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Improvement in Thermal Stability and Substrate Binding of Pig Kidney D-Amino Acid Oxidase by Chemical Modification

Mikio Bakke* and Naoki Kajiyama

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba 278-0037, Japan, E-mail: mbakke@mail.kikkoman.co.jp

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Abstract

Chemical modification was evaluated to stabilize pig kidney D-amino acid oxidase (pkDAAO), which is required for analytical determination of D-amino acids. Optimization of modification conditions was performed to obtain high recovery yield and stability, and chemical modification at 30°C for 12 h with a highly concentrated enzyme solution gave dextran-conjugated pkDAAO with a 70% yield of activity. pkDAAO was stable at less than 55°C at pH 6.0, while the conjugated enzyme was stable even at 70°C. In addition, the conjugated enzyme showed decreased K_m values for D-amino acids. Because of these outstanding characteristics, this new material is expected to be available for use as a liquid assay reagent.

Index Entries: Chemical modification; K_m value; D-amino acid oxidase; soluble dextran; stabilization.

Introduction

D-Amino acid oxidase (DAAO, D-amino acid: oxygen oxidoreductase; EC 1.4.3.3), a flavoenzyme containing flavin adenine dinucleotide (FAD) as the prosthetic group, catalyzes the oxidative deamination of D-amino acids to the corresponding imino acids and hydrogen peroxide *(1)*. The activity of DAAO has been found to be widespread, such as in mammals and microorganisms. This enzyme is of considerable practical importance, because it has been used in the pharmaceutical industry for the oxidation of cephalosporin C. In addition, the measurement of D-amino acids is drawing much attention for clinical diagnosis and analyses of food components *(1,2)*, because

*Author to whom all correspondence and reprint requests should be addressed.

an elevation in the plasma D-amino acid level was observed in a group of patients with renal disease *(1,3,4)*. D-Amino acids are also proposed to be indicators of aging because age-related accumulation was observed in lens protein and brain tissue *(1)*. Pig kidney DAAO (pkDAAO) is used for enzymatic assays of plasma D-amino acids *(3,4)*, but it is labile in solution at low enzyme concentrations owing to the monomerization of the dimer enzyme, dissociation of FAD, and subsequent denaturing of the protein *(1,5)*.

To improve the enzyme's stability, some techniques for the chemical modification and immobilization of the enzyme have been developed *(6–8)*. Tosa et al. *(6)* prepared immobilized pkDAAO by covalently binding the enzyme to a cyanogen bromide-activated insoluble polysaccharide, Sepharose 6B, but thermal stability was not improved. There are also some reports about immobilization of DAAOs from microorganisms on hydrophobic supports, yielding stabilization of the enzyme preparations *(7,8)*. These immobilized DAAOs appear to be useful for industrial production, but not to be applicable to liquid assay reagent owing to their insolubility. Previously we have been successful in attaining stabilization of L-fucose dehydrogenase by chemical modification with soluble dextran *(9)*, so we investigated the chemical modification of pkDAAO according to this method. In addition, the properties of the conjugated enzyme were compared with those of the native enzyme.

Materials and Methods

Chemicals

The lyophilized holoenzyme of pkDAAO (4 U/mg, single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis) and peroxidase from horseradish were from Kikkoman. FAD was from Tokyo Kasei. Dextran (mol wt = 40,000–70,000) was purchased from Meito Sangyo. The molecular weight marker for HPLC was purchased from Wako. All other chemicals used in this study were of reagent grade.

Enzyme Assays

An assay mixture (3 mL) containing 170 m*M* Tris-HCl (pH 8.3), 37 m*M* D-alanine, 21 m*M* phenol, 5 U/mL of peroxidase, and 1.4 m*M* 4-aminoantipyrine was preincubated at 25°C for 5 min. A pkDAAO sample was diluted with a cold 1 m*M* FAD solution, and 100 µL of sample was added to the assay mixture. The increase in absorbance was measured at 500 nm. One unit of enzymatic activity was defined as the amount of enzyme that caused an oxidation of 1 µmol of D-alanine/min under the assay conditions. An extinction coefficient of 13.8 cm^2/µmol was used for the calculation. All assays were repeated at least three times.

