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Secretion of Amylase by the Marine Bacterium, *Alteromonas rubra*

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Abstract

A. rubra possesses a constitutive α-amylase which is secreted during the stationary phase of growth when glucose (at concentrations below 5 mM) is the carbon source. The activity of the enzyme was increased about eightfold over the control level when the organism was grown on 0·2% (w/v) starch or maltose, with secretion starting in the late logarithmic phase of growth and continuing into the stationary phase. α-Amylase secretion was repressed by the addition of glucose, at concentrations in excess of 5 mM, when the organism was grown on a medium containing starch or maltose as carbon source. The enzyme was extracellular and was not released as a result of cell lysis and was of the α-type, hydrolysing starch mainly to maltose, maltotriose and maltotetraose. After dialysis against water for 16 h the enzyme lost 98% of its activity. Activity could be partially restored by Ca^{2+} (70% at 15 mM) or Cl^- (43% at 60 mM); at 15 mM Ca^{2+} and 30 mM Cl^- full activity was restored. Br^- was equally as effective as Cl^-, but Mg^{2+} was less effective than Ca^{2+}.

Chloramphenicol, at concentrations which did not affect RNA synthesis, completely inhibited amylase secretion showing that it was a *de novo* process. When actinomycin D or rifampin, at concentrations which completely and rapidly inhibited cellular RNA synthesis, was added to cultures actively secreting amylase there was no inhibition of secretion for at least 20 min, indicating the presence of a pool of mRNA specific for the enzyme.

Introduction

Secretion of extracellular enzymes is a widespread phenomenon among bacteria (Glenn 1976; Priest 1977). One of these enzymes is α-amylase and its secretion by halophilic bacteria (Good and Hartman 1970; Onishi 1972; Onishi and Hidaka 1978; Onishi and Sonoda 1979) and non-halophilic bacteria (Markovitz and Klein 1955; Tanaka *et al.* 1969; Tanaka and Iuchi 1971; McWethy and Hartman 1977; Priest 1977) has been reported. Qualitative tests have shown that a number of marine *Beneckea* spp. also secrete α-amylase (Baumann and Baumann 1977).

This paper presents the first evidence for the secretion of an extracellular α-amylase by the marine bacterium *Alteromonas rubra* and describes some of the properties of the enzyme. Evidence is also presented to support the idea that the organism accumulates a pool of mRNA specific for the enzyme. This organism was chosen because of its apparent high extracellular amylase activity compared with a range of other marine bacteria.

Materials and Methods

Culture Procedures

Alteromonas rubra R1 was grown in the following medium: artificial seawater (ASW), 250 ml; Tris-HCl buffer, pH 7·5, 50 mM; NH_4Cl, 19 mM; sodium β-glycerophosphate.$5\frac{1}{2}$ H_2O, 1·3 mM; ferric ammonium citrate, 50 mg; Bactotryptone 2·5 g and yeast extract 50 mg, made up to 500 ml with distilled water. The carbon sources were glucose, maltose or starch. ASW contained: NaCl, 600 mM; KBr 12 mM, KCl, 20 mM; $MgSO_4.7H_2O$, 100 mM and $CaCl_2.2H_2O$, 20 mM. Cultures were grown under aerobic conditions at 30°C (O'Brien and Morris 1971). The culture was maintained at pH 6·5–7·0 by the intermittent addition of sterile NaOH and growth was followed by measuring the turbidity of the culture at 700 nm after appropriate dilution in half-ASW (ASW diluted with an equal volume of distilled water). At various intervals, a 5-ml sample was collected and centrifuged to remove the bacterial cells and the supernatant fraction was assayed for amylase activity. The rest of the sample was frozen at −20°C until analysed for glucose (where appropriate).

Preparation of Cell Extracts

A. rubra was grown in 500 ml of medium containing 0·5% starch as carbon source, in which glycylglycine buffer replaced the Tris-HCl buffer, to the mid-logarithmic phase of growth and was harvested by centrifugation at 16000 *g* for 10 min at 5°C. The supernatant fraction was retained for enzyme assays. The cells were washed twice with 30 ml of half-ASW, buffered with 50 mM glycylglycine buffer (pH 7·5) and were resuspended in 7 ml of 0·1 M triethanolamine-HCl buffer, pH 7·5. The cells were disrupted by sonic oscillation (eight 30-s periods at 60 W) by using a Branson Sonifier, model B12 (Branson Sonic Power Co., Danbury, Mass., U.S.A.). Cell debris was removed by centrifugation at 29000 *g* for 15 min at 5°C and the supernatant fraction was used for enzyme assays.

Enzyme Assays

The Phadebas Amylase Test kit was used for routine measurements of amylase activity. The substrate in this kit is a water-insoluble, cross-linked starch polymer carrying a blue dye, which is hydrolysed by α-amylase to form water-soluble blue fragments. The absorbance of the blue solution is a function of the α-amylase activity in the sample. One Phadebas Amylase Test tablet was suspended in 10 ml of half-ASW containing 50 mM imidazole-HCl buffer, pH 7·5. The assay mixture contained 2 ml of this suspension and 0·1 ml of culture supernatant (suitably diluted if necessary in half-ASW containing 50 mM imidazole-HCl buffer, pH 7·5) and 0·9 ml of water. The assay was performed at 30°C for 30 min with frequent shaking and was terminated by the addition of 0·4 ml of 1 M HCl. After centrifugation in a bench centrifuge the absorbance of the supernatant fraction was measured at 620 nm. For the determination of the ion requirements for amylase the assay mixture contained: potassium phosphate buffer, pH 7·5, or imidazole-HCl buffer, pH 7·5, 12 mM; amylopectin azure, 20 mg; dialysed culture supernatant, 0·1 ml, in a final volume of 3 ml. One unit of amylase activity is defined as the amount of enzyme which produces an absorbance of 1·0 at 620 nm after incubation for 30 min.

