Production and Purification of the Metalloprotease of *Bacillus polymyxa*

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Received for publication 27 February 1973

The nutritional and environmental factors relating to the production of an extracellular protease by *Bacillus polymyxa* were investigated. The enzyme was produced in all media that supported growth of the microorganism, irrespective of the carbon source used. Arabinose and hydrolyzed starch, however, gave highest yields. The nature of the peptone had a significant effect on the level of protease produced. Calcium and manganous ions exerted a beneficial effect on protease production. Highest enzyme levels were obtained when the initial pH of the medium was within the range 5.9 to 7.0. When the pH of the medium was not controlled during the fermentation, the accumulation of the enzyme paralleled the growth of the microorganism and reached a maximum towards the end of the exponential phase. With a fixed pH of 6.8, the level of protease was only one-fifteenth of that obtained when the culture was allowed to maintain its own pH. In addition, accumulation of the protease reached a maximum somewhat earlier, i.e., in the mid-log phase of growth. A 70-fold increase in the specific activity of the protease was obtained by ammonium sulfate and acetone fractionation followed by gel filtration on Sephadex G-100. The purified protease behaved as a homogenous entity when eluted by a sodium chloride gradient from CM-cellulose at pH 6.9. An overall enzyme recovery of 60% was obtained.

Proteolytic enzymes have many uses in medicine and in the food and other industries (10, 16, 25). New applications are being found, thereby creating a greater demand for such enzymes. Many of the proteases studied to date have at least some of the properties required for particular applications. However, the production and characterization of proteolytic enzymes from new species or strains of microorganisms is constantly being investigated.

*Bacillus polymyxa* produces a starch-degrading enzyme that yields maltose in very high yield (23, 24, 27). In the course of investigations relating to the production of this enzyme (13), it was observed that this microorganism produced considerable quantities of an extracellular protease in a basal starch-peptone medium. Numerous studies have been carried out on different aspects of the proteolytic enzymes elaborated by various species of the genus *Bacillus* (1, 4, 9, 15, 19–21; K. Aunstrup, O. Andresen, and H. Ouhrup, German Patent 1,800,506). As there have been no reports relating to the production and properties of the protease of *B. polymyxa* and in view of the fact that the enzyme was produced simultaneously, in high yield, with the amyloglucosidase, it was decided to investigate various aspects of this extracellular protease. In this paper we report on some of the factors affecting the production and purification of the enzyme. A preliminary account of this work has been reported (11).

MATERIALS AND METHODS

**Materials.** Sephadex G-100 was obtained from Pharmacia (G.B.) Ltd., London. CM-cellulose ion-exchange resin was purchased from Sigma Chemical Co. (London). The azo-casein was supplied initially from Mann Research Laboratories and later from Schwarz/Mann, Orangeburg, N. Y. Folin and Ciocalteau reagent was a product of BDH Chemicals Ltd., Poole, England. Hydrolyzed starch was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, and soluble starch from May and Baker Ltd., Dagenham, England. All the salts used were "Analar" grade reagents.

**Medium.** As a result of an examination of the nutritional aspects of the organism, the following medium was devised for production of highest levels of protease activity. It consisted of, in grams per liter: KCl, 1.0; MgCl₂·6H₂O, 0.2; NaH₂PO₄, 5.4; Na₂HPO₄·2H₂O, 7.0; CaCl₂·2H₂O, 0.25; FeSO₄·7H₂O, 0.0006; MnSO₄·4H₂O, 0.001; bacteriological peptone
(Oxoid L37), 10.0; and soluble starch, 10.0. The initial pH of the medium was 7.0 except where otherwise stated.

**Cultivation conditions.** B. polymyxa NCIB 8158 was streaked on nutrient glucose agar slopes in Bijou bottles and incubated at 30 C for 2 days and then stored at 4 C as stock cultures.

Inocula were grown in 250-ml Erlenmeyer flasks containing 50 ml of nutrient glucose broth in a Gallenkamp orbital shaker at 30 C and 150 rpm for 18 h. The cells were collected by centrifugation, washed in saline, and diluted to give an optical density of about 10 at 600 nm in an EEL spectrophotometer. A 2% (vol/vol) inoculum was used in all experiments.

Experiments with shake-flask cultures were grown under similar conditions to those outlined above.

Fermentor cultures were grown in a 5-liter batch fermenter (L. H. Engineering Ltd., Stoke Poges, Bucks, England), in 3-liter working volumes, at 30 C. The pH was controlled, where necessary, by using 2 N NaOH and 2 N HCl. Foam was suppressed by the addition of antifoam A (Sigma Chemical Co.).

