The L-Amino Acid Oxidase from Silkworm Eggs (*Bombyx mori* L.)

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ABSTRACT An enzyme which catalyzes the oxidation of L-amino acids has been isolated from silkworm eggs (*Bombyx mori* L.), and purified. The activity of the enzyme preparation is about 1584 times more than that of the crude preparation. Methods of purification of the enzyme are described in detail and certain physicochemical properties of the enzyme are also described.

INTRODUCTION

Since Neubauer and Gress presented some experimental evidence for the oxidation of natural amino acids in animal tissues (Neubauer and Gress, 1910), the presence of L-amino acid oxidase has been reported in rat kidney and liver (Blanchard *et al.*, 1944), and in turkey liver (Boulanger and Osteux, 1956). The occurrence of L-amino acid oxidase in various snake venoms has been observed by several investigators (Zeller and Maritz, 1944, 1945; Singer and Kearney, 1950 *a* and *b*). There is evidence that L-amino acid oxidase activity occurs in the molds, *Penicillium* and *Aspergillus* (Knight, 1948), and is present in the culture medium (Bender *et al.*, 1949) as well as in the hyphae (Horowitz, 1944) of *Neurospora crassa.*

It is a well known fact that the L-amino acids are oxidized by many bacteria (Yagi, 1956). This activity of *Proteus vulgaris* has been studied in detail (Stumpf and Green, 1944).

In comparison with these materials, little was known about L-amino acid oxidase of insect orign. Nishizawa *et al.* (1955) have observed that the O_2 uptake by silkworm egg homogenate was increased significantly in the presence of certain L-amino acids. Their observation suggested to the author that L-amino acid oxidase should be present in the homogenate. A preliminary experiment performed to confirm this suggestion showed that the homogenate consumed a large quantity of oxygen with L-leucine, L-lysine, L-alanine, and L-glycine, under conditions that will be described later. In corroboration it

was found that NH_3 was liberated and the corresponding keto acids were produced.

This paper describes a procedure for the purification of L-amino acid oxidase from the silkworm egg homogenate and certain physicochemical properties of the enzyme.

MATERIALS AND METHODS

Silkworm eggs (*Bombyx mori* L. var. Nichi 122 × Shi 122) were chosen as source material throughout the experiment. They were purchased from the Ohiso Laboratory, Shoh-ei Sericultural Co., Ltd., Ohiso, Japan. The eggs were kept at 5°C prior to use.

5 gm of silkworm eggs were thoroughly ground with 1 gm of quartz sand and a small volume of M/15 phosphate buffer (pH 7.2). Subsequently the volume was adjusted to 30 ml by addition of the buffer solution. The mixture was allowed to stand at 5°C overnight, then filtered through a piece of filter paper. The filtrate was centrifuged at 5°C at 5,000 × *g* for 15 minutes. The supernate thus obtained is designated crude preparation.

Assay Method

The enzyme activity was determined in terms of oxygen consumption in 30 minutes by means of a Warburg manometer. For the assays in the course of purification, the conditions used were as follows: 1 ml of enzyme preparation and 0.2 ml of M/70 KCN were placed in the main chamber, 1 ml of substrate in side arm I, and 0.5 ml of 4 N H_2SO_4 in side arm II. A piece of filter paper containing 0.3 ml of 20 per cent KOH was placed in the center cup. The gas phase was air and the reaction took place at 37.0 ± 0.5°C unless otherwise specified. The KCN prevented the decomposition of H_2O_2 by catalase. The H_2SO_4 solution absorbed the NH_3 produced in the course of L-amino acid oxidation by the enzyme. KCN solution was omitted from the assay system for purified enzyme preparation because no catalase activity was present.

Protein was determined by the microKjeldahl method. The procedures were as follows: To 1 ml of enzyme preparation, 0.1 ml of 5.0 N trichloroacetic acid was added. The mixture was allowed to stand in an ice box (4°C) for 1 hour, then centrifuged at 2,000 × *g* for 30 minutes. The precipitate was washed with 0.5 N trichloroacetic acid three times. This precipitate was designated as protein. Total nitrogen of the precipitate was determined by the standard microKjeldahl method. To convert the total nitrogen value thus obtained into the protein, the nitrogen value was multiplied by the conventional factor 6.25. The protein value thus obtained was used for estimation of the QO_2 value.

