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Amylase and Protease Secretion by the Marine Bacterium *Vibrio gazogenes*

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Abstract

V. gazogenes secreted an amylase throughout the logarithmic phase of growth when starch or maltose was the carbon source for growth. The enzyme was apparently not constitutive and was repressed by glucose. The amylase was of the α-type and had an optimum pH of 6·5. Protease secretion by *V. gazogenes* occurred when the organism was grown in a defined medium containing 0·005% (w/v) yeast extract. The activity of the enzyme was increased 40-fold when the organism was grown in a medium containing peptone or Casamino Acids. Enzyme secretion started in the late logarithmic phase of growth and ceased when the cells entered the stationary phase of growth and was not repressed by glucose. The protease was neither induced nor repressed by glutamic acid, aspartic acid, alanine, serine, arginine, valine, threonine, lysine, leucine, proline or α-ketoglutarate. Tryptophan delayed, but did not inhibit, protease secretion. Both the amylase and the protease were truly extracellular and were not released as a result of cell lysis. Phenylmethylsulfonyl fluoride and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride, L-1-tosylamide-2-phenylethyl chloromethyl ketone, leupeptin, antipain and chymostatin did not markedly inhibit the protease, indicating that it is not a serine protease nor similar to trypsin, chymotrypsin, papain or cathepsin B. Inhibitors of sulfhydryl enzymes were also without effect on protease activity, but 1,10-phenanthroline, ethylenediaminetetraacetate and ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetate almost completely inhibited the protease, indicating that it requires a divalent metal ion for activity. After dialysis against water, both amylase and protease lost over 90% of their activity. The amylase was almost completely reactivated by 15 mM Cl^-, Br^- or I^-, but not by Ca^{2+} or Mg^{2+}. Protease was fully reactivated by about 30 mM Ca^{2+} or Mg^{2+} (at 70 mM concentration these ions stimulated activity to about 140% of the rate of the undialysed enzyme). Mn^{2+} and Co^{2+} partially restored protease activity. Chloramphenicol, at concentrations that did not affect RNA synthesis, completely inhibited amylase and protease secretion, showing that secretion of both enzymes was a *de novo* process. When rifampin or actinomycin D, at concentrations that completely and rapidly inhibited cellular RNA synthesis, was added to cultures actively secreting amylase or protease, there was no inhibition of secretion for periods of 6–20 min, indicating the presence of a pool of mRNA specific for each enzyme. The protease was not affected by trypsin or α-chymotrypsin during the secretion process, indicating that the enzyme had taken up its tertiary conformation before emergence from the cell membrane.

Introduction

The genus *Vibrio* contains a number of species of marine origin (Baumann *et al.* 1980) that bear a close resemblance to the terrestrial enterobacteria in a number of physiological and metabolic properties (Baumann and Baumann 1977). The production of extracellular enzymes is common among marine members of this genus (Baumann and Baumann 1977), but as yet enzyme secretion has been studied in detail in only one marine species, namely *V. alginolyticus* (Reid *et al.* 1978, 1980),

although limited studies have been carried out on two other species of marine *Vibrio* (Tanaka *et al.* 1969; Tanaka and Iuchi 1971; Dreisbach and Merkel 1978).

The present study was undertaken for two reasons. Firstly to determine whether the pattern of enzyme secretion in *V. gazogenes* is similar to that of the other *Vibrio* species (Tanaka *et al.* 1969; Tanaka and Iuchi 1971; Dreisbach and Merkel 1978; Reid *et al.* 1978, 1980) and other Gram-negative bacteria (Glenn 1976), and secondly, because *V. gazogenes* should be considered as a potential commercial source of amylase and protease as it grows rapidly in a readily available low-cost medium based on seawater.

Materials and Methods

Cultural Procedures

Vibrio gazogenes, formerly *Beneckea gazogenes* (Baumann *et al.* 1980), was grown in an artificial seawater (ASW) medium under aerobic conditions (Gavrilovic *et al.* 1982). The carbon sources were glucose, mannitol, starch or maltose at concentrations up to 0·5% (w/v). Cultures for the antibiotic experiments were grown with aeration at 35°C in a model SS30 reciprocating shaker (Grant Instruments Ltd, Cambridge, England) at about 100 cycles per minute; pH was not controlled in these cultures.

