

Antigenic compositions of enzyme-containing preparations of *Bacillus subtilis* origins

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Bacillus subtilis enzyme preparations were examined by immunologic methods. α -Amylase and proteinases were identified by the use of purified enzyme preparations and by the demonstration of enzyme activities in the immune precipitates formed in double-diffusion reactions. The presence of multiple antigenic components in the *B. subtilis* preparations, including crystalline enzyme preparations, is described. The contained proteinases included alkaline proteinases of bacillopeptidase A and bacillopeptidase B types. There is no apparent antigenic cross-reactivity between the bacillopeptidases; however, crystalline preparations of these proteinases were found to contain small quantities of the alternate proteinases. A cognizance of the heterogeneities of available preparations is required in studies of immune responses and of hypersensitivity to *B. subtilis* products.

Among the products of organisms of *Bacillus* species are a number of enzymes including a series of alkaline proteinases,¹⁻⁴ neutral proteinases,⁵ and α -amylase.⁶ Two distinct *B. subtilis* alkaline proteinases, subtillopeptidase A (SPA, subtilisin Carlsberg) and subtillopeptidase B (SPB, bacterial proteinase Novo), were initially differentiated by analyses of physicochemical and enzymatic properties.² The alkaline proteinases of *Bacillus* origins are alternately referred to as bacillopeptidases and can be resolved into bacillopeptidase A (BPA) and bacillopeptidase B (BPB) groups.^{3, 4} The proteinases of each group appear to be indistinguishable; there is antigenic cross-reactivity within the group but none between the groups.⁴ Subtilisin BPN' is a bacillopeptidase B.⁴ The production of neutral proteinases and α -amylase is said to be largely confined to bacillopeptidase B-producing organisms.⁴

Workers in the detergent industry have been found to develop a respiratory disease resulting from exposure to airborne *B. subtilis* products⁷; there is type I reagenic hypersensitivity to these products including the contained alkaline proteinases.⁸ This is, therefore, a well-defined example of allergy to bacterial products. Nonimmunologic interactions and inhibitions of *B. subtilis* alkaline proteinases with α_2 -macroglobulin and α_1 -antitrypsin of human serum have been observed.^{9, 10}

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B. subtilis preparations necessarily represent complex biological mixtures which have been processed to varying levels of purity. The present report describes analyses of a number of *B. subtilis* enzyme preparations. α -Amylase and bacillopeptidases were identified by the use of purified reference preparations in gel diffusion studies and by the use of an "imprint" technique to directly demonstrate the enzyme activities contained in precipitin lines. The presence of multiple antigenic components in the enzyme preparations including crystalline preparations is described. In most bacillopeptidase preparations, detectable quantities of the alternate bacillopeptidase were present.

MATERIALS AND METHODS

Bacillus subtilis products

Crystalline preparations of SPA (Lot Nos. 70424 and 90919) and SPB (Lot No. 101), herein designated bacillopeptidase A-1 (BPA-1) and bacillopeptidase B-1 (BPB-1), respectively, were received as gifts from Gerda Madsen of Novo Industri A/S, Copenhagen. Purified alkaline proteinase (P136624), neutral proteinase (P136700, F-3), and α -amylase (NED, P26901) were kindly provided by Sidney G. Clark of Monsanto Co., St. Louis (the alkaline proteinase was found to cross-react with crystalline SPB in Ouchterlony analysis). These preparations are referred to as BPB-2, NP, and AM-1, respectively. Results of standard assays of the enzyme activities were supplied with the preparations. BPB-2 contained 94,000 units of proteinase per gram at pH 7.0, 5,780,000 units of proteinase per gram at pH 10.3, and 1,400 units of amylase per gram at pH 6.0. NP contained 8,100,000 units of proteinase at pH 7.00, 184,000 units proteinase at pH 10.3 and 217,000 units amylase at pH 6.0. Corresponding values for AM-1 were 144,000 units of proteinase at pH 7.0, 20,000 units of proteinase at pH 10.3, and 6,700,000 units of amylase at pH 6.0. Crystalline bacterial protease type VIII (SPA, Lot No. 28B-2340) and bacterial protease type VII (subtilisin, P-5255, Lot No. 60C-1150) were purchased from the Sigma Chemical Corp., St. Louis; they are designated as BPA-2 and BPB-3, respectively. Crystalline α -amylase (Lot No. 08001) and the bacillopeptidase BPN' (Lot No. 08020) were purchased from Nagase & Co., Ltd., Omaha, Amagasaki, Japan, and are referred to as AM-2 and BPB-4, respectively. *B. subtilis* preparations which are used commercially and added to detergents were from two separate sources: bacillopeptidase A was found to be the predominant alkaline proteinase in one, which is designated "nonpurified preparation A" (NPPA); the other contained bacillopeptidase B as the principal alkaline proteinase and is termed NPPB.

