

# Evaluation of Alfalfa and Oat Polyamine Oxidase: One-Step Purification and Characteristics

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## Abstract

The polyamine oxidase activity of acetone powder from alfalfa was investigated. No enzyme activity was detected.

Oat polyamine oxidase was purified in one step isoelectric point chromatography. This method is described in detail and discussed. The enzyme was homogenously by SDS-PADE. The minimum molecular weight of the enzyme was 51,000 on SDS PAGE. The difference in the Stokes radius of native enzyme and N-glycosidase F-treated enzyme is discussed. The optimal pH of the enzyme was 6.0 for spermidine and spermine. The enzyme activity was reduced by 1 M NaCl to 70.2% and altered the optimal pH from 6.0 to 6.5.

Keywords: polyamine oxidase, alfalfa, oat, isoelectric point chromatography, one step purification

## Introduction

In plant kingdom, amine oxidases have been reported in dicotyledonous and monocotyledonous plants [1,2]. Amine oxidase in dicotyledons contains Cu and a type of quinone molecule, which attacks an amino group in diamines rather than in monoamines and polyamines [3]. In General, amine oxidase in dicotyledons is called diamine oxidase (EC 1.4.3.22), whereas in monocotyledons it is polyamine oxidase (EC 1.5.3.14). Amine oxidase in monocotyledons contains FAD, and attacks an imino group in polyamines (spermidine and spermine) similar to FAD-containing amine oxidase in animals. However, monocotyledonous and animal enzymes differ in their active part (the left or right sides of the imino group). Both type of amine oxidases in higher plants are known to be glycoproteins [1,2].

Bagga et al. reported that polyamine oxidase was present in the dicotyledonous plant alfalfa [4]. Polyamine oxidase activity in acetone powder extract of alfalfa was measured by photometric assay, whereas that of it's dialyzate was measured by radio-metric assay. Alfalfa polyamine oxidase activity decreased rapidly after dialysis. Because I was primarily interested in alfalfa polyamine oxidase, I re-examined its activity in the acetone powder extract.

Enzyme purification steps generally include ion-exchange chromatography, gel filtration, and affinity chromatography. Enzyme purification is usually time-consuming. A protein may be obtained homogeneously if two or more methods based on different principles are used. But if more purification steps are involved, only a small amount of intact (non-modified) enzyme is obtained. To achieve rapid purification, I developed a one-step purification method. This method can be added to other purification procedures as a new method with different principle. It is simple and cost-effective. It is simple because of its similarity with ion-exchange chromatography. I used this method for purification of oat polyamine oxidase.

Here I describe the re-examination of alfalfa polyamine oxidase activity and the one-step purification of oat polyamine oxidase. In addition, I describe the fundamental characteristics of oat polyamine oxidase.

## Materials and Methods

### *Plant material*

Oat seeds (*Avena sativa*) available as animal food in market were purchased. The seeds were soaked in tap water for a day, transferred to moist absorbent cotton, and grown for 13 days in the dark at 25°C. The shoots were harvested, sterilized with 0.1% benzalkonium chloride for 10 min, and thoroughly washed with deionized water. Alfalfa was purchased as sprouts in a market,

sterilized with 0.1% benzalkonium chloride for 10 min, and thoroughly washed with deionized water. Alfalfa sprouts (100 g) were ground in a mixer with 1L of cold acetone ( $-20^{\circ}\text{C}$ ) following the method of Nason [5]. The acetone powder (1 g) was ground with 30 mL of 0.2M K phosphate buffer pH 6.5 and then centrifuged at 15,000 g for 20 min. The supernatant was used as the enzyme solution.

#### *Measurement of polyamine oxidase activity*

Polyamine oxidase activity was measured by two methods. The primary method used was measurement of the increase in the absorbance at 435 nm due to the condensation of the spermidine oxidative product and *o*-aminobenzaldehyde. The incubation mixture consisted of 1.5 mL of 0.2 M potassium phosphate buffer pH 6.0, 0.2 mL of 0.1% *o*-aminobenzaldehyde (in ethanol), 0.3 mL of 10 mM spermidine, and appropriate enzyme in a total volume of 3.0 mL. The incubation temperature was  $30^{\circ}\text{C}$ . The reaction was terminated by addition of 0.1 mL of 50% TCA.

