Identification of a Pea Component Stimulatory for Heat-Stressed Putrefactive Anaerobe 59-123 Spores

D. H. ASHTON
Campbell Institute for Food Research, Camden, New Jersey 08101

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Pea extract contains a factor which improves recovery counts of heat-stressed putrefactive anaerobe spores in a complex medium up to threefold. The factor is heat-stable and nondialyzable. Most of the active principle is found in the precipitate which forms during storage of pea extract at 4 C. The precipitate disperses upon heating, is high in starch content, and retains activity after extraction with organic solvents and water. Treatment of pea extract with \( \alpha \)-amylase results in complete destruction of the active principle. These observations indicate that starch is the factor in pea extract responsible for increased recovery counts of heat-stressed putrefactive anaerobe spores.

In 1937, Curran and Evans (2) reported that spores of \textit{Bacillus} species surviving a damaging heat treatment are more exacting in their nutritional requirements for germination and outgrowth than before treatment. This observation has since been extended to include spores of \textit{Clostridium botulinum} (1, 4, 9, 10, 13) and putrefactive anaerobe (PA) 3679 (5, 11–13). Thus, when determining the thermal resistance of spores, careful selection of a recovery medium is important if underestimates of survivor numbers are to be avoided. In studies with anaerobic sporeformers, which are by nature quite fastidious, many substances have been used to enrich recovery media. Popular among these additives are peas and pea extract (1, 3, 5, 12, 13). Although the enrichment value of peas has been well established the active component has not been identified. Therefore, a study of the factor from peas stimulatory for heat-stressed PA spores was undertaken with the hope that pea extract might be replaced in recovery media by a commercially available chemical. In addition, a knowledge of the nature of the stimulant may lead to a better understanding of the metabolism and early stages of heat inactivation of anaerobic sporeformers.

MATERIALS AND METHODS

\textbf{Test organism}. A suspension of PA 59-123 spores was obtained from the National Canners Association, Washington, D.C., and was stored at 4 C. Vegetative cells present in the spore suspension were inactivated by adding an equal volume of absolute ethanol and incubating for 1 hr at 25 C (6). Spores were recovered by centrifugation. Serial dilutions of stock suspensions were made in sterile water so that 0.2 ml of a heat-stressed suspension yielded between 30 and 100 colonies on unenriched recovery medium. Heat stressing was effected by immersing 10-ml quantities of the spore suspension, contained in a screw-cap tube (20 by 150 mm), in boiling water for 8 min followed by rapid cooling in an ice bath.

\textbf{Preparation and assay of pea extract}. Pea extract was prepared as described by Andersen (1), placed in either glass dilution bottles or dialysis tubing, and autoclaved for 15 min at 121 C. The response of heat-stressed spores to various concentrations of the test material was measured in PA 3679 Agar. The composition of PA 3679 Agar was 10 g of tryptone (Difco), 5 g of dextrose (Fisher Scientific Co.), 1 g of yeast extract (Difco), 3 g of beef extract (Difco), 0.5 g of sodium thioglycollate (BBL), 1.25 g of \( \text{K}_2\text{HPO}_4 \) (Fisher Scientific Co.), 8.5 g of Troy agar (Colab) and 1 liter of deionized water. This medium is T Best Agar, described by Wheaton and Pratt (12), without the soluble starch. For assay purposes, each plate contained 10 ml of medium, spores, and sterile deionized water or test substances, or both, in a total volume of 12.2 ml. Plates were incubated at 37 C for 60 hr in Case-Anaero Jars (Case Laboratories, Inc., Chicago, Ill.) containing an atmosphere of 90% nitrogen and 10% carbon dioxide (Matheson Co., Inc.). Before incubation, jars were successively (three times) exhausted to 26 inches (66 cm) of vacuum and flushed with the gas mixture to a slight positive pressure.

To determine the relative stimulatory activities of pea extract and its fractions, all preparations were adjusted with sterile water to the volume of the original pea extract. The degree of stimulation was
measured as the fold increase in recovery count caused by 1 ml, or fractions thereof, of the test substance over the count obtained with the same heat-stressed spore suspension on unenriched PA 3679 Agar.

Fractionation of pea extract. Dialysis sacs (Fisher Scientific Co.) containing 40 ml of pea extract were suspended in open flasks and autoclaved for 15 min at 121 C. Dialysate was obtained from pea extract by one of two procedures. Agitated dialysis was carried out in 2-liter quantities of sterile deionized water for 15 hr at 4 C. Sacs were transferred to a second 2-liter quantity of sterile deionized water and dialyzed for an additional 6 hr. Dialysate was concentrated to 40 ml by flash evaporation, sterilized by membrane filtration (Millipore Corp., Bedford, Mass.), and assayed for stimulatory activity. The endo-

A white, biocululant precipitate developed in pea extract during storage at 4 C. The precipitate was removed from 20 ml of extract by centrifugation at 20,000 X g for 10 min and washed twice at 4 C with 20 ml of each of the following: acetone, anhydrous ether, 85% methanol, 95% ethanol, and water. The extracted precipitate was suspended in 20 ml of sterile water, heated for dispersion, and assayed for stimulatory activity and starch content.

