Regulation of Extracellular Collagenase Production in *Achromobacter iophagus*

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Achromobacter iophagus synthesized extracellular collagenase in a highly aerated peptone medium at the late-exponential and early-stationary phases of growth. Collagenase synthesis was subject to end-product repression and was repressed by various amino acids and ammonium ions. Glutamine caused severe repression of collagenase production. Collagenase synthesis was sensitive to catabolite repression by glucose and a number of carbon sources. Cyclic AMP, dibutyryl cyclic AMP and cyclic GMP did not relieve catabolite repression. Glucose and 2-deoxyglucose caused a severe transient repression. Although rifampicin and chloramphenicol immediately inhibited RNA and protein synthesis, respectively, they failed to inhibit collagenase production completely. No intracellular pre-formed collagenase was detected and collagenase production ceased when induced cells were washed and resuspended in buffer.

INTRODUCTION

Welton & Woods (1973, 1975) described the isolation from hides of an aerobic, collagenolytic, Gram-negative *Achromobacter iophagus* strain which produces an inducible extracellular collagenase with the highest specific activity for a collagenase (Lecroisey *et al.*, 1975). Keil-Dlouha *et al.* (1976) showed that collagenase is synthesized as the culture enters stationary phase and that it is induced by collagen and its high molecular weight fragments. Robbertse *et al.* (1978) reported the induction of collagenase by peptone but not by gelatin. Since peptone is used for the industrial production of collagenase by *A. iophagus* we have investigated the regulation of collagenase synthesis in a peptone medium. Although there are many reports of true exoprotein production by Gram-positive bacteria (Priest, 1977) there are few reports on the regulation of exoproteins in Gram-negative bacteria (Glenn, 1976), in contrast to numerous reports on periplasmic space proteins.

METHODS

All percentage compositions are w/v and all nutrients were from Difco, unless otherwise stated.

Bacteria. The collagenolytic *A. iophagus* strain isolated from hides by Welton & Woods (1973) was used. The strain was identified as *A. iophagus* using the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957), but using the eighth edition (Buchanan & Gibbons, 1974) it was found to be of uncertain taxonomic position.

Media and chemicals. The *A. iophagus* strain was maintained on the complex medium of Welton & Woods (1973). The peptone medium was 2.5% peptone in 0.1 M-Tris/HCl buffer (pH 7.6) containing 0.4 M-NaCl and 2 mM-CaCl₂. One drop of silicone anti-foaming agent was added per 100 ml broth. The Casamino acids medium was 2.5% vitamin-free acid-hydrolysed casein in similar Tris/HCl buffer. Amino acids, adenosine 3':5'-cyclic monophosphoric acid (sodium salt) (cyclic AMP), dibutyryl cyclic AMP, guanosine 3':5'-cyclic monophosphoric acid (sodium salt) (cyclic GMP) and 2-deoxy-D-glucose were obtained from Sigma. The various carbon sources were obtained from Merck.

Growth conditions and collagenase production. Samples (10 ml) of overnight aerated *A. iophagus* cultures in

Fig. 1

Fig. 2

Fig. 1. Effect of glucose and 2-deoxy-D-glucose on collagenase production in peptone medium. No additions (●); 0.4 % glucose added at 2 h (□), 4 h (■) and 6 h (○); 0.2 % glucose added at 3.5 h (▲), 0.4 % 2-deoxy-D-glucose added at 3.5 h (△). Absorbance of control culture (▽).

Fig. 2. Effect of cyclic nucleotides on glucose repression of collagenase production. Glucose and the cyclic nucleotides were added at 3.5 h. No additions (●); 0.2 % glucose (○); 0.2 % glucose plus 5 mM-cyclic AMP (△); 0.2 % glucose plus 5 mM-dibutyl cyclic AMP (■); 0.2 % glucose plus 5 mM-cyclic GMP (□); 5 mM-AMP (▲); 2 mM-GMP (▽).

the Casamino acids medium were inoculated into 100 ml peptone medium and incubated on an orbital shaker at 250 rev. min⁻¹ at 30 °C for 2.5 h. Loosely fitting aluminium foil caps were used instead of bungs to facilitate aeration. The culture was then divided into 10 ml aliquots and growth was followed turbidimetrically at 600 nm. Supernatant fractions obtained by centrifugation of the cultures were assayed for collagenase at different times. Collagenase was assayed using the synthetic substrate phenylazobenzyl-oxy-carbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (PZ-Pro-Leu-Gly-Pro-Arg) (Fluka, Buchs, Switzerland) by a modification of the method of Wünsch & Heidrich (1963) in which the Tris/HCl buffer was used instead of veronal/acetate buffer to make up the substrate. Collagenolytic activity was expressed as nkat (ml supernatant)⁻¹ (A_{600} unit)⁻¹, or as nkat (ml supernatant)⁻¹ in experiments where rifampicin or chloramphenicol were added.

