Purification and characterization of the higher plant enzyme L-canaline reductase

(l-canavanine catabolism/plant nitrogen metabolism/leguminosae)

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ABSTRACT A newly discovered enzyme, L-canaline reductase (NADPH:L-canaline oxidoreductase, EC 1.6.6. -), has been isolated and purified from 10-day-old leaves of the jack bean Canavalia ensiformis (Leguminosae). This higher plant is representative of a large number of legumes that synthesize L-canavanine, an important nitrogen-storing nonprotein amino acid. Canavanine-storing legumes contain L-canavanine, representative of a large number of legumes that synthesize the overall one-half of the total dry matter of the seed of certain legumes (9). To support their nitrogen metabolism, these plants use their arginase to release the stored nitrogen of canavanine as urea (10). canaline is a toxic by-product of this catabolic reaction. Analysis of these canavanine-storing legumes discloses the presence of only trace amounts of canaline (5, 11). To avoid the toxic effects of canaline, these plants have evolved a mechanism to efficiently catabolize the appreciable canaline formed from canavanine.

Investigation of the canavanine-producing jack bean Canavalia ensiformis (Leguminosae) has resulted in the isolation of an enzyme that used NADPH to reductively cleave the oxygen-nitrogen bond of canaline irreversibly to yield homoserine and ammonia.

H2N—O—CH2—CH2—CH(NH2)COOH

H-n-canavanine

→ HO—CH2—CH2—CH(NH2)COOH + NH3

l-homoserine

ammonia

This communication describes the purification and characterization of the enzyme L-canaline reductase (CR; NADPH:L-canaline oxidoreductase, EC 1.6.6.-), which not only detoxifies canaline but also supports the nitrogen metabolism of the plant.

MATERIALS AND METHODS

Substrate Preparation. L-Canavanine (free base) was isolated from acetone-defatted jack bean seeds by ion-exchange chromatography and purified by repetitive crystallization (12). L-Canaline (free base) was prepared from L-canavanine by the method of Rosenthal (13).

l-[U-14C]Canaline was synthesized from commercially prepared l-[U-14C]Homoserine (Amersham; 1.48 GBq/mmol). The radiochemical synthesis involved the successive synthesis of 1-2-[benzoylcarbonylamino]-4-hydroxybutyric acid, 1-2-[benzoylcarbonylamino]-4-butyrolactone, benzyl 1-2-[carboxybenzyl]amino]-4-hydroxybutyrate, and benzyl 1-2-[carboxybenzyl]amino]-4-[(p-tolylsulfo-
nyl)oxy)butyrate. The aminooxy function was introduced with the addition of benzohydroxamic acid to form benzyl 1-2[(carbobenzyloxy)amino]-4-(benzamidoxy)butyrate. This compound was deprotected by refluxing with 19% (wt/vol) ethanolic HCl followed by refluxing with 3 M HCl to form L-[U-14C]canaline (14). Scintillation medium (Ecolume) was purchased from ICN. Sigma supplied the biochemicals. All other chemicals were obtained from Aldrich.

**Canaline-Linked, Cyanogen Bromide-Activated Sepharose.** A suspension of Sepharose (40–190 μm) in deionized water was centrifuged at 600 × g for 10 min to pack the gel. Ten milliliters (settled gel volume) of the gel was suspended in 10 ml of deionized water, taken to pH 10.5–11 with 4 M NaOH, and stirred mechanically in a well ventilated hood with 1.0 g of cyanogen bromide dissolved in 15 ml of 1-methyl-2-pyrrolidinone. The pH was maintained between 10 and 11 for 12 min. The cyanogen bromide-activated gel was transferred to a sintered glass funnel and washed with 10 vol of ice-cold 100 mM sodium carbonate/bicarbonate buffer (pH 9.5) (buffer A).