RESULTS

Purification of Enzyme

The crude preparation was centrifuged at 22,000 × *g* for 30 minutes at 4°C. A one to ten volume of 0.1 M L-leucine was added to the supernatant. The

mixture was heated to 70°C, it was kept at 70°C for 5 minutes, and then was cooled to 0–3°C. The denatured protein was centrifuged off at 1,000 × *g* for 30 minutes at 4°C. The supernatant was a clear yellowish solution and was dialyzed against 1,000 volumes of distilled water at 5°C for 48 hours, changing the distilled water twice. The dialysate is called hereafter the dialyzed preparation. An equal volume of cold 98 per cent acetone was added to the dialyzed preparation in an ice bath with vigorous agitation and the mixture was allowed to stand in a cold room (4°C) for 1 hour. The precipitate was centrifuged off at 5,000 × *g* for 30 minutes. Then the precipitate was washed twice with 98 per cent acetone. After evaporating acetone *in vacuo* the prepara-

TABLE I
PURIFICATION OF L-AMINO ACID OXIDASE

Enzyme preparation	O_2 consumed	Protein	μl O_2 consumed/ 30 min./mg. protein
	µl/30 min.	*mg*	
Crude preparation	163.0 ± 3.0*	2.0×10^3	0.08
Dialyzed preparation	170.0 ± 4.0	3.0×10^2	0.57
Purified enzyme preparation	190.0 ± 3.0	1.5	126.7

Conditions: 7.0×10^{-3} M L-leucine; 0.2 ml of M/15 phosphate buffer (pH 7.2); 1 ml enzyme preparation; total volume, 3.0 ml.
* The standard error of mean. No. of measurements, 5.

tion was kept in a desiccator over $CaCl_2$ at 4°C. This preparation is designated the purified enzyme. A summary of the purification is given in Table I.

Properties of the Enzyme

Properties of the enzyme were investigated with the purified preparation. Enzyme activity on L-leucine substrate was determined over a pH range of 5.5–8.5. The buffers used were 0.1 M phosphate buffer, pH 5.5–7.7; and 0.1 M tris buffer, pH 8.0–8.5. The results are seen in Fig. 1.

The effect of temperature on enzyme activity on L-leucine was examined. The temperature range was 25–55°C. The results are seen in Fig. 2.

The effect of substrate concentration on the catalytic activity of the enzyme was determined at pH 7.2 using various concentrations of L-leucine. Plots of the results are shown in Fig. 3.

The effect of varying the concentration of enzyme on the rate of reaction was determined and the results are plotted in Fig. 4.

In order to detect any change in specificity resulting from the purification procedure, the relative rates of oxidation of various amino acids were deter-

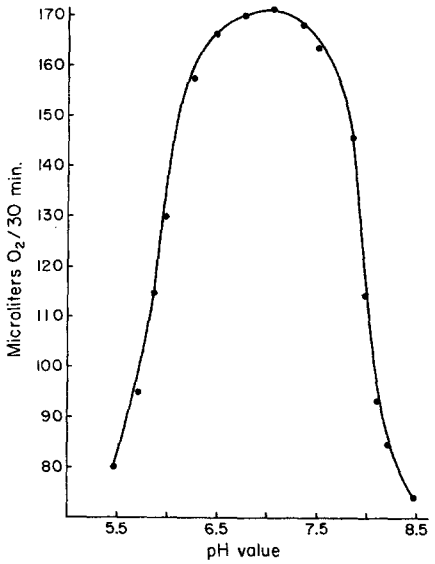

FIGURE 1. Effect of pH on enzyme activity. Conditions: 7.0×10^{-3} M L-leucine; 0.2 ml of buffer solution; 1 ml of enzyme preparation (equivalent to 1.0 mg of protein); total volume, 3.0 ml. Buffer solutions: 0.1 M phosphate buffer, pH 5.5 to 7.7; and 0.1 M tris buffer, pH 8.0 to 8.5.

