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Nature and Properties of a Cytolytic Agent Produced by *Bacillus subtilis*

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SUMMARY

The substance responsible for lysis of erythrocytes by cultures of *Bacillus subtilis*, designated subtilysin, was purified. It contained a peptide of leucine, aspartic acid, glutamic acid and valine and probably a lipid. Subtilysin was activated by Mg^{2+}, Mn^{2+} and Ca^{2+}. The rate of haemolysis was abruptly increased by chilling the reaction mixture. Haemolysis was inhibited by normal sera; the most inhibitory serum fractions contained α- and β-globulins. Haemolysis was inhibited by low concentrations of phosphatidylcholine, phosphatidylinositol, phosphatidic acid and sphingomyelin. Subtilysin possessed antibiotic properties and lysed protoplasts and spheroplasts derived from several bacterial species; subtilysin was identical with surfactin, a peptidelipid from *B. subtilis* cultures that inhibits fibrin clot formation. Kakinuma and co-workers found surfactin to be a heptapeptide having an N-terminal glutamic acid in amide linkage with the carboxyl group of 3-hydroxy-13-methyl-tetradecanoic acid. Surfactin (subtilysin) possesses some properties in common with two other cytolytic agents of bacterial origin, namely, staphylococcal δ-toxin and streptolysin S.

INTRODUCTION

In an earlier study a series of aerobic sporogenic bacilli was surveyed for production of haemolysin active against rabbit erythrocytes (Bernheimer & Grushoff, 1967). Among the species examined, broth cultures of *Bacillus cereus*, *B. alvei* and *B. laterosporus* were found to contain relatively potent lysins that were activated by SH-compounds and that belonged to the group of oxygen-labile haemolysins of which streptolysin O is the prototype (Bernheimer, 1970). *B. subtilis* produced a haemolysin which appeared to differ from these. What can be assumed to be the same lysin of *B. subtilis* was studied by Büsing (1950) and later by Williams (1957), who found the active moiety had the solubility properties of an organic acid. To establish more exactly its nature and properties, we have re-investigated this lytic agent; it is provisionally designated 'subtilysin'.

METHODS

Organism. Bacillus subtilis QMB 1228 was supplied by Dr H. S. Levinson.

Reagents. Phosphatidylcholine and diphosphatidylglycerol were bought from Sylvana Co. (Milburn, N.J.); phosphatidylserine, phosphatidylethanolamine, sphingomyelin and cerebrosides from Applied Science Laboratories, Inc. (State College, Pa.); phosphatidic acid from General Biochemicals (Chagrin Falls, Ohio); phosphatidyl-

inositol and serum fractions from Nutritional Biochemicals Corp. (Cleveland, Ohio); cholesterol from Matheson, Coleman and Bell (East Rutherford, N.J.).

Measurement of haemolytic activity. In the early part of the investigation, capacity of subtilysin to lyse washed rabbit erythrocytes was measured as for staphylococcal α-toxin (Bernheimer & Schwartz, 1963) but with 0·1 % (w/v) gelatin instead of bovine serum albumin. This method utilized phosphate-buffered saline solution and an incubation period of 30 min. at 37°; later, the method was altered by substituting for phosphate buffer, tris buffer + Mg and by incubating the mixtures of lysin and cells for 30 min. at 37° followed by 30 min. in an ice-bath. These changes resulted in titres about three times as great as those obtained under the conditions first used. In the method finally adopted, test preparations were diluted in 0·84 % (w/v) NaCl (buffered at pH 7·2) with 0·01 M-tris containing 0·1 % (w/v) gelatin + 0·01 M-$MgCl_2$. To 1 ml. of each of a series of dilutions increasing in about 30 % steps was added 1 ml. of a twice-washed suspension of rabbit red blood cells. The medium in which the cells were washed and suspended was 0·84 % (w/v) NaCl buffered at pH 7·2 with 0·01 M-tris. The concentration of the red cell suspension (about 0·7 %, v/v) was adjusted so that a sample, after lysis with saponin and addition of an equal volume of diluent, gave a spectrophotometric (Zeiss) extinction of 0·8 with light of 545 nm. and a light path of 1 cm. The mixtures of subtilysin dilutions and red blood cells were put in a 37° water-bath for 30 min., removed to an ice-bath for 30 min. and then centrifuged briefly. The colour of the haemoglobin in the supernatant fluids was compared visually with standard haemoglobin solutions, and the dilution haemolysing 50 % of the red cells in the suspension determined by interpolation. A unit of subtilysin is defined as that amount which liberates half the haemoglobin in the test red cell suspension under the conditions stated.

