Properties of Repressible Alkaline Phosphatase from Wild Type and a Wall-less Mutant of Neurospora crassa*

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SUMMARY

The repressible alkaline phosphatase of Neurospora crassa was purified from both the mycelium of a wild type strain and from the medium in which cultures of the slime mutant (which lacks the normal cell wall) had been grown. The enzyme preparations from the two sources had similar amino acid compositions, immunological properties, specific activities, thermal stabilities, and kinetic constants, but differed in a number of other properties. Both enzyme preparations contained carbohydrate, but the carbohydrate content of the enzyme isolated from slime medium was almost double that of the enzyme from wild type mycelium (24 and 14%, respectively). The molecular weight of the enzyme secreted by slime cells, estimated by gel filtration on Sephadex G-200 and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, was higher than that of the enzyme from wild type mycelium by an amount consistent with its increased carbohydrate content.

Electrophoresis at pH 4.7 and 9.5 indicated that the enzyme isolated from slime medium is more anionic than the enzyme from wild type mycelium. Chemical analysis revealed the presence of approximately 8 phosphate groups per enzyme molecule in the purified slime extracellular enzyme, whereas the wild type enzyme contained less than 0.5 phosphate molecule per enzyme molecule. The presence of phosphate in the slime extracellular enzyme, and the lack of significant amounts of phosphate in the wild type mycelial enzyme, was also demonstrated by determination of 32P associated with the enzymes isolated from the two sources following derepression in the presence of 32PO4·.

A significant portion of the repressible alkaline phosphatase produced by derepressed wild type N. crassa was found to be secreted into the growth medium. The electrophoretic mobility of the enzyme isolated from the wild type culture medium resembled that of the enzyme isolated from the slime culture medium rather than that of the enzyme isolated from wild type mycelium.

In Neurospora crassa a number of enzymes are derepressed by inorganic phosphate starvation or by growth on a limiting phosphorus source. These include an alkaline phosphatase (1), an acid phosphatase (2), a phosphate permease which has a high affinity for phosphate at high pH (3, 4), and one or more extracellular nucleases (5). The repressible alkaline phosphatase of N. crassa is a particularly attractive subject for the study of control of protein synthesis in a eukaryotic organism since its range of activity from full repression to full derepression is over 1000-fold (1, 4). In addition, it is easily and specifically assayed even in crude extracts containing other phosphatases (1), it is readily purified in good yield (6), and the purified enzyme has been characterized by physical and chemical methods (7).

Recent studies have concentrated on the isolation and characterization of possible structural gene mutants (8) and of regulatory mutants altered in the ability to repress or derepress (4, 8) the repressible alkaline phosphatase and the physiologically related enzymes mentioned above. Further progress in understanding such mutants requires knowledge of the physiological factors involved in derepression of the alkaline phosphatase and, ultimately, a knowledge of the actual mechanism of derepression. With this in mind we began a study of the kinetics of derepression in the slime mutant of N. crassa. This strain lacks the normal cell wall and grows as isolated multinucleate protoplasts rather than branching hyphae (9, 10). This fact makes it much easier to manipulate in kinetic studies than the wild type strain. It quickly became apparent that, whereas the wild type strain retains most of the enzyme in a cell-bound form, slime cultures secrete nearly all of their repressible alkaline phosphatase into the growth medium. Preliminary examination of the enzyme secreted by slime cells revealed a clear difference between it and the enzyme prepared from wild type mycelia.

In the present paper we describe the purification of the repressible alkaline phosphatase secreted into the growth medium by slime cultures and compare some of its physical and chemical properties with those of the enzyme purified from wild type mycelia. A number of distinct differences were found between the two purified enzyme preparations.

EXPERIMENTAL PROCEDURES

Chemicals—Agarose, γ-aminobutyric acid, barbital, 2-amino-2-methyl-1,3-propanediol, D-mannose, 5-bromo-4-chloro-3-indolyl phosphate, Coomassie brilliant blue, N-acetylmuramic acid (type IV), bovine serum albumin (Fraction V), alcohol dehydrogenase (yeast, crystalline), ribonuclease A (type I-A), bovine pancreas, ribonuclease T1 (Aspergillus oryzae) α-sialidase (N-acetylmuramylate glycohydrolase), type VI, Clostridium per...
fringens), alkaline phosphatase (type III, Escherichia coli), α-amylase (type I-A, hog pancreas), phosphorylase α (rabbit liver), and mucin (type I, bovine submaxillary gland) were purchased from Sigma Chemical Co., DEAE-Sephadex, and CM-Sephadex were obtained from Pharmacia Fine Chemicals. Reagents for acrylamide gel electrophoresis and AG 50W-X8 cation exchange resin were purchased from Bio-Rad; aniline blue-black from Canalco; p-nitrophenyl phosphate from Calbiochem; disodium EDTA from Fisher Chemical Co.; β-galactosidase and β-arabinosidase from Pfannstiehl Laboratories; sodium dodecyl sulfate from British Drug House Chemicals Ltd.; dextran sulfate from British Drug House Chemicals Ltd.; and carrier-free H₃PO₄ from New England Nuclear Co.