Preparation of Cell Extract

V. gazogenes was grown in 500 ml of medium containing 0·5% (w/v) starch, in which glycylglycine buffer replaced the Tris-HCl buffer. The cells were harvested and cell extracts were prepared as described by Gavrilovic *et al.* (1982).

Enzyme Assays

Protease was assayed by a modification of the method of Rinderknecht *et al.* (1968), which involves the release of a blue dye carried by a water-insoluble hide powder. The assay mixture contained 20 mg hide powder azure; 50 mM imidazole-HCl buffer, pH 6·5 (in ASW diluted to half strength); and culture supernatant, in a final volume of 3 ml. After 15 min at 30°C, the reaction was stopped by the addition of 0·4 ml of 1 M HCl. After centrifugation, the absorbance of the supernatant fraction was measured at 595 nm. One unit of activity is defined as the amount of enzyme sufficient to produce an absorbance of 1·0 at 595 nm in 15 min.

Amylase was assayed according to Gavrilovic *et al.* (1982) using 50 mM imidazole-HCl buffer, pH 6·5, or potassium phosphate buffer, pH 6·5. One unit of amylase activity is defined as the amount of enzyme that produces an absorbance of 1·0 at 620 nm after 15 min incubation.

To determine whether the amylase was of the α- or β-type the rate of disappearance of the blue iodine colour of amylose and the rate of formation of reducing sugar (measured as maltose) after the addition of enzyme were measured according to Robyt and Whelan (1968). The incubation contained 20 mM imidazole-HCl buffer, pH 6·5; 20 mM NaCl; 12 mg amylose; and 1 ml dialysed culture supernatant, in a final volume of 12 ml. The enzyme from *V. gazogenes* was compared with *Bacillus subtilis* α-amylase and sweet potato β-amylase. The assay for the *B. subtilis* enzyme was carried out in 55 mM imidazole-HCl buffer, pH 6·9, and for the sweet potato enzyme in 25 mM acetate buffer, pH 4·8.

Triosephosphate isomerase (EC 5.3.1.1) was assayed according to Gavrilovic *et al.* (1982).

Assay Procedures

Protein in cell extracts was assayed by the biuret method using bovine serum albumin as a standard (Gornall *et al.* 1949). Starch was assayed by the iodine-staining method (Robyt and Whelan 1968), and glucose by the glucose oxidase method (Sigma Technical Bulletin No. 510), with the enzymes dissolved in 0·5 M Tris-HCl buffer, pH 7·0, to inhibit maltase activity (Dahlqvist 1968). Reducing sugars (measured as maltose) were determined by the method of Somogyi (1952) with Nelson's (1944) arsenomolybdate reagent.

Protein and RNA Synthesis

Cellular protein synthesis was determined by measuring the incorporation of L-[^{14}C]phenylalanine into trichloroacetic acid (TCA)-insoluble material, and RNA synthesis was determined by measuring the incorporation of [5-^{3}H]uracil into TCA-insoluble material. The procedures used were those described by Gavrilovic *et al.* (1982).

Materials

Chloramphenicol, uracil, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), NADH, DL-glyceraldehyde 3-phosphate, phenylmethyl-sulfonyl fluoride (PMSF), *N*-α-β-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), L-1-tosyl-amide-2-phenylethyl chloromethyl ketone (TPCK), glucose oxidase enzymes, *B. subtilis* α-amylase, sweet potato β-amylase, glycerolphosphate dehydrogenase, leupeptin, antipain and chymostatin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Actinomycin D, rifampin, hide powder azure and amylopectin azure were bought from Calbiochem-Behring Corp., La Jolla, Cal., U.S.A., and Phadebas Amylase Test tablets from Pharmacia Diagnostics AB, Uppsala, Sweden. Radio-isotopes were purchased from Radiochemical Centre, Amersham, England. All other chemicals used were of analytical reagent grade.

