A new chitosanase gene from a *Nocardioides* sp. is a third member of glycosyl hydrolase family 46

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Strain N106, a newly isolated soil actinomycete classified in the genus *Nocardioides* on the basis of its chemotaxonomy, produced an extracellular chitosanase and was highly active in chitosan degradation. A gene library of *Nocardioides* sp. N106 was constructed in the shuttle vector pFD666 and recombinant plasmids carrying the chitosanase gene (*csnN106*) were identified using the 5'-terminal portion of the chitosanase gene from *Streptomyces* sp. N174 as a hybridization probe. One plasmid, pCSN106-2, was used to transform *Streptomyces lividans* TK24. The chitosanase produced by *S. lividans*(pCSN106-2) is a protein of 29.5 kDa, with a pI 8.1, and hydrolyses chitosan by an endo-mechanism giving a mixture of dimers and trimers as end-products. N-terminal sequencing revealed that the mature chitosanase is a mixture of two enzyme forms differing by one N-terminal amino acid. The *csnN106* gene is 79.5% homologous to the *csn* gene from *Streptomyces* sp. N174. At the amino acid level, both chitosanases are homologous at 74.4% and hydrophobic cluster analysis revealed a strict conservation of structural features. This chitosanase is the third known member of family 46 of glycosyl hydrolases.

Keywords: chitosan, actinomycetes, *Streptomyces*, *Nocardioides*, chitosanase

INTRODUCTION

Chitosanases (EC 3.2.1.132) – enzymes that catalyse the hydrolysis of glycosidic bonds in chitosan (a partially or totally de-acetylated derivative of chitin) were first described by Monaghan *et al.* (1973). Chitosanase activities have been detected in numerous prokaryotes, fungi, and more recently, in plants (El Ouakfaoui & Asselin, 1992). For many years, this group of enzymes remained poorly defined and the differences between the chitosanases and other enzymes able to hydrolyse partly acetylated chitosan (chitinases, lysozymes, exo-*N*-acetyl- β -D-glucosaminidases, exo- β -D-glucosaminidases) were not clear. Chitosanase was essentially distinguished from chitinase on the basis of its inability to hydrolyse fully acetylated chitin.

Abbreviations: GlcN, D-glucosamine; GlcNAc, *N*-acetyl- β -D-glucosamine; HCA, hydrophobic cluster analysis.

The GSDB accession number for the sequence reported in this paper is L40408.

Recently, Fukamizo *et al.* (1994) proposed a distinction between chitinase and chitosanase based on the specificity of cleavage of the (1,4)- β -glycosidic linkages in partially acetylated chitosan. Chitosanases were defined as glycosyl hydrolases able to hydrolyse various types of linkages in partially acetylated chitosan, but not the GlcNAc–GlcNAc bond. On the other hand, chitinases split only GlcNAc–GlcNAc and GlcNAc–GlcN linkages in chitosan. Furthermore, the chitosanases were subdivided into three subclasses, characterized by the ability to split GlcN–GlcN and GlcNAc–GlcN linkages (subclass I); only GlcN–GlcN linkages (subclass II) and, finally, GlcN–GlcN and GlcN–GlcNAc linkages (subclass III).

Nucleotide sequences (and deduced primary amino acid sequences) are available for the chitosanase from *Bacillus circulans* MH-K1 (Ando *et al.*, 1992) and from *Streptomyces* sp. N174 (Masson *et al.*, 1994). Comparison of the two amino acid sequences revealed a significant homology between the N-terminal ends of the mature proteins (amino acids 59–107 from the *B. circulans* chitosanase

precursor and amino acids 41–90 from the *Streptomyces* sp. N174 chitosanase precursor). Despite the use of several computer programs, no homology could be detected between these chitosanases and any family of chitinases, lysozymes or hexosaminidases. On that basis, it was proposed that chitosanases form a new family of glycosyl hydrolases, distinct from other families of chitosan-hydrolysing enzymes (Masson *et al.*, 1994). This family is classified under the number 46 (B. Henrissat, personal communication).