Triosephosphate isomerase (EC 5.3.1.1) was assayed according to Bergmeyer *et al.* (1974). The assay mixture contained in a final volume of 1 ml: triethanolamine-HCl buffer, pH 7·5, 100 mM; DL-glyceraldehyde-3-phosphate, 2·5 mM; NADH, 0·02 mM; glycerol-3-phosphate dehydrogenase 1·5 units and cell extract (about 0·01 mg of protein) or culture supernatant (0·2 ml).

To determine whether the amylase was of the α- or β-type the following incubation was made at 30°C in a final volume of 17 ml: imidazole-HCl buffer, pH 7·5, 50 mM; amylose, 16 mg and culture supernatant, 0·3 ml. Samples were removed at 5-min intervals up to 30 min and the blue iodine colour of the amylose and the amount of reducing sugar (as maltose) were measured according to Robyt and Whelan (1968). The enzyme from *A. rubra* was compared with *Bacillus subtilis* α-amylase (assayed in 55 mM imidazole-HCl buffer, pH 6·9) and sweet potato β-amylase (assayed in 25 mM acetate buffer, pH 4·8).

Action Pattern of Amylase

The products of the hydrolysis of starch by *A. rubra* amylase were identified by thin-layer chromatography (t.l.c.) on silica gel aluminium sheets (E. Merck, Darmstadt, Germany) using

isopropanol : acetone : 0·1 M lactic acid (4 : 4 : 2 by vol.) as the developing solvent and diphenylamine aniline phosphate as the visualizing agent (Hansen 1975). The digestion mixture contained in a final volume of 4·0 ml of half-ASW: imidazole-HCl buffer, pH 7·5, 50 mM; starch, 1·4 mg and 60 units of amylase. The reaction was carried out at 30°C and samples (0·5 ml) were removed at 5-min intervals up to 30 min and the reaction stopped by the addition of one drop of 4 M HCl. Glucose, maltose, maltotriose and maltotetraose were used as standards.

Assay Procedures

Protein was assayed by the biuret method using bovine serum albumin as a standard (Gornall *et al.* 1949). Glucose was assayed 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).

Protein Synthesis

Cellular protein synthesis was determined by measuring the incorporation of L-[^{14}C]phenylalanine into trichloroacetic acid (TCA)-insoluble material. The cells were grown in 200 ml of medium (0·3% w/v starch as carbon source) in a 500-ml Erlenmeyer flask. The medium was inoculated with 2 ml of a culture grown overnight in the same medium. The flask was shaken at 30°C on a New Brunswick model G-76 gyrotory shaker operating at 240 cycles/min. When the culture reached an absorbance (at 700 nm) of 1·5 (at which point amylase secretion starts) samples (10 ml) were removed at various times and were placed in 125-ml Erlenmeyer flasks and 6·25 μCi of L-[^{14}C]phenylalanine (specific activity 509 mCi/mmol) was added. The flasks were shaken as described above. At appropriate times 0·2-ml samples were removed and added to 3 ml of a solution of 1% (w/v) Casamino Acids and 8·9 mM uracil in 10% (w/v) TCA and kept for 15 min at 0°C. The insoluble material was collected on Whatman GF/A filters (2·5 cm) which were then washed four times with 5 ml of Casamino Acids/uracil in TCA and twice with 10 ml of 1% (w/v) acetic acid. The filters were dried in a vacuum oven at 80°C for 60 min and were counted for radioactivity.

Samples (0·75 ml) were removed at the same time as those for radioactive counting and were centrifuged in a bench centrifuge to remove bacterial cells and the supernatant fractions were assayed for amylase.

RNA Synthesis

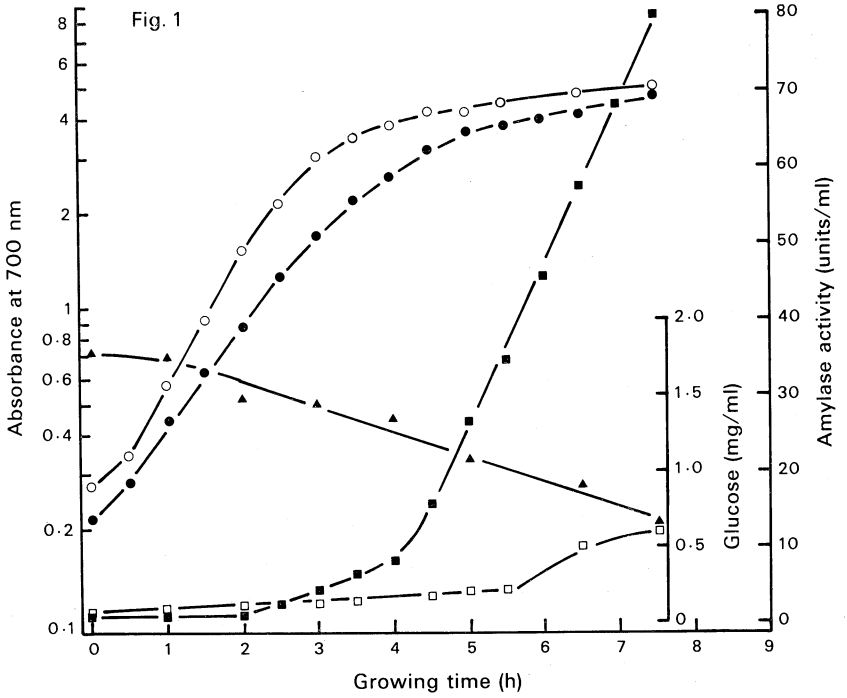
RNA synthesis was determined by measuring the incorporation of [5-^{3}H]uracil into TCA-insoluble material. The cells were grown as described above for protein synthesis and 10-ml samples of the culture were removed and 20 μCi of [5-^{3}H]uracil (specific activity 23 mCi/mmol) and unlabelled uracil (final concentration of 0·18 mM) were added. Samples (0·2 ml) were removed and treated as described for protein synthesis to recover the TCA-insoluble material. Samples were also collected for amylase assay.

