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N-Acetylglucosaminidase (chitobiase) from *Serratia marcescens*: gene sequence, and protein production and purification in *Escherichia coli*

(Expression; family 20 glycosyl hydrolases)

Ivo Tews, Renauld Vincentelli and Constantin E. Vorgias

European Molecular Biology Laboratory, c/o DESY, D-22603 Hamburg, Germany

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SUMMARY

The chitobiase (Chb) encoding gene (*chb*) from *Serratia marcescens* (*Sm*) has been cloned, sequenced and expressed in *Escherichia coli* (*Ec*). Sequencing has revealed an open reading frame encoding a protein of 885 amino acids (aa). *Ec* cells harbouring plasmids containing *chb* can produce enzymatically active *Sm* Chb protein which is secreted into the periplasm. An efficient purification scheme using cation-exchange chromatography is presented. This yields about 3 mg of >95% pure *Sm* Chb per litre of *Ec* culture. The deduced aa sequence is 27-aa longer at the N terminus than that determined by sequencing of the purified protein, suggesting that a leader sequence is removed during transport of the enzyme across the cell membrane. Comparison with the other members of the family 20 of glycosyl hydrolases revealed that Chb has a conserved central region which aligns with almost all members of this family. According to the crystal structure of *Sm* Chb, this region comprises the catalytic domain of Chb which has an α/β barrel fold.

INTRODUCTION

N-Acetylglucosaminidases (Chb) (EC 3.2.1.30) and *N*-acetylgalactosaminidases (hexosaminidases) (EC 3.2.1.52) are glycosyl hydrolytic enzymes widely distributed in prokaryotes and eukaryotes (Conzelmann and Sandhoff, 1987). Both enzymes established the family 20 of glycosyl hydrolases classified by Henrissat and Bairoch (1993). While *N*-acetylglucosaminidase digests the β,1–4 glycosidic bonds in *N*-acetylglucosamine (NAG) oligomers (mainly dimers), hexosaminidase cleaves the same bond in *N*-acetylgalactosamine oligomers. The Chb is required to complete the digestion of chitin to NAG monomers used by bacteria as a nutrient source while recycling the billions of tons of chitin debris in the marine environment (ZoBell and Rittenberg, 1937). The hexosaminidases are mainly involved in the degradation of glycolipids and glycoproteins in the human lysosomes and their deficiency leads to accumulation of G_{M2} and causes fatal gangliosidoses (for review, see Sandhoff et al., 1994).

In bacteria, the hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extracellularly by the action of chitinases. Chitinolytic enzymes from many microorganisms have been purified and characterized (Perrakis et al., 1993). Among the different chitinolytic model systems *Serratia marcescens* (*Sm*) has an important position. *Sm* chitinolytic enzymes (mainly chitinases) were shown to play an important role

Correspondence to: Dr. C.E. Vorgias, EMBL c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany. Tel. (49-40) 8990-2118; Fax (49-40) 8990-2149; e-mail: vorgias@embl-heidelberg.de

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); Chb, chitobiase(s) or *N*-acetylglucosaminidase(s); *chb*, gene encoding Chb; *Ec*, *Escherichia coli*; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; G_{M2}, ganglioside; kb, kilobase(s) or 1000 bp; LB, Luria-Bertani (medium); NAG, *N*-acetylglucosamine; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF open reading frame; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PNP, *p*-nitrophenyl; SD, Shine-Dalgarno (sequence); SDS, sodium dodecyl sulfate; *Sm*, *Serratia marcescens*; SP, signal peptide; T_m, melting temperature (50% denaturation); u, unit(s); *V.*, *Vibrio*.

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in the biological control of soil plant pathogens (Oppenheim and Chet, 1992; Chet et al., 1993; Lam and Gaffrey, 1993). Recently we have published the first structure of the bacterial chitinolytic enzyme chitinase A (Perrakis et al., 1994). The crystal structure of Chb from *Sm* was also recently solved and the manuscript is under submission (I.T., A. Perrakis, A.B. Oppenheim, Z. Dauter, K.S. Wilson and C.E.V., data not shown).

To date only two *chb* have been cloned and sequenced. One from the marine bacterium *Alteromonas* sp. O-7 (Tsujibo et al., 1994) and the other from *Vibrio harveyi* (Soto-Gil and Zysking, 1989).

The aim of this work was to clone *chb* from *Sm* into *Escherichia coli* (*Ec*), the production of the enzyme in *Ec* and its purification in adequate amounts for biochemical and structural analysis.

