Purification and Partial Characterization of a Genetically-Defined Superoxide Dismutase (SOD-1) Associated with Maize Chloroplasts

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ABSTRACT

The chloroplast-associated form of superoxide dismutase from maize (Zea mays L.) (SOD-1) has been purified by a stepwise procedure consisting of (NH₄)₂SO₄ fractionation, G-100 Sephadex gel filtration, DEAE-Sephacel chromatography, and hydroxylapatite chromatography. This procedure resulted in a single band on sodium dodecyl sulfate-polyacrylamide gels indicating that the preparation is homogeneous. The holoenzyme molecular weight was estimated at 31,000 to 33,000 by gel filtration. The subunit molecular weight of this dimeric protein was estimated at 14,500 on sodium dodecyl sulfate-polyacrylamide gels. Studies involving amino acid composition analysis, immunological cross-reactivity, in vitro subunit hybridizations, and H₂O₂ sensitivity indicate that SOD-1 differs significantly from SOD-2 and SOD-4, the other cupro-zinc forms of SOD from maize. The possible physiological role of SOD-1 within the chloroplast is discussed.

Multiple molecular forms of superoxide dismutase (Superoxide:superoxide oxidoreductase, EC 1.15.1.1; SOD) exist in maize (Fig. 1). In previous reports, we described the developmental expression, intracellular localization, and genetic control of these isozymes (4, 6, 7). In addition, the cytosolic and mitochondrial isozymes of maize SOD have been purified and partially characterized (5). These studies suggested that the chloroplast-associated isozyme, SOD-1, differs structurally from the other SOD isozymes of maize. SOD-1 is coded for by Sod1, a nuclear gene with two codominant alleles, Sod1A and Sod1B. This gene has no apparent effect on the expression of the other SOD isozymes. Genetically, SOD-1 behaves as a dimer since plants with the genotype Sod1A/Sod1B exhibit a three-banded electrophoretic pattern for SOD-1 on nondenaturing polyacrylamide gels. The isozyme of intermediate electrophoretic mobility is composed of both SOD-1A and SOD-1B subunits (7). The cyanide sensitivity of SOD-1, SOD-2, SOD-4, and SOD-5 suggests that these enzymes are copper and zinc-containing SOD (11), a supposition which is supported by metal dialysis experiments conducted with crude extracts (5). In order to make more detailed comparisons between SOD-1 and the cytosolic cupro-zinc SOD, it was necessary to obtain purified preparations of SOD-1. In this report, we describe a reliable procedure for the purification of the chloroplast-associated SOD of maize as well as some biochemical characterizations of the purified enzyme.

MATERIALS AND METHODS

Materials. Xanthine oxidase, nitro blue tetrazolium, acrylamide, N,N'-methylene-bis-acrylamide, Coomassie brilliant blue R-250, SDS, β-mercaptoethanol, and xanthine were obtained from the Sigma Chemical Company while hydroxylapatite was obtained from the Bio-Rad Company. Ultra pure ammonium sulfate was purchased from the Schwarz/Mann Company. All other chemicals were of reagent grade or better. The maize (Zea mays L.) inbred line W64A, maintained by our laboratory, was used for the purification experiments described in this paper.

Enzyme and Protein Assays. SOD was assayed spectrophotometrically at 25°C according to the procedure described by
Table 1. Purification of Chloroplast Superoxide Dismutase (SOD-I) from Maize

<table>
<thead>
<tr>
<th>Superoxide Dismutase Purification</th>
<th>units/ml</th>
<th>total units</th>
<th>mg protein</th>
<th>units/mg protein</th>
<th>fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>109</td>
<td>114,265</td>
<td>3,580</td>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td>45-65% (NH₄)₂SO₄ fraction</td>
<td>1,844</td>
<td>51,639</td>
<td>359</td>
<td>144</td>
<td>4.5</td>
</tr>
<tr>
<td>G-100 Sephadex chromatography</td>
<td>465</td>
<td>31,140</td>
<td>56</td>
<td>556</td>
<td>17.4</td>
</tr>
<tr>
<td>DEAE-Sephalic chromatography</td>
<td>148</td>
<td>16,619</td>
<td>5.4</td>
<td>3,078</td>
<td>96.2</td>
</tr>
<tr>
<td>Hydroxylapatite chromatography</td>
<td>2,000</td>
<td>4,000</td>
<td>0.366</td>
<td>10,930</td>
<td>341.6</td>
</tr>
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</table>