Repression of collagenase synthesis. The effects of the addition, at different times, of various carbon sources, amino acids (Table 1), ammonium ions, antibiotics and cyclic nucleotides on collagenase production were investigated.

RNA and protein synthesis. RNA and protein synthesis were determined by the incorporation of [³H]uracil (2 µg ml⁻¹, 0.4 µCi ml⁻¹) and [¹⁴C]leucine (2 µg ml⁻¹, 0.4 µCi ml⁻¹), respectively, into trichloroacetic acid (TCA)-precipitable material by the method of Eichenlaub & Winkler (1974). Rifampicin and chloramphenicol were each added to exponential phase cells to give a final concentration of 300 µg ml⁻¹.

Ultrasonic disintegration and toluene treatment of cells. In order to determine whether collagenase was preformed within the cell, 4 ml samples of culture were disintegrated with a MSE ultrasonic disintegrator and their collagenase activity was then compared with the activity of untreated control samples. Cells were also treated with toluene to determine whether they would release preformed collagenase or become permeable to the substrate and indicate the presence of preformed collagenase: 0.2 ml toluene was mixed with 0.5 ml samples of culture and the collagenase activity was determined in the supernatant and in uncentrifuged samples.

RESULTS

Kinetics of collagenase production. Collagenase was produced in the peptone medium when the culture reached the late-exponential and early-stationary phases of growth (6 to 12 h) and maximum yields were obtained after 10 to 12 h (Fig. 1). Production of collagenase was affected by aeration and optimum yields were only obtained under highly aerated conditions.

Table 1. *Effect of various carbon sources, amino acids and ammonium ions on collagenase production*

Carbon sources were added at 0.2% and amino acids at 0.5%. Additions were made to peptone-grown cultures 3.5 h after inoculation, and collagenase activity was assayed at 6 and 8 h after inoculation.

Addition	Activity (% of control)		Addition	Activity (% of control)	
	6 h	8 h		6 h	8 h
None	100	100	Casamino acids (1%)	1	4
Glucose	0	9	Casamino acids (0.5%)	3	47
Fructose	5	17	Glutamine	3	5
Sucrose	0	18	L-Proline	38	28
D(+)-Mannose	0	19	Histidine.HCl	17	31
Mannitol	10	22	Glycine	14	33
Maltose	9	24	Tryptophan	53	49
Glycerol	0	28	Isoleucine	100	60
Succinic acid (Na ₂ salt)	24	28	L-Hydroxyproline	100	60
Pyruvic acid (Na salt)	8	38	Leucine	64	61
Arabinose	50	63	β -Alanine	80	100
2-Oxoglutaric acid	80	70	(NH ₄) ₂ SO ₄ (100 mM-N)	65	100
Lactose	70	100	(NH ₄) ₂ SO ₄ (200 mM-N)	40	42

Glucose repression of collagenase synthesis. Addition of 0.4% glucose to the peptone culture before 6 h, the time at which collagenase production normally starts, completely inhibited the synthesis of collagenase (Fig. 1). The glucose analogue 2-deoxy-D-glucose at the same concentration (0.4%) had a transient inhibitory effect on the production of collagenase when added at 3.5 h (Fig. 1). Addition of 2-deoxy-D-glucose at 2 h, at concentrations of 0.2% and 0.4%, also resulted in a transient inhibitory effect, with 0.4% 2-deoxy-D-glucose causing more inhibition than 0.2% (results not shown). This transient effect was also observed with 0.2% glucose (Fig. 1) and 0.05% glucose had no effect on collagenase production. The addition of 0.4% glucose after 6 h caused a slight reduction in collagenase production. Various other carbon sources repressed the synthesis of collagenase (Table 1). Lactose, which the bacterium was unable to utilize (Welton & Woods, 1973), caused little repression after 6 h and no repression after 8 h.

