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

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EARL G. BURTON AND ROBERT L. METZENBERG†
From the Department of Physiological Chemistry, School of Medicine, University of Wisconsin, Madison, Wisconsin 63706

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 phosphorus 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 a 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-acetylneuraminate acid (type IV), bovine serum albumin (Fraction V), alcohol dehydrogenase (yeast, crystalline), ribonuclease A (type I-A, bovine pancreas), ribonuclease T1 (Aspergillus oryzae) sialidase (N-acetylneuraminic glycohydrolase, type VI), Clostridium per

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fringes), alkaline phosphatase (type III, Escherichia coli), 
α-amylase (type I-A, hog pancreas), phosphorylase a (rabbit 
muscle), and mucon (type I, bovine submaxillary glands) 
were purchased from Sigma Chemical Co. Sephadex G-200, 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 Calcanol; p-nitrophenyl phosphate from 
Calbiochem; disodium EDTA from Fisher Chemical Co.; α-galact-
ose and β-arabinose from Pfannstielh Laboratories; sodium do-
decyl sulfate from British Drug House Chemical Co. Sephadex 
L, DEAE-Sephadex, and carrier-free H,PO from New England Nuclear 
Co.

Cultures and Growth—The wild type strain of Neurospora crassa 
used in these studies was 74-08-1a (Fungal Genetics Stock 
Center No. 90). Other strains included osmotolerant culture 
E1120, FGSC No. 34), crisp-1, aure-1 (alleles B122, B135, FGSC No. 280), 
and the multiple mutant fusc-1, spontaneous germination, argi-
nine-1, crisp-1, aure-1, osmotic-1 (izg, arg-1, cr-1, aur, os-1) 
known as slimes. The slimes were obtained as a heterocaryon (FGSC 
No. 527) and the slime component of the heterocaryon was re-
sulted from the ultraviolet absorption spectrum of purified enzyme in 
0.1

Assay—The repressible alkaline phosphatase was 
mixed with “sample buffer” consisting of 10 mM sodium phosphate buffer (pH 7.2), 0.14 M 2-mercaptoethanol, 0.25 

M NaCl. Elution was conducted with the same 

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 Reifesh et 
al. (20). The latter system was modified by raising the pH of the 
running gel, stacking gel, and electrode buffer 0.4 unit each 
either the alkaline buffer system (pH 9.5) of Tamura and Ui (19), 
or an acidic buffer system similar to that described by Reifesh et 
al. (20). The latter system was modified by raising the pH of the 
running gel, stacking gel, and electrode buffer 0.4 unit each 

Estimation of Molecular Weight by SDS-Acrylamide Gel Electro-
phoresis—SDS-acrylamide gel electrophoresis was carried out as 
described above. The desired N. crassa alkaline phosphatase was 
purified in sample buffer and the denatured protein was subjected 
to electrophoresis along with marker peptides derived from phos-
hate, bovine serum albumin (mol wt = 67,000), a-amylase (mol wt = 40,000), 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 
fractionation 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 

Molecular Immunological Methods—One milligram of purified wild type 
alkaline phosphatase was mixed with Freund’s complete adjuvant 
and injected subcutaneously (subcapsular region) into an adult 
rat. 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 volume of 0.01 M potassium phosphate buffer, pH 7.2, sterile distilled water, and dialyzed against 20 volumes of the same buffer containing 0.1 M NaCl. The dialyzed fraction was stored frozen at -15° until needed.

Double diffusion studies of enzyme and antibody in agar gels were carried out as suggested by Ouchterlony (24) using microscope slides (1 X 3 inches) covered with a 0.85% agarose gel prepared in a buffer containing 60 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 barbital buffer, pH 8.3.

Isolation of Alkaline Phosphatase from Cells Grown in Presence of [32P]Phosphate—Cultures of the wild type or slime strains of N. crassa were labeled with [32P] by growth for 36 hours in 100 ml of phosphate-free Fries' medium supplemented with 10 mM K3HPO4 (40 mCi). At the time of harvest more than 98% of the radioactivity was associated with the cell material. The cells were disrupted by the addition of 100 volumes of 0.2 M Tris buffer (pH 7.4) by grinding with alumina powder (12). The homogenate was centrifuged 10 min at 1000 X g. The supernatant fraction precipitating between 65 and 95% saturation was collected by centrifugation, dissolved in 4 ml of 0.05 M potassium phosphate buffer, pH 7.2, and dialyzed against 1 liter of the same buffer for 3 days at 4°.