Electrophoretic Procedures. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (14) using a vertical slab gel apparatus. SOD-1 was denatured by incubation with SDS in a boiling water bath for 2 min in the presence or absence of 10 mM β-mercaptoethanol. Electrophoresis was performed in 13.5% and 15% polyacrylamide gels at 4°C with a current of 30 mamps per slab gel for approximately 4 h. The following proteins were used as mol wt standards: BSA (67,000), ovalbumin (45,000), aldolase (39,500), chymotrypsinogen (25,000), and Cyt c (12,384). Gels were stained with Coomassie brilliant blue R-250 (125 mg Coomassie brilliant blue, 85 ml 50% methanol, 4.6 ml acetic acid) overnight prior to destaining. Nondenaturing polyacrylamide gel electrophoresis and localization of SOD activity on polyacrylamide gels were performed by procedures previously described (4, 5). Starch gel electrophoresis was performed as described by Scandalios (23).

Enzyme Mol Wt Determination. The mol wt (Mₐ) of SOD-1 was determined by gel filtration using a 2.5- × 80-cm column of Sephadex G-100 equilibrated with 20 mM K-phosphate (pH 7.0), and 0.10 M KCl. BSA (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and ribonuclease A (13,700) were used as mol wt markers.

Amino Acid Analysis. The major cytosolic isozymes of maize SOD (SOD-2 and SOD-4) were purified from inbred line W64A as previously described (5). Purified fractions obtained by this procedure were analyzed by nondenaturing polyacrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis, and polyacrylamide gel electrophoresis in acetic acid-urea, and appeared to be free of impurities.

For amino acid analysis, duplicate lyophilized samples of SOD protein were hydrolyzed in 0.7 ml 6 N HCl under nitrogen in 1-ml vials (Pierce Chemical Co.) at 145°C for 2, 4, and 8 h. The contents were neutralized and dried in a NaOH vacuum desicator and analyzed with a Durrum-500 amino acid analyzer. The labile amino acids, threonine and serine, were estimated by extrapolation to zero-time hydrolysis. For the other amino acids, the estimates obtained for the 2, 4, and 8-h hydrolysis periods were averaged together because the recoveries of each amino acid were statistically the same for each sample (±10%).

Immunological Studies. Antibodies against SOD-3 and SOD-4 (5) were tested for cross-reactivity with purified preparations of SOD-1 employing the Ouchterlony double-immunodiffusion technique (20).

RESULTS

Purification of SOD-1. Maize kernels (500 g) were surface sterilized for 10 min in 1% NaOCl, rinsed thoroughly with deionized H₂O, and soaked in deionized H₂O for 24 h. The seeds were grown in the dark for 5 d and transferred to light (12 h/d) for another 7 d. Leaf tissue was harvested (165 g) and homogenized in 0.1 M K-phosphate (pH 7.0; 8 ml/g fresh weight) with 80 g polyvinylpolyPyrrolidone using a commercial Waring Blender. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 22,000 g for 20 min. The protein precipitate formed at 45 to 65% saturation (NH₄)₂SO₄ was recovered by centrifugation (22,000 g maximum) and the resulting pellet
CHLOROPLAST SUPEROXIDE DISMUTASE (SOD-1) OF MAIZE

