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Peptone Induction and Rifampin-Insensitive Collagenase Production by *Vibrio alginolyticus*

GRAHAM C. REID,† DAVID R. WOODS,*† AND FRANK T. ROBB†

Department of Microbiology, Rhodes University, Grahamstown 6140, South Africa

Vibrio alginolyticus produces an extracellular collagenase which requires specific induction by collagen or its high-molecular-weight fragments. Peptone also induces collagenase during the late exponential and early stationary growth phases. The peptone inducers have been shown to have a broad molecular weight range between 1,000 and 60,000. The peptone inducers supported slow growth of *V. alginolyticus* when supplied as the sole nitrogen source in minimal medium. Digestion of the peptone inducers with purified *V. alginolyticus* collagenase resulted in a decrease in their inducing ability, whereas digestion with trypsin or α-chymotrypsin did not. This indicated that induction by the inducers required the presence of collagenase-sensitive bonds. Prolonged digestion of the inducers with collagenase did not completely eliminate the inducing ability of the inducers. The peptone inducers acted as inhibitors of collagenase. A minimal medium induction system has been developed which involves resuspending cells at high density in a medium containing succinate, $(NH_4)_2SO_4$, KH_2PO_4, and the peptone inducer. Cells grown in minimal medium induce earlier than cells grown on peptone, Casamino Acids, or tryptone. Collagenase production was shown to occur for 30 to 60 min in the presence of rifampin at levels which completely inhibit the incorporation of [^{3}H]uracil into trichloroacetic acid-precipitable material. Chloramphenicol completely and immediately abolished collagenase production, which together with labeling studies has confirmed that collagenase production involves de novo synthesis of the enzyme. Both glucose and Casamino Acids repressed collagenase production, although synthesis of the enzyme continued for 30 to 60 min after their addition. The repression of collagenase production by glucose and Casamino Acids was more severe than the inhibition of enzyme formation due to addition of rifampin.

Welton and Woods (16, 17) described the isolation of an aerobic, halotolerant, collagenolytic, gram-negative bacterium which was originally classified as an *Achromobacter iophagus* strain from hides. The identification was originally confirmed by the National Collection of Industrial Bacteria, Aberdeen, Scotland, but has since been reinvestigated by M. Hendrie of the National Collection of Industrial Bacteria and reclassified as a *Vibrio alginolyticus* strain. This strain is of interest because it produces an inducible extracellular collagenase with the highest specific activity for a collagenase (8). The collagenase is induced by peptone, collagen, or its high-molecular-weight fragments and is synthesized as the culture enters stationary phase (7, 12, 13). Keil-Dlouha et al. (7) showed that the presence of collagenase-digestible peptide bonds in the macromolecular inducer fragments from collagen were essential for induction and suggested that the tertiary conformation of the α-helix plays an important role in the collagenase induction process. Since peptone is used for the industrial production of collagenase by *V. alginolyticus*, we investigated the nature of the inducer in peptone.

Both et al. (2) reported that extracellular protease synthesis by *Bacillus amyloliquefaciens* showed unusual responses to the transcriptional inhibitors rifampin and actinomycin D. Late-log-phase cells continued to produce extracellular protease for over 60 min in the presence of concentrations of rifampin and actinomycin D, causing 95% inhibition of uracil incorporation into RNA. Chloramphenicol rapidly inactivated protease production. A hypothesis has been postulated to account for the rifampin-insensitive protease production and involves a pool or reserve of mRNA (2, 9).

Because there are few reports of true, inducible exoprotein production by gram-negative bacteria in stationary phase, in contrast to many reports in gram-positive bacteria (5, 11), we in-

† Present address: Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa.

vestigated the mRNA pool hypothesis in our collagenase-producing *V. alginolyticus* strain. In a previous study on the regulation of collagenase production by *V. alginolyticus*, we reported a difficulty with the interpretation of antibiotic inhibition studies and collagenase production (12). Observed increases in collagenase activity with time after the inhibition of transcription or translation 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 was not possible to conclude whether exoenzyme synthesis is supported for a period in the absence of mRNA synthesis. This problem has been resolved and an experimental system has been developed which enabled us to investigate the effect of rifampin and chloramphenicol on collagenase production.

