Purification and Characterization of Diamine Oxidase (Histaminase) from Rat Small Intestine

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Diamine oxidase (DAO) was purified to homogeneity from rat small intestine, and its biochemical and immunochemical properties were studied. DAO was suggested to be a dimer of a 92 kDa subunit, and its isoelectric point was found to be 6.0. Histamine, putrescine, N'-methylhistamine, and cadaverine were good substrates, with $K_m$ values ranging from 9.4 to 16.0 $\mu$M. Spermine and spermidine were not substrates. Both an immunoprecipitation study and Ouchterlony’s double diffusion test involving antiserum against the purified DAO showed that the immunological properties of the DAOs from rat small intestine, thymus, and placenta were identical. Among small intestinal DAOs from different species, this antibody reacted to the guinea pig enzyme as strongly as to the rat enzyme, but the reaction was much weaker to the mouse enzyme than to the rat enzyme. The DAOs from rabbit and dog small intestine, pig kidney, and human placenta showed no reactivity toward this antibody.

Key words: diamine oxidase, histamine, immuno-crossreactivity, intestine.

Diamine oxidase [DAO, diamine:oxygen oxidoreductase (deaminating) (copper-containing), EC 1.4.3.6.] catalyzes the oxidative deamination of histamine and aliphatic diamines, such as putrescine and cadaverine, according to the following reaction (1-3),

$$\text{R-CH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{R-CHO} + \text{NH}_3 + \text{H}_2\text{O}_2$$

DAO is also called histaminase since the inactivation of histamine is a major physiological role of this enzyme (4, 5). The small intestine exhibits high DAO activity, and heparin is suggested to regulate the release of the intestinal DAO into plasma (6, 7). The heparin-induced DAO activity in plasma was decreased by various intestinal diseases (8-10); however, the physiological and pathophysiological roles of DAO in the small intestine have not been fully investigated. In order to clarify the role of DAO, it is necessary to characterize the small intestinal DAO. Until now, characterization of DAO has been mainly carried out on the enzymes from pig kidney (11-14) and human placenta (15-21). Although some kinetic and immunological studies have suggested that the intestinal DAO has properties which are quite distinct from those of DAOs of other sources (4, 18), the purification of DAO from the small intestine has never been fully investigated. In the present paper, we describe the purification of DAO from the small intestine to homogeneity, and report the biochemical and immunochemical properties of the purified enzyme.

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Abbreviations: ABP, amiloride-binding protein; CBB R250, Coomassie Brilliant Blue R250; DAO, diamine oxidase; NaPB, sodium phosphate buffer; PEG, polyethylene glycol.

MATERIALS AND METHODS

Chemicals—Putrescine dihydrochloride, cadaverine dihydrochloride, and histamine dihydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.). N'-Methylhistamine dihydrochloride was from Calbiochem (La Jolla, CA, U.S.A.). α-Methylglucoside was from Wako Pure Chemicals (Osaka). o-Aminobenzaldehyde and polyethylene glycol (PEG) 20,000 were from Nacalai Tesque (Kyoto). DE-52 (DEAE-cellulose) and BIO-GEL HTP (hydroxyapatite) were from Whatman (Maidstone, Kent, U.K.) and Bio-Rad Laboratories (Richmond, CA, U.S.A.), respectively. Sephacryl S-300, Con A Sepharose, Protein A Sepharose CL-4B, and molecular weight and pI standard calibration marker proteins were from Pharmacia LKB Biotecnology (Uppsala, Sweden). Other chemicals were purchased from commercial sources.

Enzyme Assays—DAO activity was measured by the method of Yamada et al. (11) using putrescine as a substrate. Briefly, incubation was performed at 37°C in 1 ml of 100 mM Na-phosphate buffer (NaPB), pH 7.0, containing 2 mM putrescine, 0.03% (w/v) o-aminobenzaldehyde, and enzyme. The reaction was terminated by adding 0.2 ml of 50% (w/v) trichloroacetic acid and 0.1 ml of ethanol. The amount of product formed by DAO was determined by measuring the absorbance at 430 nm using the molar coefficient of the quinazolinium (ε = 1.86 x 10^5 M^-1 cm^-1 at 430 nm). One unit of enzyme activity was defined as the deamination of 1 μmol of putrescine per min under the standard assay conditions.

