Immuunochemical Studies on α-Amylase

III. Immunochemical Relationships Among Amylases from Various Microorganisms

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ABSTRACT

SIRISINHA, STITAYA (University of Rochester School of Medicine and Dentistry, Rochester, N.Y.), AND PETER Z. ALLEN. Immunochemical studies on α-amylase. III. Immunochemical relationships among amylases from various microorganisms. J. Bacteriol. 90:1120-1128. 1965—Immunochemical relationships among amylases obtained from a selected group of microorganisms were examined, and a cross-reaction was detected between the α-amylases of Bacillus steaerothermophilus and B. subtilis. Immuno-diffusion and quantitative precipitin studies, as well as cross-neutralization tests, indicate that B. stearothermophilus α-amylase reacts with a portion of antibody present in antisera to crystalline B. subtilis α-amylase. Amylases from these two species thus have some aspects of structure in common. Limited data obtained by immunodiffusion suggest that groupings which confer cross-reactivity to the B. stearothermophilus enzyme are lost after exposure to mercaptoethanol in the presence of ethylenediaminetetraacetate, followed by treatment with iodoacetamide. With the antisera employed and within the concentration range examined, no immunochemical cross-reaction was observed among amylases from Aspergillus oryzae, B. subtilis, B. polymyxa, B. macerans, Pseudomonas saccharophila, and Euglena sanguinis. Immuno-electrophoresis of partially purified B. stearothermophilus α-amylase by use of antiserum to the crude enzyme, together with localization of amylase activity in immunoelectrophoretic plates, suggests that B. stearothermophilus α-amylase is antigenic in the rabbit.

Immuunochemical studies on the antigenicity and specificity of α-amylases have been carried out by several investigators. McGeachin, Reynolds, and Huddleston (1961), McGeachin (1963), and McGeachin and Reynolds (1959, 1961) demonstrated that both human salivary and hog pancreatic α-amylases are antigenic for rabbits. The anti-amyrase sera obtained were found to inhibit almost completely the α-amylase activity of homologous preparations when starch was employed for the assay of residual enzymatic activity. These antisera were found, in addition, to cross-react and to neutralize partially the activity of α-amylases from closely related animal species.

Similar findings were obtained by Wada and Nomura (1958) and by Wada (1959), who examined various microbial amylases with antisera to crystalline α-amylase from Aspergillus oryzae. By use of both immunodiffusion and enzyme neutralization tests, these antisera were found to cross-react only with microbial α-amylase preparations from the genus Aspergillus. Amylases obtained from other genera of molds or from Bacillus subtilis failed to show any cross-reaction.

In the present study, immunochemical relationships among amylases produced by a selected group of microorganisms were examined. Antisera prepared against crystalline B. subtilis α-amylase were found to cross-react with partially purified amylase obtained from B. stearothermophilus. The cross-reaction was examined, and its specificity was established by use of immunodiffusion, immunoelectrophoresis, quantitative precipitation, and cross-neutralization tests. In addition, immunoelectrophoretic findings suggest that the α-amylase of B. stearothermophilus is antigenic in the rabbit.

MATERIALS AND METHODS

B. subtilis α-amylase. Crystalline enzyme was prepared from a commercial crude enzyme concentrate of B. subtilis, HT Concentrate (Miles

1120
Chemical Co., Elkhart, Ind., lot no. B-248), according to the procedure of Stein and Fisher (1961). The purification and crystallization were carried out in the presence of 10^{-4} M DFP (diisopropylfluorophosphate; Sigma Chemical Co., St. Louis, Mo.). After four or five recrystallizations, the enzyme was examined by diethylaminoethyl (DEAE)-cellulose column chromatography, immunodiffusion, and immunoelectrophoresis, and was found to consist of a single component. The extinction coefficient, \( \varepsilon_{280}^{\text{max}} \), of the five-times crystallized enzyme in 0.002 M glycerophosphate-\( \text{HCl} \) buffer (pH 5.9) was found to be 1.281.