The activated gel in buffer A was treated with 100 mg of L-canaline and agitated gently overnight at 23°C. After washing the canaline-linked Sepharose with buffer A, it underwent reaction with 3 vol of 1 M 2-aminoethanol (pH 9.5) for 60 min at 23°C. After transferring the treated gel to a sintered glass funnel, it was washed thoroughly with buffer A and stored in 50 mM sodium acetate (pH 4.0) with a few crystals of sodium azide at 4°C.

**Enzyme Assay.** Crude CR activity was determined by measuring the degradation of L-canaline to L-homoserine in the presence of a reduced NADP-regenerating system. The assay mixture (1.0 ml) consisted of 15 mM L-canaline (pH 7.3), 100 mM sodium Tricine (pH 7.3), 2.5 mM NADP, 25 mM glucose 6-phosphate, 150 μg of glucose 6-phosphate dehydrogenase (300 units per mg of protein), and no more than 0.065 unit of CR. All assays were conducted in triplicate at 37°C for 60 min. The reaction was terminated by the addition of 2.0 ml of 3.5 M perchloric acid, neutralized with 2.0 ml of 3.5 M KOH and placed on ice for several minutes. After clarifying the assay mixture by centrifugation, the supernatant solution was assayed colorimetrically for the disappearance of canaline. Controls consisted of appropriate reaction mixtures to which boiled enzyme was added.

Purified CR was assayed by monitoring the decrease in absorbance at 340 nm resulting from the oxidation of NADPH. The assay mixture (1.0 ml) included 15 mM L-canaline (pH 7.3), 100 mM sodium Tricine (pH 7.3), 0.1 mM NADPH, and no more than 0.05 unit of CR. Assays were conducted at 23°C in a Gilford response recording spectrophotometer that determined the absorbance 10 times per min. One unit of enzyme catalyzed the reduction of 1 μmol of canaline per min under the described reaction conditions.

**Colorimetric Assay.** Canaline depletion was determined colorimetrically; canaline was carbamoylated with cytosine to generate ureidohomoserine prior to color development (15). The neutralized enzyme assay mixture (0.5 ml) was diluted with an equal vol of 100 mM sodium acetate buffer (pH 4.0) and treated with 1.0 ml of 1:1 (vol/vol) oxime/semidine reagent and then 0.5 ml of concentrated sulfuric acid. The tubes were placed into boiling water for exactly 2.5 min; the resulting chromogen was read immediately at 542 nm (15).

**Identification of Homoserine.** The standard enzyme assay mixture, containing a 10-fold excess of reagents and 0.37 MBq of L-[U-14C]canaline, underwent reaction at 37°C for 4 h. Afterwards, the reaction mixture was deproteinized as described above, taken to pH 3.5 with 2 M HCl, and applied to a column (20 × 75 mm) of Dowex 50 (H+). After washing the column with 1 liter of deionized water, it was developed with 0.5 liter of 150 mM ammonia. The column effluent was concentrated by rotary evaporation in vacuo, the residue was dissolved in deionized water, and the process was repeated twice. Finally, the 14C-bearing residue was taken up in a minimum amount of deionized water and reacted with sufficient 2-oxoglutaric acid to convert any unreacted [14C]canaline to its radiolabeled canaline-2-oxoglutarate oxide. Unlike canaline, this oxide can be quantitated since it is stable to the buffers used in automated amino acid analysis.

The 14C-bearing effluent was subjected to automated amino acid analysis in which the column effluent was collected without reacting with ninhydrin. The column effluent was then assayed by liquid scintillation spectroscopy. Radiola- beled homoserine (retention time, 32 min) accounted for 98% of the 14C of the column effluent not residing in [14C]canaline-2-oxoglutarate oxide (retention time, 14 min).