Preparation of antisera

Rabbits were immunized by the subcutaneous injection of antigen preparations generally incorporated into complete Freund's adjuvant. The antiserum designated AS13 was obtained from a rabbit immunized 3 times with 150 mg. of NPPB. AS21 was from a rabbit immunized 2 times with 150 mg. of NPPB. AS16 was an antiserum pooled from two rabbits immunized with NPPA. AS125 resulted from immunization with Sephadex G-50-purified BPA-1. For the preparation of AS30, immunization was with 24 isolated immunoelectrophoresis (IEP) arcs formed between NPPB and AS13. AS1001 resulted from 3 courses of immunization of a rabbit with 60 isolated precipitin lines formed in double-diffusion reactions between BPB-1 and AS13. The antisera used were chosen from a larger number because they allowed the detection of the largest number of antigenic components in *B. subtilis* products (AS13, AS21, and AS16) or, alternatively, because of the apparent complete or almost complete specificity for an individual antigenic component (AS125, AS30, and AS101). Antisera were concentrated approximately threefold by two cycles of ammonium sulfate precipitation.

Immunologic techniques

Tests by the Ouchterlony technique were performed in duplicate in 1.5 per cent agar (Difco Labs., Detroit, Mich., special agar Noble) in Petri dishes. IEP was performed in 1.5

per cent agar or one per cent agarose in veronal buffer, pH 8.2, on microscopic slides placed in a 6 slide holder (Gelman Instrument Co.). In some instances, a modification of the IEP procedure was used wherein antigen preparations were added to wells in the agar after electrophoresis (Fig. 1). All gel diffusion tests were stored at 4° C. until read.

BPA-1, BPB-1, and AM-2 were used as reference standards to determine the relative concentrations of these proteins in the other preparations by the single radial diffusion method.¹¹ To test the possibility that the enzymatic activities of the preparations might alter the results in single radial diffusion tests, small quantities of bacillopeptidase were mixed with concentrated preparations of the alternate bacillopeptidase. The small quantities were detected in appropriate concentrations in single radial diffusion tests, indicating that under the conditions of study the tests were not invalidated by the proteolytic activities of concentrated enzyme solutions.

Double-diffusion and single radial diffusion reactions employing specific antisera, normal rabbit sera, or immune rabbit sera to nonrelated antigens were compared to confirm the specificities of precipitation reactions observed.

Enzymatic techniques

Proteinase and amylase activities in precipitin lines were demonstrated by the use of casein and starch substrates, respectively, in an "imprint" technique.¹² For the preparation of casein dishes, a mixture of 9 ml. of 0.2M Tris buffer, pH 8.7, 1.0 ml. of 0.01 per cent Ca acetate, pH 6.0, and 2.0 ml. of a 2 per cent purified casein (36-005, Lot No. 5, Miles Laboratories, Kankakee, Ill.) solution¹³ was heated to 55 to 60° C. This was mixed with 10 ml. of 3 per cent agar in saline previously adjusted to 55 to 60° C. and poured into Petri dishes. Starch dishes were prepared by the addition of 10 ml. of 3 per cent agar to 1 ml. of hydrolyzed starch, one per cent (Connaught Medical Research Laboratories, Toronto), which had been boiled and added to 9.0 ml. of Tris buffer, pH 7.4.

Enzyme activity in precipitin lines was demonstrated by the careful application of dried preparations of washed precipitin patterns in agar to the top of casein or starch-containing agar in Petri dishes. The dishes were incubated at 37° C. for 10 to 20 minutes. The precipitin patterns were then peeled off and returned to the original Petri dishes. Casein dishes were then treated with a methanol-acetic acid fixative and stained with amido black. Starch plates were stained with a 0.03 per cent iodine solution containing 0.3 per cent potassium iodide. A clear line in the appropriate location seen against a blue-colored background of stained casein or starch indicated the presence of the corresponding enzyme activity in precipitin lines.