Counting of Radioactivity

Radioactivity was measured by liquid scintillation in a Kontron MR300 Liquid Scintillation System (W and W Electronic Scientific Instruments, Basel, Switzerland) with automatic quench correction by the external standards ratio method. The instrument was programmed to measure ^{3}H and ^{14}C simultaneously. The scintillation fluid contained 3 g of 2,5-diphenyloxazole and 0·1 g of 1,4-bis-[(4-methylphenyloxazol-2-yl)] benzene per litre of toluene.

Materials

The following materials were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.: glycerol-3-phosphate dehydrogenase, DL-glyceraldehyde-3-phosphate, *B. subtilis* α-amylase, sweet potato β-amylase, glucose oxidase enzymes, uracil, chloramphenicol, NADH and maltotriose. Maltotetraose was obtained from Pierce Chemicals, Rockford, Ill., U.S.A. Amylopectin azure, rifampin and actinomycin D were obtained from Calbiochem-Behring Corp., La Jolla, Calif., U.S.A. and Phadebas Amylase Test tablets from Pharmacia Diagnostics AB, Uppsala, Sweden. Bactotryptone and Casamino Acids were supplied by Difco Laboratories, Detroit, Mich., U.S.A. Radioactive chemicals were obtained from The Radiochemical Centre, Amersham, England. All other chemicals were of analytical reagent grade.



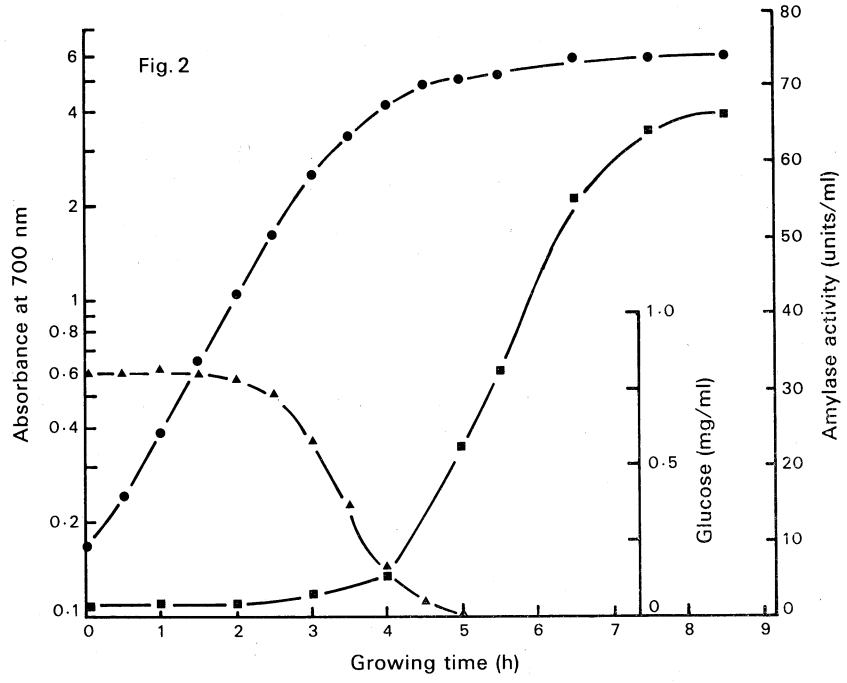


Fig. 1. Growth of *A. rubra* on 0·2% (w/v) glucose (○) and 0·2% (w/v) starch (●) and amylase secretion. □ Amylase secretion by glucose-grown cells. ■ Amylase secretion by starch-grown cells. ▲ Glucose utilization.

Results

Growth of the Organism and Secretion of Amylase

The growth of *A. rubra* and secretion of amylase when the organism was grown on glucose or starch as carbon source are shown in Fig. 1. Growth was slightly faster with glucose as carbon source (mean generation time 45 min) than with starch as carbon source (mean generation time 54 min). Amylase was secreted by the glucose-grown cells after the culture had entered the stationary phase of growth and when the glucose concentration in the medium was about 1 mg/ml (about 5 mM). The amount of amylase in the culture fluid of starch-grown cells at 7·5 h was some eightfold greater than for glucose-grown cells. Secretion commenced at the end of the logarithmic phase of growth and increased rapidly as the culture went into the stationary phase of growth.

When *A. rubra* was grown on a mixture of glucose and starch as carbon sources (Fig. 2) the mean generation time was 45 min. Amylase secretion started at the same stage of growth and was produced at the same rate as in cultures growing on starch as sole carbon source (Fig. 1) and before the culture was depleted of glucose. These results indicated that glucose was not a repressor of amylase secretion at concentrations of about 1 mg/ml. However, addition of glucose [2·2 mg/ml (or about 12 mM), final concentration] to a culture growing on starch as sole carbon source caused an inhibition of amylase secretion within 15 min (Fig. 3). The inhibition was relieved when the glucose concentration fell to about 1 mg/ml or 5 mM. Addition of 5 mM (final concentration) glucose to cultures grown with starch as carbon source did not inhibit amylase secretion.