However, the lack of homology in the middle and C-terminal segments between the *B. circulans* MH-KI and *Streptomyces* sp. N174 chitosanases raises several new questions. Can these two chitosanases be considered as the first members of two distinct subfamilies? Is there any relationship between these sequence-defined subfamilies and the subclasses proposed by Fukamizo *et al.* (1994) on the basis of their cleavage specificity?

To answer these questions, more chitosanase genes and enzymes should be available for molecular and enzymological studies. In this paper, we describe a new chitosanase protein and its encoding gene from the actinomycete *Nocardioide*s sp. N106, isolated from soil.

METHODS

Micro-organisms and culture conditions. Strain N106 ('N' for neutral pH soil) was isolated from soil during a screening programme for chitosanolytic organisms. The enrichment, detection and long-term maintenance methods were described previously (Boucher *et al.*, 1992). For total DNA isolation, 10^6 spores of N106 were used to inoculate 250 ml Tryptic Soy Broth (TSB; Difco) and cultivated for 36 h. Cells were harvested before the fragmentation stage.

The strains *Escherichia coli* DH5 α F' [F' ϕ 80 d*lac*ZM15 Δ (*lac*ZY*A*-*arg*F)U169 *deoR* *recA1* *endA1* *hsdR17*(r_K⁻ m_K⁺) *supE44* λ^- *thi-1* *gyrA96* *relA1*], obtained from Life Technologies, and *Streptomyces lividans* TK24, obtained from Professor D. A. Hopwood, were used as cloning hosts. *E. coli* strains were grown in Luria-Bertani medium (Sambrook *et al.*, 1989). *S. lividans* strains were routinely propagated on TSB. For the propagation of plasmid-containing strains, media were supplemented with 50 μ g ml⁻¹ ampicillin or kanamycin. The media for *S. lividans* protoplast preparation and regeneration were as described by Hopwood *et al.* (1985).

For chitosanase production, spores of *S. lividans*(pCSN106-2) were used to inoculate 100 ml TSB supplemented with 50 μ g kanamycin ml⁻¹ and grown for 3 d at 30 °C with shaking. Cells were harvested by 5 min centrifugation in a clinical centrifuge and a quantity of cells corresponding to 3.5 ml pellet volume was used to inoculate 500 ml chitosanase production medium. This consisted of 1.5% (w/v) chitosan (practical grade, Sigma), 0.5% (w/v) malt extract (Difco), 0.2% (v/v) olive oil and 0.035% (v/v) antifoam agent (Antifoam A, Sigma), supplemented after sterilization with 50 ml filter-sterilized 0.1 M MOPS buffer, pH 7, and 0.5 ml filter-sterilized riboflavin solution (1 mg ml⁻¹). The culture was incubated for 72 h at 30 °C with shaking (250 r.p.m.). Kanamycin was not needed in the chitosanase production medium.

Enzyme purification. The procedure of Boucher *et al.* (1992) was simplified, allowing rapid purification of chitosanase in two chromatographic steps. Cultures in chitosanase production

medium were centrifuged. The supernatant was acidified to pH 4 with 5 M acetic acid. Conductivity was adjusted to 9 mS with distilled water. The adjusted supernatant was then applied to a 1.6 $\times$ 20 cm S-Sepharose Fast Flow column (Pharmacia LKB), previously equilibrated with 50 mM sodium acetate buffer, pH 4 (buffer A). The flow rate was 100 ml h⁻¹. The column bed was protected by an on-line, hollow fibre filter (MediaKap-5, 0.2 μ m, Microgon). Unbound protein was washed from the column with 50 ml buffer A, then a linear 160 ml gradient from 0 to 0.6 M NaCl in buffer A was applied. Chitosanase-containing fractions were identified by the standard chitosanase assay, then pooled (total volume 30 ml) and concentrated threefold by ultrafiltration. The next step, size-exclusion chromatography, was as described by Boucher *et al.* (1992).