Effect of cyclic nucleotides. Repression of collagenase synthesis by glucose was not relieved by 5 mM-cyclic AMP, 5 mM-dibutyryl cyclic AMP or 5 mM-cyclic GMP (Fig. 2). The addition of 5 mM-cyclic AMP actually inhibited collagenase production whereas 2 mM-cyclic GMP had no effect (Fig. 2).

Effect of amino acids. Casamino acids (0.5 and 1%) and various individual amino acids (0.5%) inhibited collagenase production (Table 1). Glutamine caused severe repression of collagenase synthesis but other amino acids inhibited collagenase production to a lesser extent. Ammonium ions (100 to 200 mM-N) also repressed collagenase synthesis.

Effect of antibiotics. The addition of 200 μ g rifampicin ml⁻¹ and 500 μ g chloramphenicol ml⁻¹ at 3 h prevented the production of collagenase (Fig. 3). When these antibiotics were added at 6 h, collagenase production was not inhibited completely and significant increases in activity occurred. However the effect of 300 μ g rifampicin ml⁻¹ and 300 μ g chloramphenicol ml⁻¹ on the incorporation of [³H]uracil and [¹⁴C]leucine, respectively, into TCA-precipitable material when added at 6 h indicated that rifampicin inhibited RNA synthesis and chloramphenicol inhibited protein synthesis immediately (Fig. 4).

Collagenase assays and peptone inhibition. Cell-free peptone culture filtrates obtained after 6 and 8 h incubation were diluted in the Tris/HCl buffer and in peptone medium, i.e. the Tris/HCl buffer containing 2.5% peptone. The filtrates obtained at 6 and 8 h and diluted in the Tris/HCl buffer showed 3- and 11-fold higher collagenase activities, respectively, than

Fig. 3

Fig. 4

Fig. 3. Effect of rifampicin and chloramphenicol on collagenase production. No additions (●); 200 μg rifampicin ml^{-1} added at 3 h (Δ) and 6 h ($\blacktriangle$); 500 μg chloramphenicol ml^{-1} added at 3 h ($\square$) and 6 h ($\blacksquare$).

Fig. 4. Effects of rifampicin and chloramphenicol on the incorporation of [^3H]uracil and [^{14}C]leucine, respectively, into TCA-precipitable material. [^3H]Uracyl incorporation: no additions ($\circ$); 300 μg rifampicin ml^{-1} added at 6 h (Δ). [^{14}C]Leucine incorporation: no additions ($\bullet$); 300 μg chloramphenicol ml^{-1} added at 6 h ($\blacktriangle$).

Table 2. *Effect of ultrasonic disintegration and toluene treatment on collagenase activity*

Age of culture (h)	Collagenase activity (nkat ml^{-1})				
	Ultrasonic disintegration		Toluene treatment		
	Untreated	Treated	Untreated	Treated: supernatant	Treated: uncentrifuged
4	0.58	0.58	0.47	0.55	0.55
6	9.11	7.95	3.53	2.89	2.84
8			6.46	7.20	5.79
10			10.60	8.20	7.21

equivalent filtrates diluted in Tris/HCl buffer plus peptone. The peptone therefore appeared to inhibit the collagenase. Furthermore the collagenase activity of a cell-free supernatant increased 2.6-fold over a 2 h period suggesting that degradation of an inhibitor of the enzyme occurred.

Ultrasonic disintegration and toluene treatment. Ultrasonic disintegration or toluene treatment of the cells did not result in an increase in collagenase activity (Table 2).

Release of collagenase by washed cells. Experiments were carried out to determine whether induced cells which were producing collagenase in the peptone medium would continue to release collagenase in buffer after washing. Washed induced cells did not produce collagenase in buffer.

DISCUSSION

The production of collagenase by *A. iophagus* requires a specific inducer and only occurs at the late-exponential and early-stationary phases of growth in highly aerated cultures. This pattern of exoenzyme synthesis in a complex medium is similar to protease, nuclease and cell wall-lytic enzyme production by *Bacillus* strains (Priest, 1977). The growth of cells

to late-exponential phase and the precise time of collagenase synthesis, at 6 h, facilitated studies of the regulation of enzyme formation.