Table II. Amino Acid Compositions

<table>
<thead>
<tr>
<th>Cupro-Zinc Superoxide Dismutases</th>
<th>Maize SOD-1*</th>
<th>Maize SOD-2*</th>
<th>Maize SOD-4*</th>
<th>Wheat isozyme I®</th>
<th>Wheat isozyme II®</th>
<th>Green pea®</th>
<th>Spinach®</th>
<th>Bovine erythrocyte®</th>
<th>Chicken®</th>
<th>Neurospora crassa®</th>
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<tbody>
<tr>
<td>Aspartic acid†</td>
<td>36.2 ± 0.8</td>
<td>38.3 ± 0.4</td>
<td>38.2 ± 0.5</td>
<td>28</td>
<td>28</td>
<td>45</td>
<td>35</td>
<td>35</td>
<td>32</td>
<td>36</td>
</tr>
<tr>
<td>Threonine</td>
<td>26.8 ± 0.6</td>
<td>17.0 ± 0.2</td>
<td>24.2 ± 0.3</td>
<td>33</td>
<td>30</td>
<td>45</td>
<td>28</td>
<td>26</td>
<td>18</td>
<td>26</td>
</tr>
<tr>
<td>Serine</td>
<td>12.8 ± 0.1</td>
<td>20.3 ± 0.2</td>
<td>21.9 ± 0.3</td>
<td>15</td>
<td>12</td>
<td>14</td>
<td>10</td>
<td>20</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Glutamic acid†</td>
<td>28.0 ± 0.1</td>
<td>29.8 ± 0.6</td>
<td>21.2 ± 0.4</td>
<td>21</td>
<td>26</td>
<td>19</td>
<td>20</td>
<td>24</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>Proline</td>
<td>19.7 ± 0.8</td>
<td>15.5 ± 0.6</td>
<td>17.0 ± 0.5</td>
<td>19</td>
<td>19</td>
<td>14</td>
<td>17</td>
<td>14</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Glycine</td>
<td>48.1 ± 1.1</td>
<td>68.0 ± 0.9</td>
<td>65.0 ± 0.8</td>
<td>55</td>
<td>43</td>
<td>56</td>
<td>42</td>
<td>50</td>
<td>50</td>
<td>39</td>
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<tr>
<td>Alanine</td>
<td>27.7 ± 0.5</td>
<td>26.8 ± 1.0</td>
<td>29.6 ± 0.5</td>
<td>28</td>
<td>25</td>
<td>21</td>
<td>23</td>
<td>21</td>
<td>22</td>
<td>20</td>
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<tr>
<td>Half-cystine</td>
<td>2.2 ± 0.6</td>
<td>3.4 ± 0.3</td>
<td>3.6 ± 0.8</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>14</td>
<td>3</td>
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<tr>
<td>Valine</td>
<td>28.8 ± 0.2</td>
<td>30.8 ± 1.2</td>
<td>27.8 ± 1.1</td>
<td>31</td>
<td>34</td>
<td>21</td>
<td>28</td>
<td>28</td>
<td>22</td>
<td></td>
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<tr>
<td>Methionine</td>
<td>0</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Isoleucine</td>
<td>11.6 ± 0.2</td>
<td>11.4 ± 0.2</td>
<td>12.1 ± 0.2</td>
<td>13</td>
<td>10</td>
<td>20</td>
<td>6</td>
<td>17</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Leucine</td>
<td>30.1 ± 0.2</td>
<td>18.3 ± 0.3</td>
<td>19.9 ± 0.3</td>
<td>22</td>
<td>31</td>
<td>21</td>
<td>22</td>
<td>20</td>
<td>16</td>
<td>11</td>
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<tr>
<td>Tyrosine</td>
<td>4.0 ± 0.1</td>
<td>4.4 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Phenylalanine</td>
<td>8.4 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>7</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
<td>14.5 ± 0.1</td>
<td>15.7 ± 0.4</td>
<td>17.6 ± 0.1</td>
<td>19</td>
<td>15</td>
<td>18</td>
<td>14</td>
<td>16</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.5 ± 0.2</td>
<td>10.8 ± 0.2</td>
<td>12.3 ± 0.3</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>13</td>
<td>22</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.2 ± 1.1</td>
<td>5.4 ± 0.2</td>
<td>5.2 ± 0.6</td>
<td>8</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>9</td>
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<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<td></td>
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</tbody>
</table>

* The number of residues was calculated for 32,000 Mr.
† Reference 9.
‡ Reference 22.
§ Reference 2.
¶ Reference 13.
© Reference 24.
† Reference 18.
# Amide content not determined.