Amylase activity was assayed by the dinitrosalicylic acid procedure described by Stein and Fisher (1961) with the exception that the optical density at 540 \( \text{m\mu} \) was converted to milligrams of maltose from a standard curve established with chromatographically pure d(+)-maltose in the presence of 0.5% starch rather than in a plain glycerophosphate-\( \text{HCl} \) buffer (Dahlqvist, 1962).

\textit{B. stearothermophilus} \( \alpha \)-amylase. A partially purified enzyme preparation employed as antigen was prepared from culture medium of \textit{B. stearothermophilus} ATCC 7953 (American Type Culture Collection, Washington, D.C.). The purification procedure employed was a modification of the scheme originally employed by Manning and Campbell (1961) to obtain crystalline enzyme. Ion-exchange chromatography was employed in this modified procedure because the enzyme failed to crystallize under conditions employed in this laboratory. Organisms were grown in a culture medium made up as originally described. The 36 to 48-hr culture was filtered, precipitated with ammonium sulfate and sodium sulfate, dissolved, and then precipitated twice with acetone as described by Manning and Campbell (1961).

The crude enzyme preparation obtained after a second acetone precipitation was further purified by ion-exchange chromatography. Enzyme solution was applied to a DEAE-cellulose column (4 by 45 cm), previously equilibrated with 0.01 M phosphate (pH 8.0) starting buffer. Bound proteins not eluted by starting buffer were removed by gradient elution by use of a gradient of decreasing pH and increasing molarity obtained by means of the cone-sphere assembly described by Tombs et al. (1961). Two volumes of starting buffer were placed in a round mixing chamber, and one volume of 0.3 M monosodium phosphate limit buffer was placed in an Erlenmeyer flask. The optical density of each fraction eluted was measured at 280 \( \text{m\mu} \). The chromatogram obtained (Fig. 1a) showed two major protein fractions. A smaller, enzymatically active protein fraction was eluted with starting buffer (tubes 11 to 24), and an enzymatically inactive fraction, comprising the bulk of the 280 \( \text{m\mu} \) absorbing material applied to the column, was more firmly bound and could be removed only after the gradient was applied.

Fractions possessing enzymatic activity (Fig. 1a, shaded area) were pooled, dialyzed against water, lyophilized, and dissolved in a minimal amount of 0.01 M sodium acetate buffer (pH 5.0). This partially purified enzyme solution was then applied to a carboxymethyl cellulose column (2.5 by 30 cm), previously equilibrated with a starting sodium acetate buffer. Starting buffer was run through the column until the absorption at 280 \( \text{m\mu} \) had returned to a base line value. A gradient of increasing pH and molarity obtained by use of the cone-sphere assembly previously described was then applied to the column. The gradient system consisted of 0.1 M sodium acetate buffer (pH 5.0) and 1 M sodium acetate as starting and limiting buffer solutions, respectively. The protein fraction eluted by starting buffer (Fig. 1b) showed no

![Fig. 1](http://jb.asm.org/)
enzymatic activity, whereas fractions emerging from the column upon application of the gradient were found to possess amylase activity. Fractions showing α-amylase activity were pooled, dialyzed against water, lyophilized, and reconstituted in 0.02 M calcium acetate. This partially purified enzyme preparation was found by immunological analysis to contain at least two antigenic components in addition to α-amylase. The α-amylase activity of this thermostable enzyme was assayed by the method of Manning and Campbell (1961). Although the properties of α-amylase from \textit{B. stearothermophilus} ATCC 7954 have been reported (Campbell and Cleveland, 1961; Campbell and Manning, 1961; Manning, Campbell, and Foster, 1961).

\textit{B. polymyza} amylase. Partially purified amylase from \textit{B. polymyza} was prepared according to the procedure described by Robyt and French (1964). Evaporation of volatile products present in the bacterial filtrate as recommended in the original procedure was omitted because of the high viscosity of the culture fluid. A crude enzyme was obtained directly from the culture fluid by ammonium sulfate precipitation, and the precipitate obtained was further purified essentially as described in the original procedure.

