Growth of *Clostridium perfringens* in Food Proteins Previously Exposed to Proteolytic Bacilli¹

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Proteolytic sporeforming bacteria capable of surviving processing heat treatments in synthetic or fabricated protein foods exhibited no antagonistic effects on growth of *Clostridium perfringens*, but instead shortened the lag of subsequent growth of *C. perfringens* in sodium caseinate and isolated soy protein. *Bacillus subtilis* A cells were cultured in 3% sodium caseinate or isolated soy protein solutions. The subsequent effect on the lag time and growth of *C. perfringens* type A (strain S40) at 45°C was measured by colony count or absorbance at 560 nm, or both. *B. subtilis* incubation for 12 h or more in sodium caseinate reduced the *C. perfringens* lag by 3 h. Incubation of 8 h or more in isolated soy protein reduced the lag time by 1.5 h. Molecular sieving of the *B. subtilis*-treated sodium caseinate revealed that all molecular sizes yielded a similar reduced lag time. Diethylaminoethyl-Sephadex ion exchange fractionation and subsequent amino acid analysis indicated that the lag time reduction caused by *B. subtilis* incubation was not related to charge of the peptides nor to their amino acid composition. Apparently the shortened *C. perfringens* lag in these *B. subtilis*-hydrolyzed food proteins was a result of the protein being more readily available for utilization by *C. perfringens*.

*Clostridium perfringens* food-borne illness continues to be a major concern in the food industry (4). Dramatic food processing advances and significant changes in consumer attitudes and marketing approaches have resulted in public acceptance of fabricated synthetic foods utilizing soy protein or casein as the protein base. The production of fabricated foods by using pasteurization processes combined with good sanitation and modern manufacturing practices can result in the removal of common spoilage microorganisms leaving sporeforming bacteria (i.e., *Clostridium* sp. and *Bacillus* sp.) without major competition. Earlier studies have shown that small amounts of hydrolyzed proteins in conjunction with soy proteins in synthetic meats were stimulatory to the growth of *C. perfringens* (15). In view of known responses of *C. perfringens* to partially hydrolyzed protein, a study of the influence of proteolytic enzymes or microorganisms in food proteins appeared warranted. This study was conducted to characterize the effects of the prior growth of proteolytic *Bacillus subtilis* in sodium caseinate and isolated soy protein on the subsequent growth of *C. perfringens*.

**MATERIALS AND METHODS**

**Test cultures.** *C. perfringens* strain S40 was obtained from H. E. Hall (National Center for Urban and Industrial Health, Cincinnati, Ohio). Stock cultures were maintained at −20°C in cooked meat medium (BBL) + 10% glycerol.

The *B. subtilis* A culture was taken from our laboratory culture collection.

**B. subtilis-treated food proteins.** An 18-h culture of *B. subtilis* strain A was used as an inoculum for a 3% sodium caseinate (Land O'Lakes, Inc., Domestic Sodium Caseinate, Minneapolis, Minn.) or isolated soy protein (Promine D, Central Soya, Chicago, Ill.) solution containing 2.5 g of NaCl (Merck & Co.) per liter, and 1.5 g of K₂HOP₄ (J. T. Baker Chemical Co.) per liter. The initial population was 10⁷/ml. Incubation was carried out with agitation for periods ranging from 0 to 48 h at 35°C.
Effects on C. perfringens growth. To determine any subsequent effect on the growth of C. perfringens, the B. subtilis-cultured sodium caseinate (BSC) and isolated soy protein (BSP) were centrifuged, and the supernatant fluid was added to the growth medium at a concentration equal to 10% of the total sodium caseinate present in the C. perfringens growth medium (i.e., 0.2% BSC added to 1.8% sodium caseinate). The final protein concentration was 2%. Trypticase (BBL), substituted for BSC, served as a reference. Sterile sodium thiglycollate (BBL) (0.6 g/liter), sodium sulfite (Allied Chemical Corp.) (0.2% g/liter), NaCl (Merck & Co.) (2.5 g/liter), and K$_2$HOP$_4$ (J. T. Baker Chemical Co.) (1.5 g/liter) were aseptically added. The volume was adjusted with sterile deionized water to 10 ml for absorbance determinations or 500 ml for colony count determinations. The growth medium was steamed for 20 min before inoculation with C. perfringens. Lack of contamination or B. subtilis growth in the test medium was verified by testing for any viable cells in un inoculated control samples.