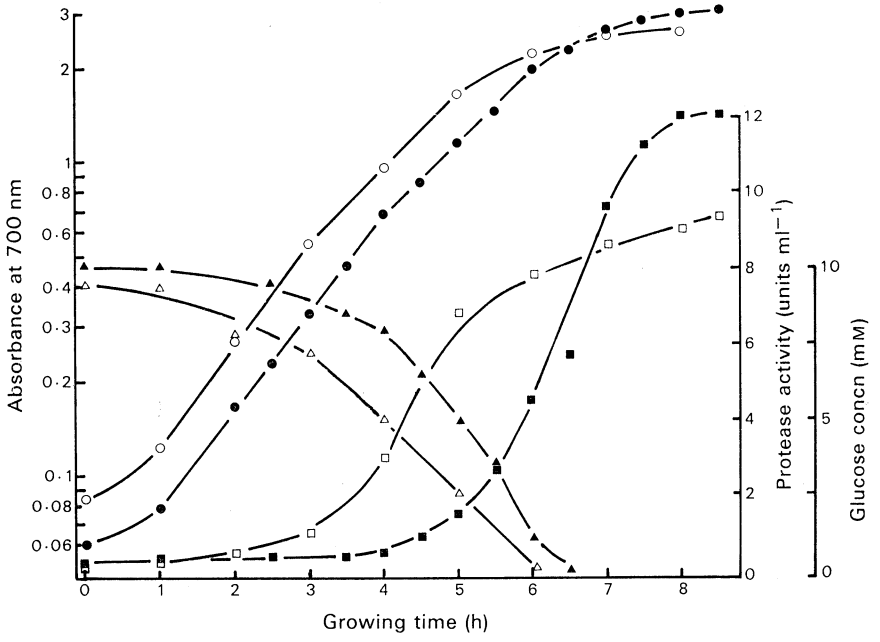


Fig. 1. Growth of *V. gazogenes* on 0·2% (w/v) glucose+tryptone (●) or on 0·2% (w/v) glucose+Casamino Acids (○), protease secretion in the presence of tryptone (■) or Casamino Acids (□), and glucose utilization in the presence of tryptone (▲) or Casamino Acids (△).

Results

Growth of the Organism and Secretion of Protease and Amylase

When *V. gazogenes* was grown on glucose medium containing tryptone, secretion of protease started in the late exponential phase of growth and reached a maximum value as the organism went into the stationary phase of growth (Fig. 1). A small amount of enzyme (less than 0·3 units per millilitre of culture) was secreted by cells growing in a medium devoid of tryptone. It was not possible to determine whether the small amount of yeast extract present in the medium was responsible for the

induction of the protease, as in the case of *Pseudomonas maltophilia* (Boethling 1975), because *V. gazogenes* would not grow if yeast extract was omitted from the medium. When Casamino Acids replaced tryptone in the medium, protease was induced about 2 h earlier (during the mid-exponential phase of growth), but to a lesser extent than when tryptone was the inducer (Fig. 1). When protease production commenced, it was always accompanied by a change in the growth rate of the organism, with a change of the mean generation time from 60 to 75 min.

Amino acids have been reported as both inducers (Keen and Williams 1967; Daatselaar and Harder 1974) and repressors (Litchfield and Prescott 1970; Reid *et al.* 1978) of extracellular proteases in Gram-negative bacteria. α-Ketoglutarate also acts as a repressor of protease in *P. maltophilia* (Boethling 1975). Experiments carried out with *V. gazogenes* showed that glutamic acid, aspartic acid, alanine, serine, arginine, valine, threonine, lysine, leucine, proline or α-ketoglutarate [at 0·5% (w/v) final concentration] did not repress protease secretion when the organism was grown on a glucose–tryptone medium, nor did any of these compounds induce protease secretion when *V. gazogenes* was grown on a glucose–NH_4Cl medium devoid of tryptone. Glycine (0·5% w/v) completely inhibited growth of the organism. The amino acids tested were the major amino acids in the Casamino Acids used in growth studies, as determined on an amino acid analyser. The delay in protease secretion observed when peptone, instead of Casamino Acids, was the nitrogen source may have been due to the presence of tryptophan in the peptone. When tryptophan [at 0·2% (w/v) final concentration] was added to medium containing Casamino Acids as nitrogen source, secretion of protease was delayed for 30 min. However, once secretion started, it proceeded at the same rate as in cultures free of tryptophan. At concentrations equivalent to those in cultures with peptone as nitrogen source, tryptophan did not affect protease secretion.

Growth of *V. gazogenes* in a medium in which 0·2% (w/v) maltose was the carbon source led to secretion of an amylase (Fig. 2). The organism did not grow at a logarithmic rate in this medium but did so when the maltose concentration was raised to 0·4% (data not shown). Secretion of amylase started in the early phase of growth (early in the logarithmic phase of growth when 0·4% maltose was used) and, although not shown in Fig. 2, continued until the organism stopped growing. Similar results were observed when starch was the carbon source for growth (data not shown). The ratio of the amount of enzyme secreted (whether protease or amylase) to the number of cells (based on the absorbance of the culture) was constant throughout the period of maximum enzyme secretion.