The other method involved measurement of hydrogen peroxide: the increase in absorbance at 470 nm due to formation of tetraguaiacol by the coupling reaction of peroxidase with guaiacol and polyamine oxidase at  $30^{\circ}\text{C}$ . This method was used for the determination of the optimal pH of the enzyme. The reaction mixture contained 1.5 mL of 0.2 M K Pi buffer, 0.2 mL of 10 mM substrate, 0.2 mL of 20 mM guaiacol, 0.2 mL peroxidase (0.5 mg/10 mL), and appropriate enzyme in a total volume of 3.0 mL. To evaluate the effects of 1 M NaCl on enzyme activity, 1 mL of 3 M NaCl to the reaction mixture was added.

Protein was determined by the method of Bensadoun and Weinstein [6] with BSA as a standard.

#### *Purification of polyamine oxidase*

All operations were performed at  $4^{\circ}\text{C}$ . Oat shoots (approximately 50–70g) were homogenized with 200 mL of 0.5 M Tris succinate buffer pH 7.5, and 5  $\mu\text{M}$  FAD. The homogenate was squeezed through a nylon mesh (74  $\mu\text{m}$ ) and then centrifuged at 15,000g for 20 min. The enzyme solution was brought to 400 mL by addition of cold distilled water to the supernatant (step 1). To the diluted enzyme solution (step 1) was added CM-Sephadex c 50 (wet weight 1g) equilibrated with 0.25 M Tris succinate buffer pH 7.5 and 2.5  $\mu\text{M}$  FAD. After addition, the CM-Sephadex was stirred gently for 3 h, it was recovered using a Buchner funnel and washed with 200 mL of 0.25 M Tris succinate buffer pH 7.5, and 2.5  $\mu\text{M}$  FAD. The CM-Sephadex was packed into a glass cylinder as a column (diameter: 2.2 cm). After washing with 100 mL of the same buffer, the enzyme was eluted with 0.5 M Tris succinate buffer pH 7.5, and 0.25  $\mu\text{M}$  FAD. Active fractions were gathered and stored at  $4^{\circ}\text{C}$  until use (step 2).

#### *SDS-PAGE and enzyme staining*

SDS-PAGE was performed as described by Laemmli [7]. As standard protein markers, Mark11 (Tefco) was used. Proteins were visualized with a silver staining kit (Nacalai Tesque).

#### *N-Glycosidase F treatment*

The purified enzyme (3.7  $\mu\text{g}$ ) was denatured with 1% SDS and 5% 2-mercaptoethanol for 10 min at  $100^{\circ}\text{C}$  and the denatured enzyme (0.74  $\mu\text{g}$ ) was then deglycosylated with N-glycosidase F (10 U) in 0.1 M potassium phosphate pH 7, 25 mM EDTA, 1% n-octylglucoside, 0.1% SDS, and 0.5% 2-mercaptoethanol in a total volume of 0.1 mL for 20 h at  $37^{\circ}\text{C}$ . Proteins were analyzed by SDS-PAGE with the silver staining method.

#### *Optimal pH of polyamine oxidase*

Optimal pH of oat polyamine oxidase was determined by the measurement of hydrogen peroxide (guaiacol–peroxidase reaction). Oat polyamine oxidase was reported to be activated by a high concentration of salts [8]. Although Yanagisawa *et al.* reported a similar feature of trumpet lily polyamine oxidase [9], I investigated whether a high concentration of salt (1 M NaCl) activates oat polyamine oxidase purified by a different method.

## **Results and Discussion**

#### *Enzyme activity of alfalfa*

The enzyme assay performed was similar to a method [4], by Bagga *et al.*: a measurement of the increase in absorbance at 435 nm due to the condensation of the spermidine oxidative product and *o*-aminobenzaldehyde.

The reaction mixture revealed increased yellow color, despite the complete absence of substrate (spermidine). A compound in the acetone powder extract passing through dialysis reacted with *o*-aminobenzaldehyde. Consequently the reaction mixture exhibited the absorption maximum at 382.5 nm. No peak except that at 382.5 nm was seen in the assay including spermidine. Thus, TCA did not stop but delayed the reaction. Though radiometric assay revealed that spermidine was decomposed in the dialyzate, it is necessary to consider the roles of bacterial contamination and other enzyme systems.