**Enzyme assay.** Proteolytic activity was determined by a slight modification of the method of Charney and Tomarelli (5). A 2.5% (wt/vol) solution of azo-casein was prepared in 0.02 M tris(hydroxymethyl)ammoniummethane (Tris)-hydrochloride buffer, pH 7.0. A 0.2-ml amount of the enzyme was added to 1.0 ml of the azo-casein solution after equilibration in a water bath at 37 C. The enzymatic reaction was stopped after 30 min by the addition of 8.0 ml of 5% (wt/vol) trichloroacetic acid. After standing for 10 min, the mixture was filtered through a Whatman no. 1 filter paper. A 0.5-ml amount of 0.5 N NaOH was added to 5.0 ml of this filtrate, and the color intensity was measured at 440 nm. A blank was prepared by adding the trichloroacetic acid to the substrate prior to the addition of the enzyme solution. One unit of activity was defined as the activity which caused an increase in optical density (OD) of 0.1 OD unit per ml of enzyme under the conditions of the test.

Protein was measured using the Folin-Ciocalteau reagent (17) and expressed as casein equivalents.

**Measurement of biomass.** Biomass in shake-flask cultures was measured at 600 nm in an EEL Spectra colorimeter by using a sample of the uninoculated growth medium as a blank. In the batch fermenter studies the cells were collected by centrifugation, washed in saline, and dried to a constant weight at 80 C. An OD measurement of 70 is equivalent to 3.6 mg of cells (dry wt) per ml.

**Protease purification.** All purification procedures were carried out at 0 to 4 C. The protease in the cell-free supernatant fluid of the culture liquor was precipitated by the slow addition of ammonium sulfate to a saturation level of 65%. After 2 h the precipitate was removed by centrifugation and suspended in a minimum volume of 0.02 M Tris-hydrochloride buffer, pH 7.0. It was dialyzed against two changes of 52 liters of the same buffer over 18 h. The precipitate, which formed during dialysis, was removed by centrifugation and discarded. The supernatant fluid was then assayed for enzyme activity, and the protein content was determined. After the ammonium sulfate precipitation step, the resulting enzyme solution was then treated by the slow addition of 2 vol of acetone (which had been previously cooled to –20 C). The precipitate that formed was collected by centrifugation and resuspended in 0.01 M Tris-hydrochloride buffer, pH 7.0, and dialyzed for 6 h against the same buffer.

The active protein preparation was further fractionated by gel filtration. The protein was applied to a column (2.5 by 80 cm) of Sephadex G-100 which had been equilibrated previously with 0.02 M Tris-hydrochloride buffer, pH 7.0. Elution of the protein was carried out with the same buffer. Fractions (5.0 ml) were collected.

Ion-exchange chromatography of the protease from the acetone precipitation step was carried out on CM-cellulose ion-exchange resin. The protease solution in 0.01 M Tris-hydrochloride buffer, pH 7.0, was dialyzed against distilled water for 1 h and applied to a CM-cellulose column (2 by 20 cm), which had been equilibrated previously with 0.01 M phosphate buffer, pH 6.9. After the column was washed with the buffer, the enzyme was eluted by using a sodium chloride gradient in the same buffer. The eluant was fractionated into 20.0-ml portions.

**RESULTS**

**Protease production and nitrogen sources.** As reported previously (24), B. polymyxa grew only in the presence of complex nitrogen sources. Peptone (1% wt/vol), skim milk powder, and yeast extract, in that order, gave highest protease production. Poor enzyme yields were obtained when casein or Casamino Acids were used as nitrogen sources (Table 1). The nature of the peptone used in the medium had a significant effect on growth and enzyme production (Table 2). Oxoid L37 and Difco 0120 were shown to be the most suitable. The amino acid composition of the individual peptones may offer some explanation of these results.

**Protease production and carbon sources.**

The protease was produced with all the carbohydrates that supported growth (Table 3). Arabinose and hydrolyzed starch (1%, wt/vol) have been used in previous studies (24) to improve the yield of the enzyme. As previously stated, casein and Casamino Acids were the nitrogen sources of choice.

**Table 1. Effect of nitrogen source on protease production by B. polymyxa**

| Nitrogen source (%, wt/vol) | Biomass (OD at 600 nm) | Protease activity (U/ml) |
|---------------------------|------------------------|-------------------------|
| Peptone, 1.0              | 70                     | 1,412                   |
| Casein, 1.0               | 20                     | 250                     |
| Casamino Acids, 1.0       | 10                     | 250                     |
| Skim milk powder, 1.0     | 52                     | 860                     |
| Yeast extract, 1.0        | 48                     | 400                     |
| Yeast extract, 0.5        | 42                     | 350                     |

