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Physiological and Nutritional Factors Affecting Synthesis of Extracellular Metalloproteases by *Clostridium bifermentans* NCTC 2914

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Extracellular protease production by *Clostridium bifermentans* NCTC 2914 occurred throughout the growth phase in batch culture. In both glucose-excess and -limited chemostats, protease formation was inversely related to the dilution rate, over the range $D = 0.03$ to $0.70\ h^{-1}$. At high dilution rates ($D > 0.25\ h^{-1}$), protease activities were greatest under excess glucose conditions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chemostat culture effluents showed the presence of up to 18 bands of protease activity at low dilution rates, with apparent molecular masses ranging from about 36 to 125 kDa. High-performance liquid chromatography gel filtration of culture supernatants gave four peaks of activity at 34, 42, 60, and 102 kDa. Glucose, peptone, and phosphate stimulated protease formation, but ammonia concentrations up to 10 g liter^{-1} had little effect on the process. Culture pH in glucose-excess chemostats strongly influenced protease synthesis, which was maximal during growth at pH 6.4. The optimal pH of protease activity was 7.0. Although a wide variety of proteins were hydrolyzed by *C. bifermentans* proteases, none of the enzymes were collagenolytic. Of 21 different *p*-nitroanilide, β-naphthylamide, and N-carbobenzoyl substrates tested, none were hydrolyzed. With the exception of Ca^{2+}, divalent metal ions inhibited proteolysis. Experiments with protease inhibitors demonstrated that 1 mM EDTA inhibited protease activities in culture supernatants by over 90%, indicating that the enzymes were principally of the metalloprotease type.

Approximately 12 g of protein enter the human large intestine each day (8), consisting mainly of a mixture of dietary residues and pancreatic secretions (9). In the large bowel, pancreatic proteases are rapidly inactivated (6, 21), and as a consequence, a large proportion of the protein degraded is carried out by proteases produced by the colonic microflora (14, 19). Bacterial proteases are particularly important because they have different substrate specificities to pancreatic proteases and are able to hydrolyze highly globular proteins which would otherwise escape digestion (14).

The significance of proteolysis in the large intestine lies in the fact that the peptide and amino acid products of protein hydrolysis are fermented to a variety of toxic metabolites including ammonia, amines, phenols, and indoles (9) by the gut microflora.

Although much is now known of the proteolytic enzymes formed by mixed populations of colonic bacteria (19, 20), information on proteases produced by individual gut species is limited. However, studies have indicated that clostridia are among the principal proteolytic bacteria in the colon (20). We have previously investigated the factors that control protease formation in *Clostridium perfringens* (1) and *Clostridium sporogenes* (2) and in this communication extend these studies to *Clostridium bifermentans*.

This bacterium is an amino acid-fermenting species which is also able to utilize glucose. It is part of the normal colonic flora, with typical log cell population densities of 8.8/g (dry weight) of gut contents (11). *C. bifermentans* is also of interest in that it is a causative agent of gas gangrene in humans (22) and is associated with enteritis and enterotoxemia in animals (7).

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The objectives of this study were to determine the effects of culture characteristics on protease formation by *C. bifermentans*, including nutrient availability, growth rate, and pH, and to investigate some of the physiological and biochemical properties of the enzymes.

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MATERIALS AND METHODS

Bacterium. *C. bifermentans* NCTC 2914 was obtained from the National Collection of Type Cultures, Public Health Laboratory Service, London, England.

Growth in batch culture. The bacterium was routinely maintained in Wilkins-Chalgren anaerobe broth (Oxoid). In batch culture experiments, *C. bifermentans* was grown at 37°C in 0.28-liter (working volume) glass reaction vessels, on a medium containing the following (in grams per liter): Na_2HPO_4, 0.8; NaH_2PO_4, 0.2; NaCl, 4.5; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.05; sodium thioglycolate, 0.4; yeast extract (Oxoid), 2.5; peptone water (Oxoid), 5.0; NH_4Cl, 1.0; glucose, 2.0; and Tween 80, 0.5 ml; and trace elements solution, 10 ml (4). Anaerobic conditions were maintained by sparging the vessels with oxygen-free nitrogen (0.6 liter h^{-1}). Culture pH (6.5) was controlled by the automatic addition of 2 M NaOH by a Modular Fermentor pH Controller (Gallenkamp). In the growth experiments, 4-ml samples of culture were taken at intervals for measurements of growth (change in A_{650}) and protease activity.

