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REGULATION OF AMYLASE SYNTHESIS BY *VIBRIO PARAHAEMOLYTICUS*

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SUMMARY Amylase synthesis in *Vibrio parahaemolyticus* was induced by starch, dextrin and maltose in chemically defined medium. The enzyme was also sensitive to catabolite repression caused by glucose or other carbohydrates added in the medium, and showed marked diauxic growth when a mixture of starch and glucose was used as the carbon source.

The preferential production of the enzyme in the post-logarithmic growth phase, a common phenomenon among extracellular enzymes, was also observed in this organism. This phenomenon could be interpreted as the result of interaction of two regulation mechanisms, namely induction and catabolite repression.

INTRODUCTION

A characteristic of many microorganisms is the production of extracellular enzymes, i.e. exoenzymes, and there are many reports on this (Pollock, 1962; Lampen, 1965). Although this phenomenon is of wide occurrence and also has some practical importance in taxonomy, in putrefaction of foods and in industrial enzyme production, the mechanisms of production of exoenzymes and even their physiological roles are still not clear.

Recently, Coleman (1967) studied the regulation of extracellular enzyme formation by *Bacillus subtilis* and confirmed that their formation mainly occurred in the stationary growth phase rather than in the logarithmic phase, a fact originally described by Nomura et al. (1956).

The present work is on extracellulr amylase production by *Vibrio parahaemolyticus* and some basic mechanisms regulating synthesis of the enzyme.

MATERIALS AND METHODS

1. *Organism*

Strain EB-101 of *Vibrio parahaemolyticus*, originally found by Fujino et al. (1965), was supplied by the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. The strain was maintained on Brain Heart Infusion Broth (Difco) agar slants supplemented with 3% NaCl.

2. *Medium*

The basal mineral medium was as described previously (Tanaka et al., 1967a; b) except for the addition of 3% NaCl, and the pH was adjusted to 8.0.

3. *Growth conditions*

The organism was grown with shaking at 37 C in a nepherometric flask containing 10 ml of basal

mineral medium supplemented with various sugars at a concentration of 0.2% (1.0 % in the case of starch) as the sole carbon and energy source. Growth was monitored by turbidometry using a Shimazu, Bausch & Lomb Spectronic 20 Colorimeter at 550 mμ.

4. *Dry weight of bacterial cells*

Cell dry weight was determined by the method of Coleman and Elliott (1962).

5. *Assay of amylase activity*

Amylase activity was assayed by a modification of the method of Coleman and Elliott (1962). The standard procedure was as follows. A sample of 1.0 ml of suitably diluted culture supernatant was added to 3.0 ml of mixture containing soluble starch, sodium chloride and phosphate buffer (pH 6.0). The final concentrations of these components were 0.5%, 0.1% and 0.1 M, respectively. The resulting reaction mixture was incubated at 37 C for 1 hr, and the reaction was stopped by adding 0.8 ml of 1 N HCl. To a 1.0 ml aliquot of the above mixture were added 0.5 ml of 1 N HCl and 0.4 ml of iodine reagent, and the mixture was diluted to 50.0 ml with water. The extinction at 620 mμ was measured immediately. The enzyme activity was expressed as mg of starch decomposed per hour per mg dry weight of bacterial cells.

6. *Enzyme induction*

Basal mineral medium supplemented with 1% casamino acids was used throughout. Each carbohydrate tested was added at a final concentration of 0.02% to the above medium. After 5 hours growth, when the culture had reached the stationary phase, amylase activity in the supernatant was assayed.

7. *Catabolite repression*

To logarithmically growing culture (OD_{550} approximately 0.30) on 1% starch as the sole carbon source, each carbohydrate was added at a final concentration of 0.2%. Incubation was further continued for 2 hours, then amylase activity in the supernatant was assayed.

8. *Chemicals*

Soluble starch was obtained from Junsei Pure Chemicals Co., Ltd., Tokyo; dextrin and casamino acids from Difco Laboratories, Detroit, Michigan, U.S.A. and maltose from Wako Pure Chemical Industries, Ltd., Osaka.

RESULTS

1. *Growth on defined medium*

The generation times with various carbon sources in the basal mineral medium are shown in Table 1.

The organism grew rapidly with various carbohydrates and organic acids, such as glucose, galactose, fructose, mannose, L-arabinose, mannitol, glycerol, maltose, pyruvate and succinate, as the sole carbon and energy source.

Sufficient amylase seemed to be produced during growth with starch as the sole carbon source, because there was no difference in the generation times with maltose and with starch.

2. *Inducible synthesis of amylase*

There was little amylase activity in the supernatant of cultures grown on 1% casamino acids in basal mineral medium or nutrient

TABLE 1. *The generation times of Vibrio parahaemolyticus, strain EB 101 on various carbon sources*

Carbon source	Generation time (min.)
Glucose	51
Galactose	53
Fructose	58
Mannose	51
L-Arabinose	59
Mannitol	58
Sorbitol	no growth
Glycerol	72
Lactose	no growth
Sucrose	no growth
Maltose	57
Pyruvate	67
Citrate	no growth
Succinate	74
Starch	56

Growth media are basal mineral medium supplemented with various carbohydrates as the sole source of carbon.

broth supplemented with 3% NaCl. The effects of addition of various carbohydrates at a final concentration of 0.02% to casamino acids medium on the synthesis of amylase are shown in Table 2.