To verify the identity of homoserine, a portion of the radiolabeled effluent from the Dowex 50 column was refluxed with 19% (wt/vol) ethanolic HCl for 90 min. After removing the solvent by rotary evaporation in vacuo, the residue was allowed to dry in vacuo at 55°C for an additional 30 min after solvent removal. Deionized water was added to the residue and the drying process was repeated twice. The residue was then dissolved in 3 M HCl and refluxed for 90 min at 115°C. Afterwards, the HCl was removed by exhaustive rotary evaporation in vacuo and the residue was dissolved in deionized water, taken to pH 3.5 with 1 M ammonia, and placed on a column (20 × 75 mm) of Dowex 50 (NH4+). The column was washed with 0.7 liter of deionized water and developed with 0.5 liter of 200 mM ammonia. The column effluent was concentrated by rotary evaporation in vacuo.

The above procedures convert homoserine stoichiometrically to homoserine lactone; unlike homoserine, the latter compound is basic. Free homoserine, unreacted canaline, neutral compounds, and acidic compounds cannot bind to this resin in the NH4+ form. Homoserine lactone is converted to homoserine in situ when the column is developed with 200 mM ammonia. All of the 14C adhering to the exhaustively washed column eluted with 200 mM ammonia. Automated amino acid analysis of the column effluent established that homoserine was the sole 14C-bearing compound.

**Identification of Ammonia.** Ammonia production resulting from CR-catalyzed reduction of canaline was established by monitoring the oxidation of NADH in the presence of gluta- mic acid dehydrogenase and 2-oxoglutaric acid as described by Tabor (16).

**Competitive Inhibitor Kinetics.** The KI value for the competitive inhibitors aminooxyacetic acid and hydroxylamine was determined from a series of Lineweaver–Burk reciprocal plots of 1/V vs. 1/[S] in the presence of various inhibitor concentrations. The slope of the reciprocal plots when inhibitor is present can be represented as:

\[
\text{slope} = \frac{K_m}{V_{max}K_i}/(1 + \frac{K_m}{V_{max}}).
\]

The slope obtained from each of the reciprocal plots was then plotted as a function of the inhibitor concentration. The x intercept of the resulting curve is \(-K_i\).

**Protein Assay.** Protein was determined in crude preparations by the method of Lowry et al. (17). The absorbance of purified CR was determined with the exhaustively dialyzed enzyme that was dried to constant weight in vacuo at 100°C. A 0.1% (wt/vol) solution of CR had a A280 of 1.05.

**Polycarboxyamide Gel Electrophoresis.** CR was analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions by the method of Davis (18). The protein, treated with SDS under reduced conditions for 3 min at 100°C, was also analyzed by the method of Laemmli (19). All gels were stained with 0.5% (wt/vol) Coomassie blue. Commercially prepared proteins (ranging from 14 to 181 kDa) served as the standards (Sigma SDS-6 and SDS-7 standards).
Amino Acid Analysis. CR samples were hydrolyzed in 6 M HCl with 0.02% (vol/vol) 2-mercaptoethanol and 0.01% (wt/vol) phenol for 24 h at 100°C. The amino acid composition of the hydrolyzate was determined by ion-exchange chromatography using a Dionex D-300 automated amino acid analyzer equipped with a lithium citrate physiological buffer system and ninhydrin detection. Tryptophan and half-cystine were determined as described (20). Amino acids were standardized with Pierce amino acid standards. Peak area was determined by a Spectra physics model 4270 automated area integrator.

Absorbance. Absorbance spectra were obtained with a solution (0.5 mg/ml) of purified enzyme that was dialyzed overnight against standard buffer. A sample of the dialyzed enzyme was diluted twice with 0.1% (vol/vol) phenol for chromatography using HCl with 1782 Biochemistry: Rosenthal

RESULTS

Preparation of CR. Jack bean, C. ensiformis [Leguminosae], seedlings were greenhouse-grown from seeds for 10 days as described (21). Frozen 10-day-old jack bean leaves (75 g) were ground with a Sorvall Omni mixer at full power for 30 sec with 300 ml of buffer. Unless otherwise indicated, the buffer was 50 mM sodium Tricine (pH 7.3) containing 0.1% (vol/vol) 2-mercaptoethanol and 1 mM dithiothreitol. This buffer provided the most stable storage condition while yielding maximum enzymatic activity.