Maltose was equally as effective as starch in inducing higher activities of amylase. The ability of glucose to repress amylase secretion in these cultures was similar to that observed with the starch cultures (data not shown). Secretion of amylase (2–3·4 units/ml of culture) by cells grown on 0·5% (w/v) mannitol, citrate or malate as sole carbon source or on glucose when the glucose concentration was below 5 mM indicates that the enzyme is constitutive.

To demonstrate that the amylase activity was truly extracellular and not released by cell lysis the activity of the enzyme in the cell-free culture supernatant and in extracts of whole cells was measured. The activity of triosephosphate isomerase, an intracellular enzyme, was also measured. A high activity of triosephosphate isomerase was found in the cell extract (1·5 μmol glyceraldehyde-3-phosphate converted per minute per milligram protein), but could not be detected in the culture supernatant. On mixing the cell extract with the culture supernatant there was no change in the activity of triosephosphate isomerase, indicating that failure to detect the enzyme in the supernatant was not due to an extracellular inhibitor. Tris-HCl buffer inhibits triosephosphate isomerase and hence for this experiment the culture was buffered with glycylglycine instead of Tris-HCl. Lysis of as little as 10% of the bacterial cells would release enough triosephosphate isomerase to be easily detected (equivalent to an activity of about 0·04 μmol glyceraldehyde-3-phosphate converted per minute per millilitre of culture supernatant). The amylase activity of the culture

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Fig. 2. Growth of *A. rubra* on 0·08% (w/v) glucose plus 0·1% (w/v) starch (●). ■ Amylase secretion. ▲ Glucose utilization.

supernatant (total of 1560 units of activity) was not altered by the addition of the cell extract showing that the latter did not contain amylase inhibitors. The small amount of amylase activity found in the cell extract (total of 25 units of activity) may have been due to inadequate washing of the cells or to some enzyme adhering to cell membranes being 'solubilized' by sonic oscillation.

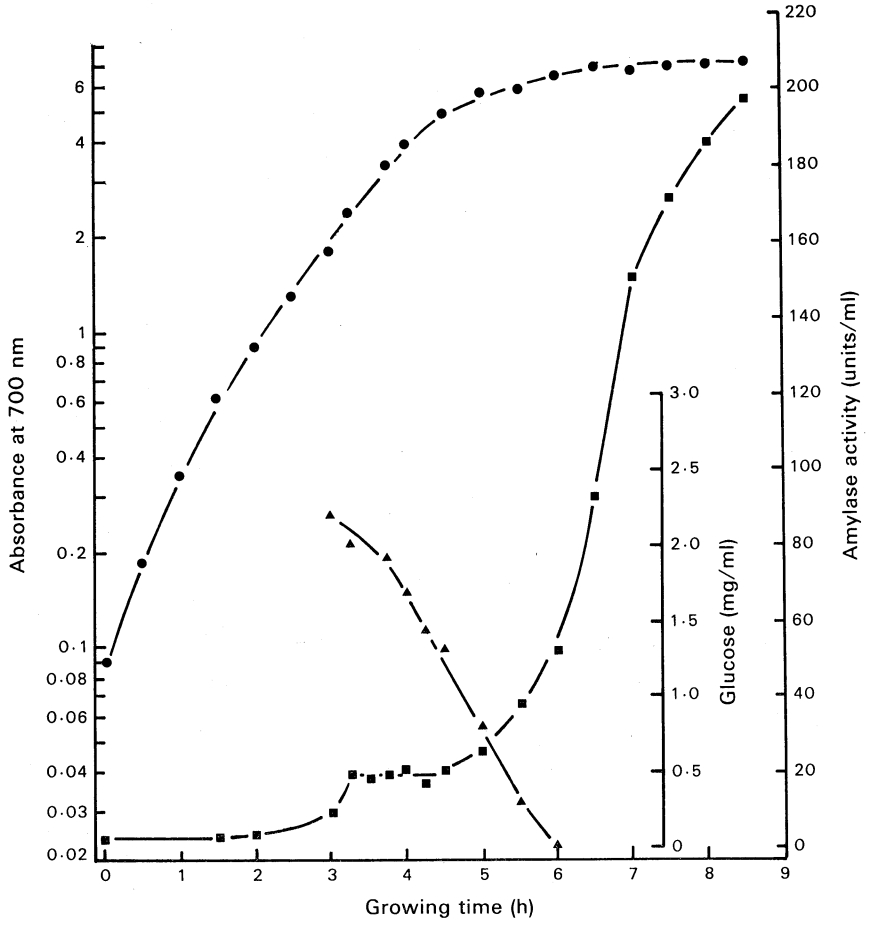


Fig. 3. Effect of adding glucose to 12 mM final concentration (after 3 h of growth) on amylase secretion by cells growing on 0·2% (w/v) starch. ● Growth. ■ Amylase secretion. ▲ Glucose utilization.