**Cultures and Growth**—The wild type strain of Neurospora crassa used in these studies was 74-OR8la (Fungal Genetics Stock Center No. 9072). Other strains included osmotrophs A, large cuticles E1120, FGSC No. 34), crisp-1, cr-1 (alleles B122, B135, FGSC No. 280), and the multiple mutant facesy, spontaneous germination, arginine-1, crisp-1, aurescent, osmosis-1 (jz, sp, arg-1, cr-1, aar, os-1) known as slime. The slime was obtained as a heterocaryon (FGSC No. 321) and the slime component of the heterocaryon was reversed by inoculating a medium consisting of 4.9 M sorbitol and 0.01 M ammonium hydroxide (to detector), 35 cc per min; oxygen (to detector), 300 cc per min; thermostat range, 10; electrometer attenuation, 16.

Samples containing approximately 1 mg of alkaline phosphatase in distilled water were prepared and hydrolyzed in 0.01 M HCl in the presence of Dowex 50-X8 as described by Luhrmann and Winzler (17). An internal standard was provided by the addition of 0.10 ml of 2.1 mM β-arabinose and the neutral sugar fraction was then isolated, reduced with NaBH₄, and acetylated as described above.

To calibrate the detector and calculate results, 50-μl aliquots of a mixture containing 8.06 mm β-arabinose, 8.27 mm d-mannose, and 8.22 mm β-galactose were subjected to the above procedure and chromatographed on the same day as the enzyme samples. Amino sugars were isolated from the same hydrolysates described above and were determined with the amino acid analyzer (18).

**Electrophoresis**—Disc electrophoresis was carried out using either the alkaline buffer system (pH 9.5) of Tamura and Ui (19), or an acidic buffer system similar to that described by Reisfeld et al. (20). The latter system was modified by raising the pH of the running gel, stacking gel, and electrode buffer 0.1 unit each (4.5 to 9.0) and substituting 0.005 M barbituric acid for 0.01 M barbital buffer, pH 8.5, which also contained 0.01 M EDTA. Staining was accomplished with washing with several changes of distilled water and the washed gels were stored in distilled water at 4°C.

Gels were stained for protein by immersing them for 45 min in a solution containing 0.05% (w/v) of both aniline blue-black and benzidine HCl. The mixture was then allowed to stand at 28°C for the duration of this study by daily transfers on Fries' medium for enzyme purification were grown in 3-liter Fernbach flasks containing 1.5 liters of medium. Large cultures were used in these studies was 74-OR8la (Fungal Genetics Stock Center No. 9072) and the slime component of the heterocaryon was reversed by inoculating a medium consisting of 4.9 M sorbitol and 0.01 M ammonium hydroxide (to detector), 35 cc per min; oxygen (to detector), 300 cc per min; thermostat range, 10; electrometer attenuation, 16.

**Amino Acid Composition**—Amino acid analyses were performed with the Beckman model 120 automatic amino acid analyzer using a single column procedure. Samples of purified alkaline phosphatase were dialyzed against four changes, 400 volumes each, of distilled water for 48 hours. Aliquots containing approximately 0.5 μg of protein (0.5 A₂₈₀) were dialyzed against 100 ml of 0.1 M KCl, 50 ml of 5% (v/v) mercaptooctanol, 0.25 M sucrose, and 0.1% (w/v) sodium dodecyl sulfate, and then heating 2 min in a boiling water bath. The rest of the electrophoretic procedure was exactly as described by Laemmli (21). The acrylamido concentration used was 10%.

**Estimation of Molecular Weight by SDS-Acrylamide Gel Electrophoresis**—SDS-acrylamide gel electrophoresis was carried out as described above. The desired N. crassa alkaline phosphatase was prepared in sample buffer and the denatured protein was subjected to electrophoresis along with marker peptides derived from phosphorylase a (mol wt = 97,400), bovine serum albumin (mol wt = 66,900), bovine serum albumin (mol wt = 66,900), and E. coli alkaline phosphatase (mol wt = 40,000). The gels were stained for protein as described earlier and the mobility of each band was calculated as suggested by Weber and Osborn (22).

**Estimation of Molecular Weight by Gel Filtration**—Analytical gel filtration of the purified alkaline phosphatases was carried out as suggested by Andrews (23) using a column (1.5 X 82 cm) of Sephadex G 200 equilibrated with 0.05 M Tris-HCl buffer, pH 8.3, containing 0.1 M NaCl. Elution was conducted with the same buffer. Samples were applied in a volume of 1.5 ml and fractions of 1.0 ml were collected at a flow rate of approximately 12 ml per hour. Bovine serum albumin (mol wt = 67,000), E. coli alkaline phosphatase (mol wt = 40,000), and yeast alcohol dehydrogenase (mol wt = 134,000) were mixed with the desired alkaline phosphatase sample prior to each run to provide internal standards for molecular weight estimation.

**Immunological Methods**—One milligram of purified wild type alkaline phosphatase was mixed with Freund's complete adjuvant and injected subcutaneously (subscapular region) into an adult rabbit. Injection of the same mixture was repeated three times at weekly intervals. Two weeks after the last injection the rabbit was bled and serum was prepared from the collected blood. The crude serum was fractionated with solid (NH₄)₂SO₄. The fraction precipitating between 20 and 50% saturation which contained all

*The abbreviation used is: SDS, sodium dodecyl sulfate.*
of the detectable antibody activity, was dissolved in one-half the original volume of 0.1 M potassium phosphate buffer, pH 7.2, and dialyzed against 20 volumes of the same buffer containing 0.1 M NaCl. The dialyzed fraction was stored frozen at −15°C until needed.