An 18-h culture of C. perfringens S40 grown in thiglycollate medium without added dextrose (BBL) was centrifuged (4,080 x g for 10 min) and washed in 6.25 x 10$^{-4}$ M potassium phosphate buffer. The washing procedure was repeated once. The initial inoculum was 10/ml for colony count determinations (1) and 10/ml for absorbance measurements at 650 nm (model 330 spectrophotometer, G. K. Turner Associates, Palo Alto, Calif.). The lag time was estimated from the intercept of the exponential growth slope with the initial inoculation level (10). Lag time estimations were determined from data of three replicates.

Colorimetric determination of partial hydrolysis of food proteins by B. subtilis. Partial hydrolysis was measured according to a modification of the method developed by Hull (8). To 4 ml of the protein sample, 4 ml of 0.72 N trichloroacetic acid (Fisher Scientific Co.) was added, and the mixture was filtered. To 1 ml of the filtrate, 3 ml of 7.5% Na$_2$CO$_3$ and 1 ml of a 1:3 dilution of Folin-Ciocalteau phenol reagent (Fisher Scientific Co.) was added. Color change was measured at 650 nm (Beckman Acta III spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.) and converted to milligrams of tyrosine per milliliter from a standard curve.

Molecular sieving of nontreated and B. subtilis-treated food proteins. BSC, BSP, 3% nontreated sodium caseinate, or 3% nontreated isolated soy protein (5-ml sample) was sieved by using gel filtration (Sephadex G-25 fine, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The method used was a modification of a method by Nekvasilova et al. (14). Size of column was 2.5 by 36 cm, and elution buffer was 0.05 M ammonium bicarbonate (J. T. Baker Chemical Co.) (pH 7.6). The fractions were monitored at 280 nm with a flow-through cell (Beckman Acta III) and collected automatically. The flow rate was 0.5 ml/min.

For large volumes of sample, 100 ml of 3% nontreated or B. subtilis-treated sodium caseinate or isolated soy protein was fractionated by using a Sephadex G-25 column (5 by 86 cm) with a flow rate of 2.5 ml/min and collected at 10 ml/tube.

The fractions forming a peak were combined, flash-evaporated at 40 C (Laboratory Glass and Instruments Corp., N.Y.), and freeze-dried (Virtis Research Equipment, Gardiner, N.Y.).

Fractionation of active peptide groups on ion exchange gel. BSC, the second peak of gel filtration (Sephadex G-25), 22-h BSP, and untreated sodium caseinate control were fractionated by ion exchange gel. Freeze-dried samples (1 g) were fractionated by gradient elution with a linear pH change according to Carnegie (2). A column (2.5 by 36 cm) was filled with diethylaminoethyl (DEAE)-Sephadex A-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) in acetate form and brought to equilibrium with 0.1 M collidine (2, 4, 6-trimethyl pyridine; Eastman Kodak) acetate buffer (pH 8.55). Elution was affected with collidine acetate buffer at pH 8.55. A pH gradient was obtained by gradual mixing in gradient chambers with 0.1 M acetic acid. Flow rate was 0.75 ml/min. After 1 liter was eluted, 1 M acetic acid was added to the gradient chamber. Approximately 200 fractions consisting of 12 ml/fraction were collected automatically. Detection of protein fractions was made from 1-ml samples from each tube with ninhydrin reagent (Eastman Kodak Co.) by using the Cocking and Yemm modification (3) of Moore and Stein's technique (11). Color change was measured with a Beckman Acta III spectrophotometer at 570 nm and recorded as milligrams of alanine per milliliter by conversion from a standard curve.