was resuspended in approximately 20 ml of 20 mM K-phosphate buffer (pH 7.0). This was then dialyzed overnight against 4 L of the same buffer and applied to a 2.5 × 80-cm Sephadex G-100 column previously equilibrated with the dialysis buffer. Fractions (5 ml) were collected at a flow rate of 20 to 40 ml/h and assayed for SOD activity. Those fractions containing SOD activity (principally SOD-1) were pooled and applied directly to a 2.5 × 30-cm DEAE-Sephacel column equilibrated with 20 mM K-phosphate buffer (pH 7.0). Under these conditions, SOD-2 and SOD-4 did not bind to the column and were detected in the void volume. SOD-1 was eluted from the column using a 20 to 250 mM linear gradient of K-phosphate (pH 7.0; 1 L, total). Fractions (10 ml) were collected at a flow rate of 30 to 50 ml/h and assayed for SOD activity. The SOD-1 fraction was pooled, dialyzed against 4 L of 20 mM K-phosphate buffer (pH 7.0) overnight, and passed through a 1.5 × 30-cm column of hydroxylapatite equilibrated with the same buffer. SOD-1 did not bind to the column under these conditions and was recovered in the column wash. These fractions were pooled, dialyzed against 4 L of deionized H₂O overnight, and lyophilized. The lyophilized powder was resuspended in approximately 2 ml of 20 mM K-phosphate buffer (pH 7.0). The purification of SOD-1 is summarized in Table I.

Purity of the Isozyme Preparation. The SOD-1 preparation yielded a single protein band on nondenaturing polyacrylamide gels which coincided with SOD activity (Fig. 2). Purified SOD-1 comigrates with SOD-1 obtained from crude extracts on nondenaturing gels (not shown). When treated with 10 mM β-mercaptoethanol and SDS, this isozyme yielded a single protein band on SDS-polyacrylamide gels (Fig. 2), indicating that the preparation is homogeneous.

Mol Wt and Subunit Composition. The holoenzyme mol wt (Mr) of SOD-1 was estimated to be 33,500 ± 1,500 by gel filtration on Sephadex G-100 (Fig. 3). This estimate compares well with the mol wt range of 31,000 to 33,000 calculated for SOD-2 and SOD-4. The subunit mol wt (Mr) of SOD-1 was determined to be 14,500 by SDS-polyacrylamide gel electrophoresis on 13.5% and 15% gels (Fig. 2). In the absence of β-mercaptoethanol, the mobility of SOD-1 was slightly increased in SDS gels, suggesting the presence of intrachain disulfide bonds which affect the conformation of the polypeptide molecules (data not shown; 5). It was concluded that SOD-1 has a mol wt of approximately 31,000 to 33,000 and is composed of two, apparently equal, subunits lacking interchain disulfide bonds.

Amino Acid Composition Analysis. The amino acid compositions of SOD-1, SOD-2, and SOD-4 are shown in Table II along with amino acid composition data for several cupro-zinc SOD purified from other eukaryotes. SOD-2 and SOD-4 appear to be the most similar of the three maize isozymes, differing significantly only in their glutamic acid, threonine, and tyrosine content. SOD-1 differs dramatically from both SOD-2 and SOD-4 in serine, glycine, and leucine content. Similarities among all of the SOD (Table II) can be observed, reinforcing the notion that they may be evolutionarily conserved (17). Although we have not determined tryptophan content, it is clear that cupro-zinc SOD are generally deficient in this amino acid, particularly those from plants (Table II; 3, 18, 24). The UV absorption spectrum of SOD-1, like those of SOD-2 and SOD-4, is typical of cupro-zinc SOD in that there is not clear absorption maximum at 280 nm, suggesting a deficiency in tyrosine and tryptophan.