Double diffusion studies of enzyme and antibody in agar gels were carried out as suggested by Ouchterlony (24) using microscope slides (1 × 3 inches) covered with a 0.85% agarose gel prepared in a buffer containing 0.5 mM sodium borate (pH 8.3); 0.85% (w/v) NaCl, and 0.05% (w/v) sodium azide. Precipitin lines were allowed to develop for 72 hours at room temperature. Nonprecipitated protein was removed by washing with several changes of borate-saline (0.85% NaCl solution) buffer and the gels were then stained for protein by immersing for 1 min in a solution of 1% (w/v) aniline blue-black in 7.5% (v/v) acetic acid. Alternatively, the gels were stained for enzyme activity using a 0.05% (w/v) solution of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M barbitral buffer, pH 8.3.

Isolation of Alkaline Phosphatase from Cells Grown in Presence of [32P]Phosphate— Cultures of the wild type or slime strain of N. crassa were labeled with [32P] by growth for 36 hours in 100 ml of phosphate-free Fries' medium supplemented with 10 mM KH₂PO₄ (40 μCi). At the time of harvest more than 98% of the radioactivity was in the supernatant fluid and the centrifuged cells were washed twice with 500 ml of 0.2 M Tris buffer (pH 7.2) and were stored frozen at −15°C. The mycelium (246 g wet weight) was mixed with crushed Dry Ice and the mixture was ground to a fine powder (approximately 50% of the original volume). The homogenate was centrifuged 15 min at 5,000 × g and the spent medium, which contained more than 98% of the total alkaline phosphatase, was further clarified by filtering through a 1.2-μm Millipore filter and stored frozen at −15°C.

The dialyzed enzyme preparation was applied to a column (4.5 × 50 cm) of DEAE-Sephadex A-50 which had been equilibrated with the 0.01 M Tris buffer, pH 7.8, and dialyzed for 24 hours against 1 liter of 100 mM NaCl (adjusted to pH 7.8 with 1 N NaOH). This concentration of salt was sufficient to elute the enzyme from the column but not to wash out the ammonium sulfate (6) except that the second CM-Sephadex chromatography step was omitted.

Enzyme Purification—Tables I and II summarize the purification scheme for the repressible alkaline phosphatase from wild type mycelia and slime culture media, respectively. The enzyme from wild type mycelia is bound to CM-Sephadex at pH 8.3 but is not bound to DEAE-Sephadex at pH 7.8; the phosphatase from slime culture media behaves in the opposite manner on the two types of ion exchange media, indicating a more
TABLE I
Purification of repressible alkaline phosphatase from N. crassa 7A-OR3-1a mycelia
The starting material was 246 g (wet weight) of mycelium from 20 liters of low phosphate (0.25 mM KH₂PO₄) culture medium.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein*</th>
<th>Enzyme</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
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<tr>
<td>Crude extract</td>
<td>880</td>
<td>4,750</td>
<td>5,980</td>
<td>1.26</td>
<td>100%</td>
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<tr>
<td>60 to 96% saturation with (NH₄)₂SO₄</td>
<td>66</td>
<td>920</td>
<td>5,500</td>
<td>6.03</td>
<td>92%</td>
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<tr>
<td>Carboxymethyl-Sephadex eluate (concentrated)</td>
<td>5.5</td>
<td>68.6</td>
<td>3,070</td>
<td>57.3</td>
<td>51%</td>
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<tr>
<td>Sephadex G-200 eluate</td>
<td>19.5</td>
<td>39.4</td>
<td>2,420</td>
<td>61.3</td>
<td>40%</td>
</tr>
</tbody>
</table>

* Based on colorimetric determination (13).

TABLE II
Purification of extracellular repressible alkaline phosphatase from slime mutant of N. crassa
The starting material was enzyme released into 12.8 liters of medium.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein*</th>
<th>Enzyme</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
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<tr>
<td>Cell-free medium</td>
<td>12,400</td>
<td>1,550</td>
<td>4.410</td>
<td>16.4</td>
<td>(100)</td>
</tr>
<tr>
<td>Concentrated medium</td>
<td>393</td>
<td>86</td>
<td>1,410</td>
<td>16.4</td>
<td>91%</td>
</tr>
<tr>
<td>60 to 90% saturation with (NH₄)₂SO₄</td>
<td>11.7</td>
<td>70</td>
<td>1,300</td>
<td>16.5</td>
<td>84%</td>
</tr>
<tr>
<td>DEAE-Sephadex eluate</td>
<td>2.9</td>
<td>19.1</td>
<td>1,050</td>
<td>55.0</td>
<td>68%</td>
</tr>
<tr>
<td>Sephadex G-200 eluate</td>
<td>22</td>
<td>15.4</td>
<td>842</td>
<td>62.8</td>
<td>54%</td>
</tr>
</tbody>
</table>

* Based on colorimetric determination (13).

acidic nature for the enzyme from this source. The final specific activity of both enzyme preparations was essentially identical and was very similar to the value reported earlier by Kadner et al. (6).

