Enzymic Conversion of Agmatine to Putrescine in *Lathyrus sativus* seedlings

**PURIFICATION AND PROPERTIES OF A MULTIFUNCTIONAL ENZYME (PUTRESCINE SYNTHASE)**

(Received for publication, November 21, 1980)

Kalkunte S. Srivenugopal† and P. Radhakantha Adiga

From the Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India

The participation of a multifunctional enzyme (a single polypeptide with multiple catalytic activities) has been demonstrated in the conversion of agmatine to putrescine in *Lathyrus sativus* seedlings. This enzyme (putrescine synthase) has inherent activities of agmatine iminohydrolase, putrescine transcarbamylase, ornithine transcarbamylase, and carbamate kinase. The enzyme has been purified to homogeneity and has a specific activity of 55,000.

In the presence of inorganic phosphate, the enzyme catalyzed the stoichiometric conversion of agmatine and ornithine to putrescine and citrulline, respectively. The different activities associated with the enzyme co-purified with near constancy in their specific activity. The enzyme catalyzed phosphorolysis and arsenolysis of *N*-carbamyl putrescine. The multifunctionality of putrescine synthase was also supported by 1) activity staining, 2) intact transfer of the ureido group from labeled *N*-carbamyl putrescine to ornithine to form citrulline, and 3) the affinity of the enzyme toward structurally and functionally related affinity matrices.

An agmatine cycle is proposed wherein *N*-carbamyl putrescine arising from the agmatine iminohydrolase reaction is converted to putrescine and citrulline, with the ureido group of *N*-carbamyl putrescine being transferred intact to ornithine. Preliminary results indicate that this series of reactions is also present in other plants.

### EXPERIMENTAL PROCEDURES

#### RESULTS

In terms of subcellular distribution, most of putrescine synthase was associated with the cytosolic fraction and hence, the postmitochondrial supernatant was routinely used for enzyme purification (for details, see Miniprint). The different reactions catalyzed by the enzyme are represented in Table 1.

**Co-purification of the Different Activities Associated with Putrescine Synthase**

The purification of putrescine synthase was monitored by assaying the different component activities, as well as the side reactions.

---

* The abbreviations and trivial names used are: putrescine synthase, the multifunctional enzyme with associated activities of agmatine iminohydrolase (agmatine deiminase, EC 3.5.3.12), putrescine transcarbamylase (carbamoyl phosphate: putrescine carbamoyltransferase, EC 2.1.3.12), ornithine transcarbamylase (carbamoyl phosphate: ornithine carbamoyltransferase, EC 2.1.3.3), and carbamate kinase (ATP:carbamate phosphotransferase, EC 2.7.2.2). The terms multifunctional, polycephalic, and chimeric enzyme used in the text represent a single polypeptide with several catalytic activities (14). CH-Sepharose, carboxyhexyl Sepharose; NBT, nitrobluetetrazolium chloride; PMS, phenazine methosulfate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; CNT, cyanogen bromide.

---

**Footnotes:**

1 Portions of this paper (including "Experimental Procedures," part of "Results," Tables II, VII, and VIII, and Figs. 1, 3, and 10) are presented in miniprint at the end of this paper. Figs. 4 and 6 and Tables IV and X appear in the text in miniprint as prepared by the author. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 950 Rockville Pike, Bethesda, MD 20814. Request Document No. 80M-2469, cite authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations and trivial names used are: putrescine synthase, the multifunctional enzyme with associated activities of agmatine iminohydrolase (agmatine deiminase, EC 3.5.3.12), putrescine transcarbamylase (carbamoyl phosphate: putrescine carbamoyltransferase, EC 2.1.3.12), ornithine transcarbamylase (carbamoyl phosphate: ornithine carbamoyltransferase, EC 2.1.3.3), and carbamate kinase (ATP:carbamate phosphotransferase, EC 2.7.2.2). The terms multifunctional, polycephalic, and chimeric enzyme used in the text represent a single polypeptide with several catalytic activities (14). CH-Sepharose, carboxyhexyl Sepharose; NBT, nitrobluetetrazolium chloride; PMS, phenazine methosulfate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; CNT, cyanogen bromide.

---

Biosynthesis of putrescine, the obligatory precursor of spermidine and spermine involves different steps in different biological systems. In contrast to microorganisms and animals, in higher plants the principal source of putrescine is arginine (1, 2), although detection of ornithine decarboxylase has been reported in some plants (3-6). Earlier, Smith (7-9) and Smith and Garray (10) proposed that in higher plants agmatine is converted to putrescine in two discrete steps, with *N*-carbamyl putrescine as the intermediate. It has been suggested that *N*-carbamyl putrescine is probably an enzyme-bound intermediate and is rapidly degraded to putrescine (2, 11).