Production of subtilysin. In preliminary experiments the quantity of subtilysin produced in cultures utilizing a variety of media was estimated by titrating culture supernatant fluids for haemolytic activity; stationary and shaking cultures were used. The casein basal medium of Knight & Proom (1950) supplemented with a small amount of yeast extract gave satisfactory titres of subtilysin. 10 g. of Casamino acids (Difco Laboratories, Detroit, Michigan), 5 g. KH_2PO_4, 1 g. NaCl, 100 mg. tryptophan and 10 mg. cystine were dissolved in a litre of water, and the mixture adjusted to pH 7·0, the medium brought to a boil, filtered through paper, and distributed in 50 ml. amounts/250 ml. Erlenmeyer flask. Sterilization was at 123° for 20 min. To each flask was added 0·13 ml. 20 % (w/v) solution of yeast extract (Difco Laboratories, Detroit, Michigan). Growth and subtilysin formation in this medium are illustrated by the data of Table 1 derived from flasks which received an inoculum of 5 ml. and incubated at 37° in a shaking water-bath. Within limits of error of measurement the haemolytic activity paralleled the degree of growth. Addition to the medium of glucose, sodium ribonuclease or serum did not yield significantly higher titres.

For routine production of subtilysin a seed culture was prepared by adding a loopful of growth from an agar plate to 50 ml. medium and incubating in a shaking water-bath to an extinction of about 1·0 at 650 nm. Eight flasks containing 50 ml. medium were each inoculated with 1 ml. of a 10^4 dilution of seed culture and incubated in a shaking water-bath at 37° for 17 h. Supernatant fluids of the cultures usually contained 20 to 40 subtilysin units/ml.

RESULTS

Purification of subtilysin

Unless otherwise noted purification was at room temperature. The combined supernatant fluids from a series of cultures (stage 1, Table 2) were concentrated seven- to tenfold by pervaporation through Cellophane sacks. The decrease in volume was accompanied by formation of a precipitate (stage 2) which was collected by centrifuga-

Table 1. *Growth of Bacillus subtilis and course of appearance of subtilysin*

Time of incubation (hr)	Growth as extinction at 650 nm.	Subtilysin in culture supernatant fluid*
4	0·62	4
7	2·1	13
16	3·5	18
24	5·2	23

* Units of haemolytic activity/ml. obtained by titrations utilizing phosphate-buffered saline and incubation time of 30 min.

Table 2. *Purification and recovery of subtilysin*

	Volume (ml.)	Total units of subtilysin	Recovery of activity (%)
Stage 1. Culture supernatant fluid	1,055	35,000	100
Stage 2. Precipitate from pervaporation	134	35,000	100
Stage 3. Acid precipitate	30	15,000	43
Stage 4. Salt precipitate	30	18,300	52
Stage 5. Dialysed and lyophilized product, 234 mg.	—	20,100	57

tion and which contained virtually all the subtilysin activity. The supernatant fluid, containing less than 10 units of subtilysin/ml. was discarded. The precipitate was washed twice with 30 ml. 10 % (w/v) NaCl and then dissolved in 30 ml. distilled water. A small amount of insoluble material was removed by centrifugation, and the supernatant fluid was adjusted with N-HCl to pH 4·0. The precipitate which formed was washed twice with 30 ml. 0·1 N-acetate buffer (pH 4), and then suspended in 30 ml. 0·05 N-tris (pH 7·5). Sufficient NaOH was added to the mixture to bring to slightly above neutral pH and solution was left to occur overnight in the cold (stage 3).