Amino acid analysis. The amino acid composition of protein fractions was determined by ion exchange chromatography (12). Hydrolysis of the protein samples was carried out in vacuo at 110°C for 24 h under standard conditions described by Moore and Stein (13).

RESULTS AND DISCUSSION

The effects of added BSC on C. perfringens growth was determined by adding BSC (0.2% final concentration) to the regular sodium caseinate medium. The C. perfringens growth responses were determined by colony count and by turbidity measurements. In Fig. 1, representative growth curves for C. perfringens in a sodium caseinate medium with added BSC treated for 10 to 48 h are shown. BSC from incubations of 12 h or more markedly shortened the lag time. The BSC samples (more than 10 h) produced an effect similar to added Trypticase. Ten-hour or less BSC had little or no influence on C. perfringens lag time (data not presented).

Similar results were obtained by the addition of BSP. Incubation periods of 8 h or longer yielded BSP that resulted in a C. perfringens shortened lag time. A shortened lag time also was observed with the addition of the control
Fig. 1. Effect of 10- to 48-h BSC added to sodium caseinate medium on the growth of C. perfringens (absorbance at 650 nm). One gram of BSC was added to 9 g of sodium caseinate per 500 ml. Trypticase was substituted for BSC to serve as a reference.

(no B. subtilis treatment) isolated soy protein, but not to the extent of 8-h or greater BSP (data not presented). Representative growth curves of 8- to 48-h BSP are shown in Fig. 2. Isolated soy protein also increased the growth rate of C. perfringens when added to sodium caseinate. This increased rate of growth was consistent with earlier findings (1).

Colony count determinations were made to confirm absorbance data (Fig. 3A, B). In this study, 12 and 24 h-treated BSC or 10- and 34-h BSP shortened the lag time of C. perfringens. In earlier reported data (P. F. Busta, L. B. Smith, and D. J. Schroder, in Spore Research, in press), BSC initiated germination and outgrowth of C. perfringens spores (>90% in 8 h) to a greater extent than did the sodium caseinate control. Those findings and these presented here indicated that incubation of B. subtilis in food proteins exhibited no antagonistic effects but resulted in altered protein and a shortened lag time for C. perfringens. This may have been the result of either producing stimulative peptides or the addition of some factor to the protein. Cell constituents were eliminated as the stimulative factors by a study on the addition of B. subtilis cell material to a medium. No effect was observed by the addition of B. subtilis cells cultured in buffer and added to a sodium caseinate medium (data not presented). A 260- to 280-nm scan of stimulative protein did not reveal the presence of nucleic acids in the stimulative medium.

We postulated that B. subtilis incubation hydrolyzed the food proteins and produced stimulative peptides. The following relevant literature supports this hypothesis. Numerous studies have reported that certain fractions derived from hydrolyzed protein were effective in promoting growth or alpha-toxin production by C. perfringens (7, 9, 14). The production of toxins has been shown to increase with increasing length of peptide chains while growth remained essentially the same (7). The largest molecules of a molecular-sieved commercial enzymatic casein hydrolysate had been shown to stimulate growth but gave only minimal alpha-toxin production (14). The toxin-stimulating capacity of some of 29 synthetic

Fig. 2. Effect of 8- to 48-h BSP added to sodium caseinate medium on the growth of C. perfringens (absorbance at 650 nm). One gram of BSP was added to 9 g of sodium caseinate per 500 ml. Trypticase was substituted for BSP to serve as a reference.

Fig. 3. A, Effect of 12- and 24-h BSC added to sodium caseinate on the growth of C. perfringens (colony count determinations). One gram of BSC was added to 9 g of sodium caseinate per 500 ml. B, Effect of 10- and 34-h BSP added to isolated soy protein on the growth of C. perfringens (colony count determinations). One gram of BSP was added to 9 g of isolated soy protein per 500 ml.
peptides, especially glycyl-L-asparagine, was demonstrated (9). These and other earlier studies were concerned with toxin production and the concurrent extent of growth, not the rate of growth nor the initiation of growth (lag time).

