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Tube growth process in the deep-sea hydrothermal vent tubeworm *Riftia pachyptila* (Vestimentifera): synthesis and degradation of chitin

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Abstract: Enzymatic activities involved in chitin metabolism were studied in the giant tubeworm *Riftia pachyptila* in order to understand the tube growth process. Experiments performed on repressurized worms provided tissues, from the vestimentum and trunk, that were in a secretion state. Analysis of the composition of the tube newly secreted during these experiments showed a chitin content of about 12% and a protein content of about 22%, approximately twofold less than in mature tubes. Chitin synthase assays demonstrated the presence of an enzymatic activity functioning with UDP-GlcNAc as a substrate. This activity was sensitive to polyoxin D, insensitive to proteolysis by trypsin and stimulated by GlcNAc. Notable results are the lack of a Mg^{2+} requirement and stimulation by chitobiose and chitotriose. The activity, in our experimental conditions, was shown to be identical in tissues from repressurized animals and from non repressurized animals rapidly dissected after collection. A chitinolytic activity was demonstrated in the opisthosome and in the plume. These results support the model of tube growth at both ends (Gaill et al., 1997).

Résumé : *Processus de croissance du tube chez* Riftia pachyptila (Vestimentifera) : *synthèse et dégradation de chitine.* Les activités enzymatiques impliquées dans le métabolisme de la chitine ont été étudiées chez *Riftia pachyptila* afin d'élucider les processus de croissance du tube. Des expériences menées sur des animaux repressurisés ont procuré des tissus du vestimentum et du tronc en état de sécrétion. L'analyse de la composition du tube fraîchement sécrété pendant ces expériences montre un contenu en chitine d'environ 12 %, et un contenu en protéines d'environ 22 %, ce qui est approximativement deux fois inférieur aux données obtenues pour le tube mature. Les dosages d'activité de chitine synthétase montrent la présence d'une activité enzymatique ayant l'UDP-GlcNAc pour substrat. Cette activité est sensible à la polyoxine D, insensible à la protéolyse par la trypsine et stimulée par le GlcNAc. Les résultats notables sur l'activité de la chitine synthétase sont l'insensibilité au Mg^{2+} et la stimulation par le chitobiose et le chitotriose. L'activité détectée, dans nos conditions d'expérimentation, s'est révélée identique dans les tissus d'animaux repressurisés et ceux d'animaux non repressurisés mais rapidement disséqués après leur récolte. Une activité chitinolytique a été mise en évidence dans l'opisthosome et dans la branchie, ce qui renforce l'hypothèse du modèle de croissance du tube aux deux extrémités (Gaill et al., 1997).

Keywords: Vestimentifera - Growth process - Extracellular Matrix - Chitin synthase - Chitinase.

Introduction

Vestimentifera, and particularly *Riftia pachyptila* Jones, 1980 are representative of the communities associated to

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deep-sea hydrothermal vents. These organisms, unusual by their anatomy and gigantism, live in tubes which play a skeletal role and allow the animal's gill (the plume) to be exposed in the hydrothermal fluid. This tube, composed of chitin associated with proteins (Gaill & Hunt, 1986), grows at high rates reaching up to 85 cm.year^{-1} (Lutz et al., 1994). How can *Riftia* produce such a great quantity of tube material ? Previous studies have partially answered this question, by showing that, in *Riftia*, tube's secretion is achieved through a considerable increase of the secreting surfaces that exist at different scales: anatomical, tissular, cellular and subcellular (Gaill, 1993). This secretion takes place in specialized structures, the chitin secreting glands, which constitute a very efficient system (Shillito et al., 1993).

This high growth rate can be viewed in term of chitin synthesis. Several assays, using protocols adapted to fungi (Gay et al., 1989; Bulone et al., 1992), have previously failed to detect such a chitin synthesis activity (Gaill, unpublished). The first objective of this work was to detect this activity, using varying experimental conditions on tissues from animals maintained in pressure vessels, which are known to exhibit an enhanced chitin secretion (Shillito et al., 1995b). The tube newly secreted during these experiments was then removed and analysed for its composition in chitin and proteins.

Another question about this giant cylindrical tube is related to its growth process. A model of tube growth has been proposed which is a moulting-like process (Gaill et al, 1997). This model implies a tube dissolution which would involve a chitinase activity. In order to test this assumption, the second goal of this work was to assay the chitinolytic activity in a chitin secreting tissue.

Material and methods

I. Animal collection and experiments in pressure vessels

Specimens of *Riftia pachyptila* used in this study were collected at 2600 m depth during the French / US HOT 96 cruise (February - March 1996) at the 9°N and 13°N sites on the East Pacific Rise. Sampling was done with the submersible *Nautile* from the oceanographic ship *Nadir* (IFREMER, France). During the submersible ascent, the worms were kept in an isothermal container (4°C). As soon as they arrived on board, the animals were transferred to the oceanographic laboratory ship *Wecoma* (National Science Foundation; Oregon State University). Some animals were directly dissected and samples of tissues from vestimentum and body wall of trunk were stored in liquid nitrogen until further investigations.

