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Chitovibrin: a chitin-binding lectin from *Vibrio parahemolyticus*

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A novel 134 kDa, calcium-independent chitin-binding lectin, 'chitovibrin', is secreted by the marine bacterium *Vibrio parahemolyticus*, inducible with chitin or chitin-oligomers. Chitovibrin shows no apparent enzymatic activity but exhibits a strong affinity for chitin and chito-oligomers >dp9. The protein has an isoelectric pH of 3.6, shows thermal tolerance, binds chitin with an optimum at pH 6 and is active in 0–4 M NaCl. Chitovibrin appears to be completely different from other reported Vibrio lectins and may function to bind *V. parahemolyticus* to chitin substrates, or to capture or sequester chito-oligomers. It may be a member of a large group of recently described proteins in Vibrios related to a complex chitinoclastic (chitinivorous) system.

Keywords: chitin-binding lectin; 'chitovibrin'; *Vibrio parahemolyticus*.

Abbreviations: $(GlcNAc)_2$, *N,N'*-diacetylchitobiose; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PTS, phosphotransferase system.

Introduction

Marine vibrios produce a large number of proteins related to chitin degradation and assimilation including cell surface Ca^{2+}-dependent lectins [1, 2], *endo*- and *exo*-chitinases [3, 4], *endo*-chitodextrinases [5] *exo-β-N*-acetylglucosaminidases (*N,N'*-diacetylchitobiose hydrolases) [6–9], permeases [5] and chemotactic proteins [10]. Normally found in environments rich in chitin originating from plankton, crustaceans, insects or fungi, these bacteria are chitinoclastic (chitinivorous), and have been reported to utilize *N*-acetylglucosamine as efficiently as *E. coli* metabolizes glucose [5]. After approaching a chitin nutrient source, it has been proposed [1, 2] that a cell surface calcium-dependent lectin temporarily attaches the Vibrio to chitin keeping the organism in the vicinity, while a secreted chitinase produces chitodextrins [$(GlcNAc)_n$] from chitin that diffuse into the periplasmic space where the chitodextrinase degrades the oligomers to dimers [5]. Either an *N,N'*-diacetylchitobiose permease transports this disaccharide to the cytoplasm for hydrolysis by a separate cytoplasmic *exo-β-N*-acetylglucosaminidase (*N,N'*-diacetylchitobiose) followed by phosphorylation [5], or, alternatively, in the periplasm an *endo*-chitodextrinase and a periplasmic *exo-β-N*-acetylglucosaminidase splits *N,N'*-diacetylchitobiose to *N*-acetylglucosamine [5, 11–13] which is a phosphotransferase system (PTS) sugar [5].

We report here a novel, soluble *Vibrio parahemolyticus* chitin-binding protein of unknown function, inducible and copiously secreted in similar amounts and in concert with endo-chitinase. This lectin appears to be completely different from the calcium-dependent mannose-favouring cell surface lectin described by Yu *et al.* [1, 2] in *V. parahemolyticus* or another GlcNAc-specific lectin studied in *V. furnissii* [1, 2]. This new lectin is soluble, secreted and does not bind small chitin oligomers, all of which are properties different from the *V. furnissii* lectin. Yu *et al.* [1, 2] reported on an adhesion/de-adhesion apparstus in *V. furnissii* where a proposed calcium-dependent, pili-associated lectin with a broad sugar specificity mediates the binding of the bacteria to glycosides of *N*-acetylglucosamine, glucose or mannose linked to agarose beads. That protein has not been isolated or characterized; however, its properties, as described, differ considerably from the lectin in this report.

Lectins are variously defined as carbohydrate-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates [14] or simply as carbohydrate-binding proteins of non-immune origin [15]. They have been isolated from nearly every class of organism including microbes [16], invertebrates and vertebrates [17, 18], and plants [19]. Often, lectins are glycosylated and composed of homo- or heterodimers with

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one binding site per subunit [18]. An unusual chitin-binding lectin from *Urtica dioica* (stinging nettle) reportedly consists of one polypeptide chain [20, 21]. It is expressed in this organism simultaneously with a chitinase [22].

Very few non-catalytic chitin-binding lectins have been isolated from microorganisms. *Chlamydia trachomatis*, *Bordetella bronchiseptica*, *Pasteurella multocida* and *Streptococcus salivarius* have been shown to express specificity of binding to GlcNAc [16]. The association of these activities with a particular cellular structure has not been established. A number of plant lectins are specific for GlcNAc such as wheat germ agglutinin (WGA) and tomato lectin, although they bind other structures besides chitin. A number of plant chitin-binding proteins have been described, some with insecticidal and fungicidal properties [23–27].

We report isolation and partial characterization of a novel, calcium independent chitin-binding lectin 'chitovibrin' from *V. parahemolyticus*, which is secreted into the growth media, inducible upon addition of chitin, chitin oligomers or cellobiose.

Materials

Organisms

Wild-type *V. parahemolyticus* isolates (ATCC 27969 and similar strains*) were grown on 804 medium (0.75 g l^{-1} KCl, 6.9 g l^{-1} $MgSO_4$, 23.4 g l^{-1} NaCl, 1 g l^{-1} tryptone, 1 g l^{-1} yeast extract) and 0.05% (w/v) swollen commercial chitin. Cells were harvested after chitin was hydrolysed. Isolates were preserved at −70 °C in 804 medium with 30% glycerol.

