The Effect of Magnesium and Manganese Ion Concentrations and Medium Composition on the Production of Extracellular Enzymes by *Lysobacter enzymogenes*

By RICHARD G. VON TIGERSTROM

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

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The effect of medium composition on the production of four types of extracellular enzymes by *Lysobacter enzymogenes* was investigated. The nuclease, RNAase, alkaline phosphatase and proteases were produced in good yield after growth in tryptone broth. Much higher yields of the proteases, but low yields of the other three enzymes, were obtained using a skim milk/yeast extract or a chemically defined medium. The addition of NH_4^+ , HPO_4^{2-} , ribonucleosides, Mg^{2+} or Mn^{2+} to tryptone broth reduced the production of some of the enzymes rather specifically. Of 12 monovalent and divalent metal ions tested, Mg^{2+} and Mn^{2+} had the greatest effect. Mg^{2+} at concentrations greater than about 0.1 mM inhibited the production of the nuclease, RNAase and the phosphatase but increased the proteases two- to threefold. Mn^{2+} at concentrations greater than about 0.01 mM inhibited production of the three enzymes more severely but did not stimulate protease synthesis. The extracellular enzymes produced with or without added Mg^{2+} or Mn^{2+} were analysed by PAGE and the activities associated with the cells and a shock fluid were determined. In addition, the effect of adding Mg^{2+} or Mn^{2+} to a growing, extracellular enzyme-producing culture was determined. The results suggest that the nuclease, RNAase and phosphatase are produced by a different mechanism than the proteases and that the metal ions interfere specifically with their production rather than with their release or by causing inhibition.

INTRODUCTION

Bacteria of the genus *Lysobacter* are unicellular gliding bacteria of the class *Flexibacteriae* (Reichenbach, 1981). In addition to their unique means of locomotion, these organisms play an important role in biodegradation (Christensen & Cook, 1978; Reichenbach, 1981). Various species of *Lysobacter* produce extracellular enzymes, e.g. proteases (Jackson & Matsueda, 1970; Whitaker, 1970), chitinase (Hedges & Wolfe, 1974), glucanases and galactosidase (Guntermann *et al.*, 1975), nucleolytic enzymes and phosphatase (von Tigerstrom, 1980, 1981) and the antibiotic myxin (for references see Christensen & Cook, 1978). In addition, these organisms, particularly *L. gummosus*, produce large amounts of slime (Christensen & Cook, 1978). Thus, the organisms produce a number of products of commercial or potential commercial value. So far, however, few systematic studies have been carried out to determine the physical and nutritional factors which influence the production of the extracellular products by these organisms.

During studies of the two nucleolytic enzymes of *L. enzymogenes* 495 (von Tigerstrom, 1980, 1981) it was found that medium composition greatly affected enzyme yields. The effects of medium composition, and in particular the effect of Mg^{2+} and Mn^{2+} , on extracellular endonuclease, RNAase, phosphatase and protease production, are described in the present paper.

METHODS

Organism, media and culture conditions. *Lysobacter enzymogenes* UASM 495 (ATCC 2987) was obtained from Dr F. D. Cook, University of Alberta, Canada and maintained as previously described (von Tigerstrom, 1980). For enzyme production the organism was grown routinely in 0.4% (w/v) tryptone broth at 25 °C on an orbital shaker at 250 r.p.m. The flasks were filled to one-fifth of their capacity. The medium was supplemented with other nutrients as indicated in the text. Carbon and nitrogen sources were added to 0.2% (w/v), except for the nucleosides which were used at 0.05% (w/v). It was assumed that 0.4% tryptone broth contains about 75 μM - Mg^{2+} and less than 0.007 μM - Mn^{2+} (Difco Manual, 1953). Monovalent and divalent metal ions (NaCl , KCl , LiCl , RbCl , AgNO_3 , CaCl_2 , MgCl_2 , MnCl_2 , FeSO_4 , ZnSO_4 , SnCl_2 , CoCl_2 , BaCl_2 , CuSO_4 , SrCl_2 , lead acetate and HgCl_2) were used at 0.1 mM and 1 mM unless stated otherwise.

The other liquid media used were: trypticase soy broth; 0.5% (w/v) skim milk/0.05% (w/v) yeast extract; and a defined medium containing inorganic salts, aspartate and maltose (Guntermann *et al.*, 1975). Cultures were usually grown until the stationary phase of growth was reached. The cells were then removed by centrifuging at 16300 g for 15 min.

Enzyme assays. The endonuclease, RNAase and protease determinations have been described (von Tigerstrom, 1980). The protease was not stimulated by the addition of MgCl_2 to the assay solution. Alkaline phosphatase activities were measured in 0.9 M-Tris/HCl, 0.2 mg *p*-nitrophenyl phosphate ml^{-1} , pH 8.0. One unit of enzyme is the amount which generates 1 μmol of acid-soluble products (nucleolytic enzymes) or 1 μmol *p*-nitrophenol (phosphatase) min^{-1} at 37 °C. One unit of protease is the amount which solubilizes one A_{370} unit of azocasein min^{-1} at 37 °C.