Earlier, arginine decarboxylase from *Lathyrus sativus* was purified to homogeneity (12). During the purification of agmatine iminohydrolase, we found that, only when the assay mixture contained inorganic phosphate, significant amounts of putrescine (besides *N*-carbamyl putrescine) were produced. On the basis of the obligatory involvement of inorganic phosphate in the *N*-carbamyl putrescine → putrescine conversion, it was surmised that the *N*-carbamyl-putrescine amidohydrolase (producing putrescine, CO₂, and NH₃) might in fact represent phosphorolytic cleavage of this intermediate by a putrescine transcarbamylase-mediated reaction (functioning in the reverse direction) on lines suggested in *Streptococcus faecalis* growing on agmatine as the sole carbon source (13). Further experiments have revealed that the purified enzyme is in fact a versatile multifunctional enzyme (for reviews on this class of enzyme, see Refs. 14-17) with agmatine iminohydrolase, putrescine transcarbamylase, ornithine transcarbamylase, and carbamate kinase activities associated with a single polypeptide chain and that the metabolic conversion of agmatine to putrescine is apparently linked to citrulline production in *L. sativus.*
activity ratios remaining more or less constant throughout the Table or N-carbamyl putrescine.

ble difference in terms of ornithine transcarbamylase activity purification steps. However, the enzyme exhibited considera-
conceivably be due to more than one ornithine transcar-
bamylase activity in the plant cell-free extracts (45, 46) and the association of only one of them with putrescine synthase. Additional activities associated with the purified enzyme are 1) that related to the synthesis of N-carbamyl putrescine owing to the inherent putrescine transcarbamylase activity, which had a specific activity of 33 units; 2) carbamoyl kinase activity, assayed in the direction of ATP synthesis, with a specific activity of 2.5 units. Generation of ATP from agmatine + ADP + Pi, and N-carbamyl putrescine + ADP + Pi, combina-
tions (i.e. the overall reactions linked to carbamoyl kinase) could also be demonstrated. However, these component activities of putrescine synthase were not quantitated during the purification.

TABLE I
Constituent activities of putrescine synthase

Reactions
1) Agmatine + H₂O → N-carbamyl putrescine + NH₃
2) N-Carbamyl putrescine + Pₐ → putrescine + carbamyl phosphate
3) Carbamyl phosphate + ornithine → citrulline + Pᵢ
Overall reaction
4) Agmatine + ornithine + H₂O + Pᵢ → putrescine + citrulline + NH₄ + Pᵢ
Additional reaction
5) Carbamyl phosphate + ADP + H₂O → ATP + CO₂ + NH₃

Enzymes
1) agmatine iminohydrolase
2) putrescine transcarbamylase
3) ornithine transcarbamylase
4) putrescine synthase
5) carbamoyl kinase

Physicochemical Properties

Purity of the Isolated Enzyme—The final preparation obtained by both purification procedures, i.e. the organomercurial-Sepharose step followed by fractionation on DEAE-Sephadex (procedure I) and the putrescine-CH-Sepharose affinity step (procedure II), showed a single sharp band on polyacrylamide gel electrophoresis at pH 8.3 (Fig. 2, a and b) and at pH 4.0 (Fig. 2c). The fast moving band in basic gel systems was coincident with the different enzyme activities associated with the protein as evidenced by activity scanning after gel

FIG. 2. Polyacrylamide gel electrophoresis of putrescine synthase under nondenaturing conditions. The staining pattern of the purified enzyme at pH 8.3 by procedure I (a); procedure II (b); protein from procedure II at pH 4.0 (c); and protein from procedure II (d) (purified in the presence of 1 mm phenylmethylsulfonyl fluoride) at pH 8.3. Protein used in a and b, 50 µg and in c and d, 100 µg, O, origin.

TABLE III
Co-purification of the different catalytic activities of putrescine synthase and comparison of their specific activities

