Protease Production by *Bacteroides amylophilus* Strain H 18

By T. H. BLACKBURN

The Rowett Research Institute, Bucksburn, Aberdeen

(Accepted for publication 7 March 1968)

SUMMARY

Bacteroides amylophilus strain H18 produced protease(s), active at pH 7.0, when grown anaerobically in a simple maltose, ammonium sulphate, cysteine, sodium bicarbonate and salts medium. Protease production was neither induced nor repressed by a wide range of nutrients. Protease was synthesized by exponentially growing organisms and 20% of it was liberated into the growth medium. The cell-bound protease was completely accessible to the protein substrate. Protease production was proportional to ammonium sulphate and maltose concentration in the ranges in which these nutrients were growth limiting.

INTRODUCTION

The breakdown of dietary protein in the rumen is a process which has a significant effect on the retention of nitrogen by ruminants (Blackburn, 1965). Rumen micro-organisms are completely responsible for this proteolysis and bacteria have been shown to be actively implicated (Blackburn & Hobson, 1960*a*). Early attempts to isolate the bacteria effecting this protein breakdown resulted in the growth of facultative anaerobes (Appleby, 1955; Blackburn & Hobson, 1960*b*) but the application of more rigorous anaerobic methods resulted in the isolation of true rumen anaerobes which had considerable proteolytic activity (Blackburn & Hobson, 1962). Most of these bacteria were Gram-negative species, which was considered to be unusual since, with the exception of members of *Pseudomonas* and *Vibrio* genera (Pollock, 1962), most bacteria which produce exo-enzymes are Gram-positive. Protease production by Gram-negative anaerobic bacteria has been little investigated; its study thus seemed to be of general interest and it was also hoped that knowledge of the mode of action of these proteases might enable protein hydrolysis in the rumen to be controlled. *Bacteroides amylophilus* was selected for investigation principally because it rapidly hydrolyses casein in growth media, but it also has other features of interest. Most of the strains examined produced relatively small quantities of free amino acid from protein, indicating the absence of peptidases, and in common with other proteolytic rumen bacteria they appeared to utilize ammonia in preference to preformed amino acids and peptides (Abou Akkada & Blackburn, 1963). The growth requirements of *B. amylophilus* are very simple and rumen fluid is not essential for its growth, but it has an absolute requirement for maltose or for α -1,4-linked glucose polymers (Blackburn & Hobson, 1962). It produces an active amylase and its capacity to hydrolyse casein is not diminished by subculture. In practical terms the object of this work was to define the conditions for the growth of *B. amylophilus* which would give maximum protease production, so that the proteolytic enzyme(s) might be studied in more detail. To define these conditions the following questions had to be answered: is

protease production inducible or repressible? At what stage of the growth cycle is proteolytic activity produced, is it liberated free into the growth medium, is it cell bound or is it located intracellularly? If it is liberated free into the growth medium is its appearance associated with cell lysis?

METHODS

Organism. *Bacteroides amylophilus* strain H18 was isolated from a dilution of $1/10^8$ in a roll tube of casein + rumen fluid medium (Blackburn & Hobson, 1962), from sheep no. 6 (Blackburn & Hobson, 1960c). This strain was identical to previous isolates (Blackburn & Hobson, 1962) and conformed to the description of the type species (Hamlin & Hungate, 1956).