PAGE. Electrophoresis was carried out for 6 h at 4 to 5 °C in 7.5% (w/v) polyacrylamide gels (Maizel, 1971) prepared in 0.5×11 cm tubes. To concentrate the extracellular products, $(\text{NH}_4)_2\text{SO}_4$ was added to culture supernatants to 70% saturation. After 60 min stirring at 0 °C, the suspensions were centrifuged for 30 min at 20000 g. The pellicles and pellets were dissolved in 10 mM-Tris/HCl, pH 8.0, using 1/10 of the original volume. After dialysis at 0 °C against 10 mM-Tris/HCl, pH 8.0, 100 μl of the concentrated preparations were applied per gel tube. The gels were stained with Coomassie blue after electrophoresis and duplicate gels were sliced for the determination of enzyme activities (von Tigerstrom, 1980).

Preparation of shock fluid and cell extract. *Lysobacter enzymogenes* was grown in 0.4% tryptone or 0.4% tryptone plus 0.1 mM- MnCl_2 . The cells and the supernatants were obtained by centrifuging and the cells were washed once with fresh medium by resuspension and centrifuging. The shocking procedure was essentially that of Neu & Heppel (1965) except that the cells, suspended in 30 mM-Tris/HCl, containing 1 mM-EDTA and 20% (w/v) sucrose, pH 8.0 [1 ml (g wet weight cells) $^{-1}$], were added to cold 0.1 mM- MgCl_2 , 0.1 mM- ZnCl_2 [80 ml (g wet weight cells) $^{-1}$]. After 10 min stirring at 0 °C the cells were separated from the shock fluid. They were suspended in 10 mM-Tris/HCl, 50 mM- NaCl , 1.5 mM- MgCl_2 , pH 7.4, and passed through a French pressure cell at 103.35 MPa to obtain the cell extract.

RESULTS AND DISCUSSION

Growth and extracellular enzyme production in different media

Lysobacter nuclease and RNAase were produced in good yield when the organism was grown in 0.4% tryptone broth (von Tigerstrom, 1980). In this medium the organism has a generation time of 2.5 h and grows to a cell density corresponding to an A_{600} of 2.2. In addition to the two nucleolytic enzymes, the organism excretes an alkaline phosphatase, two proteases and possibly other enzymes. The production of the enzymes starts after the exponential growth phase and they appear to be typical extracellular enzymes (Glenn, 1976; Priest, 1977) which are excreted while the organism is still intact.

The enzyme activities usually obtained in 0.4% tryptone broth are given in Table 1. In skim milk/yeast extract medium the organism grew as well as in 0.4% tryptone but only 0.006 units of nuclease, 0.06 units of RNAase and 0.02 units of phosphatase were produced per A_{600} unit; however, the protease activity was increased to 0.59 units per A_{600} unit. Similar enzyme activities were obtained with the inorganic salts/aspartate/maltose medium in which the organism had a generation time of about 8 h and grew to an A_{600} of 2.6. In trypticase soy broth the cells grew well (to an A_{600} of approximately 8) but enzyme production was generally poor. For further studies 0.4% tryptone was used as the basic medium.

The addition to 0.4% tryptone broth of carbon sources such as glucose, fructose, mannose, lactose, sucrose, glycerol, succinate, malate and starch, or nitrogen sources such as NH_4Cl and KNO_3 , or of compounds such as glutamate, urea and nucleosides which could serve as both

Table 1. *Effect of Mg²⁺ and Mn²⁺ on the activities of enzymes detected in the culture supernatants of L. enzymogenes*

Addition to 0.4% tryptone	Enzyme activities (units per A ₆₀₀ unit)			
	Nuclease	RNAase	Phosphatase	Protease
None	0.75	0.29	0.36	0.16
1.0 mM-MgCl ₂	0.022	0.21	0.12	0.42
0.1 mM-MnCl ₂	0.015	0.038	0.066	0.17

nitrogen and carbon sources, generally produced little change in the cell yields and extracellular enzyme production. However, RNAase activity was decreased somewhat by malate and cytosine and both RNAase and nuclease activities were reduced by NH₄⁺ (von Tigerstrom, 1980) and guanosine. Adenosine, on the other hand, reduced protease production to less than 50% whereas the other three enzyme activities were slightly elevated.

The effect of 50 mM-phosphate on the production of the nuclease and RNAase has been reported (von Tigerstrom, 1980). In the present work it was found that this phosphate concentration had little effect on protease production but the alkaline phosphatase activity was reduced by 98%.