Chemicals and reagents

Protein assay reagents, ion-exchange and gel filtration resins were from Bio-Rad (Richmond, CA). Chitin, chitosan, buffers, electrophoresis standards, dipalmitoylphospatidylethanolamine, and p-nitrophenyl butyrate were from Sigma (St Louis, MO). Acrylamide: bisacrylamide solution (37.5:1) was from Amresco (Solon, OH). Tritiated acetic anhydride and $Na^{125}I$ were from ICN Biomedicals (Costa Mesa, CA). Iodo-beads used in protein radioiodination were from Pierce (Rockford, IL). Thin-layer chromatography silica gel G plates were from Analtech (Newark, DE). Sepharose 4B, 6B and Sephadex G-75 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Materials

Pretreatment of chitin

Swollen chitin was prepared from crab chitin (Sigma, practical grade) with phosphoric acid, following published procedures [11].

Tritium-labelled chitin

Tritiated chitin was prepared by *N*-acetylation of chitosan with [^{3}H]-acetic anhydride [28]. Before acetylation, commercial chitosan usually had to be further deacetylated in order to make it more reactive [29]. Specific activity was 0.5 μCi mg^{-1} chitin, corresponding roughly to 0.1 μCi μmol^{-1} GlcNAc.

Chitin oligomers

Chito-oligomers were obtained by hydrolysis of purified, commercial chitin with HF or HCl. Chito-tetramer, -pentamer and -hexamer were purchased from Seikagaku (Ft Lauderdale, FL). Larger chitin oligomers were prepared by hydrolysis of chitin, either with HF [30] or HCl [31]. The latter method produced partial de-*N*-acetylation of the resulting oligomers. Contaminating amino acids and salts are removed during the first two steps of elution from activated charcoal, which is used to trap the oligomers after dilution of the hydrolysate. Chitin oligomer preparations were fractionated on a Bio-Gel P-6DG desalting gel column (3.5 × 83 cm) and eluted with 50 mM ammonium acetate to minimize clustering of oligomers, under low ionic strength conditions [30]. A finer purification of the oligomers was accomplished by repeated chromatography on a Bio-Gel P-4 gel column (1 × 113 cm).

Immobilization of chitin oligomers

After the Bio-Gel P-6DG purification step, chitin hydrolysate fractions containing mixtures of higher oligomers were pooled and bound to aminobutylamino-agarose by reductive amination. Twenty mg of high molecular weight Chito-oligomers (MW ranging from 1000 to 3000) were resuspended in 0.2 M borate buffer, pH 9.5 and incubated for 42 h at 50 °C with 6 ml of aminobutylamino-agarose gel and 25 mg cyanoborohydride. After acidification with 0.5 M HCl and drying under a vented hood, boric acid was removed by co-evaporation with methanol, and the gel was washed extensively with 10 mM phosphate buffer, pH 6.

Chitinase activity

Chitinase activity was measured as described by Molano *et al.* [32] and Cabib [28], with minor modifications. One hundred and ninety μl of tritiated chitin (adjusted to 90 000 cpm μmol^{-1} GlcNAc) was incubated at 45 °C with 10 μl of enzyme preparation for 10 min. The reation was stopped by the addition of 200 μl 20% trichloroacetic acid, centrifuged for 2 min, and 100 μl of the clear supernatant fraction was radioassayed.

Antibodies

Contaminating 95 kDa *V. parahemolyticus* chitinase was removed from purified chitovibrin concentrates on Sepharose-chitinase antibody affinity chromatography. Antichitinase polyclonal antisera were obtained from rabbits

inoculated with cloned chitinase to avoid any chitovibrin-specific antibodies. Antisera were purified as described by Chua *et al.* [33] and by Maurer and Callahan [34]. Antichitinase antibody purification was accomplished on immobilized cloned chitinase: 5 mg of cloned chitinase dissolved in 20 ml of 0.1 M sodium bicarbonate was attached to 25 ml of wet Sepharose 4B by cyanogen bromide activation, packed in a column (1.5 × 3.5 cm), washed and used to affinity-purify and anti-chitinase IgG.

Immobilization of anti-chitinase antibodies

A 22 ml preparation of affinity-purified anti-chitinase antibodies (0.439 mg ml^{-1}) was immobilized on 50 ml of wet Sepharose 4B activated with 1.4 mg of cyanogen bromide [35], and 100 ml of 1 M glycine was used to block the remaining active sites; the gel was washed with 10 mM phosphate buffer, pH 6. Protein content of the original preparation and solutions after binding showed nearly quantitative immobilization of antibodies on Sepharose.