The putrescine-CH-Sepharose affinity step (procedure II) was employed for purification. Enzyme activity has been expressed in total enzyme units and the numbers within parentheses refer to the specific activity (µmol of product/mg of protein/h). The specific activity of carbamoyl kinase in the final preparation was 2.5 µmol of ATP formed/ mg of protein/h.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Agmatine iminohydrolase</th>
<th>Putrescine transcarbamylase (N-carbamyl putrescine arsenosynthesis)</th>
<th>Ornithine transcarbamylase</th>
<th>Agmatine + ornithine → citrulline</th>
<th>N-Carbamyl putrescine + ornithine → citrulline</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>4680</td>
<td>28 (0.01)</td>
<td>449 (0.10)</td>
<td>1220 (0.26)</td>
<td>267 (0.06)</td>
<td>330 (0.07)</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂ treatment</td>
<td>3450</td>
<td>27.6 (0.01)</td>
<td>414 (0.12)</td>
<td>1000 (0.29)</td>
<td>214 (0.06)</td>
<td>270 (0.08)</td>
<td>1.3</td>
<td>91</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>1050</td>
<td>21 (0.02)</td>
<td>409 (0.39)</td>
<td>950 (0.9)</td>
<td>180 (0.17)</td>
<td>231 (0.22)</td>
<td>3.6</td>
<td>75</td>
</tr>
<tr>
<td>Putrescine-CH-Sepharose affinity step</td>
<td>5</td>
<td>7 (1.40)</td>
<td>110 (21)</td>
<td>140 (28)</td>
<td>59.5 (11.9)</td>
<td>70 (14.2)</td>
<td>230</td>
<td>25</td>
</tr>
</tbody>
</table>

* Calculated for agmatine iminohydrolase.

* Represents the ratio of relative specific activities with respect to agmatine iminohydrolase.
Multifunctional Enzyme in Putrescine Biosynthesis in Plants

Electrophoresis (Fig. 4). No additional component could be detected even when 100 μg of enzyme were applied to the acrylamide gels and run under both acidic and basic conditions. Furthermore, the antiserum raised against the purified enzyme gave a single precipitin line on immunodiffusion analysis with the homogeneous preparation (not shown).

Electrophoresis on Denaturing Gels—Polyacrylamide gel electrophoresis of the enzyme in the presence of 0.1% SDS, 5% mercaptoethanol (40) reproducibly showed a single protein species (Fig. 5a) with an estimated $M_r$ of 55,000 (Fig. 6).

Denaturation of the protein under more severe conditions, namely employing 8 M guanidine hydrochloride followed by treatment with urea, SDS, and mercaptoethanol prior to electrophoresis (41), also revealed the presence of a single protein band on SDS gels (Fig. 5b). These results are consist-

![Fig. 4. Distribution of enzyme activity in polyacrylamide gel electrophoresis of putrescine synthase. After electrophoresis (6.5 h), the gels were stained with Coomassie brilliant blue.](image)

**Fig. 4.** Distribution of enzyme activities in polyacrylamide gel electrophoresis of putrescine synthase. After electrophoresis (6.5 h), the gels were stained with Coomassie brilliant blue. The activities were monitored by staining the gels and extracting at 4°C with TCA in imidazole buffer. The enzyme activities were determined in the supernatant after removing the gel pieces by centrifugation. A-Sase, P-Tase, and O-Case stand for agmatine iminohydrolase, putrescine transcarbamoylase and ornithine transcarbamoylase respectively.

**Fig. 5.** Sodium dodecyl sulfate-disc gel electrophoretic pattern of putrescine synthase. a, purified enzyme (30 μg) electrophoresed for 2 h and b, the enzyme protein (100 μg) subjected to guanidine hydrochloride, sodium dodecyl sulfate, and urea treatment and electrophoresed for 4 h. The protein treated with 8 M guanidine hydrochloride and 2-mercaptoethanol was alkylated with iodoacetate (41). The sample was then dialyzed against 8 M urea, 0.1% sodium dodecyl sulfate in Tris-Cl (pH 8.0) and subjected to gel electrophoresis. O, origin.

**Fig. 6.** Standard curve for molecular weight determination by SDS gel electrophoresis. For molecular weight of marker proteins used: chicken an-giotensinogen A (23,000), chicken a-2-mercaptoethanol (34,000), ovalbumin (45,000), BSA monomer (68,000) and dimer (136,000).

**Fig. 7.** Activity staining to demonstrate ATP synthesis in the carbamate kinase-linked overall reaction catalyzed by putrescine synthase. a, agmatine + ADP + P; b, N-carbamyl putrescine + ADP + P; c, purified putrescine synthase stained with Coomassie brilliant blue. Polyacrylamide gel electrophoresis of putrescine synthase was carried out in the cold (4°C) at pH 8.3. Following the equilibration of the gels in 50 mM imidazole-Cl (pH 7.5) buffer, they were transferred to a solution (2.5 ml) containing 1 mM dithiothreitol, 5 mM agmatine, or N-carbamyl putrescine, 3 mM ADP, 5 mM NaHPO$_4$, 20 mM glucose, 5 mM MgSO$_4$, 0.5 mM NADP$^+$, 0.5 mM KCI, 150 units of hexokinase, 100 units of glucose-6-phosphate dehydrogenase, 0.5 mg of phenazine methosulfate, 1.5 mg of nitroblue tetrazolium chloride and were dissolved in 50 mM imidazole-Cl buffer and incubated at 37°C for 1 h along with corresponding blank gels (i.e. minus substrate). The gels were stored in 7% acetic acid after development of the formazan band. O, origin.
Multifunctional Enzyme in Putrescine Biosynthesis in Plants

ent with the view that the four different enzyme activities assayed reside in a single polypeptide, thereby substantiating the multifunctional nature of putrescine synthase.