Furthermore, three animals were placed in pressure vessels as soon as they arrived on the laboratory ship. They were kept for 3 days in the vessels in conditions close to that of their original habitat (pressure, 210 atm; temperature, 15°C; pH, 6.1; $[O_2]$ = 220 µM; $[CO_2]$ = 5300 µM; $[H_2S]$ = 202 µM; $[N_2]$ = 150 µM). Before pressurization, a polyethylene ribbon was inserted in between the worm and its tube, at the level of vestimentum and trunk (as previously described by Gaill et al., 1997), in order to remove the fresh tube material that will be laid down by the animal during its stay in the vessel. After three days the worms were removed from the pressure vessels and separated from their tube. Fresh tube material was gently scraped off from the polyethylene ribbon with a scalpel, lyophilized and weighed prior to protein and chitin contents analysis. Parts of mature tube were also removed. Secreting tissues from the animals were dissected and stored in liquid nitrogen for enzymatic assays.

II. Tube composition : Chitin and protein content analyses

These analyses were done as described in Shillito et al. (1995a). Pieces of the freeze-dried tube of one specimen (fresh tube material and mature tube material) of 2.5 mg (lyophilized dry weight) were subjected to two successive 3 hours treatments with 0.5 M NaOH at 100°C. Proteins were assayed in these NaOH extracts with the BCA kit (Pierce). Chitin was enzymatically assayed in residues, after NaOH extraction, according to a protocol derived from Jeuniaux (Jeuniaux, 1963; Jeuniaux, 1966). The material was suspended in 2.5 ml of chitinase solution (1 mg ml^{-1}; SIGMA C-6137), incubated at 37°C for eight hours, then centrifuged 10 min at 2000*g* and the supernatant was recovered. This step was repeated three times (until total enzymatic degradation of the material). 1 ml of supernatant was then removed and added to 1 to 4 ml of lobster blood serum diluted 10 times with water (used as a chitobiase solution). After two hours of incubation at 37°C, the mixture was boiled for 10 min to stop the reaction and was centrifuged for 10 min at 2000*g*. The supernatant was recovered for GlcNAc (N-acetyl-D-glucosamine) assay (Reissig et al., 1955). Taking into account the dilution factor, the result was expressed as a quantity of chitin by multiplying the quantity of GlcNAc by the correction factor 0.92 (weight of GlcNAc in the chitin molecule / weight of free GlcNAc) (Jeuniaux, 1966).

III. Chitin synthase assays

Tissues used for chitin synthase assays were dissected from vestimentum and trunk body wall of three animals of approximatively the same length (20 to 30 cm) collected at 13°N site. Similar tissues were dissected on one specimen maintained in pressure vessel.

1. Enzyme extraction:

tissues (from 3 to 10 g) were ground with a Virtis homogenizer or with a Potter homogenizer in 3 to 10 ml of extraction buffer (HEPES 25 mM / NaCl 0.5 M; pH 7.4) or *Riftia* saline pH 7.5 (HEPES 30 mM; NaCl 0.4 M; KCl 2.6 mM; $MgSO_4$, 7 H_2O 30 mM; $CaCl_2$, 2 H_2O 10 mM) (Fisher et al., 1988). This crude extract was centrifuged at 2000*g* for 15 min. The 2000*g* pellet (cell fragments) was then resuspended in extraction buffer (1 or 2 ml); the supernatant was centrifuged at 48,000*g* for 30 min. Mixed membrane fraction (48,000*g* pellet) and supernatant were subjected to further experimentation.

2. Solubilization of the enzyme with digitonin:

2000*g* and 48,000*g* pellets (obtained as described above) were incubated with 10 mg ml^{-1} digitonin prepared in extraction buffer for 30 min at 4°C (Gay et al., 1992). The suspension was then centrifuged at 48000*g* for 35 min. All fractions were collected and assayed for enzyme activity.

3. Incubation:

chitin synthase activity was determined according to a procedure previously described by Gay et al. (1989). Standard reaction mixture consisted of 100 µl of tissues extracts, 100 µl of substrate solution (10 mM GlcNAc; 1 mM UDP-[^{12}C]-GlcNAc (Uridine diphosphate-[^{12}C]-GlcNAc); 0.5 µM UDP-[^{14}C]-GlcNAc (Amersham, U-K; 2.5 nCi.μl^{-1} (nanoCurie.μl^{-1}); 155 nCi in each reaction mixture); 10 µg ml^{-1} trypsin; HEPES 25 mM / NaCl 0.5 M / $MgCl_2$ 10 mM, pH 7.4 or *Riftia* saline pH 7.5 (Fisher et al., 1988) and 20 µl digitonin (50 mg ml^{-1}) i.e. a concentration of 4.5 mg ml^{-1} in incubation mixture. The mixture was incubated for 1 hour at room temperature and the reaction was stopped with 2 ml of ethanol 95% or 3 drops of glacial acetic acid. Reaction products were filtered on glass-fiber filters (GF/C Whatman) and washed successively with 5 ml NaOH 0.5 M then twice with a mixture of 2 per 8 volumes of ethanol 95% and acetic acid 1M and finally twice with 5 ml of ethanol 95%. Radioactivity was determined by scintillation spectrometry. Activity was expressed as pmoles (picomoles) UDP-GlcNAc incorporated per minute.

