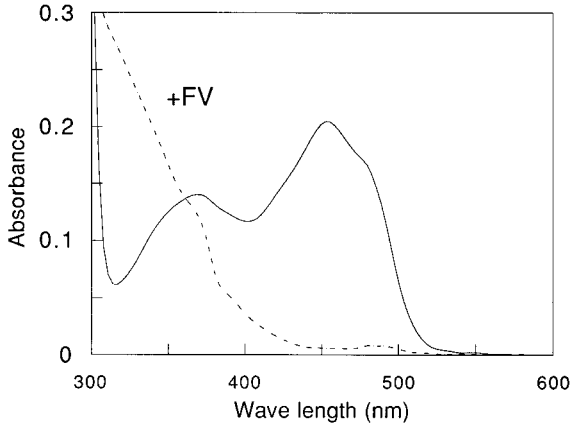

FIG. 1. Absorption spectra of FAOD. ——, purified FAOD (0.91 mg/ml) in 33 mM KPD buffer; – – –, purified FAOD (0.91 mg/ml) preincubated with 0.2 mM FV in 33 mM KPD buffer at 30°C for 3 h.

and 7.5, respectively (Fig. 2A and B, respectively). The residual activity of the enzyme was measured after incubation for 10 min at various temperatures and pHs. In these experiments, more than 90% of activity was retained over a temperature range of 25 to 40°C and a pH range of 6.0 to 9.0 (data not shown). The enzyme was incubated with an inhibitor or a metal salt at 30°C for 10 min, and then the remaining activity was assayed under the standard conditions. The enzyme activity was sensitive to sulfhydryl reagent; 0.1 mM *p*-chloromercuribenzoate (>99% inhibition), 1 mM dithiobis(2-nitrobenzoate) (87%), and 1 mM $HgCl_2$ (98%): and carbonyl compounds; 1 mM phenylhydradine (99%), 1 mM hydradine (88%). The

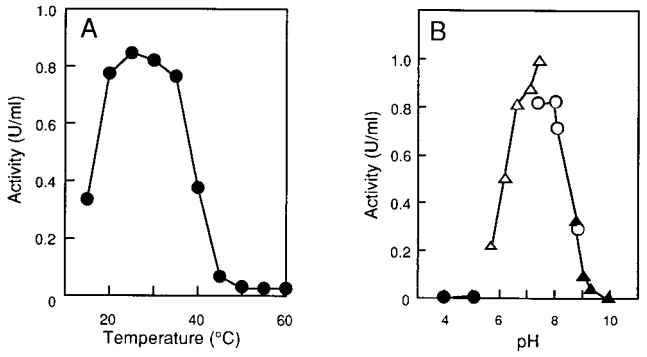

FIG. 2. Effects of temperature and pH on FAOD activity. Enzyme activities were measured under the standard conditions at various temperatures (A) and in various buffers (B). Symbols in panel B: ●, acetate buffer; △, potassium phosphate buffer; ○, Tris-HCl buffer; ▲, glycine-NaOH buffer.

enzyme was also susceptible to several metal salts at a concentration of 1 mM (each) $ZnSO_4$ (94% inhibition), $CoCl_2$ (87%), $CuCl_2$ (49%), $BaCl_2$ (34%), and $CaCl_2$ (26%). The enzyme activity was not affected by the addition of 1 mM iodoacetate, NaN_3, α,α′-dipyridyl, *o*-phenanthroline, semicarbazide, deprenyl, aminoguanidine, NaCl, $MgCl_2$, $FeSO_4$, or $FeSO_3$. From these compounds, at least the sulfhydryl and carbonyl groups of the enzyme were essential for catalytic activity. The apparent K_m of the purified enzyme was determined by reciprocal plots of the initial velocity versus the substrate concentration: 0.19 mM for ε-FL, 0.62 mM for α-FL, and 0.52 mM for FV. The maximal reaction velocities for ε-FL, α-FL, and FV were 5.15, 149, and 52.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. Although the K_m values for α-glycated amino acids, FV and α-FL, were slightly higher than that for ε-FL, the maximal reaction velocities for the former were much higher than that for ε-FL. These findings suggested that FAOD from *P. janthinellum* AKU3413 was more active at the glycation site in α amino group. No enzyme activity was found for N^{ε}-methyl-L-lysine (at the concentration of 1.67 mM), fructosyl poly-L-lysine (0.17% [wt/vol]), fructosyl bovine serum albumin (0.17%), and fructosyl human serum albumin (0.17%). Even when these glycated proteins were digested with trypsin, the digested products did not become substrates of the enzyme. This is consistent with the substrate specificity of the enzyme, because albumin is mostly glycated at the ε amino group of lysine residues, and the enzyme has low activity with ε-FL. It is evident that the substrate specificity of the enzyme from *P. janthinellum* is distinguishable from that of the enzyme from *F. oxysporum* S-1F4, which is most active with ε-FL and is active with the tryptic glycated proteins (8).

A variety of enzymatic methods for diagnostic analyses are coupled with the colorimetric determination of H_2O_2 formed by the oxidase reaction. From this respect, FLOD is suitable for the assay system of glycated proteins. The amount of HbA_{1c} can be an index for a long-term glycemic control, while that of glycated albumin can be an index for a comparatively short-term glycemic control. The proper use of the two indices according to the conditions of the diabetic patients is important diagnostically; pregnant patients need the index for a shorter-term glycemic control than normal diabetic patients. Glycated albumin can be determined by the use of FLOD from *F. oxysporum* S-1F4, and the enzyme from *P. janthinellum* is a most suitable candidate for the specific determination of HbA_{1c} in blood.

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