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Regulation of Protease Production in *Clostridium sporogenes*

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The physiological and nutritional factors that regulate protease synthesis in *Clostridium sporogenes* C25 were studied in batch and continuous cultures. Formation of extracellular proteases occurred at the end of active growth and during the stationary phase in batch cultures. Protease production was inversely related to growth rate in glucose-excess and glucose-limited chemostats over the range D = 0.05 to 0.70 h^{-1}. In pulse experiments, glucose, ammonia, phosphate, and some amino acids (tryptophan, proline, tyrosine, and isoleucine) strongly repressed protease synthesis. This repression was not relieved by addition of 4 mM cyclic AMP, cyclic GMP, or dibutyryl cyclic AMP. Protease formation was markedly inhibited by 4 mM ATP and ADP, but GTP and GDP had little effect on the process. It is concluded that protease production by *C. sporogenes* is strongly influenced by the amount of energy available to the cells, with the highest levels of protease synthesis occurring under energy-limiting conditions.

Clostridium sporogenes is classified as a proteolytic member of the genus *Clostridium* on the basis of its ability to ferment amino acids and liquefy gelatin (17, 34). The bacterium is ubiquitous in many natural environments and is of some economic and medical importance. It is commonly found as a spoilage organism in canned foods (13, 32) and dairy products (3), and recent interest has focused on its biotechnological potential as a producer of commercially useful enzymes (2, 33). Although *C. sporogenes* cannot be regarded as being one of the major clostridial pathogens of humans, its role as an etiological agent in anaerobic cellulitis is well established (22).

Protease formation has been correlated with food spoilage (27) and pathogenicity (16) in a number of clostridia. Protease production by the saccharolytic species *C. histolyticum* and *C. perfringens* has been investigated in some detail (1, 4, 6, 37), but few workers have examined the factors that regulate protease synthesis by amino acid-fermenting clostridia such as *C. sporogenes*.

We isolated *C. sporogenes* C25 during experiments on the protease-producing bacteria that occur in the human large intestine. The isolate was particularly notable because of the exceptionally high levels of protease secreted into the culture medium. This paper reports studies on the physiology and regulation of protease production by the bacterium.

MATERIALS AND METHODS

Organism. *C. sporogenes* C25 was isolated from human feces by direct plating as described by Macfarlane et al. (21) and was identified according to Gram reaction, morphology, fermentation products formed during growth in peptone-yeast extract-glucose broth, and biochemical tests as described in Holdeman et al. (17).

Growth in batch culture. The bacterium was routinely maintained in Wilkins-Chalgren anaerobe broth (Oxoid). In batch culture experiments, *C. sporogenes* was grown in 0.5-liter (working volume) glass reaction vessels on a medium containing in grams per liter: Na_2HPO_4, 0.9; KH_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), 4.0; NH_4Cl, 0.4; glucose, 5.0; and trace elements solution, 1 ml (12). Anaerobic conditions and temperature (37°C) were maintained as described by Englyst and Macfarlane (11). Culture pH (6.8) was controlled with a Modular Fermentor pH Controller (Gallenkamp). In the growth experiments, samples of culture (5 ml) were taken at timed intervals for measurements of growth (change in optical density at 650 nm) and protease activity.

Continuous cultures. *C. sporogenes* was grown in the basal medium described previously, which contained either 0.5 or 5.0 g of glucose $liter^{-1}$ for glucose-limited and glucose-excess growth, respectively. The bacteria were grown at various dilution rates in glass reaction vessels with a working volume of 0.28 liter. Anaerobic conditions, pH (6.8), and temperature (37°C) were maintained as described above.

Pulse experiments. Batch cultures (20 ml) were grown in 58-ml-volume serum bottles on Wilkins-Chalgren broth diluted 1:1 with distilled water (pH 7.1) and incubated at 37°C on a rotary shaker. Growth and extracellular protease activities were monitored for 12 h. Protease-producing cultures were pulsed with either sterile saline (0.9%, wt/vol) or sterile solutions of glucose, glucose plus cyclic AMP (cAMP), ammonia, ammonia plus cAMP, NaH_2PO_4/K_2HPO_4 buffer (pH 7.1), glucose plus dibutyryl cAMP, glucose plus cyclic GMP (cGMP), GDP, GTP, ADP, or ATP. Final concentrations of the nucleotides, glucose, and ammonia were 4, 100, and 50 mM, respectively. Phosphate buffer was added to give a final phosphate concentration of approximately 0.5 M. In experiments to test the effect of amino acids on protease production, various amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) were autoclaved at 121°C for 10 min in culture bottles under an atmosphere of CO_2. Protease-producing bacteria, grown as described previously, were added to each bottle (20-ml culture) and incubated at 37°C on a rotary shaker. The final concentration of each amino acid was 50 mM.