The enzymatic activity of the \textit{B. polymyza} amylase was determined by the same assay procedure employed for the \textit{B. stearothermophilus} enzyme, except that 0.067 M phosphate buffer (pH 6.8) was used and enzymolysis was carried out at 40°C rather than at 65°C. One unit of activity is defined as the amount of enzyme producing a 10% reduction in blue value of a 10% soluble starch solution at 40°C in 1 min (Robyt and French, 1964).

\textit{B. macerans} α-amylase. A concentrated preparation of crude α-amylase was obtained from the culture fluid of \textit{B. macerans} ATCC 7069 grown 0.2% in a medium described by Pazur and Ando (1962). After approximately 2 weeks of incubation at 40°C, culture media were centrifuged at 4,000 × g for 1 hr to remove any insoluble residue. The clear cell-free supernatant fluid was dialyzed overnight against several changes of water, lyophilized, and reconstituted in a minimal amount of water. This concentrated enzyme preparation was used directly, without further purification. Assay for amylase activity was done by the procedure of Tilden and Hudson as described by Pazur and Ando (1962).

\textit{Pseudomonas saccharophila} α-amylase. An enzymatic solution containing 500 μg of protein per ml was a gift from L. Levine of Brandeis University. It was purified and crystallized from a culture of \textit{P. saccharophila} as described by Markovitz, Klein, and Fischer (1956).

\textit{Euglena sanguinis} amylase. Crystalline amylase prepared from \textit{E. sanguinis} was also obtained from L. Levine. An enzyme suspension was made in saline to contain approximately 2 mg of protein per ml.

\textit{Aspergillus} α-amylase. Five-times crystallized enzyme was prepared by the procedure of Stein (1954). This preparation, shown to consist of a single antigenic component, was previously described in detail (Sirisinha and Allen, Arch. Biochem. Biophys., in press).

\textit{Antienzyme sera.} Antisera to various crude and crystalline α-amylase preparations were prepared in 2- to 3-kg New Zealand white rabbits. Pre-immunization bleedings were obtained from all animals and served as serum controls.

\textit{Antiserum to crude} \textit{B. subtilis} α-amylase. Antisera to crude \textit{B. subtilis} α-amylase were prepared by immunizing rabbits with mother liquors obtained from the first crystallization. Animals were intravenously injected, over a course of 4 weeks, with a total of 19 mg of nitrogen of alum-precipitated antigen. The first injection of this series was given subcutaneously, the second intramuscularly, and the last two intravenously. Animals were bled 1 week after the last injection to provide hyperimmune sera R4-2C and R5-2C.

\textit{Antisera to crystalline} \textit{B. subtilis} α-amylase. Antisera to crystalline α-amylase were prepared by injecting into the foot pads a total of 0.5 mg of protein nitrogen in complete Freund’s adjuvant. Animals were bled after 3 weeks, and first course immune sera R57-1C, R79-1C, and R196-1C were obtained. After 10 months, the animals were boostered with a total of 0.8 mg of nitrogen of alum-precipitated antigen. The first injection of this series was given subcutaneously, the second intramuscularly, and the last two intravenously. Animals were bled 1 week later to provide hyperimmune sera R79-2C and R196-2C. Antiserum R57-1C contained 213 μg of antibody nitrogen per 0.25 ml, and antisera R196-2C and R79-1C, respectively, contained 144 and 121 μg of antibody nitrogen per ml.

\textit{Antiserum to crude} \textit{B. stearothermophilus} α-amylase. A single rabbit received by foot-pad injections over a course of 2 weeks, a total of 0.4 mg of nitrogen of crude enzyme preparation in complete Freund’s adjuvant. Crude enzyme used as antigen consisted of the \textit{B. stearothermophilus} α-amylase preparation obtained after the second acetone precipitation in the fractionation procedure already described. The animal was bled 3 weeks later to provide immune serum R4206-1C. After 1 month the animal was boostered with 0.8 mg of nitrogen of alum-precipitated antigen preparation, by use of the schedule described above for \textit{B. subtilis}. Hyperimmune serum R4206-2C was obtained 1 week after the last injection.