Biochemical procedures. Chitosanase activity was assayed as described by Boucher *et al.* (1992) using the neocuproine reagent (Dygert *et al.*, 1965) for reducing sugar determination. One unit (U) of enzyme is defined as the amount that liberated 1 μ mol D-glucosamine equivalent per min under the assay conditions. For chitosanase substrate-specificity studies, the substrates were prepared as 2 mg ml⁻¹ solutions or suspensions in 50 mM sodium acetate buffer, pH 5.5. Protein concentration was estimated by the method of Bradford (1976), using the BioRad protein assay reagent and a BSA (Boehringer Mannheim) standard. Thin-layer chromatography (TLC) of end-products of chitosan degradation was performed as described by Neugebauer *et al.* (1991). The N-terminal amino acid sequence of chitosanase was determined by Edman degradation with an Applied Biosystems 473A protein sequencer.

Chemotaxonomical procedures. The diaminopimelic acid form in the cell wall and the predominant sugars in whole-cell hydrolysates were determined by TLC according to Stanek & Roberts (1974). Mycolic acids were analysed according to Tomiyasu & Yano (1984).

Cloning of the chitosanase gene from *Nocardioide*s sp. N106. Chromosomal DNA from *Nocardioide*s sp. N106 was prepared according to the method of Chater *et al.* (1982), digested with *Bgl*II and analysed by Southern blotting using a 0.38 kb *Sph*I-*Sac*II fragment from the chitosanase gene of *Streptomyces* sp. N174 (Masson *et al.*, 1994) as the hybridization probe. A strong signal was obtained in the 5–8 kb portion of the *Bgl*II-digested DNA. A partial library of *Nocardioide*s sp. N106 genes was constructed by ligating the 5–8 kb *Bgl*II fragments eluted from an agarose electrophoresis gel to *Bam*HI-linearized and dephosphorylated vector pFD666 (Denis & Brzezinski, 1992). Competent *E. coli* DH5 α F' cells were transformed with the ligation mixture. Approximately 700 transformants were recovered on LB plates supplemented with 50 μ g kanamycin ml⁻¹. These were screened by colony hybridization using the same probe. Four positive clones were kept for further analysis. Standard methods (Sambrook *et al.*, 1989) were used for DNA manipulation, *E. coli* transformation and hybridization.

DNA sequencing. The nucleotide sequence of the *csnN106* gene was determined on both strands by the chain-termination method (Sanger *et al.*, 1977) with double-stranded templates obtained after subcloning appropriate segments in the pUC118 vector (Vieira & Messing, 1987). Unidirectional deletions (Henikoff, 1984) were created to sequence segments not bordered by restriction sites. Some gaps in the sequence were filled in using synthetic oligodeoxyribonucleotides as sequencing primers. Computer-assisted analysis of the sequence was done with the University of Wisconsin Genetics Computer Group package of programs (Devereux *et al.*, 1984). The hydrophobic cluster analysis (HCA) program (Gaboriaud *et al.*, 1987) was obtained from Doriane.

RESULTS AND DISCUSSION

Characterization of the N106 isolate

Actinomycete N106 was one of the most active chitosan-hydrolysing micro-organisms isolated during an extensive soil screening programme (Boucher *et al.*, 1992). Strain N106 grows abundantly on Tryptic Soy Agar at 30 °C, forming aerial mycelium. Old primary hyphae break up into short, rod-like fragments with polyphosphate inclusions. Mycelium fragmentation is observed in a variety of liquid media. The LL-form of diaminopimelic acid is present in the cell walls of vegetative as well as fragmented mycelium. Galactose, glucose, mannose, ribose and rhamnose were identified in whole-cell hydrolysates. The organism is strictly aerobic. Mycolic acids were not detected. From these data, N106 was classified in the genus *Nocardioide*s (Prauser, 1976) and will be referred as *Nocardioide*s sp. N106.