A small amount of insoluble material was removed by centrifugation, and solid NaCl was added to 10 % (w/v). After standing 60 min. the mixture was centrifuged for 10 min. at 10,000 rev./min. The opalescent supernatant fluid, which contained 50 units subtilysin/ml., was discarded. The precipitate was stirred with 30 ml. 0·05 N-tris (pH 7·5) allowing several hours for solution to occur (stage 4). The solution was dialyzed at 4° against two changes of distilled water (1800 ml.) over about 20 hr, and then freeze-dried. A white solid (234 mg.) was obtained (stage 5). This product had a specific activity of 86 haemolytic units/mg. The specific activities of a series of such products varied from 55 to 95 haemolytic units/mg. Further purification was

attempted by iso-electric focusing and by use of a Sephadex G-100 column. Upon iso-electric focusing, subtilysin precipitated as it moved into the region pH 4 to 5; in Sephadex no further increase in specific activity was achieved.

Physical and chemical properties

Subtilysin was soluble in slightly alkaline water, in ethanol, acetone and chloroform; it was precipitated from aqueous solution by 10 % NaCl (w/v) or by acidification to pH 4; it did not diffuse through Cellophane. Solutions retained their haemolytic activity at 100° for 15 min. at pH 8·2 or 9·6, but at pH 3·0 two-thirds of the activity disappeared. The haemolytic activity of subtilysin was not affected by treatment with trypsin, papain or pronase. These properties suggested that the active material was of relatively large molecular size but was apparently not a protein.

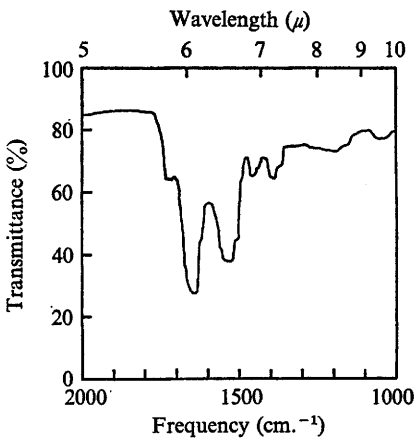

Fig. 1

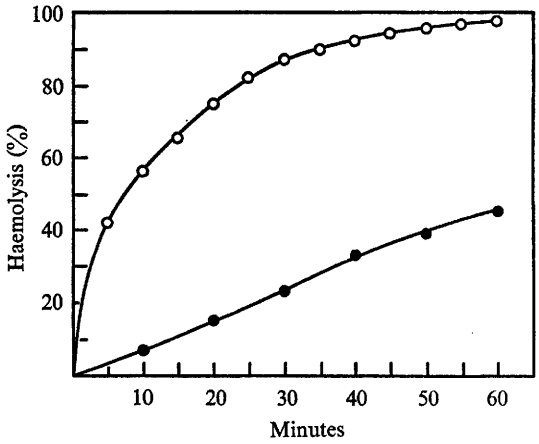

Fig. 2

Fig. 1. Infrared spectrum of subtilysin.

Fig. 2. Course of haemolysis in presence of 0·005M-$MgCl_2$ (open circles) and in absence of added Mg^{2+} (solid circles).

A solution containing 7 mg. subtilysin/ml. 0·05M-tris buffer (pH 7·5) appeared to be homogeneous in the analytical ultracentrifuge, giving a single peak having an $S_{20\,w}$ of about 1·2. Using a column of Sephadex G-100 (superfine) equilibrated with 0·03M-sodium borate buffer (pH 8·5) containing 0·1M-KCl, and calibrated with bovine serum albumin, ovalbumin and cytochrome *c*, elution volumes plotted according to Andrews (1964) gave a molecular weight of about 50,000.