\textit{Antiserum to Aspergillus} α-amylase. The preparation and characterization of rabbit antisera to crude (R63-1C) and crystalline (R75-1C) \textit{Aspergillus} α-amylase were previously described in detail (Sirisinha and Allen, in press).

Quantitative precipitin studies. Quantitative
precipitin analyses were carried out essentially as described by Kabat and Mayer (1961). Total nitrogen of washed specific precipitants was determined by the Markham micro-Kjeldahl method (Kabat and Mayer, 1961). The nitrogen content of the partially purified B. stearothermophilus α-amylase preparation employed as antigen in quantitative precipitation was also determined by micro-Kjeldahl analysis.

Localization of α-amylase activity. Localization of α-amylase activity of B. stearothermophilus preparations was made on immunoelectrophoresis plates after arcs of specific precipitate had fully developed. This was done by carefully placing in contact with the agar plate a strip of filter paper impregnated with 1% soluble starch in 0.3 M acetic acid buffer (pH 4.6) containing 0.1 M CaCl₂. The plate and strip were then incubated in a moist chamber at 55°C for 60 to 90 min. At the end of the incubation period, the strip was left in situ, and iodine solution containing 3 mg of iodine and 30 mg of potassium iodide per ml was applied to the paper. Areas in which α-amylase activity was present in the agar appear on the paper as light-yellow spots surrounded by a dark-blue background of the undigested starch-iodine complex. The center of curvature of the yellow amylase spot on filter-paper strips was always found to coincide with the center of curvature of the most cathodal migrating antigen component of B. stearothermophilus preparations.

Enzyme neutralization assays. Inhibition of α-amylase activity by antisera was estimated by incubating 0.5 units of B. stearothermophilus enzyme with 0.5 ml of serum at 37°C for 1 hr and comparing the residual activity of the amylase-antiamylase mixture with that of an equal amount of free enzyme. Enzyme incubated with normal rabbit serum was also included. Under these conditions of assay, no visible precipitation with antisera was observed. The trace amount of serum amylase normally present in rabbit sera made no significant contribution to the total amylolytic activity of the mixtures. Assay of amylase activity was carried out as described by Manning and Campbell (1961). The degree of neutralization by any antisera was estimated from the residual activity expressed as a percentage of the original activity.

Ion-exchange chromatography, gel filtration, and immunoelectrophoretic analyses were carried out by procedures already described (Sirisinha and Allen, in press). Double diffusion in two dimensions was carried out by a modified Ouchterlony (1962) method. About 13 ml of a molten 0.5 M glycine solution containing 1% I onagar (Colab Laboratories, Inc., Chicago Heights, III.), 0.85% sodium chloride, and 0.0001% Merthiolate were pipetted onto a glass slide (80 by 100 mm). After the agar had solidified, a central well (11 mm in diameter) to contain antisera was cut with a no. 8 cork borer. Wells (6 mm in diameter) for antigen were cut with a no. 3 cork borer around the central well at a distance of 5 mm. Slides were kept at room temperature over moistened filter paper inside of covered petri plates, and were photographed after bands had formed. Agar diffusion analyses with antisera R196-2C were carried out with an optimal amount of B. subtilis α-amylase (50 to 75 alters of solution containing 500 µg of nitrogen per ml), so as to obtain sharp, narrow, well-defined bands of precipitation.

RESULTS

Characterization of anti-B. subtilis α-amylase sera. Antiserum to six-times crystallized B. subtilis α-amylase (R57-1C, R79-1C, R196-1C, and R196-2C) were examined by immunodiffusion (Table 1) and immunoelectrophoresis (Fig. 2). These sera gave only a single band of precipitation with either crude or crystalline B. subtilis α-amylase preparations. Whereas antisera to crude B. subtilis α-amylase (R4-2C and R5-2C) could detect seven or eight components in preparations of crude enzyme, these sera showed the presence of only one component in preparations of crystalline enzyme (Fig. 2). Quantitative precipitin studies carried out with antisera to crystalline enzyme R196-2C gave a typical precipitin curve with crystalline amylase (Fig. 5) and showed the presence of 144 µg of antibody nitrogen per 0.25 ml of serum. In the region of antibody excess, none of the α-amylase activity added as antigen could be found in supernatant fractions of the reaction mixture; moreover, this antisem could inhibit the enzymolysis of starch by homologous antigen.