When *V. gazogenes* was grown on a medium containing both glucose and starch as carbon sources, growth was diauxic with glucose being the first carbon source to be used. About 1 h after the depletion of glucose, starch consumption started and coincided with the secretion of amylase. Further evidence that glucose represses amylase synthesis was obtained by adding glucose to a culture growing on maltose as carbon source (Fig. 2). Enzyme secretion ceased 15 min after the addition of the glucose and did not resume until the glucose had been consumed. Protease secretion was not affected by the addition of glucose.

To demonstrate that the protease and the amylase were truly extracellular and were not released by cell lysis, the activity of both enzymes in the culture medium and in cell extracts was measured. Triosephosphate isomerase, an intracellular enzyme, was also measured. Triosephosphate isomerase was present in cell extracts

(specific activity 3·1 μmol glyceraldehyde 3-phosphate converted per minute per milligram protein), but was not found in the culture medium. On mixing the cell extract with the culture medium, there was no change in the activity of triosephosphate isomerase, indicating that failure to detect the enzyme in the medium was not due to endogenous inhibitors. Protease and amylase activities were high in the culture medium and constituted 99 and 95%, respectively, of the total activity in the medium plus cell extract. The activity of the two enzymes in the medium was not altered by the addition of cell extract, showing that the latter lacked inhibitors.

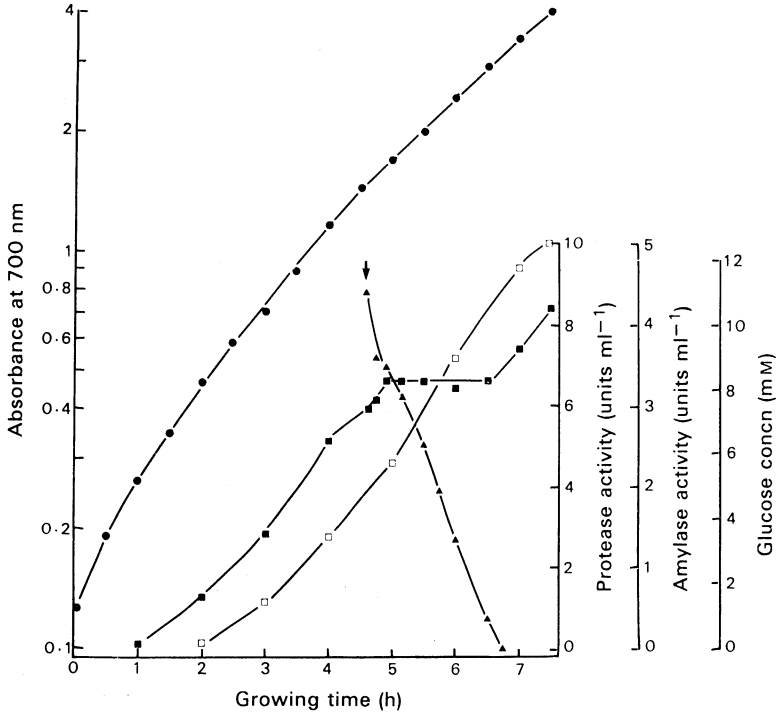


Fig. 2. Effect of adding glucose (at the time indicated by the arrow) on amylase (■) and protease (□) secretion by *V. gazogenes* growing on 0·2% (w/v) maltose. Growth (●) and glucose utilization (▲) are also shown.

Optimum pH for Enzyme Activity

The optimum pH for the activity of both amylase and protease (when assayed as described in Materials and Methods) was 6·5.

Type of Amylase

An experiment was conducted, as described in Materials and Methods, to determine whether the amylase was of the α- or β-type. The results obtained were similar to those for the amylase of *Alteromonas rubra* (Gavrilovic *et al.* 1982), indicating that the amylase of *V. gazogenes* is of the α-type.

Effect of Inhibitors on Protease Activity

Metal-chelating agents, such as EDTA, EGTA and 1,10-phenanthroline but not 2,2′-dipyridyl, inhibited protease activity by 98%. The sulfhydryl inhibitors *p*-chloro-

mercuribenzoate, *N*-ethylmaleimide and iodoacetate, had no effect on protease activity. PMSF (a serine protease inhibitor), TLCK (a trypsin inhibitor), TPCK (a chymotrypsin inhibitor) and the protease inhibitors antipain, chymostatin, and leupeptin had only a slight inhibitory effect (7–11%) on protease activity.

