Serinol Phosphate as an Intermediate in Serinol Formation in Sugarcane

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ABSTRACT

A novel compound, serinol phosphate, was identified in sugarcane (Saccharum officinarum) clone 51NG97. It was produced by an enzyme-mediated transamination of dihydroxyacetone phosphate with either alanine, glutamate, aspartate, or glutamine serving equally well as an amino donor. Some detectable phosphatase activity was present in crude leaf enzyme preparation that hydrolyzed serinol phosphate. A proposal for a pathway of the biosynthesis of serinol in sugarcane was formulated.

Serinol can serve as an "activator" of toxin production in attenuated cultures of the sugarcane pathogen Helminthosporium sacchari and it is present in susceptible clone 51NG97. Resistant clone H50-7209 does not possess serinol and likewise dihydroxyacetone phosphate transaminase activity could be demonstrated in enzyme preparations of this clone. The concept of toxin activation in attenuated fungus cultures is briefly discussed relative to disease resistance and susceptibility.

Materials and Methods

Plants. Sugarcane (Saccharum officinarum) clones 51NG97, susceptible to H. sacchari, and H50-7209, resistant to this fungus, were obtained from R. Coleman, USDA, Beltsville, Md. The stalks were grown in large plastic pots at 22 ± 5°C under greenhouse conditions. Leaves used in this study were taken from the upper parts of the stalk after they had matured.

Enzyme Preparation. Crude enzyme preparations were obtained from leaves that had been deribbed, cut into pieces (2 × 2 mm) with scissors, placed in 0.2 m-K-phosphate buffer (4 g of leaf material/15 ml) (pH 8), 1 mM EDTA, 1% PVP, and ground in a Sorvall homogenizer at top speed for 1 min. The crude leaf juice was centrifuged at 3,000g for 10 min. A 2-ml aliquot was then transferred to a column of Sephadex G-25 (1.5 × 6 cm) in a plastic syringe whose bottom support end was equipped with a small mesh nylon net. The whole syringe was centrifuged at 1,000g for 1 min and the fluid that passed through the column was taken as the crude enzyme preparation (1.8 mg of protein/ml). Each step in the procedure was performed at 0 to 4°C in precooled vessels. This technique effectively removed many of the harmful phenols and quinones from the preparation.

Substrates. The method of Bublitz and Kennedy (3) was used for the preparation of 14C-dihydroxyacetone phosphate after several modifications. The reaction mixture contained 42.2 μmol of dihydroxyacetone and 5 units of glycerol kinase, (EC 1.7.1.30) which had been desalted immediately before use. At 7 min intervals on four separate occasions, 10 μmol of ATP (Na salt) and 10 μmol of MgCl2 were added. The final preparation of 14C-dihydroxyacetone phosphate possessed a specific radioactivity of 0.78 μCi/μmol. Serinol was prepared by the method of Szammer (17) with the following modifications. The tritylation and final removal of trityl groups were done according to Amiard et al. (2). The reduction was done in tetrahydrofuran that was dried with LiAlH4. The produce was purified as described by Pinkerton and Strobel (13). The over-all yield of the reaction was 30%.

Enzyme Assay. Dihydroxyacetone phosphate transaminase activity was measured in 0.22 ml of 0.2 m-K-phosphate (pH 8), which contained 1% FVP, 1 mM EDTA, 360 μg of protein, 10 mM alanine, 1 mM pyridoxal phosphate, and 4.5 mM 14C-dihydroxyacetone phosphate (0.01 μCi) at 25°C unless otherwise indicated. At the termination of the experiment, 0.2 ml of ethanol (−18°C) was added, and the solution centrifuged at 1,000g for 5 min. The supernatant liquid was applied to Whatman No. 3 paper and developed in solvent system (a).

Chromatography. Descending chromatography was performed on Whatman No. 3MM and 541 papers. The 541 paper was washed with H2O and dried at room temperature prior to being used. The latter paper was specifically used in preparing 14C-dihydroxyacetone phosphate and serinol phosphate. The following solvent systems were used: (a): 1-butanol-acetic acid-

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2 Supported by a grant from the Deutsche Forschungsgemeinschaft. Present address: Fachbereich Biologie, Universität Regensburg, West Germany.