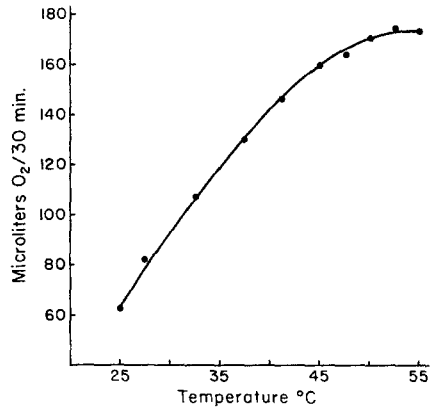

FIGURE 2. Effect of temperature on enzyme activity. Conditions: 7.0×10^{-3} M L-leucine; 0.2 ml of 0.1 M phosphate buffer (pH 7.5 at 25°C); 1.0 ml of purified enzyme preparation (equivalent to 1.5 mg of protein); total volume 3.0 ml.

mined both with the crude preparation and the purified enzyme (Table II). The following compounds were not oxidized with the crude or the purified enzyme preparation: D-methionine, D-proline, β-alanine, L-glycylglycine, and D-serine. No amino acids were determined except those described here and in

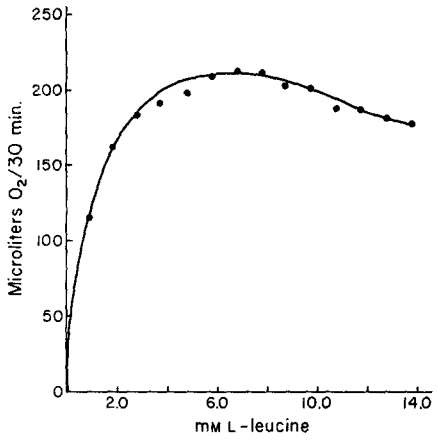

FIGURE 3. Effect of substrate concentration on enzyme activity. Conditions: 0.2 ml of 0.1 M phosphate buffer (pH 7.2); 1 ml of purified enzyme solution (equivalent to 1.5 mg of protein); total volume, 3.0 ml.

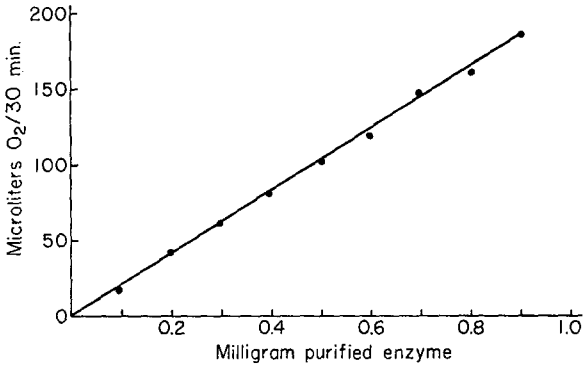

FIGURE 4. Effect of enzyme concentration on enzyme activity. Conditions: 7.0×10^{-3} M L-leucine; 0.1 M phosphate buffer (pH 7.2). The purified enzyme preparation was dissolved in 0.1 M phosphate buffer (pH 7.2) in a desired concentration; total volume, 3.0 ml.

Table II. Optimum concentrations of substrates with both crude and purified enzyme preparations were determined for L-leucine, L-glycine, L-asparagine, L-lysine, L-glutamine, and L-glutamic acid prior to the study of specificity of the enzyme preparation. The results showed almost the same relative saturation of enzyme at 7.0×10^{-3} M. So the concentration of 7.0×10^{-3} M was employed for the other amino acids and peptides without determination of individual optimum concentrations.

Several enzyme inhibitors against the purified enzyme preparations were tested in the presence of 0.2 M phosphate buffer (pH 7.2) instead of 1/15 M phosphate buffer. No appreciable inhibition was observed with KCN, $CuSO_4$,

TABLE II
SPECIFICITY OF THE ENZYME PREPARATION

Substrate	Relative rates of oxidation by	
	Crude enzyme	Purified enzyme
L-Leucine	100	100
L-Glycine	99	98
L-Asparagine	99	89
L-Lysine	95	97
L-Glutamine	87	85
L-Alanine	75	89
L-Valine	78	81
L-Norvaline	77	88
L-Cysteine	71	75
L-Cystine	70	72
L-Glutamic acid	21	19
L-Arginine	46	30
L-Histidine	9	13
DL-Proline	73	72
DL-Phenylalanine	71	70
DL-Methionine	17	26
DL-Aspartic acid	4	2

Conditions: 7.0×10^{-3} M of substrates; 0.2 ml of M/15 phosphate buffer (pH 7.2); 1 ml of enzyme solution; total volume 3.0 ml.