Polyacrylamide Gel Electrophoresis—The results of gel electrophoresis of the purified phosphatase at pH 4.7 and 9.5 are shown in Fig. 1. A single, somewhat broad, protein band was observed for both enzyme preparations at both pH values. The slime enzyme migrated less rapidly toward the cathode for tubes A and B and toward the anode for tubes C and D. The gels were all stained for protein.

Fig. 2 (center). SDS-polyacrylamide gel electrophoresis of purified wild type and slime repressible alkaline phosphatases. Electrophoresis was carried out as described under "Experimental Procedures." The sample for tube A contained 3 µg of wild type enzyme. The sample for tube B contained 3 µg of slime enzyme. The gels were stained for protein.

Fig. 3 (right). Double diffusion analysis in agar gels of purified repressible alkaline phosphatases and antiserum prepared against the purified wild type enzyme. The center well contained 20 µl of ammonium sulfate fractionated antiserum. Peripheral wells marked A contained approximately 4 µg of wild type enzyme. Peripheral wells marked B contained approximately 4 µg of slime enzyme. Precipitin lines in I were stained for protein with aniline blue-black. Precipitin lines in D were stained to reveal enzyme activity with 5-bromo-4-chloro-3-indolyl phosphate.

was tested by double diffusion in agar gels against antiserum prepared against the wild type mycelial enzyme. A sharp, fully connecting precipitin line was observed on gels stained for either protein or enzyme activity (Fig. 3). In addition a second, much weaker, precipitin line for the slime enzyme was observed on gels stained for protein.

Amino Acid Composition—Amino acid analysis of the wild type and slime repressible alkaline phosphatases gave the results shown in Table III. The initial results are expressed on the basis of the amount of each amino acid corresponding to 1 A of enzyme at 280 nm as defined under "Experimental Procedures." These initial results have been used to calculate the number of residues of each amino acid per 136,000 g of protein. This value is the molecular weight of the protein moiety of the native wild type enzyme, as calculated from the data of Kadner et al. (6). The observed amino acid composition of the two enzyme preparations do not appear to differ significantly; the small differences for some of the amino acids are probably close to or within the experimental error.

Carbohydrate Content—Quantitative determination of the neutral and amino sugars present in the purified enzymes was carried out on samples hydrolyzed under mild conditions as described under "Experimental Procedures." The results are incorporated into the calculations for the composition of the two enzymes, and are shown in Table III. The values observed for the slime enzyme in this study are fairly similar to those reported by Kadner et al. (6), but the values observed for the slime en-

![Fig. 1](http://example.com/fig1.png)

![Fig. 2](http://example.com/fig2.png)

![Fig. 3](http://example.com/fig3.png)
zyme differ markedly, the content of mannose and galactose being much higher in the latter enzyme. From the data in Table III the carbohydrate content of the wild type and slime phosphatases are calculated to be 13.7 and 23.8%, respectively, of the total weight of the enzymes.

**Phosphate Content**—Samples of the purified enzyme preparations were hydrolyzed in 6 x HCl and the inorganic phosphate content of the hydrolysates was measured by the method of Ames and Dubin (27) as modified by Bloch and Schlesinger (28). This method indicated less than 1 phosphate group present per native enzyme molecule of the wild type phosphatase (Table IV), whereas the slime phosphatase was found to contain approximately 8 phosphate groups per enzyme molecule.

The occurrence of phosphate in the repressible alkaline phosphatase from cultures derepressed in the presence of $^{32}P_4$ was further examined by isolation of enzyme from cultures derepressed in the presence of $^{32}P_4$. The isolation procedure, described in detail under "Experimental Procedures," involved precipitation of enzyme from crude preparations with specific antiserum, solubilization of the antibody-enzyme complex in sodium dodecyl sulfate solution, and polyacrylamide gel electrophoresis in the presence of the detergent. Standards consisting of the respective purified alkaline phosphatases were carried through the same procedure. The results of staining one set of gels for protein are shown in Fig. 4. The distribution of $^{32}P$ on the gels, and a diagram of the position of the protein bands of the respective purified alkaline phosphatases on duplicate gels, are shown in Fig. 5. In agreement with the results of direct chemical analysis of the purified enzymes, the enzyme isolated from wild type mycelia has only very low levels of $^{32}P$ (<0.5 phosphate per enzyme molecule) associated with it, whereas the enzyme isolated from slime media coincides with a large peak of $^{32}P$ activity.