3 Supported by a grant from the Max Kade Foundation. Present address: Biologisches Institut II der Universität Freiburg, West Germany.

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H₂O, 4:1.5, v/v; (b) 1-pentanol-88% formic acid-H₂O, 48.8:48.8:2.4, v/v; (c) ethyl acetate-88% formic acid-H₂O, 55:25:15, v/v; and (d) 2-propanol-H₂O-HCl, 68:18:4.16, v/v. TLC was performed on precoated silica gel plates (Merck AG, Darmstadt, West Germany). The solvent systems used were: (e) 1-butanol-acetic acid-H₂O, 4:1:1, v/v; and (f) 1-propanol-H₂O 7.3, v/v. The plates were heated at 110 C for 30 min prior to use. Amino compounds were detected with alcohoholic ninhydrin and phosphate esters with moylebdate (ammonium)-perchloric acid (7).

Radioactivity measurements were cut from the chromatograms and placed in 5 ml of scintillation solution containing 6 g of 2,5-diphenyloxazole and 75 mg of 1,4-bis[2-(5-phenyloxazole)-benzene/l of toluene. Measurements were obtained from a Packard liquid scintillation spectrometer model 3360. The channels ratio method was used to correct for quenching. The counting efficiency was approximately 70% for [14C]. Radioautograms of thin layer plates after two-dimensional chromatography were made using Kodak no screen x-ray film after a 2-week exposure.

High voltage paper electrophoresis was carried out in 0.1 M ammonium formate (pH 4) at 12.5 v/cm for 60 min on a Shandon model L24 apparatus. Water-washed Whatman No. 3 paper (22 x 56 cm) was used.

Inorganic phosphate was determined by the method of Lowry and Lopez (11). Protein was determined by the method of Lowry et al. (12) using BSA as a standard.

[U-14C]Dihydroxyacetone was purchased from ICN, and [3-14C]serine from New England Nuclear. [14C]Dihydroxyacetone phosphate was prepared fresh every month, and stored as its cyclohexylamine salt, dimethyl ketal-H₂O. Alkaline phosphatase was purchased from Boehringer Mannheim Corporation. The pyridoxal-phosphate (Schiff's base) of serinol was prepared by incubating equal molar amounts of the two at room temperature.

RESULTS

[14C]Dihydroxyacetone did not replace [14C]dihydroxyacetone phosphate as an amino acceptor in the standard enzyme reaction mixture (minus pyridoxal phosphate) (Fig. 1A). However, when [14C]dihydroxyacetone phosphate was used in the standard reaction mixture (minus pyridoxal phosphate), a new radioactive product appeared at the origin on the chromatogram (Fig. 1B).

This product was not present in the boiled enzyme control solution. This compound was eluted from the origin and chromatographed on Whatman No. 541 paper in solvent system (a). Subsequently, it was eluted with H₂O and treated with alkaline phosphatase for 5 hr and then chromatographed in solvent system (a); it yielded a product with the same mobility as authentic serinol (Fig. 1C). Furthermore, when a portion of the phosphatase reaction mixture was co-chromatographed with authentic serinol by two-dimensional TLC in solvent systems (e) and (f), followed by exposure to an X-ray film, the single ninhydrin-positive spot on the thin layer plate had the same size, shape, and location relative to the single spot on the developed X-ray film [Rₚ 0.15 solvent (a), Rₚ 0.16 solvent (f)].

Pyridoxal phosphate was included as part of the standard reaction mixture and the solution subsequently treated with alkaline phosphatase and chromatographed by two-dimensional TLC in solvents (e) and (f). A single UV fluorscencing spot appeared on the thin layer plate having the same chromatographic properties as the authentic pyridoxal phosphate derivative of serinol, Rₚ 0.15 and 0.3 in solvents (e) and (f), respectively. These results further support the idea that a transaminase activity is present in the enzyme preparation and that the phosphorylated product (serinol phosphate), when treated with phosphatase to release phosphate, forms a pyridoxal-phosphate complex with free serinol in the reaction medium.