$HgCl_2$, and ethylenediaminetetraacetic acid (EDTA) at 1.0×10^{-4} M. At a concentration of 1.0×10^{-3} M, $CuSO_4$ inhibited enzyme activity about 15 per cent, $HgCl_2$ inhibited about 20 per cent, while KCN and EDTA did not affect the enzyme activity at the same concentration (1.0×10^{-3} M). They inhibited the enzyme activity 20 per cent (KCN) and 28 per cent (EDTA) at 1.0×10^{-2} M. The activity was remarkably inhibited with riboflavin, 77 per cent at

TABLE III
INHIBITION OF THE ENZYME

Inhibitor	Final concentration Molar	O_2 consumed μl O_2/mg of protein/30 min.	Inhibition
			per cent
Riboflavin	1.0×10^{-4}	40.0	77
	1.0×10^{-3}	2.5	99
Isoriboflavin	1.0×10^{-4}	50.0	72
	1.0×10^{-3}	8.6	95
Control	None	178.0	0

Conditions: 7.0×10^{-3} M L-leucine; 0.2 ml of M/15 phosphate buffer (pH 7.2); 0.5 ml of inhibitor; total volume 3.0 ml.

1.0×10^{-4} M and 99 per cent at 1.0×10^{-3} M; and with isoriboflavin, 72 per cent at 1.0×10^{-4} M and 95 per cent at 1.0×10^{-3} M (Table III).

DISCUSSION

The procedure described resulted in the production of a purified L-amino acid oxidase from silkworm eggs. The activity of the purified enzyme was 1584 times that of the crude preparation and 222 times that of the dialyzed preparation (Table I).

As in the case of L-amino acid oxidase from snake venom (Singer and Kearney, 1950 *a* and *b*) and from *Neurospora* (Burton, 1951), the enzyme in the presence of substrate (L-leucine) proved to be stable against heat. Activity of the enzyme increases with temperature and reaches a maximum at 55°C.

Because L-leucine showed the highest O_2 absorption (Table I), the following characteristics of the enzyme were determined on *L-leucine*. The optimum pH was found to be 7.2 (Fig. 1). The phosphate inhibition reported by Kearney and Singer (1949) was not observed with the purified enzyme.

Several investigators (Singer and Kearney, 1950; Zeller and Maritz, 1944, 1945) observed that higher concentrations of substrate inhibited the activity of the L-amino acid oxidase from snake venom. The same inhibition was also observed with L-amino acid oxidase from silkworm eggs. The apparent optimum substrate concentration was observed to be about 7.0×10^{-3} M (Fig. 3). Estimation of the Michaelis-Menten constant of this enzyme was difficult because a relatively high substrate concentration is required for this measurement. An approximate K_m value, 0.7×10^{-3} M, was deduced from Fig. 3.

It was established that the rate of the reaction was linearly proportional to the enzyme concentration under the conditions of the enzyme assay (Fig. 4).

In a study of the specificity of the enzyme, no remarkable difference was observed between the activities of the crude and the purified enzyme preparations (Table II). The enzyme was highly active on L-leucine, L-glycine, L-asparagine, L-lysine, L-glutamine, L-alanine, L-valine, and L-norvaline; and less active on L-glutamic acid, L-arginine, L-histidine, DL-methionine, and DL-aspartic acid. The enzyme showed a moderate activity on L-cysteine, L-cystine, DL-proline, and DL-phenylalanine.

Riboflavin and isoriboflavin inhibited the enzyme activity 77 per cent and 72 per cent at 1.0×10^{-4} M, and 99 per cent and 95 per cent at 1.0×10^{-3} M, respectively (Table III), due perhaps to inhibition by a competitive activity of prosthetic group analogs, flavin compounds, as described by Singer and Kearney (1950). Other inhibitors, such as KCN, $HgCl_2$, $CuSO_4$, and EDTA (ethylenediaminetetraacetic acid), required a relatively high concentration to inhibit the activity more than 30 per cent.

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