Media. The basal medium, based on that of Hungate (1950), contained (g./l.): K_2HPO_4 , 0.45; KH_2PO_4 , 0.45; $(NH_4)_2SO_4$, 0.9; NaCl, 0.9; $MgSO_4$, 0.09; $CaCl_2$, 0.09; resazurin, 0.001; L-cysteine hydrochloride, 0.5; $NaHCO_3$, 5.0. The mineral and resazurin solutions plus any other additions to the medium were placed in a screw-capped bottle and the volume made to 900 ml. with distilled water. The medium was autoclaved at 120° for 15 min. and the cap screwed tight immediately on removal from the autoclave; this resulted in a partial vacuum in the bottle when it had cooled. Autoclaved 1.0% (w/v) L-cysteine hydrochloride (50 ml.) and 10% (w/v) sodium bicarbonate solution (50 ml.) which had been sterilized by heating to 100° were added to the medium under a stream of oxygen-free CO_2 . The medium could then be dispensed into tubes closed with rubber-bungs; this and subsequent steps were done under an atmosphere of CO_2 . Other media constituents were added at 3.0 g./l., except for casein (5.0 g./l.) and agar (25.0 g./l.). Yeast extract (Difco) and Tryptose (Bacto) were from Bacto Laboratories, Detroit, U.S.A.; vitamin-free acid hydrolysed casein (Casamino acids) from Allen and Hanbury Ltd., London, E. 2; soluble starch and maltose were AR grade from B.D.H. Ltd., Poole, Dorset; proteose peptone from Oxoid, London, E.C. 4; cysteine hydrochloride from L. Light Ltd., Colnbrook, Bucks.; corn steep liquor (MX9A) from Brown and Polson, Trafford Park, Manchester 11. Casein was prepared from Glaxo casein C (Glaxo Ltd., Greenford, Middlesex) by the acid precipitation method of Blackburn & Hobson (1962). Throughout this paper media are named by their additional constituents, thus maltose medium refers to basal medium + 0.3% (w/v) maltose.

Maintenance. A large number of lyophilized suspensions of *Bacteroides amylophilus* H18 was prepared, the suspending fluid being 10% (w/v) maltose in sterile horse serum. The bacteria have remained viable for 4 years. The usual drying medium containing 10% (w/v) glucose did not yield viable dried bacteria. Stock cultures were maintained on slopes of casein maltose agar medium. Transfers were made each week to fresh slopes by stab inoculation. All cultures were grown at 38° and inocula generally consisted of 0.01 vol. of log phase bacteria in maltose medium.

Concentration of bacterial cultures. The concentration of bacterial growth was read on an EEL nephelometer (Evans Electroselenium Ltd., Halstree, Essex) set to read 100 against an arbitrary standard of glass fragments suspended in agar. EEL readings were converted into equivalent dry weights of bacteria from a calibration curve, 100 EEL units = equiv. 0.49 mg. dry wt bacteria/ml.

Assay of proteolytic activity. The assay procedure was a modification of the Anson

(1938) method. Enzyme solution (1 ml.) was incubated at 38° with 4.0 ml. of 2.0% (w/v) casein, 0.01% thiomersalate, in 0.1 M-KH₂PO₄ adjusted to pH 7.0 with NaOH. Immediately after adding the enzyme 2.0 ml. of the digest was removed and mixed with 2.0 ml. of 0.72 N-trichloroacetic acid. The remaining digest was incubated for up to 24 hr when a further 2.0 ml. was precipitated by 2.0 ml. of 0.72 N-trichloroacetic acid. At least 30 min. were allowed for the tubes to stand at room temperature and precipitated protein then filtered off on Whatman no. 42 paper. The soluble tyrosine was estimated by adding 1.0 ml. of filtrate to 5.0 ml. of 0.55 M-sodium carbonate and making up to 10.0 ml. with distilled water. One ml. of 1/3 Folin-Ciocalteu reagent (B.D.H. Ltd., Poole, Dorset) was added and mixed in rapidly. The tubes were incubated at 38° for 30 min. and extinctions were read at 650 m μ on an EEL Spectra colorimeter (Evans Electroselenium Ltd., Halstree, Essex). The extinction at zero time was subtracted from the extinction of the test and the value compared with a standard tyrosine curve; 1 μ g. tyrosine was equivalent to E_{650} 0.0061. The plot of extinction against enzyme concentration was linear up to E_{650} 0.25 (Fig. 1); extinctions higher than this were corrected to the corresponding value on the extrapolation of the linear portion of the curve. The assay of trypsin ($\times 2$ crystallized, salt free, from L. Light and Co. Ltd., Colnbrook, Bucks.) at pH 8.0 under similar conditions gave a similar curve. It was calculated that 1.0 ml. of the culture (Fig. 1) contained proteolytic activity equivalent to 0.91 μ g. trypsin. It was because of this relatively low activity that long incubations had to be used. When assaying the proteolytic activity of whole cultures or of supernatant fluids of cultures it was necessary to oxidize the cysteine with iodine, with starch as an indicator (Tracey, 1948). This decreased the blank value considerably and gave more reliable results, since cysteine was oxidized to some extent in the enzyme digest but to a lesser extent in the zero time control. A unit of proteolytic activity was defined as the amount of enzyme which would solublize the equivalent of 1.0 μ g. tyrosine in 1 min. Different times of incubation were used, up to 24 hr for preparations of low proteolytic activity, but there was a linear relationship between time of incubation and tyrosine solubilized.