Anticipated responses to specific materials prompted the following study to determine whether the incubation of food proteins with *B. subtilis* produced specific peptides that decreased the lag time of *C. perfringens*.

**Relationship between extent of food protein hydrolysis and *C. perfringens* growth.** Sodium caseinate was supplemented with 0.2% casein hydrolysates (Trypticase and Casamino Acids) or amino acids of similar composition to determine the effect on *C. perfringens* growth. The addition of Trypticase, Casamino Acids, or amino acids to sodium caseinate (10% of total protein) showed an increased lag time with increased hydrolysis (Fig. 4). This implied that peptides were important initiators of *C. perfringens* growth in a sodium caseinate medium. This is in agreement with studies by Hauschild (6) which showed the incorporation of $^{14}$C from peptides into protein by *C. perfringens* was greater than incorporation of $^{14}$C from amino acids.

**Fractionation of BSC and BSP.** BSC and BSP samples were fractionated to characterize protein hydrolysis resulting from incubation with *B. subtilis*. Figure 5 presents the Sephadex G-25 gel filtration patterns of BSC and BSP. The change in elution pattern of sodium caseinate from the control to 12 and 24 h of *B. subtilis* incubation is evident.

The extent of protein hydrolysis was determined chemically by using Folin-Ciocalteau phenol reagent. The extent of hydrolysis of sodium caseinate and isolated soy protein during incubation with *B. subtilis* is shown in Fig. 6. In both protein media, the maximal extent of hydrolysis was reached at approximately 28 h.

**Fig. 4.** Effect of Trypticase, Casamino Acids, and amino acids added to sodium caseinate on the growth of *C. perfringens*. One gram of test protein was added to 9 g of sodium caseinate per 500 ml (growth measured by absorbance at 650 nm).

**Fig. 5.** Sephadex G-25 elution patterns of sodium caseinate and isolated soy protein. Elution patterns run from right to left and show the protein breakdown corresponding to incubation time with *B. subtilis* (absorbance at 280 nm). Trypticase is shown as a reference.
The incubation time required for minimal stimulation of *Clostridium perfringens* (12-h BSP and 10-h BSP) approximately the time of the first detectable protein breakdown shown in Fig. 6. These results can be compared with data on the Trichloracetic acid-soluble fraction of Trypticase resulting in 0.90 mg of tyrosine per ml. They indicate that the shorted lag of *C. perfringens* was due to partial hydrolysis of the protein. The next step was to determine whether specific peptides or protein fractions were responsible for the shortened lag. BSC and control samples were fractionated on Sephadex G-25. Fractions were isolated, flash-evaporated, freeze-dried, and reincorporated as 10% of the total sodium caseinate in the *C. perfringens* medium. BSC was used exclusively in this study. The growth curve for each fraction is shown in Fig. 7. All six Sephadex fractions added singly or added to sodium caseinate in combination shortened *C. perfringens* lag. Each fraction representing a specific size range of hydrolyzed protein exhibited similar effects. Fraction 2 initiated growth in 2.37 h, compared with 2.25 h for Trypticase and 3.75 h for the sodium caseinate control. These data indicated that the stimulative effect of the hydrolyzed protein appeared to be nonspecific with regard to molecular size of the protein fraction.

**Ion exchange chromatography of Sephadex fractions.** The nonspecificity of molecular size of the hydrolyzed protein fractions prompted studies to determine whether this

stimulation was due to peptides of a specific charge.