K_m values were determined as follows. The first reagent contained 266 m*M* Tris-HCl (pH 8.3), 32 m*M* 3-hydroxy-2,4,6-triiodobenzoic acid, 7.6 U/mL of peroxidase, and 2.1 m*M* 4-aminoantipyrine. The second reagent contained D-amino acids at 10 concentrations ranging from 1.28 to

30.1 m*M*. The assay was performed on a Hitachi 7070 autoanalyzer for a rate assay measuring absorbance at 546/660 nm at 37°C with 6 µL of pkDAAO sample, 240 µL of the first reagent, 63 µL of the second reagent, and 57 µL of water. The amount of absorbance change between 18 and 20 points was evaluated, and K_m values were calculated from Hanes plots.

Screening of Optimum Conditions for Preparation of Dextran-pkDAAO Conjugate

The basic conditions were based on a previous report *(9)*. To 200 mL of a 10% (w/v) dextran solution, $NaIO_4$ (14.3 g, 0.6 eq. of $NaIO_4$/glucose unit) was dissolved by stirring. The mixture was left in the dark at 4°C for 12 h. $NaHSO_3$, 40% (w/v), was slowly added to a cold mixture until the mixture turned colorless, and the pH was adjusted to 4.0 with NaOH. The resulting solution was dialyzed against water and concentrated to 100 mL.

To 500 µL of the oxidized dextran solution, 330 µL of water, 100 µL of 1 *M* acetate buffer (pH 6.0), 5 mg (20 U) of lyophilized pkDAAO, and 20 µL of borane-pyridine complex were added and left at 25°C for 3 h. Reaction temperature, reaction time, concentration of the enzyme and activated dextran, and amount of borane-pyridine complex were examined to increase the yield and stability. Subsequently, 50 µL of 2 *M* glycine was added, the mixture was left at 25°C for 1 h, and then the product was dialyzed against 10 m*M* potassium phosphate buffer (pH 6.0).

Preparation of conjugated enzyme under optimum conditions in a large scale was carried out as follows. To 7.5 mL of the oxidized dextran solution, 3 mL of water, 1.5 mL of 1 *M* acetate buffer (pH 6.0), 450 mg (1.8 KU) of pkDAAO, and 0.6 mL of borane-pyridine complex were added and left at 30°C for 12 h. Subsequently, 0.75 mL of 2 *M* glycine was added and the mixture was left at 25°C for 1 h. The product was dialyzed against 10 m*M* potassium phosphate buffer (pH 6.0) and concentrated to 22 mL. The prepared solution was centrifuged and filtered with a membrane (pore size of 0.20 µm; Nalgene).

Evaluation of Conjugation by Gel Permeation Chromatography

A sample of the dextran-conjugated pkDAAO was applied to a TSK-GEL G3000 SWXL column (7.5 × 600 mm; Tosoh) and eluted with potassium phosphate buffer (50 m*M*, pH 6.5) containing 150 m*M* NaCl. The flow rate was 1.0 mL/min and the detection wavelength was 280 nm. The activities of each fraction were checked to determine the peak of pkDAAO.

Results and Discussion

Preparation of Dextran-pkDAAO Conjugate

According to previous reports *(9)*, pkDAAO was coupled to periodate-oxidized soluble dextran by reductive alkylation. pkDAAO was stable at about pH 6.0, so the modification was conducted with this condition. At the

Table 1
Identification of Optimum Conditions
for Preparation of Dextran-Conjugated pkDAAO[a]

pkDAAO (U)[b]	Reaction temperature (°C)	Reaction time (h)	Yield of activity (%)[c]	Residual activity (%) after incubation[c]
20	25	3	59	41
20	25	12	64	65
20	30	12	67	72
60	30	12	68	81
120	30	12	72	86

[a]All modification reactions were carried out as described in Screening of Optimum Conditions for Preparation of Dextran-pkDAAO Conjugate.

[b]The quantity of pkDAAO relative to 500 µL of oxidized dextran solution.

[c]The activity before the modification corresponded to 100%.