RESULTS

Protease assay

Proteolytic activity was measured at pH 7.0 but there was evidence (Fig. 2) that two proteolytic enzymes with optima at pH 6.5 and 8.0 were produced. The relative proportions of these two enzymes varied in different cultures and often there appeared to be a broad optimum from pH 6.5 to 8.0.

Ammonia dependence

The nutritional requirements of *Bacteroides amylophilus* H18 are very simple and neither growth nor protease production was stimulated by adding to the maltose medium any of the following substances (Bryant & Robinson, 1962): thiamine HCl, Ca D-pantothenate, nicotinamide, haemin, riboflavin, pyridoxal (2 mg./l. each); *p*-aminobenzoic acid (1 mg./l.); biotin, folic acid, DL-thioctic acid (50 μ g./l. each); cobalamin (20 μ g./l.); sodium acetate. 3H₂O (3.06 g./l.); sodium isobutyrate (17 mg./l.); sodium valerate, sodium isovalerate, DL- α -methyl-*n*-butyrate (19 mg./l. each). The addition of rumen fluid (20%, v/v) or corn steep liquor (0.01–5.0%, v/v) was also

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Protease concentration curve. The digests contained 80.0 mg. casein, 40.0 μ g. thiomersal and 0.4 mmoles KH_2PO_4 adjusted to pH 7.0 with NaOH. An 18 hr culture of *Bacteroides amylophilus* H18 in maltose medium was added in amounts up to 2.0 ml. and the final volumes were adjusted to 5.0 ml. The digests were incubated at 38° for 20 hr and the trichloroacetic acid-soluble tyrosine was assayed as described in Methods.

Fig. 2. Protease activity of *Bacteroides amylophilus* H18 protease as affected by pH. One ml. amounts of an 18 hr culture of *B. amylophilus* H18 in maltose medium were incubated with 80.0 mg. casein, 40.0 μ g thiomersal and 0.4 mmoles tris adjusted to the correct pH with maleic acid (pH 5.5–7.0) or HCl (pH 7.5–10.5). The final volume of the digest was 5.0 ml. and the proteolytic activity was assayed as described in Methods.

Fig. 3. Ammonium sulphate concentration and protease production by *Bacteroides amylophilus* H18. Ammonium sulphate was omitted from the basal medium and was added as a sterile solution to the tubed maltose (○—○) and maltose-tryptose medium (□—□) in the concentrations shown. The tubes (20 ml.) were inoculated with a loop from a young culture of *B. amylophilus* H18 in maltose medium. The tubes were incubated for 18 hr. and the protease activity assayed as described in Methods.

Fig. 4

Fig. 5

Fig. 4. Rate of protease production by *Bacteroides amylophilus* H18. 250 ml. maltose medium was inoculated with 2.0 ml. of 18 hr culture of *B. amylophilus* H18 grown in maltose medium. Ten ml. amounts were removed at time intervals and the cell densities were read (○—○). The proteolytic activity of the whole culture (□—□) and of the cell free supernatant (29,000 g 10 min.) (Δ—Δ) were determined as described in Methods.