Effect of metal ions

Relatively low concentrations of Mg²⁺ had a significant effect on the production of extracellular enzymes by *L. enzymogenes* and so other monovalent and divalent metal ions were tested for possible effects on growth and enzyme production. Of the 17 compounds listed in Methods, CoCl₂, HgCl₂, lead acetate and AgNO₃ appeared to be very toxic to the organism and were not investigated further. The other metal ions were used at either 1 mM or 0.1 mM and did not affect the growth of the organism significantly at these concentrations.

Of the metal ions tested, Mg²⁺ and Mn²⁺ caused the greatest changes in the production of the extracellular enzymes (Table 1). With the addition of ≤0.01 mM-MgCl₂ there was no effect on enzyme production, whereas an inhibition similar to that shown in Table 1 was obtained with 0.1 to 4.0 mM-MgCl₂. Manganese had no effect at 0.001 mM but 0.01 to 2.0 mM-MnCl₂ strongly inhibited the production of three enzymes. The 0.4% tryptone medium contains some magnesium but very little manganese (see Methods) and, therefore, the exact quantities of the divalent metal ions required to produce inhibition might be expected to vary somewhat from one batch of medium to another. The addition of 1.0 mM-MgCl₂ increased the cell yield by approximately 25% whereas 0.1 mM-MnCl₂ decreased the cell yield slightly.

The reduced levels of enzyme activity in the presence of Mg²⁺ and Mn²⁺ could be due to a lowered synthesis or excretion, to inhibition by substances produced in response to the metal ions, or degradation by a protease. Several experiments were carried out in an attempt to find the correct explanation.

PAGE of the extracellular products

Lysobacter enzymogenes was grown in 0.4% tryptone without added metal ions or with Mg²⁺ or Mn²⁺ until the stationary phase was reached. The cells were removed by centrifuging and the extracellular products were prepared for electrophoresis as described in Methods. Electrophoresis was carried out under non-denaturing conditions (Fig. 1). The enzymes were eluted from the slices and assays were carried out to correlate the positions of the stained bands in the gel with the individual enzymes. Figure 2 shows the enzyme activities obtained from the gel of the supernatant from cells grown in 0.4% tryptone. The alkaline phosphatase was present in one peak at position II (slice 7) and the RNAase in one peak at position III (slice 12). The nuclease was seen as a major and a minor peak at position IV (slice 16) and position V (slice 21). The presence of two components with nuclease activity has been discussed previously (von Tigerstrom, 1980). The protease activity was present in two peaks at position I (slice 5) and position VI (slice 32). Both protease peaks showed increased activity in the gel containing the

Fig. 1. PAGE of extracellular products of *L. enzymogenes*. The organism was grown to stationary phase in 0.4% tryptone (A), 0.4% tryptone + 1 mM-MgCl₂ (B) and 0.4% tryptone + 0.1 mM-MnCl₂ (C). The extracellular products were concentrated and used for electrophoresis as described in Methods.

Fig. 2. Location of extracellular enzymes after PAGE. A duplicate gel to the one shown in Fig. 1 (A) was sliced into 2 mm slices and the enzymes were eluted. The nuclease (●), RNAase (○), alkaline phosphatase (■) and protease (□) activities were determined as described in Methods.

sample from cells grown with MgCl₂, but the two peaks are probably due to different enzymes (Whitaker, 1970).

The changed intensities of the bands in Fig. 1 generally agree well with the enzyme activities listed in Table 1 except at position V (slice 21). Most of the protein at this position is probably not due to the nuclease but to an unidentified protein. Also, the proteins in the bands below position III and IV and the minor bands below position II and between V and VI have not been identified. One can conclude, however, that the reduced enzyme activities seem to be due to a

Table 2. *Distribution of enzyme activities after bacteria had been subjected to osmotic shock*

Bacteria were grown in medium A (0.4% tryptone) or B (0.4% tryptone plus 0.1 mM-MnCl₂) and then subjected to osmotic shock as described in Methods.

Component	Percentage of total enzyme activity							
	Nuclease		RNAase		Phosphatase		Protease	
	A	B	A	B	A	B	A	B
Culture supernatant	98	96	88	85	77	63	87	96
Wash	1	1	4.8	3	1.7	0.3	4.5	0.2
Shock fluid	0.4	1.1	5.2	9.1	0.8	0.4	0	0
Cell extract	0.8	1.6	1.8	3.2	21	36	8.9	3.5

lowered synthesis or excretion of the enzymes from the cells or to extensive degradation, rather than to some inhibition of the enzymes or to partial cleavage or dissociation of the enzymes in the presence of Mg²⁺ or Mn²⁺.

Enzyme activities associated with the cells and the shock fluid

Divalent metal ions have, among other functions, a role in the integrity of the outer membrane of Gram-negative organisms (Osborn, 1979). If the nucleic acid-degrading enzymes and the phosphatase are periplasmic constituents they may leak through the outer membrane when these ions become limiting.