Growth in continuous culture. *C. bifermentans* was grown in the medium described above plus either 1.0 or 10.0 g of glucose liter^{-1}, for glucose-limited and -excess growth, respectively. The bacteria were grown over a range of

dilution rates in glass reaction vessels (0.5-liter working volume). Anaerobic conditions and pH (6.5) were maintained as described above. In experiments to determine the effect of pH (range 5.1 to 8.0) on protease synthesis, the bacteria were grown under conditions of carbon excess at $D = 0.10\ h^{-1}$. Samples were taken after at least eight culture turnovers to ensure that the chemostats were in steady state.

Effect of nutrients on protease production. The bacteria were grown in batch culture as described above. Glucose, ammonia, phosphate, and peptone concentrations were individually varied as shown in Table 1.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using cell-free supernatants of chemostat cultures grown over a range of dilution rates. The samples were run on gels, with and without gelatin (0.25% [wt/vol]) in the presence of 0.1% (wt/vol) SDS. A 4.0% polyacrylamide stacking gel and 7.5% polyacrylamide separating gel were used. After the gels were run, they were renatured by incubation in 25 mM Tris buffer (pH 8.0) containing 0.5% (wt/vol) Lubrol and 0.5% (vol/vol) Triton X-100 for 2 h at room temperature. The buffer was discarded and replaced, and the gels were left at 4°C for 14 h. This was followed by a further incubation with 100 mM Tris buffer (pH 7.0). The gels were then wrapped in clear plastic film and incubated for 4 h at 37°C. They were subsequently stained with amido black (0.1% [wt/vol] in 40% methanol–10% acetic acid–50% water). After the gels were destained, bands of protease activity where the gelatin had been hydrolyzed could be visualized. Protein bands on gels which did not contain gelatin were detected by silver staining. The protein standards used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

Gel filtration chromatography. Proteases in cell-free culture supernatants were separated by high-performance liquid chromatography (HPLC). A TSK SW Ultropac column (0.8 by 30 cm) (LKB) was used. Isocratic elution was carried out using 0.1 M sodium phosphate buffer (pH 7.0) at a flow rate of 0.2 ml min^{-1}. The column effluent was monitored at 208 nm for protein, and 0.5-ml fractions were collected. The system was calibrated by using the following protein standards: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa).

Assay of proteolytic activity. Cell-free culture supernatants were obtained from protease-producing cultures by centrifugation (20,000 × *g*, 20 min) and assayed for protease activity at pH 7.0 (azocoll substrate) using methods previously described by Macfarlane et al. (19). Protease activity was also measured at other pH values over the range 5.0 to 9.0 using 0.1 M Tris buffer. The proteases were preincubated in the buffers for 15 min at 37°C before addition of substrate. The A_{475} of the azo dye released was measured spectrophotometrically. One unit of protease activity is equal to 1 mg of azocoll hydrolyzed h^{-1}. Initial control experiments showed that *C. bifermentans* did not produce either a cell-bound or extracellular azoreductase and confirmed the stability of the azo dye in the assay mixtures. In the inhibition experiments, a variety of protease inhibitors were individually included in the protease assays as shown in Table 2. In studies to determine the effects of sulfhydryl reagents and divalent metal ions on proteolysis, dithiothreitol and mercaptoethanol (both 5 mM), and $CaCl_2$, $MnCl_2$, $CoCl_2$, $FeCl_2$, $ZnCl_2$, and $CuCl_2$ (all 1.0 to 10 mM) were included in the assay mixtures, as described by Gibson and Macfarlane (13).

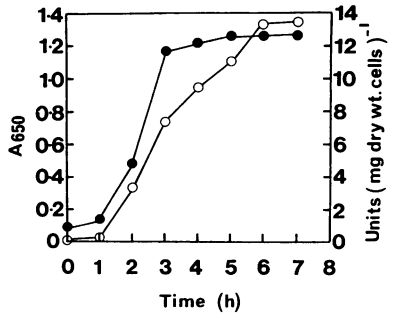

FIG. 1. Protease production by *C. bifermentans* in batch culture. Symbols: ●, culture A_{650}; ○, azocoll hydrolysis. Results are mean values from three experiments.