As a qualitative test for the presence of enzyme activity in solutions, antigen preparations in concentrations of 5 mg. per milliliter or less were placed in wells cut directly in starch or casein plates.⁹ These were left at 37° C. for 10 to 20 minutes and then stained. A clear zone surrounding the well in the stained plates indicated the presence of corresponding enzyme activity.

RESULTS

Serial dilutions of the antigen preparations were used in double-diffusion reactions with the various antisera to determine optimal conditions for the identification of the maximum number of lines of precipitation (Table I) and the demonstration of antigenic relationships between individual lines of precipitation. In double-diffusion tests with serial dilutions of the bacillopeptidase and α -amylase preparations, single lines of precipitation observed at higher concentrations were often totally or partially split at lower concentrations. Preparations with a single antigenic component demonstrable in IEP reactions with a particular antiserum often exhibited splitting of precipitin lines in double-diffusion tests with the same antiserum. In addition, in tests with a variety of antisera and with a variety of antigen preparations in adjacent wells, it was

TABLE I. Lines of precipitation formed in reactions between the various *B. subtilis* enzyme preparations and selected antisera

Enzyme preparations	Antisera					
	AS13	AS21	AS16	AS125	AS30	AS1001
NPPA	2*	2	≥ 4 †	<u>1</u>	1	1
BPA-1x‡	1	ND§	<u>1</u>	<u>1</u>	0	1
BPA-2x	1	1	<u>1</u>	ND	0	1
NPPB	≥ 5	≥ 4	2	1	1	<u>1</u>
BPB-1x	<u>1</u>	<u>1</u>	1	1	0	<u>1</u>
BPB-2	<u>4</u>	<u>3</u>	ND	0	0	<u>1</u>
BPB-3x	<u>4</u>	<u>4</u>	ND	1	1	<u>1</u>
BPB-4x	<u>4</u>	<u>4</u>	ND	1	1	<u>1</u>
NP	ND	<u>3</u>	ND	1	1	<u>1</u>
AM-1	≥ 3	2	1	0	1	<u>1</u>
AM-2x	ND	2	ND	1	1	1

*The number of lines of precipitation formed in double-diffusion reactions is recorded. Additional evidence of the multiplicity of the antigenic components is provided in the text.

†Underline indicates that the antigen preparation was reacted with an antiserum resulting from immunization with an antigen which contained the corresponding bacillopeptidase.

‡x Indicates a crystalline preparation.

§ND = Not done.

not possible to demonstrate the presence of multiple antigens as an explanation of the concentration-dependent split lines. They are, therefore, expressed as single lines in Table I. The multiplicity of antigenic components in the preparations outlined in Table I were confirmed in IEP tests with many of the antisera, and the numbers of lines recorded are taken to indicate the minimum number of antigenic components present. Fig. 1 illustrates an IEP pattern which demonstrates the presence of five separate antigen components in NPPB.

In reactions of the industrial preparations NPPA and NPPB with corresponding antisera, four or more lines of precipitation formed. The heterogeneity of some crystalline bacillopeptidase preparations illustrated in Table I is particularly noteworthy. There were four well-defined lines of precipitation between AS13 and BPB-3 and BPB-4. High concentrations of antigen preparations were generally required to demonstrate the presence of components which reacted with rabbit antisera formed in response to preparations of the alternate bacillopeptidase. Thus, a single line was formed by the reaction of AS13 with 0.15 to 2.5 mg. per milliliter of solutions of PBP-1 or 50 to 100 mg. per milliliter solutions of BPA-1.

Proteinase- and α -amylase-containing lines of precipitation were identified by the "imprint" technique. Fig. 2 illustrates a test for the presence of amylase in precipitin lines formed by the reactions of four antigen preparations with AS30, an antiserum to α -amylase. In separate Ouchterlony tests, α -amylase was found to be present in NPPA and AM-1 but not in BPA-1 or BPA-2. In this particular test, the imprint illustrates more clearly than does the Ouchterlony pattern the presence or absence of α -amylase in the proteinase preparations.