It appears that amylase synthesis in this organism is inducible in nature, being induced by starch, dextrin or maltose. No other carbohydrates, such as glucose, galactose, mannose, fructose, L-arabinose, mannitol, glycerol, pyruvate or succinate caused induction of amylase.

TABLE 2. *Inducible synthesis of amylase in Vibrio parahaemolyticus*

Carbohydrate added	Specific activity
None	0.7
Starch	8.8
Dextrin	11.6
Maltose	8.0
Glucose	2.6
Galactose	1.7
Mannose	2.0
Fructose	2.9
Mannitol	3.1
L-Arabinose	1.8
Glycerol	1.9
Pyruvate	1.7
Succinate	0.9
Lactose[a]	1.5
Sucrose[a]	0.8
Citrate[a]	1.9

Each carbohydrate was added to basal mineral medium supplemented with 1% casamino acids. Specific activities of amylase are expressed as mg of starch decomposed per mg dry cell weight per hour at 37 C.
a: *not utilized for growth by this organism.*

3. *Kinetics of amylase production*

Many workers have reported that extracellular enzymes are produced at much higher rates in the post-logarithmic phase than in the logarithmic phase of growth. This was also observed in this organism.

When the medium contained maltose, which served as an inducer as well as a carbon and energy source, the specific activity of amylase rapidly increased when growth of cells reached the stationary growth phase. Fig. 1 shows amylase production in diauxic growth with glucose plus maltose as carbon sources. Here again, the enzyme was produced in the final stationary phase when the cell density was approximately 1.0. Another peak of amylase production was observed in the interphase between two logarithmic phases, i.e. after exhaustion of glucose. There was little amylase

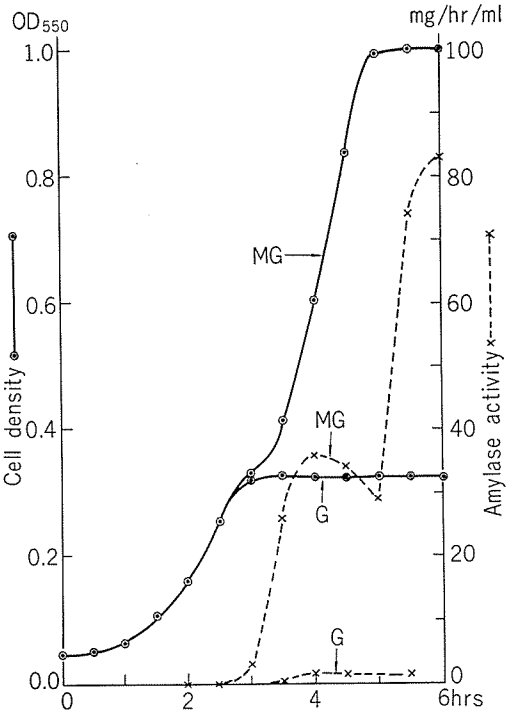

FIGURE 1. *Kinetics of amylase production during growth on maltose plus glucose.*

Growth curves on 0.2% maltose plus 0.05% glucose (MG) and 0.05% glucose alone (G) are shown by solid lines. Amylase activities, expressed as mg starch decomposed per hour per ml of culture supernatant, are shown by broken lines.

production in the control culture grown on glucose alone even in the stationary growth phase. The slight decrease observed in the amylase activity after the first peak of production was probably the result of inactivation of the enzyme.

4. *Diauxic growth*

Fig. 2 shows the growth curves on 1% starch in basal mineral medium with or without glucose. In the presence of glucose, the curve was diauxic and the first logarithmic phase corresponded to growth on glucose alone. The starch concentration in the medium decreased abruptly when glucose became exhausted. A similar effect was observed with other sugars, such as mannitol or galactose, but not with glycerol.

These phenomena strongly suggest that synthesis of amylase is controlled by a mechanism called catabolite repression (Magasanik, 1961; Loomis and Magasanik, 1967; Epstein et al., 1966).

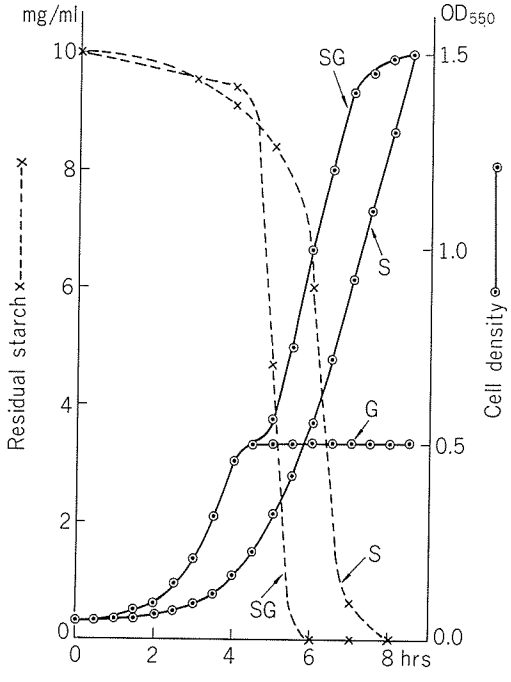

FIGURE 2. *Diauxic growth on starch in the presence of glucose and kinetics of starch consumption.*

Growth curves on 1% starch plus 0 1% glucose (SG), 1% starch alone (S) and 0 1% glucose alone (G) in basal mineral medium are shown by solid lines. The amounts of residual starch in the medium are shown by broken lines. Starch concentrations are expressed as mg starch per ml of medium.