Effects of Ions on Protease and Amylase Activity

The effect of chelating agents on protease indicated it was dependent on divalent ions for activity. Dialysis of the enzyme against distilled water for 16 h resulted in a loss of 98% of its activity. About 50% of the activity could be restored by 15 mM Ca^{2+} or Mg^{2+}; full activity was restored by 30 mM Ca^{2+} or Mg^{2+}. The enzyme could be re-activated to about 140–150% of the original activity by 70 mM Ca^{2+} or Mg^{2+}. Mn^{2+} and Co^{2+} at 30 mM reactivated the protease by 55 and 30%, respectively. Cu^{2+}, Zn^{2+} and Ni^{2+} did not activate the enzyme.

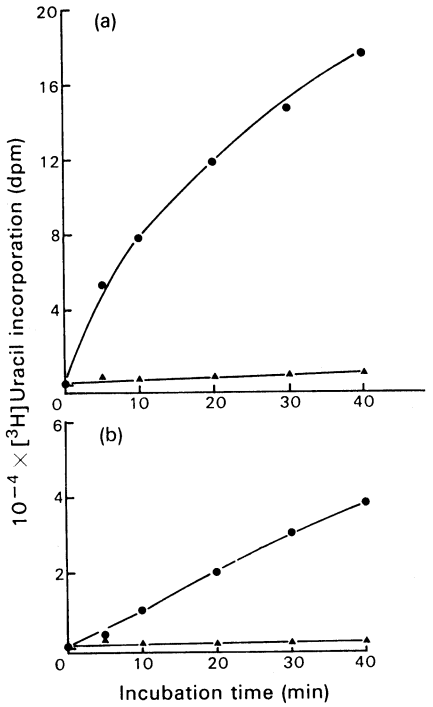
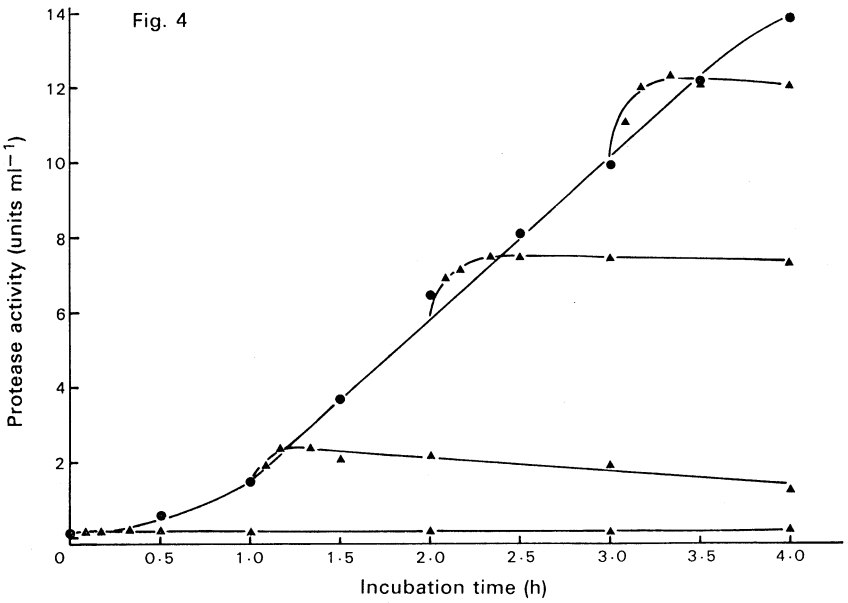
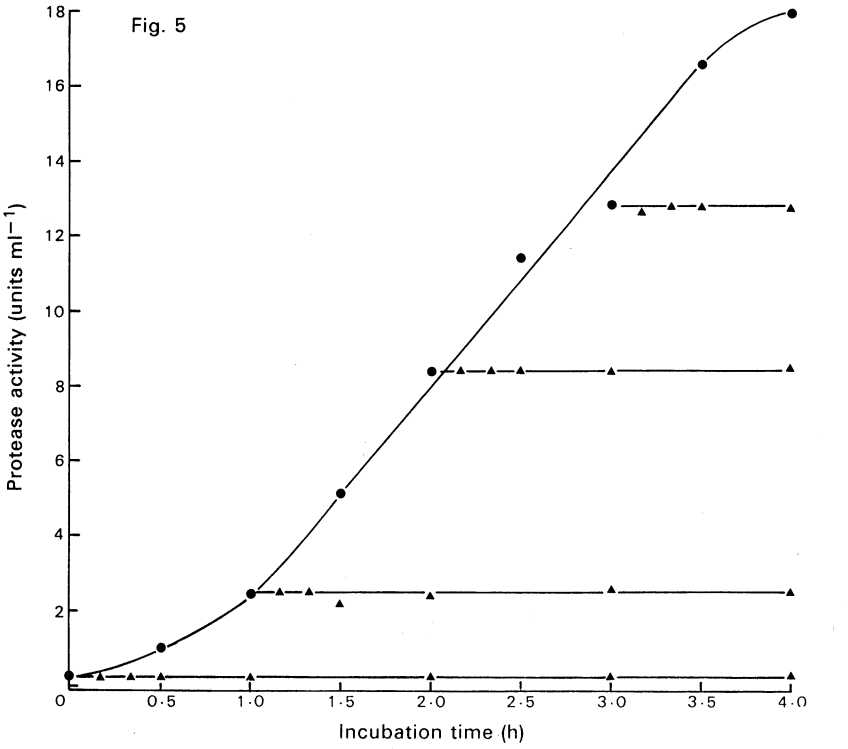