Protein Determination—Protein concentrations were determined by the method of Lowry et al. (22) with bovine serum albumin (BSA) as a standard, or by measuring the absorbance at 280 nm.
Purification of DAO from Rat Small Intestine—All purification steps were carried out at 4°C.

Step 1. Crude extract preparation: Rat small intestine tissue (150 g) was homogenized with a Polytron homogenizer (Kinematica, Steinhofhalde, Switzerland) in 5 volumes of 100 mM NaPB, pH 7.4. A crude extract was obtained by ultracentrifugation of the homogenate at 100,000 \( \times g \) for 1.5 h.

Step 2. Ammonium sulfate fractionation: The crude extract was saturated with 30% ammonium sulfate for 2 h, and then centrifuged at 10,000 \( \times g \) for 1 h. The supernatant was brought to 60% ammonium sulfate saturation, left for 2 h, and then centrifuged at 10,000 \( \times g \) for 1 h. The precipitate, which contained DAO activity, was dissolved in a minimal amount of 50 mM NaPB, pH 7.4, and then dialyzed against the same buffer.

Step 3. DEAE-cellulose column chromatography: The dialyzed extract was applied on a DE-52 column (3.4 \( \times 69 \) cm) equilibrated with 50 mM NaPB, pH 7.4. The enzyme was eluted with a linear gradient of NaCl (0 to 0.3 M) in the equilibration buffer. DAO-containing fractions were pooled, concentrated with PEG 20,000, and then dialyzed against 100 mM NaPB, pH 7.4.

Step 4. Hydroxylapatite column chromatography: The dialyzed enzyme was applied on a BIO-GEL HTP column (2.2 \( \times 13 \) cm) equilibrated with 100 mM NaPB, pH 7.4. The enzyme was eluted with a linear gradient of ammonium sulfate (0 to 0.5 M) in the equilibration buffer.

Step 5. Sephacryl S-300 gel filtration: The DAO fraction from step 4 was concentrated with PEG 20,000, and then applied on a Sephacryl S-300 column (1.6 \( \times 110 \) cm) and eluted with 100 mM NaPB, pH 7.4, containing 0.2 M NaCl. The fractions containing DAO were concentrated with PEG 20,000.

Step 6. Con A Sepharose column chromatography: The enzyme was incubated overnight in 1 ml of a suspension of Con A Sepharose equilibrated with 100 mM NaPB, pH 7.4, containing 0.2 M NaCl. The gel was packed into a column and then washed with the equilibration buffer. The enzyme was eluted with the equilibration buffer containing 2 M \( \alpha \)-methylglucoside.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) —SDS-PAGE was performed by the method of Laemmli (23) using a ready-made gradient gel (4 to 20%; Daiichi Pure Chemicals, Tokyo). The gel was stained with Coomasie Brilliant Blue R250 (CBB R250) or a silver stain kit (Daiichi Pure Chemicals).

Isoelectric Focusing—Isoelectric focusing was carried out with a Pharmacia flat bed IEF system on a 4% polyacrylamide gel containing 6% ampholine (pH 5 to 8) at 12°C under N\(_2\) gas. The protein band corresponding to DAO was detected by immunoblotting using antiserum against the purified enzyme.

Anti-DAO Antiserum Preparation—An emulsion of the purified DAO (20 \( \mu \)g, 1 ml) in an equal volume of Freund’s complete adjuvant (Wako Pure Chemicals) was injected into a female rabbit. An emulsion of the enzyme (15 \( \mu \)g) and 150 \( \mu \)g of adjuvant peptide (Peptide Institute, Osaka) with Freund’s incomplete adjuvant (Wako Pure Chemicals) was injected as the first and second boosters at 2 and 4 weeks, respectively, after the first injection. Anti-DAO antiserum was collected 5 days after the second booster.

Immunoprecipitation Study—DAO samples from various sources, having equal activity of 0.6 mU, were incubated overnight at 4°C with a series of 2-fold dilutions of the anti-DAO antiserum. The antigen–antibody complex was co-precipitated by incubation at 4°C for 5 h with 200 \( \mu \)l of 10% protein A Sepharose CL-4B. The co-precipitate was removed by centrifugation, and the remaining DAO activity in the supernatant was measured.