Activity Staining—ATP formation in situ was detected by coupling with hexokinase + glucose-6-phosphate dehydrogenase + NADP and the NADPH thus generated was in turn reacted with neotetrazolium chloride and phenazine methosulfate to give rise to formazan. A blue formazan band corresponding to the protein stain was observed on gels when incubated with either agmatine + ADP + P; or N-carbamyl putrescine + ADP + P. (Fig. 7, a, b, and c). This finding further strengthens the polypehelic nature of putrescine synthase, since it demonstrates that agmatine iminohydrolase, putrescine transcarbamylase, and carbamate kinase activities (Table I) (generating ATP as one of the products) reside in a single protein species. The formazan band was not detected in the incubation mixtures from which the substrates were omitted (not shown).

Molecular Weight—The purified enzyme migrated with an apparent $M_r \approx 56,000$ on Sephadex G-200 (data not shown). The $M_r$ determined by gel filtration is in close agreement with that from SDS-gel electrophoretic analysis (Fig. 6). Furthermore, it is significant that all of the component activities of putrescine synthase were recovered as a single protein peak when eluted from the molecular sieve (Fig. 8), thus providing additional evidence for the chimeric nature of the enzyme.

Stoichiometry of the Reactions

Among the various partial reactions catalyzed by putrescine synthase, the following were shown to occur stoichiometrically.

1) Agmatine Iminohydrolase Reaction—The new assay developed for agmatine iminohydrolase earlier by us (20) facilitated the quantification of N-carbamyl putrescine as the reaction product. Parallel estimation of ammonia was also carried out for comparison. As the purification progressed, the relative amount of NH$_3$ produced decreased proportionately, finally reaching approximately 1:1 stoichiometry of both the products.

2) Overall Reaction I (Agmatine + Ornithine + H$_2$O + P; $\rightarrow$ Citrulline + Putrescine + NH$_3$ + P) — The stoichiometric conversion of metabolites in the above overall reaction could be easily demonstrated (Table IV). Paper chromatographic analysis of the amine fraction isolated from the above reaction mixture revealed the presence of residual agmatine and the other product, putrescine. Omission of inorganic phosphate led to accumulation of significant amounts of N-carbamyl putrescine as revealed by chromatographic analysis.

3) Overall Reaction II (Agmatine $\rightarrow$ N-Carbamyl Putrescine + ATP + 2NH$_3$ + CO$_2$): In the carbamate kinase-linked reaction, ATP synthesis was stoichiometrically coupled to the amount of agmatine degraded. Fig. 9 shows the formation of ATP in the reaction mixture containing agmatine (or N-carbamyl putrescine) + ADP + P. Since the commercial ADP sample (Sigma) was contaminated with ATP to a small extent, a small increase in absorbance at 340 nm was observed with ADP alone. The ATP accumulated in the incubation mixture resulted in a steep increase in A$_{260}$ nm in the coupled assay used to measure ATP (Fig. 9). These observations were confirmed with the more sensitive luciferase procedure (27).

Evidence for the Intact Transfer of the Carbamyl Group from N-Carbamyl Putrescine to Ornithine and Requirements for the Overall Reaction

Availability of synthetic [ureido-'$^{14}$C]N-carbamyl putrescine permitted demonstration of the transfer of the carbamyl group of carbamyl phosphate to ornithine to form citrulline during the overall enzyme reaction (Table V). No liberation of $^{14}$CO$_2$ could be detected when the above reaction was performed in a closed Warburg flask. Furthermore, both Mg$^+$ and P, are required for this reaction. The requirement for these components in the enzymatic production of citrulline was also evidenced.