From these results, I conclude that polyamine oxidase is not contained in acetone powder of alfalfa. Assays of oxygen absorption, the corresponding aldehyde, 1,3-diaminopropane, and hydrogen peroxide have been performed as measurement methods for polyamine oxidase.

*Enzyme purification*

One step purification was accomplished using isoelectric point, chromatography. Each protein has a unique isoelectric point. The pI value of oat polyamine oxidase was reported to be 4.3 [10], but I found it to be 9 or higher from the result of isoelectric focusing (data not shown). A well-known isoelectric point (absolute value) has been determined by different experiments. We were not able to elute each protein from a cation-exchange resin at a precise isoelectric point (absolute value).

The pH of protein elution in a system is not predictable even in an expensive system. The method described here is capable to elute each protein at a precise isoelectric point (absolute value), as well as to determine the isoelectric point (absolute value) of a new protein. Cation-exchange resins tend to adsorb common proteins weakly and to adsorb glycoproteins tightly in Tris succinate buffer. Therefore, these two protein types must be handled separately. Because polyamine oxidase is a glycoprotein, I used 0.25 M buffer as an adsorption buffer and CM-Sephadex, based on trial experiments, to purify oat polyamine oxidase. The high ionic strength of the adsorption buffer prevented absorption of some common proteins.

Thus, this method is suitable for many glycoproteins adsorbed by a cation-exchange resin in Tris succinate buffer; although, it is necessary to predetermine the best ionic strength of the adsorption and elution buffers. Tris succinate buffer is composed of a

Table 1 Comparison of cation-exchange chromatography and isoelectric point chromatography

|                 | cation-exchange chromatography  | isoelectric point chromatography  |
|-----------------|---|---|
| action          | cation-exchange resin + protein (ionic bond)  | cation-exchange resin + protein (ionic bond)  |
| elution methods | 1. pH gradient<br>elution dependent to a system<br>→<br>isoelectric point peculiar to a system<br>Because isoelectric point can not be predicted.<br><br>2. buffer concentration increase | 1. pH gradient (11,12)<br>elution independent to a system<br>(A protein can be eluted by the same pH even if caion-exchange resin of the system is different.)<br>→<br>universal isoelectric point<br>Because isoelectric point can be predicted.<br><br>2. buffer concentration increase (this work) |
| used buffer     | bis-Tris HCl, phosphate Na, etc.  | Tris succinate  |
| comment         | Separation of common proteins and glycoproteins is impossible.  | Cation-exchange resins tend to adsorb weakly common proteins and tightly glycoproteins.   |

weak acid and weak base, and its buffering action is especially weak near neutral pH. Buffers composed of a weak acid and weak base has not been used until recent owing to their weak buffering action; however, they may be used to obtain exact information of the proteins. A comparison of cation-exchange chromatography and isoelectric point, chromatography is presented in Table 1.

When FAD was not involved during purification of oat polyamine oxidase, the enzyme activity decreased by approximately 50% in two days, but the addition of 2.5 μM FAD this trend is prevented (Fig. 1).

Accordingly, 2.5 μM FAD was added during purification.

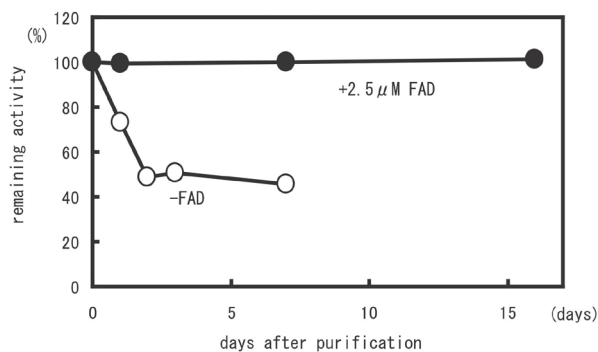


Fig. 1 Time course of oat polyamine oxidase activity after purification. in the presence of 2.5 μM FAD during purification (● closed circles), no addition of FAD (○ open circles).