IV. Chitinolytic activity assays

Tissues chosen for chitinase activity assays were the opisthosome and the branchial lamellae from respectively three and two animals (20 to 30 cm in length), collected at 13°N site. Commercial chitin was used for these assays (Sigma ref. C 3641).

1. Enzyme extraction:

tissues were ground in liquid nitrogen, and resuspended in extraction buffer (Na_2HPO_4 1.2 M; citric acid 0.6 M pH 5.2). These crude extracts were centrifuged at 2000*g* for 10 min and supernatants were assayed for their chitinolytic activity.

2. Activity assay:

chitinase activity was determined according to a procedure described by Jeuniaux (1966). The standard mixture consisted of 1 volume of *Riftia* saline buffer (Fischer et al., 1988), 1 volume lobster blood serum diluted 10 times (chitobiase solution), 1 volume of chitin suspension (5 mg ml^{-1}) and 1 volume of tissues extract. After 0, 90 and 180 min of incubation, 1 ml of reaction mixture was pipetted into a tube containing 1 ml of boiling distilled H_2O, and placed at 100°C for 10 min. The suspension was then centrifuged for 10 min at 2000*g* and the supernatant was used for GlcNAc measurements (Reissig et al., 1955). The activity was expressed as mg GlcNAc released per hour and per milliliter.

3. Assays with Streptomyces griseus *chitinase (Sigma C 6137)*:

procedure was similar to that described above except for the incubation buffer (Na_2HPO_4 1.2 M; citric acid 0.6 M pH 5.2) and tissues extracts which were replaced by commercial chitinase solution (1 mg ml^{-1}).

Results

I. Tube composition

After their removing from the pressure vessel, the tubes exhibited a gelatinous collar at their apex, corresponding to freshly laid tube material. All the inner surface of the tubes was also covered by this fresh translucid material. Samples from the apex of one of the tubes, from the inner-tube fresh material laid on the polyethylene sheet (see Material and methods), and fractions of the tube (one facing the polyethylene ribbon, one at the middle of the tube and the last one at the bottom) were taken. These pieces of tube were assayed for chitin and protein contents (Table 1).

In the mature tube wall material, the chitin percentage ranged from 19.4 to 24.7%. This percentage range was equivalent along the tube (Student test, $n = 9$, $p<0.05$). In the fresh tube, the chitin percentage ranged from 9.1 to 12.8% and was about twofold lower than in the mature tube wall (Table 1).

The protein content of the mature tube ranged from 37.9 to 53.4% and it was not statistically different along the tube (Student test, $n = 9$, $p<0.05$). For the fresh tube, the protein percentage ranged from 19.9 to 23%, i.e., it was about twofold lower than in the mature tube material (Table 1).

Table 1. Chitin and protein contents of fresh tube and mature tube material. All values except values of fresh tube on ribbon) represent the mean of 3 assays and are given with their standard deviation.

Tableau 1. Composition en chitine et en protéines du tube fraîchement sécrété et du tube mature. Toutes les valeurs (sauf les valeurs obtenues pour le tube frais sur le ruban de polyéthylène) représentent la moyenne de 3 dosages dont les écarts types sont indiqués.

		Chitin content (% of dry weight)	Proteins content (% of dry weight)
Fresh tube material	Apex of the tube	11.5 ± 1.3 %	20.41 ± 2.57
	Fresh tube on ribbon	9.1 %	19.86 %
Mature tube material	Tube at ribbon level	22.7 ± 2 %	43.44 ± 0.94 %
	Middle	19.9 ± 0.5 %	42.66 ± 4.78 %
	Bottom	22.5 ± 2 %	50.11 ± 2.78 %

II. Enzymatic activity of secreting tissues : chitin synthase assays

Properties of enzymatic activity

The vestimentum, a tissue secreting high amounts of chitin (Gaill et al., 1997) was chosen to determine the optimal experimental conditions for chitin synthase assays. Effects of different parameters, such as incubation buffer, pH, temperature and substrate concentration were tested on vestimentum extracts in order to obtain optimal conditions for activity detection. In addition, trypsin, (GlcNAc)n and polyoxin D effects were tested in order to discriminate the type of activity detected.