Protease substrates. The ability of *C. bifermentans* proteases to hydrolyze a variety of native and diazotized proteins was determined by using methods described by Gibson and Macfarlane (12). The following substrates were tested: casein, bovine serum albumin, ovalbumin, gelatin, elastin-orcein, azocasein, azoalbumin, azocoll, azo soybean flour, and collagen types I, II, and VI. A range of L-amino acids, *p*-nitroanilide, β-naphthylamide, and carboxypeptidase substrates were also tested including: benzoyl-arginine *p*-nitroanilide, valylalanine *p*-nitroanilide, leucine *p*-nitroanilide, succinylalanylalanyl alanine *p*-nitroanilide, glycylproline *p*-nitroanilide, glutamyl *p*-nitroanilide, glutarylphenylalanine *p*-nitroanilide, benzyltyrosine *p*-nitroanilide, benzylcysteine *p*-nitroanilide, lysine *p*-nitroanilide, pyrrolidonyl β-naphthylamide, alanine β-naphthylamide, proline β-naphthylamide, cystine β-naphthylamide, serine β-naphthylamide, valine β-naphthylamide, N-carbobenzoyl (N-CBZ) glycylalanine, N-CBZ glutamylphenylalanine, N-CBZ valylalanine methyl ester, N-CBZ proline, and N-CBZ aspartate. The *p*-nitroanilide and β-naphthylamide assays were carried out as described by Gibson and Macfarlane (12), and carboxypeptidase measurements using the N-CBZ substrates were made by using the methods of Appel (3).

Dry weight measurements. Measurements of culture dry weights were made using procedures described by Keith and Herbert (16).

Chemicals. Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co.

RESULTS

Protease formation in batch culture. *C. bifermentans* secreted proteases throughout the exponential phase of growth in batch culture (Fig. 1). Protease production ceased in the stationary phase. Cell-associated protease activity was not detected at any time, and microscopic examination of the cultures indicated no correlation of protease formation with sporulation.

Protease production in continuous cultures. Protease synthesis was inversely related to growth rate in glucose-limited and glucose-excess chemostats, at dilution rates between 0.03 and 0.70 h^{-1} (Fig. 2). Except at very low dilution rates, protease production was maximal during growth under glucose-excess conditions. SDS-PAGE of culture supernatants from glucose-limited chemostats produced 17 bands of gelatin hydrolysis at $D = 0.10\ h^{-1}$ and 13, 10, and 12 bands at $D = 0.35$, 0.54, and 0.70 h^{-1}, respectively (Fig. 3). In the glucose-excess chemostats (Fig. 4), 18 bands were found at $D = 0.09\ h^{-1}$, and 14, 7, and 9 bands at $D = 0.35$, 0.50, and

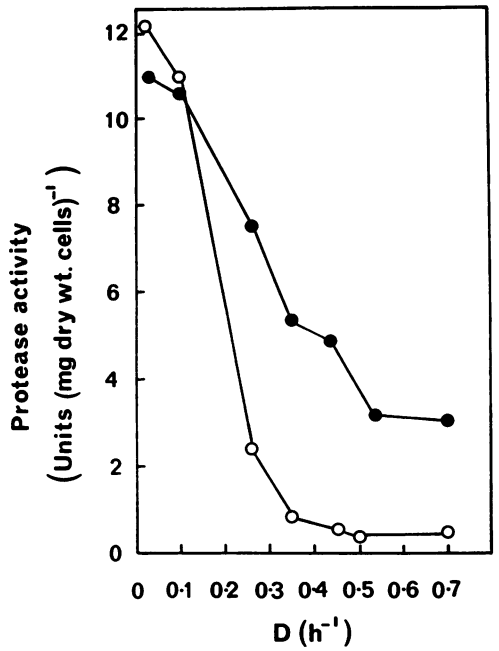

FIG. 2. Effects of growth rate and nutrient availability on protease formation by *C. bifermentans* grown in glucose-limited (○) and glucose-excess (●) continuous cultures.