Cloning of the chitosanase gene from *Nocardioide*s sp. N106

In our previous work (Masson *et al.*, 1994), we showed that the two chitosanases for which amino acid sequences were deduced from DNA sequences were rather distantly related to each other but that a reasonably well conserved module of about 50 amino acids could be identified. The nucleotide sequence encoding this conserved module is entirely included in a 0.38 kb *Sph*I–*Sac*II fragment from the cloned *csn* gene of *Streptomyces* sp. N174 (Masson *et al.*, 1994). This DNA fragment was used as a hybridization probe with total *Nocardioide*s sp. N106 DNA digested with various restriction enzymes.

We detected one positive hybridization band per digest indicating that *Nocardioide*s sp. N106 contains a putative chitosanase gene at least partially homologous to the *Streptomyces* sp. N174 DNA probe (data not shown). The 5–8 kb fragments from a *Bgl*II digest of *Nocardioide*s sp. N106 DNA were eluted from an agarose electrophoresis gel and cloned into the *Bam*HI site of the vector pFD666 (Denis & Brzezinski, 1992). After *E. coli* transformation, positive clones were identified by colony hybridization with the *Streptomyces* sp. N174 DNA probe. Four positive clones were recovered. Restriction mapping and hybridization analysis showed that these carried the same 6.5 kb insert in alternative orientations. Plasmid pCSN106-2 was chosen for further studies because the orientation of the insert towards the kanamycin resistance gene of the vector was the same as in the previously described plasmids pRL226 and pRL270 (Masson *et al.*, 1993). These directed efficient expression of the *Streptomyces* sp. N174 chitosanase gene in a heterologous host, *S. lividans* TK24.

Complementation of *S. lividans* TK24 with pCSN106-2

S. lividans TK24 is an appropriate host for the study of heterologous chitosanase genes because it seems to lack its own chitosanase. Even on a medium containing chitosan as sole carbon source, it does not produce a typical

chitosanase (i.e. an enzyme acting on chitosan but not on chitin, Neugebauer *et al.*, 1991), and the low activity of this strain against chitosan is probably due to chitinase activities (Miyashita *et al.*, 1991) that poorly recognize chitosan as a substrate. The absence in the *S. lividans* TK24 genome of DNA sequences homologous to known chitosanase genes was confirmed by hybridization experiments using the same DNA probe as above.

When tested on chitosanase detection medium (Boucher *et al.*, 1992), *S. lividans* TK24 gave only a weak degradation zone (Fig. 1). In contrast, the transformant TK24(pCSN106-2) hydrolysed chitosan with only slightly lower efficiency than the donor strain, *Nocardioide*s sp. N106. It was concluded that pCSN106-2 carried a chitosanase-encoding gene. Several possibilities explain why the degradation zone around TK24(pCSN106-2) is smaller than that around the donor strain. *Nocardioide*s could produce several chitosan-hydrolysing enzymes and only one of the corresponding genes was cloned on plasmid pCSN106-2. Multiple chitosanase activities were found in *Bacillus megaterium* P1 (Pelletier & Sygusch, 1990). Alternatively, poor inducibility by chitosan of heterologous chitosanase production by *S. lividans* TK24(pCSN106-2) may be responsible (I. Boucher & R. Brzezinski, unpublished data). Factors necessary for chitosanase gene induction could be limiting in *S. lividans*.

Production and purification of the chitosanase

A total quantity of 24 mg N106 chitosanase was purified from 500 ml culture supernatant of *S. lividans* TK24(pCSN106-2). The yield was 16% and the purification factor was 1.5. In zymograms (El Ouakfaoui & Asselin, 1992), the purified chitosanase co-migrated with the chitosanase produced by *Nocardioide*s sp. N106. (not shown). The proteins also had the same isoelectric point (see below).

