Regulation of Extracellular Alkaline Protease Activity by Histidine in a Collagenolytic *Vibrio alginolyticus* Strain

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Vibrio alginolyticus synthesized an inducible extracellular collagenase in a peptone medium during the stationary growth phase. These cultures also possessed extracellular alkaline serine protease activity. The alkaline protease activity did not require a specific inducer and it was produced in tryptone or minimal media. The collagenase was not produced in either the tryptone or minimal media. The alkaline protease activity was sensitive to catabolite repression by a number of carbon sources, including glucose, and by amino acids and ammonium ions. Cyclic AMP, dibutyryl cyclic AMP and cyclic GMP did not relieve catabolite repression. Histidine and urocanic acid stimulated the production of alkaline protease activity in tryptone and minimal media. Other compounds associated with the histidine utilization (*hut*) pathway did not increase alkaline protease activity. Histidine reversed the repression of alkaline protease activity by glucose or $(\text{NH}_4)_2\text{SO}_4$ in minimal medium. Histidine and the compounds associated with the *hut* pathway inhibited collagenase production.

INTRODUCTION

Welton & Woods (1973, 1975) described the isolation from hides of an aerobic, halotolerant, collagenolytic, Gram-negative bacterium which was originally classified as *Achromobacter iophagus* but has recently been reclassified as *Vibrio alginolyticus* (Reid *et al.*, 1980). This strain is of interest as it produces an inducible extracellular collagenase with a high specific activity (Lecroisey *et al.*, 1975). The enzyme is synthesized as the culture enters stationary growth phase and is induced by collagen and peptone (Keil-Dlouha *et al.*, 1976; Reid *et al.*, 1978, 1980; Robbertse *et al.*, 1978). Reid *et al.* (1978) investigated the regulation of collagenase production and found that collagenase synthesis was repressed by various amino acids and by ammonium ions.

Crude collagenase preparations were assumed to contain an additional protease as the preparations possess caseinolytic activity, which also affected the activity of the collagenase (Lecroisey *et al.*, 1975). Although it was reported that the caseinolytic activity was inhibited by *p*-chloromercuribenzoate (Lecroisey *et al.*, 1975), which did not affect the collagenase, no studies have been carried out on the production of the additional protease. The production and purification of collagenase would be greatly enhanced and facilitated if the production of the additional protease activity could be controlled or inhibited. An attempt to increase collagenase production by the isolation of mutants with no caseinolytic activity was unsuccessful as all the caseinolytic mutants had no or markedly reduced collagenase activity.

METHODS

Bacterial strain, media and chemicals. The collagenolytic strain previously isolated and classified as *Achromobacter iophagus* by Welton & Woods (1973) but recently reclassified as *Vibrio alginolyticus* was used (Reid *et al.*, 1980). It was maintained on the complex medium of Welton & Woods (1973). The peptone medium which contained the collagenase inducer has been described previously (Reid *et al.*, 1980). The tryptone medium contained (g l⁻¹): tryptone, 25; NaCl, 23.4; in 0.1 M-Tris/HCl buffer (pH 7.6). The minimal medium contained (g l⁻¹): NaCl, 23.4; K₂HPO₄, 10.6; KH₂PO₄, 4.56; trisodium citrate, 0.48; (NH₄)₂SO₄, 1.0; MgSO₄·7H₂O, 0.1; glucose, 2.5. The succinate minimal medium contained (g l⁻¹): succinate, 0.54; (NH₄)₂SO₄, 0.132; KH₂PO₄, 0.136; in 0.1 M-Tris/HCl buffer (pH 7.6). Amino acids, cyclic AMP, dibutyryl cyclic AMP, cyclic GMP, L-histidine, urocanic acid, imidazole acetic acid (sodium salt), formimino-L-glutamic acid and L-glutamic acid were obtained from Sigma. All percentage compositions were w/v and all nutrients were from Difco unless otherwise specified.

Growth conditions for enzyme studies. The growth conditions for the production of collagenase and alkaline protease in the peptone medium have been described previously (Reid *et al.*, 1980). Studies on the regulation of the extracellular proteases were done by inoculating overnight minimal medium cultures (10 ml) of *V. alginolyticus* into 90 ml of fresh minimal medium. The cultures were aerated on an orbital shaker at 250 rev. min⁻¹ for 4 h at 30 °C. The cells were harvested by centrifugation, washed and resuspended in 10 ml of the succinate minimal medium at an A₆₀₀ of 8.0. Samples were removed at intervals, centrifuged and the supernatants were assayed for protease or collagenase activity.