Assay of proteolytic activity. Cell-free supernatants were obtained from protease-producing cultures by centrifugation (20,000 × *g*, 20 min) and assayed for proteolytic activity (azocoll substrate) by using methods described by Macfar-

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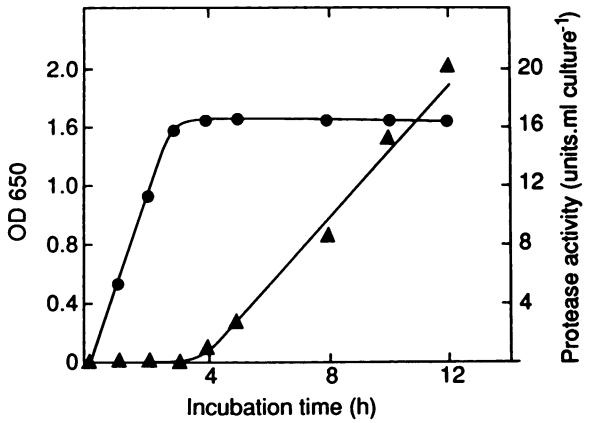

FIG. 1. Extracellular protease production by *C. sporogenes* during growth in batch culture. Symbols: optical density at 650 nm (OD_{650}; ●); azocoll hydrolysis (▲). Values are means from two experiments.

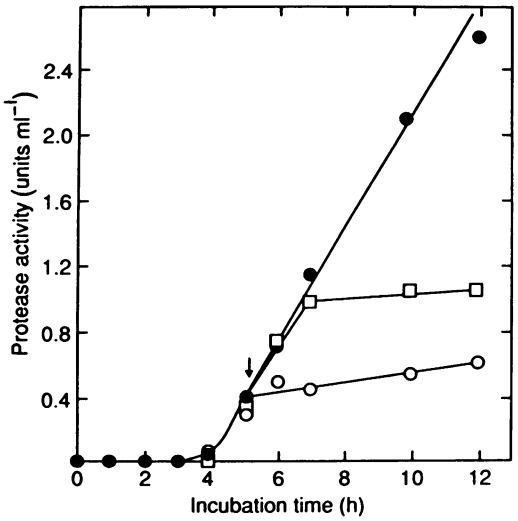

FIG. 2. Effect of addition (arrow) of rifampin (□) and chloramphenicol (○) on protease secretion by *C. sporogenes*. Saline was added to control cultures (●). Data are average values from three experiments.

lane et al. (21). The A_{475} of the azo dye released was measured spectrophotometrically. One unit of protease activity is equivalent to 1 mg of azocoll hydrolyzed h^{-1}. Initial control experiments showed that *C. sporogenes* C25 did not produce either a cell-bound or extracellular azoreductase and confirmed the stability of the azo dye in the assay mixtures.

Chemical analyses. Glucose was determined in culture supernatants by the dinitrosalicylic acid method (8). Ammonia was measured by the phenolhypochlorite method (34).

Dry weights. Dry weight determinations were carried out with procedures described by Keith and Herbert (18).

Chemicals. All chemicals were supplied by Sigma Chemical, except the formulated bacteriological media (Oxoid).

RESULTS

Protease production in batch culture. *C. sporogenes* produced extracellular protease towards the end of growth and in the stationary phase of batch culture (Fig. 1). Microscopic examination of the cultures indicated no correlation of protease secretion with sporulation.

Inhibition of protease synthesis by rifampin and chloramphenicol. Protease activity was not detected in cell extracts of *C. sporogenes* either before or during active protease secretion. Studies were carried out with inhibitors of protein synthesis and mRNA transcription to determine whether protease was synthesized intracellularly as a precursor protein during active growth. Results showed that the addition of chloramphenicol (40 μg ml^{-1}) immediately inhibited protease production (Fig. 2). In contrast, cultures pulsed with rifampin (1 μg ml^{-1}) continued to synthesize protease at control levels for at least 60 min after the time required for full inhibition of mRNA translation.

Protease production in continuous culture. Chemostat studies demonstrated that proteolysis was inversely related to growth rate in both glucose-limited and glucose-excess cultures (Table 1). At growth rates between D = 0.05 and 0.35 h^{-1}, protease production was approximately twice as great in the glucose-limited vessels.