Preparation of neoglycolipids

The procedure described by Stoll *et al.* [36], was used with modifications due to low solubility of higher chitin oligomers in non-aqueous solvents. Five μmol of lyophilized *N*-acetyl glucosamine oligomer were dissolved at 65 °C in 0.5 ml of 5% lithium chloride in *N,N'*-dimethylacetamide for 12 h (DMAc) [37]. The oligomer solution was mixed with 5.24 ml of a 5 mg ml^{-1} solution of dipalmitoylphosphatidylethanolamine in chloroform:methanol (1:1), sonicated for 10 min and incubated for 2 h at 65 °C. After adding sodium cyanoborohydride (1.2 mg) in methanol (120 μl), the mixture was incubated at 65 °C for 16 h. Excess cyanoborohydride was destroyed with 0.5 M HCl, the sample was neutralized with ammonium bicarbonate and boric acid was co-evaporated with methanol under nitrogen. The dried product was redissolved in chloroform:methanol:H_2O (15:70:30) (Solvent A) and applied to a disposable silica-C18 column washed with the same solvent and eluted with chloroform:methanol:H_2O (60:35:8) (Solvent B), according to Lawson *et al.* [38]. Binding activity was tested with radiolabelled wheat germ agglutinin (WGA) in a 96-well plate. Yield of neoglycolipids was determined with p-dimethylamino-benzaldehyde (DMAB), as described by Reissig *et al.* [39], with modifications for hexosamines: 100 μl aliquots of the samples were dried at room temperature and incubated at 37 °C for 4 h with 100 μl of a chitobiase solution. After adding 20 μl of 0.8 M potassium tetraborate, pH 9.5, samples were boiled for 3 min, cooled and 750 μl of DMAB reagent was added. After incubation at 37 °CC for 30 min, absorbance at 585 nm was measured.

Ninety-six-well plate binding assay

Neoglycolipid solutions were air-dried in triplicate in 96-well plates, and incubated for 2 h at 25 °C on a shaker after addition of 300 μl per well of 1.5% BSA blocking solution. The wells were washed with dH_2O and incubated for 2 h with 60000 to 100000 cpm of ^{125}I-chitovibrin in 300 μl 1.5% BSA solution. After removing the labelled solution, wells were washed for 20 min with 250 μl PBS, rinsed with dH_2O and 200 μl of 6 M guanidine-HCl were added to each well and incubated for 10 min, removed and radioassayed.

pH-dependent binding assays

One hundred μl of an aqueous 2 g l^{-1} suspension of regenerated chitin was centrifuged for 2 min in a minifuge, the supernatant fraction was removed, 50 μl of buffer at the desired pH was added and the suspension was mixed by vortexing. Radiolabelled chitovibrin was added, the sample was vortexed and incubated at 4 °C for 30 min. After centrifuging, the pellet was rinsed with water and boiled with loading buffer + 2-mercaptoethanol, and electrophoresed. The gel was stained, destained and dried. After autoradiography, relative binding was assessed by densitometry.

Western-blot analysis

Proteins were transferred on to polyvinylidene fluoride membranes as described by Matsudaira [40]. Proteins were electrophoresed on 6% SDS polyacrylamide gel after a 20 min prerun with sodium thioglycolate. The transfer was accomplished at 40 V and 0.68 Ampere-hour. Membranes were incubated with diluted (1:100) antiserum for 90 min, treated wuth radiolabelled protein A [41], and allowed to expose X-ray film at −70 °C for 3–18 h.

Column chromatography

Chitin affinity chromatography

To test for chitin binding, protein preparations were passed through regenerated chitin, prepared as described above.

Gel permeation chromatography. Size-exclusion columns were eluted with 10 mM potassium phosphate buffer, pH 6. A rough separation of chitin oligosaccharides was performed on P-6DG desalting-gel (4.5 × 42 cm). Further purification was accomplished on Bio-Gel P-4 (0.8 × 85 or 113 cm).

Hydrophobic interaction chromatography. 3 ml of Phenyl-Sepharose-CL 4B was packed into a column (0.8 × 6 cm). Sodium chloride concentration was brought to 4 M before samples were loaded on to the columns, which was equilibrated with 4 M-salt buffer. Samples were eluted with gradients of decreasing salt concentration [42].

Radiolabelling of proteins

Chitovibrin: 100 μl of 0.18 mg ml^{-1} was incubated for 5 min with 1 mCi of Na^{125}I and 2 Iodo-beads in 100 μl buffer, pH 7.5 (10 mM Tris-HCl). A Pharmacia PD-10 column, Sephadex G-25 M, bed height 5 cm, was used collecting 0.6 ml fractions. Three μl samples were analysed for ^{125}I and specfic activity was 3.2×10^6 cpm per μg protein.

Isoelectric focusing

IEF gels were prepared with 2.8 g sucrose, 3.1 ml 40% acrylamide/bis (37.58 l), 2 ml of ampholines (pH 3–7), 80 μl ammonium persulphate and H_2O to 25 ml. After electrophoresis, the gel was washed to remove ampholines, and stained [43].

Induction of chitinase and chitovibrin

Cultures (35 ml) were grown in 125 ml Erylenmeyer flasks with 804 M or M9CA (minimal) medium supplemented with sugars at 2.5 mg ml^{-1}. After 15 h, supernatant fractions were analysed for chitinase after precipitation with 50% ammonium sulphate, and dialysis. Proteins were separated on 6% SDS-PAGE to detect chitovibrin.

General methods

Protein content: method of Bradford [44], using 1 ml of a 5 × diluted Bio-Rad protein assay reagent and 100 μl of sample. The SDS-PAGE experiments (usually 7.5% or 6%) were according to Laemmli [43]. Gels were stained for 30–60 min with 0.1% Coomassie Blue R in 50% methanol: H_2O. Destaining used several changes of methanol:acetic acid:H_2O (2:1:7).