Activity was only detected in fractions containing cell membranes. It was clearly associated with cell fragments (2000*g* pellet) and mixed membrane fraction (48000*g* pellet) since 80 to 90% of the total activity was recovered in these fractions. No activity was detected in the soluble fraction, i.e., 48000*g* supernatant, of tissues extracts. Membrane-containing fractions were used for activity characterization. Activity was stimulated twofold by 4.5 mg ml^{-1} digitonin (Fig. 1). In an attempt to solubilize the enzyme, tissues extracts were incubated with high concentration of digitonin (10 mg ml^{-1}). These tissues extracts were then incubated with the substrate. Activity in membrane-containing fraction was twofold lower than that of the control without digitonin (Fig. 1). No activity was detected in the soluble fraction (result not shown).

In assays with or without 0.5M NaCl in Tris/HCl or HEPES buffers at pH 7.4, activity was higher in saline HEPES buffer (results not shown).

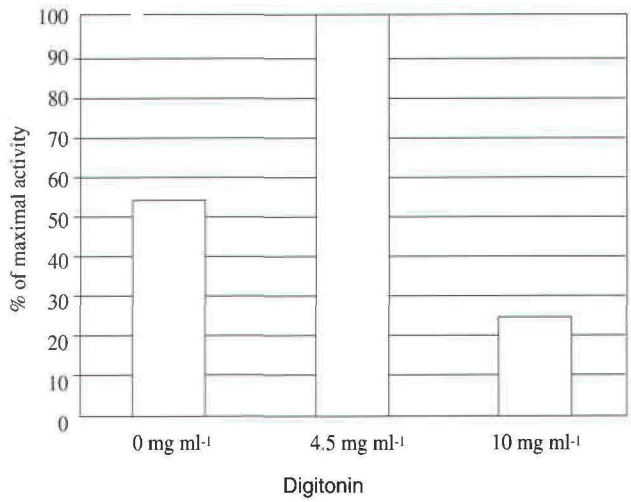

Figure 1. Digitonin effect on chitin synthase activity in membrane containing fraction from the vestimentum of *Riftia*. Activities are given in % of maximal values recorded (about 8 pmoles min-1 100 μl^{-1} extract); maximal values were obtained with 4.5 mg ml^{-1} digitonin. Each value is the mean of two measurements.

Figure 1. Effet de la digitonine sur l'activité chitine synthétase d'une fraction membranaire du vestimentum de *Riftia*. Les activités sont présentées en % des valeurs maximales mesurées (environ 8 pmoles min^{-1} 100 μl^{-1} d'extrait) ; les valeurs maximales ont été obtenues avec 4,5 mg ml^{-1} de digitonine. Chaque valeur représente la moyenne de deux mesures.

Presence or absence of $MgCl_2$ did not affect the activity (results not shown).

The activity was assayed at pH values ranging from 6 to 8 (Fig. 2) and a maximal value was obtained at pH 7.4. Deviations from this optimal condition resulted in a sharp decrease of the enzyme activity since about 3/4 of the activity were lost at pH 8 and 6.5. Activity was also assayed under standard conditions at temperatures of 4, 14, 24 and 37°C (Fig. 3). The optimal temperature for enzymatic activity was ca. 24°C.

The influence of substrate concentration on the activity is shown on Fig. 4. The apparent Km value obtained using UDP-[^{12}C]-GlcNAc as substrate was 0.23 mM.

Enhancement of activity by partial proteolysis is a property of most fungal chitin synthases. Assays were conducted in the presence of various amounts (from 5 to 100 $\mu g.ml^{-1}$) of trypsin. No effect of trypsin could be observed (results not shown).

Some chitin synthases need primers to begin the synthesis (allosteric stimulators). Enzymatic activity was assayed in incubation mixtures containing GlcNAc, $(GlcNAc)_2$, i.e. chitobiose, or $(GlcNAc)_3$, i.e. chitotriose. The activity of *Riftia* tissues increased about twofold with 10 mM GlcNAc and about threefold with chitobiose or chitotriose (Fig. 5).

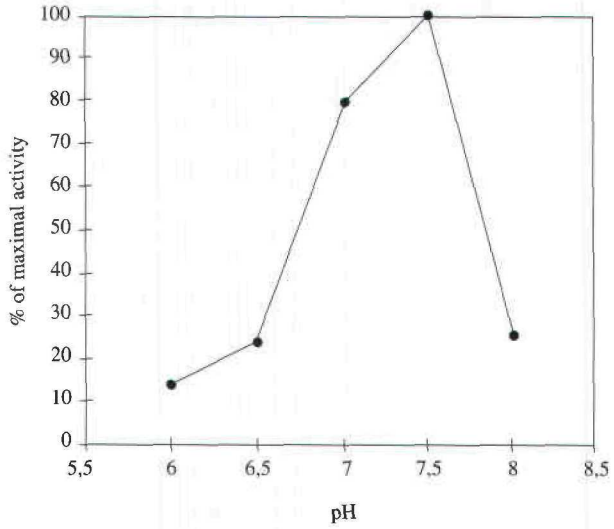

Figure 2. Effect of pH on chitin synthase activity in membrane containing fraction from the vestimentum of *Riftia*. Activities are given in % of maximal values recorded (about 10 pmoles min-1 100 µl-1 extract); maximal values were obtained with pH 7.4. Each value is the mean of two measurements.