**Stalldase Treatment**—Multiple electrophoretic forms of glycoproteins have been shown in several instances to be caused by occurrence of different amounts of sialic acid in the several forms of the proteins (29-32). The possible occurrence of sialic acid

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**Table III**

<table>
<thead>
<tr>
<th>Amino acid or carbohydrate</th>
<th>Residues</th>
<th>Calculated residues per 136,000 g of protein</th>
<th>Nearest integer</th>
<th>Residues</th>
<th>Calculated residues per 136,000 g of protein</th>
<th>Nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic acid</td>
<td>0.0492</td>
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<td>9</td>
<td>0.055</td>
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<td>Aspartic acid</td>
<td>0.782</td>
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<td>146</td>
<td>0.830</td>
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<td>Threonine</td>
<td>0.583</td>
<td>103</td>
<td>103</td>
<td>0.583</td>
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<td>103</td>
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<tr>
<td>Serine</td>
<td>0.472</td>
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<td>83</td>
<td>0.462</td>
<td>81.3</td>
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<tr>
<td>Glutamic acid</td>
<td>0.559</td>
<td>98.4</td>
<td>98</td>
<td>0.554</td>
<td>97.5</td>
<td>98</td>
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<td>Proline</td>
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<td>0.415</td>
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<td>Glycine</td>
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<td>125</td>
<td>0.702</td>
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<tr>
<td>Alanine</td>
<td>0.577</td>
<td>102</td>
<td>102</td>
<td>0.574</td>
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<td>Valine</td>
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<td>0.401</td>
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<td>Methionine</td>
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<td>16</td>
<td>0.0885</td>
<td>15.6</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<td>95</td>
<td>0.556</td>
<td>97.9</td>
<td>98</td>
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<tr>
<td>Tyrosine</td>
<td>0.274</td>
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<td>48</td>
<td>0.270</td>
<td>47.6</td>
<td>48</td>
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<tr>
<td>Phenylalanine</td>
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<td>29</td>
<td>0.111</td>
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<td>Lysine</td>
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<td>86</td>
<td>0.493</td>
<td>82.5</td>
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<td>Arginine</td>
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<td>0.198</td>
<td>34.9</td>
<td>35</td>
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<td>Tryptophan</td>
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<td>19</td>
<td>0.108</td>
<td>19.0</td>
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<tr>
<td>Glucosamine</td>
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<td>18.1</td>
<td>18</td>
<td>0.108</td>
<td>19.0</td>
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<td>Mannose</td>
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<td>90.7</td>
<td>91</td>
<td>0.530</td>
<td>164</td>
<td>164</td>
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<tr>
<td>Galactose</td>
<td>0.133</td>
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<td>23</td>
<td>0.438</td>
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<tr>
<td>Glucose</td>
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<td>0.019</td>
<td>3.3</td>
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**Table IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amount*</th>
<th>Phosphate</th>
<th>Calculated amount of enzyme*</th>
<th>Ratio of phosphate to enzyme</th>
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<tr>
<td>Wild type</td>
<td>1.33</td>
<td>4.1</td>
<td>9.8</td>
<td>0.42</td>
</tr>
<tr>
<td>Slime</td>
<td>2.15</td>
<td>5.3</td>
<td>15.8</td>
<td>0.84</td>
</tr>
</tbody>
</table>

* Based on colorimetric determination (13).

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![Fig. 4](http://www.jbc.org/content/fig/4.jpg)
Fig. 5. Incorporation of $^{32}$P into repressible alkaline phosphatase synthesized during derepression. Enzyme from cultures of the wild type (x--x) and slime (o--o) strains of Neurospora crassa derepressed in medium containing 0.10 mM KH$_2$PO$_4$ (40 µCi) was isolated by precipitation with antiseraum and subjected to SDS-polyacrylamide gel electrophoresis as described under “Experimental Procedures.” Duplicates of Gels B and C shown in Fig. 4 were cut into 1.75-mm slices and the radioactivity in each slice was determined as described. A diagrammatic representation of the position of bands of the wild type mycelial enzyme (A) and the slime medium enzyme (B) on gels stained for protein (Fig. 4) is shown for comparison.

in the repressible alkaline phosphatases purified in this study was tested by incubating 15 µg of each purified phosphatase with 50 µg of sialidase (N-acetylneuraminic glycolhydrolase) for 24 hours at 30°C in 0.12 ml of 0.1 M potassium acetate buffer, pH 5.0 (33). This amount of sialidase released 22 µg of sialic acid from 500 µg of bovine submaxillary mucin in parallel incubations under the same conditions. The recovery of alkaline phosphatase activity was greater than 90% for both the wild type and slime enzymes at the end of the sialidase treatment. Disc gel electrophoresis of the treated alkaline phosphatases and of untreated controls was carried out at pH 4.7 and 9.5. The electrophoretic mobility of the treated samples was identical with that of the respective controls indicating the absence of terminal sialic acid residues in both forms of the enzyme.

Molecular Weight—The molecular weights of the wild type and slime enzymes were estimated by gel filtration, along with protein standards of known molecular weight, on a Sephadex G-200 column as described under “Experimental Procedures.” The peak elution volume of the slime alkaline phosphatase (Fig. 6) was less than that of the wild type enzyme, in agreement with the larger size expected of the former enzyme due to its increased carbohydrate content (Table III). The apparent molecular weights, estimated from the line formed by plotting elution volume against log molecular weight of the standards, were 165,000 for the wild type enzyme and 178,000 for the slime enzyme.

The native form of the N. crassa repressible alkaline phosphatase is a dimer (9). The molecular weight of the subunits was estimated by gel electrophoresis of the N. crassa alkaline phosphatases and standards of known molecular weight in the presence of sodium dodecyl sulfate as described under “Experimental Procedures.” The results are shown in Fig. 7. The molecular weights of the subunits, estimated from the standard curve, were 85,000 for the wild type enzyme and 90,000 for the slime enzyme.