Results

When *V. parahemolyticus* is grown in 804 medium supplemented with chitin, 95 kDa and 134 kDa polypeptides appear in the medium 36 h after inoculation, as analysed on SDS-polyacrylamide gel electrophoresis (Fig. 1A) concomitant with chitinase activity as shown in Fig. 1B. These proteins could be purified by chitin affinity chromatography as shown in Fig. 2. The presence of calcium was not necessary for binding. Induction of these proteins by various saccharides was tested on 15 h *V. parahemolyticus* cultures grown on media supplemented with 1.25 mg ml^{-1} of various saccharides, as described in Methods. (Inducibility with oligosaccharides was much more rapid than with chitin.) The protein and activity profiles are shown in Fig. 1B. Among a large number of saccharides tested in place of chitin, only cellobiose also induced expression of chitinase and chitovibrin, although neither protein showed affinity for cellulose (data not shown).

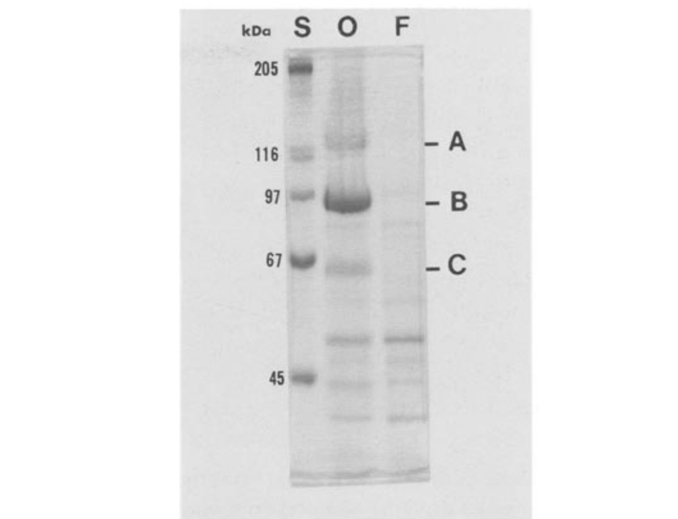
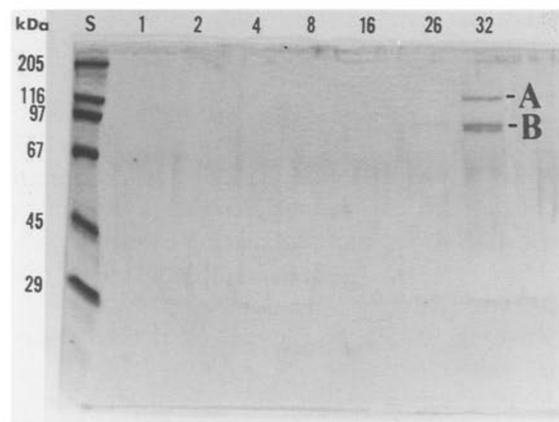
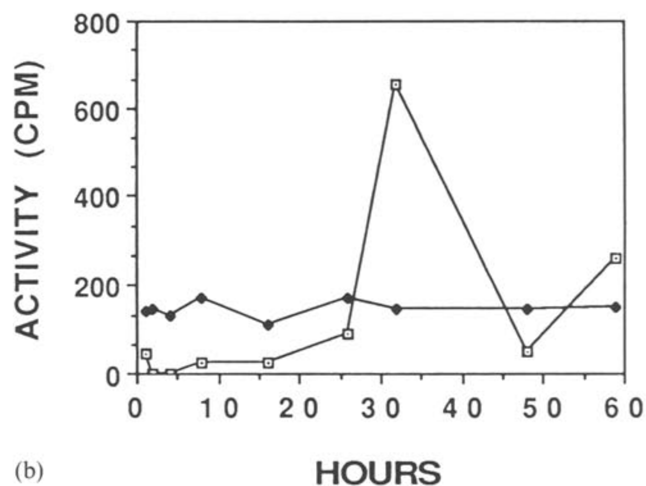


Figure 2. Chitin affinity. A 2 ml (3 mg) sample of crude extract was passed through a 2 ml (wet volume) swollen chitin column. Proteins from original (O) and flow through (F) fractions were analysed on 6% SDS-PAGE. A, chitovibrin; B, 95 K chitinase; C, 65 K chitinase fragment; S, high MW standards.





(b)

Figure 1. (a) Chitin inducibility of chitovibrin and chitinase. Time-course of secreted proteins, supplemented with chitin. Proteins were analysed on 7.5% SDS-PAGE, using 100 μl supernatant fraction. Numbers on top of figures correspond to hours of incubation. S, high MW standards; A, chitovibrin; B, chitinase. (b) Chitin inducibility. Chitinase activity of fractions at different sampling times for cultures with chitin (□—□—□—□—) or no chitin (—◆—◆—◆—◆—) added. Activity, expressed in cpm was determined for 100 μl samples (see Methods).