Figure 2. Effet du pH sur l'activité chitine synthétase d'une fraction membranaire du vestimentum de *Riftia*. Les activités sont présentées en % des valeurs maximales mesurées (environ 10 pmoles min-1 100 µl-1 d'extrait) ; les valeurs maximales ont été obtenues à pH 7,4. Chaque valeur représente la moyenne de deux mesures.

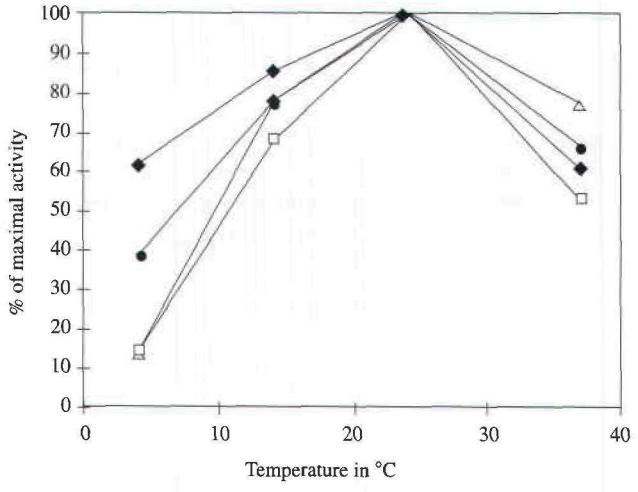

Figure 3. Effect of temperature on chitin synthase activity in membrane containing fraction from the vestimentum of *Riftia*. Activities are given in % of maximal values recorded (about 18 pmoles min-1 100 µl-1 extract); maximal values were obtained with 24°C. Four independant experiments are presented. Each value is the mean of 2 measurements.

Figure 3. Effet de la température sur l'activité chitine synthétase d'une fraction membranaire du vestimentum de *Riftia*. Les activités sont présentées en % des valeurs maximales mesurées (environ 18 pmoles min-1 100 µl-1 d'extrait) ; les valeurs maximales ont été obtenues à 24°C. Quatre expériences indépendantes sont présentées. Chaque valeur représente la moyenne de 2 mesures.

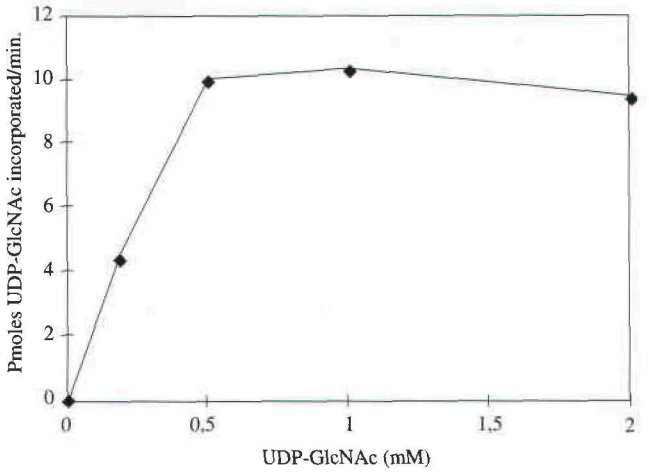

Figure 4. Activity of chitin synthase in membrane containing fraction from the vestimentum of *Riftia*, as a function of substrate concentration. Activity is given in pmoles UDP-GlcNAc incorporated min-1. Each value is the mean of two measurements.

Figure 4. Activité de la chitine synthétase d'une fraction membranaire du vestimentum de *Riftia* en fonction de la concentration en substrat. L'activité est donnée en pmoles d'UDP-GlcNAc incorporées min-1. Chaque valeur représente la moyenne de deux mesures.

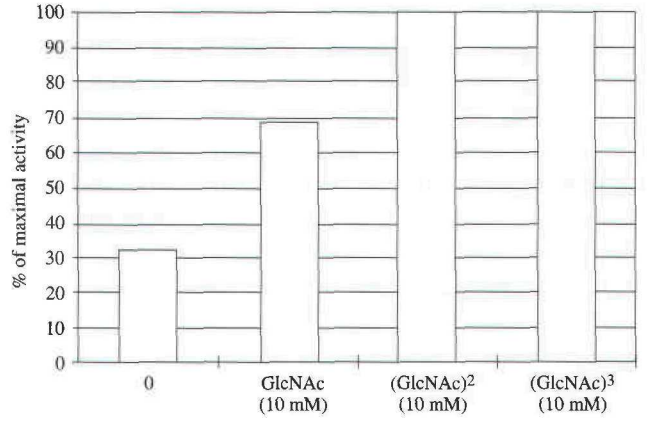

Figure 5. Effect of (GlcNAc)n on chitin synthase activity membrane containing fraction from the vestimentum of *Ri* Activity is given in % of maximal value recorded (about 20 pmoles min-1 100 µl-1 extract); maximal values were obtained with chitobiose (GlcNAc)2 and chitotriose (GlcNAc)3. Each value is the mean of two measurements.