Enzyme assays. Collagenase was assayed using the synthetic substrate phenylazobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Pz-Pro-Leu-Gly-Pro-Arg) (Fluka, Buchs, Switzerland) as previously described (Wünsch & Heidrich, 1963; Reid *et al.*, 1978). Collagenase activity was expressed as nkat ml⁻¹ where 1 kat is the amount of enzyme that converts 1 mol substrate s⁻¹ (Florkin & Stotz, 1973). Alkaline protease activity was assayed using the synthetic substrate azocasein (Sigma) at a concentration of 1% in 0.1 M-Tris/HCl buffer (pH 9). The sample (1 ml) was added to 2 ml of the azocasein solution and the mixture was incubated at 40 °C for 30 min. The reaction was stopped by the addition of 10% trichloroacetic acid. After standing at 4 °C for 10 min the mixture was filtered and 2 ml 0.4 M-NaOH was added to 2 ml of the filtrate. One unit of alkaline protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0.1 at 440 nm in 30 min at 40 °C. Each sample was assayed in duplicate and experiments were repeated at least three times.

Alkaline protease characterization. The optimum conditions for the activity and stability of the alkaline protease activity in culture supernatants were investigated by assaying the enzyme activity at different temperatures and pH values between pH 5 and 11. Samples of culture supernatants were assayed for alkaline protease activity in the following buffers (0.1 M): acetic acid (pH 5), sodium phosphate (pH 6 to 8), sodium carbonate (pH 9 to 10) and phosphate/sodium hydroxide (pH 11). Inhibition of the alkaline protease activity by *p*-chloromercuribenzoate (pCMB) and phenylmethylsulphonyl fluoride (PMSF) was determined over the concentration range of 10⁻² to 10⁻⁵ M. In order to determine whether the alkaline protease activity was present within the cell, 4 ml samples of culture were treated with a MSE ultrasonic disintegrator for 2 min at 4 °C and their alkaline protease activity was compared with the activity of untreated control samples. Cells were also treated with toluene to determine whether they would release preformed alkaline protease or become permeable to the substrate and indicate the presence of preformed alkaline protease. Toluene (0.2 ml) was mixed with 0.5 ml samples of culture and the alkaline protease activity was determined in the supernatant and in uncentrifuged samples.

RESULTS

Collagenase and alkaline protease activity

Collagenase and alkaline protease activity of *V. alginolyticus* was determined in peptone medium at different times in the bacterial growth cycle (Fig. 1). Both enzyme activities were produced during the stationary growth phase. The collagenase was produced first and maximum activity was detected after 10 h. There was a 4 h difference between the production of the two enzyme activities and the alkaline protease activity reached a peak after 14 h. The production of the alkaline protease activity was associated with the inactivation of the collagenase. The addition of minimal medium solutions with alkaline protease activity to collagenase solutions resulted in a rapid decrease in collagenase activity. Control collagenase solutions showed no decrease in activity.

Since the production of collagenase requires a specific inducer (collagen or peptone) (Reid *et al.*, 1980), the production of alkaline protease activity was investigated in tryptone medium and succinate minimal medium (Fig. 2). Alkaline protease activity was produced in both

Fig. 1. Collagenase and alkaline protease activity throughout the growth cycle in peptone medium. Collagenase (○), alkaline protease (●) and turbidity of the culture (□).

Fig. 2

Fig. 3

Fig. 2. Collagenase and alkaline protease activity in stationary phase cultures after resuspension in tryptone medium and succinate minimal medium. Alkaline protease in tryptone (■) and minimal (□) media. Collagenase in tryptone (●) and minimal (○) media.

Fig. 3. Catabolite repression of alkaline protease activity by glucose and the effect of cyclic AMP and cyclic GMP on alkaline protease activity. No addition (●); 0.4% glucose (▽); 0.4% glucose and 5 mM-cyclic AMP (▼); 0.4% glucose and 5 mM-dibutyl cyclic AMP (□); 0.4% glucose and 5 mM-cyclic GMP (■); 5 mM-cyclic AMP (△); 5 mM-cyclic GMP (▲). All additions were made at 30 min (arrowed).

media but higher activity was obtained in the tryptone medium. Collagenase activity was not detected in either the tryptone or minimal medium. Purified collagenase showed no caseinolytic activity (Welton & Woods, 1975). It was therefore possible to study the production of alkaline protease activity independently of collagenase production.