Some Properties of the Amylase

The optimum pH for the enzyme was 7·5 using imidazole-HCl buffer. Dialysis of the culture supernatants against distilled water resulted in the loss of 98% of the amylase activity. In the presence of 5 mM calcium acetate there was 50% recovery of activity, increasing to 70% at 15 mM (Fig. 4*a*). Replacement of calcium acetate with calcium chloride led to a better recovery of activity with 50% at 3·5 mM and 98% at 15 mM calcium chloride. Magnesium sulfate was less effective than the Ca^{2+} salts giving only 45% recovery at 15 mM (Fig. 4*a*). The enzyme was not activated

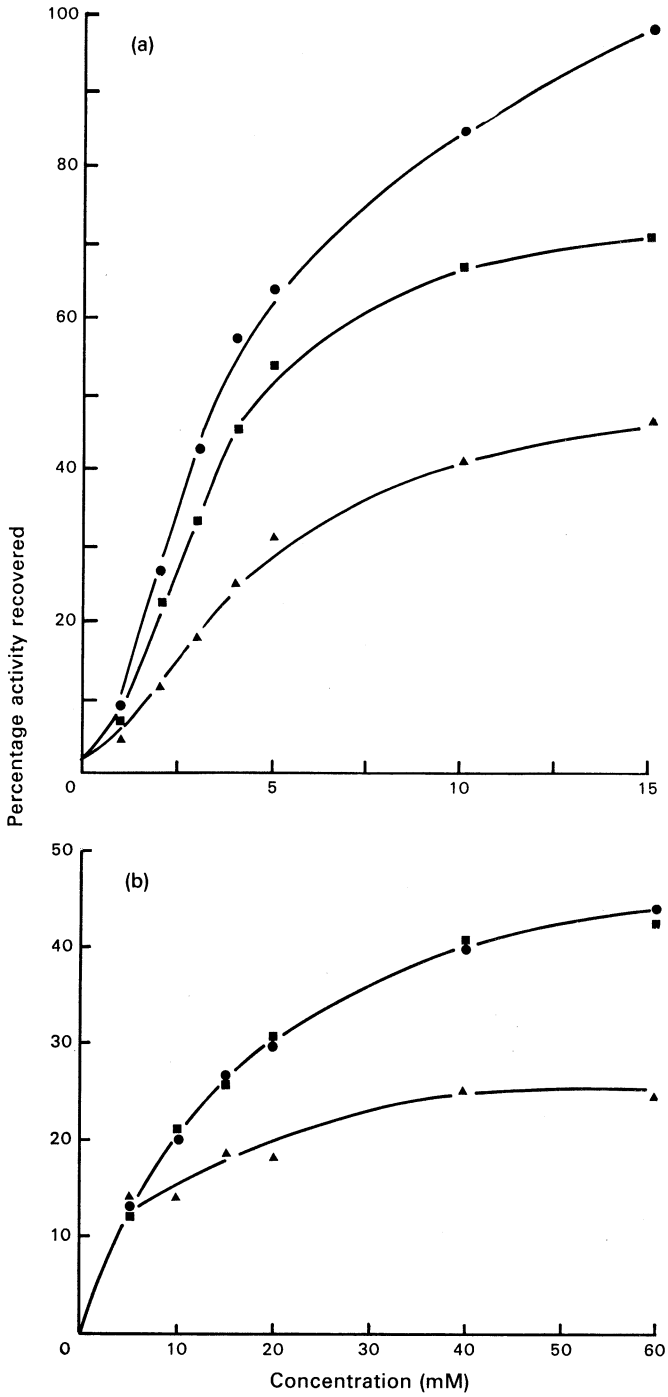


Fig. 4. Effect of (*a*) calcium chloride (●), calcium acetate (■), and magnesium sulfate (▲) and of (*b*) sodium chloride (●), potassium bromide (■) and potassium iodide (▲) on the activity of amylase after dialysis of the culture supernatant (5 ml) against 1 litre of water at 5°C for 16 h. The assays were carried out in 12 mM imidazole-HCl buffer, pH 7·5 (*a*) and in 12 mM potassium phosphate buffer, pH 7·5 (*b*). The activation is calculated as the percentage of the activity of the undialysed culture supernatant kept at 5°C for 16 h.

by sodium sulfate or sodium acetate. These results indicated that the enzyme had a requirement for both Ca^{2+} and Cl^-. The data in Fig. 4*b* show that Cl^- and Br^- were able to restore about 43% of the original activity at 60 mM; I^- was less effective giving only 25% activation at 60 mM. At 5, 10 and 15 mM the activation by Cl^- was 14, 21 and 26%, respectively, which was in agreement with the results in Fig. 4*a* showing that Cl^- increases the activation over that given by Ca^{2+}. Potassium chloride was equally effective as sodium chloride as an activator, but potassium sulfate had no effect.

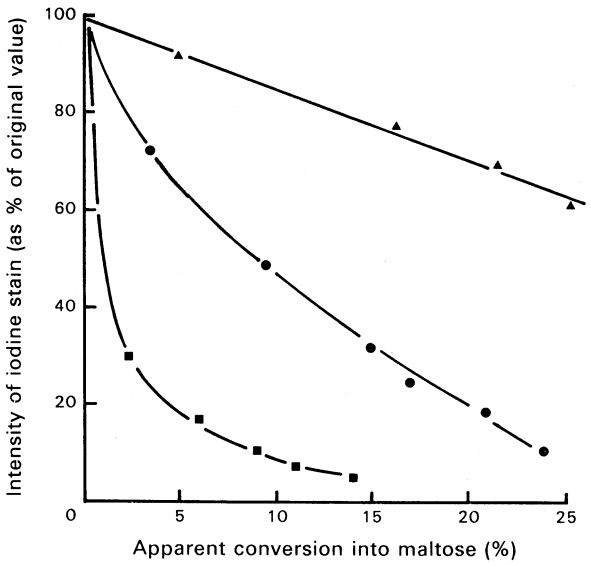


Fig. 5. Decrease in the intensity of the iodine stain of amylose plotted against the conversion of amylose into reducing sugar (measured as maltose) during the hydrolysis of amylose by *A. rubra* amylase (●), *Bacillus subtilis* α-amylase (■) and sweet potato β-amylase (▲).