In order to determine whether Mn²⁺ affects the excretion of the enzymes from the cells and whether any of the enzymes appeared to be periplasmic, an experiment was carried out to measure the activities obtained in the culture supernatant, in the wash of the cells, in the shock fluid and in the cell extracts of cultures grown with and without Mn²⁺ (Table 2). Except for the alkaline phosphatase, very little enzyme activity was associated with the shock fluid or the cells. Characterization of the *Lysobacter* phosphatases suggests that the activity associated with the cells is due to a different enzyme than that found in the supernatant (unpublished results).

Although there was a slightly higher proportion of the enzymes in the shock fluid and the cells when Mn²⁺ was used, the results do not warrant the conclusion that any of the enzymes are periplasmic proteins. In addition, if lack of integrity of the outer membrane resulted in overproduction of the enzymes, one would expect Ca²⁺ also to affect the enzyme levels but, as mentioned above, this was not the case.

Addition of divalent metal ions during growth

The effect on enzyme production of adding the divalent metal ions at various times during growth of the organism was determined. A culture was grown in 0.4% tryptone to an A₆₀₀ of 1, which represents about half the maximum yield. At this point extracellular enzyme synthesis has not started (von Tigerstrom, 1980). The cells were resuspended in fresh medium and divided into three cultures. One was grown without any addition, one with the addition of Mg²⁺ at zero time and one with the addition of Mg²⁺ at 5 h. Samples of the cultures were removed at intervals to determine the A₆₀₀ and the enzyme activities in the culture supernatants (Fig. 3). An identical experiment was carried out with Mn²⁺ and the results were very similar except the inhibition of RNAase production was more pronounced and the production of the protease took place at a reduced rate in the presence of Mn²⁺.

Comparing the results in Fig. 3 (a to d) it is evident that the nuclease, RNAase and alkaline phosphatase were produced starting at 2 h, when the growth rate was decreasing. The protease, however, was not synthesized until about 5 h. The nuclease and the phosphatase productions were severely inhibited when Mg²⁺ was present from zero time and the synthesis of both enzymes was stopped immediately when Mg²⁺ was added at 5 h. The effect of Mg²⁺ on RNAase production was less pronounced.

The rate of synthesis of the proteases was increased about twofold when Mg²⁺ was present. It did not result in earlier production when added at zero time but caused an almost immediate

Fig. 3. Production of extracellular enzymes by *L. enzymogenes* during growth in the absence and presence of added $MgCl_2$. The enzyme activities were determined during growth of the organism in 0.4% tryptone without any addition ($\bullet$), with the addition of 1 mM- $MgCl_2$ at zero time ($\circ$) and with the addition of 1 mM- $MgCl_2$ at 5 h ($\blacksquare$). Samples were removed at the times indicated to determine (a) the cell density (A_{600}) and nuclease activity, (b) RNAase activity, (c) alkaline phosphatase activity and (d) protease activity. The growth of the organism without added $MgCl_2$ (line 1), with $MgCl_2$ added at zero time (line 2) and with $MgCl_2$ added at 5 h (line 3) is shown in (a).

effect when added at 5 h. Increased levels of protease activity in the presence of excess Mg^{2+} (Hanlon *et al.*, 1982), and the Mg^{2+} - or Ca^{2+} -dependent production of active protease (Sanders & May, 1975) have been reported in *Bacillus* species. A similar mechanism could explain the increased production of the *Lysobacter* protease.

Although there is much information on the various roles of Mg^{2+} in bacterial cells (Jasper & Silver, 1977) it is not often an inhibitor at physiological concentrations. Less is known about the roles of Mn^{2+} (Silver & Jasper, 1977; Weinberg, 1977; Williams, 1982). It is a cofactor for certain enzymes and may be essential or inhibitory for the production of secondary metabolites, but the mode of action is not known.

From this study one can conclude that Mg^{2+} and Mn^{2+} either inhibit some step in the synthesis of the nuclease, RNAase and phosphatase, or that in the presence of excess Mg^{2+} or Mn^{2+} the three enzymes are inactivated (for example by proteases) as they emerge from the cytoplasmic membrane and before excretion of the functional enzyme is complete. The extracellular proteases could not account for the reduced production of the other three enzymes, although they may have some effect on the final yield.

The fact that the nuclease, the RNAase and the alkaline phosphatase are produced much earlier than the proteases and are affected differently by the divalent metal ions suggests that their synthesis is controlled by different factors than the proteases. This is in agreement with current thinking that there is more than one mechanism for the biosynthesis of extracellular enzymes (Kreil, 1981). The control of the biosynthesis of extracellular enzymes in bacteria is still poorly understood (Glenn, 1976; Priest, 1977) and much work remains to be done with *L. enzymogenes*.

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