Fig. 2. TLC of end-products of chitosan Sigma (degree of acetylation = 0.21) hydrolysis. Lanes: 1, *N,N'*-diacetylchitobiose; 2, GlcNAc; 3, GlcN; 4, mixture of dimeric and trimeric chitooligosaccharides (end-product generated by chitosanase from *Streptomyces* sp. N174); 5, end-product generated by chitosanase from *Nocardioideis* sp. N106.

Preliminary characterization of the purified chitosanase

The specific activity of the N106 chitosanase was 63 U mg^{-1} . The apparent molecular mass of the N106 chitosanase, determined by SDS-PAGE, was estimated to be 29.5 kDa by comparison with reference proteins. Analytical isoelectric focusing of the enzyme on a pH gradient of 6–10 indicated a pI of 8.1.

The N106 chitosanase was specific for chitosan degradation. No hydrolysis of colloidal chitin, CM-cellulose, Avicell or heparin could be detected. Fully de-acetylated chitosan was hydrolysed at a rate of 176% that of Sigma chitosan (degree of acetylation = 0.21). The main chitosan hydrolysis products detected by TLC co-migrated with the products obtained after chitosan hydrolysis with chitosanase from *Streptomyces* sp. N174, identified previously (Boucher *et al.*, 1992) as dimeric and trimeric chitooligosaccharides (Fig. 2). The absence of detectable monomeric products (either GlcN or GlcNAc) indicated that the enzyme is an endoglycosidase.

The biochemical properties of the *Nocardioideis* sp. N106 chitosanase are thus very similar to those of the *Streptomyces* sp. N174 chitosanase (Boucher *et al.*, 1992). However, the *Streptomyces* sp. N174 chitosanase is a slightly less alkaline protein (pI 7.5) and its preference for fully de-acetylated chitosan is less marked (rate of hydrolysis is 123% of that of Sigma chitosan).

The first 16 amino acids at the N-terminus of the *Nocardioideis* chitosanase were determined by the Edman

method. This revealed that the purified chitosanase is a mixture of two polypeptides differing by one amino acid. About 84% of the molecules had the N-terminal sequence AAVGLDDPHKKDIAMQ, while the remaining 16% had the sequence AVGLDDPHKKDIAMQ.

Sequence analysis of the *csnN106* gene

Fig. 3 shows the sequence of a 1059 bp DNA fragment containing the *csnN106* gene. The ORF of 837 bp starts with an ATG triplet (nt 128–130) and corresponds to a 278 amino acid polypeptide. A potential RBS (GGAG) is present 7–10 nt upstream of the initiation codon. A putative streptomycete promoter (Strohl, 1992) consisting of a –35 box TTGCGC and a –10 box TTCAAT (with a spacer of 18 nt) was also found (nt 21–26 and 45–50).

An extensive inverted-repeat sequence, perfectly homologous to the sequence upstream from the *csn* gene of *Streptomyces* sp. N174, was located between the putative promoter and the RBS. This conserved sequence lies in the middle of an otherwise poorly conserved DNA segment. Similar sequences may be involved in regulating initiation of transcription. Experiments are in progress to ascertain the role of this inverted repeat.

The N-terminus of the mature chitosanase is located at amino acid +1 (Fig. 4), determining a 41 amino acid signal peptide. However, a minority (16%) of mature molecules begin with the amino acid at +2. At present, we cannot determine if this heterogeneity is due to ambiguity in the recognition of the processing site by the signal peptidase or if it is due to some post-secretory modifications. Neither of the observed processing sites obey the (–3, –1)-rule (von Heijne, 1986). Applying the algorithm of von Heijne, the highest scores are obtained for links between amino acids –18 and –17 (highest score) or –17 and –16 (second best score). The actual processing sites are predicted with a very low probability by the algorithm. This is reminiscent of the situation found for the *Streptomyces* sp. N174 chitosanase (Masson *et al.*, 1994). Other features of the N106 chitosanase signal peptide are typical: the *n*-region consisting of nine amino acids (three of them being positively charged) is followed by a hydrophobic segment of ten amino acids and a rather long *c*-region, the latter being characteristic of actinomycete signal peptides (von Heijne & Abrahmsén, 1989) with a helix-breaking residue (proline) at position –7.