0.70 h^{-1}, respectively. The apparent molecular masses of the proteases ranged from 36 to 125 kDa. The relative intensities of the high-molecular-mass protease bands increased with increasing dilution rates in glucose-excess and glucose-limited cultures. Silver staining of samples from the glucose-limited chemostats showed that proteases were the principal secretory proteins of *C. bifermentans*.

Gel filtration chromatography. Four peaks of protease activity were detected after gel filtration chromatography (HPLC) of culture supernatants. The proteases which gave

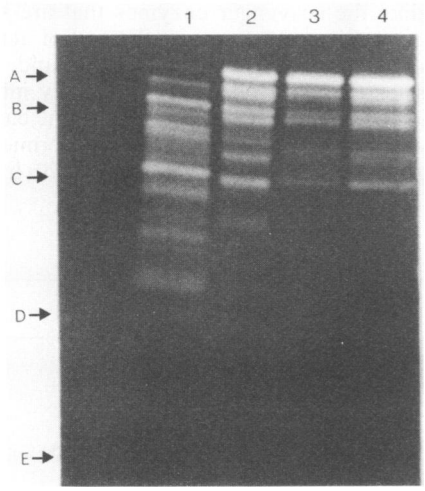

FIG. 3. SDS-PAGE of proteases obtained from glucose-limited chemostats at different dilution rates. Lane 1, $D = 0.10\ h^{-1}$; lane 2, $D = 0.35\ h^{-1}$; lane 3, $D = 0.54\ h^{-1}$; lane 4, $D = 0.70\ h^{-1}$. Molecular mass markers are indicated by the arrows: A, β-galactosidase (116 kDa); B, phosphorylase *b* (97.4 kDa); C, bovine serum albumin (66 kDa); D, egg albumin (45 kDa); E, carbonic anhydrase (29 kDa).

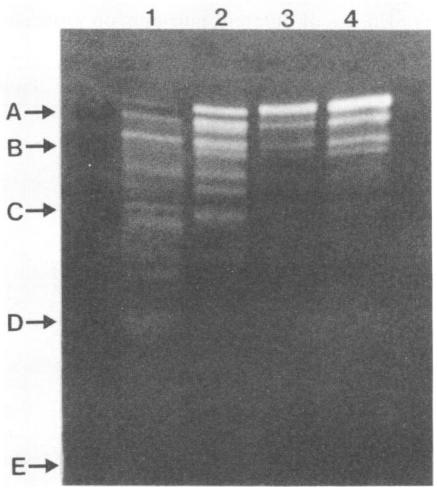

FIG. 4. SDS-PAGE of proteases obtained from glucose-excess chemostats at different dilution rates. Lane 1, $D = 0.09\ h^{-1}$; lane 2, $D = 0.35\ h^{-1}$; lane 3, $D = 0.50\ h^{-1}$; lane 4, $D = 0.70\ h^{-1}$. See the legend to Fig. 3 for molecular mass markers.

the major peaks of activity had estimated molecular masses of 34 and 42 kDa (Fig. 5). Two smaller peaks with apparent molecular masses of about 60 and 102 kDa were found.

Effect of nutrient availability and pH on protease formation. Results in Table 1 show that ammonia had little effect on protease production by *C. bifermentans*. In contrast, protease formation increased with increasing availability of glucose and peptone. In particular, raising peptone concentrations from 5 to 20 g $liter^{-1}$ resulted in stimulation of protease synthesis by a factor of 30. Raising phosphate concentrations from 1 to 5 g $liter^{-1}$ increased protease synthesis by a