Spent-medium analyses showed only trace levels of glucose in effluent from the glucose-limited chemostats, irrespective of dilution rate (Fig. 3, top). However, in cultures grown under conditions of glucose excess, residual glucose increased concomitantly with growth rate and at D = 0.7 h^{-1}; 95% of the glucose in the feed medium was recovered in culture spent media. Ammonia concentrations in glucose-limited chemostats decreased with increasing growth rate, but lower levels were found in the glucose-excess cultures, which were relatively unaffected by culture dilution rate (Fig. 3, bottom).

Effect of glucose, ammonia, and phosphate on protease production. The inhibitory effects of a variety of nutrients on

TABLE 1. Effect of growth rate and nutrient availability on protease formation by *C. sporogenes* grown in continuous culture[a]

Dilution rate (h^{-1})	Glucose availability	Culture dry wt (mg ml^{-1})	Protease activity[b]	
			Vol activity (U ml of culture^{-1})	Sp act (U mg [dry wt] of cells^{-1})
0.05	Limiting	0.22	0.41	1.86
	Excess	0.59	0.70	1.19
0.10	Limiting	0.25	0.40	1.60
	Excess	0.68	0.57	0.83
0.20	Limiting	0.27	0.39	1.44
	Excess	0.56	0.31	0.55
0.35	Limiting	0.28	0.32	1.14
	Excess	0.52	0.27	0.51
0.50	Limiting	0.32	0.11	0.34
	Excess	0.48	0.24	0.50
0.70	Limiting	0.20	0.10	0.50
	Excess	0.22	0.12	0.55

[a] Bacteria were grown under glucose-limited (0.5 g liter^{-1}) or glucose-excess (5 g liter^{-1}) conditions at various dilution rates. Anaerobic conditions were maintained by sparging cultures with a gas mixture containing 10% CO_2, 10% H_2, and 80% N_2. Results are mean values from two experiments.

[b] 1 U of protease activity = 1 mg of azocoll hydrolyzed h^{-1}.

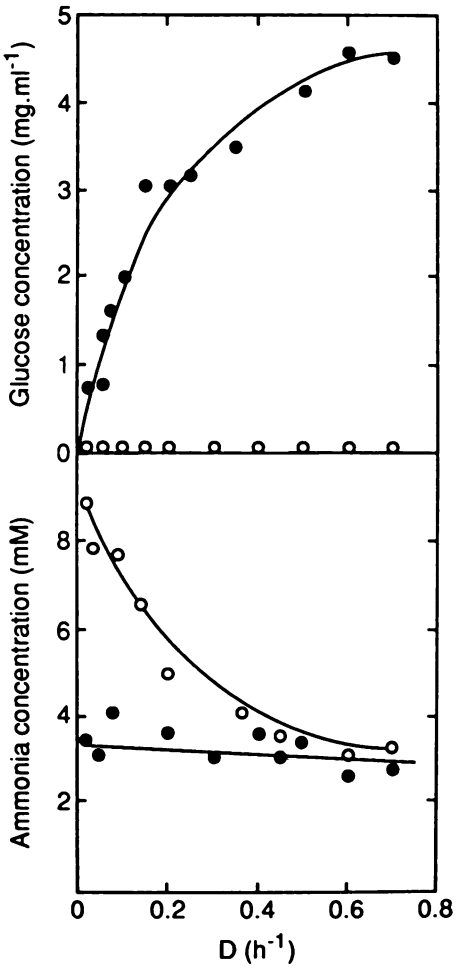

FIG. 3. Residual glucose (top) and ammonia (bottom) concentrations in spent media from continuous cultures of *C. sporogenes* growing under glucose-limited (○) and glucose-excess (●) conditions.

protease synthesis by *C. sporogenes* were shown in batch culture experiments when protease formation was repressed when the bacteria were pulsed with 100 mM glucose, 50 mM ammonia, or 0.5 M phosphate (Fig. 4). Inclusion of 4 mM cAMP, cGMP, or dibutyryl cAMP did not relieve these inhibitory effects (data not shown).