Fig. 5. The relationship between cell density and protease production. The data from Fig. 4 are replotted, total protease (○—○) and cell-free protease (□—□).

without effect. Growth and protease production were dependent on the presence of ammonium ions in the growth media. Figure 3 shows that protease production in maltose medium was proportional to ammonium sulphate concentration up to $3.5 \mu\text{-moles (NH}_4)_2\text{SO}_4/\text{ml.}$ medium when maltose became limiting; tryptose did not replace ammonium sulphate as a growth factor. The bacterial concentrations showed a similar relationship with ammonium sulphate concentration but were more irregular due to the tendency of *B. amylophilus* to lyse when the bacteria ceased to multiply. The relative rates of production of bacteria and protease are better illustrated in Figs. 4 and 5. Figure 4 shows plots of bacterial concentration, total and free protease, against age of culture in maltose medium. Protease production began early in the logarithmic phase of growth and continued until the bacteria ceased to divide. The cell-free protease constituted at a maximum 20 % of the total protease and no immediate increase in free protease was associated with the decrease in bacterial concentration. The data in Fig. 4 are replotted in Fig. 5 to show more clearly the relationship of bacterial concentration and protease production. The cell-free protease was proportional to the bacterial concentration but the total protease was not, especially during the mid-logarithmic growth phase. The proportion of cell-free (29,000 g; 10 min.) protease increased in cultures on further incubation to 42, 50 and 87 % after 24, 48 and 72 hr, respectively. The total protease, after 72 hr, had fallen to 0.31 protease units/ml. which showed a loss of 54 % of the highest activity observed. In the logarithmic phase of growth most of the activity was associated with the intact bacteria. Treatment with toluene or ultrasonic disintegration did not result in an increase of protease, indicating that all the protease was superficially located at the bacterial surface and directly accessible to the casein molecules.

Table 1. *Effect of organic nitrogen sources on growth and protease production of Bacteroides amylophilus* H18

Media compositions and protease assay are described in Methods. The media were incubated for 24 hr and centrifuged 29000 g for 10 min. Unhydrolysed casein was measured by a Biuret method (Layne, 1957). Cell densities could not be measured in casein-containing media, due to opacity.

	Medium							
	Maltose	Maltose tryptose	Maltose proteose-peptone	Maltose Casamino-acids	Maltose casein	Maltose Tryptose casein	Maltose Proteose-peptone casein	Maltose Casamino-acids casein
Total protease (units/ml.)	0.62	0.72	0.63	0.27	0.63	0.67	0.68	0.36
Total protease in cell-free supernatant (%)	44	39	50	21	25	28	29	20
Casein hydrolysed (%)	—	—	—	—	80	83	83	87
Cell density (EEL units)	77	60	96	49	—	—	—	—

Effect of organic nitrogen

It was observed (Fig. 3) that the presence of tryptose did not stimulate protease production, nor did mixtures of tryptose, proteose peptone and Casamino acids with casein (Table 1). The bacterial concentrations in 24 hr cultures have little significance

but there was definite inhibition of growth by 0.3 % (w/v) Casamino acids. In experiments not reported this inhibition by 0.3 % (w/v) or more Casamino acids was confirmed, while lower concentrations were without effect in influencing growth and protease production. A considerable proportion of the casein originally present (50 mg./ml. growth medium) was hydrolysed in all the media, irrespective of their composition. That tryptose and proteose peptone were without effect on growth rate and protease production is illustrated in Fig. 6. The mean generation time in all media was 68 min. The addition (5 g./l.) of glutamic acid, aspartic acid, lysine or arginine did not allow *Bacteroides amylophilus* H18 to grow in the basal medium and had no effect on growth or protease production in the maltose medium.

Fig. 6. Protease production by *Bacteroides amylophilus* H18. Three 20 ml. 16 hr cultures of *B. amylophilus* H18 in maltose medium were sedimented at 3000 g for 10 min. and the cells inoculated directly into 100 ml. bottles of: maltose medium ($\square$ — $\square$), maltose-tryptose medium ($\circ$ — $\circ$) maltose-proteose and peptone medium (∇ — ∇). The bottles were incubated and samples removed for cell density and protease determinations as described in Methods.

Table 2. *The fermentation products of Bacteroides amylophilus* H18

The four media were inoculated and incubated for 24 hr. The fermentation products were separated and assayed by the method of Wiseman & Irwin (1957).