Figure 5. Effet de (GlcNAc)n sur l'activité chitine synthétase d'une fraction membranaire du vestimentum de *Riftia*. L'activité est donnée en % des valeurs maximales mesurées (environ 20 pmoles min-1 100 µl-1 d'extrait) ; les valeurs maximales ont été obtenues avec le chitobiose (GlcNAc)2 et le chitotriose (GlcNAc)3. Chaque valeur représente la moyenne de deux mesures.

The effects of various concentrations of polyoxin D, a competitive inhibitor of chitin synthase activity (Endo et al.,

1970), was studied (Fig. 6). An almost total inhibition of the activity (94% inhibition) was observed for a 10 µg ml^{-1} polyoxin D concentration.

It has previously been shown that animals placed in pressure vessels secrete great quantities of tube material (Gaill et al., 1997), and it was interesting to check if this experimental condition affected the activity detected in vitro. Body wall fractions from 2 animals of same length and sampled at the same site (Genesis, 13°N), one of which maintained for three days in pressure vessels, the other immediately dissected and stored in liquid nitrogen after its arrival on board, were compared. The specimen which has been placed in pressure vessel had secreted fresh tube material (see Results, Tube composition). No difference in chitin synthase activity was found between the two samples.

III. Chitinolytic activity

Two types of tissues were assayed for their chitinolytic activity: opisthosomes from animals with bifid tubes (which exhibited a degradation of the basal part of their tube), and branchial lamellae from the plume (which does not secrete chitin and was chosen as a control).

Activity was assayed in a phosphate buffer of pH 5.2 (Jeuniaux, 1966) or the *Riftia* saline HEPES buffer pH 7.5 (Fisher et al., 1988). Activity increased ca. sevenfold with *Riftia* saline HEPES buffer (results not shown).

Chitinolytic activities of *Riftia* tissues, i.e. opisthosome and plume, were compared to that of *Streptomyces griseus* purified chitinase (Sigma) at a concentration of 1 mg ml^{-1}, as a standard (Fig. 7). Chitinolytic activity recorded in the opisthosome was about 40% of *S. griseus* chitinase activity. Activity in branchial lamellae was surprisingly very variable from one specimen to another. Indeed, it could represent 15 to 120% of *S. griseus* chitinase activity. The activity recorded in three different opisthosome extracts showed similar values, whereas activity of branchial lamellae extracts was very variable in the two specimens investigated and can reach threefold that of the opisthosome or half of it (Fig. 7).

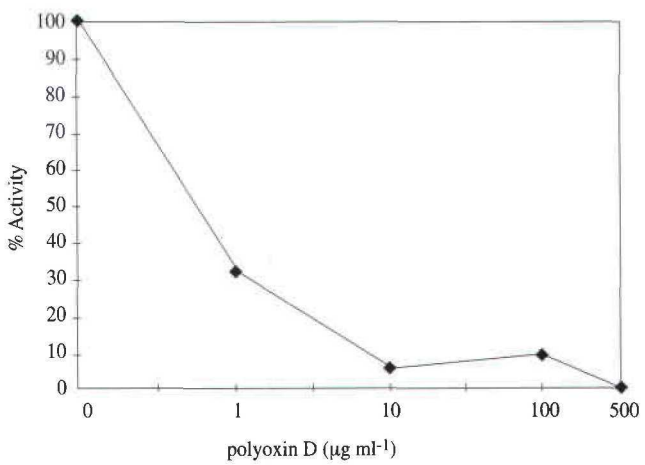

Figure 6. Effect of polyoxin D on chitin synthase activity in membrane containing fraction from the vestimentum of *Riftia*. Activity is given as % of the control. Each value is the mean of two measurements.

Figure 6. Effet de la polyoxine D sur l'activité chitine synthétase d'une fraction membranaire du vestimentum de *Riftia*. L'activité est exprimée en % de l'activité contrôle. Chaque valeur représente la moyenne de deux mesures.

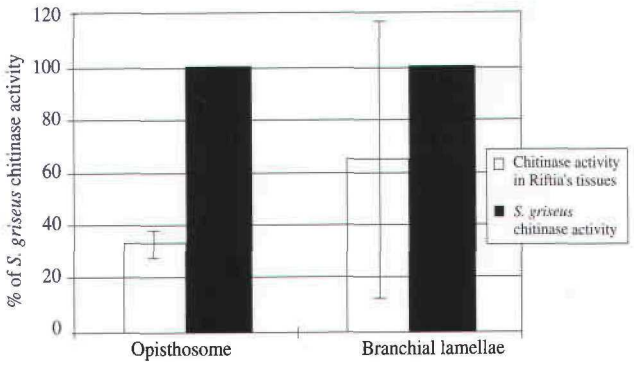

Figure 7. Comparison of chitinolytic activities in opisthosome and plume of *Riftia* with *Streptomyces griseus* chitinase activity. Activities, obtained after 3 hours incubation with crab chitin (SIGMA), are given in % of control (*S. griseus* chitinase: 1 mg ml^{-1}). The opisthosome value is the mean of three measurements, the plume value is the mean of two measurements. They are given with their mean deviation.