Characterization of anti-B. stearothermophilus α-amylase sera. Antiserum to crude preparations of B. stearothermophilus α-amylase (R4206-1C and R4206-2C) were found, by immunodiffusion and immunoelectrophoresis, to contain precipitins for at least nine antigenic constituents of crude enzyme (Table 1). These sera were able to detect the presence of three antigenically distinct components in B. stearothermophilus amylase preparations partially purified by ion-exchange chromatography. The presence of antienzyme in serum R4206-2C is indicated from the association of amylolytic activity with that antigenic component showing the most cathodal migration in immunoelectrophoresis (Fig. 4).

Characterization of anti-Aspergillus oryzae α-amylase sera. Antiserum to crude enzyme (R63-1C) gave at least seven bands of precipitation in immunodiffusion when diffused against crude enzyme, whereas only one band was obtained with crystalline enzyme. Antiserum to crystalline enzyme (R75-1C) gave one band of precipitation with both crude and crystalline en-
Table 1. Reactivity of various amylases by immunodiffusion analysis

<table>
<thead>
<tr>
<th>Amylase prep employed as antigen</th>
<th>Amt of amylase tested</th>
<th>Anti-Aspergillus</th>
<th>Anti-Bacillus subtilis</th>
<th>Anti-B. stearothermophilus, R196-2C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus oryzae</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crystallized</td>
<td>25 mg/ml</td>
<td>1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Crude</td>
<td>25 mg/ml</td>
<td>7 (1)</td>
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<td>0</td>
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<td>Pseudomonas saccharophila</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Crystallized</td>
<td>0.5 mg/ml</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Euglena sanguinis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystallized</td>
<td>2 mg/ml</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystallized</td>
<td>3 mg/ml</td>
<td>0</td>
<td>0</td>
<td>8 (1)</td>
</tr>
<tr>
<td>Crude</td>
<td>11 mg/ml</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<td>B. stearothermophilus</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Partially purified crude</td>
<td>0.3 mg/ml</td>
<td>0</td>
<td>2</td>
<td>1 (1)</td>
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<tr>
<td>B. macerans</td>
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<td></td>
</tr>
<tr>
<td>Crude</td>
<td>25 units/ml</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B. polymyza</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>5 units/ml</td>
<td>0</td>
<td>0</td>
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</table>

* Results are expressed as the number of bands obtained with antiamylase serum as determined by the Preer method (Kabat and Mayer, 1961). Values in parentheses indicate additional faint or barely detectable bands.

Values represent highest concentration of amylase tested in antigen dilution series; lowest concentrations tested range from 1 to 0.1% of amount listed.

Antiserum to crude enzyme preparations.

Antiserum to crystalline enzyme.

zyme (Table 1); precipitins present in this serum have been shown to be antienzyme (Sirisinha and Allen, 1964, in press).

Examination of cross-reactivity by immunodiffusion findings obtained with various microbial amylases by use of several antiamylase sera are summarized in Table 1. As indicated in Table 1, with the antiserum employed and within the range of antigen concentrations examined, the only detectable cross-reaction found to occur was between the B. stearothermophilus enzyme and antisera to B. subtilis α-amylase. Agar-diffusion results (Fig. 3) with antiserum (R196-2C) to crystalline B. subtilis α-amylase clearly demonstrate that the partially purified B. stearothermophilus enzyme preparation gives only a single band of precipitation which shows a reaction of interference or "partial identity" (Ouchterlony, 1962) with the band given by crystalline B. subtilis α-amylase.