MATERIALS AND METHODS

All percentage compositions are ratios (wt/vol), and all nutrients were from Difco unless otherwise specified.

Bacteria. The collagenolytic strain previously isolated and classified as *Achromobacter iophagus* by Welton and Woods (16) but recently reclassified as a *V. alginolyticus* strain (NCIB 11038) was used.

Media. Unless otherwise specified, the bacterium was grown and maintained as reported previously (12). Minimal medium contained (in grams per liter): NaCl, 23.4; K_2HPO_4, 10.6; KH_2PO_4, 4.56; trisodium citrate, 0.48; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; and glucose, 2.5.

In induction experiments, cells were resuspended at high cell density after growth in minimal medium, 2.5% peptone, 2.5% Casamino Acids, or 2.5% tryptone in Tris-hydrochloride buffer (pH 7.6) as reported previously. Cells were washed with the medium in which they were to be resuspended. The medium designated as high SNP medium (nitrogen and carbon rich) contained 20 mM disodium succinate, 10 mM $(NH_4)_2SO_4$, and 1 mM KH_2PO_4 in Tris-hydrochloride buffer (pH 7.6). The medium designated as low SNP medium (nitrogen and carbon limiting) contained 2 mM disodium succinate, 1 mM $(NH_4)_2SO_4$, and 1 mM KH_2PO_4 in the Tris-hydrochloride buffer.

Collagenase assay. Collagenase was assayed as described previously (12, 18) with the synthetic collagenase substrate phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (PZ-Pro-Leu-Gly-Pro-Arg) (Fluka, Buchs, Switzerland). Each sample was assayed in duplicate, and experiments were repeated at least twice. The standard error for collagenase assays was less than 10% but usually lower (<5%).

Growth conditions. *V. alginolyticus* cells were grown from an overnight culture in the appropriate medium for 4 h (optical density of 3 at 600 nm, early stationary phase). Cells were harvested by centrifugation, washed once with the medium in which they were to be resuspended, and finally resuspended in 10 ml of the medium to give an absorbance at 600 nm of 8. The purified peptone inducer was used at a final concentration of 0.5%.

Fractionation of peptone. Peptone (2 g) was dissolved in 5 ml of 0.01 M NH_3-NH_4Cl buffer (pH 8.5) and layered on a Sephadex G25 column (80 by 2.5 cm). Samples (10 ml) were eluted with the same buffer at a flow rate of 30 ml h^{-1}. Fractions under the same peaks were pooled, dialyzed against two changes of distilled water at 4°C, and lyophilized. The collagenase-inducing fraction was termed the peptone inducer. The molecular weight of the peptone inducer was determined by applying 0.7 g in 2 ml of buffer to a Sephadex G100 column (80 by 2.5 cm) and eluting 3-ml fractions at a flow rate of 10 ml h^{-1}. The column was calibrated with ovalbumin (molecular weight, 43,000), cytochrome *c* (molecular weight, 11,700), and insulin (molecular weight, 6,000).

Enzymatic digestion of peptone and peptone inducer. Unfractionated peptone and the peptone inducer in low SNP medium were subjected to digestion by purified *V. alginolyticus* collagenase, trypsin, or α-chymotrypsin at enzyme-to-substrate ratios of 1:50 or 1:100 for 13 to 39 h at 30°C. After digestion the enzymes were inactivated by placing the flasks in a boiling-water bath for 30 min. These flasks were then innoculated with washed minimal medium-grown cultures to give an absorbance at 600 nm of 8. The collagenase activity was expressed as a percentage of the control activity at the peak of production. The control consisted of heat-inactivated enzyme added to undigested peptone.

Purification of *V. alginolyticus* collagenase. Collagenase was purified by the method of Lecroisey et al. (8) and purified collagenase with a specific activity of 750 nkat mg^{-1} was obtained.