Purification of CR. The jack bean leaf homogenate was expressed through cheesecloth and then centrifuged for 20 min at 12,500 × g. A total of 225 g of frozen leaves were processed. The supernatant solution, combined from the three grindings, was taken to 55% saturation by the addition of a liquid, saturated ammonium sulfate solution (pH 7.6) and allowed to sit at 4°C for at least 90 min. The turbid solution was centrifuged at 12,500 × g for 20 min. The pellet was dissolved in a minimum amount of buffer and dialyzed overnight against 2 × 2 liters of the same buffer. Centrifugation was used to remove protein that precipitated during dialysis.

DEAE-Cellulose Chromatography. The dialyzed enzyme solution was diluted with an equal volume of deionized water immediately before its application to a column (28 × 375 mm) of Whatman DEAE-cellulose (DE32) equilibrated with 20 mM buffer. The column was washed with 200 ml of 75 mM buffer.

CR was obtained by developing the washed column with a linear gradient consisting of 100 ml of 100 mM buffer and an equal volume of 400 mM buffer. The effluent, at a flow rate of 1.5 ml/min, was collected at 2-min intervals. Assays were conducted with 100 μl of each fraction; the five most active fractions were pooled.

G-200 Sephadex Chromatography. The concentrated effluent was applied to a column (10 × 420 mm) of Sephadex G-200 equilibrated with buffer. Fractions (1 ml) were collected at 12-min intervals. Assays were conducted with 50 μl of each fraction; the six most active fractions were pooled, concentrated as described above, and stored at -60°C.

Affinity Chromatography. The concentrated enzyme solution (1.0 ml) was applied to a column (7 × 40 mm) of canaline-linked Sepharose equilibrated with 20 mM buffer. The column was washed with the same buffer and developed with 200 mM buffer containing 100 mM KCI; 1.0-ml fractions were collected. After concentrating the three most active fractions by Amicon filtration using a 10-ml cell, the enzyme was diluted twice with 50 mM buffer and concentrated each time to 1–2 ml; 0.2-ml fractions were stored at −60°C (Table 1). Enzyme assays were conducted with protein purified through this step.

High-Performance Liquid Chromatography. Traces of contaminant protein were further removed by injecting the concentrated affinity column effluent (200 μl) into a reverse-phase C18-μP column (Bakerbond) and the protein was eluted over a period of 40 min with a linear gradient of solution A [0.1% (vol/vol) aqueous trifluoroacetic acid] and solution B [80% acetonitrile and 20% (vol/vol) solution A]. Fractions of 1.0 ml were collected at 1-min intervals. The effluent was monitored at 280 nm with an SSI model 500 variable-wavelength spectrophotometer.

CR is a labile enzyme that loses activity when maintained on ice and it is highly unstable in the absence of protective mercaptans. CR maintained in 50 mM buffer lost virtually all of its activity overnight at 3°C; in the presence of suitable protecting mercaptans, this loss was reduced by about one-half. Purified CR was stored as a pellet under liquid-saturated ammonium sulfate at −60°C; the frozen enzyme was used within a few weeks of its preparation.

Characterization of CR. Characterization of the reaction. Analysis of the standard enzyme assay mixture containing [14C]-canaline revealed that homoserine was the sole radiolabeled reaction product. Separate analysis established ammonia as another reaction product of canaline reduction. Isolation and identification of these reaction products were conducted as described. This reaction appears to be irreversible as reaction of CR with L-homoserine and such nitrogen donors as ammonia, glutamine, asparagine, or hydroxylamine failed to produce any detectable canaline. The limit of canaline detectability was 10 nmol.