A second, putative translation initiation in-frame ATG codon is localized 30 nt upstream from the proposed initiation codon (Fig. 3). This codon is also preceded by a putative RBS, GGAGA, situated at an optimal distance from the ATG triplet. The alternative initiation triplet would determine a 51 amino acid signal peptide with a 19 amino acid *n*-region including six positively charged and two negatively charged amino acids. Such a long signal peptide would still resemble some other signal peptides described for extracellular enzymes in actinomycetes; for instance that of xylanase C (Shareck *et al.*, 1991) or β -galactosidase (Eckhardt *et al.*, 1987), both from *S. lividans*. However, the proposed initiation codon determines a

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      -35          -10  <----->
1  GCAACATGGCGCCAGGGCCTTGCGCGGTGGTGGCGTGAACGCTTCAATCTAGTTAGGAACTTTCTTACTCTCTCA
      RBS
81  TGGGTCCGGAGACCCCCATGCCCGGACCAGGAAACGAGCGCCCCATGCGTCTCAAGCACCCACCGCCCGGCTCGCC
      ****      ***                      M R L K H P T A R L A -31
                                     NotI
161 CTGCGCGCCCTGCTGGTTCGCGCTCCCGCGCAGTGTGCGCGCGCGCGCACCGTCCACGCGCCCGGCCCGCCGCGCGC
      L A A L L V A V P R S V A A A G T V H A A P A P A G A -4
241 CACCCGGCTCGCGCGCGCTCGGGCTGGACGACCCGCACAAGAAGGACATCGCCATGCAGCTGGTGTCCAGCGCCGAGAAT
      T R L A A V G L D D P H K K D I A M Q L V S S A E N S 24
      -1 1
321 CCTCCCTCGACTGGAAGTCCAGTACAAGTACATCGAGGACATCAAGGACGGCGCGGTACACCGCCGGCATCATCGGC
      S L D W K S Q Y K Y I E D I K D G R G Y T A G I I G 50
                                     SacII
401 TTCTGTTCCGGCACCGCGCATGCTCGACCTGGTTCGCGAGTACACCGACCTCAAGCCGGGCAACATCCTCGCCAAGTA
      F C S G T G D M L D L V A D Y T D L K P G N I L A K Y 77
481 CCTCCCGCGCTCGCGCAAGGTCAACGGCACCGAGTCGCACCGCGCTCGCGTCCGCCTTCGAGAAGGACTGGGCCACCG
      L P A L R K V N G T E S H A G L A S A F E K D W A T A 104
                                     SacII
561 CCGCCAAGGACAGCGTCTTCCAGCAGGCCAGAACGACGAGCGGACCGCTCGTACTTCAACCCCGGTCACACGAGCC
      A K D S V F Q Q A Q N D E R D R S Y F N P A V N Q A 130
641 AAGGCGAGCTGCGCGCGCTCGGCCAGTTTCGCTACTACGACGCGATCGTCATGCACGGCCCGCGGACGCTCGGACG
      K A S L R A L G Q F A Y Y D A I V M H G P G D S S D S 157
721 CTTCGGCGGCATCCGCAAGGCCCGCATGAAGAAGGCCAAGACCCCGCCAGGGCCGCGACGAGGCCACCTACCTCAAGG
      F G G I R K A A M K K A K T P A Q G R D E A T Y L K A 184
                                     SalI
801 CCTTCTGGCGGCCCGCAAGACCGTGATGCTGAAGGAGGAGGCCACTCGGACACCGCGGTTCGACACCGAGCAGACG
      F L A A R K T V M L K E E A H S D T S R V D T E Q T 210
881 GTGTTCTCAACGCCAAGAACTTCGACCTCAACCCCGCGCTGAAGTGAAGGTCTACGGGGACAGCTACGCCATCAACAG
      V F L N A K N F D L N P P L K W K V Y G D S Y A I N S 237
961 CTGACGGTCCGCCGGGTGGCCGGAGGCGCGCGCGCGCGCGCTGCCGTGTCCCGAACGCACGCACGGCGCACCGTAAT
      *
      <----->
1041 TCGGATGACGGTCAACGG