Subtilysin was estimated to contain 8·8 % nitrogen (micro-Kjeldahl analysis), less than 0·3 % phosphorous (Lohmann & Jendrassik, 1926) and less than 1 % carbohydrate as glucose by the anthrone reaction (Colowick & Kaplan, 1957). When assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951) it gave a colour equivalent to 4 % of that produced by an equal weight of ovalbumin. Subtilysin after hydrolysis for 22 hr at 110° in 6·5N-HCl yielded a ninhydrin colour equivalent to 75 % of that given by an equal weight of ovalbumin.

Analysis for amino acids (Blackburn, 1968) showed leucine, aspartic acid, glutamic acid and valine in molar ratio near 4:1:1:1. Infrared absorption (KBr) yielded a

spectrum (Fig. 1) consistent with lipid as well as of a peptide. The presence of lipid could also account for the solubility properties, the nitrogen content and the low sedimentation coefficient in relation to molecular size as determined by gel filtration. The formula weight of subtilysin is 1036; therefore the material formed aggregates of large size. Consistent with this was a tendency for subtilysin to give solutions that were opalescent.

Haemolysis and inhibition of haemolysis

Divalent cations. The following experiment shows the effect of $MgCl_2$ on haemolysis. 15 ml. tris-buffered saline (0·84 %, w/v, NaCl at pH 7·2 with 0·1 M-tris) containing 35 haemolysin units was mixed with 15 ml. 0·7 % (v/v) washed rabbit erythrocytes in tris-buffered saline; both solutions were pre-warmed to 37°; a similar mixture was prepared with 0·005 M-$MgCl_2$ present. Samples were taken at intervals and immediately centrifuged, and the percentage haemolysis estimated from light absorption readings at 545 nm. The results (Fig. 2) show that Mg substantially increased the rate of lysis. A similar effect was produced when Ca or Mn were substituted for Mg.

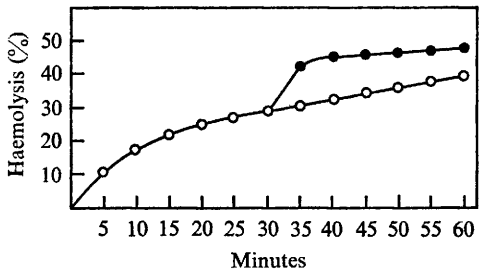

Fig. 3. Effect of chilling on course of haemolysis. Open circles: reaction mixture at 37° throughout: solid circles: reaction mixture at 37° for 30 min. and then chilled.

'*Hot–cold*' *effect.* The experimental conditions were like those described, but the concentration of subtilysin was about two-thirds as great, and 0·005 M-$MgCl_2$ was in each of two flasks of reaction mixture: one was at 37° for the duration of the experiment: the other was at 37° for 30 min. and then chilled in an ice-bath. The results (Fig. 3) show that chilling increased abruptly the rate of lysis.

Source of erythrocytes. Assays of the haemolytic activity of a single preparation of subtilysin with erythrocytes from four different rabbits agreed within 10 %; the sensitivity of erythrocytes derived from man and guinea pig resembled that of rabbit cells. About four times as much subtilysin was required to produce 50 % lysis of erythrocyte suspensions derived from calf, sheep and goat as for rabbit erythrocytes. The sensitivity of red cells from horse, pig, cat and chicken was less than that of rabbit cells but greater than that of cells from calf, sheep and goat.

Inhibition of haemolysis by serum and serum fractions. The capacity of serum to inhibit the haemolytic activity of subtilysin was assayed by mixing decreasing concentrations with a fixed amount (3 haemolysin units) of subtilysin, allowing the mixtures of 1 ml. each to stand for 10 min. at 20°, and then adding 1 ml. 0·7 % (v/v) washed rabbit red cells. After 30 min. at 37° followed by 30 min. in an ice-bath, the mixtures were centrifuged briefly and the haemoglobin in the supernatant fluids estimated colorimetrically at 545 nm. The tests were done in tris-buffered saline containing

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