In common with exo-protease synthesis by bacteria of a number of genera, including *Bacillus*, *Sarcina* and *Arthrobacter*, collagenase production is subject to control by end-product repression (Glenn, 1976). Various amino acids and ammonium ions repressed collagenase production. The severe repression by glutamine suggests that the enzymes involved in glutamine metabolism may be implicated in the regulation of collagenase (Streicher *et al.*, 1974; Friedrich & Magasanik, 1977), possibly in similar roles to those occupied by glutamine synthetase in the induction of histidase. Experiments are in progress to isolate glutamine auxotrophs and study the effects of the mutations on the regulation of collagenase. It is interesting that glycine, which constitutes one-third of all the amino acids in collagen, strongly repressed collagenase synthesis. Lecroisey *et al.* (1975) reported that histidine inhibited purified collagenase and part of the apparent repression caused by histidine may be due to the inactivation of the collagenase. Isoleucine and proline, which have been identified as the most common repressive amino acids in other bacteria (Glenn, 1976), also repressed collagenase synthesis but not as severely as glutamine.

In addition to end-product repression the synthesis of collagenase is subject to catabolite repression by a number of different growth substrates. However, the addition of exogenous cyclic AMP, dibutyryl cyclic AMP [which stimulates the synthesis of β -galactosidase in *Caulobacter crescentus* (Shapiro *et al.*, 1972)] or cyclic GMP did not relieve catabolite repression. The failure of cyclic AMP to overcome the glucose effect has been reported for extracellular enzyme synthesis in *Vibrio parahaemolyticus* (Tanaka & Iuchi, 1971), *Pseudomonas maltophilia* (Boethling, 1975), *Pseudomonas lemoignei* (Stinson & Merrick, 1974), *Staphylococcus aureus* (Yoshikawa *et al.*, 1974) and many *Bacillus* species (Priest, 1977). Cyclic AMP actually inhibited the synthesis of collagenase. A similar inhibitory effect of cyclic AMP was reported for the synthesis of extracellular 1,3- β -glucanase in a *Streptomyces* strain (Lilley *et al.*, 1974). As well as inducing catabolite repression the addition of 0.2% glucose or 0.4% 2-deoxy-D-glucose resulted in a severe transient repression similar to the transient repression observed with the *lac* operon in *Escherichia coli* (Tyler *et al.*, 1967). Higher concentrations of glucose (0.4%) resulted in a complete repression of collagenase synthesis. The isolation by Robbertse *et al.* (1978) of a *colG* mutant, which is insensitive to glucose but repressed by amino acids, and a *colA* mutant, which is insensitive to glucose but requires amino acids, suggests that the glucose and amino acid effects are mediated differently.

Rifampicin and chloramphenicol added at 6 h failed to inhibit collagenase production completely and increases in activity occurred. Rifampicin inhibited RNA synthesis and chloramphenicol inhibited protein synthesis immediately. The increase in activity of the chloramphenicol culture could be due to the release of preformed enzyme. However this seems unlikely as ultrasonic disintegration or treatment of cells with toluene did not result in an increase in collagenase activity. Furthermore Keil-Dlouha *et al.* (1976) also reported that no zymogen or cell-accumulated enzyme is present during exponential growth. Some of the increase in activity with time could have been due to preformed collagenase reducing the concentration of an inhibitor (possibly inducer molecules) in the peptone culture. This would result in a decrease in the concentration of the inhibitor in the medium and an increase in collagenase activity. Because of this effect it is not possible to conclude whether exoenzyme synthesis is supported for a period in the absence of messenger synthesis, as was shown for protease secretion in *P. maltophilia* (Boethling, 1975). These results emphasize the care that must be taken when studying the regulation of inducible enzymes in complex media.

Induced cells which have been washed do not continue to synthesize collagenase in buffer. This may be due to the removal of the inducer. Alternatively the failure to continue synthesis of collagenase may be due to the absence of an energy source. Attempts are being made to

isolate a constitutive mutant in order to study the control of induction of collagenase by the large inducer molecule. Synthesis of collagenase by *A. iophagus* provides an ideal model for studying the regulation of exoenzyme production which requires the specific induction by a large macromolecule such as collagen.

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