Inhibition of collagenase by peptone. Purified collagenase at a concentration of 4.2 nkat ml^{-1} was incubated with various concentrations of peptone (0.25% to 2.5%) in Tris-hydrochloride buffer at 30°C. Samples were withdrawn at intervals and assayed for collagenase activity.

RNA and protein synthesis. RNA and protein synthesis were determined by the incorporation of [^{3}H]uracil (2 μg ml^{-1}, 1 μCi ml^{-1}) and [^{3}H]leucine (2 μg ml^{-1}, 0.6 μCi ml^{-1}), respectively, into trichloroacetic acid-precipitable material by the method of Eichenlaub and Winkler (4). Rifampin and chloramphenicol were each added to give a final concentration of 100 μg ml^{-1}.

Effect of rifampin and chloramphenicol on collagenase production. Peptone (2.5%)-grown cultures were harvested at 4 h, washed once with 0.25% peptone, and resuspended at an absorbance at 600 nm of 8. Rifampin and chloramphenicol at concentrations of 100 μg ml^{-1} were added at intervals to the cultures. Because of the critical effect of aeration on collagenase production, separate cultures had to be used.

[^{3}H]leucine labeling of collagenase. A washed 4-h peptone culture was resuspended in 0.25% peptone and incubated with shaking for 1.5 h before the addition of rifampin (100 μg ml^{-1}). Incubation was contin-

ued for 15 min before the addition of [^{3}H]leucine (2 μCi ml^{-1}) (specific activity, 146 Ci mmol^{-1}). Incubation was continued for 1 h before collagenase was purified as described previously (6, 8). Collagenase was eluted stepwise from a DE 52 cellulose column (7.5 by 1.5 cm) by subsequent application of three Tris-hydrochloride buffers (pH 7.0): 0.2 M–0.3 M–0.3 M made 1.0 M in NaCl. The flow rate was 12 ml h^{-1}, and 2-ml fractions were collected. Fractions were assayed for collagenase activity and radioactivity.

RESULTS

Fractionation of peptone. Peptone fractionated on a Sephadex G25 column gave three major peaks (Fig. 1). Samples from peak 1 induced collagenase, whereas samples from peaks 2 and 3 did not induce the production of the enzyme. Samples from peak 1 were pooled and added to a Sephadex G100 column (Fig. 2). A broad peak was obtained, and the resolution was not improved by the addition of smaller quantities of material to the column. The molecular weight ranged between 1,000 and 60,000. Samples taken from different positions within the peak all induced collagenase production with equal efficiency. This material was used for subsequent induction experiments and is referred to as the peptone inducer.

Enzymatic digestion of and induction by peptone inducer. Digestion of the peptone inducer and unfractionated peptone by purified *V. alginolyticus* collagenase caused a marked decrease in the inducing ability of both the inducer and peptone (Table 1). Digestion by collagenase (35 nkat ml^{-1}) for 39 h did not completely destroy the inducing ability of the peptone inducer. Collagenase was stable and lost no activity under these conditions over 22 h as shown in Fig. 5. Digestion with α-chymotrypsin and trypsin had little or no effect on the inducing ability of the purified inducer or peptone. Both α-chymotrypsin and trypsin were active under the same con-

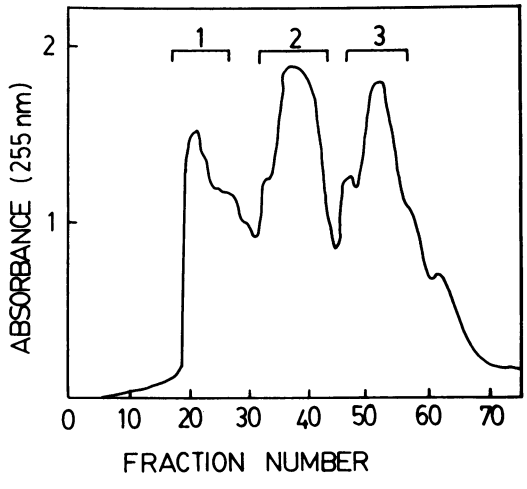

FIG. 1. *Gel filtration of peptone on Sephadex G-25. Fractions under each peak were pooled as shown in the figure (brackets). Only the first peak induced collagenase. Void volume, fraction 17; fully included volume, fraction 72.*