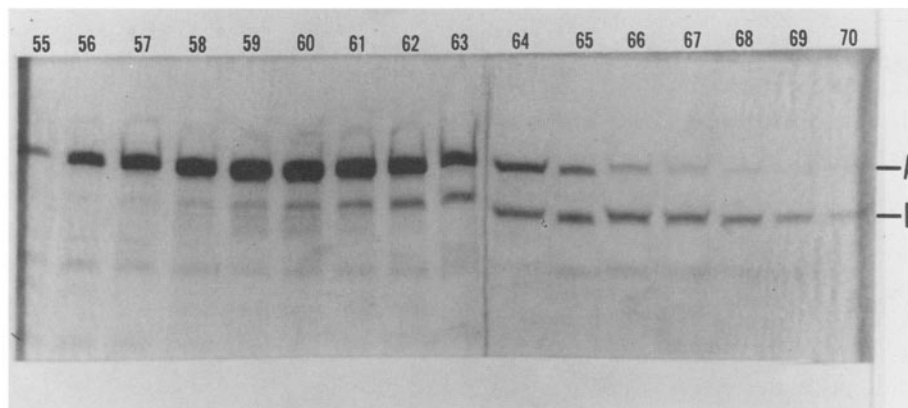


Figure 3. P-200 gel permeation chromatography. Bio-Gel P-200 column was pre-equilibrated with 50 nm potassium phosphate buffer, pH 6.0. The elution of proteins was observed on 7.5% SDS-polyacrylamide gel electrophoresis and absorbance at 280 nm. A, chitovibrin; B, 95 K chitinase.

Gel filtration; Bio-Gel P-200

The 95 kDa chitinase and the 134 kDa chitovibrin were difficult to resolve by gel permeation. Separation of the proteins could be partially accomplished on Bio-Gel P-200 (1.5 × 110 cm) eluted with 50 mM potassium phosphate buffer, pH 6, collecting 2.8 ml fractions as shown in Fig. 3. Both proteins eluted from the column with larger apparent sizes than values obtained through gel electrophoresis, but not high enough to implicate multimers at low salt concentration. Elution of the proteins with 0.3% octyl β-glucoside gave somewhat better separation indicating possible hydrophobic interaction between the proteins. Chitovibrin stained with Coomassie blue as a 134 kDa protein band on SDS polyacrylamide gel (Fig. 4).

Immunoaffinity chromatography

To remove traces of chitinase activity from chitovibrin, gel filtration purified samples were chromatographed on immobilized, affinity-purified anti-chitinase antibodies. Polyclonal antibodies (rabbit) against the cloned chitinase were found specific for chitinase by blots on PAGE containing both chitinase and chitovibrin (data not shown). Most of the chitinase in the original preparation was removed with Sepharose-antichitinase. Proteins were bound to the affinity gel and released with 6 M guanidine-hydrochloride. A 65 K proteolytic *N*-terminal chitinase fragment with poor binding to polyclonal antichitinase was observed (data not shown). Ouchterlony double-diffusion gels supported the notion that chitinase and chitivobrin are antigenically different (data not shown). At this point of purification, chitovibrin was electrophoretically homogeneous (Fig. 4). Overall yields for chitovibrin were <10% due to difficulty of separation from chitinase.

Affinity to chitin and chitin-oligomers

A 1 ml column of regenerated chitin was used to chromatograph a chitovibrin-enriched sample that had been

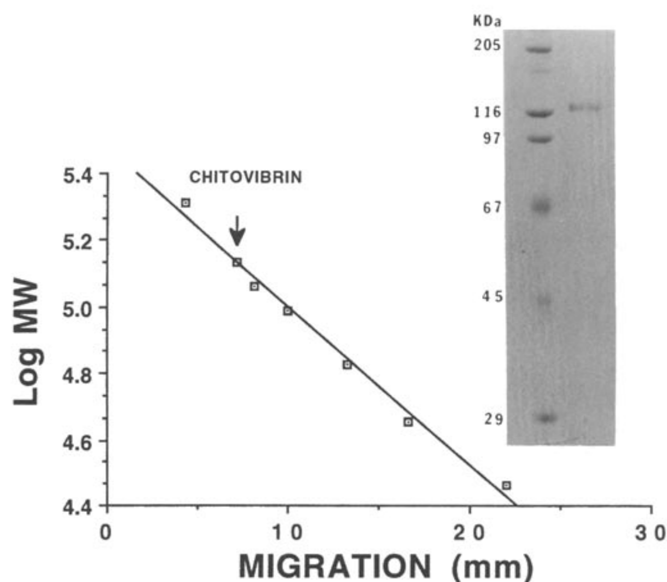
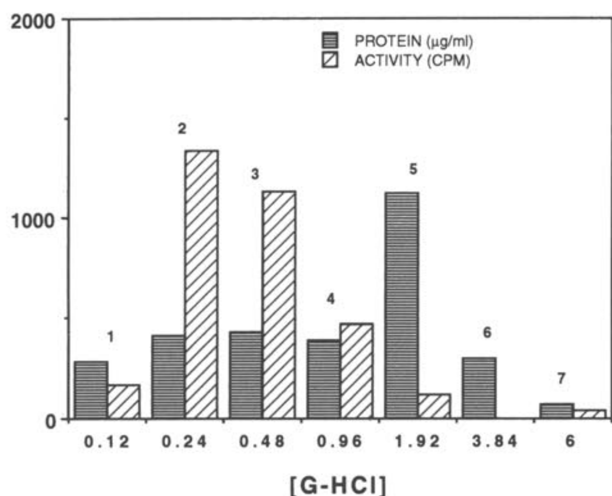


Figure 4. Molecular mass of electrophoretically pure chitovibrin. Relative mobility on SDS-PAGE is shown plotted against molecular weight standards.

radioabelled with ^{125}I. The autoradiogram showed binding of chitovibrin, which could be eluted with concentrated chitin oligosaccharides at approximately 30 mg ml^{-1} (about 20–30 mM) (data not shown). Calcium was not required to effect binding in any of the chitin-binding assays (data not shown).