Figure 7. Comparaison des activités chitinolytiques de l'opisthosome et des lamelles branchiales de *Riftia* avec l'activité de la chitinase de *Streptomyces griseus*. Les activités, obtenues après 3 heures d'incubation avec de la chitine de crabe (SIGMA), sont exprimées en % de l'activité du contrôle (chitinase de *S. griseus* : 1 mg ml^{-1}). La valeur pour l'opisthosome représente la moyenne de trois mesures, celle de la plume représente la moyenne de deux mesures. Les écarts moyens sont indiqués.

Discussion

I. Tube composition

Chitin and protein composition of the mature tube was close to that previously determined by Gaill et al. (1992a, b) and later confirmed by Shillito et al. (1995a) who found chitin and protein content values in the same range, i.e. 24% of chitin and 41-37% of proteins (dry weight). This led to a chitin to protein ratio of about 0.5.

On the other hand, the fresh tube secreted in pressurized vessels contains ca. twofold less chitin and protein in % of dry weight than the mature tube, i.e. ca. 11% of chitin and

22% of proteins. This led again to a chitin to protein ratio of about 0.5. Recently, contents of 25% for chitin and 39.3 - 50.8% for proteins have been found (Gaill et al., 1997). It was therefore concluded that chitin and protein content of the fresh tubes were similar to contents measured on mature tubes by Shillito et al. (1995a). The new results here obtained with fresh tube and mature tube materials should be more reliable because they were collected from the same animal. Our results corroborate those of Shillito et al. (1997) in showing that there is a difference in the relative composition of chitin between fresh and mature tube: the chitin to protein ratio is preserved from fresh to mature tube, but for a same amount of tube material there is twice as much chitin and protein in the mature tube. It can be hypothesized that maturation processes would act on the non-characterized componants of the tube. A 50% loss (in % of total dry weight) of these unknown components during maturation would result in an increase of the chitin and protein content (in % of dry weight) without changing the relative proportion of these two compounds. This loss of tube material could be due to a compaction process as described by Shillito et al. (1997), who demonstrated a ten times as much compaction of chitin microfibrils in mature tube compared to fresh tube.

II. Chitin synthesis activity

Our data demonstrate that *Riftia* tissues extracts incorporate UDP-GlcNAc into a product of a sufficient length to be retained on a GF/C filter, which means that they exhibit a N-acetylglucosamine transferase activity. Results are summarized in Table 2 and compared to other data on chitin synthases.

The crude enzyme preparation has an optimum pH of about 7.5, which is in the same range of most chitin synthases (Cabib, 1987), and an optimal temperature of about 24°C, which could be related to the maximal body temperature sustained by *Riftia* (Dahlhoff & Somero, 1991). From the data of Fig. 4 an apparent Km value of 0.23 mM was calculated. This value is in the lower range of what was found in other organisms, i.e., 82 µM for *Chironomus* cells (Londershausen et al., 1988) to 33.7 mM for *Chironomus* extracts (Ludwig et al., 1991), and 1 to 3 mM for fungi (Cabib, 1987).

To characterize the enzymatic activity, usual properties of chitin synthases were tested, such as proteolytic activation, GlcNAc and Mg^{2+} stimulation and polyoxin D inhibition. The enhancement of activity by partial proteolysis is a property of most fungal chitin synthases (Cabib, 1987) while a slight enhancement may occur in insect chitin

Table 2. Chitin synthases properties. Results obtained for *Riftia* are compared with data from the literature (indicated by numbers with corresponding authors).

Tableau 2. Propriétés des chitine synthétases. Les résultats obtenus pour *Riftia* sont comparés avec les données de la littérature (indiquées par des chiffres avec les auteurs correspondants).

[1,2,3] Cabib (1987) ; Gay et al. (1989); Bulone et al. (1992)

[4,5,6] Cohen (1987) ; Ludwig et al. (1991) ; Londershausen (1988)

[7] Horst (1981 et 1993)