Effect of amino acids on protease formation. Twenty different amino acids were tested for ability to inhibit protease synthesis by *C. sporogenes*. Tryptophan, isoleucine, tyrosine, and proline almost totally repressed protease production (Fig. 5), whereas alanine, serine, and valine were neither inhibitory nor stimulatory (results not shown). The other amino acids (arginine, threonine, glutamate, glycine, lysine, cysteine, histidine, aspartate, asparagine, methionine, glutamine, leucine, and phenylalanine) were inhibitory to varying degrees. cAMP, cGMP and dibutyryl cAMP did not relieve amino acid repression of protease synthesis (data not shown).

Repression of protease production by ATP and ADP. Addition of 4 mM ATP or 4 mM ADP to protease-producing cultures reduced rates of protease synthesis by four- and threefold, respectively (Fig. 6). However, protease formation was only partly inhibited when 4 mM GTP or GDP was added.

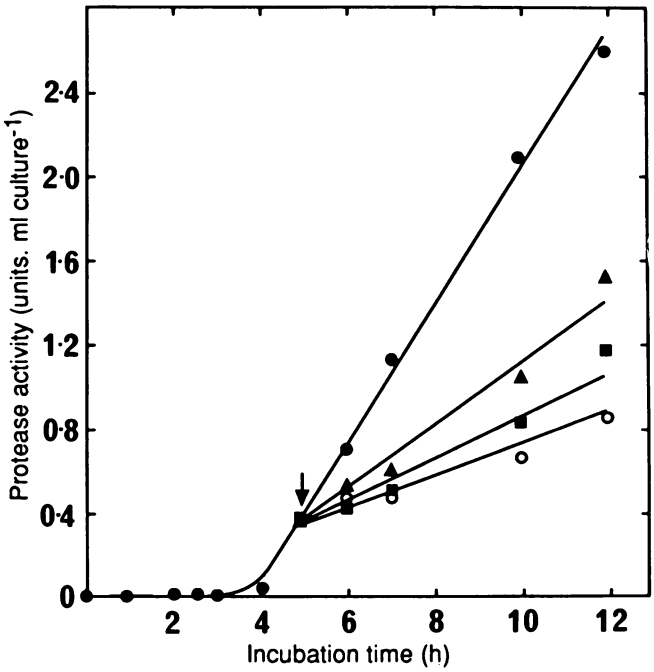

FIG. 4. Effect of glucose, ammonia, and phosphate on protease formation by *C. sporogenes*. Compounds were given as a pulse (arrow) to protease-producing bacteria. Control cultures were pulsed with saline (●; standard error of the mean rate of protease production [SEM] = 0.007). Glucose (▲; SEM = 0.010), ammonia (■; SEM = 0.007), and phosphate (○; SEM = 0.011) were added to give final concentrations of 100 mM, 50 mM, and 0.5 M, respectively. Results are means of values from three experiments.

DISCUSSION

Extracellular protease synthesis was strictly regulated in *C. sporogenes* C25, occurring only after active growth had ceased in batch culture (Fig. 1). Repression of enzyme synthesis during the early stages of growth, followed by derepression in late exponential or early stationary phase, is a common feature of extracellular enzyme formation in bacteria (29). Examples of this phenomenon in protease production have been documented for many gram-negative species, including vibrios (7, 20), *Achromobacter iophagus* (31), *Aeromonas hydrophila* (26), and a number of pseudomonads (24, 39). Exoprotease synthesis has been correlated with sporulation in *Bacillus subtilus* (23), but the complete absence of spores in protease-producing cultures of *C. sporogenes* and the secretion of proteases in continuous cultures indicate that the process is not associated with spore formation in this bacterium.

The addition of chloramphenicol immediately stopped protease formation by *C. sporogenes* (Fig. 2), showing that the appearance of extracellular protease in stationary-phase cultures resulted from rapid export of a product of de novo protein synthesis, rather than secretion of protease that had accumulated intracellularly during growth. In contrast, protease synthesis proceeded for some time after addition of the transcription inhibitor rifampin. Boethling (5) similarly found that protease synthesis by *Pseudomonas maltophila* occurred for at least 30 min in the absence of RNA synthesis, while collagenase formation by *Vibrio alginolyticus* continued for between 30 and 60 min after addition of rifampin (31). The authors attributed these effects to the presence of a stable pool of exoprotease mRNA. While this could explain