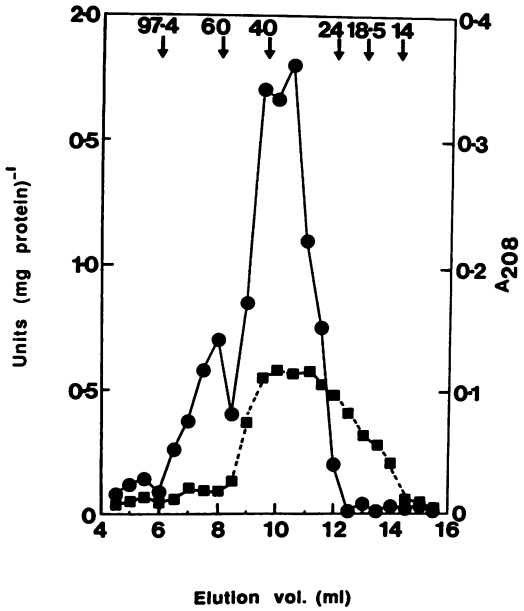

FIG. 5. HPLC gel filtration of *C. bifermentans* proteases. Symbols: ●, azocoll hydrolysis; ■, protein A_{208}. Molecular mass markers are phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), and lyzozyme (14 kDa).

TABLE 1. Effects of different nutrients on protease formation by *C. bifermentans*[a]

Nutrient	Concn (g liter^{-1})	Protease activity[b]
Ammonium chloride	1.0	2.1 ± 0.2
	2.5	2.8 ± 0.1
	5.0	1.9 ± 0.2
	10.0	1.6 ± 0.1
Glucose	2.0	2.1 ± 0.2
	5.0	3.9 ± 0.4
	10.0	12.0 ± 1.5
	20.0	15.6 ± 0.3
Peptone	5.0	2.1 ± 0.2
	10.0	8.4 ± 2.7
	15.0	44.8 ± 0
	20.0	61.4 ± 4.0
Sodium phosphate	1.0	2.1 ± 0.2
	5.0	19.1 ± 2.8
	10.0	15.7 ± 0.1
	15.0	11.4 ± 0.5

[a] Bacteria were grown for 14 h in batch culture.
[b] Results are mean values (in units milligram [dry weight] of cells^{-1}) of two experiments ± standard deviations.

factor of about 10, but higher concentrations of phosphate did not enhance this effect. Culture pH strongly influenced protease synthesis when *C. bifermentans* was grown in continuous culture ($D = 0.1$ h^{-1}). Over the pH range tested (5.1 to 8.0), protease formation was optimal at pH 6.4 (data not shown).

Substrates of *C. bifermentans* proteases. Many different types of protein were hydrolyzed by proteases produced by *C. bifermentans*, including casein, ovalbumin, bovine serum albumin, gelatin, azocasein, azocoll, azoalbumin, and the proteins in azo soybean flour. The proteases did not exhibit elastase or collagenase activities, as evidenced by their inability to degrade elastin-orcein and a range of native collagens. Twenty-one different chromogenic and synthetic substrates (see Materials and Methods) were tested for their ability to act as substrates for *C. bifermentans* proteases, but none were hydrolyzed.

Inhibition experiments. *C. bifermentans* proteases were strongly inhibited by EDTA, an inhibitor of metalloproteases (Table 2). The thiol protease inhibitor thimerosal and the serine protease inhibitor phenylmethylsulfonyl fluoride were also effective inhibitors. The proteases did not have characteristics of trypsin-, chymotrypsin-, or elastase-like enzymes, as shown by the inability of soybean trypsin inhibitor, chymostatin, or elastinal to affect proteolysis. The effects of various divalent metal ions (Table 3) and sulfhydryl reagents on protease activity were also investigated. The results showed that Ca^{2+} slightly stimulated proteolysis and that Mn^{2+} and Zn^{2+} at concentrations as low as 1.0 mM were strongly inhibitory. Co^{2+}, Cu^{2+}, and Fe^{2+} also inhibited proteolysis to various degrees. The addition of 5 mM Ca^{2+} or Zn^{2+} to proteases inhibited by 1 mM EDTA did not restore protease activities. Moreover, 5 mM EDTA did not appreciably restore activity to proteases inhibited by divalent metal ions. Mercaptoethanol and dithiothreitol had no effect on protease activity at concentrations up to 5 mM.

TABLE 3. Effects of divalent metal ions on *C. bifermentans* proteases

Concn (mM)	% Activity[a] (mean ± SD)					
	Ca^{2+}	Co^{2+}	Mn^{2+}	Fe^{2+}	Cu^{2+}	Zn^{2+}
1.0	109 ± 8	66 ± 7	45 ± 10	100 ± 3	73 ± 11	39 ± 11
2.5	112 ± 3	42 ± 9	22 ± 6	94 ± 9	44 ± 12	25 ± 5
5.0	116 ± 2	37 ± 2	15 ± 4	52 ± 11	10 ± 3	0
10.0	111 ± 1	26 ± 3	13 ± 4	16 ± 2	0	0

[a] Values are presented as percentages of control assays which contained no divalent metal ions. Results are from two separate experiments.