*The assays were carried out after an incubation period of 30 h.*
TABLE 2. Effects of various commercial peptones on protease production by B. polymyxa

| Peptone type | Biomass (OD at 600 nm) | Protease activity (U/ml) | Final pH |
|--------------|------------------------|--------------------------|---------|
| Oxoid        |                        |                          |         |
| L37 Peptone  | 70                     | 1,250                    | 5.9     |
| L41 Casein hydrolyzate (acid) | 23 | 250 | 5.7 |
| L47 Tryptone | 62                     | 1,150                    | 5.9     |
| L42 Tryptone | 60                     | 1,150                    | 6.0     |
| L49 Peptone P | 50                | 850                       | 6.1   |
| L46 Protease peptone | 45 | 650 | 5.9 |
| L40 Peptone mycolical | 70 | 1,200 | 6.2 |
| Difco        |                        |                          |         |
| 0120 Protease peptone | 70 | 1,250 | 6.1 |
| 0125 Tryptone | 62                     | 750                      | 5.7     |
| 0118 Peptone  | 62                     | 350                      | 5.6     |
| Oxoid L37 Beef extract | 50 | 600 | 5.8 |

TABLE 3. Effect of various carbon sources on protease production by B. polymyxa

| Carbohydrate 1% (wt/vol) (except where stated) | Biomass (OD at 600 nm) | Protease activity (U/ml) | Final pH |
|-----------------------------------------------|------------------------|--------------------------|---------|
| Control (no addition)                         | 30                     | 500                      | 6.1     |
| Hydrolyzed starch (1%)                        | 60                     | 1,800                    | 6.2     |
| Arabinose                                     | 76                     | 1,600                    | 6.0     |
| Hydrolyzed starch (2%)                        | 70                     | 1,500                    | 4.9     |
| Cellobose                                     | 76                     | 1,450                    | 6.4     |
| Dextrin                                       | 72                     | 1,400                    | 6.0     |
| Fructose                                      | 80                     | 1,400                    | 6.1     |
| Maltose                                       | 74                     | 1,400                    | 6.1     |
| Starch                                        | 70                     | 1,400                    | 6.0     |
| Glucose                                       | 52                     | 1,300                    | 6.1     |
| Hydrolyzed starch (3%)                        | 70                     | 1,300                    | 4.7     |
| Raffinose                                     | 75                     | 1,250                    | 6.2     |
| Lactose                                       | 75                     | 1,000                    | 6.1     |
| Xylose                                        | 70                     | 950                      | 5.9     |
| Glycerol                                      | 75                     | 750                      | 6.2     |
| Sodium acetate                                | 46                     | 300                      | 7.5     |
| Sodium citrate                                | 50                     | 250                      | 6.6     |

Glycerol gave the highest biomass; however, the yield of protease was 45% less than that obtained with arabinose.

Effect of metals on protease production. Of the metals included in the basal medium, the yield of protease was most sensitive to the levels of calcium and manganese. The presence of manganese ions caused a proportionate increase in biomass and protease production, the optimum level for maximum enzyme yield being 1 mg of MnSO₄·4H₂O per liter of medium. Similarly, the presence of calcium chloride dihydrate (0.025%, wt/vol) was found necessary for maximum enzyme production. In addition, calcium had a stabilizing effect on the enzyme in the fermentation liquor.

pH and protease production. High protease yields were obtained when the initial pH was varied within the range 5.9 to 7.0 (Fig. 1). The fall in the level of enzyme produced was more rapid when the pH was varied below 5.9 than above 7.0. Exoprotease production parallels the growth of the microorganism and reaches a maximum towards the end of the exponential phase of growth (Fig. 2). At the time of maximum enzyme yield the pH of the medium had fallen from an initial pH of 7.0 to 5.9.

Protease production in batch culture in a fermenter. As a consequence of the results obtained in shake-flask cultures and observations made during studies on amylase elaboration by this organism (13), experiments were undertaken in a batch fermenter to study the

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FIG. 1. Influence of initial pH of the culture medium on protease production. Analyses were carried out after 48 h of incubation. Symbols: Δ, biomass; ●, protease.
effect of controlled pH on protease production. An aeration rate of 600 ml per liter per min and a stirring rate of 450 rpm was established as the optimum for protease production. Controlling the pH at a fixed value during the fermentation resulted in poor yields of the protease being obtained (Table 4). When the pH was controlled at 6.8 during the fermentation, protease production reached a maximum in the mid-log phase of growth. No activity could be detected in the cell-free fluid towards the end of the exponential phase. Protease synthesis or elaboration is therefore associated with the fall in pH that occurs during the early stages of growth. The overall yield of enzyme obtained under these conditions (initially pH 6.8) was only one-fifteenth of that obtained when the culture was allowed to establish its own value.