NADPH served as a highly effective reductant for the conversion of canaline to homoserine. Spectral analysis of CR showed that the reduced coenzyme could bind to the enzyme without canaline being present (Fig. 1); NADPH was present in the blank. NADH did not function as an effective agent for canaline reduction. ATP/Mg2+ did not enhance the formation of homoserine from canaline.

Mass. The molecular mass of the CR subunit was obtained by SDS/polyacrylamide gel electrophoresis with proteins of known mass used as the standard. This procedure yielded a single band with an apparent molecular mass of 82 ± 3 kDa.

The molecular mass of jack bean CR was determined to be 170 ± 5 kDa by polyacrylamide gel electrophoresis under nondenaturing conditions. Molecular mass evaluation by Sephadex G-200 chromatography yielded a value of 167 ± 6 kDa. These data suggest that jack bean CR exists as a dimer composed of two 82-kDa units.

Automated amino acid analysis. Automated amino acid analysis of CR disclosed a high proportion of aspartate/asparagine and glutamate/glutamine in the protein. Aromatic residues were limited but the proportion of hydrophobic residues was high. There were 12 cysteine residues (Table 2). Ellman titration of the native protein disclosed 8 sulphydryl groups.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity, units</th>
<th>Protein, mg</th>
<th>Specific activity, units/mg</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>3000</td>
<td>460</td>
<td>6.5</td>
<td>—</td>
</tr>
<tr>
<td>Ammonium sulfate (0–55% saturation)</td>
<td>2875</td>
<td>135</td>
<td>21.3</td>
<td>96</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>860</td>
<td>30</td>
<td>28.7</td>
<td>29</td>
</tr>
<tr>
<td>Sephadex G-200 chromatography</td>
<td>290</td>
<td>6.8</td>
<td>42.6</td>
<td>10</td>
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<tr>
<td>Affinity chromatography</td>
<td>165</td>
<td>0.27</td>
<td>611.1</td>
<td>3</td>
</tr>
</tbody>
</table>

Experimental details are provided in the text.
groups; this value increased to no more than 10 when CR was treated with 6 M guanidine.

Kinetic parameters. Kinetic analysis of CR indicated that the apparent $K_m$ values for L-canaline and NADPH were 0.76 (Fig. 2) and 0.16 mM, respectively. The highest specific activity CR gave a turnover number of $1.224 \times 10^4$ µmol per min per µmol of CR.

Inhibitor studies. Kinetic analysis of CR revealed that the $K_i$ values for the competitive inhibitors aminoxyacetic acid and hydroxylamine were 4.9 and 7.9 mM, respectively (Fig. 3).

Sulfhydryl inactivation. Treatment of CR with N-ethylmaleimide established the sensitivity of the enzyme to this sulfhydryl-group inhibitor. Exposure of CR to 1 mM N-ethylmaleimide for 20 min inactivated the enzyme (Fig. 4). Even exposure to 1 µM inhibitor severely affected CR activity (Fig. 4).

DISCUSSION

CR, purified from the leaves of C. ensiformis, mediates an NADPH-dependent, stoichiometric reduction of L-canaline to L-homoserine and ammonia. CR has a molecular mass of about 167 kDa and is composed of dimers with a mass of 82 kDa. It is a labile enzyme that rapidly loses its catalytic activity if not protected by mercaptans.