To determine whether the amylase activity was of the α- or β-type it was incubated with starch and samples were taken to measure the blue iodine colour of the starch (as a measure of its hydrolysis) and the amount of reducing sugar produced (as maltose). These two parameters are plotted against one another in Fig. 5. The enzyme was compared with sweet potato β-amylase and α-amylase from *Bacillus subtilis*. A comparison of the curves shows that the amylase from *A. rubra* is of the α-type. The products of starch hydrolysis by *A. rubra* amylase were identified by t.l.c. After incubation for 10 min the main product was maltotetraose, with lesser amounts of maltotriose and maltose. By 30 min the main products were maltose and maltotriose; no glucose was detected.

Effect of Chloramphenicol on Amylase Secretion

When chloramphenicol (5 μg/ml) was added at zero time to cells actively secreting amylase there was an immediate and complete inhibition of amylase secretion. The same concentration of chloramphenicol inhibited the incorporation of [^{14}C]phenylalanine into TCA-insoluble material by more than 90%, but had no inhibitory effect on [5-^{3}H]uracil incorporation into TCA-insoluble material. The actinomycin D- and rifampin-insensitive amylase production (see below) were also completely inhibited by the addition of chloramphenicol.

Effect of Actinomycin D on Amylase Secretion

When actinomycin D (100 μg/ml final concentration) was added to cell suspensions at 1, 2 or 3 h after enzyme secretion had started there was a rapid (within 5 min) inhibition of [5-^{3}H]uracil incorporation into RNA (Fig. 6) and [^{14}C]phenylalanine

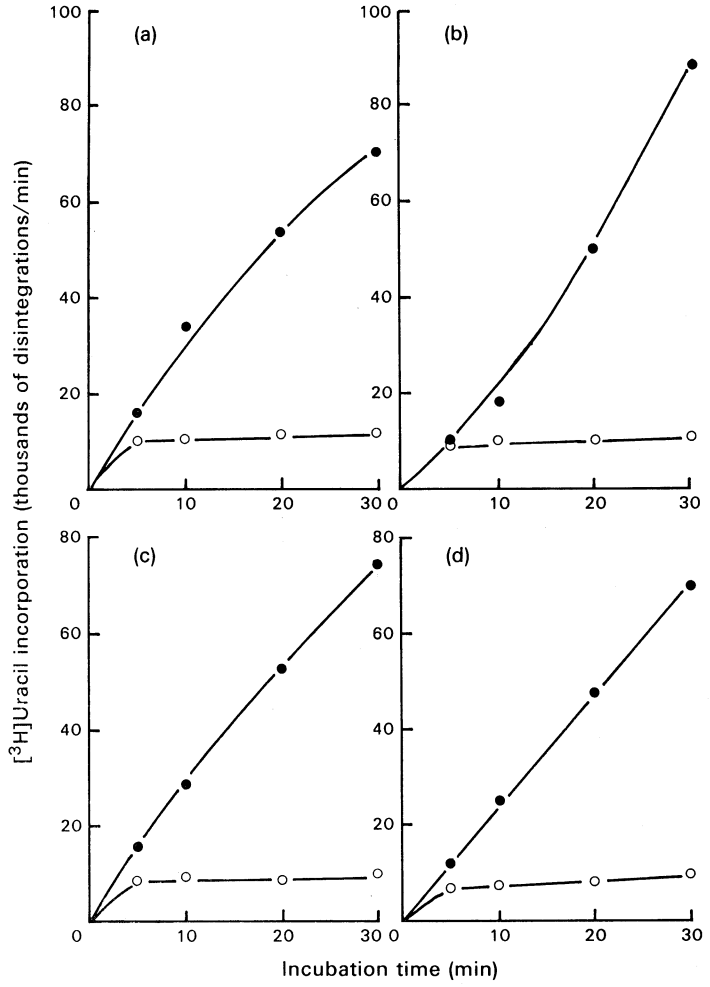
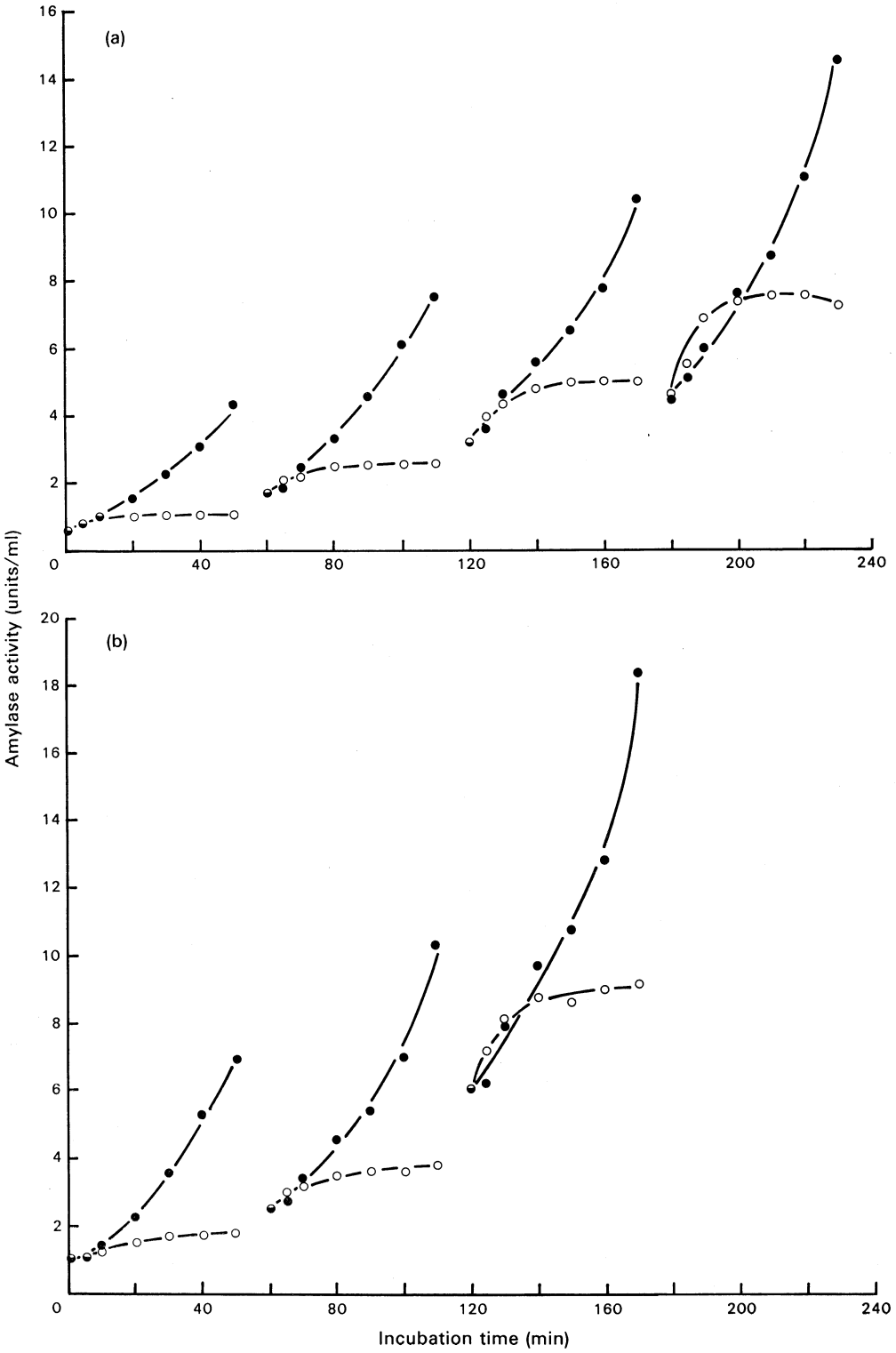