TABLE IV

<table>
<thead>
<tr>
<th>Agmatine utilized (pmol)</th>
<th>Products (pmol) in the reaction mixture</th>
<th>Putrescine</th>
<th>NH$_3$ released</th>
<th>Citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I (0.20)</td>
<td>0.16</td>
<td>0.18</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Experiment II (0.25)</td>
<td>0.21</td>
<td>0.23</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 8 (left).** Gel filtration of homogeneous putrescine synthase on Sephadex G-200. Gel filtration of the purified enzyme was carried out on a Sephadex G-200 column (1.8 x 89 cm), using 50 mM Tris-Cl buffer (pH 8.0) containing 0.1 M KCl. Fractions of 3 ml were collected and the constituent activities associated with putrescine synthase were assayed as described under "Experimental Procedures." AHHase, agmatine iminohydrolase; PTCase, putrescine transcarbamylase; CKase, carbamate kinase.

**Fig. 9 (right).** Generation of ATP in the carbamate kinase-linked overall reaction catalyzed by putrescine synthase. The assays were carried out under standard conditions ("Experimental Procedures") using the purified enzyme, with agmatine or N-carbamyl putrescine (NCP) as substrates. The reaction was terminated with 0.1 ml of 10% perchloric acid followed by neutralization of the solution with 3 M KOH. ATP in an aliquot of the supernatant was quantitated using the hexokinase-glucose-6-phosphate dehydrogenase coupled assay procedure (26).
**Stability of the Enzyme**

The purified enzyme was highly unstable even in the presence of glycerol, dithiothreitol, and MgCl₂; it lost all the component activities within 48 h after purification when stored at 4 °C. Prolonged dialysis and freeze-thawing also led to a considerable loss of activity. The carbamate kinase activity was the most labile, since dialysis of the crude extract for more than 10 h rendered this activity unstable. However, with the purified preparations, (NH₄)₂SO₄ at 1 M concentration preferentially stabilized the carbamate kinase activity. This observation is in agreement with that recorded with the purified carbamate kinase from *S. faecalis* in which case the SO₄²⁻ ion was shown to be responsible for the stabilization (50). It was consistently found that all of the component activities associated with putrescine synthase were stabilized in dilute solutions (<50 µg of protein/ml) for about 3–4 h at 37 °C by bovine serum albumin at a 250 µg/ml concentration.

**Interaction of Putrescine Synthase with Different Affinity Matrices: Additional Evidence for its Polypehcal Nature**

Further evidence for the multifunctionality of putrescine synthase was obtained by affinity chromatography. During the preliminary studies on putrescine biosynthesis in *L. sativus*, the specific affinity of agmatine iminohydrolase toward homoaarginine- and citrulline-Sepharose was repeatedly observed. Furthermore, in view of the presence of a nucleotide (ADP) binding site ascribable to the inherent carbamate kinase activity, the affinity of this chimeric enzyme to blue Sepharose was expected. These observations were exploited and the (NH₄)₂SO₄ fraction (step 3) was adsorbed onto homoaarginine-, citrulline-, organomercurial-, and blue Sepharose affinity columns (10 × 1 cm). This was followed by extensive washing with 50 mM imidazole-Cl buffer (pH 8) to remove unadsorbed proteins. The proteins held with high affinity were eluted with their respective ligands in imidazole buffer (i.e., 4 mM homoaarginine or citrulline in cases of the first two affinity columns, 5 mM ATP in the case of blue Sepharose and 20 mM 2-mercaptoethanol in the case of organomercurial affinity matrix). The eluates were dialyzed against 20 mM imidazole buffer thoroughly to remove the respective soluble ligands. This ligand-specific elution resulted in the recovery of all the component activities inherent in putrescine synthase, with an identical elution profile. The specific interaction of this chimeric protein toward the affinity adsorbents possessing structural analogies to homoaarginine and citrulline, particularly with regard to guanido function and hydrocarbon backbone.

The ureido group of N-carbamyl putrescine, a transient intermediate in the overall reaction, and ornithine transcarbamylase activity inherent in the protein are probably responsible for the affinity of putrescine synthase to citrulline-Sepharose. It is significant to note that the dialyzed eluates from all affinity matrices exhibited ratios of specific activity of the different component activities, similar to that corresponding to the purified enzyme, while still reflecting the different degrees of enzyme purity achieved by these affinity procedures (Table IX).

Polyacrylamide gel electrophoresis of the above four eluates revealed the presence among others of a fast moving protein band corresponding in position on gels to the purified putrescine synthase (not shown). Evidence for the presence of putrescine synthase in the eluates from the four affinity matrices was also obtained immunologically. The antiserum raised...
Multifunctional Enzyme in Putrescine Biosynthesis in Plants

Specific activities of the component reactions of putrescine synthase in the eluates from different affinity Sepharoses

The enzyme activities were assayed under the standard conditions described under "Experimental Procedures," using the dialyzed enzyme eluates from different affinity Sepharoses. The values represent the specific activities of constituent reactions of putrescine synthase.