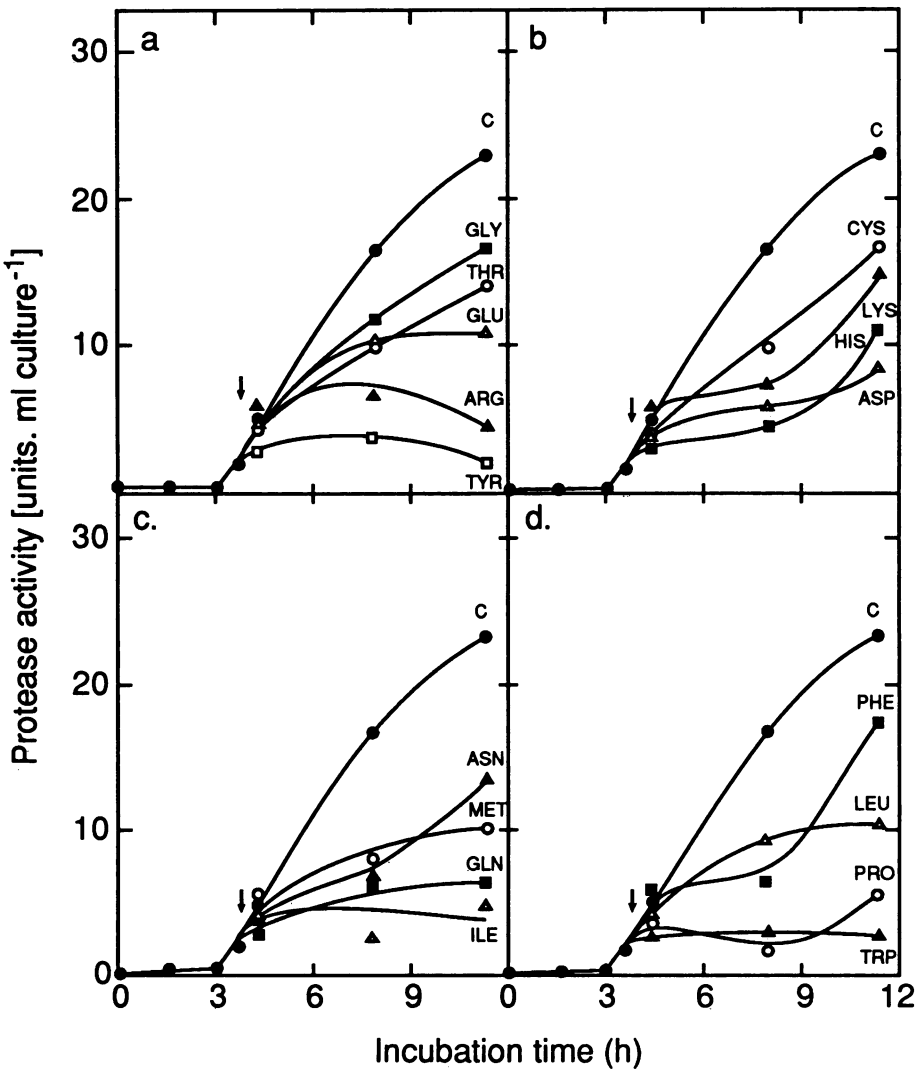

FIG. 5. Effect of amino acids on protease formation by *C. sporogenes*. Bacteria were grown in batch culture and pulsed (arrow) with the various amino acids (50 mM final concentration) during the early stages of protease production. Control cultures (c) were pulsed with saline. Values are means from two experiments.

the results obtained with *C. sporogenes*, the lag in inhibition of protease synthesis seen with rifampin may also be due to a slower rate of uptake of the inhibitor by the bacteria.

Continuous culture studies have shown that high growth rates repress exoprotease production in some bacteria (39, 41). This was also the case with *C. sporogenes*. Protease formation occurred maximally at low dilution rates, especially during glucose-limited growth (Table 1). Measurements of residual glucose in spent media indicated that at dilution rates between $D = 0.05$ and 0.35 h^{-1}, products of glucose metabolism were involved in the repression of protease synthesis. The specific inhibitory effect of glucose was confirmed in subsequent pulse experiments (Fig. 4). These batch culture studies also demonstrated that ammonia inhibited protease formation.

In the chemostats, however, spent medium ammonia concentrations were lowest at high dilution rates, where repression of protease synthesis was greatest. Conversely, glucose progressively accumulated in the glucose-excess cultures when dilution rates were increased (Fig. 3), suggesting that energy generation was occurring almost completely as a result of amino acid fermentation at high growth rates. Since concentrations of extracellular ammonia, levels of glucose fermentation, and protease activities were almost identical at high dilution rates in both glucose-limited and glucose-excess chemostats (Table 1, Fig. 3), the marked decline in protease production was probably due to repression resulting from increased amino acid availability.