Immunological Cross-Reactivity. Antibodies raised against purified SOD-4 have been found to cross-react identically with purified SOD-2, yielding fused precipitin lines on immunodiffusion plates (5). SOD-3, the mitochondrial isozyme, resembles manganese-containing SOD and is antigenically distinct from cupro-zinc SOD, namely SOD-2 and SOD-4 (5). To determine
the antigenic relationship of SOD-1 to the other SOD in maize, antibodies raised against SOD-3 and SOD-4 were tested for cross-reactivity with SOD-1 antigen on Ouchterlony plates. According to this analysis, SOD-1 is antigenically distinct from SOD-2, SOD-4, and SOD-3 (Fig. 4, A and B).

**DISCUSSION**

In this report, we describe a convenient procedure for the purification of SOD-1, the genetically defined (7) SOD isozyme associated with maize chloroplasts. The pure enzyme preparation appears homogeneous as judged by nondenaturing and SDS-polyacrylamide gel electrophoresis.

SOD-1 behaves biochemically and genetically as a dimer and apparently requires copper for enzymic activity (5). The protein has a holoenzyme mol wt of 31,000 to 33,000 and is composed of apparently equal subunits, not bound by interchain disulfide bonds. Its amino acid composition is similar to those of SOD-2 and SOD-4 and to those of other cupro-zinc SOD purified from a wide range of eukaryotic organisms (Table II; 3, 18, 24). The results of our amino acid composition analyses, H$_2$O$_2$ sensitivity, immunological cross-reactivity, in vitro subunit hybridization, and subcellular compartmentation experiments indicate that SOD-1 is unique among the cupro-zinc SOD in maize.

Previously, we demonstrated that a mutation at the Sod4 structural gene locus affects the expression of SOD-4 but not that of SOD-2, suggesting that the two proteins are encoded in separate genes (4, 7). The amino acid analyses presented in this communication demonstrate that SOD-2 and SOD-4 are distinct proteins even though they appear to exhibit similar biochemical properties (5). Until an electrophoretic variant for SOD-2 is
found, it will be difficult to determine with certainty the genetic relationship between these two isozymes. It is not clear at this time what the biological significance is of having two similar cytosolic superoxide dismutases produced in the same organism.

The observation that SOD-1 is more resistant to \( \text{H}_2\text{O}_2 \) inactivation than the cytosolic SOD of maize is intriguing in light of the fact that this isozyme is localized in chloroplasts (4, 10), an organelle in which \( \text{H}_2\text{O}_2 \) is generated during photosynthesis (1). It seems possible that the increased resistance to \( \text{H}_2\text{O}_2 \) inactivation represents an adaptation to this particular subcellular environment. The chloroplast-associated SOD of wheat (10) has been reported to be more resistant to \( \text{H}_2\text{O}_2 \) inactivation than the corresponding cytosolic isozyme (9), again suggesting that this increased resistance to \( \text{H}_2\text{O}_2 \) inactivation has a functional significance. Asada et al. (2) suggest the possibility that chloroplast-associated SOD catalyzes the disproportionation of superoxide radicals, generated by the chloroplast upon illumination, to form \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \). Therefore, it appears that the enzyme’s resistance to \( \text{H}_2\text{O}_2 \) may be a protective adaptation to its own action.

Other instances of SOD activity in association with chloroplasts have been reported. Soluble cyanide-sensitive SOD are associated with the chloroplasts of spinach (2, 12, 16), Brassica campestris (21), green pea, and sorghum (10). All appear to be distinct from SOD localized in the cytosol. In some cases, it appears that a specific isozyme is localized within the chloroplast (10, 12). The present case, however, is the first instance of a specific, genetically defined, SOD isozyme associated with chloroplasts.

The purification of this isozyme is an initial step towards an understanding of the process whereby SOD-1 is selectively compartmentalized into the chloroplast, instead of the other cupro-zinc isozymes, SOD-2 and SOD-4. Also, it is the first step towards understanding the evolutionary relationship between SOD-1 and the other cyanide-sensitive isozymes of maize. Finally, it is now possible to obtain monospecific antibodies against the pure protein which can be utilized in future studies concerning subcellular compartmentation and developmental expression of this isozyme.

Acknowledgments—We thank C. Young for providing advice and facilities for the amino acid analyses, and Stephanie Rusza and Lorraine Siebenaler for expert technical assistance.

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