<table>
<thead>
<tr>
<th>Affinity Sepharose</th>
<th>Agmatine iminohydrolase</th>
<th>Putrescine transcarbamylase (N-carbamyl putrescine aminolyisis)</th>
<th>Ornithine transcarbamylase</th>
<th>Putrescine transcarbamylase (N-carbamyl putrescine synthesis)</th>
<th>Agmatine + ornithine → citrulline</th>
<th>N-Carbamyl putrescine + ornithine → citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline-Sepharose</td>
<td>1.20</td>
<td>19</td>
<td>23.5</td>
<td>39</td>
<td>9.6</td>
<td>11.4</td>
</tr>
<tr>
<td>Homoarginine-Sepharose</td>
<td>0.80</td>
<td>11.2</td>
<td>15.2</td>
<td>24</td>
<td>6.4</td>
<td>7.3</td>
</tr>
<tr>
<td>Blue Sepharose</td>
<td>0.42</td>
<td>5.46</td>
<td>7.8</td>
<td>12.6</td>
<td>3.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Organomercurial Sepharose</td>
<td>0.54</td>
<td>8.0</td>
<td>10.8</td>
<td>17.3</td>
<td>4.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Putrescine-CH-Sepharose (pure enzyme)</td>
<td>1.40</td>
<td>21</td>
<td>28</td>
<td>47</td>
<td>11.3</td>
<td>14</td>
</tr>
</tbody>
</table>

- Represents the specific activity ratio of various activities relative to agmatine iminohydrolase.
- Procedure II (Table III).

Table IX

Specific activity

μmol product formed/mg protein/h

against putrescine synthase cross-reacted with the protein fractions eluted from the different affinity matrices, exhibiting a single precipitin line in each case (not shown).

Evidence for the Association of Putrescine Biogenesis with Citrulline Production in Other Higher Plants

The data described above show that the conversion of agmatine to putrescine in L. sativus is interlinked with the production of citrulline and is mediated by a multifunctional enzyme. The question then arose whether this series of reactions also occurs in other plants. For this purpose, the overall enzymatic reaction catalyzing the ammatine → putrescine conversion, namely agmatine (or N-carbamyl putrescine) + ornithine + P, → citrulline + putrescine was assayed in the dialyzed crude extracts of seedlings of different plants. The results obtained clearly indicate the occurrence of similar reactions in other plants examined (Table X).

Discussion

The most significant feature of the present study is the evidence for a novel multifunctional enzyme involved in agmatine to putrescine conversion in L. sativus and its functional significance. Two highly reproducible purification procedures, one involving an organomercurial affinity step followed by DEAE-Sephadex chromatography and the other employing affinity chromatography on putrescine-CH Sepharose, were used to purify the enzyme to a homogeneous state. In contrast to a two-step hydrolytic scheme proposed earlier for the conversion of agmatine to putrescine, involving agmatine iminohydrolase and N-carbamyl putrescine-aminohydrolase in plants (9,10), the present study demonstrates that putrescine transcarbamylase rather than N-carbamyl putrescine-aminohydrolase catalyzes putrescine production. The most likely explanation for this discrepancy is that a crude extract prepared in phosphate buffer was incubated for long periods in the earlier studies (9); consequently, putrescine transcarbamylase acting in the reverse direction might have degraded N-carbamyl putrescine into putrescine and carbamyl phosphate, the latter undergoing further hydrolysis to CO₂ and NH₃ (52). The detection of putrescine transcarbamylase activity in the pea (49) and the phospholytic cleavage of N-carbamyl putrescine in S. faecalis (13) are consistent with our findings.

Several lines of evidence prove the multifunctionality of putrescine synthase from the plant system: (a) the purified enzyme exhibits a single protein band on SDS-polyacrylamide gel electrophoresis, with M₅, 55,000; (b) co-purification of the different constituent activities with near constancy of specific activity ratios; (c) the stoichiometric conversion of the metabolites in the overall reactions catalyzed by the enzyme; (d) all the constituent activities band in a single region corresponding to the protein stain on polyacrylamide gel electrophoresis and co-elute as a single protein peak from Sephadex G-200; (e) activity staining (Fig. 7) for ATP synthesis is a result of interaction between the constituent activities; (f) the intact transfer of the ureido group from N-carbamyl putrescine to ornithine as a result of interaction between the two transcarbamylase components of the enzyme (Table V); (g) the affinity toward different functionally and structurally related affinity matrices and recovery of all of the activities during elution (with similar ratios of specific activities as found with the pure enzyme + immunological and electrophoretic evidence for the enzyme protein in these eluates.