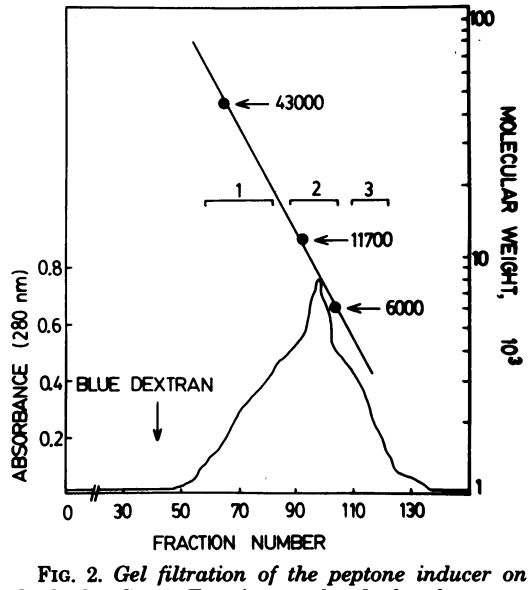

FIG. 2. *Gel filtration of the peptone inducer on Sephadex G-100. Fractions under the brackets were pooled. Fractions 1, 2, and 3 all induced collagenase equally. Molecular weight markers used were ovalbumin (43,000), cytochrome c (11,700), and bovine insulin (6,000).*

TABLE 1. *Effect of enzymatic digestion of peptone and the peptone inducer on their ability to induce collagenase*[a]

Substrate	Digestion time (h)	Enzyme/substrate ratio	% Control collagenase activity after digestion of substrate with:		
			Collagenase	α-Chymotrypsin	Trypsin
0.5% Peptone inducer	13	1:100	68	94	107
0.5% Peptone	20	1:100	51	102	90
0.25% Peptone	39	1:50	44	111	

[a] The standard error of these data is 2.4%, and duplicate samples were assayed in each case.

ditions as used for the digestion of peptone, when assayed by an azocasein assay. The induction of collagenase by the peptone inducer was determined after resuspending cells in SNP medium and 0.5% inducer (Fig. 3). No collagenase was produced in the absence of the inducer. When the inducer concentration was reduced from 0.5 to 0.1% the collagenase activity decreased by 60%; 0.05% inducer showed slight induction of collagenase (0.27 nkat ml^{-1} absorbance unit at 600 nm^{-1}), whereas induction by 0.025% inducer was negligible (0.02 nkat ml^{-1} absorbance unit at 600 nm^{-1}). The *V. alginolyticus* strain was able to grow in a minimal medium containing the peptone inducer as the sole nitrogen source.

The production of collagenase was also affected by the nature of the growth medium before inoculation into the SNP induction medium. Cells grown in minimal medium induced more rapidly and gave higher yields of collagenase than cells grown in peptone, Casamino Acids, or tryptone media (Fig. 3). High SNP medium repressed collagenase synthesis whereas low SNP medium enhanced collagenase production (Fig. 3). The addition of succinate, $(NH_4)_2SO_4$ and KH_2PO_4 (i.e., low SNP) to the 0.5% inducer in Tris-hydrochloride buffer markedly enhanced the production of collagenase, although the rate of collagenase production in the absence of low SNP medium is the same as in the presence of low SNP medium but a longer lag period occurs in the former medium (Fig. 4).

Inhibition of collagenase by peptone. Collagenase activity assayed with the synthetic substrate PZ-Pro-Leu-Gly-Pro-Arg was inhibited with increasing concentrations of peptone (Fig. 5). At an enzyme concentration of 4.2 nkat ml^{-1}, no inhibition was observed at 0.25% peptone compared with 65% inhibition by 2.5% peptone. Decreasing the collagenase concentration to 0.86 nkat ml^{-1} resulted in 35% inhibition by 0.25% peptone. The inhibitory effect of peptone can be removed by continued incubation with collagenase (Fig. 5). Thus, 65% inhibition by 2.5% peptone was decreased to 5% inhibition by incubation with 4.2 nkat of collagenase per ml for 4 h.