Fig. 3. Effect of rifampin on [5-^{3}H]uracil incorporation into RNA. Cells from the mid-logarithmic phase of growth were resuspended in fresh maltose (0·5% w/v) medium containing uracil (2 μg ml^{-1}) and incubated at 35°C. After 1 h (*a*) or 3 h (*b*), incubation samples (5 ml) of the culture were removed and incubated in shake culture at 35°C with 10 μCi [5-^{3}H]uracil plus 50 μg uracil carrier, in the absence (●) or presence (▲) of rifampin (final concentration 50 μg ml^{-1}).

Since a number of halophilic bacteria secrete amylases that have requirements for particular ions or for a medium with a high ionic strength (Good and Hartman 1970; Onishi 1972; Onishi and Hidaka 1978; Onishi and Sonoda 1979; Gavrilovic *et al.* 1982), we examined the ion requirements for the amylase of *V. gazogenes*. The crude enzyme was dialysed against water, which resulted in the loss of activity. Half of the activity was recovered by the addition of about 2·5 mM NaCl and 92% activation was achieved at 15 mM NaCl. The enzyme was not activated by Na_2SO_4, indicating that Cl^- was the true activator; SO_4^{2-} was not inhibitory. Br^- and I^- were more effective activators than Cl^-, with 50% activation at a concentration of 1–1·5 mM. NO_3^- gave 50% activation at 3 mM, but only 70% activation at 15 mM, whereas





Figs 4 and 5. Effect of rifampin (*4*) and chloramphenicol (*5*) on protease secretion. Cells from the mid-logarithmic phase of growth (with mannitol as carbon source) were resuspended in fresh mannitol (0·5% w/v) medium containing 2 μg ml^{-1} uracil. After 0, 1, 2 and 3 h incubation, 10 ml of the culture was removed and rifampin (50 μg ml^{-1} final concentration) or chloramphenicol (1 μg ml^{-1} final concentration) was added. Samples were removed, centrifuged and assayed for protease activity. All incubations were at 35°C. ● No rifampin or chloramphenicol. ▲ Plus rifampin or chloramphenicol.

Br^- and I^- at this concentration gave recoveries in excess of 90%. The amylase appeared to have no requirement for Ca^{2+} or Mg^{2+}. EDTA and EGTA (15 mM) did not inhibit the activity of the undialysed enzyme.