[d]Each sample was heated in acetate buffer (100 m*M*, pH 6.0) containing 1 m*M* FAD at 70°C for 10 min and assayed for residual activity. The activity of the unheated sample was 100%. Under the conditions, native enzyme was inactivated completely.

first preparation, the yield of activity after the modification process was 59% (Table 1). The thermal stability was evaluated at pH 6.0 after 10 min at 70°C, and the modified enzyme retained 41% of its activity, whereas the native enzyme was inactivated completely. The addition of dextran alone did not enhance the stability, so the improvement in stability likely needed covalent bonds between the enzyme and dextran. To check the covalent modification between pkDAAO and dextran, the high-performance liquid chromatography analysis of gel filtration was performed, and the conjugated sample showed a peak with an *Rt* of 11.5 min (Fig. 1) without a peak of native enzyme (*Rt* = 23.1 min). A molecular weight marker indicated that the molecular weight of the conjugated pkDAAO was more than 290,000 (glutamate dehydrogenase), and it was much larger than the sum of two molecules of pkDAAO (about 40,000) and dextran (40,000–70,000). Highly functionalized dextran and the protein allow multiple covalent bonds, so intramolecular and intermolecular crosslinked polymers were likely formed *(10)*.

Since it was suggested that chemical modification is effective for the stabilization of pkDAAO, the screening of optimum conditions was carried out as follows (Table 1). To further increase the stability, first the time and temperature for the reaction were examined. When the reaction time was extended from 3 to 12 h, the yield was improved by 5 percentile points (64%), and the residual activity at pH 6.0 after 10 min at 70°C increased from 41 to 65%. Next, the temperature for the modification was elevated from 25 to 30°C, and residual activity after the same heat treatment enhanced to 72% with almost the same yield.

The proportion of enzyme and activated dextran was next examined. When only activated dextran was reduced in the same reaction volume

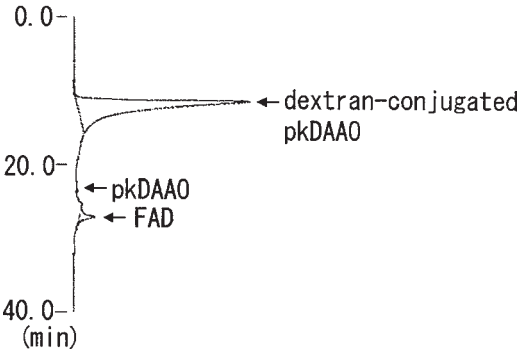

Fig. 1. Gel permeation chromatography of conjugated pkDAAO.

without changing the quantity of enzyme, the yield of activity and the stabilization effect decreased (data not shown). On the contrary, a three- and a sixfold concentration of enzyme were added to the fixed amount of activated dextran, and then the yields of activity were retained or improved a little, to 68 and 72%. Furthermore, the residual activity after thermo-incubation at pH 6.0 for 10 min at 70°C was elevated to 81 and 86%, respectively. A ninefold concentration was examined, but the reaction mixture became a gel owing to hard crosslinking. In a similar experiment, Chae et al. *(11)* also found that the recovery for immobilization of protease was improved when the process was conducted in the minimum volume required to soak the support, which gave a high probability of contact between the enzyme and support. Also in our case, the improvements of the yield and the stability were achieved by the treatment of the enzyme with a high concentration.

Reducing the amount of borane-pyridine complex to one-quarter was found not to effect the yield or stability of the dextran-pkDAAO conjugate (data not shown). Under the optimum conditions, we scaled up the modification reaction and prepared 1.3 KU of dextran-modified pkDAAO (57 U/mL, 22 mL) from 1.8 KU of native enzyme. The 70% yield of the activity could be reproducible in the large scale.

Properties of Dextran-pkDAAO Conjugate Prepared Under Optimum Conditions

The thermal stabilities of native and dextran-modified pkDAAO were investigated in 0.1 *M* acetate buffer (pH 6.0) containing 1 m*M* FAD (Fig. 2). It should be noted that the modified enzyme was stable to 70°C compared with 55°C for the native enzyme. Thus, the thermal stability of the modified enzyme was 15°C higher than that of the native form.