The finding that antiserum R196-2C completely absorbed with crystalline B. subtilis α-amylase failed to give a band of precipitation with the B. stearothermophilus enzyme also suggests that the band obtained with unabsorbed serum was due to antienzyme present in the unabsorbed serum. That the band of precipitation observed in the cross-reaction is associated with the amylase component of the partially purified B. stearothermophilus preparation and not with one of the antigenic impurities shown to be present was further demonstrated by immunoelectrophoresis. As shown in Fig. 4, localization of α-amylase activity on starch-impregnated filter-paper imprints of plates showed amylase activity to be associated with the most slowly migrating antigen component which also shows cross-reaction with antiserum R196-2C. The center of curvature of the α-amylase spot on starch-paper coincides with the center of curvature of the single antigen components of R196-2C. Further evidence for the specificity of the cross-reaction was obtained from cross-neutralization tests.

Neutralization of amylase activity. Anti-B. subtilis sera were capable of neutralizing the amylase activity of partially purified B. stearothermophilus preparations (Table 2). The ability of anti-B. subtilis serum R196-2C to almost completely neutralize the amylase activity added provides additional support for the specificity of the cross-reaction between anti-B. subtilis amylase and the B. stearothermophilus enzyme.

An increase in the cross-neutralizing ability of rabbit antienzyme with prolonged immunization is also apparent. After booster stimulation (Table 2), the cross-neutralizing ability of serum from
rabbit R196 increased from 59% (R196-1C) to 96% (R196-2C). Whereas anti-*B. subtilis* sera R196-1C, R196-2C, and R57-1C showed cross-reactivity with the *B. stearothermophilus* enzyme, a reciprocal cross-reaction between anti-*B. stearothermophilus* serum R4206-2C and *B. subtilis* amylase was not observed. No arc of precipitation was found (Fig. 4) when the *B. subtilis* enzyme was diffused against antiserum R4206-2C, nor did this serum show any ability to neutralize *B. subtilis* α-amylase activity. Both anti-*B. subtilis* and anti-*B. stearothermophilus* sera failed to inhibit activity of *B. macerans* α-amylase.

**Quantitative precipitin study.** A quantitative precipitin analysis was carried out with partially purified *B. stearothermophilus* α-amylase, by use of antiserum to six-times crystallized *B. subtilis* α-amylase R196-2C. A quantitative precipitin study of the cross-reaction (Fig. 5) could be conducted, despite the presence of three components in the antigen preparation, because immunodiffusion (Fig. 3), immunoelectrophoresis (Fig. 4), and cross-neutralization (Table 2) showed that only the amylase component of the mixture was involved in the cross-reaction with this serum. In addition, the only antibody present in this antiserum was shown to be anti-*B. subtilis* α-amylase (Table 1 and Fig. 2). Due to the presence of impurities in the antigen preparation, the amount of cross-reacting antibody could not be directly determined by subtracting the antigen nitrogen added from the total nitrogen found in washed specific precipitates. Supernatant analyses were therefore carried out by the addition of crystalline *B. subtilis* enzyme to quantitatively recover unreacted antibody. Since the homologous antibody nitrogen content of the serum was known (144 µg of antibody nitrogen per 0.25 ml), the amount of cross-reacting antibody could be estimated from this recovery. Analysis of supernatant fractions of the cross-reaction shown in Fig. 5 indicated that only 18% (27 µg) of the total antibody nitrogen to the *B. subtilis* enzyme present in this serum could be precipitated by the cross-reacting *B. stearothermophilus* enzyme. A similar study with serum R57-1C showed the presence of only 12 µg of cross-reacting antibody nitrogen per 0.25 ml.

**Effect of reduction in the presence of ethylenediaminetetraacetic acid (EDTA)** and alkylation on cross-reactivity. Partially purified *B. stearother-
FIG. 3. Agar diffusion showing the formation of a band with spurring by partially purified Bacillus stearo-thermophilus α-amylase with antiserum to crystalline B. subtilis amylase (R196-2C) in central well. Wells 1 and 4 contain crystalline B. subtilis α-amylase; wells 2, 5, and 6 contain partially purified B. stearo-thermophilus α-amylase; well 3 contains reduced and alkylated B. stearo-thermophilus α-amylase.