EXPERIMENTAL AND DISCUSSION

(a) Cloning and sequencing of the *chb* from *Sm*

The initial cloning of *chb* and identification of Chb enzymatic activity is described by Kless et al. (1989). Two clones of 3.7 and 2.8 kb of *Sm* genomic DNA have been subcloned into pEMBL18 and used for sequencing. Each of these fragments contains a part of the *chb*. The clones were named pCB13 and pCB15, respectively. Further fragmentation of the gene and subcloning into pUC vectors allowed us fast access to several regions of the *chb* using external primers.

Fig. 1 shows the complete sequence of the *chb* and the deduced aa sequence of the Chb. Computer translation of the nt sequence yielded a large ORF of 2655 nt coding for a 885-aa protein. A ribosome-binding site was found upstream from the ATG start codon. However, the N terminus of the purified protein is identical to the aa sequence starting at aa 28 of the deduced aa sequence. This finding suggests the presence of a 27-aa leader sequence, which is cleaved off probably during protein secretion. The cleavage occured between Ala^{27} and Asp^{28}. In preliminary experiments it was found that, in *Ec*, the major part of the enzymatic activity is transported to the periplasm (Kless et al., 1989).

(b) Comparison of the nt sequence of the *chb* with the other members of family 20 of glycosyl hydrolases

As a first step to identify primary structure similarities between the *Sm* Chb and the other members of the family