The subsequent purification of the repressible alkaline phosphatase from the above extract was carried out exactly as described by Kudner et al. (6) except that the second CM-Sephadex chromatography step was omitted.

Purification of Repressible Alkaline Phosphatase from Slime Strain of N. crassa—Cultures of slime (800 ml per flask, 16 flasks) were grown for 18 hours (late log phase) in standard Fries' medium and were then harvested by centrifugation (5 min at 500 X g), washed once with phosphate-free Fries' medium (1/4 original culture volume), and then suspended in the original volume of fresh phosphate-free Fries' medium and incubated 6 hours at 30°. Cells were removed by centrifugation for 5 min at 600 X g and the spent medium, which contained more than 96% of the total repressible alkaline phosphatase, was further clarified by filtering through a 1.2-μm Millipore filter. The clarified supernatant was concentrated approximately 30-fold using a hollow fiber ultrafilter (Bio-Fiber 80 Bioek, Bio-Rad Laboratories, Richmond, Cal.). All further steps were carried out at 0°-4°.

The concentrated protein solution from the medium was brought to 60% saturation by the addition of 275 mg of solid (NH4)2SO4 per ml. The mixture was stirred 30 min and then centrifuged 20 min at 13,000 X g. The precipitate was discarded and the supernatant solution was brought to 95% saturation by the addition of 257 mg of solid (NH4)2SO4 per ml. The mixture was stirred 60 min and then centrifuged as above. The supernatant solution was discarded and the precipitate was dissolved in 10 ml of 0.01 M Tris buffer, pH 7.8, and dialyzed for 24 hours against 1 liter of the same buffer.

The dialyzed enzyme preparation was applied to a column (4.5 X 50 cm) of DEAE-Sephadex A-50 which had been equilibrated with the 0.01 M Tris buffer, pH 7.8, and the column was washed free of unbound protein with 800 ml of the same buffer. The phosphatase was then eluted by application of a linear gradient consisting of 2000 ml of 0.01 M Tris buffer, pH 7.8, in the mixing chamber and 2000 ml of the same buffer containing 0.25 M NaCl in the reservoir. Fractions (10 ml each) were collected and assayed for phosphatase activity. Fractions 83 to 125 (1590 to 2380 ml of the gradient) contained the bulk of the activity (81%); these were pooled and concentrated to 18 ml by use of a Diaflo ultrafilter equipped with a UM-10 filter and then further concentrated to 2.9 ml using a collodion bag ultrafilter (Schleicher and Schuell Co, Keene, N.H.). These fractions were grown for 18 hours in standard Fries' medium (25 g wet weight) and then harvested by centrifugation at 10,000 X g. The pellets were washed with 200 ml of distilled water as above and the supernatant solution was saved. The residue was extracted with 200 ml of ice-cold distilled water by brief homogenization in the Waring Blender. The homogenate was centrifuged 15 min at 5000 X g and the supernatant solution was saved. The residue was extracted with 200 ml of distilled water as above and the supernatant solution after centrifugation was combined with the supernatant solution after precipitation with 65% saturation was collected by centrifugation, dissolved in 4 ml of 0.05 M potassium phosphate buffer, pH 7.2, and dialyzed against 1 liter of the same buffer for 24 hours.

The repressible alkaline phosphatase was precipitated from the dialyzed solutions by the addition of 0.4 ml of partially purified antiserum. The tubes were incubated 45 min at room temperature, and then centrifuged 5 min at 1500 X g to sediment the antibody-enzyme complex. The clear supernatant solution was combined with the pellet, dissolved in 0.05 M potassium phosphate buffer, pH 7.2, and dialyzed against 1 liter of the same buffer for 10 min at 105,000 X g. The pellet was discarded. The concentrated supernatant solution was centrifuged 60 min at 105,000 X g. The pellet was discarded. The supernatant solution was fractionated by the addition of solid (NH4)2SO4. The enzyme solutions centrifuged overnight at 10,000 X g and 100 μg of deoxyribonuclease were added to each, and the preparations were incubated for 30 min at 37°. The enzyme solutions were then fractionated by the addition of solid (NH4)2SO4; the fraction precipitating between 65 and 95% saturation was collected by centrifugation, dissolved in 4 ml of 0.05 M potassium phosphate buffer, pH 7.2, and dialyzed against 1 liter of the same buffer for 24 hours.