Products	Medium			
	Maltose	Maltose Tryptose	Maltose casein	Maltose Tryptose casein
Acetic acid (μ moles/ml.)	11.7	15.9	11.7	12.7
Formic acid (μ moles/ml.)	11.5	11.4	8.1	14.4
Succinic acid (μ moles/ml.)	14.2	13.5	12.1	12.8

Maltose utilization

The final products of protein degradation in the rumen include branched- and straight-chain volatile fatty acids (Blackburn, 1965) but the data in Table 2 do not suggest that tryptose or casein were degraded in this way by *Bacteroides amylophilus* H18, as no increment was found in acetic or formic acid concentrations in media con-

Table 3. *Effect of maltose concentration on growth yields and protease of Bacteroides amylophilus H18*

Maltose was added as a sterile filtered solution to the basal medium to give the concentrations indicated below. The maltose concentration before and after 16 hr growth of *B. amylophilus* was measured by the anthrone method of Fairbairn (1953). The protease assay is described in Methods. The dry weight of cells was calculated from the cell density.

Initial maltose concentration (mg./ml.)	Final maltose concentration (mg./ml.)	Maltose used (mg./ml.)	Cell density (EEL/units)	Protease (units/ml.)	Cell yield (g. cells/mole maltose)	Protease yield (units/mg. maltose)
1.16	0.32	0.84	20	0.01	39.8	0.01
2.30	0.46	1.84	41	0.39	37.4	0.21
3.21	0.48	2.73	51	0.65	31.4	0.24
4.88	1.38	3.50	100	0.74	47.6	0.21
6.50	1.90	4.60	102	0.92	37.2	0.20
7.62	3.60	4.02	98	0.78	40.8	0.19
8.89	4.83	4.06	100	0.82	40.6	0.20
9.45	5.85	3.60	101	0.78	47.0	0.22

taining tryptose or casein, and no other volatile fatty acids were produced. Carbon dioxide was an essential growth factor for *B. amylophilus* H18 and the fermentation products suggest that: maltose + 2CO₂ → 2 acetic acid + 2 formic acid + 2 succinic acid. Yields of bacteria and protease were proportional to the amount of maltose fermented and the total yield of both, expressed per ml. culture, increased up to a maltose concentration of 0.6% (w/v) (Table 3). The average yield of bacteria was equivalent to 40 g. dry wt/mole maltose. A high concentration of maltose remained unfermented even where it was growth-limiting.

Utilization of other energy sources

Growth and protease production occurred to the same extent in starch medium as in maltose medium and the addition of casein, tryptose or Casamino acids did not stimulate further protease production. Casein was hydrolysed in starch media and autolysis of the bacteria also occurred. No energy source other than α-1,4-linked glucose polymers was found to support growth, and the presence of α-oxoglutaric acid, pyruvic acid, succinic acid, acetic acid, butyric acid or formic acid (5 g./l.) did not affect growth or protease production.

Bacteroides amylophilus H18 produced small colonies in roll tubes of casein agar medium containing maltose at 0.2 g./l. These colonies were surrounded by a dense zone of opacity and when transferred to casein maltose slopes they produced a similar dense opacity in the casein. A total of five transfers on slopes of this medium did not produce an inoculum capable of growth in a liquid medium of the same composition.

Effect of pH value of medium

The pH value of the basal medium, which contained 0.5% (w/v) NaHCO_3 , was pH 6.7 when equilibrated with a 100% CO_2 gas-phase. The buffering capacity of this medium was very poor and *Bacteroides amylophilus* H 18 when supplied with a surplus of maltose, lowered the medium to pH 5.5 after 26 hr. As shown in Table 4 the presence of twice the usual NaHCO_3 concentration resulted in an increase to only pH 6.9, but after 26 hr the pH value had decreased less than in the normal medium. The protease yields in this experiment were very low and the yield at 26 hr was not greatly increased by the additional buffering of the medium, but on further incubation the yield almost doubled in the well buffered medium while it fell in the normal medium.