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ctgcagccctgcaagtcggtgctgaacgtgctgaaacaagaaaaacagcaccagcagtttgctgagcaggaaacggtgcgggtgatggattatcagaactgcattatggcggttcacagcggtaacggtcaggcttatgacgccaagtgc 150
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                                                   M  N  A  F  K  L  S  A  L  A  R  L  T  A  T  M  G  F  L  G  G  M  G  S  A  M  A  D  Q  Q  L  V  D   33
CAACTGAGCCAGCTGAAGCTGAACGTGAAAATGCTGGATAACCGCGCCGGCGAAAACGGCGTGGATTGCGCGGCGCTGGGCGCTGACTGGGCTTCTTGCAACCGGGTGTTGTTCACCCTCAGCAACGACGGCCAGGCGATCGACGGCAAA 450
Q  L  S  Q  L  K  L  N  V  K  M  L  D  N  R  A  G  E  N  G  V  D  C  A  A  L  G  A  D  W  A  S  C  N  R  V  L  F  T  L  S  N  D  G  Q  A  I  D  G  K   83
GACTGGGTCATCTATTTCCACAGCCCGCGGCAGACCTTGCGGGTGGACAACGACCAGTTCAAGATCGCGCATCTGACCGGCGATCTGTACAAGCTGGAGCCGACCGCCAAATTCAGCGGTTTCCCGGCCGGTAAGGCGGTGGAAATCCCG 600
D  W  V  I  Y  F  H  S  P  R  Q  T  L  R  V  D  N  D  Q  F  K  I  A  H  L  T  G  D  L  Y  K  L  E  P  T  A  K  F  S  G  F  P  A  G  K  A  V  E  I  P   133
GTGGTCGCCGAATACTGGCAGCTGTTCAGAAACGACTTCCTGCCGCGTTGGTATGCCACCTCCGGCGACGCCAAGCCGAAAATGCTGGCGAATACCGACACCGAAAACCTGGATCAGTTTGTGGCGCCGTTCACCGGCGACCAGTGGAAG 750
V  V  A  E  Y  W  Q  L  F  R  N  D  F  L  P  R  W  Y  A  T  S  G  D  A  K  P  K  M  L  A  N  T  D  T  E  N  L  D  Q  F  V  A  P  F  T  G  D  Q  W  K   183
CGCACCAAGGACGACAAAAACATTCTGATGACGCCGGCTTCGCGCTTTGTCAGCAATGCCGATCTGCAGACGCTGCCCGCCGGCGCGCTGCGCGGCAAGATCGTGCCGACGCCGATGCAGGTGAAGGTCCACGCACAGGACGCCGACCTG 900
R  T  K  D  D  K  N  I  L  M  T  P  A  S  R  F  V  S  N  A  D  L  Q  T  L  P  A  G  A  L  R  G  K  I  V  P  T  P  M  Q  V  K  V  H  A  Q  D  A  D  L   233
CGCAAAGGGGTGGCGCTGGATCTGAGCACGCTGGTCAAGCCGGCGGCGGACGTCGTCAGTCAGCGTTTCGCGCTGCTGGGCGTGCCGGTTCAGACCAACGGTTACCCGATCAAGACCGATATCCAGCCGGGCAAGTTTAAAGGCGCGATG 1050
R  K  G  V  A  L  D  L  S  T  L  V  K  P  A  A  D  V  V  S  Q  R  F  A  L  L  G  V  P  V  Q  T  N  G  Y  P  I  K  T  D  I  Q  P  G  K  F  K  G  A  M   283
GCGGTATCGGGCGCCTATGAGCTGAAAATCGGCAAGAAAGAGGCGCAAGTGATCGGCTTCGATCAGGCCGGGGTGTTCTACGGCCTGCAGTCGATCCTGTCGTTAGTGCCGAGCGACGGCAGCGGCAAGATCGCCACGCTGGACGCCAGC 1200
A  V  S  G  A  Y  E  L  K  I  G  K  K  E  A  Q  V  I  G  F  D  Q  A  G  V  F  Y  G  L  Q  S  I  L  S  L  V  P  S  D  G  S  G  K  I  A  T  L  D  A  S   333
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ATCGAGATCCCCGGCTTGCCTGAGTTGACGGAAGTCGGCGGCCAGCGCTGCCACGATCTGAGCGAAACCACCTGCCTGCTGCCGCAGTACGGCCAGGGGCCGGACGTCTACGGCGGCTTCTTCAGCCGTCAGGACTACATCGACATCATC 1500
I  E  I  P  G  L  P  E  L  T  E  V  G  G  Q  R  C  H  D  L  S  E  T  T  C  L  L  P  Q  Y  G  Q  G  P  D  V  Y  G  G  F  F  S  R  Q  D  Y  I  D  I  I   433
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K  Y  A  Q  A  R  Q  I  E  V  I  P  E  I  D  M  P  A  H  A  R  A  A  V  V  S  M  E  A  R  Y  K  K  L  H  A  A  G  K  E  Q  E  A  N  E  F  R  L  V  D   483
CCGACCGATAACTCCAACACAACCTCCGTGAAGTTCTTTAACCGCCAGAGATACCTGAACACGTGCCTGGATTCCTCCCAACGCTTTGTCAACAAGGTGAACGGCGAGATAGCCCAGATGAATAAAGAAGACGGGCAGCCAATCAAGACC 1800
P  T  D  T  S  N  T  T  S  V  Q  F  F  N  R  Q  S  Y  L  N  P  C  L  D  S  S  Q  R  F  V  D  K  V  I  G  E  I  A  Q  M  H  K  E  A  G  Q  P  I  K  T   533
TGGCACTTTGACGGCGATGAAGCGAAAAACATCCGCCTGGACGCCGGCTAAACCGACAAGACGAAACCGGAACCGGGTAAAGGCATCATTAACCAGAGTAATGAAGACAAACCGTGGGCCAAGTCGCAGGAGTGCCAGACAATGATCAAA 1950