Table 2. Amino acid composition of C. ensiformis CR

<table>
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<tr>
<th>Amino acid</th>
<th>Mol %</th>
<th>Residues per mol</th>
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<tbody>
<tr>
<td>Asx</td>
<td>10.42</td>
<td>149</td>
</tr>
<tr>
<td>Thr</td>
<td>4.32</td>
<td>70</td>
</tr>
<tr>
<td>Ser</td>
<td>4.20</td>
<td>79</td>
</tr>
<tr>
<td>Glx</td>
<td>16.40</td>
<td>209</td>
</tr>
<tr>
<td>Pro</td>
<td>4.68</td>
<td>79</td>
</tr>
<tr>
<td>Gly</td>
<td>3.10</td>
<td>89</td>
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<tr>
<td>Ala</td>
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<tr>
<td>Cys</td>
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<td>12</td>
</tr>
<tr>
<td>Val</td>
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<td>105</td>
</tr>
<tr>
<td>Met</td>
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<tr>
<td>Ile</td>
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</tr>
<tr>
<td>Leu</td>
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</tr>
<tr>
<td>Tyr</td>
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<tr>
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</tr>
<tr>
<td>Lys</td>
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</tr>
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</tr>
<tr>
<td>Arg</td>
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</tr>
<tr>
<td>Trp</td>
<td>2.16</td>
<td>19</td>
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</table>

Total, 1467 residues; mass, 163,875 Da. Experimental details are provided in the text.

CR is a unique enzyme in two ways. First, it is the only enzyme able to metabolize canaline, which cannot function with ornithine. Previous reports of enzymes capable of metabolizing canaline were limited strictly to proteins for which canaline served as a substrate only by virtue of its structural analogy to ornithine. For example, ornithine carbamoyltransferase (EC 2.1.3.3) not only carbamylates ornithine to citrulline but also carbamylates canaline to form O-ureido-L-homoserine (22, 23). In contrast, CR metabolizes canaline but in vitro experiments with CR and ornithine reveal no demonstrable catabolism of ornithine. Thus, this enzyme is not reactive with ornithine. Second, CR is the only presently known enzyme able to use NADPH to reductively cleave an O—N bond.

Canaline reductase has a moderately high affinity for canaline, as judged by the apparent $K_m$ value for canaline of 0.76 mM. This substrate affinity would facilitate catabolism of low levels of canaline and may contribute significantly to the paucity of canaline in canavanine-storing plants (5). Many leguminous plants store massive amounts of canavanine and they also possess appreciable arginase activity (2). Canaline

Fig. 1. Absorbance spectra of CR. The absorbance spectrum of CR (•) and enzyme reacted with 30 µM NADPH (■) were determined as described in the text.

Fig. 2. The course of L-canaline reduction by CR. (Inset) Kinetic analysis of CR. Reduction of canaline and determination of the apparent $K_m$ value were conducted as described in the text. Velocity is in nmol/min.

Fig. 3. Competitive inhibitor kinetic analyses of CR. The $K_i$ values for hydroxylamine (■) and aminoxyacetic acid (●) were determined from the slope value obtained from a series of plots of 1/v vs. 1/[I] at the indicated inhibitor concentration. See text for additional experimental details.

Fig. 4. Enzyme activity in CR (a) and its inactivated with N-ethylmaleimide (b). See text for details.
must form in these plants. CR provides a highly effective means of detoxifying this potent antimetabolite.

Higher plants need to conserve nitrogen because this element is often a rate-limiting nutrient for growth (22). CR supports the vital nitrogen metabolism of legumes such as jack bean in two important ways. First, it provides a means to release the nitrogen stored in the aminoxy moiety of canaline as ammonia. Thus, plants that can convert canavanine to canaline and then canaline to homoserine gain all three of the nitrogens of the guanidinooxy group of canavanine (directly as well as through urea hydrolysis). Second, homoserine is a vital precursor in the sulfur metabolism of legumes (23). Higher plants phosphorylate homoserine to phosphohomoserine; the latter functions in the biosynthesis of such critically important sulfur-containing metabolites as cystathionine, homocysteine, and methionine (23). CR not only functions in canaline detoxification, thereby contributing to the low natural abundance of canaline, but it also serves as an important bridge between canavanine and homoserine. This is a particularly important function because CR provides a means for a metabolite such as canavanine, which is primarily part of the organism’s secondary metabolism, to support the primary metabolic reactions of canavanine-containing legumes.

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