Table II illustrates the results of qualitative tests for the presence of proteinase and amylase activities in the individual antigen preparations. The result of assays of concentrations of enzyme components of the preparation are also

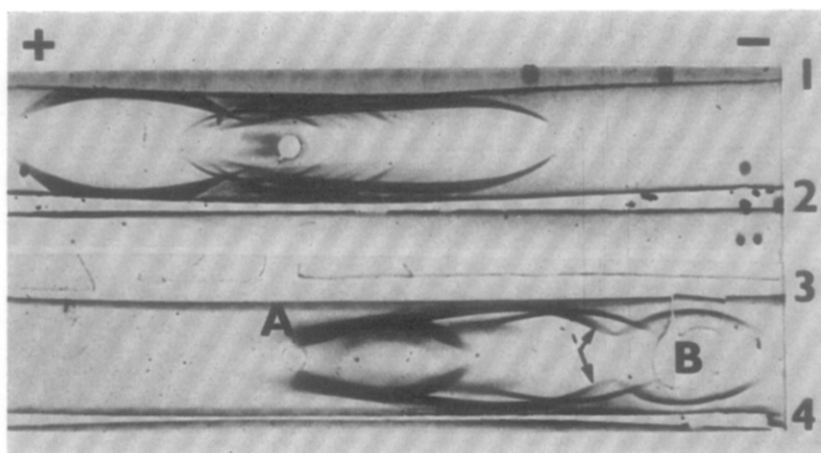


FIG. 1. Immunoelectrophoresis of whole human serum and NPPB: Whole human serum was placed in the upper well and NPPB in the lower well. Following electrophoresis, antiserum to whole human serum was placed in Troughs 1 and 2, AS13 was placed in Troughs 3 and 4, and the crystalline bacillopeptidase B preparation, BPB-1, was placed in the well at B. Five individual antigenic components of NPPB are readily identified. A is adjacent to a dense anionic arc which contained α -amylase. The arrows point to BPB arcs which can be seen to fuse with the arcs of precipitation formed by the BPB-1 placed in Well B.

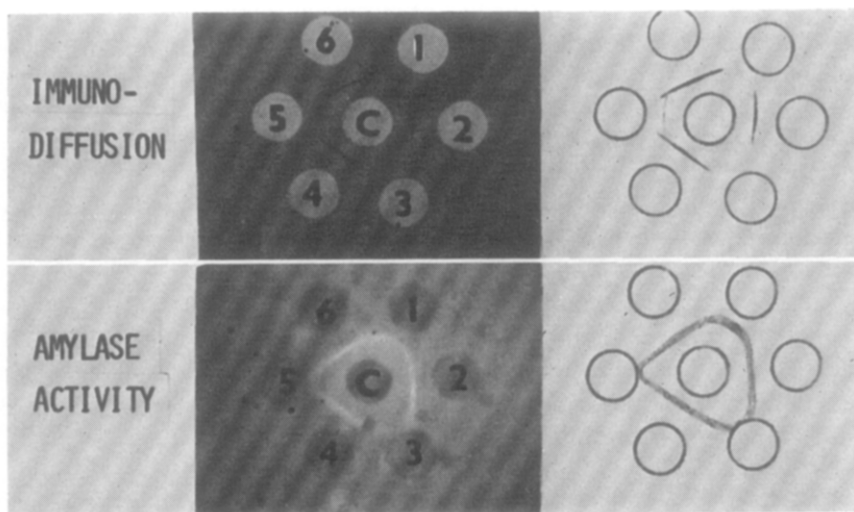


FIG. 2. Amylase activity in precipitin lines containing α -amylase: A double-diffusion test is at upper left and the corresponding starch plate demonstrating amylase activity in the precipitin lines is illustrated at lower left. The patterns are illustrated in diagrams on the right. The wells were filled as follows: Well C—AS30, an antiserum to α -amylase; Well 1—NPPA, a solution formed by the addition of 2 Gm. of NPPA per milliliter of buffer; Wells 2, 4, and 6—AM-1, 0.15 mg. per milliliter; Well 3—BPA-1, 100 mg. per milliliter; Well 5—BPA-2, 100 mg. per milliliter. The presence of amylase in precipitin lines is demonstrated. The barely visible line opposite Well 5 contained neither amylase nor proteinase. The presence of α -amylase in NPPA and absence in BPA-1 and BPA-2 are more clearly demonstrated by the starch plate than by the precipitin pattern.

TABLE II. Enzyme components identified in *B. subtilis* products

Enzyme preparations	Presence of caseinolytic activity*	Presence of amylase activity†	Identified components (%)		
			BPA	BPB	α -Amylase
NPPA	Yes	Yes	14.8‡	< 0.001	< 0.001
BPA-1x§	Yes	No	Ref	< 0.04	UD
BPA-2x	Yes	No	100.0	< 0.04	UD
NPPB	Yes	Yes	< 0.001	18.0	6.1
BPB-1x	Yes	No	6.0	Ref	UD
BPB-2	Yes	No	UD	48.0	UD
BPB-3x	Yes	Yes	0.2	93.0	0.8
BPB-4x	Yes	Yes	2.2	88.0	0.1
NP	Yes	Yes	UD	17.0	0.3
AM-1	Yes	Yes	UD	0.6	72.0
AM-2x	Yes	Yes	0.05	1.4	Ref

*Qualitative test for proteinase activity.