Effect of rifampin and chloramphenicol on collagenase production. Rifampin (100 μg ml^{-1}) completely inhibited the incorporation of [^{3}H]uracil into trichloroacetic acid-precipitable material at 1 and 3 h after washed cell suspensions were resuspended in 0.25% peptone (Fig. 6). Chloramphenicol (100 μg ml^{-1}) completely inhibited the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material at 1 and 3 h. *V. alginolyticus* was resistant to acti-

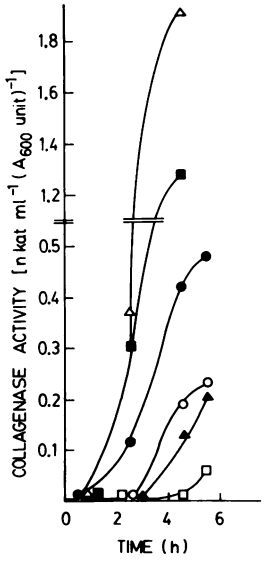

FIG. 3. *Induction of collagenase by the peptone inducer after growth in different media. Cells were resuspended in high SNP medium after growth in minimal medium (●), peptone (○), Casamino Acids (▲), and tryptone (□). Cells were resuspended in low SNP medium after growth in minimal medium (△) and peptone (■). The peptone inducer was included at a concentration of 0.5%.*

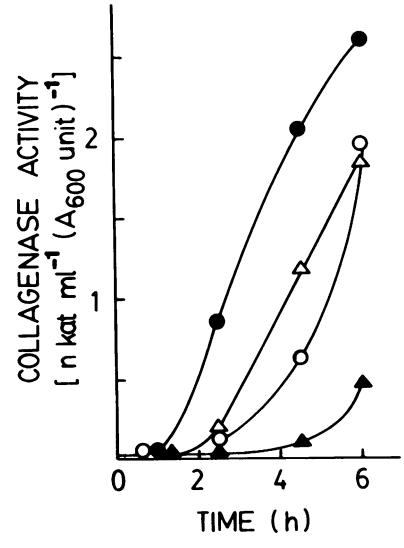

FIG. 4. *Induction of collagenase by the peptone inducer in the presence and absence of low SNP medium. Cells were grown in minimal medium and resuspended in low SNP medium (●) or Tris-hydrochloride buffer (○). Cells were grown in peptone and resuspended in low SNP medium (△) or Tris-hydrochloride buffer (▲). The peptone inducer was included at a concentration of 0.5%.*

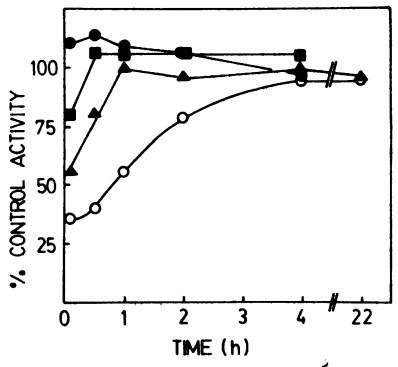

FIG. 5. *Inhibition of collagenase by peptone. Peptone at final concentrations of 2.5% (○), 1% (▲), 0.5% (■), and 0.25% (●) was incubated with purified V. alginolyticus collagenase (final concentration, 4.2 nkat ml^{-1}) at 30°C. Collagenase activity was assayed at intervals as described in the text.*

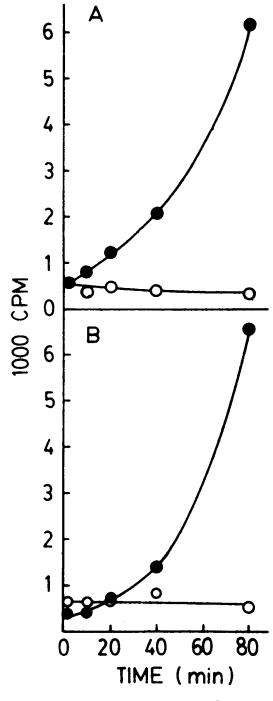

FIG. 6. *Effect of rifampin on [^{3}H]uracil incorporation. (A) [^{3}H]uracil was added at 1 h. No addition of drug (●); rifampin (100 μg ml^{-1}) was added at 1 h (○). (B) [^{3}H]uracil was added at 3 h. No addition of drug (●); rifampin (100 μg ml^{-1}) was added at 3 h (○).*