Influence of pH on protease activity. The effect of pH on protease activity was tested over the range pH 5.0 to 9.0 (Fig. 6). *C. bifermentans* proteolysis was optimal at neutrality, but over 50% of maximal protease activity remained at pH 5.0. In contrast, however, proteolysis was sensitive to alkaline conditions, with only 8% of control protease activity detectable at pH 9.0.

DISCUSSION

The production of extracellular proteases by exponential-phase cultures of *C. bifermentans* (Fig. 1) indicates that the enzymes have an important nutritional function, in making available peptides and amino acids for growth. They are clearly unlike the scavenger enzymes that are secreted by many other species of bacteria at the end of active growth (27). Although protease formation occurred under all growth conditions tested, the process was markedly influenced by the nutritional and cultural environment of the bacteria. This was shown in the continuous culture experiments, where increasing dilution rates from 0.03 to 0.70 h^{-1} reduced

TABLE 2. Effects of inhibitors on proteolysis in cell-free culture supernatants of *C. bifermentans*[a]

Protease inhibitor	Concn	% Inhibition (mean ± SD) of proteolysis	Type of protease inhibited	Reference
EDTA	1.0 mM	91.1 ± 3.1	Metalloproteases	23
Cysteine	2.0 mM	0	Metalloproteases	28
PMSF[b]	3.0 mM	28.5 ± 2.2	Serine proteases	5
Iodoacetate	5.0 mM	5.3 ± 0.4	Thiol proteases	5
Thimerosal	5.0 mM	39.2 ± 5.9	Thiol proteases	31
Elastinal	8.5 μM	0.2 ± 0.1	Elastase	33
Soybean trypsin inhibitor	100 μg ml^{-1}	0	Trypsin	15
Chymostatin	100 μg ml^{-1}	0	Chymotrypsin	33
Pepstatin A	50 μg ml^{-1}	0	Aspartic proteases	33

[a] Bacteria were grown for 24 h in batch culture. Results are from three separate determinations.
[b] PMSF, phenylmethylsulfonyl fluoride.

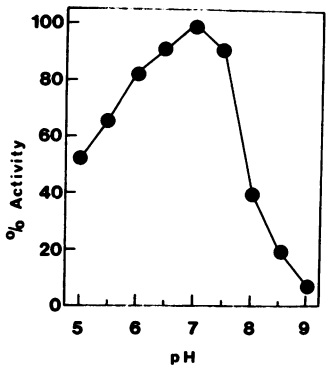

FIG. 6. Influence of pH on the activities of *C. bifermentans* proteases. Results are mean values from three experiments.

protease production by 3- and 10-fold, respectively, in glucose-excess and -limited chemostats (Fig. 2). This pattern of protease synthesis is characteristic of that associated with inducible rather than constitutive enzymes, and in view of the very strong stimulation of protease formation observed with peptone (Table 1), it seems probable that the principal inducers are amino acids. Unlike many other bacteria that produce extracellular proteases, including *Clostridium botulinum* (26), *Vibrio alginolyticus* (17), *Aeromonas hydrophila* (25), and *Pseudomonas aeruginosa* (32), ammonia did not significantly repress protease synthesis in *C. bifermentans*.

Like many clostridia, *C. bifermentans* is highly proteolytic. Comparison of the nutritional and physiological factors affecting protease formation in other clostridia shows that *C. bifermentans* exhibits both similarities and differences in regulation. In *C. sporogenes*, for example, protease synthesis is strictly controlled and occurs only in the stationary phase of batch culture, after active growth has ceased. Moreover, glucose, ammonia, amino acids, and phosphate all repress protease formation (2). Like *C. sporogenes*, *C. histolyticum* produces proteases toward the end of active growth (30); however, protease formation by *C. perfringens* resembles *C. bifermentans* in that proteases are secreted during exponential growth and that peptone is stimulatory (1).