Table 2 purification of oat polyamine oxidase

| step                                       | protein (mg) | total activity ( $\mu$ moles/min) | specific activity ( $\mu$ moles/min/mg) | purification (-fold) | yield (%) |
|--|--------------|-----------------------------------|---|----------------------|-----------|
| 1. crude extract                           | 233.1        | 397.3                             | 1.70                                    | 1                    | 100       |
| 2. isoelectric point chromatography eluate | 2.65         | 107.9                             | 40.52                                   | 23.78                | 27.2      |

The results of oat polyamine oxidase purification are shown in Table 2. The purpose of extraction for 3 h in the batch method was to improve the yield. The purification yield was approximately 23%, and the value generally considered good for protein, purification but approximately 3/4 of the enzyme activity was not adsorbed to the cation resins, despite the single purification step, the purified enzyme was homogeneously by SDS-PAGE (Fig. 2).

#### SDS-PAGE

The minimum molecular weight of the enzyme was determined to be 51,000 using SDS-PAGE. The value was lower than the reported value of 63,000 [13]. I explain this large difference in molecular weight as follows. The intact enzyme has many polysaccharides that bind amino acids of the protein. If amino acids combinable with polysaccharides are present some distance apart and are drawn together, some bend or compression of the protein will result. Thus, the Stokes radius of the enzyme will be small. In contrast, the Stokes radius of the artifact enzymes, with only few polysaccharides that do not bind most amino acids of the protein, will be large. Thus, the difference in the Stokes radius may explain the difference in molecular weights.

I verified my hypothesis about N-glycosidase F treatment of the enzyme.

#### N-Glycosidase F treatment

I performed SDS-PAGE of the enzyme and N-glycosidase F-treated enzyme (Fig. 3). N-glycosidase F deglycosylates most glycoproteins. N-glycosidase F treatment changed oat polyamine oxidase into enzyme groups with molecular weights 51,000–65,000. The minimum molecular weight of the enzyme increased on SDS-PAGE as polysaccharides were removed from the enzyme. This result revealed that polysaccharides of oat polyamine oxidase reduced the radius, assuming that the enzyme has an ideal spherical form. The radius of the native enzyme was smaller than that of the artifact (N-glycosidase F-treated) enzyme. These results are in agreement with my hypothesis. When the number of polysaccharides decreases on the surface of protein, as in this example: glycoproteins, may result in altered chemical characteristics.

#### Optimal pH of polyamine oxidase

The result is shown in (Fig. 4). Oat polyamine oxidase activity was not activated by 1 M NaCl but decreased to 70.2%, though Smith reported that oat polyamine oxidase activity was activated by high ionic strength of salts [8]. Since peroxidase

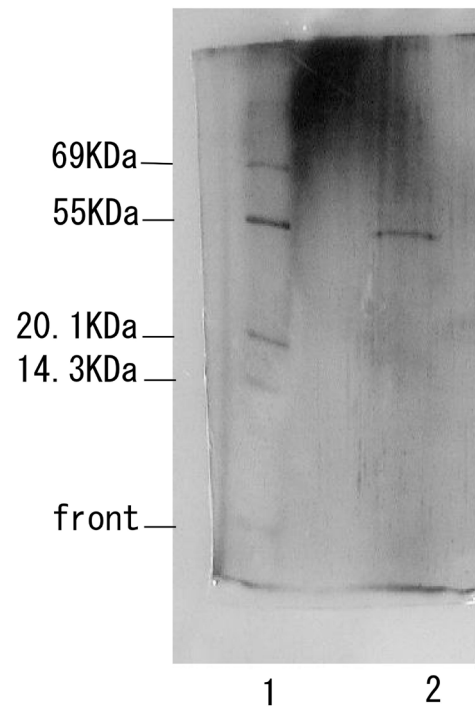


Fig. 2 SDS PAGE of oat polyamine oxidase. Lane 1, markers; lane 2, polyamine oxidase (84 ng)