FIG. 4. Immunoelectrophoretic patterns of five-times crystallized Bacillus subtilis α-amylase (C) and partially purified B. stearo-thermophilus α-amylase (B). Arcs developed with anti-B. subtilis (R196-2C) and anti-B. stearo-thermophilus (R4206-2C) sera. Localization of amylolytic activity of the B. stearo-thermophilus preparation, on iodine-stained starch-filter paper imprint (A). Arrow in A shows that center of curvature of enzymatic activity of B. stearo-thermophilus α-amylase corresponds to center of curvature of arcs of precipitation located closest to cathode in B.

mophilus enzyme in phosphate-buffered saline (pH 7.3) was treated with 0.2 M mercaptoethanol in the presence of 0.02 M EDTA and a few drops of toluene at 37 C for 48 hr. A slight excess of iodoacetamide in phosphate buffer (pH 7.5) was then added. The reduced and alkylated preparation was applied to a Sephadex G-50 column and

<table>
<thead>
<tr>
<th>Serum</th>
<th>OD at 620 μμ*</th>
<th>ΔOD</th>
<th>Per cent inhibition of added activity†</th>
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<tbody>
<tr>
<td>Normal rabbit serum</td>
<td>.125</td>
<td></td>
<td></td>
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<tr>
<td>Normal rabbit serum + enzyme</td>
<td>.032</td>
<td>.093</td>
<td>0</td>
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<tr>
<td>Anti-B. subtilis R75-1C</td>
<td>.120</td>
<td></td>
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<td>Anti-B. subtilis R75-1C + enzyme</td>
<td>.065</td>
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<td>38</td>
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<tr>
<td>Anti-B. subtilis R196-1C</td>
<td>.116</td>
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<tr>
<td>Anti-B. subtilis R196-1C + enzyme</td>
<td>.080</td>
<td>.036</td>
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<td>.107</td>
<td></td>
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<tr>
<td>Anti-B. subtilis R196-2C + enzyme</td>
<td>.104</td>
<td>.003</td>
<td>96</td>
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<tr>
<td>Acid-inactivated enzyme</td>
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<td></td>
<td></td>
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<tr>
<td>Active enzyme</td>
<td>.018</td>
<td>.088</td>
<td>0</td>
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</table>

* Results of a single determination.
† Residual amylase activity was determined after 0.5 unit of B. stearo-thermophilus (partially purified) amylase had been incubated with 0.5 ml of antiserum for 1 hr at 37 C. Inhibition was calculated from the formula (0.088 minus OD test, divided by 0.088) × 100 equals per cent inhibition.
eluted with water. Only a single protein fraction showing 280-mu absorption was eluted which failed to show enzymatic activity. This fraction also failed to give a band in immunodiffusion (Fig. 3) or immunoelectrophoresis with antisera to the B. subtilis enzyme. The immunodiffusion behavior of enzyme after exposure to EDTA in the absence of mercaptoethanol, followed by treatment with iodoacetamide, was not examined. Treatment of crystalline \( \alpha \)-amylases from B. subtilis and A. oryzae with EDTA under comparable conditions, however, has been shown not to destroy the ability to form a band with antibody in agar diffusion (Sirisinha and Allen, in press).

**DISCUSSION**

Immunoochemical relationships among various amyloses obtained from a selected group of microorganisms were examined. The ability of enzyme preparations to interact with rabbit antisera to the amyloses of B. subtilis, B. stearothermophilus, and A. oryzae was studied by means of immunodiffusion, immunoelectrophoresis, quantitative precipitation, and neutralization of amylolytic activity. Immunodiffusion results summarized in Table I show that, apart from the formation of a band by B. stearothermophilus \( \alpha \)-amylase with antisera to crystalline B. subtilis \( \alpha \)-amylase, no other immunoochemical cross-reaction was observed.