Fig. 6. Effect of actinomycin D (*a*, *b*) and rifampin (*c*, *d*) on [5-^{3}H]uracil incorporation into RNA. Actinomycin D was added 2 h (*a*) and 3 h (*b*), respectively, after amylase secretion had started. Rifampin was added 1 h (*c*) and 2 h (*d*), respectively, after amylase secretion had started. *a*, *b*, No actinomycin D (●); plus actinomycin D (○). *c*, *d*, No rifampin (●); plus rifampin (○).

incorporation into protein. The effect of actinomycin D on amylase secretion is shown in Fig. 7*a*. When the antibiotic was added to cells which were just starting to secrete amylase there was a very rapid inhibition of secretion. If actinomycin D was added 1, 2 or 3 h after secretion had begun inhibition of secretion did not occur until about 20 min after the addition of the antibiotic. A similar effect was observed

Fig. 7


(a)
(b)
Amylase activity (units/ml)
Incubation time (min)
0
40
80
120
160
200
240
2
4
6
8
10
12
14
16
18
20

at 4 h (data not shown in Fig. 7*a*) with the activity of the amylase increasing from 6 units/ml to 9·5 units/ml before inhibition occurred. When added at 3 h, actinomycin D caused a transient stimulation of amylase synthesis, before inhibition occurred after 4 h.

Effect of Rifampin on Amylase Secretion

When rifampin (1·5 μg/ml final concentration) was added to cell suspensions at 1, 2 or 3 h after enzyme secretion had started there was a rapid (within 5 min) inhibition of [5-^{3}H]uracil incorporation into RNA (Fig. 6) and [^{14}C]phenylalanine incorporation into protein. The effect of rifampin on amylase secretion is shown in Fig. 7*b*. When the antibiotic was added to cells which were just starting to secrete amylase there was virtually complete inhibition of secretion. Cells incubated in the absence of rifampin readily synthesized amylase. However, if rifampin was added 1 or 2 h after secretion of amylase had begun inhibition of secretion did not occur until about 20 min after addition of the antibiotic. A similar effect was seen at 3 h (data not shown in Fig. 7*b*) with the activity of amylase in the presence of rifampin increasing from 11 units/ml to 16·2 units/ml before inhibition occurred. As with actinomycin D it was observed that rifampin added after 2 h (Fig. 7*b*) and 3 h (data not shown) caused a slight, transient stimulation of enzyme secretion.

The data in Figs 6 and 7*a* show that the rate of amylase secretion by the main suspension of cells was slower than that by the 10 ml samples transferred to 125-ml flasks. For example, in Fig. 7*a* at 1 h the main culture contained about 2 units/ml of amylase, whereas the transferred culture had 4·2 units/ml of amylase after 50 min. This discrepancy can, probably, be attributed to the better supply of oxygen (and hence better growth and secretion of enzyme) to the cells in the 125-ml flask.