One of the basic features of the reaction catalyzed by putrescine synthase is the intact transfer of the carbamyl moiety of N-carbamyl putrescine to ornithine, due to coupled activities of the two transcarbamylase components of the enzyme. In the absence of ornithine, the putrescine transcarbamylase component acting in the reverse direction can conceivably catalyze the phosphohytic cleavage of N-carbamyl putrescine to putrescine and carbamyl phosphate, provided carbamyl phosphate is depleted from the catalytic site to drive
the reaction in favor of putrescine synthesis. That the higher plant efficiently uses ornithine transcarbamylase activity for this purpose to channel carbamyl phosphate generated in situ to citrulline production is clearly evident from the data presented. Of relevance in this context is that, in *S. foecalis*, ornithine transcarbamylase activity is intrinsic to the purified putrescine transcarbamylase (48). Thus, it would appear that an "agmatine cycle" (Fig. 11) functions in the higher plants; one of the essential tenets of this cycle is the intact transfer of reactions comprising the agmatine cycle are functional in other the higher plants. Preliminary data indicate that these reactions comprising the agmatine cycle are functional in other plants also. The above series of reactions is similar to those of the arginine dihydrolase pathway in microorganisms although three separate enzymes catalyze the latter reactions (53).

The higher catalytic efficiency (approximately 10 times) of putrescine synthase in the overall reaction compared to agmatine iminohydrolase assayed in isolation (Table III) is in line with the cooperativity in multienzyme systems (15). The requirement of Mg²⁺ in the ornithine transcarbamylase-linked overall reaction is also consistent with its known stabilizing influence on carbamyl phosphate (54). It is intriguing that the putrescine synthase with $M_r = 55,000$ harbors four functionally discrete reaction domains on its single chain polypeptide backbone. This is not surprising since several such multienzyme proteins with similar molecular size were reported earlier (14). While a subunit structure for the purified putrescine synthase can be ruled out, the possibility of an oligomeric assembly at pH 8.3 (Fig. 2 d) *vía-* *vía* the protein purified in the absence of phenylmethylsulfonyl fluoride (Fig. 2, a, b, and c). The relatively larger size of the enzyme prepared in the presence of the proteolytic inhibitor was also evident during gel filtration on Sephadex G-200 since the activity was eluted in the void volume itself.

Thus, it is clear that the putrescine synthase of *L. sativus* is another example of a highly organized chimeric protein, catalyzing a set of sequential reactions (14). The coexistence of both the coupling activities (carbamate kinase and ornithine transcarbamylase) in putrescine synthase is not easily understood at present. Both of these activities catalyze reversible reactions, with their $K_m$ favoring useful anabolic reactions and thus are ideally suited for coupling mechanisms, which in turn enhance the catalytic efficiency in a multistep reaction. The transfer of the carbamyl group in the series of reactions leading to citrulline synthesis (Table V) is one of the few examples of its kind; the other two instances are (a) generation of citrulline from carbamyl oxamate and ornithine in *Streptococcus allantoicus* cell-free extracts (56) and (b) synthesis of arginine from carbamyl aspartate and ornithine in the crude extracts of wheat seedlings (57).

REFERENCES

30. Ramakrishna, S., and Adiga, P. R. (1973) *J. Chromatogr.* 68, 214-218
zymol. 34, 544–547
ENZYMATIC CONVERSION OF ARGININE TO PUTRESCINE IN ANIMAL MUSCLE SEEDING: PURIFICATION AND PROPERTIES OF A MULTIFUNCTIONAL ENZYME (PUTRESCINE SYNTHESE)

I.K. Silvennoinen and P.E. Albersheim

EXPERIMENTAL PROCEDURE

Materials - [Details not specified]

The preparation of crude extracts and the purification of the enzyme were carried out essentially as described previously (13, 22). The concentration of protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard. The activity of the enzyme was measured by the method of Huber et al. (23), and the amount of ornithine was estimated by the colorimetric method of Rouser et al. (24).

Detection of Putrescine Synthase - The enzyme was detected as described by Huber et al. (23). The reaction mixture contained 50 mM Tris-Cl buffer (pH 7.5), 1.5 mM MgCl2, 50 mM KCl, 150 mM NaCl, and 50 mM Na2HPO4. The enzyme was incubated at 30°C for 30 minutes, and the absorbance was measured at 570 nm.

RESULTS

Purification of putrescine synthase

The enzyme was purified by gel filtration on Sephadex G-200 (25). The enzyme was then applied to a column of CM-Sepharose (26) and eluted with a linear gradient of 0-500 mM NaCl. The enzyme activity was assayed by the method of Huber et al. (23).