5. *Catabolite repression*

Table 3 shows the effects of various carbohydrates on the induced synthesis of amylase. The details of the procedures used are described in Materials and Methods. Each sugar or organic acid was added to cultures of logarithmically growing cells with 1% starch as the sole carbon source. The amylase activity at the time of addition of carbohydrate was negligible, because the enzyme was produced mainly in the stationary growth phase.

TABLE 3. *Catabolite repression of amylase synthesis by Vibrio parahaemolyticus*

Carbohydrate added	Specific activity
None	20.0
Glucose	9.7
Galactose	12.1
Mannose	11.3
Fructose	15.4
Mannitol	12.7
L-Arabinose	12.3
Glycerol	19.3
Pyruvate	18.7
Succinate	18.5
Lactose[a]	18.8
Citrate[a]	17.2

Each carbohydrate was added to a growing culture on basal mineral medium supplemented with 1% starch as sole carbon source. Specific activies are expressed as mg of starch decomposed per mg dry cell weight per hour at 37 C.

a: *not utilized for growth by this organism.*

Among several carbohydrates tested, glucose, galactose, mannose, fructose, mannitol and L-arabinose repressed amylase synthesis, while glycerol, pyruvate and succinate had little effect.

DISCUSSION

There are only a few papers on regulation of extracellular enzymes. Especially, catabolite repression of exoenzymes was rarely described. Fukumoto et al. (1958) reported an inverse relationship between the rate of metabolism of carbon sources and the amylase activity produced in their presence. This probably indicates that amylase production in his strain of *Bacillus subtilis* was sensitive to catabolite repression. More recently, Welker and Campbell (1963a ; c) reported that amylase production in their partially constitutive strain of *B. stearothermophilus* was repressed by sucrose, but they could not conclude that the phenomenon was catabolite repression because their strain only grew on a limited number of carbon sources. Furthermore, Coleman (1967) concluded that constitutive amylase synthesis by *B. subtilis* was not sensitive to catabolite repression because he could observe no decrease of amylase production after adding of various carbohydrates in the stationary growth phase. The present report clearly shows that induced amylase synthesis of *Vibrio parahaemolyticus* is sensitive to catabolite repression caused by many carbohydrates including glucose.

Amylase of *Vibrio parahaemolyticus* was induced by starch, dextrin and maltose. Similar observations were reported with *Pseudomonas saccharophila* (Markovitz and Klein, 1955) and with *B. stearothermophilus* (Welker and Campbell, 1963a; b). Since enzyme induction is generally believed to occur intracellularly, induction by starch would be indirect, i.e. after degradation into smaller products which could enter into the cells.

A unique feature of most extracellular enzymes so far reported is their preferential synthesis in the stationary growth phase, that is, enzyme synthesis is repressed during the logarithmic growth phase and ceases to be repressed in the stationary growth phase. Most examples so far reported are constitutive enzymes. The present paper shows that even inducible exoenzyme synthesis is repressed during the logarithmic growth phase. The first peak of amylase production immediately after exhaustion of glucose shown in Fig. 1, indicates amylase synthesis of the organism is controlled by at least two independent mechanisms, namely the growth phase and the existence of an inducer, because there is little amylase synthesis in the control culture grown on glucose alone even in the stationary growth phase. Enzyme production is repressed again in the second logarithmic growth phase when the exogenous inducer, maltose, which is also a carbon and energy source, is utilized and finally exhausted. Probably at least some steps of induction are established during the repressed stage. Catabolite repression is the most probable cause of repressed synthesis during the logrithmic growth phase. Exhaustion of metabolite(s) after the logarithmic growth would release the synthesis of these enzymes from the effect of catabolite repressor(s), resulting in their rapid syntheses. The same situation was reported in the regulation of enzymes of the tricarboxylic acid cycle in *B. sutilis* and *B. cereus*. These enzymes were also repressed until glucose was exhausted (Hanson et al., 1963 ; 1967 ; Cox and Hanson, 1968).

Growth with starch as the sole carbon and energy source suggests at least one of the physiological roles of amylase and probably of other extracellular enzymes. Provided microorganisms are under conditions where no carbon source other than macromolecules, starch for example, is available, they would degrade the macromolecules and utilize them as carbon and energy sources for their multiplication. This mechanism seems reminiscent of the most primitive form of digestion and absorption most prominent in the intestinal canal of higher organisms.

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