Effect of Rifampin and Actinomycin D on Protein Synthesis and Enzyme Secretion

Reid *et al.* (1980) reported that extracellular collagenase secretion by *V. alginolyticus* showed an unusual response to rifampin, in that secretion continued for 30–60 min after the addition of antibiotic, even though RNA synthesis was inhibited. When rifampin (at 50 μg ml^{-1} final concentration) was added to cell suspensions of *V. gazogenes* at 1 or 3 h after incubation of the cells in fresh medium, there was an immediate inhibition of [5-^{3}H]uracil incorporation into RNA (Fig. 3) and [^{14}C]phenylalanine incorporation into protein (data not shown). When rifampin was added at zero time to cells actively secreting protease and resuspended in fresh medium, enzyme secretion was completely inhibited. However, when the antibiotic was added at 1, 2 or 3 h after resuspension of the cells, enzyme secretion continued for 6–20 min before secretion was inhibited (Fig. 4). Similar effects by rifampin on amylase secretion were observed (data not shown). Actinomycin D (at 70 μg ml^{-1} final concentration) had the same effect on [5-^{3}H]uracil incorporation into RNA and on protease and amylase secretion as did rifampin (data not shown). The results in Fig. 3 discount the idea that the failure of the antibiotics to inhibit enzyme secretion was due to the cells becoming resistant or impermeable to the antibiotic because RNA synthesis was immediately inhibited by the antibiotics.

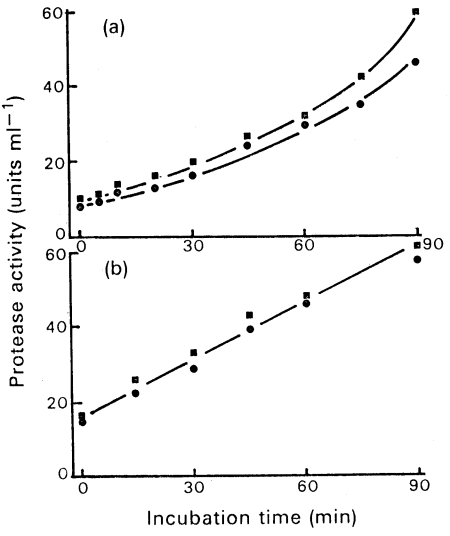


Fig. 6. Effect of α-chymotrypsin (*a*) and trypsin (*b*) on protease secretion by intact cells. Cells from the mid-logarithmic phase of growth (with mannitol as carbon source) were resuspended in fresh mannitol (0·5% w/v) medium in the absence and presence of 100 μg ml^{-1} α-chymotrypsin or 100 μg ml^{-1} trypsin. Protease activity was measured in the presence of 50 μg ml^{-1} TPCK or 50 μg ml^{-1} TLCK to inhibit α-chymotrypsin and trypsin, respectively. ● No α-chymotrypsin or trypsin. ■ Plus α-chymotrypsin or trypsin.

Effect of Chloramphenicol on Protein Synthesis and Enzyme Secretion

Chloramphenicol (1 μg ml^{-1} final concentration) instantly and completely abolished the incorporation of [^{14}C]phenylalanine into TCA-insoluble material. Control experiments showed that incorporation of [^{3}H]uracil into TCA-insoluble material was unaffected by this concentration of chloramphenicol (data not shown). When chloramphenicol was added to cells actively secreting protease after resuspension in fresh medium at 1, 2 or 3 h, there was an immediate inhibition of protease

secretion (Fig. 5). A similar effect on the secretion of amylase by chloramphenicol was observed (data not shown). None of the antibiotics had any inhibitory effect on the activity of the protease or the amylase.

Effect of Trypsin and α-Chymotrypsin on Protease Secretion

To determine whether the protease was susceptible to attack by proteolytic enzymes, secretion was measured in the presence of trypsin (100 $\mu g\ ml^{-1}$) or α-chymotrypsin (100 $\mu g\ ml^{-1}$) (Fig. 6). The results show that the activity of the secreted protease was not affected by either of the enzymes.

Discussion

Protease secretion by *V. gazogenes* occurred when the organism was grown on a medium containing tryptone or Casamino Acids and was not repressed by glucose or ammonium ion. Glucose represses extracellular protease production by *V. parahaemolyticus* (Tanaka and Iuchi 1971) and *V. alginolyticus* (Reid *et al.* 1980), although in the latter organism repression did not occur until about 20 min after the addition of the glucose. The behaviour of *V. gazogenes* is in contrast to that of *V. parahaemolyticus* (Tanaka and Iuchi 1971) and *V. alginolyticus* (Reid *et al.* 1978, 1980) in that Casamino Acids repress protease synthesis by these two bacteria. The nature of the true inducer of protease in *V. gazogenes* was not determined. Glutamic acid, aspartic acid, alanine, serine, arginine, valine, threonine, lysine, leucine and proline failed either to induce or repress protease syntheis. Leucine and proline inhibit collagenase production by *V. alginolyticus* (Reid *et al.* 1978). Tryptophan delayed protease synthesis but did not inhibit the rate of secretion. With *V. alginolyticus*, tryptophan inhibited protease secretion by about 50% (Reid *et al.* 1978). It was not possible to determine whether the protease is a constitutive enzyme because *V. gazogenes* would not grow on a completely defined medium free of inducer. Certainly, a higher activity of enzyme is induced by peptone or Casamino Acids, but it is not clear whether the constitutive and induced protease activities observed are due to the same enzyme. It is possible that two (or more) proteins with protease activity are produced.