In all paper chromatographic systems indicated in Table I, the purified enzyme reaction product, taken to be serinol phosphate, yielded a single ninhydrin-positive spot that was coincident with a positive molybdate-reacting spot. Table I also shows the Rₚ values of dihydroxyacetone phosphate. Electrophoresis of the serinol phosphate at pH 4 gave a single band 25 mm/hr toward the positive pole and it was 1.25 times as mobile as dihydroxyacetone phosphate.

Dihydroxyacetone, glyceraldehyde, glyceroldehyde phosphate, and iso-serinol conceivably could have originated in the enzyme reaction mixture, but all of these compounds proved to have a different migration behavior than the radioactive compound in solvent system (a) (Fig. 1B).

Ideally, the specific radioactivity of serinol phosphate formed in the enzyme reaction mixture should be the same as that of the dihydroxyacetone phosphate used as the starting material. With this assumption, equal molar amounts of both products should yield the same molar amounts of inorganic phosphate, that is if the putative serinol phosphate is a monophosphoric ester. Alkaline phosphatase treatment for 5 hr of 1 µmol of dihydroxyacetone phosphate yielded 0.93 µmol of inorganic phosphate. One µmol, determined by its specific radioactivity, of serinol phosphate (from the enzyme reaction mixture minus pyridoxal phosphate), when treated with alkaline phosphatase, yielded 0.90 µmol of inorganic phosphate and 0.70 µmol of recoverable serinol. No additional phosphate was released from the product either after prolonged treatment (22 hr) with alkaline phosphatase, or after acid hydrolysis. Collectively, the results were taken as compelling evidence that the product of the enzyme-mediated transamination of dihydroxyacetone phosphate was serinol phosphate. No attempts were made to ascertain the stereochemistry of the amino group on carbon No. 2 of serinol phosphate isolated from the transaminase reaction.
Transaminase Activity. Although the enzyme preparation used was crude leaf protein, it was nevertheless possible to demonstrate certain characteristics of the dihydroxyacetone phosphate transaminase activity. It is apparent from Table II that the reaction was the result of enzymic activity since neither boiled enzyme nor the reaction mixture minus the enzyme preparation resulted in significant amounts of serinol phosphate. Only a small increase (21%) in the product was noted when the reaction mixture was supplemented with pyridoxal phosphate, suggesting that this cofactor is already present in the preparation, probably bound to the transaminase (8, 15). There was some inhibition of activity when α-ketoacids were present in the reaction mixture.

The formation of serinol phosphate under standard assay conditions was proportional to the enzyme protein concentration up to 360 μg (Fig. 2). The amount of serinol phosphate found at pH 7.5 catalyzed by 360 μg of protein increased rapidly for 10 min, then leveled off (Fig. 2). The decrease in enzyme activity observed after 10 min was not due to substrate unavailability inasmuch as both substrates were still present in saturating amounts. An incubation period of 2 min was therefore used for all kinetic assays in order to insure the optimum production of serinol phosphate. The apparent Km values for alanine and dihydroxyacetone phosphate were obtained by Lineweaver and Burk plots and were 10⁻³ M and 10⁻⁴ M, respectively.

The optimum pH (7.5) for the formation of serinol phosphate was determined using K-phosphate and Na-borate buffer systems (Fig. 3). The pH optimum for many plant aminotransferases is slightly alkaline (6, 14, 18).

A number of other transaminase activities were also present in the crude enzyme preparation including: glutamate:oxaloacetate, alanine:α-ketoglutarate, and alanine:oxaloacetate. Transaminase activities were measured by complexing the corresponding α-ketoacid formed with dinitrophenylhydrazine and chromatographing the products in solvent (a). The amount of the dinitrophenylhydrazone salt formed was measured spectrophotometrically at 360 nm. No glutamate:pyruvate transaminase activity could be detected in the preparation.