The minimal medium system developed for the study of alkaline protease synthesis involved allowing the cells to reach stationary phase before concentrating, washing and resuspending them in a succinate minimal medium at a high cell density. At this high cell concentration, the medium was limiting and the cells remained in stationary phase. Succinate was a non-repressing carbon source and alkaline protease production of the washed stationary phase cells could be determined routinely from a fixed time (time zero, Figs. 2 to

5). The addition of glucose, $(\text{NH}_4)_2\text{SO}_4$ or histidine and its degradation products did not stimulate or reinitiate growth.

Alkaline protease characterization

The alkaline protease activity was maximal at pH 9, zero at pH 11 and half-maximal at pH 5. Culture filtrates of the alkaline protease were stable at 4 °C, showed a 20% loss in activity after 5 d at 22 °C and a 50% loss in activity after 15 min at 60 °C. Alkaline protease filtrates were treated with various concentrations of enzyme inhibitors for 2 h before assay. It was found that 10^{-3} M-PMSF, a serine protease inhibitor, markedly inhibited the alkaline protease activity (11% of the control activity remaining after treatment). Higher concentrations of the SH-blocking agent pCMB were required to inactivate the alkaline protease, and after treatment with 10^{-2} M-pCMB 70% of the control activity remained. Ultrasonic disintegration or toluene treatment of the cells did not result in an increase in alkaline protease activity.

Repression and stimulation of alkaline protease activity

The addition of 0.4% glucose to the concentrated cells in the limiting succinate minimal medium at 30 min prevented further production of alkaline protease (Fig. 3). The addition of 0.4% glucose at 1 h resulted in a 36% inhibition of alkaline protease activity. Repression of alkaline protease activity was not relieved by 5 mM-cyclic AMP, 5 mM-dibutyryl cyclic AMP or 5 mM-cyclic GMP (Fig. 3). The addition of 5 mM-cyclic AMP alone actually inhibited alkaline protease production, whereas 5 mM-cyclic GMP had no effect. Various other carbon sources repressed the production of alkaline protease activity (Table 1). Succinate and arabinose had no effect and lactose caused only a slight repression in alkaline protease activity.

Casamino acids (0.5 and 1%), various individual amino acids (0.2%) and ammonium ions (100 and 200 mM-N) repressed alkaline protease activity in the succinate minimal medium (Table 1). However, the amino acid L-histidine markedly enhanced the production of alkaline protease activity. The stimulation of alkaline protease production by histidine occurred in both tryptone medium and succinate minimal medium (Fig. 4). Histidine was able to reverse the repression by glucose in the succinate minimal medium, but not in the tryptone medium or when 200 mM- $(\text{NH}_4)_2\text{SO}_4$ was added along with 0.4% glucose to the succinate minimal

Table 1. *Effect of various carbon sources, amino acids and ammonium ions on alkaline protease activity*

Carbon sources were added at 0.2% and amino acids at 0.5%. Additions were made to cultures 30 min after resuspension in succinate minimal medium and alkaline protease activity was assayed at 2.5 h after resuspension.

Addition	Activity (% of control)	Addition	Activity (% of control)
None	100	Casamino acids (1%)	26
Glycerol	36	Casamino acids (0.5%)	62
Mannose	36	Leucine	44
Sucrose	36	Isoleucine	44
Fructose	40	Glutamine	46
2-Oxoglutarate	44	Tryptophan	66
Maltose	50	Alanine	76
Mannitol	54	Proline	80
Glucose	55	Glycine	80
Lactose	96	Histidine. HCl	210
Arabinose	100	$(\text{NH}_4)_2\text{SO}_4$ (100 mM-N)	65
		$(\text{NH}_4)_2\text{SO}_4$ (200 mM-N)	40

Fig. 4

Fig. 5

Fig. 4. Effect of histidine on glucose repression of alkaline protease activity in tryptone medium (a) and on glucose and $(\text{NH}_4)_2\text{SO}_4$ repression in succinate minimal medium (b). No addition (●); 0.5% histidine (○); 0.4% glucose (■); 0.4% glucose and 0.5% histidine (□); 200 mM- $(\text{NH}_4)_2\text{SO}_4$ (▲); 200 mM- $(\text{NH}_4)_2\text{SO}_4$ and 0.5% histidine (▽); 0.4% glucose and 200 mM- $(\text{NH}_4)_2\text{SO}_4$ and 0.5% histidine (▼). All additions were made at 30 min (arrowed).