Thermal Stability—The rate of loss of activity during heat denaturation of the two enzyme preparations was measured at three pH values. The enzymes were prepared in the indicated buffer by dialysis against a large excess of the buffer, followed by dilution to a concentration of 2 µg per ml in the same buffer. Aliquots of the enzyme solutions were heated at the indicated temperature for appropriate lengths of time, cooled rapidly, and surviving enzyme activity was determined in the standard enzyme assay. The half-life of each enzyme under the indicated conditions of temperature and pH were derived from the linear plots of incubation time against the logarithm of the surviving enzyme activity. As shown in Table V, the half-lives of the wild type and slime enzymes did not differ significantly under the conditions tested. It is interesting to note however that, whereas a precipitate formed in solutions of the wild type enzyme heated
alkaline phosphatase activity in slime culture was found almost a significant fraction of this enzyme appeared to be liberated into

0.05 summarized in Table VI. As already mentioned, the repressible media from each culture were carried out and the results are determinations on both the cell extracts and the cell-free culture in conical tubes. The dried material was dissolved by the ad-
tation was observed in similar solutions of the above the denaturation temperature, no coagulation or precipi-
tation was observed in similar solutions of the enzyme even after heating at 100° for several hours.

Kinetic Properties—$K_m$ values for $p$-nitrophenylphosphate, using the standard 0.3 M glycinate buffer (pH 9)-1 mM EDTA assay system, were calculated from Lineweaver-Burk plots (34). The values obtained were 1.72 $\times$ 10$^{-4}$ M for the wild type enzyme and 1.56 $\times$ 10$^{-4}$ M for the slime enzyme. The observed $K_i$ for phosphate under the same conditions was 1.31 $\times$ 10$^{-4}$ M for the wild type enzyme and 1.23 $\times$ 10$^{-4}$ M for the slime enzyme.

Incubation of Enzyme in Highly Concentrated Solution—The possibility that the phosphatase secreted by slime cells might be capable of a self-modification process in which neighboring en-
zyme molecules would liberate phosphate from each other was tested by prolonged incubation of highly concentrated enzyme solutions. Samples of enzyme, 670 µg each, were prepared by dialysis against distilled water and then dried by lyophilization in conical tubes. The dried material was dissolved by the ad-
dition of 5 µl of Fries’ salts (minus phosphate). The concen-
trated enzyme solutions were incubated 24 hours at 37°C in a closed container over moistened filter paper and then diluted to a concen-
tration of 2 mg per ml with 0.2 M Tris-HCl buffer, pH 7.4. Enzyme assays indicated a recovery of greater than 95% of the enzyme activity. Disc electrophoresis at pH 4.7 revealed no observable change in the electrophoretic mobility of either en-
zyme.

Distribution of Repressible Alkaline Phosphatase between Cells and Media of Wild Type and Several Mutant Strains of N. crassa—The repressible alkaline phosphatase of N. crassa has been considered to be primarily, if not exclusively, cell-bound (1, 2) rather than excreted into the media as is the repressible acid phos-
phatase of the same organism (2, 35). The finding that in cultures of the slime strain the repressible alkaline phosphatase is pri-
marily free in the medium, prompted us to do a quantitative examination of the distribution of this enzyme in cultures of wild type and of several mutant strains affected in cell wall synthesis (36). Cultures were grown for 24 hours in 100 ml of phosphate-
free Fries medium supplemented with either 0.05 mM KH$_2$PO$_4$ or 2 mM O-phosphorylethanolamine; this latter phosphorus source allows essentially maximal rates of growth of the organism, but at the same time gives quite a high degree of derepression of alkaline phosphatase (37). Cells were harvested and extracts were prepared by homogenization with alumina powder (12) in 0.05 M sodium acetate buffer, pH 5.0 (2). Alkaline phosphatase determinations on both the cell extracts and the cell-free culture media from each culture were carried out and the results are summarized in Table VI. As already mentioned, the repressible alkaline phosphatase activity in slime culture was found almost exclusively in the culture medium. However, even in wild type a significant fraction of this enzyme appeared to be liberated into

The values obtained were 1.72 $\times$ 10$^{-4}$ M for the wild type enzyme and 1.56 $\times$ 10$^{-4}$ M for the slime enzyme. The observed $K_i$ for phosphate under the same conditions was 1.31 $\times$ 10$^{-4}$ M for the wild type enzyme and 1.23 $\times$ 10$^{-4}$ M for the slime enzyme.