Chitovibrin binding to chitin was further analysed by eluting with a step gradient of guanidine-HCl. A 10 ml sample of chitinase/chitovibrin was loaded on a 3 ml column of regenerated chitin which was washed with 8 ml of PBS and eluted with 1 ml batches ranging from 0.12 to 6 M guanidine-hydrochloride. Fractions were dialysed against H_2O. Proteins analysed on 6% SDS-PAGE, shown in Fig. 5B, indicate that chitinase is released at lower concentrations of guanidine-hydrochloride than chitovibrin.



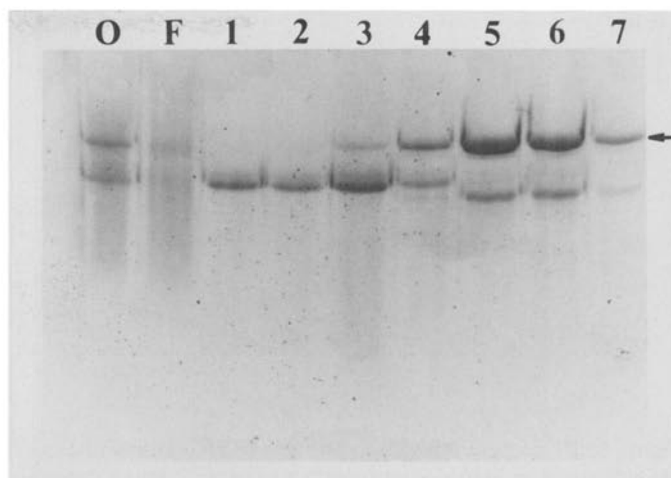


Figure 5. (A) Elution of chitovibrin from chitin with guanidine-hydrochloride. Protein content and chitinase activity of fractions eluted from chitin column with batch-wise gradient of guanidine-hydrochloride. (B) SDS-PAGE of fractions from guanidine-HCl elution. The arrow points to the chitovibrin protein band on the 6% SDS-polyacrylamide gel. O, original sample; F, flow through.

An 80–85 kDa protein, not seen either in the original sample, or in affinity experiments with pure chitinase, coelutes with chitovibrin and may be an equally strong binding proteolytic breakdown product of this protein (unconfirmed). Bound chitovibrin could not be released from chitin with 4 M NaCl or by changing pH from 4 to 10 (data not shown).

Sepharose-chitooligomer affinity gel (1 ml) was prepared with chitin mixed oligomers, dp5–20. ^{125}I-labelled chitovibrin was loaded on to the column, washed with a low-salt buffer, and eluted stepwise with 2 N NaCl and 6 M guanidine-hydrochloride. Fractions, analysed by autoradiography indicated a strong, salt-resistant binding of chitovibrin to chitodextrins (data not shown). Rechromatography showed that chitodextrin binding capacity of chitovibrin, lost by denaturation in 6 M guanidine-hydrochloride, could be recovered after dialysis (data not shown).

Binding to chitodextrin neoglycolipids

The binding affinity of chitovibrin to chitodextrins according to degree of polymerization was examined using synthetic chitin oligomer neoglycolipids. Binding of radiolabelled chitovibrin was examined at pH 8.8 on 96-well plates, as described in Methods. Each well in triplicate was coated with chitodextrin neoglycolipids ranging from dp3 to dp12. A chitovibrin sample from fraction 60 (Fig. 3) was radiolabelled in the presence of chitin oligomers to protect the binding site, and 50 µl of the labelled protein (100 cpm) were used for the binding in each well. A neoglycolipid prepared with lactose was used as an irrelevant control (no binding, not included in Fig. 3). Chitovibrin appeared to bind most avidly to chitodextrin neoglycolipids with dp > 10, or intact oligomers of dp > 9 (the reducing terminal is linearized by the reductive amination). Significant binding occurred with dp8 and dp9 neoglycolipids and dp4 gave increased binding

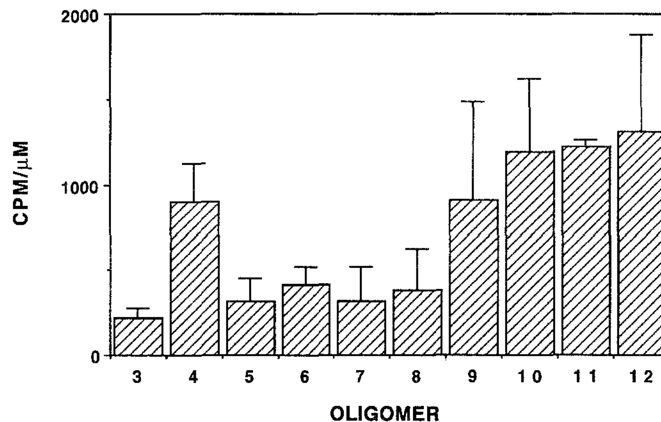


Figure 6. Binding of chitovibrin to neoglycolipids. Samples containing chitovibrin were incubated in 96-well plates coated with chitovibrin neoglycolipids of dp3–12, using lactose-neoglycolipid as control. See Methods for details.