Fig. 5. Effect of compounds associated with the *hut* pathway on collagenase activity in peptone medium (a) and on alkaline protease activity in succinate minimal medium (b). No addition (●); 0.25% histidine (○); 0.25% urocanic acid (□); 0.25% imidazole acetic acid (■); 0.25% formimino-L-glutamic acid (Δ); 0.25% glutamic acid (▲). All additions were made at 30 min (arrowed).

medium (Fig. 4). The repression by 200 mM- $(\text{NH}_4)_2\text{SO}_4$ in the succinate minimal medium was also relieved by histidine but not in the presence of 0.4% glucose (Fig. 4).

Effect of *hut* degradation products on collagenase and alkaline protease activity

Studies on the effect of compounds associated with the histidine utilization (*hut*) pathway showed that both histidine (0.25%) and urocanic acid (0.25%) stimulated the production of alkaline protease activity in the succinate minimal medium (Fig. 5). Formimino-L-glutamic acid (0.25%) caused a small decrease in activity and glutamic acid (0.25%) caused a slight repression of alkaline protease production. Imidazole acetic acid (0.25%) inhibited the production of alkaline protease activity. Histidine and its degradation products had no effect on the alkaline protease activity of cell-free culture filtrates and did not activate or inhibit the enzyme. These substances therefore affected the production of the alkaline protease

activity. Collagenase synthesis in peptone medium was inhibited by histidine and the other compounds associated with the *hut* pathway (Fig. 5).

DISCUSSION

Vibrio alginolyticus produces a collagenase in peptone medium during the stationary growth phase. The cultures also possess extracellular alkaline serine protease activity. These two enzyme activities are not under the same control system as the alkaline protease activity does not require a specific inducer and it is produced in a minimal medium. The alkaline protease inactivates the collagenase and the production of the alkaline protease in a peptone medium causes a decrease in the collagenase activity. As the enzymes are produced at different times it should be possible to obtain peptone culture filtrates with high collagenase activity and low protease activity. The results of the studies with enzyme inhibitors suggest that the alkaline protease activity could be due to more than one enzyme. Preliminary results involving the electrophoretic analysis of crude and purified enzyme preparations indicate that at least two alkaline proteases are produced by *V. alginolyticus* (unpublished results).

The pattern of alkaline protease production by *V. alginolyticus* is similar to collagenase production by the same strain (Reid *et al.*, 1978, 1980) and to protease production by *Bacillus* strains (Priest, 1977). The alkaline protease, the collagenase and the *Bacillus* exoprotease (Glenn, 1976) are all subject to control by end-product repression, and various amino acids and ammonium ions repressed their production. A surprising discovery was the stimulation of the production of the alkaline protease activity in *V. alginolyticus* by histidine.

The stimulation of alkaline protease production by histidine and urocanic acid suggests that the *hut* system may be involved in the regulation of the alkaline protease. In the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*, the *hut* enzymes are induced by urocanic acid and histidine which is an inducer by virtue of its conversion to urocanic acid (Smith & Magasanik, 1971; Smith *et al.*, 1971; Hagen & Magasanik, 1973). The nature of the involvement of the *hut* pathway in the regulation of the alkaline protease activity will be investigated by the isolation of *hut* mutants. Histidine and urocanic acid do not stimulate collagenase activity and this is further evidence for the separate control of the collagenase and alkaline protease activities in *V. alginolyticus*.

Both the alkaline protease and the collagenase activities (Reid *et al.*, 1978) are subject to catabolite repression by a number of different substrates. This catabolite repression is not relieved by cyclic AMP or cyclic GMP. 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* (Yosmikawa *et al.*, 1974) and many *Bacillus* species (Priest, 1977). Cyclic AMP actually inhibited the production of both the protease and collagenase activities (Reid *et al.*, 1978) and a similar effect of cyclic AMP was shown for the synthesis of extracellular 1,3- β -glucanase in a *Streptomyces* strain (Lilley *et al.*, 1974). The repressing effects of glucose and ammonia can be relieved by histidine, but where the concentrations of glucose and ammonia are high enough histidine has no effect.

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