Ouchterlony’s Double Diffusion Test—Ouchterlony’s double diffusion test (24) was performed in a 1% agarose gel with the anti-DAO antiserum (15 \( \mu \)l) placed in the central well. In the surrounding wells, DAO samples from various sources with equal activity of 0.05 mU were placed. After 5-day incubation at 4°C, the precipitates were stained with CBB R250.

**RESULTS AND DISCUSSION**

Distribution of DAO Activity in Rat Tissues—DAO activities from various rat tissues are shown in Table I. Relatively high DAO activity was found in the small intestine (1.81 ± 0.41 mU/mg protein) and the placenta (2.11 ± 0.38 mU/mg protein), followed by in the thymus and stomach (0.06 ± 0.03 and 0.024 mU/mg protein, respectively). A small amount of DAO activity (0.003 mU/mg protein) was found in the lung, but no DAO activity was detected in the liver, kidney, or whole brain. These results indicate that the small intestine and placenta are two major tissues with high DAO activities in the rat. Thus it is suggested that DAO plays a major role in histamine inactivation in the small intestine, particularly in non-pregnant rats. The data obtained in this study coincide with those reported by Shaff and Beaven (25).

Purification of Rat Small Intestinal DAO—From 150 g of rat small intestine tissue, DAO was purified to homogeneity, with a 4.7% recovery after the purification steps (Table II). The enzyme was purified 2,500-fold, and the purified enzyme gave a single protein band on SDS-PAGE (Fig. 1A). Among the purification steps, the hydroxyxylap.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DAO activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>2.11 ± 0.38</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.81 ± 0.41</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.024</td>
</tr>
<tr>
<td>Lung</td>
<td>0.003</td>
</tr>
<tr>
<td>Liver</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
</tr>
<tr>
<td>Whole brain</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Mean ± SD. 'Not detectable.

**TABLE II. Summary of purification of DAO from rat small intestine.**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Activity* (U)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>10.4</td>
<td>9.100</td>
<td>0.0011</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>6.29</td>
<td>2.030</td>
<td>0.0021</td>
<td>60.6</td>
<td>1.82</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>2.38</td>
<td>0.298</td>
<td>0.008</td>
<td>22.9</td>
<td>7.01</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>1.48</td>
<td>12.1</td>
<td>0.114</td>
<td>14.3</td>
<td>89.7</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>1.12</td>
<td>2.0</td>
<td>0.55</td>
<td>10.8</td>
<td>489</td>
</tr>
<tr>
<td>Con A Sepharose</td>
<td>0.488</td>
<td>0.175</td>
<td>2.81</td>
<td>4.7</td>
<td>2,460</td>
</tr>
</tbody>
</table>

*U, \( \mu \)mol/min. +Start from 150 g of rat small intestine.

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tite step was the most effective, with 14-fold purification. The Con A Sepharose step was another effective one, which verified the DAO from rat small intestine was a glycoprotein. DAOs from other sources have been reported to be glycoproteins (13, 17, 20, 26-28). Previously, the DAOs from pig kidney (11-14), human placenta (15-21), and kidney (27, 29), and seminal plasma (30) were purified to homogeneity. However, DAOs from the small intestines of rabbit, dog, pig, and man could only be purified partially because of their instability (26, 31, 32). Similar to the DAO from human small intestine, the rat intestinal DAO was reported to be unstable (26, 33). In our study, DAO from the rat small intestine was also unstable, especially in the first three steps of the purification. So we had to finish the first three steps of the purification as fast as possible. However, the enzyme became stable after the fourth step of the purification. Heat treatment was a very useful procedure for purification of the pig kidney (12, 13, 34) and human kidney (27) enzymes, but we found that this treatment was not suitable for purification of the rat small intestinal DAO (data not shown).