above background. While we do not understand the dp4 binding, we may speculate that 2–3 dp4 units from the multimeric neoglycolipid surface fit synergistically into the binding site. Cellulose oligomers were not prepared because chitovibrin showed no binding to cellulose (data not shown). β-mannans were not tested. The results in Fig. 6 are expressed in cpm μM^{-1}.

pH optimum and pI

Binding of chitovibrin to chitin was examined at pH 3.6 to 10.8 with radiolabelled, purified chitovibrin as described in Methods (Fig. 7).

Isoelectric pH of chitovibrin

Figure 8 indicates that chitovibrin as a pI near 3.6.

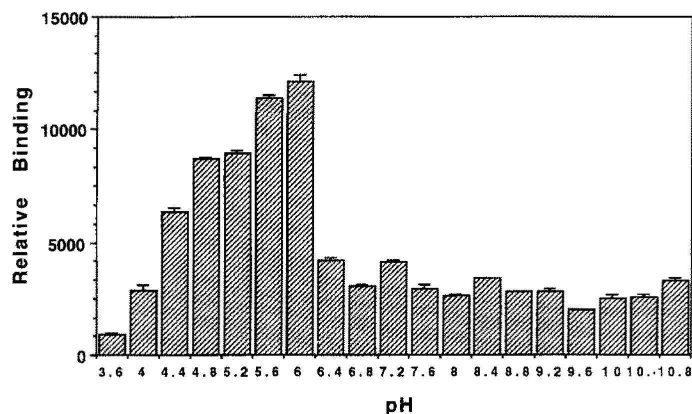


Figure 7. pH and chitin-binding. Binding of chitovibrin to chitin was examined at pH ranging from 3.6 to 10.8, using autoradiography of SDS-PAGE gels and densitometry as assay.

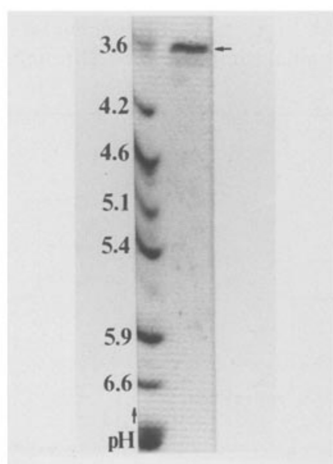


Figure 8. Isoelectric pH. The pI value of chitovibrin was examined on a flat-bed isoelectric focusing assay. The arrow indicates location of chitovibrin.

Other purification schemes

Neither ion-exchange nor phenyl-Sepharose chromatography were useful to separate chitinase and chitovibrin (not shown).

N-terminal sequence

The sequence AVDAAPLEVYDSNKVYNGGDQVQHE was determined after transfer on to polyvinylidene fluoride membranes [40]. The N-terminal 25 amino acids were compared with sequences in the Genebank. The 25aa segment showed up to 73% homology with internal sequences from tubulin from several organisms, and of more interest, high homology with internal sequences from (1) a sucrose-6-phosphate hydrolase (sucrase from *V. alginolyticus*; Poisson probability $p = 0.99$; (2) a pilus biogenesis protein from *V. cholera*; $p = 0.99$; (3) a chitinase from an Alteromonas species; $p = 0.74$ and lower homologies with other proteins. No homology was apparent with the already determined complete sequence of the *V. parahemolyticus* soluble endo-chitinase (to be published elsewhere), and tryptic peptide maps (HPLC) of the two proteins showed no similarity (data not shown).

Discussion

A novel 134 kDa lectin 'chitovibrin' from *V. parahemolyticus*, behaving as a monomeric protein in gel filtration, appears to be secreted simultaneously with a chitinase, after induction by GlcNAc, chito-oligomers or chitin. Chitovibrin, with a pI of 3.6 exhibits a strong and specific affinity to chitin and chitodextrins >dp9 over a broad range of pH, temperature and salt concentrations and independent of calcium. Because of its inducibility with chitin fragments, this protein is probably not responsible for initial binding of the organism to chitin, unless expressed in a different form attached to the cell surface. The N-terminal sequence of the secreted protein begins with alanine, an indication that a signal sequence cleavage has occurred, consistent with its secreted nature, and arguing against simple release from dying cells. The *V. parahemolyticus* and other *Vibrio* cell surface lectins reviewed and examined by Yu *et al.* [2] are all as yet uncharacterized calcium-dependent cell surface carbohydrate binding molecules which may be responsible for the initial binding of chitin substrates. It is not clear yet why this Vibrio makes another chitin-binding protein with a specificity for >dp9, which it secretes along with chitinase. Lerner and Raikhel [22] reported a chitinase and a chitin-binding lectin being expressed simultaneously in a plant, the stinging nettle *Urtica dioica.* A number of plants appear to produce chitin-binding lectins in addition to chitinases, and these have been shown to be insecticidal and fungicidal or fungistatic [26, 27].