Fig. 1. CM-Sepharose chromatography of putrescine synthase.

The protein was eluted from the column with a 0-500 mM NaCl gradient. The enzyme activity was assayed by the method of Huber et al. (23).
Multifunctional Enzyme in Putrescine Biosynthesis in Plants

Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein Total Solubility</th>
<th>Fold Recovery</th>
<th>Enzyme purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Aelr extract</td>
<td>960</td>
<td>56.4</td>
<td>0.0027</td>
</tr>
<tr>
<td>2. NaCl precipitation</td>
<td>699, 69.9</td>
<td>0.31</td>
<td>1.4</td>
</tr>
<tr>
<td>3. IMAC-Sepharose in equilibration</td>
<td>560</td>
<td>0.08</td>
<td>4.2</td>
</tr>
<tr>
<td>4. IMAC-Sepharose in affinity</td>
<td>75</td>
<td>2.52</td>
<td>50.0</td>
</tr>
<tr>
<td>5. Gdn-Sepharose chromatography</td>
<td>7.7</td>
<td>0.1</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Purification of multifunctional enzyme by affinity chromatography using putrescine-Im-Sepharose (Procedure I)

A specific putrescine-Im-Sepharose affinity step was later developed for the rapid purification of the chimeric enzyme with good recovery of all associated activities, in line of the more laborious ion exchange affinity step plus Gdn-Sepharose chromatography (Procedure I, step 5). The dialyzed protein fraction (step 2) was subjected to affinity chromatography on the putrescine-Im-Sepharose column. It is clear from Fig. 3 that the putrescine-Im-Sepharose selectively eluted the protein as a single peak without losing any activity. The pool was pooled, dialyzed and concentrated protein fraction of this step shown on polyacrylamide gel electrophoresis. A single protein species identified with and inseparable from the one obtained by the procedure 1. Therefore, in further studies, this affinity procedure was exclusively utilized for enzyme purification.

Initial velocity studies on synthetic analogues - The enzyme exhibited a typical Michaelis-Menten kinetics, when the velocity of the reaction (mM) was plotted against the concentration of agmatine. A higher activity was observed for the 

\[
\text{Initial velocity } \left( V_{\text{max}} \right) \text{ for agmatine in the direction of putrescine synthesis and is in agreement with the results of other studies.}
\]

The kinetics of the putrescine transcarbamylase reaction followed by the Michaelis-Menten equation for the determination of the Lineweaver-Burk plot, is given as:

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \frac{1}{[S]}
\]

where:
- \( v \) is the initial velocity
- \( V_{\text{max}} \) is the maximum velocity
- \( K_m \) is the Michaelis constant
- \([S]\) is the substrate concentration

Table II

<table>
<thead>
<tr>
<th>Compounds tested (mm)</th>
<th>Relative activity to agmatine</th>
<th>Compounds tested (mm)</th>
<th>Relative activity to agmatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfuryl blockers</td>
<td></td>
<td>Sulfuryl blockers</td>
<td></td>
</tr>
<tr>
<td>NaCN</td>
<td>1.5</td>
<td>NaCN</td>
<td>1.5</td>
</tr>
<tr>
<td>NaSO₃</td>
<td>1.0</td>
<td>NaSO₃</td>
<td>1.0</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.5</td>
<td>NaNO₃</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2</td>
<td>NaCl</td>
<td>0.2</td>
</tr>
<tr>
<td>NaF</td>
<td>0.1</td>
<td>NaF</td>
<td>0.1</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.1</td>
<td>Na₂SO₄</td>
<td>0.1</td>
</tr>
<tr>
<td>( p \end{pmatrix} \text{carbamoyl phosphate} )</td>
<td>1.0</td>
<td>( p \end{pmatrix} \text{carbamoyl phosphate} )</td>
<td>1.0</td>
</tr>
<tr>
<td>2-mercaptopropanol</td>
<td>0.2</td>
<td>2-mercaptopropanol</td>
<td>0.2</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0.2</td>
<td>2-mercaptoethanol</td>
<td>0.2</td>
</tr>
<tr>
<td>GSH</td>
<td>0.2</td>
<td>GSH</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Initial velocity studies on synthetic analogues - The enzyme exhibited a typical Michaelis-Menten kinetics, when the velocity of the reaction (14Cp) was plotted against the concentration of agmatine. A higher activity was observed for the higher concentrations of agmatine. The initial velocity studies were performed with substrates containing different concentrations of agmatine. The reaction was followed by the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stopped with the addition of substrate to the enzyme at substrate concentrations ranging from 0.1 to 10 mM. The reaction was stoppe