W  H  F  G  G  D  E  A  K  N  I  R  L  G  A  G  Y  T  D  K  A  K  P  E  P  G  K  G  I  I  D  Q  S  N  E  D  K  P  W  A  K  S  Q  V  C  Q  T  M  I  K   583
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E  G  K  V  A  D  M  E  H  L  P  S  Y  F  G  Q  E  V  S  K  L  V  K  A  H  G  I  D  R  M  Q  A  W  Q  D  G  L  K  D  A  E  S  S  K  A  F  A  T  S  R   633
GTGGGCGTCAACTTCTGGGAAACCCTGTATAGGGGCGGTTACGACAGCGTAAACGACTGGACCAATAAAGAGTATGAAGTAGTGGTCTCCAACCCGGACTACGTCTACATAGACTTCCCTAACGAGGTGAATCCGGACGAACGCGGTTAC 2250
V  G  V  N  F  W  D  T  L  Y  W  G  G  F  D  S  V  N  D  W  A  N  K  G  Y  E  V  V  V  S  N  P  D  Y  V  Y  M  D  F  P  Y  E  V  N  P  D  E  R  G  Y   683
TACTGGGGCAACCGCTTCAGAGACGAGCGCAAGGTGTTCAACTTCGCGCCAGACAACATGACGCAGAACGAGGAAACCTCAGTCGACCGCAACGGCAACCACTTCAACGCAAAAAGCGACAAGCCGTGGCAGGGCGCCTAAGGTCTGTCC 2400
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GCTCAGCTGTAGAGCGAAACACAGCGCACCAATCCGCAGAAGGAATACATAATCTTCCCGAGCGCGCTTTAGGTGGCGGAAcggtCCTGGAACCGCGCCGATTGGGAGCAAGACTATCGCACCGGCCGCGAATACAAAGGAGGGGAAACC 2550
A  Q  L  W  S  E  T  Q  R  T  D  P  Q  M  E  Y  M  I  F  P  R  A  L  S  V  A  E  R  S  W  H  R  A  G  W  E  Q  D  Y  R  A  G  R  E  Y  K  G  G  E  T   783
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H  F  V  D  T  Q  A  L  E  K  D  W  L  R  F  A  N  I  L  G  Q  R  E  L  A  K  L  D  K  G  G  V  A  Y  R  L  P  V  P  G  A  R  V  A  A  G  K  L  E  A   833
AATATCGCGCAGCCGGGATTAGGCATCGAGAATTCCACCGACGGCGGCAAACAGTGGCAGAGCTATGACGACAAGGCCAAACCGGCGGTCACCGGTGAAGAGCAGGTGCGATCGGTCAGCACGGACGGTAAACGCTACAGACGCGCCGAG 2850
N  I  A  L  P  G  L  G  I  E  Y  S  T  D  G  G  K  Q  W  Q  R  Y  D  A  K  A  K  P  A  V  S  G  E  V  Q  V  R  S  V  S  P  D  G  K  R  Y  S  R  A  E   883
AAGGTCTAGgtctggctccggggcccgtggttctggcggcaggcacatcaaaatgaagggagaatgcgttagcccccgcgagtgccggcgaggggcggatattgaagtgaaagcagtacgaggagtcgccatgccgctgcaatggtagta 3000
K  V  *                                                                                                                                                      885
ttcatgacaagatctacccacggcttgtcaggattttgacgtgacgcaaggataaaaaacgcccaa 3070
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Fig. 1. Nucleotide sequence of the *chb* and the deduced aa sequence. Genomic DNA of *Sm* was partially cleaved with *Sau*3AI and ligated with the vector λD69 at the unique *Bam*HI site. A 4.5-kb *Hin*dIII fragment was isolated and cloned into the pEMBL18 plasmid. Both DNA strands were sequenced, using a number of subclones and oligo primers. DNA sequencing was carried out using synthetic internal primers. G+C-rich regions were sequenced several times. Both strands of the entire *chb* were sequenced using the Sequenase® kit from US Biochemical (Cleveland, OH, USA) according to the instructions of the manufacturers (Sanger et al., 1977). Using the Strider 1.2 program the coding region of *chb* was translated to the single aa code. The SD is underlined. The stop codon is indicated by an asterisk (*). Lower-case nt indicate sequence areas with serious problems to resolve the correct ORF. These areas were verified according to the aa primary structure of the protein revealed from the crystal structure. The nt sequence data reported in this paper has been deposited with GenBank under accession No. L43594.