The effect of temperature on the enzymatic activity of the native and conjugated pkDAAOs was evaluated (Fig. 3). Native enzyme had an optimum temperature of 40–50°C, whereas that of the modified enzyme was

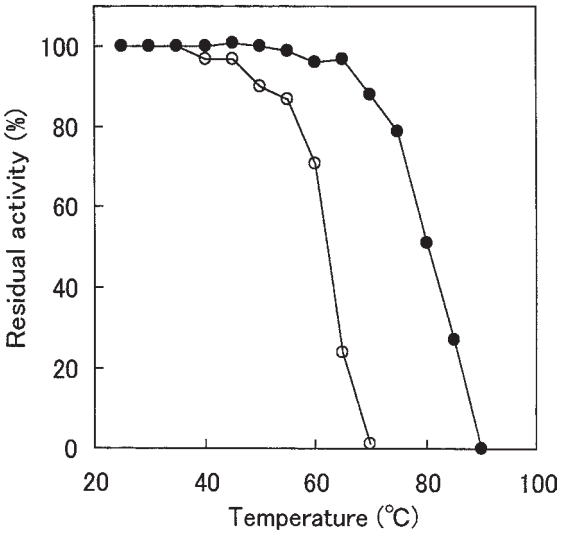

Fig. 2. Thermal stability of native and conjugated pkDAAO. The pkDAAO samples (4 U/mL) in 0.1 *M* acetate buffer (pH 6.0) containing 1 m*M* FAD were heated for 10 min and assayed for residual activity. The activity of unheated sample was 100%. Native enzyme (○); conjugated enzyme (●).

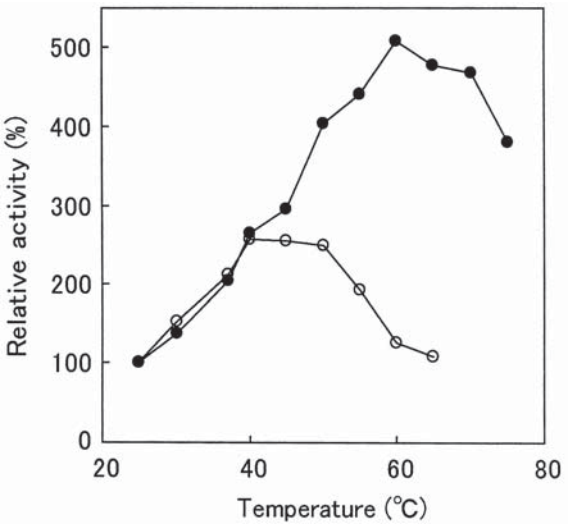

Fig. 3. Effect of temperature on activity of native and conjugated pkDAAO. The activity of each sample at 25°C was 100%. Native enzyme (○); conjugated enzyme (●).

60°C. This increase in optimum temperature was most likely owing to the enhanced thermal stability *(12)*.

The pH stability of the conjugated pkDAAO was compared with that of the native enzyme in the pH range of 4.0–11.0 at 25°C (Fig. 4). The range of pH for the stability of modified enzyme was between 4.5 and 9.5, which was slightly wider than that of native enzyme (pH 5.5–9.0).

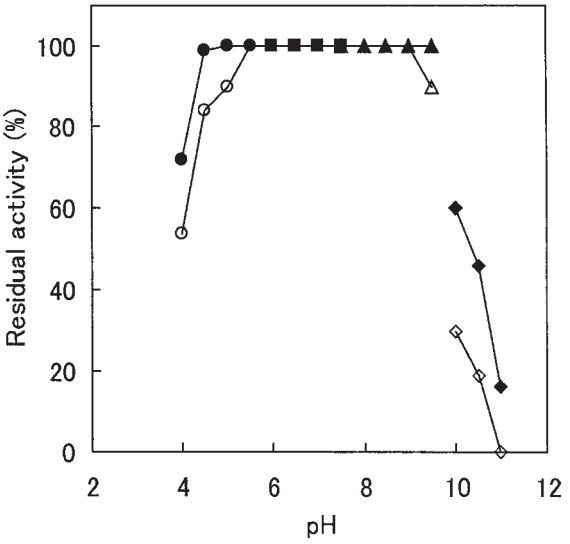

Fig. 4. pH stability of native and conjugated pkDAAO. The pkDAAO samples (4 U/mL) were incubated at various pH values in buffers containing 1 m*M* FAD and 0.1% NaN_3 for 24 h at 25°C and assayed for residual activity. The buffers used were 0.1 *M* acetate buffer (○, ●), potassium phosphate buffer (□, ■), Tris-HCl buffer (△, ▲), and CAPS-NaOH buffer (◇, ◆). Open symbols: native enzyme; closed symbols: conjugated enzyme. The activity of each sample before the incubation was 100%.