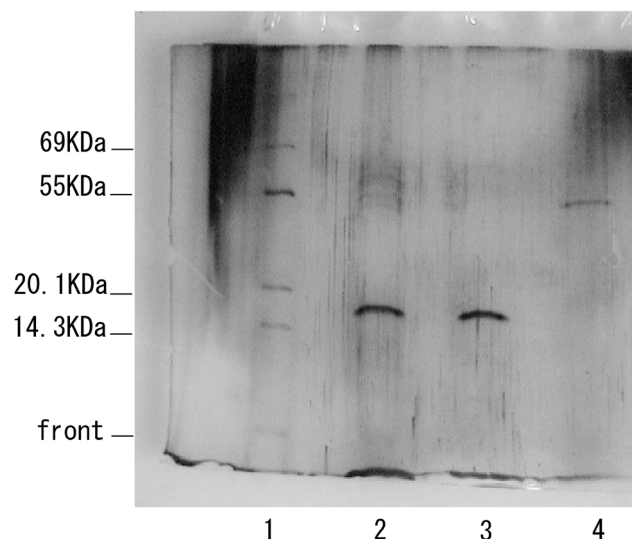


Fig. 3 SDS PAGE of oat polyamine oxidase and N-glycosidase F-treated enzyme. Lane 1, makers; lane 2, polyamine oxidase (84 ng) with N-glycosidase F treatment for 20 h at 37°C; lane 3, N-glycosidase F; lane 4, polyamine oxidase (84 ng).

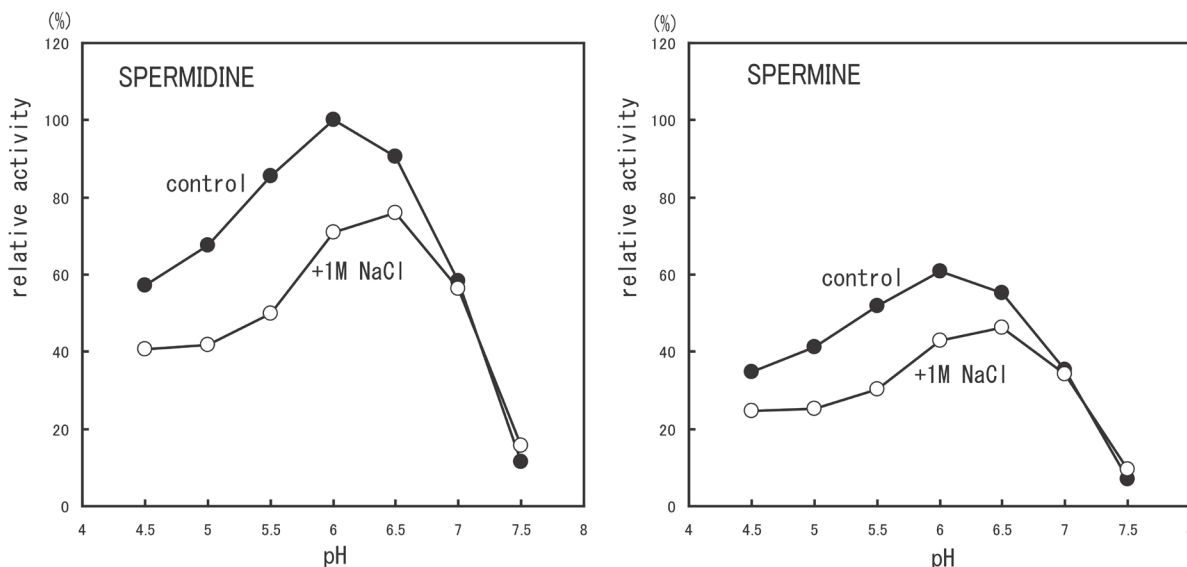


Fig. 4 Influence of pH on oat polyamine oxidase activity. control (● closed circles), in the presence of 1 M NaCl (○ open circles)

activity was not inhibited by 1 M NaCl, oat polyamine oxidase appears to be influenced directly by 1 M NaCl (data not shown). The decisive difference between the two experiments was the enzyme purification method. The purification method this time required one step for homogeneity and caused minimal damage to the enzyme. In contrast, Smith used a partially purified enzyme to investigate the influence of pH [8]. The absence of one or more sugar chain(s) may be related to enzyme activation.

Smith and Federico et al. reported that the optimal pH of oat polyamine oxidase was 6.5 for both spermidine and spermine [8,13]. This value is the same as that for 1 M NaCl addition to the purified enzyme.

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