20 of glycosyl hydrolases we applied a computational search performed at the NCBI using the BLASTP network service (Altschul et al., 1990). The search has revealed 16 different entries with identities ranging from 16 to 54%. Among those, only one Chb appeared, the other 15 were hexosaminidases.

From the crystal structure of Chb (I.T., A. Perrakis, A.B. Oppenheim, Z. Dauter, K.S. Wilson and C.E.V., data not shown) four distinct structural and functional domains were identified. These domains differ in length and fold. Briefly, domain I is 154-aa long (aa 28–181), domain II is 122-aa long (aa 214–335), domain III is the major catalytic domain with an α/β barrel fold and is 483-aa long (aa 336–818) and finally domain IV is only 67-aa long (aa 819–885). Although the structure of each individual domain is known, only the function of the major catalytic domain III has been assigned.

In an attempt to elucidate the function of the other domains of Chb the same computational tool was used as described above. The results can be summarized as follows: (*i*) Domain I of Chb shows a substantial identity (51%) to the N terminus (aa 36–178) of endo-β-*N*-acetylglucosaminidase from *V. harveyi* (P13670) (Soto-Gil and Zysking, 1989) and 34% to the N terminus (aa 35–139) of β-*N*-acetylhexosaminidase (A48228) from *V. vulnificus* (Sommerville and Colwell, 1993). (*ii*) Domain II of Chb is missing in eukaryotic hexosaminidases. This domain shows a substantial identity (34%) to a protein segment between aa 212 and 329 of endo-β-*N*-acetylglucosaminidase from *V. harveyi* and 36% to a protein segment between aa 212 and 329 of β-*N*-acetylhexosaminidase from *V. vulnificus*. (*iii*) Domain III of Chb aligns (67% identity) with only one bacterial endo-β-*N*-acetylglucosaminidase from *V. harveyi* (aa 330–816). The structural identity of this domain to β-*N*-acetylhexosaminidase from *V. vulnificus* is about 50% (aa 313–779). The sequence identity between the eukaryotic hexosaminidases and domain III of *Sm* is about 26%. A detailed analysis proposing that eukaryotic hexosaminidases have essentially an α/β barrel catalytic domain and similar, if not identical, catalytic mechanism is not presented here (I.T., A. Perrakis, A.B. Oppenheim, Z. Dauter, K.S. Wilson and C.E.V., data not shown). (*iv*) Finally, domain IV of Chb shows a substantial identity (41%) to the C

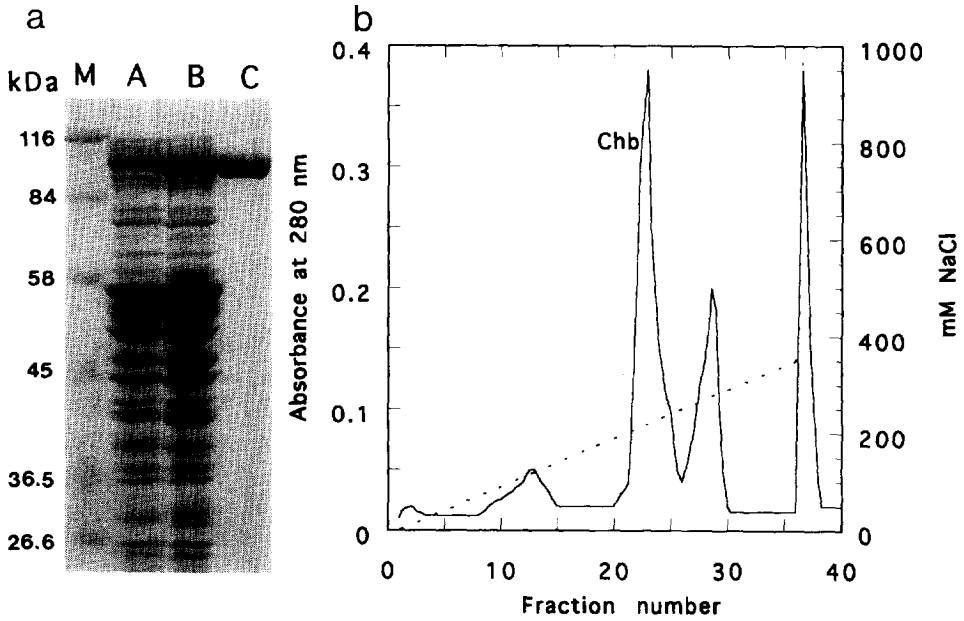

Fig. 2. Production, fractionation and purification of Chb by column chromatography. (**a**) Chb production and fractionation by 0.1% SDS-10% PAGE. Lanes: **A**, total *Ec* periplasmic fraction of the clone A5441 producing Chb; **B**, 55–85% saturated $(NH_4)_2SO_4$ fraction of the periplasmic fraction; **C**, the Chb peak fraction after SP-Sepharose HP column; **M**, molecular mass markers. (**b**) Cation-exchange column chromatography of Chb. The elution profile of the $(NH_4)_2SO_4$-fractionated bacterial periplasmic extract on SP-Sepharose HP (Pharmacia) column at pH 6.4 in buffer B (see Methods) is presented. Chb eluted between 200 and 250 mM NaCl. **Methods:** *Ec* cells producing Chb were resuspended in 5 ml per g cell paste in buffer A (100 mM Tris-HCl pH 8.0/0.5 mM EDTA/0.5 M sucrose/0.1 mM PMSF/1 mM DTT). 100 μl of 2 mg/ml lysozyme solution was added per 1 ml of suspension and the mixture incubated on ice for 20 min. An equal volume of ice-cold water was added and kept on ice for the next 20 min. Finally the extract was adjusted to 5 mM $MgCl_2$ and centrifuged at 15 000 × *g* for 20 min. The supernatant containing the periplasmic fraction of the *Ec* cells was used as the initial fraction for the final purification of Chb. Chb was precipitated between 55–85% saturated $(NH_4)_2SO_4$. The protein was dissolved in a large volume of buffer B (10 mM Na-phosphate pH 6.4/1 mM EDTA/0.1 mM PMSF/1 mM DTT) and the Chb-enriched fraction was directly applied on a SP-Sepharose HP (Pharmacia), equilibrated in buffer B. Bound protein was eluted with 20 column volumes of a 0–0.5 M NaCl gradient in buffer B, at a flow rate of 60 ml/h and 10 ml fractions were collected. The Chb activity assay was used to select and combine the appropriate fractions. The purified enzyme was boiled in the presence of SDS and β-mercaptoethanol and separated by 0.1% SDS-10% PAGE (Laemmli, 1970). Staining with Coomassie blue revealed a single protein band with an estimated molecular mass of about 98 kDa.