The effect of pH on the activity of the native and modified pkDAAOs was determined using various buffers (Fig. 5). At higher pH, the relative activity of the modified enzyme was slightly higher than that of the native enzyme, but the optimum pH of both preparations was about the same, at 8.5–9.5.

The activity of modified pkDAAO toward various D-amino acid substrates was compared with that of native enzyme (Table 2). Both enzyme preparations showed a similar tendency in terms of substrate specificity.

Finally, the Michaelis constant for D-alanine was evaluated with Hanes plots (Table 3), and the K_m value was found to have decreased with the chemical modification. K_m values for the D-alanine of native and modified pkDAAOs were 2.1 and 0.9 m*M*, respectively. Next, D-methionine and D-serine were investigated, and apparently decreased K_m values after the modification were also observed (Table 3). In general, immobilization results in an increase in K_m on account of the resistance to the mass transfer of the substrate into the immobilization support, hindering the substrate from accessing the active site of the enzyme *(12)*. In addition, a decrease in the flexibility of the molecule caused by multiple fixation of the enzyme was also thought to affect the affinity of enzyme toward its substrate *(12)*. As an example of the decrease in the K_m value after immobilization, Tiller et al. *(13)* reported an immobilized *Aspergillus* glucose oxidase whose K_m value was 20 times smaller than that of native enzyme. The reason for this decrease was not clear, but we assume that it was attributable to a partition-

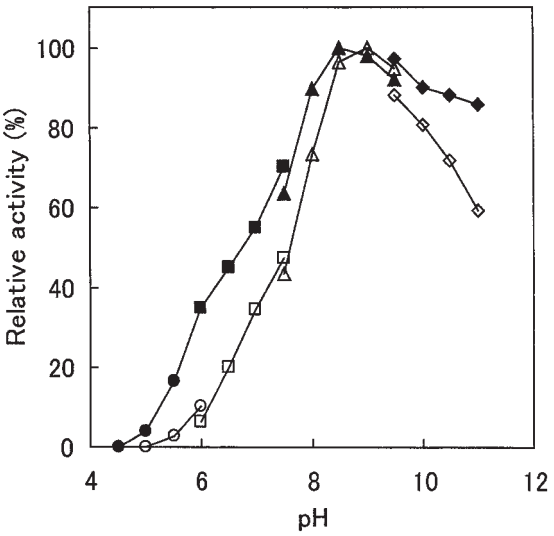

Fig. 5. Effect of pH on activity of native and conjugated pkDAAO. The buffers used were 0.1 *M* acetate buffer (○, ●), potassium phosphate buffer (□, ■), Tris-HCl buffer (△, ▲), and CAPS-NaOH buffer (◇, ◆). Open symbols: native enzyme; closed symbols: conjugated enzyme. The activity of each sample at optimum pH was 100%.

Table 2
Comparison of Substrate Specificities
Between Native pkDAAO and Conjugated Enzyme

	Relative activity (%)[a]	
Substrate	Native pkDAAO	Conjugated pkDAAO
D-Alanine	100	100
D-Methionine	187	149
D-Serine	33	84
D-Phenylalanine	130	300
D-Valine	49	67
D-Proline	269	308
D-Tryptophan	13	47
Glycine	0	0
L-Alanine	0	0

[a]The activity for D-alanine was taken as a control (100%).

Table 3
Comparison of Michaelis Constants (K_m Value)
Between Native pkDAAO and Conjugated Enzyme

	K_m value (m*M*)	
Substrate	Native pkDAAO	Conjugated pkDAAO
D-Alanine	2.1 ± 0.2	0.9 ± 0.1
D-Methionine	0.9 ± 0.1	0.2 ± 0.1
D-Serine	7.7 ± 0.8	5.0 ± 0.4

ing effect and the conformational change caused by the covalent binding to dextran.

Conclusion

The modified pkDAAO was found to have advantageous characteristics: excellent stability and higher affinity for substrates. This new material is suitable as a liquid assay reagent in view of storage and reactivity and therefore might expand the possible applications of pkDAAO in analytical chemistry.

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