Enzyme Purification—Tables I and II summarize the purification of the repressible alkaline phosphatase from wild type mycelia and slime cultures, respectively. The enzyme from the two sources had similar solubility properties in ammonium sulfate solutions and similar elution profiles on Sephadex G-200 columns, but differed in their behavior on ion exchangers. 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

\[ M \]

\[ M \]

\[ M \]

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\[ M \]
TABLE I
Purification of repressible alkaline phosphatase from N. crassa 74-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>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>880</td>
<td>4,750</td>
<td>5,980</td>
<td>1.26</td>
<td>(100)</td>
</tr>
<tr>
<td>60 to 96% saturation with</td>
<td>66</td>
<td>920</td>
<td>5,500</td>
<td>6.03</td>
<td>92</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxymethyl-Sephadex</td>
<td>5.5</td>
<td>59.6</td>
<td>3,070</td>
<td>57.3</td>
<td>51</td>
</tr>
<tr>
<td>eluate (concentrated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
<tbody>
<tr>
<td>Cell-free medium</td>
<td>12,400</td>
<td>1,550</td>
<td>1,410</td>
<td>16.4</td>
<td>(100)</td>
</tr>
<tr>
<td>Concentrated medium</td>
<td>303</td>
<td>86</td>
<td>1,300</td>
<td>16.5</td>
<td>91</td>
</tr>
<tr>
<td>60 to 96% saturation with</td>
<td>11.7</td>
<td>70</td>
<td>1,050</td>
<td>55.0</td>
<td>68</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex eluate</td>
<td>2.9</td>
<td>19.1</td>
<td>842</td>
<td>62.8</td>
<td>54</td>
</tr>
<tr>
<td>(concentrated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-200 eluate</td>
<td>22</td>
<td>13.4</td>
<td></td>
<td></td>
<td></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 at pH 4.7 and more rapidly toward the anode at pH 9.5 than did the wild type enzyme. The differences in the electrophoretic behavior of the two enzymes are consistent with their different chromatomic behavior on ion exchange media as noted above, with the slime enzyme behaving as the more acidic species in all cases. Duplicate gels stained for alkaline phosphatase activity (not shown) exhibited exactly the same banding pattern as the gels stained for protein (see Fig. 1).

The purified enzymes were further compared by electrophoresis at pH 8.8 in the presence of 0.1% sodium dodecyl sulfate (21), a procedure generally assumed to separate proteins on the basis of size rather than charge differences. Only a single band of protein was observed with either preparation (Fig. 2) and the mobility of the wild type enzyme was slightly greater than that of the slime enzyme.

Immunodiffusion—The immunological similarity of the repressible alkaline phosphatases purified from the two sources 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 wild type enzyme in this study are fairly similar to those reported by Kadner et al. (6), but the values observed for the slime en-
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 N 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 phosphatases from the two sources was further examined by isolation of enzyme from cultures derepressed in the presence of $^{32}$P$_{04}$-.

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.