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Fig. 3. Nucleotide sequence and deduced amino acid sequence of the *csnN106* gene from *Nocardioides* sp. N106. Underlined amino acids were determined by N-terminal amino acid sequencing of the purified chitosanase. Amino acids are numbered from the first residue of the mature chitosanase form. The putative RBS is indicated. Asterisks indicate an alternative in-frame initiation codon and the corresponding RBS. An inverted-repeat sequence upstream from the coding sequence as well as the terminator-like sequence downstream of the coding sequence are indicated by horizontal dashed arrows.

signal peptide that is much more similar to a typical actinomycete signal peptide (von Heijne & Abrahmsén, 1989). A putative transcription terminator was found immediately downstream from the ORF (Fig. 3).

Homology with other chitosanases

The nucleotide sequence of the *csnN106* gene shown on Fig. 4 is 79.5% homologous to the corresponding DNA segment of the *csn* gene of *Streptomyces* sp. N174 (nt 106–1164; Masson *et al.*, 1994). At the amino acid level, the homology determined by the BESTFIT program at standard settings (Devereux *et al.*, 1984) is 74.4%. Thus the N106 chitosanase is much more closely related to the *Streptomyces* N174 chitosanase than to the chitosanase from *B. circulans* MH-K1 (Ando *et al.*, 1992), which was only 25–29% (depending of the setting of the BESTFIT program) homologous to the N174 chitosanase.

The close relationship between chitosanases from *Nocardioides* sp. N106 and *Streptomyces* sp. N174 becomes even more apparent when the sequences of both mature proteins are examined by the HCA program (Fig. 4). There is strict conservation of a clear majority of the

hydrophobic clusters visualized by the program, which can be interpreted in terms of a strict conservation of the structural features in both proteins (Gaboriaud *et al.*, 1987; Lemesle-Varloot *et al.*, 1990).

Concluding remarks

With the addition of the chitosanase from *Nocardioides* sp. N106, family 46 of glycosyl hydrolases becomes better defined, including at least two members with high (~75%) homology at the amino acid level. The sequence of the chitosanase from *B. circulans* MH-K1 can be aligned with the two actinomycete chitosanases to give a similarity of 45–49% according to the BESTFIT program, but these alignments are highly sensitive to the program settings and we do not consider them significant.

The only segment that is clearly homologous in all three chitosanases is the previously identified N-terminal module (Masson *et al.*, 1994). Interestingly, all the amino acids in this module that are conserved between the *B. circulans* MH-K1 chitosanase and the *Streptomyces* sp. N106 chitosanase are also conserved in the *Nocardioides* sp. N106 chitosanase. The consensus pattern we propose as the

Fig. 4. Alignment of HCA plots of chitosanases from *Streptomyces* sp. N174 and *Nocardioideis* sp. N106. Numbering starts from the first amino acid of the mature protein. Symbols as in Gaboriaud *et al.* (1987): ★, proline; ◆, glycine; □, serine; □, threonine.

'family 46 signature' is (using the PROSITE notation; Bairoch, 1992): GLD-x(6)-I-x(0,1)-M-x-L-x(4)-E-x(3)-L-x-W-x(3)-Y-x-Y-x-E-D-I-x-D-x-R-G-Y-T-x-G-x(2)-G. Recent evidence from site-directed mutagenesis experiments in our laboratory indicate that this module includes amino acids essential for the catalytic activity of chitosanase (I. Boucher & R. Brzezinski, unpublished observations).

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