Chitovibrin could be separated from the 95 K form of secreted *V. parahemolyticus* chitinase through: (1) selective elution from chitin matrix columns; (2) gel permeation; or (3) immunoaffinity chromatography. Western-blot results (data not shown) indicate that chitovibrin is not a pre- or pro-protein precursor of chitinase, since it is not recognized by polyclonal rabbit anti-chitinase antisera. The lack of similarity of HPLC tryptic peptide maps and lack of N-terminal sequence homology substantiated this notion. No precipitin line was observed when chitovibrin was tested against antichitinase on Ouchterlony double-diffusion (not shown).

Neoglycolipids have been successfully used to detect and analyse carbohydrate-binding activities [36]. Construction of neoglycolipids of chitin oligosaccharides with $dp = 4$ was reported by Lawson *et al.* [38]. By synthesis of a number of novel higher chitodextrin neoglycolipids, we found that the minimum effective binding site of chitovibrin may be as large as 9 sugars. Neoglycolipids made from the decamer showed significantly higher binding than smaller sizes, although 4, 8 and 9-mers gave an increase over background. Radiolabelling chitovibrin with ^{125}I appears to reduce its

binding capacity, consistent wih tyrosine being important in the binding site. Lucas *et al.* [45] observed that the amino-terminal region of chitinase from bean leaves bears resemblance to the carbohydrate binding domain of wheat germ agglutinin which includes a conserved tyrosine.

In one view, the bacterial cell surface is a mass of negatively charged tangled fibres of polysaccharides that enables it to adhere to desirable surfaces [46]. Binding relies heavily on divalent cations and pili lectins which form a bridge-like structure. Chitovibrin might function to form a network, binding both bacterial carbohydrate and chitinase, confining the hydrolytic enzymes and released nutrients near the cells.

Yu *et al.* [1] proposed that an unusual lectin with broad sugar specificity produced by *V. furnissii* was part of a cellular adhesion-deadhesion apparatus. Metabolic links allowed the system to monitor the surrounding environment, by comprising a nutrient sensorium. Chitovibrin may function similarly in *V. parahemolyticus* specific for chitin.

For some bacterial cellulases, various components are found in tightly associated multimolecular complexes [47, 48]. The cellulase complex of *Clostridium thermocellum* has at least 14–18 different polypeptides forming a very stable extracellular structure termed cellulosome [49]. At least eight of the cellulosome components exhibit cellulase activity while a non-catalytic binding factor (lectin?), termed S1 by Lamed *et al.* [49], seems to mediate the high affinity of the cellulosome for cellulose. While no 'chitosome' complex has yet been described in Vibrios or other organisms, the number of proteins involved in chitinoclastic behaviour is increasing with further research, and chitovibrin is a secreted chitin-binding lectin which shows some hydrophobic affinity for its brother protein chitinase especially in high salt characteristic of a marine environment. While we did not detect other inducible proteins secreted by *V. parahemolyticus* in as large amounts as chitovibrin and chitinase, a chitin degrading complex is not beyond the realm of possibility.

Cellulose, chitin and other polysaccharide present a common problem to bacteria who have no mechanism for endocytosis. Once found, the cell needs to remain near its substrate for purposes of diffusion and must secrete hydrolytic enzymes to degrade the polysaccharide to diffusable oligomers, which are eventually broken down, perhaps in the periplasmic space, to transportable di- or monosaccharides. We now speculate that chitin binding proteins and chitinases of Vibrios somehow comprise a complex which participates in the first two tasks. An additional function for chitin-binding lectins besides cell-surface binding might be to help keep the chitinase in proximity of chitin.

We have also isolated and cloned a soluble and entirely cytoplasmic chitobiase from *V. parahemolyticus* [51], which may be responsible for the cytoplasmic enzyme activity described by Bassler *et al.*[5], in *V. furnissii.* In an alternate pathway, $(GlcNAc)_2$ is transported into *V. parahemolyticus*, via a permease before being cleaved to GlcNAc. Soto-Gill *et al.* [9] have cloned a membrane-bound chitobiase which is apparently transported to the outer membrane of *V. harvei*. The membrane-bound version of *N,N'*-diacetylchitobiase found by Bassler *et al.* [5] in *V. furnissii*, showed very different substrate specificity than the cytoplasmic version. In this alternate pathway some Vibrios degrade oligomers to GlcNAc outside the plasma membrane before using the PTS system to transport and phosphorylate GlcNAc. Roseman and his colleagues have also isolated and cloned a chitodextrinase which is located in the periplasmic space [5]. Chemotactic sensors perform the earliest functions in the complex chitinoclastic system [52], followed by induction of secreted chitinases, and perhaps chitin-binding proteins. The periplasmic proteins perform mid-term functions of the chitin nutrient assimilation system of Vibrios, probably followed either by a PTS transport system or by a permease and hexokinase where phosphorylation locks the monomer to the cytoplasmic space. One curious result of this report is that the chitinase and chitovibrin seem to be most efficiently produced and secreted in mid-late stationary phase. Could the Vibrios be desperate to find another source of chitin? To help identify the function of the new chitin binding lectin described here, it will be interesting in future work to study the interaction of vibrios (and their chitinases) with chitin in mutants lacking either or both cell surface type [2] and chitivobrin-type lectins.

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