nomycin D, and this antibiotic therefore could not be used as an inhibitor of mRNA synthesis. Washed cell suspensions resuspended in peptone (0.25%) synthesize collagenase linearly for 2 to 3 h after an initial lag of approximately 1 h (Fig. 7). When rifampin was added at 1, 2, or 3 h after resuspension, collagenase synthesis continued for 30 to 60 min after its addition (Fig. 7). When rifampin was added immediately after resuspension, no collagenase was produced. Our results indicate that there is an increase in the amount of rifampin-insensitive collagenase produced the later rifampin is added, although as yet we have been unable to show a linear increase with time. During induction from 1 to 3 h, the optical density increased from 8.6 to 9.6, whereas the amount of rifampin-insensitive collagenase produced during the same period almost doubled. This increase in cell density cannot fully account for the increase in rifampin-insensitive collagenase production. The addition of chloramphenicol at 0, 1, 2, and 3 h after washed cell suspensions were resuspended in peptone medium completely and immediately abolished the synthesis of collagenase (Fig. 8).

Effect of glucose and Casamino Acids on collagenase production. Both 0.4% glucose and 0.5% Casamino Acids repressed collagenase synthesis, although repression was not immediate (Fig. 9). This repression was not increased by the addition of 0.8% glucose or 1% Casamino Acids. The amount of collagenase produced after the addition of both glucose or Casamino Acids was less than after the addition of rifampin but more than after the addition of chloramphenicol. Glucose (0.4%) caused greater repression than

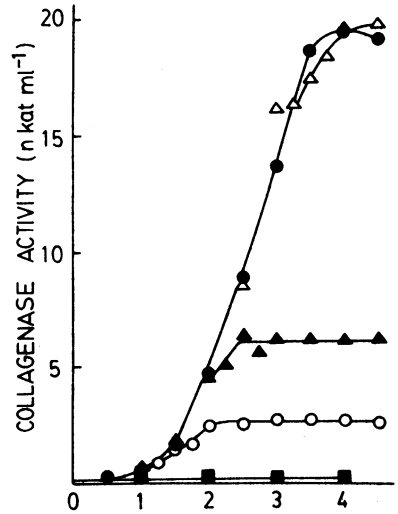

FIG. 7. *Effect of rifampin on collagenase production. No additions (●). Rifampin (100 μg ml^{-1}) was added at 0 (■), 1 (○), 2 (▲), and 3 h (△) after the resuspension of washed cells. When there was no difference between the activities of the control and experimental cultures, the symbols were omitted.*

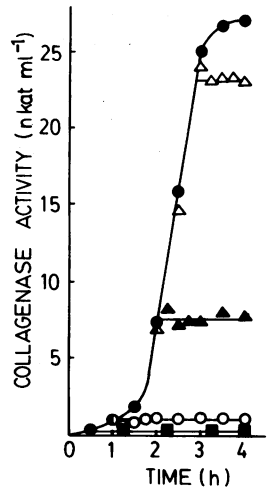

FIG. 8. *Effect of chloramphenicol on collagenase production. No additions (●). Chloramphenicol (100 μg ml⁻¹) was added at 0 (■), 1 (○), 2 (▲), and 3 h (△) after the resuspension of washed cells. When there was no difference between the activities of the control and experimental cultures the symbols were omitted.*