Starch determination. Starch concentrations were determined by a modification of the method described by McCready and Hassid (7). For standard curve construction, known amounts of corn starch (Stein, Hall & Co., Inc., New York, N.Y.) were permitted to hydrate for 10 min in 1 ml of water. A 5-ml quantity of 1 N NaOH was added, and the mixture was incubated at 55 C for 5 min. Excess NaOH was neutralized with 0.5 N HCl. The volume was diluted to 100 ml with boiling water, and the temperature was maintained at 90 to 100 C for 5 min. A 3-ml sample was added to 96 ml of water and developed with 1 ml of iodine reagent (0.2% iodine, 2% potassium iodide). Absorbance at 650 nm was measured by using a Beckman DB spectrophotometer.

Starch hydrolysis. Starch in fresh pea extract was hydrolyzed by the addition of 40 µg of α-amylase (Nutritional Biochemicals Corp., Cleveland, Ohio) per ml of extract. The reaction mixture was incubated at 25 C for 30 min. Disappearance of starch was followed by testing with iodine reagent. The reaction was terminated by autoclaving at 121 C for 15 min. Boiled enzyme added to pea extract and untreated pea extract served as controls. α-Amylase-treated and untreated pea extracts were assayed for the ability to increase recovery counts of heat-stressed PA 59-123 spores on PA 3679 Agar.

RESULTS AND DISCUSSION

Initial experiments demonstrated that boiling for 8 min reduced the colony count of PA 59-123

Table 1. Distribution of starch and stimulatory activity in fractions of a typical pea extract

| Fraction                  | Per cent | Per cent | Per cent |
|---------------------------|----------|----------|----------|
|                           | of starch| of original starch | of original stimulatory activity |
| Pea extract               | 0.60     | 100      | 100      |
| Dialyzed pea extract      | 0.60     | 100      | 100      |
| Supernatant               | 0.06     | 10       | 7        |
| Reconstituted precipitate | 0.52     | 87       | 78       |
| Extracted precipitate     | 0.43     | 75       | 57       |

Fig. 1. Effect of pea extract enrichment of PA 3679 Agar on colony count of heat-stressed (○) and unheated (●) PA 59-123 spore suspensions.

Fig. 2. Effect of α-amylase-treated (●) and untreated (○) pea extract on colony count of heat-stressed PA 59-123 spores in PA 3679 Agar.
spore suspensions on PA 3679 Agar by approximately 75% (Fig 1). Inclusion of pea extract in the medium increased the count of heat-stressed spores by as much as threefold. Concentrations of pea extract greater than 20% in PA 3679 Agar had no additional effect. Unheated spores showed no response to pea extract.

The stimulatory component of pea extract was stable to boiling for 1 hr and autoclaving. Dialysis of pea extract did not diminish its activity (Table 1). Dialysate failed to improve recovery counts, suggesting that classical germinants such as L-alanine, glucose, and salts are not responsible for the pea extract effect. The precipitate which formed in pea extract during storage at 4°C is high in starch content and contains most of the stimulatory activity. Small amounts of starch and stimulatory activity remained in the supernatant. The precipitate retained 85% of its starch content and 73% of its stimulatory capacity after washing with a variety of organic solvents and water. None of the washings possessed the ability to improve recovery counts of heat-stressed PA 59-123 spores.

The stimulatory activity of pea extract was compared with that of corn starch (Stein, Hall & Co., Inc.) and soluble starch (Difco). Maximal recovery counts were achieved with 0.15% corn starch, 0.25% soluble starch, or 20% pea extract (0.12% pea starch) present in PA 3679 Agar. Incorporation of combinations of suboptimal levels of pea extract, corn starch, and soluble starch into the recovery medium resulted in an additive effect up to a maximal count. The maximal count achieved with combinations of starches did not exceed the count obtainable with optimal additions of the individual starches.

Starch in fresh pea extract was completely hydrolyzed by the action of α-amylase. Pea extract treated with α-amylase possessed no ability to improve recovery counts of heat-stressed PA 59-123 spores on PA 3679 Agar (Fig 2). Pea extract to which boiled enzyme had been added and untreated pea extract contained high levels of starch and stimulated recovery counts of heat-stressed spores approximately threefold. Glucose and maltose, hydrolysis products of starch, had no effect on recovery counts when added to PA 3679 Agar. Thus, starch appears to be the sole component of pea extract responsible for increased recovery counts.

Starch (8, 9, 10), charcoal, or serum albumin (8, 10) are known to improve survivor counts of heat-stressed *Bacillus* and *Clostridium* spores. These additives are thought to act by adsorbing inhibitors from the medium (10). The nature of the inhibitor has not been established conclusively, but long-chain, unsaturated fatty acids (4) or their oxidation products (11) have been implicated. In the present study, slightly higher levels of corn starch and soluble starch than of pea starch were required for maximal stimulation. This discrepancy may be due to an underestimate of the amount of starch in pea extract or to the fact that pea starch contains a relatively high proportion of amylase (14) which is more active than amylpectin in improving recovery counts. Alternatively, extraction and drying procedures may render commercially available soluble and corn starches less effective.

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