Biochemical Properties of the Purified DAO from Rat Small Intestine—The purified small intestinal DAO was biochemically characterized in order to compare its properties with those of DAOs from other sources. The purified DAO showed a single band at 92 kDa and a band at 180 kDa on SDS-PAGE under reducing and non-reducing conditions, respectively (Fig. 1A). Immunoblot analysis using the antiserum raised against the purified DAO showed that both the 92 and 180 kDa bands were immunoreactive to the antiserum (Fig. 1B). On Sephacryl S-300 gel filtration, the molecular mass of the purified DAO was estimated to be 180 kDa (data not shown). These results suggest that the DAO from rat small intestine is a dimer of 92 kDa subunits, which are bound through a disulfide bond. This molecular mass and subunit structure are similar to those of the DAOs from pig kidney (11, 13) and human placenta (21). The isoelectric point of the purified DAO was 6.0 (data not shown), and this value was identical with that of the pig kidney enzyme, but higher than that of the placental enzyme (pI=4.9-5.5) (21, 34). The substrate specificity of the purified DAO from rat small intestine was investigated. The $K_m$ values for histamine, putrescine, $N^\prime$-methylhistamine, and cadaverine were 9.4, 13.4, 13.0, and 16.0 μM, respectively. The purified enzyme deaminated putrescine at the highest reaction velocity ($V_{max}=2.8 \mu$mol/min/mg protein). Histamine, $N^\prime$-methylhistamine, and cadaverine were deaminated at 70-75% of the velocity for putrescine. The $V_{max}/K_m$ value for histamine was the highest, followed by those for putrescine, $N^\prime$-methylhistamine, and cadaverine (data not shown). The purified small intestinal DAO did not deaminate spermine or spermidine. Such a substrate specificity was also reported for human intestinal DAO (26). But the enzymes from other sources, including kidney and placenta, oxidized these polyamines (35, 36), so this...
seems to be one of the specific characteristics of the intestinal enzyme. These results indicate that histamine is the best substrate for the rat small intestinal DAO among the substrates tested, putrescine being a relatively good substrate. The substrate specificity pattern of the purified DAO resembled that of the other intestinal enzymes (26, 32), the enzyme showing higher affinity toward histamine than putrescine. This suggests that the intestinal DAO plays specific roles in the gastrointestinal tract, especially in histamine metabolism. Recently, Imamura showed that the intestinal DAO played crucial roles in both endogenous and exogenous histamine metabolism in the rat (37). The kinetic data obtained in this study strongly support this observation.

Immunoochemical Properties of the Rat Small Intestinal DAO—Immunoprecipitation studies showed that the antiserum raised against the purified small intestinal DAO equally inhibited the activities of DAOs from rat small intestine, placenta and thymus (Fig. 2A). Furthermore, Ouchterlony’s double diffusion test showed this antiserum formed a single precipitation line against each of the three rat DAOs, without any spur formation (Fig. 3A). Although the immunochemical properties of DAOs from the human placenta and small intestine have been reported to be different (18, 19), our results indicate that the DAOs from these rat tissues are immunochemically identical. We also examined small intestinal DAOs from different species. As shown in Fig. 2B, the antiserum against the rat enzyme inhibited the guinea pig enzyme activity as equally as it did that of the rat enzyme. This antiserum also forms a single precipitin line against the guinea pig enzyme, without any spur formation between the guinea pig and rat enzymes (Fig. 3B). These results suggest that the guinea pig and rat enzymes are immunochemically identical. The mouse small intestinal DAO crossreacted with the antiserum in a weaker manner than the rat and guinea pig DAOs did (Fig. 2B), and no precipitin line was observed in the double diffusion study (Fig. 3). Both findings indicate that the mouse enzyme exhibits partial immuno-crossreactivity toward the rat enzyme. The dog and rabbit small intestinal DAOs did not crossreact with this antiserum, neither did the pig kidney or human placental DAO (Figs. 2 and 3). The immunoprecipitation study and the double diffusion test revealed that the immunochemical properties of DAO vary among species, and these results are consistent with the previous report (18). Recently, Novotny et al. reported that the expressed human kidney amiloride-binding protein (ABP) exhibits DAO activity, and placental DAO is the same protein as ABP (38). A cDNA encoding a rat homolog of human ABP was also cloned from a colon cDNA library, and it was revealed that the rat ABP transcript was present in the duodenum, colon, placenta, and thymus (39). This localization of ABP was similar to that of DAO reported here. So the intestinal enzyme is probably identical to ABP, although the primary structure of the rat intestinal DAO is not yet known. Our immunochemical study data supported the identity of the intestinal and placental enzymes, but the kinetic data did not. We will now attempt determination of the molecular structure of the intestinal DAO to clarify this discrepancy.

In conclusion, we first purified DAO from the rat small intestine to homogeneity, and determined its biochemical and immunochemical properties. The purified DAO and the antiserum against this enzyme can be useful tools for studying gastrointestinal functions as to exogenous and endogenous histamine.

REFERENCES


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