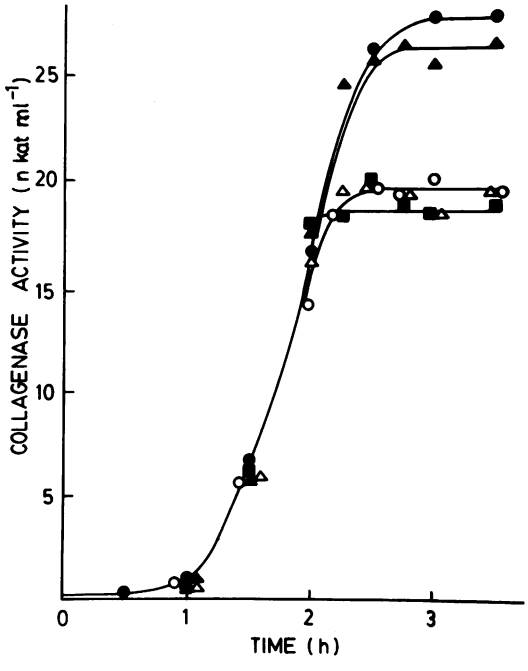

FIG. 9. *Effect of glucose and Casamino Acids on collagenase production. No additions (●); Glucose, 0.4% (△); Casamino Acids, 0.5% (○); chloramphenicol, 100 μg ml⁻¹; (■); and rifampin, 100 μg ml⁻¹ (▲) were added at 2 h after the resuspension of washed cells.*

0.5% Casamino Acids. The level of repression was the same whether glucose or Casamino Acids were added separately or together with rifampin (100 μg ml⁻¹).

Incorporation of [³H]leucine into collagenase in the presence of rifampin. The elution pattern of [³H]leucine-labeled collagenase gave three peaks of radioactivity and one peak of collagenase activity (Fig. 10). The first peak of radioactivity coincided with the elution position of the neutral protease produced by *V. alginolyticus* (7). The second peak of radioactivity coincided with the elution position of an autolytic degradation product of collagenase which shows reduced collagenase activity (6; our unpublished data). The third peak of radioactivity coincided exactly with the elution position of collagenase.

DISCUSSION

The collagenase inducer molecules in peptone have a molecular weight between 1,000 and 60,000 which is similar to that reported by Dreisbach and Merkel (3) for collagen fragments (molecular weight, 1,000 to 10,000) required for the induction of a *Vibrio* B-30 strain isolated from seawater. Keil-Dlouha et al. (7) showed that collagenase induction in *V. alginolyticus* required the macromolecular fraction of digested collagen (molecular weight ± 8,000 to 30,000).

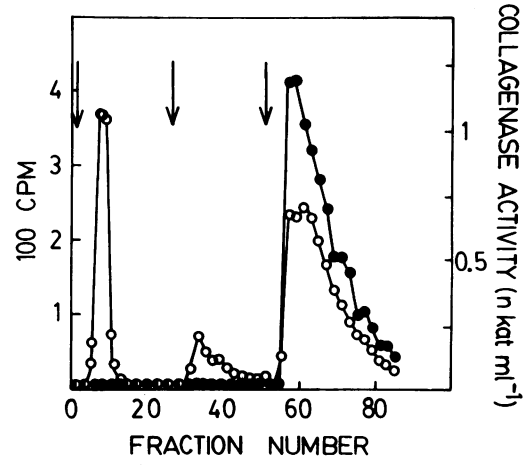

FIG. 10. *Purification of labeled collagenase on DE 52 cellulose. The arrows mark the stepwise elution as described in the text. Collagenase activity (●), counts per minute (○). The first radioactivity peak coincides with the elution position of the neutral protease, whereas the second coincides with the elution position of an autolytic degradation product of collagenase. The third peak is pure collagenase.*

Digestion of either peptone or the peptone inducer by purified *V. alginolyticus* collagenase for 13 to 39 h did not completely eliminate its inducing ability but reduced it by 32 to 56%. In contrast Keil-Dlouha et al. (7) reported that digestion of high-molecular-weight fragments of collagen with collagenase at a lower enzyme-to-substrate ratio for 2 h prevented the induction of collagenase in the same strain. They suggested that the collagenase-sensitive bond and tertiary structure of collagen were required for induction. The reduction in the inducing activity of peptone by digestion with collagenase supports this suggestion, but peptone differs from collagen as an inducer in that its reducing ability is not completely eliminated by extensive digestion with collagenase. Dreisbach and Merkel (3) observed that digestion of collagen improved its inducing ability, but this could be due to incomplete digestion which generates collagen fragments with an increased capacity to induce.