Glutamate, aspartate, glutamine, and alanine all served as amino donors to dihydroxyacetone phosphate with similar efficiency. However, dihydroxyacetone could not replace dihydroxyacetone phosphate as the amino acceptor (Fig. 1). The stoichiometry of the products of the transamination reaction in a standard reaction mixture incubated for 2 min was 0.49 μmol of serinol phosphate produced/mg protein/min and 0.5 μmol of pyruvate. The pyruvate produced was determined using the lactate dehydrogenase assay system (4).

The transaminase activity of the enzyme preparation was stable for several hr; however, a fresh preparation was normally used for every experiment.

Transaminase in Resistant Cane. Enzyme assays were performed exactly as described under "Materials and Methods," but using resistant clone H50-7209 as the source of leaf material for the enzyme preparation. After either 10 or 30 min of incubation of the reaction mixture followed by chromatography

![Fig. 2. Influence of time and protein concentration on production of serinol phosphate via aminotransferase reaction. Assays were carried out under standard conditions except for variations in time and protein concentration, respectively, in the latter case the incubation time was 2 min. O—O: time dependence; ---: protein dependence.](image)

![Fig. 3. Effect of pH on the production of serinol phosphate in the aminotransferase reaction. Standard enzyme assay (2 min) was used. Enzyme preparations were dialyzed against 0.2 M K-phosphate (O—O) and 0.2 M sodium borate (O—O) buffers, respectively, at different pH values. Equal amounts of protein were used for each assay.](image)

Table II. Requirements and inhibitors of the aminotransferase reaction with DNH to produce serinol-P

<table>
<thead>
<tr>
<th>Condition</th>
<th>Serinol-P</th>
<th>Influence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2661</td>
<td></td>
</tr>
<tr>
<td>Without enzyme</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Enzyme, 5 min 100 C</td>
<td>126</td>
<td>+2</td>
</tr>
<tr>
<td>Pyridoxal phosphate, 0.1 mM</td>
<td>3011</td>
<td>+13</td>
</tr>
<tr>
<td>Pyridoxal phosphate, 1 mM</td>
<td>3212</td>
<td>+21</td>
</tr>
<tr>
<td>Pyruvate, 0.02 M</td>
<td>3248</td>
<td>-8</td>
</tr>
<tr>
<td>Oxaloacetate, 0.02 M</td>
<td>2137</td>
<td>-20</td>
</tr>
<tr>
<td>α-Ketoglutarate, 0.02 M</td>
<td>1947</td>
<td>-27</td>
</tr>
</tbody>
</table>

Transaminase reactions are known involving substrates other than α-ketoacids as the amino acceptor. These are exemplified by the following: a) glutamine + fructose-6-phosphate → glutamate + N-acetyl glucosamine-6-phosphate (10); b) aliphatic aldehydes + amino acids → monoamines and keto acids (18); c) pyruvate oxime + acetone → pyruvate + acetone oxime (19); and d) phenylacyl carbino + glutamate → α-

**DISCUSSION**
Serinol phosphate was shown to possess toxin activation properties in attenuated cultures of *H. sacchari*; however, it is conceivable that the fungus first cleaved the phosphate from serinol phosphate resulting in serinol which had previously been shown to be an activator (13). This point deserves further study.

There is no question about the fact that dihydroxyacetone phosphate transaminase activity could not be demonstrated in clone H50-7209. This supports the previous work of Pinkerton and Strobel (13) who were unable to demonstrate serinol in this cane clone that is resistant to eye-spot disease. Although resistance to the toxin in clone H50-7209 has been related to the absence of an active binding protein (16), it seems apparent from this study that other relationships may be involved in the eye-spot disease of cane. For instance, it is apparent that the pathogen is able to take chemical cues in order to regulate the amount of toxin that it can produce in culture. We could therefore predict that there are cane clones in the world that could be quite susceptible to *H. sacchari* by virtue of an active binding protein; however, such clones could conceivably escape infection by an attenuated isolate of *H. sacchari* if a toxin activator substance were not present. It seems possible that many pathogenic organisms could use key host metabolic intermediates as compounds to regulate aspects of their pathogenicity, such as toxin production.

LITERATURE CITED