Antiserum to crystalline B. subtilis \( \alpha \)-amylase R196-2C was shown to contain only antienzyme by the formation of a single band of precipitation in immunodiffusion and immunoelectrophoresis. Supernatant analysis from the quantitative precipitin curve obtained with serum R196-2C (Fig. 5) showed that, in the region of antibody excess, none of the B. subtilis \( \alpha \)-amylase activity added as antigen remained behind in the supernatant fraction. Moreover, this antiserum gave 85% inhibition of hydrolysis when starch was added to amylase-antiserum mixtures.

Partially purified \( \alpha \)-amylase prepared from a culture filtrate of B. stearothermophilus by salt and acetone fractionation followed by ion-exchange chromatography was found to contain at least three antigenic components. This was shown by immunoelectrophoresis and immunodiffusion. That the \( \alpha \)-amylase component of the B. stearothermophilus preparation is involved in the cross-reaction with antiserum R196-2C is established by the following evidence. (i) The only precipitin present in cross-reacting sera R196-1C and R196-2C was shown to be antiamylase (Fig. 2, 3, 4, and Table 1). (ii) Serum R196-2C shows cross-neutralizing ability and inhibits 96% of the \( \alpha \)-amylase activity of partially purified B. stearothermophilus (Table 2). (iii) Diffusion of B. stearothermophilus enzyme against serum R196-2C gives only one band, which shows a reaction of interference or "partial identity" with the B. subtilis-anti-B. subtilis \( \alpha \)-amylase system (Fig. 3). (iv) The cross-reacting system shows only one arc of precipitation in immunoelectrophoresis (Fig. 4B). (v) The mobility of the cross-reacting antigen differs from that of other components present and corresponds to the mobility of the amylolytic component present (Fig. 4A and B).

The heterogeneity of antibodies produced by rabbits in response to immunization with crystalline B. subtilis \( \alpha \)-amylase is evident from the extent of cross-reaction obtained with different antisera. Quantitative precipitin studies showed that, whereas only 6% of the total antibody nitrogen was precipitable from antiserum R57-1C by the B. stearothermophilus amylase preparation, 18% of the total antibody nitrogen could be removed from antiserum R196-2C (Fig. 5).

Although the \( \alpha \)-amyloses of B. subtilis and B. stearothermophilus show marked differences in their physical and chemical properties (Stein, 1954; Stein and Fischer, 1961; Campbell and Cleveland, 1961; Campbell and Manning, 1961; Manning et al., 1961), the immunoochemical data presented establish that these two proteins share some antigenic determinants in common. Limited data obtained from immunodiffusion (Fig. 3) show that structural groupings which confer cross-reactivity to the B. stearothermophilus enzyme are lost upon exposure to mercaptoethanol in the presence of EDTA followed by treatment with iodoacetamide.

Immunoochemical relationships among bacterial enzymes may not necessarily follow strict taxonomic classification. Pollock (1956, 1963) demonstrated that penicillinases produced by different strains of B. cereus were not immunologically identical although constitutive and induced enzyme from given strains could not be immunologically distinguished. Moreover, the penicillinases of B. cereus and B. subtilis are immunologically unrelated (Manson, Pollock, and Tridgell, 1984), Monod and Cohn (1952) failed to find any immunoochemical or biochemical differences among \( \beta \)-galactosidases of Escherichia coli, Aerobacter aerogenes, or Shigella sonnei. Thus, the taxonomic significance of the present immunoochemical findings is not clear.

The localization of \( \alpha \)-amylase activity with the most slowly migrating component giving an arc of precipitation in immunoelectrophoresis (Fig. 4A and B).
4B) provides evidence for the antigenicity of the α-amylase of B. \textit{stearothermophilus}. The significance of the failure to obtain a reciprocal cross-reaction, i.e., a reaction between \textit{B. subtilis} α-amylase and anti-\textit{B. stearothermophilus} serum R4206-2C, is not known. Whether this is attributable to a low antienzyme content or some other property of the antibody of this particular antiserum remains to be established.

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**Literature Cited**