	Fungi/yeasts[1,2,3]	**Insects[4,5,6]**	**Crustaceans[7]**	***Riftia pachyptila***
Localisation	Associated with membranes	?	Associated with membranes	Associated with membranes
Optimum pH	neutral	neutral	neutral	**about 7,5**
Optimal temperature	30-32°	30°C (22°C *for Tribolium*)	37°C	**about 24°C**
Km for UDP-GlcNAc	1-3 mM	82 µM - 33,7 mM	?	**about 0,23 mM**
Stimulation				
GlNAc	3-10 mM	17 mM	No	**10 mM**
$(GlcNAc)_{2,3}$	?	?	?	**10 mM**
Mg^{2+}	1-5 mM	10-30 mM	30 mM	**No**
Proteolyse (Trypsin)	Yes	Slight	No	**No**
Inhibition				
Polyoxin D	Yes	Yes	Yes	**Yes**

synthases (Cohen, 1987). *Riftia* tissues extracts do not react to proteolytic treatment as reported for *Artemia salina* chitin synthase (Horst, 1981 and Horst et al., 1993). The enzymatic activity was almost totally abolished in the presence of 10 $\mu g\ ml^{-1}$ of Polyoxin D. Approximatively a double amount is needed to obtain 73% of inhibition for *A. salina* chitin synthase (Horst, 1981 and Horst et al., 1993). The enzymatic activity is not Mg^{2+}-enhanced, while such enhancement generally occur for most chitin synthases (Cabib, 1987; Cohen, 1987; Horst, 1981 and Horst et al., 1993). Free GlcNAc stimulates chitin synthases from several fungi and insects (Cabib, 1987 and Cohen, 1987), whereas no effect was reported in the case of *Artemia salina* (Horst, 1981 and Horst et al., 1993). Enhancement of *Riftia* tissues extracts activity was recorded in the presence of 10 mM GlcNAc. The enzymatic activity was also significantly enhanced in the presence of 10 mM of chitobiose and chitotriose. These compounds could act as primers for the enzyme, and this suggests that the enzyme doesn't carry its own primer as it was previously reported for *Artemia salina* and *Saccharomyces cerevisiae* (Horst et al., 1993).

It has been shown that the chitin synthase activity in *Riftia* is sensitive to polyoxin D as it is known for all chitin synthases, and that it shares other characteristics with some chitin synthases (insensitivity to proteolysis as in *A. salina*, and GlcNAc-stimulation as in fungi and insects). What is new compared to the available data on chitin synthases from various organisms is the Mg^{2+} needlessness and the chitobiose and chitotriose stimulation.

Some preliminary studies suggested that the reaction products were sensitive to chitinase (results not shown). The chain length of these products is greater than the one of chitobiose (comparison of migration on Thin Layer Chromatography). An increase of enzymatic activity, in order to get enough material for chemical analysis, is necessary before further characterization of these products.

Tissues from repressurized animals do not exhibit a different in vitro activity compared to tissues from animals frozen directly after sampling. Therefore, the enhanced secretion observed in pressurized aquaria is not reflected by an increase of the enzyme quantity or activation of the enzyme. The origin of this enhancement requires further investigation. Anyway, even if the increase of chitin secretion in pressure vessels does not seem to change the behaviour of the enzyme in vitro (in our experimental conditions), it is of great interest to study tissues of repressurized worms which are in good physiological conditions and in secretion state.

In conclusion, *Riftia* chitin synthase seems to be quite different from other chitin synthases concerning some of the tested parameters, especially its high affinity for its substrate, as reflected by the low Km. This could play a part in the high recorded rate of chitin synthesis, and may partly explain how *Riftia* can produce such great quantities of tube material.

III. Chitinolytic activity

A chitinolytic activity was detected in *Riftia* tissues extracts. Optimal activity was obtained at pH 7.5 in *Riftia* saline buffer, whose composition is close to that of seawater. This pH value is higher than the value previously reported for marine invertebrates chitinases, i.e. about 5 (Flach et al., 1992).

Opisthosome extracts activity represents 40% of 1 $mg\ ml^{-1}$ *Streptomyces griseus* chitinase activity. Considering that we compare activity from a tissue extract and a purified enzyme, opisthosome activity was probably underestimated. It can therefore be assumed that opisthosome chitinolytic activity is important. A model of *Riftia* growth process has been postulated which assumes that the tube grows at both ends (Gaill et al., 1997). This involves a tube dissolution process by the opisthosome, to allow the synthesis of a new tube base. In fact, it implies that opisthosome could secrete proteases and chitinase. As chitin is associated with proteins in the tube, degradation of the tube probably involves both chitinolytic and protease activity. Thus, tube proteins and *Riftia* proteases have to be studied before further investigations on the tube growth process are undertaken.

Results obtained with the plume were unexpected. As it is a non chitin-secreting tissue (Shillito et al., 1993), it was considered as a control for our studies. But surprisingly it exhibits a chitinolytic activity which could represent 15 to 120% of *S. griseus* chitinase activity. The nature of the enzyme responsible for the chitinolytic activity detected in the opisthosome and in the plume remains to be demonstrated. It is possible that this chitinolytic activity is due to a lysozyme, as lysozymes are known to degrade chitin (Jollès & Jollès, 1984). Furthermore, Boetius & Felbeck (1995) demonstrated the occurrence of lysozyme activity in all *Riftia*'s tissues (except the opisthosome which was not tested). Whether the enzyme is a chitinase or a lysozyme, it exhibits a chitinolytic activity that could be involved in tube growth and tube shape modifications.

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