Stalactae 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

**TABLE III**

Composition of alkaline phosphatases

<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>Cysteic acid</td>
<td>0.0492</td>
<td>8.7</td>
<td>9</td>
<td>0.055</td>
<td>9.3</td>
<td>9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.382</td>
<td>146</td>
<td>146</td>
<td>0.380</td>
<td>146</td>
<td>146</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.583</td>
<td>103</td>
<td>103</td>
<td>0.588</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>Serine</td>
<td>0.472</td>
<td>83.2</td>
<td>83</td>
<td>0.462</td>
<td>81.3</td>
<td>81</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.659</td>
<td>98.4</td>
<td>98</td>
<td>0.554</td>
<td>97.5</td>
<td>98</td>
</tr>
<tr>
<td>Proline</td>
<td>0.398</td>
<td>70.1</td>
<td>70</td>
<td>0.415</td>
<td>73.1</td>
<td>73</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.710</td>
<td>125</td>
<td>125</td>
<td>0.702</td>
<td>124</td>
<td>124</td>
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<tr>
<td>Alanine</td>
<td>0.577</td>
<td>102</td>
<td>102</td>
<td>0.574</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>Valine</td>
<td>0.384</td>
<td>69.4</td>
<td>69</td>
<td>0.401</td>
<td>70.6</td>
<td>71</td>
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<tr>
<td>Methionine</td>
<td>0.0888</td>
<td>15.6</td>
<td>16</td>
<td>0.0885</td>
<td>15.6</td>
<td>16</td>
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<tr>
<td>Isoleucine</td>
<td>0.274</td>
<td>48.3</td>
<td>48</td>
<td>0.281</td>
<td>49.5</td>
<td>50</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.540</td>
<td>95.1</td>
<td>95</td>
<td>0.550</td>
<td>97.9</td>
<td>98</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.274</td>
<td>48.3</td>
<td>48</td>
<td>0.270</td>
<td>47.6</td>
<td>48</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.330</td>
<td>58.2</td>
<td>58</td>
<td>0.338</td>
<td>59.7</td>
<td>60</td>
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<tr>
<td>Histidine</td>
<td>0.103</td>
<td>28.4</td>
<td>28</td>
<td>0.101</td>
<td>28.4</td>
<td>28</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.488</td>
<td>86.0</td>
<td>86</td>
<td>0.490</td>
<td>82.5</td>
<td>83</td>
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<tr>
<td>Arginine</td>
<td>0.204</td>
<td>35.9</td>
<td>36</td>
<td>0.198</td>
<td>34.9</td>
<td>35</td>
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<tr>
<td>Tryptophan</td>
<td>0.110</td>
<td>19.4</td>
<td>19</td>
<td>0.108</td>
<td>19.0</td>
<td>19</td>
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<tr>
<td>Glucosamine</td>
<td>0.103</td>
<td>18.1</td>
<td>18</td>
<td>0.108</td>
<td>19.0</td>
<td>19</td>
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<tr>
<td>Mannose</td>
<td>0.515</td>
<td>90.7</td>
<td>91</td>
<td>0.530</td>
<td>164</td>
<td>164</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.133</td>
<td>23.4</td>
<td>23</td>
<td>0.438</td>
<td>77.1</td>
<td>77</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.012</td>
<td>2.1</td>
<td>2</td>
<td>0.019</td>
<td>3.3</td>
<td>3</td>
</tr>
</tbody>
</table>

**TABLE IV**

Phosphate content of purified repressible alkaline phosphatase preparations

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amount* (mg)</th>
<th>Phosphate (nmol)</th>
<th>Calculated amount of enzyme (nmol)</th>
<th>Ratio of phosphate to enzyme (nmol/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
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<tr>
<td></td>
<td>2.15</td>
<td>5.3</td>
<td>15.8</td>
<td>0.44</td>
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<tr>
<td></td>
<td>0.579</td>
<td>21.9</td>
<td>2.76</td>
<td>7.0</td>
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<tr>
<td>Slime</td>
<td>0.935</td>
<td>35.4</td>
<td>4.37</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* Based on colorimetric determination (13).

† Calculated assuming a molecular weight of 136,000 for the protein moiety of the repressible alkaline phosphatase, based on the data of Kadner et al. (6).
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 glycohydrolase) 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 negligible. A significant fraction of this enzyme appeared to be liberated into the media from each culture were carried out and the results are determinations on both the cell extracts and the cell-free culture solutions. Enzyme assays indicated a recovery of greater than 95% of the observable change in the electrophoretic mobility of either enzyme. The values obtained were 1.72 x 10^4 M for the wild type enzyme and 1.56 x 10^4 M for the slime enzyme. The observed K<sub>i</sub> for phosphatase under the same conditions was 1.31 x 10^4 M for the wild type enzyme and 1.23 x 10^4 M for the slime enzyme.

Kinetic Properties—K<sub>i</sub> 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 x 10^4 M for the wild type enzyme and 1.56 x 10^4 M for the slime enzyme. The observed K<sub>i</sub> for phosphatase under the same conditions was 1.31 x 10^4 M for the wild type enzyme and 1.23 x 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 enzyme 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 addition of 5 µl of Fries' salts (minus phosphate). The concentrated enzyme solutions were incubated 24 hours at 37°C in a closed container over moistened filter paper and then diluted to a concentration 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 enzyme.

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 phosphatase of the same organism (23, 35). The finding that in cultures of the slime strain the repressible alkaline phosphatase is primarily 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<sub>2</sub>PO<sub>4</sub> 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 medium (20 to 30% under the conditions tested). The proportion of the phosphatase released into the medium by the cell wall mutants (os-l, os-<i>c</i>, or os, cr) was intermediate between the value observed for the wild type and slime cultures.