Discussion

The amylase produced by *A. rubra* is a true extracellular enzyme as shown by the finding that an intracellular enzyme, triosephosphate isomerase, was present only in cell extracts and not in the culture supernatant, whereas the reverse was the case with amylase. The amylase was of the α-type hydrolysing starch to mainly maltose, maltotriose and maltotetraose. The enzyme appears to be constitutive since small amounts were secreted when the bacterium was grown on glucose, citrate, malate or mannitol. However, a higher activity (about eightfold) was induced by growth of the organism on starch or maltose. The induction was subject to catabolite repression by glucose, but this effect appeared to be dependent on the concentration of glucose since the effect was only observed when the glucose concentration exceeded 1 mg/ml of culture (about 5 mM). Repression of extracellular α-amylase secretion by glucose has been observed in *Pseudomonas saccharophila* (Markovitz and Klein 1955), *Vibrio parahaemolyticus* (Tanaka *et al.* 1969), *Micrococcus halobius* (Onishi 1972), *Bacillus* spp. (Priest 1977) and *Acinetobacter* (Onishi and Hidaka 1978). Secretion

Fig. 7. Effect of (*a*) actinomycin D and (*b*) rifampin on amylase secretion by cells growing on starch as carbon source. The antibiotic was added to the cell suspension at 0, 1, 2 and 3 h (*a*) and at 0, 1, and 2 h (*b*) after secretion of amylase had begun. Samples were removed, centrifuged and assayed for amylase. (*a*) No actinomycin D (●); plus actinomycin D (○). (*b*) No rifampin (●); plus rifampin (○).

of amylase by *A. rubra* commenced in the late phase of logarithmic growth and continued during the stationary phase, a property also shown by other halophilic bacteria (Onishi 1972; Onishi and Hidaka 1978). It is not clear whether the constitutive and induced amylase activities observed are due to the same enzyme. It is possible that two (or more) proteins with α-amylase activity are produced, and clarification of this point will have to await the results of further studies.

The amylase (after dialysis) required Ca^{2+} and Cl^- for activity. When either ion was added alone reactivation was only partial, with Ca^{2+} being more effective than Cl^-. However, the reactivation by the two ions was additive, so that at 15 mM Ca^{2+} and 30 mM Cl^- the enzyme was fully reactivated. Br^- could replace Cl^-, but Mg^{2+} could only partially replace Ca^{2+}. There was no requirement for Na^+ or K^+. A requirement for high concentrations of NaCl or KCl (0·2–0·75 M) was found for the extracellular amylases of two species of halophilic bacteria (Onishi 1972; Onishi and Hidaka 1978; Onishi and Sonoda 1979). The amylase from one of these species (*Micrococcus*) also required Ca^{2+} for activity and stability. The extracellular amylase of *Halobacterium halobium* was fully activated by about 50 mM Cl^-, but apparently did not require Ca^{2+} (Good and Hartman 1970). A requirement for Ca^{2+} has been shown for the amylase from *Bacteroides amylophilus* (McWethy and Hartman 1977).

The rapid and complete inhibition of amylase production by chloramphenicol, at concentrations which did not affect RNA synthesis, showed that the secretion of the enzyme was the result of *de novo* synthesis. This, together with the finding that there was no active intracellular amylase, supports the hypothesis that the enzyme is synthesized on the cytoplasmic membrane (May and Elliott 1968). The pattern of amylase secretion and the inhibition of RNA synthesis after rifampin was added at various times to secreting cells suggested that a pool of mRNA specific for the enzyme was built up after induction had begun. An alternative hypothesis is that *A. rubra* contains a unique mRNA polymerase specific for extracellular α-amylase, which is more slowly inactivated by rifampin than the enzyme responsible for the transcription of the bulk of the cellular RNA. That this is not the case was shown by the rapid inhibition of RNA synthesis by actinomycin D while amylase secretion continued. Since actinomycin D inhibits transcription by acting directly on the cellular DNA, it is clear that the resistant polymerase hypothesis is not tenable. Similar findings have been reported for extracellular enzyme secretion by Gram-positive bacteria (Both *et al.* 1972; Semets *et al.* 1973; O'Connor *et al.* 1978) and Gram-negative bacteria (Stinson and Merrick 1974; Boethling 1975; Reid *et al.* 1980).

The cause of the transient stimulation of amylase synthesis when rifampin and actinomycin D were added to suspensions of cells after 2 and 3 h, respectively, is unknown but is reminiscent of the findings of Coleman and Elliott (1965) who observed a stimulation of the synthesis of extracellular ribonuclease by a *Bacillus* sp. after addition of actinomycin D to cells actively secreting the enzyme. However, the stimulation they observed lasted several hours.

Two hypotheses have been proposed which are consistent with the present data. Both *et al.* (1972) postulated that the mRNA pool for extracellular enzymes in *Bacillus amyloliquefaciens* resulted from a positive imbalance of transcription over translation. An alternative hypothesis was proposed by O'Connor *et al.* (1978) who suggested that there were two forms of mRNA for the extracellular protease produced by *B. amyloliquefaciens*, a short-lived form immediately available for

translation, and a relatively stable non-translatable form. They suggested that in the presence of inhibitors of transcription the stable form was converted into the translatable species, which would account for the continued production of the enzyme in the absence of transcription. The present data are compatible with both hypotheses, and the question will only be resolved when the mRNA for *A. rubra* α-amylase is purified and its structure examined.

The reason for the production of an mRNA pool for extracellular α-amylase is unknown. It was postulated by Both *et al.* (1972) that the mRNA pool found in *B. amyloliquefaciens* is part of a primitive transport mechanism in which excessive amounts of mRNA are produced to ensure that sufficient mRNA reaches the translation-extrusion sites. This argument is reasonable on teleological grounds if the mRNA for the extracellular enzyme is a short-lived species, as is the bulk of the cellular mRNA. Nevertheless, the present work and that of the other authors cited support the hypothesis that mRNA accumulation is a general property of extracellular enzyme synthesis, both in Gram-positive and Gram-negative bacteria.

Acknowledgment

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