**Purification of the protease.** Ninety percent of the protease activity together with a sevenfold increase in specific activity was obtained on recovery of the precipitate obtained at 65% ammonium sulfate saturation level. Subsequent treatment with acetone increased the specific activity a further twofold. The elution pattern of the protease from a Sephadex G-100 column after the acetone treatment (Fig. 3) indicates that most of the inactive protein was eluted in fractions 29 to 34 and yielded a 4.9-fold increase in specific activity. On rechromatography of the protease (fractions 35–43, pooled and concentrated by salt precipitation, followed by dialysis) on the same Sephadex bed, the elution pattern of the protease and protein were coincidental. A summary of the purification procedures is given in Table 5 and indicates that an overall enzyme recovery of 60% was obtained. In addition there was a 70-fold increase in specific activity compared with that in the original cell-free fluid. The enzymatically active protein from the gel-filtration process was applied to a column (2 by 20 cm) of CM-cellulose ion exchange resin. The column was washed with excess 0.01 M phosphate buffer, pH 6.9, and the enzyme was eluted using a sodium chloride gradient in the same buffer. It was recovered as a single homogeneous entity (Fig. 4). Recovery of the enzyme from this purification step was 80%. There was no increase in specific activity.

**DISCUSSION**

The effect of the nature of the nitrogen source on protease production by *B. polymyxa* is consistent with the reports on enzyme production by other microorganisms (2, 3, 6, 8, 14, 22). Cohen (6) showed that neopeptone prevented protease formation by a Group A *Streptococcus*, but yeast extract actually stimulated its production. Bovallius (2) demonstrated that production of a cholinesterase-liberating factor varied with the different commercial media. Later Bovallius and Zacharias (3) found this variation was dependent solely on the Mg²⁺ concentration in the media.

Manganous ions have been reported as being essential for protease production by *B. subtilis* (26). As the addition of manganese to the basal-starch medium caused an almost proportionate increase in biomass and protease production, it appears to have no stimulating effect on protease production per se. It could be argued, however, that the increased biomass and protease production was the result of increased protease activity giving more assimila-

**TABLE 4. Effect of controlling pH at different values on protease production by *B. polymyxa***

| pH  | 10 h | 24 h |
|-----|------|------|
|     | Biomass (g/liter) | Protease activity (U/ml) | Biomass (g/liter) | Protease activity (U/ml) |
| 5.9 | 0.09 | 100  | 1.12 | 440  |
| 6.5 | 0.11 | 130  | 1.72 | 600  |
| 6.8 | 0.14 | 150  | 2.41 | 0    |
| 7.2 | 0.12 | 50   | 2.31 | 0    |
| 7.5 | 0.12 | 0    | 2.10 | 0    |
The Secretion of proteases during various stages of growth is characteristic of many bacteria, particularly the spore-formers. This has been fairly well studied in the genus Bacillus. B. megaterium (20), like B. polymyxa, produces a protease in the log phase of growth, others like B. cereus (22) and B. subtilis (18) produce the protease in the stationary phase between growth and sporulation.

The broad initial pH optimum (5.9 to 7.1) recorded here for protease production by B. polymyxa (Fig. 1) is consistent with the observation of Davies (7) that most microbial extracellular enzymes are produced at or near the optimum pH for activity of the enzyme. The optimum pH for protease activity extends over the range 6.0 to 7.2 (12). The broad pH pattern for production and activity of the protease help to explain why, in shake-flask cultures, the rate of accumulation of the enzyme in the medium parallels growth and is not affected by change in pH. This finding is in contrast to observations on amylase production by this microorganism (13) where it was observed that amylase activity was low during the logarithmic phase of growth but rose rapidly in the culture fluid when growth ceased. In addition, it was shown that control of pH was critical to the establishment of conditions which yielded maximum levels of amylase. It is significant that calcium and manganese were the metals which exerted a beneficial effect on protease production, in the light of earlier observations on the stimulatory effect of these cations on amylase production by B. polymyxa (13).

Purification of the protease in the cell-free fluid gave an enzyme preparation which upon chromatography on Sephadex G-100 and on CM-cellulose gave a homogeneous product. The proteolytic enzymes produced by the Bacilli are either neutral, alkaline, or a mixture of both types (16). B. polymyxa is similar to B. thermo-proteolyticus, B. megaterium, and B. cereus, in that it only produces a neutral protease. The types produced by B. subtilis vary with the strain (16). In another communication we will report on the characteristics and properties of the protease of B. polymyxa (12) and demonstrate that the enzyme is a typical metalloprotease.

ACKNOWLEDGMENTS

The authors wish to thank the Irish Committee for the International Biological Programme, Academy House, Dublin, Ireland, for a grant in support of this work. They also acknowledge the skilled technical assistance of James Gray.

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