The first fraction (Sephadex fraction 1) of the sodium caseinate control and Sephadex fractions 1 and 2 of 22-h BSC were further fractionated by charge by using a DEAE-Sephadex A-25 column. The DEAE elution patterns for the sodium caseinate control and the BSC fractions appeared similar. The DEAE fractions of the first main peak and the second peak from each sample were collected, evaporated, freeze-dried, and reincorporated into a sodium caseinate medium at a concentration of 10% of the total sodium caseinate. The effects of these DEAE fractions on the growth of *C. perfringens* are listed in Table 1. The supplementation of a

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**Table 1. Effect of molecular-sieve and ion exchange fractions of sodium caseinate and 22-h BSC added to sodium caseinate on *C. perfringens* lag time**

| Sample                              | *C. perfringens* lag time (h) |
|-------------------------------------|------------------------------|
| Control (no additions)              | 3.5                          |
| Addition of:                        |                              |
| Control sodium caseinate            | 4.2                          |
| Control Sephadex fraction 1         | 2.9                          |
| Control DEAE peak 1                 | No growth                    |
| Control DEAE peak 2                 | 3.6                          |
| BSC                                 | 1.9                          |
| BSC Sephadex fractions 1 and 2      | 1.3                          |
| BSC DEAE peak 1                     | No growth                    |
| BSC DEAE peak 2                     | 2.1                          |

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* Average of three experiments. A 1-g fraction was added to 9 g of sodium caseinate per 500 ml. Growth was measured by absorbance at 650 nm.
sodium caseinate medium by the original BSC, Sephadex fractions 1 and 2 of the BSC, and the last BSC DEAE fraction (from the BSC fractions 1 and 2) all reduced the C. perfringens lag time. In comparison, the suppression of the sodium caseinate control by control Sepha-
dex fraction 1 and control DEAE fraction 2 did not reduce the C. perfringens lag time to the extent observed with the BSC samples (Table 1). No growth was observed from the DEAE fraction 1 in either the BSC samples or the control. The difficulty of removing collidine could have been responsible for this.

All fractions tested were analyzed for amino acid composition. No composition differences between the sodium caseinate control fractions or the BSC fractions were observed. The decreased lag demonstrated by the BSC fractions, therefore, did not appear to be due to the amino acid composition of the active component(s), because DEAE peak 2 of the control and BSC were of similar composition but differed in their effect on the lag time of C. perfringens. To confirm the lack of specificity of size of the protein molecule or charge of the molecules on shortening the C. perfringens lag, the complete BSC sample was fractionated on the DEAE-
Sephadex column. The fractions representing the last DEAE peak were incorporated into a sodium caseinate medium and found to decrease the C. perfringens lag similar to the complete BSC. The fractions representing the last DEAE peak were then separated by gel chromatography on Sephadex A-25. The similarity of the fractions representing the last DEAE peak and the original BSC indicated that the fractions in the last DEAE peak contained all of the original molecular size range (data not presented).

These results have demonstrated that a shortened C. perfringens lag resulted from the addition of B. subtilis-treated sodium caseinate and isolated soy protein. The shortened lag was not due to a specific molecular size of protein formed from hydrolysis. The amino acid composition of the BSC peptide fractions that short-
ened the lag was similar to the composition of the untreated control sodium caseinate fractions that did not shorten the C. perfringens lag time. Therefore, this effect was not due to a particular amino acid composition. Nor was it due to the charge of the protein fragments or peptide molecules, since BSC fractions with a net negative charge (acidic peptide) were stimulatory, whereas samples of the sodium caseinate control with a similar charge were not. There was no apparent difference in the charge pat-
tern of the BSC and control sodium caseinate.

Peptides of a specific size, charge, or amino acid composition were not solely responsible for the shortened C. perfringens lag period. Therefore, this lag time reduction caused by B. subtilis action was due to hydrolysis of the food protein, apparently making it readily available for utilization by C. perfringens.

The inadvertant modification of food proteins by B. subtilis can increase the potential of that protein for supporting growth of C. perfringens. These results indicate the importance of associ-ative growth of proteolytic bacilli with C. perfringens in a food protein medium that is normally inadequate for the growth of C. perfringens. The shortening of the lag time for C. perfringens in a food product has great public health significance. Food processes in which soy proteins are treated with microbial enzymes to improve acceptability, especially through the formation of plasmin (5), could result in initiat-
ing the growth of C. perfringens in that me-
dium. A food processor fabricating protein foods who uses soy protein or sodium caseinate should be aware of the ramifications of the partial hydrolysis of that protein.

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