It was evident from the gel electrophoresis experiments that proteases were important secretory products of *C. bifermentans*. Many bacteria produce more than one extracellular protease; for example, *Butyrivibrio fibrisolvens* produces up to ten extracellular proteases (29), and *C. sporogenes* secretes six major extracellular proteases (2a). SDS-PAGE indicated that a large number of different proteases were present in culture supernatants of *C. bifermentans* (Fig. 3 and 4). The number of bands of protease activity on the gelatin gels decreased in samples taken from chemostats run at high dilution rates. This might be expected from the results shown in Fig. 2; however, it was of interest to note that some of these bands of activity in the region of the 97.4- and 116-kDa marker proteins were considerably more active in samples from the high-dilution-rate cultures and that the lower-molecular-weight proteases, which were only detected at low dilution rates, were weakly active against gelatin. The implications of these observations are that the low-molecular-mass proteases were responsible for the high levels of protease activity found in low-dilution-rate chemostats in which azocoll was used as the proteolytic substrate and that although synthesis of these enzymes was repressed at high dilution rates, increased levels of the high-molecular-weight enzymes were expressed. These enzymes appear to efficiently digest gelatin, but not azocoll. The HPLC results (Fig. 5) provide supporting evidence for this hypothesis, because low-molecular-mass proteases in the region of the 34- to 60-kDa protein markers were highly active against azocoll. It is not known whether all of the proteases detected on gelatin gels were individual gene products, since it is possible that some may have been modified by proteolytic cleavage extracellularly, while still retaining a degree of protease activity. Further work is needed to clarify this.

The lack of ability of *C. bifermentans* proteases to hydrolyze synthetic N-CBZ, *p*-nitroanilide, and β-napthylamide substrates indicates that they are true endopeptidases. Since they do not hydrolyze benzoyl arginine *p*-nitroanilide, *C. bifermentans* proteases are clearly different from the trypsin-like enzymes produced by *C. perfringens* (19) and *C. histolyticum* (24). Their neutral pH optima (Fig. 6) and sensitivity to metal chelaters such as EDTA (Table 2) show that they are principally of the metalloprotease type (23, 27). It was surprising, however, that cysteine, which also inhibits metalloproteases (28) had no effect on proteolysis. Although phenylmethylsulfonyl fluoride and thimerosal also inhibited protease activity to some extent, confirmation that the enzymes were predominantly metalloproteases was obtained by incubating gelatin gels after SDS-PAGE with 5 mM EDTA, which completely inhibited proteolysis (results not shown). *C. bifermentans* proteases are therefore similar in many respects to certain of the metalloproteases produced by bacteria of the genus *Bacillus*, which exhibit maximal activity at or near pH 7.0, are inhibited by metal chelating agents and are stabilized by calcium ions (27).

Proteases produced by *C. bifermentans* and similar species may play a significant role in protein turnover in the colons of some individuals, since about 20% of the extracellular protease activity in feces can be attributed to metalloproteases (19). The inability of *C. bifermentans* proteases to degrade collagen or elastin would not be important in the large gut, since these proteins are efficiently digested by pancreatic proteases. However, the activities of proteases such as those formed by *C. bifermentans* may be qualitatively important, since they are able to hydrolyze highly globular proteins, as typified by bovine serum albumin, which cannot be broken down by pancreatic enzymes (14). One possible example of this could be the antigen-antibody complexes that are recirculated to the gut by the hepatobiliary system.

The human large intestine contains high levels of organic nitrogen containing compounds throughout its length (20). The pH of gut contents increases distally from about 5.5 in the proximal colon to around 6.7 in the distal gut (10), and studies have shown that the pH optima of extracellular proteases in feces are 6.4, 7.0, and 7.4 (18). In view of the sensitivity of *C. bifermentans* protease synthesis to environmental pH, as reported here, it is unlikely that the process would be favored in the proximal colon. Although extrapolating results obtained with pure cultures of bacteria in the laboratory to predict their responses to various conditions in the colon should be made with care, the availability of peptides and proteins in the distal colon and its pH all suggest that the nutritional and physical environment in this region of the gut are particularly suitable for the synthesis and activities of *C. bifermentans* proteases.

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