Electrophoretic Mobility of Repressible Alkaline Phosphatase Isolated from Wild Type Culture Medium—Medium from a derepressed culture of wild type N. crassa (grown for 20 hours on 0.05 mM KH<sub>2</sub>PO<sub>4</sub> medium) was concentrated approximately 30-fold with a Diaflo ultrafilter (UM-10 membrane). A further 10-fold concentration was achieved by the addition of Lyphogel (Gelman Instrument Co., Ann Arbor, Mich.). The final concentrated solution (0.3 ml) contained 0.10 unit of repressible alkaline phosphatase activity (an extract of the mycelium harvested from this medium contained 0.90 unit of enzyme). This solution was dialyzed overnight against 0.01 M Tris-HCl buffer, pH 7.4, and was then subjected to electrophoresis in the pH 9.5 buffer system. Standards consisting of the purified wild type mycelial and slime medium enzymes were run at the same time. The bands of enzyme activity observed following electrophoresis are shown in Fig. 8. The repressible alkaline phosphatase isolated from wild type culture medium migrates at a rate more similar to that of the slime enzyme than that of the enzyme purified from wild type mycelium.

Properties of the Residual Repressible Alkaline Phosphatase in Washed Slime Cells—Although the bulk of the repressible alkaline phosphatase in slime cultures is found free in the medium (Table VI), approximately 5% of the total activity is found still associated with the cells even after they have been washed with fresh medium. Attempts to purify the enzyme from extracts of washed slime cells by ammonium sulfate fractionation revealed that a considerable portion (15 to 25% depending on how the cells were washed) of the activity was precipitated in 65% saturated solutions of the salt. Less than 5% of the enzyme isolated from slime medium or wild type mycelium is precipitated under the same conditions. The electrophoretic mobility at pH 0.5 of this "65% ammonium sulfate precipitable" alkaline phosphatase is less than that of the enzyme isolated from either slime medium or wild type mycelium as shown in Fig. 9. The structure of this less soluble, more basic form of the enzyme was shown to be immunologically similar to the other repressible alkaline phosphatase species by precipitation of the expected number of enzyme units with graded amounts of the antisem prepared against the wild type mycelial enzyme (Table VII).
Precipitation of slime intracellular repressible alkaline phosphatase by antiserum prepared against enzyme purified from wild type mycelia

The indicated volume of 20-fold diluted antiserum was added to 0.10-ml aliquots of either the purified wild type mycelial enzyme (diluted to 8.7 µg per ml) or the 0 to 65% ammonium sulfate precipitable fraction of an extract of washed, derepressed slime cells. The total volume was adjusted to 0.20 ml by addition of borate-saline buffer. Tubes were incubated 60 min at room temperature, centrifuged 15 min at 1500 × g, and repressible alkaline phosphatase remaining unprecipitated was determined in the standard assay system using 0.025-ml aliquots of the supernatant solutions.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Enzyme remaining in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.538 0.513 0.513</td>
</tr>
<tr>
<td>0.01</td>
<td>0.438 0.440 0.440</td>
</tr>
<tr>
<td>0.02</td>
<td>0.135 0.126 0.126</td>
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<tr>
<td>0.03</td>
<td>0.019 0.015 0.015</td>
</tr>
<tr>
<td>0.04</td>
<td>0.010 0.012 0.012</td>
</tr>
<tr>
<td>0.05</td>
<td>0.006 0.013 0.013</td>
</tr>
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</table>

**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-I (38) and T9 (39) mutants of N. crassa appear to be simultaneously affected in cell wall synthesis, and they synthesize glucoamylase with altered gel filtration (30) 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 nitritotriacetic acid, a procedure which removes tightly bound zinc ions from the enzyme. The alkaline phosphatase isolated from slime cells labeled with $^{32}$P0$_4^-$ retained most of its bound $^{32}$P during an isolation procedure which included dialysis against phosphate buffer, heating at 100° 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 (14).

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 thoroughness with which the cells were washed) of this residual cell-bound enzyme clearly differs in its solubility and electrophoretic properties (Fig. 9) from both the purified enzyme from slime medium and the purified enzyme from wild type mycelia. 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 antiserum 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.

Acknowledgment—The authors are grateful to Dr. Roger Totten for help and advice with the SDS-acrylamide gel electrophoresis.

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

Earl G. Burton and Robert L. Metzenberg


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