Dist. Phosphate source wet

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phosphate source</th>
<th>Wet weight</th>
<th>Alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>KH$_2$PO$_4$ (0.05)</td>
<td>0.4</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>PE (2)</td>
<td>1.6</td>
<td>7.13</td>
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<tr>
<td>Slime</td>
<td>KH$_2$PO$_4$ (0.05)</td>
<td>0.2</td>
<td>0.119</td>
</tr>
<tr>
<td></td>
<td>PE (2)</td>
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<td>0.103</td>
</tr>
<tr>
<td>os-1</td>
<td>KH$_2$PO$_4$ (0.05)</td>
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<td>1.51</td>
</tr>
<tr>
<td></td>
<td>PE (2)</td>
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<td>0.660</td>
</tr>
<tr>
<td>os, cr</td>
<td>KH$_2$PO$_4$ (0.05)</td>
<td>0.3</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>PE (2)</td>
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<td>3.56</td>
</tr>
<tr>
<td>os, cr, aur</td>
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</tr>
<tr>
<td></td>
<td>PE (2)</td>
<td>1.4</td>
<td>2.60</td>
</tr>
</tbody>
</table>

* PE, O-phosphorylethanolamine.
Fig. 8 (left). Polyacrylamide gel electrophoresis of the repressible alkaline phosphatase isolated from wild type culture medium. Repressible alkaline phosphatase present in the medium of a derepressed culture of wild type Neurospora crassa was isolated as described in the text and subjected to polyacrylamide gel electrophoresis at pH 9.5 (tube B). The results of electrophoresis of purified wild type mycelial enzyme (tube A) and purified slime medium enzyme (tube C) are shown for comparison. Bands of enzyme activity on the gels were located by staining with 5-bromo-4-chloro-3-indolyl phosphate.

Fig. 9 (right). Polyacrylamide gel electrophoresis of cell-bound repressible alkaline phosphatase from slime. Cells from derepressed slime cultures were harvested by centrifugation and washed once with fresh medium. Washed cells from 200 ml of medium were suspended in 5 ml of 0.05 M sodium acetate buffer, pH 5.0, and homogenized with alumina powder (12). The extract obtained after centrifugation at 27,000 × g for 15 min was fractionated with solid ammonium sulfate. The fraction precipitating between 0 to 65% saturation was dissolved in 1 ml of 0.01 M Tris-HCl, pH 7.4, and dialyzed overnight against the same buffer (100 ml). Samples containing approximately 0.02 enzyme unit were subjected to electrophoresis at pH 9.5, along with samples of purified wild type mycelial enzyme and purified slime medium enzyme. Following electrophoresis, enzyme activity was located on the gels by staining with 5-bromo-4-chloro-3-indolyl phosphate.

The samples used were (A) slime medium enzyme, (B) wild type mycelial enzyme, (C) slime cell-bound, 0 to 65% ammonium sulfate-precipitable enzyme.

DISCUSSION

The repressible alkaline phosphatase secreted by the slime mutant was purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis at pH 8.8 in the presence of sodium dodecyl sulfate, and at pH 4.7 and pH 9.5 in the absence of the detergent. The enzyme was also purified from the mycelium of a wild type strain by the procedure of Kadner et al. (6). The two purified enzyme preparations exhibited essentially identical specific activities (Tables I and II) based on protein determination by either the colorimetric procedure of Lowry et al. (13) or by absorbance at 280 nm. However, the specific activity of the phosphatase in crude concentrated medium from slime cultures was 13-fold higher than that in the crude extract of wild type mycelium. In fact, the results of the purification procedure indicate that the enzyme represents about 25% of the total protein present in the cell-free medium of derepressed slime cultures. This selective appearance of the enzyme in the culture medium clearly indicates active secretion by the slime cells rather than liberation by lysis of cells during starvation for phosphate.

The repressible alkaline phosphatases purified from the two sources were very similar in a number of properties. Amino acid analysis revealed no significant differences; the small differences for some of the amino acids (Table III) are in the range of experimental error. More detailed studies, e.g. peptide mapping, will be required, however, to rule out a possible difference of one or a few residues in the primary structure of the two forms of the enzyme. Further evidence of the similarity of the slime and wild type enzymes is provided by the following: (a) apparent identity in immunodiffusion studies (Fig. 3); (b) similar heat stability at three pH values (Table V); (c) nearly identical molecular weight (after correction for carbohydrate content) both in the native form (Fig. 5) and after dissociation into subunits (Fig. 6); and (d) similar affinities for the substrate, p-nitrophenylphosphate, and the inhibitory product, orthophosphate.

In contrast to the similarities noted above are the striking differences observed between the slime and wild type enzymes with respect to electrophoretic mobility, phosphate content, and carbohydrate content.

The repressible alkaline phosphatase of N. crassa was shown by Kadner et al. (6) to be a glycoprotein. In their study the carbohydrate content of the purified enzyme was found to be approximately 11.5% of the total weight. In the present study the carbohydrate content of the purified wild type enzyme was found to be approximately 13.7%. The relative amounts of glucosamine, mannose, and galactose found in the wild type enzyme are very similar to those found by Kadner et al. (6). The somewhat higher total amounts found in this study may be due to differences in the wild type strains used in the two studies. The slime enzyme purified in this study was found to contain a
much larger per cent of carbohydrate than the wild type enzyme, and the relative amounts of glucosamine, mannose, and galactose were also quite different. The glucosamine content of the slime enzyme was almost identical with that of the wild type enzyme, but the mannose content was approximately 80% higher and the galactose content was over 3-fold higher. No other carbohydrate, other than probably insignificant traces of glucose, was found in either enzyme preparation.