TABLE I

Purification scheme of Chb from a 10 g batch of overproducing *Ec* cells

Step	Volume (ml)	Concentration (mg/ml)[a]	Total amount (mg)	Spec. activity (u/mg)[b]	Total u[b]	Yield (%)
Periplasmic fraction	50	8.2	410	2800	1 148 000	100
Ammonium sulfate precipitation 55–85%	60	2.4	144	5600	806 400	70.2
S-Sepharose HP at pH 6.4	30	0.3	9	62750	564 750	49.2

[a] Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as standard.

[b] Enzyme activity was measured by mixing 100 µl appropriately diluted Chb fraction with 900 µl of 50 µM PNP-β-*N*-acetylglucosamine dissolved in mM Na·phosphate buffer pH 8.0 and the liberation of *p*-nitrophenol was monitored at $A_{405\ nm}$. One unit (u) of Chb was defined as the amount of enzyme that liberated 1 µmol of PNP in 1 min at 37°C.

terminus (aa 817–883) of endo-β-*N*-acetylglucosaminidase from *V. harveyi*.

It is interesting to note that although between Chb from *V. harveyi* and *Sm* a substantial primary structure similarity exists (55.6% identity), the Chb from marine bacterium *Alteromonas* sp. (Tsujibo et al., 1994) does not seem to have any structural similarity to the other Chb. The sequence comparisons were obtained using the CLUSTALW program (Higgins et al., 1992).

(c) Production and purification of Chb in *Ec*

For preparative purposes a 5 L culture of A5441 clone containing the entire *chb*, constitutively expressed, was grown at 30°C in LB medium supplemented with 50 µg Ap/ml (Kless et al., 1989). The cells were collected by low speed centrifugation and stored frozen (−80°C) until use. Preliminary experiments have shown that the enzyme was secreted into the periplasm. Therefore an initial purification step is the preparation of bacterial spheroplasts and recovering the protein from the periplasmic fraction. Fig. 2 shows an electrophoretic analysis of the representative steps of the Chb purification scheme. Table I summarizes the purification procedure for Chb which is practically one step purification with a very good yield of a highly active enzyme.

(d) Properties of purified Chb

Optimal enzymatic activity degrading PNP-β-*N*-acetylglucosamine was found at pH 6–8, in 50 mM Na·phosphate buffer. The highest activity was obtained at 52°C. The K_m for the synthetic substrate was estimated as 165 µM. As determined using circular dichroic spectroscopy, the T_m was 60°C. PNP-β-*N*-acetylglucosamine was the best substrate for the enzyme among several tested oligosaccharides. The substrate specificity of *Sm* Chb is essentially identical to that of Chb from *Alteromonas* sp. (strain O-7).

(e) Conclusions

(*1*) The *chb* from *Sm* encoding a Chb was sequenced. The ORF encodes a 885-aa protein.

(*2*) When expressed in *Ec* a 27-aa signal sequence is removed probably during the secretion of the protein to the periplasmic space.

(*3*) The recombinant Chb has been purified in adequate amounts for biochemical and structural studies.

(*4*) Chb shares a high degree of similarity with only one bacterial Chb. According to the crystal structure of the *Sm* Chb the main catalytic domain of the enzyme is domain III with α/β barrel fold. This domain is conserved among the members of family 20 of glycosyl hydrolases.

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