The finding that peptone acts as an inhibitor explains previous results in which linear increases in collagenase activity were observed for up to 4 h after the addition of rifampin or chloramphenicol at concentrations of 300 μg ml^{-1} (12). The use of 0.25% peptone to induce collagenase ensured that the increase in collagenase production after the addition of rifampin cannot be due to the digestion of peptone, which inhibits collagenase.

Inhibition of translation by chloramphenicol and transcription by rifampin affected collagenase production in a way similar to exoenzyme production in late log phase in *B. amyloliquefaciens* (9), *Bacillus subtilis*, (14) and *Pseudomonas lemoignei* (15). Chloramphenicol rapidly inhibited collagenase production and protein synthesis, indicating that de novo protein synthesis is required for normal collagenase production. Collagenase can be labeled 15 min after the addition of rifampin, which indicates that rifampin-insensitive collagenase synthesis also involves de novo synthesis of the enzyme. There is a peak of radioactivity, without any collagenase activity at a position coinciding with the elution position of an autolytic degradation product of collagenase. This could be expected as the degradation products of collagenase show reduced collagenase activity (6) and the detection of radioactivity is far more sensitive than the collagenase assay.

V. alginolyticus cells continued to produce collagenase for 30 to 60 min in the presence of rifampin at levels which inhibited RNA synthesis. This type of observation in *B. amyloliquefaciens* has been used to support a hypothesis which involves a pool or reserve of mRNA (2, 9). The results obtained with our *Vibrio* strain are similar to those obtained with *B. amyloliquefaciens*, where it was shown that the later rifampin was added, the greater was the amount of rifampin-insensitive protease produced. When rifampin was added after 3 h of incubation, at which time maximum rifampin-insensitive collagenase production occurs, [^{3}H]uracil incorporation was no less sensitive to the drug than at 1 h. This indicates that the cells did not become progressively more resistant to rifampin. O'Connor et al. (9) showed that when *B. amyloliquefaciens* is transferred from a 0.5% Casamino Acids medium to a 0.025% Casamino Acids medium an mRNA pool (rifampin-insensitive secretion) builds up and reaches in 75 min a value several times greater than that found in the harvested cells. Our results suggest that an apparent mRNA pool builds up during the first hour after resuspension and increases over 1 to 2 h.

Our results on the production of collagenase also support the hypothesis of O'Connor et al. (9) which postulates that short-lived mRNA is produced for immediate translation and, in addition, a reserve of stable mRNA is laid down in a nontranslatable form. In the absence of transcription, reserve mRNA is converted to translatable mRNA and accounts for the continued production of enzyme in the presence of rifampin.

The partial repression of rifampin-insensitive collagenase production by glucose and Casamino Acids suggests that both glucose and Casamino Acids repress collagenase synthesis at a posttranscriptional but pretranslational stage. It cannot, however, be excluded that glucose or Casamino Acids do not inhibit transport or release of collagenase. Priest (10) showed that glucose represses α-amylase synthesis in *B. subtilis* at transcription but does not repress rifampin-insensitive α-amylase synthesis. This repression by glucose was not relieved by cyclic AMP. Boethling (1), however, showed that rifampin-insensitive protease secretion by *Pseudomonas maltophilia* could be repressed by either α-ketoglutarate or chloramphenicol, suggesting that α-ketoglutarate is coupled to a translational or posttranslational stage. If the effect of α-ketoglutarate on protease secretion is a reflection of a mechanism similar to cAMP-mediated catabolite repression, this finding is anomalous since catabolite repression is primarily thought to be a transcriptional control. These and our previous results (12) suggest that collagenase synthesis, like other exoenzymes, is not regulated by classical catabolite repression.

Our results support the hypothesis that

mRNA accumulation is a general property of bacterial extracellular enzyme synthesis, whether it be produced by gram-positive or gram-negative bacteria and be constitutive or inducible.

ACKNOWLEDGMENTS

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