The electrophoretic mobility of a protein in polyacrylamide gel is determined by both the size and the net charge on the protein. The fact that the slime phosphatase is the faster species when migration is toward the anode (pH 9.5) and is the slower species when migration is toward the cathode (pH 4.7) indicates that it is separated from the wild type enzyme primarily due to a difference in charge. The behavior of the two enzyme preparations on DEAE- and CM-Sephadex during purification supports this conclusion. The possibility that the difference between the slime and wild type enzymes could be at least partially due to a difference in amino acid composition cannot be completely ruled out at this time. As pointed out above, amino acid analysis is not sensitive enough to show conclusively a difference of only one, or a few, residues in such a large molecule.

However, wild type N. crassa also secretes a significant amount of repressible alkaline phosphatase into the growth medium (Table VI), and the electrophoretic mobility of this secreted wild type enzyme more nearly resembles that of the secreted slime enzyme than that of the enzyme retained by wild type mycelia (Fig. 8). This secreted wild type enzyme has not been obtained in sufficient quantity to allow its purification and chemical analysis, but its similarity to the secreted slime phosphatase strongly suggests that the secreted wild type enzyme and the mycelial wild type enzyme differ in degree of post-translational modification rather than differing in their primary amino acid structure. There is precedent for such a phenomenon: the exo-1-T9 (39) mutants of N. crassa appear to be simultaneously affected in cell wall synthesis, and they synthesize glucoamylase with altered gel filtration (38) or isoelectric focusing (39) properties.

Sialic acid, which is known to account for the electrophoretic heterogeneity of a number of enzymes (29-32), is probably not present in either the wild type or slime repressible alkaline phosphatases. Treatment of the purified enzymes with a large excess of sialidase failed to alter their respective electrophoretic mobilities, a result which would have been expected if terminal sialic acid residues had been present.

The purified slime phosphatase was found to contain approximately 8 phosphate groups per enzyme molecule (Table IV) whereas the wild type enzyme contained less than 0.5 phosphate group per enzyme molecule. This difference in phosphate content would seem to provide a sufficient explanation for the electrophoretic difference between the two forms of the enzyme. Bloch and Schlesinger (28) have shown that the purified, native E. coli alkaline phosphatase contains 1.6 to 2.1 moles of tightly bound inorganic phosphate per mole of enzyme. This phosphate is removed by dialysis against nitrioltriacetic acid, a procedure which removes tightly bound zinc ions from the enzyme. The alkaline phosphatase isolated from slime cells labeled with 18OPO42- retained most of its bound 32P during an isolation procedure which included dialysis against phosphate buffer, heating at 100°C in phosphate buffer containing 0.1% sodium dodecyl sulfate, and SDS-polyacrylamide gel electrophoresis (Fig. 4). Retention of the radioactive phosphate under these conditions indicates that in this case it is probably present as covalently bound organic phosphate rather than tightly bound inorganic phosphate.

The subcellular localization of the repressible alkaline phosphatase of N. crassa has not been studied, although the enzyme has been considered to be primarily cell-bound rather than secreted into the medium (1, 2). The analogous enzyme in several bacteria is known to be located in the periplasmic space between the cell membrane and cell wall (40-43). The fact that the slime mutant, which lacks the normal cell wall, secretes approximately 95% of the repressible alkaline phosphatase into the medium strongly suggests that this enzyme also is normally located in the periplasmic space. A similar distribution of enzyme between cells and growth medium in slime cultures has recently been observed for invertase (10), an enzyme previously shown to be primarily located in a position external to the plasma membrane (44).

If the similar electrophoretic mobility of the phosphatase secreted into the medium by both wild type mycelia and slime cells (Fig. 8) is a reflection of similar structures, then the slime enzyme may represent a natural stage in maturation rather than an "abnormal" form resulting from the mutant phenotype. It is even possible that this is the form of the enzyme as it initially enters the periplasmic space. According to this hypothesis the bulk of this enzyme, which is retained in the periplasmic space, would then be further modified, perhaps by alkaline phosphatase itself, to produce the "wild type" form of the enzyme. The enzyme secreted by slime would escape this modification due to its rapid dilution into the surrounding medium. Incubation of the purified slime phosphatase at a concentration (approximately 50%, w/v) which might occur if all of the enzyme were localized in the periplasmic space did not result in "self-modification" as judged by lack of change in electrophoretic mobility. Further studies will be required to determine whether enzyme(s) capable of converting either the cell-bound or secreted form of the enzyme to the alternative form can be isolated from N. crassa.

A small amount of repressible alkaline phosphatase activity remains associated with the slime cells even after they have been washed with fresh medium. Much of this activity is indistinguishable from the enzyme found secreted into the medium. However, a significant proportion (15 to 75%, depending on the conditions) of this residual cell-bound enzyme clearly relates to that of the other repressible alkaline phosphatase properties (Fig. 9) from both the purified enzyme from slime medium and the purified enzyme from wild type mycelium. The structure of this less soluble, more basic form of the enzyme is clearly related to that of the other repressible alkaline phosphatase species since it is precipitated by antiseraum prepared against the purified wild type mycelial enzyme (Table VII). It seems possible that this form of the phosphatase may represent enzyme which has not yet been modified by the attachment of the carbohydrate residues.

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Earl G. Burton and Robert L. Metzenberg


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