The insensitivity of the protease to PMSF, TLCK, TPCK, chymostatin, leupeptin, antipain and reagents that react with sulfhydryl groups and its sensitivity to chelating agents indicate that it is a neutral protease (Matsubara and Feder 1971). This is borne out by the enzyme showing maximum activity at pH of 6·5. After dialysis, the enzyme lost its activity and was fully reactivated by Ca^{2+} or Mg^{2+} and was partially reactivated by Mn^{2+} or Co^{2+}. These ion requirements are similar to those of a protease from a marine *Pseudomonas* species with the exception that this enzyme had maximum activity at 18% sodium chloride concentration (Qua *et al.* 1981), whereas the protease from *V. gazogenes* was not activated by sodium chloride.

Sanders and May (1975) have shown in *Bacillus amyloliquefaciens* that protease and amylase are secreted through the intact cell wall in a form insentitive to proteolytic enzymes and also to its own purified protease. In the case of *V. gazogenes*, the secreted protease was insensitive to both trypsin and α-chymotrypsin, indicating that the protease takes up its tertiary conformation before being secreted through the outer membrane.

Extracellular amylase production by *V. gazogenes* was induced by maltose or starch and was repressed by glucose. This is in contrast to another marine bacterium

A. rubra, which possesses a constitutive amylase whose production is inhibited by glucose only when the concentration of glucose is in excess of 5 mM (Gavrilovic *et al.* 1982). When glucose was added to a culture of *V. gazogenes* actively secreting amylase, repression of enzyme synthesis did not occur until 15 min after glucose addition. The same delayed repressive effect was observed when rifampin was added to actively secreting cells (see below). This implies that glucose exerts its effect at the transcriptional stage of amylase synthesis, a finding also observed with amylase secretion by *Bacillus subtilis* (Priest 1975). A similar effect of glucose on amylase secretion was observed with *A. rubra* (Gavrilovic *et al.* 1982). Induction of extracellular α-amylase synthesis by maltose or starch and repression by glucose has been observed in *V. parahaemolyticus* (Tanaka *et al.* 1969).

The amylase (after dialysis) required low concentrations of Cl^-, but not Ca^{2+}, for activity and thus differs from the amylase secreted by *A. rubra*, which required both of these ions for activity (Gavrilovic *et al.* 1982), and also differs from the amylases of two species of halophilic bacteria, which required sodium or potassium chloride at concentrations ranging from 0·2 to 0·75 M (Onishi 1972; Onishi and Hidaka 1978).

Chloramphenicol rapidly and completely inhibited protein synthesis and enzyme secretion by *V. gazogenes*, showing that amylase and protease secretion were the result of *de novo* protein synthesis. Inhibition of transcription by rifampin or actinomycin D at the commencement of enzyme induction also inhibited further enzyme secretion. However, rifampin, at concentrations that inhibited transcription, took about 6–20 min to inhibit secretion of amylase and protease when added 1, 2 or 3 h after secretion had started. This effect was not due to an increasing impermeability of the cells to the antibiotic because there was an instantaneous inhibition of RNA synthesis after the addition of rifampin, nor was it due to an increasing resistance of the RNA polymerase to rifampin. The same pattern of protease production was observed in the presence of actinomycin D. As this antibiotic acts directly on the DNA, one would expect an immediate inhibition of the protease secretion if the RNA polymerase was simply becoming less susceptible to inactivation during the period of secretion. The results suggest an apparent accumulation of two mRNA species specific for the extracellular protease and the extracellular amylase, respectively, which allows synthesis of the enzymes in the absence of transcription. The existence of mRNA pools specific for extracellular protease was first reported by Both *et al.* (1972). Similar results have been observed with protease secretion by *V. alginolyticus* (Reid *et al.* 1980) and with amylase secretion by *A. rubra* (Gavrilovic *et al.* 1982).

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