A variety of factors have been identified that are involved in regulating protease production in bacteria, including enzyme induction, product inhibition, and catabolite repression of enzyme synthesis (9). In classical catabolite repression, glucose or some other readily metabolizable nutrient causes repression of enzyme synthesis (25). Glucose (20, 24, 41), ammonia (19, 20, 31), and amino acids (26, 38) have variously been reported to repress bacterial protease production.

C. sporogenes can ferment glucose to a variety of products, but its preferred substrates are amino acids, which are fermented by Stickland reactions. Alanine, valine, and leucine serve as electron donors, while glycine, proline, hydroxyproline, arginine, and ornithine act as electron accep-

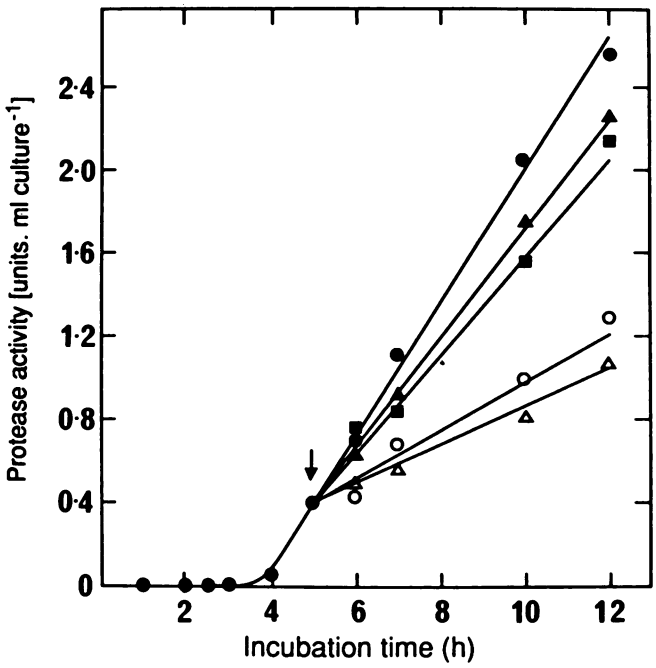

FIG. 6. Influence of different nucleotides on protease synthesis. Protease-producing cultures were pulsed (arrow) with saline (●; standard error of the mean rate of protease production [SEM]= 0.011); ATP (△; SEM = 0.009); ADP (○; SEM = 0.011); GDP (■; SEM = 0.010); or GTP (▲; 0.008). Final nucleotide concentrations were 4 mM. Results are average values from three experiments.

tors (15, 35). Many different amino acids repressed protease production by *C. sporogenes*, especially tryptophan, isoleucine, tyrosine, and proline; however, alanine, valine, and serine had no significant effect (Fig. 5), while no amino acid stimulated protease production. In other bacteria, isoleucine and proline appear to be the commonest amino acids involved in repression of extracellular enzyme synthesis (14).

Inhibition of protease formation in *C. sporogenes* by glucose, phosphate, ammonia, or amino acids was not relieved by cAMP, dibutyryl cAMP, or cGMP. Failure of cAMP to overcome repression of protease production has been shown to occur in many bacteria (5, 20, 30, 36, 38).

Regulation of protease formation in *C. sporogenes* differs from that in other clostridia. For example, *C. botulinum* is very similar to *C. sporogenes* with respect to a number of nutritional, morphological, biochemical, and genetic characteristics (10), but recent studies have shown that *C. botulinum* synthesizes an intracellular and an extracellular protease during active growth that were repressed by arginine and ammonia (28) but not glucose. Regulation of extracellular protease production in *C. sporogenes* also differs from that in the saccharolytic species, *C. perfringens*, in which protease formation occurs throughout the growth cycle and is stimulated by carbohydrate and high growth rates (1).

Protease production by *C. sporogenes* was initiated by nutrient limitation. Since no exogenous inducer was necessary for protease synthesis to occur and protease was secreted in the absence of protein substrates, the principal regulatory mechanism appears to be through repression of enzyme synthesis. Wiersma et al. (40) considered that an intermediate of energy metabolism was involved in regulation of protease production by *Vibrio* SAI and postulated that protease synthesis was repressed when the energy status of the cells was high. A similar mechanism for regulation of protease production occurs in *Pseudomonas aeruginosa* (39). The repressive effects of increased substrate availability, ATP, and ADP indicate that the energy status of the cells also plays a role in the regulation of protease synthesis in *C. sporogenes*.

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