Table 4. *Effect of additional buffering on protease production*

2.0% maltose was used instead of the usual 0.3% to ensure that maltose was not growth limiting. Twice the normal concentration of NaHCO_3 was added to one medium. 100 ml. amounts of the media were inoculated and samples withdrawn at 26 and 52 hr for pH, cell density and protease determinations, as described in Methods.

Hr	Basal medium + 2.0% (w/v) maltose			Basal medium + 2.0% (w/v) maltose + additional 0.5% (w/v) NaHCO_3		
	pH	Cell density (EEL units)	Protease (units/ml.)	pH	Cell density (EEL units)	Protease (units/ml.)
0	6.7	0	0	6.9	0	0
26	5.5	94	0.20	6.4	93	0.25
52	5.5	88	0.18	5.3	122	0.47

Effect of E_h value of media

The addition of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.025%) to the maltose medium (containing cysteine) did not affect growth or protease production. A similar result was obtained when sodium dithionite (0.003%) was used. The concentration of sodium dithionite used reduced phenosafranine; the measured reduction potential of this medium was -410 to -470 mV (Pt and calomel electrodes).

Bacteroides amylophilus H 18 grew in media in which cysteine was replaced by sodium dithionite or by sodium sulphide + ascorbic acid (0.05%). L-Methionine (0.05%) could not replace cysteine in media reduced by ascorbic acid. Protease production was similar in all media in which growth occurred.

Effect of cations in media

A fivefold increase in the normal concentration of Ca^{2+} or of Mg^{2+} did not increase either growth or protease production by *Bacteroides amylophilus* H 18. Further supplementation of the maltose medium with similar concentrations (0.45 g./l.) of MnCl_2 , CoCl_2 or FeSO_4 was without effect.

DISCUSSION

The curve for activity against pH of the *Bacteroides amylophilus* protease suggested the presence of two enzymes, although Matsubara *et al.* (1958) showed that the crystalline protease of *Bacillus subtilis* strain N has an irregular plateau of activity

from pH 7.0 to pH 11.0. Production of total protease began with bacterial division and ceased when growth stopped and a small, but constant, proportion (20 %) of the protease was released into the medium. This may be truly extracellular (Pollock, 1962) as was the extracellular pH 5.7 micrococcus protease of McDonald (1965). However, on prolonged incubation the total protease content of a culture decreased but the proportion of cell-free protease increased and this and the very sharp decrease in culture density at the end of logarithmic growth, a characteristic of many rumen bacteria (Bryant & Robinson, 1961), might indicate that a proportion of bacteria were lysing during growth and that this became apparent only when growth ceased.

The protease production was not subject to either product repression or induction since protein, peptides or amino acids in the medium did not influence its formation, nor was a general metabolic repression demonstrated. The growth requirements of *Bacteroides amylophilus* are simple and additional nutrients did not affect either growth or protease production. The observations of Hobson, McDougall & Summers (1968) that some 93 % of the cell nitrogen could be accounted for by ammonia-¹⁵N disappearance in media with and without additional identified sources of nitrogen confirmed the results of Abou Akkada & Blackburn (1963) and explain the fact that additional organic nitrogen sources did not influence growth, except that the presence of 0.1 % (w/v) tryptose in the medium did decrease the lag period, probably by stabilizing the E_h value.

The products of maltose fermentation, acetic, formic and succinic acids were those found by Hamlin & Hungate (1956). The absolute dependence of *Bacteroides amylophilus* H18 on maltose or starch as energy source made it impossible to investigate the effect of different energy sources on protease production. Presumably the cell is impermeable to a wide range of energy sources but is permeable to α -1,4-linked polymers of glucose as is the *Micrococcus* sp. ATCC 407 (Williams & McDonald, 1966). *Bacteroides amylophilus* H18 lacks the capacity to utilize maltose at concentrations less than 0.32 mg. maltose/ml. Hobson & Summers (1967) also noted this property of *B. amylophilus* H18, and to a lesser extent *Ruminococcus albus* (Hungate, 1963) showed an impaired ability to concentrate, in this case, cellobiose.

I thank Dr P. N. Hobson for helpful discussion and Mrs N. Cowie for skilled technical assistance.

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