†Qualitative test for amylase activity.

‡The estimate of the percentage content, by weight, of individual enzymes in the *B. subtilis* preparations are based upon comparisons with reference preparations (Ref) in single radial diffusion assays.

§x Indicates a crystalline preparation.

||UD indicates that the enzyme was undetectable in the preparation with the methods used.

recorded. The presence of these components in the preparations was confirmed by the use of purified antigen for reference in Ouchterlony and IEP tests and, in most instances, by tests for the presence of corresponding enzyme activity in the precipitin lines. Two of the three crystalline BPB preparations contained α -amylase, which was detected immunologically and enzymatically. An unexpected finding was the observed presence in all five crystalline bacillopeptidase preparations of small quantities of the alternate bacillopeptidase (Table II). Crystalline BPA preparations contained less than 0.04 per cent BPB by weight; BPA comprised 0.2 to 6 per cent by weight of BPB preparations.

DISCUSSION

All enzyme-containing preparations examined in this study contained multiple components. As many as five antigenic components could be detected in crystalline products. BPB-3 produced four lines of precipitation in reactions with AS13 and AS21 (Table II). The presence of this multiplicity of components was confirmed by comparisons with other preparations and by IEP procedures. The additional presence of BPA in trace quantities in BPB-3 was observed with an antiserum to BPA (Table II). *B. subtilis* enzyme preparations are produced for commercial purposes by the growth of selected strains of the organisms on grain media. The possible origin from the culture media of some of the antigenic components of the products tested cannot be excluded. An additional potential source of the observed antigenic components is the enzyme-dependent production of degradation products. If this was the case, they nevertheless appeared to be antigenically distinct on immunoelectrophoresis. Regardless, the use, for the purpose of reference, of numerous purified enzyme preparations from different sources and the demonstration of appropriate enzyme activities in precipitin lines provided positive identification of α -amylase and the bacillopeptidases.

The source(s) of the unexpected presence of trace quantities of the alternate bacillopeptidase in the enzyme preparations tested is not known. A cross-contamination in the laboratory or during the course of production cannot be excluded. Preparations which were received following the initial observations were handled with special care to reduce the possibility of cross-contamination within our laboratory. Berson and Yalow¹⁴ have commented on an observed cross-contamination during the production of preparations of bovine and porcine insulin used in early studies of immunologic responses to insulin therapy. The presence of the alternate bacillopeptidase in five crystalline proteinase preparations from a total of three different sources suggests that this was not the result of accidental contamination.

An absence of cross-reactivity between BPA and BPB in double-diffusion precipitation tests but the presence of partial cross-inhibition of enzyme activity by rabbit antisera have been noted previously.³ No evidence of cross-reactivity between BPA and BPB were observed in the present studies. Hyperimmunization with *B. subtilis* alkaline proteinase preparations might, however, result in the stimulation of the formation of antibodies to trace quantities of the contained alternate proteinase. The observed cross-inhibition previously noted might have been the result of the unrecognized presence of antibodies to the homologous proteinase. An alternate explanation for the inhibition of a bacillopeptidase by antiserum to the alternate bacillopeptidase is the nonimmunologic inhibition of *B. subtilis* alkaline proteinases by serum components.⁹

It is evident that industrial workers are exposed to *B. subtilis* products which contain numerous antigenic components. In skin tests of sensitized industrial workers with NPPA, NPPB, and the purified products BPB-2, NP, and AM-1, there was a diversity in the spectra of cutaneous responses.¹⁵ Thus a number of the antigenic components contained in *B. subtilis* products appear to contribute to the sensitization.

Prausnitz-Küstner activity to the crystalline preparation BPA-1 and a binding of labeled BPA-1 by antibodies have been observed in the sera of some sensitized subjects.¹⁵ Since the estimated content of BPB in BPA-1 was 0.04 per cent by weight, it is unlikely that the observed antibody activity had a specificity for BPB. In contrast, a similar use of BPB-1 which contained 6 per cent BPA might well result in the detection of antibodies with BPA specificity. These considerations illustrate the caution that is required in the interpretations of investigations which utilize these bacterial products.

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