Comparative studies of chitinases A and B from Serratia marcescens

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*Serratia marcescens* produces several chitinolytic enzymes, including chitinase A (ChiA) and chitinase B (ChiB). In this study, ChiB was purified to homogeneity using a newly developed protocol based on hydrophobic interaction chromatography. Subsequently, characteristics of ChiB and of the hitherto only partly characterized ChiA were determined and compared. Pure ChiA and ChiB shared several characteristics such as a broad pH optimum around pH 5.0, and a temperature optimum between 50 and 60 °C. Both enzymes were fairly stable, with half-lives of more than 10 d at 37 °C, pH 6.1. Analyses of the degradation of various N-acetylglucosamine oligomers, fluorogenic substrates and colloidal chitin showed that both enzymes cleave chitobiose ([GlcNAc]₂) from (GlcNAc)ₙ and thus possess an exo-N,N'-diacetylchitobiohydrolase activity. Both enzymes were also capable of producing monomers from longer (GlcNAc)ₙ substrates, indicating that they also have an endochitinase (ChiA) or exo-N,N',N''-triacetylchitotriohydrolase (ChiB) activity. Kinetic analyses with 4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside, an analogue of (GlcNAc)₂, showed cooperative kinetics for ChiA, whereas for ChiB normal hyperbolic kinetics were observed. ChiA had a higher specific activity towards chitin than ChiB and synergistic effects on the chitin degradation rate were observed upon combining the two enzymes. These results, together with the results of sequence comparisons and previous studies of the cellular localization of the two chitinases in *S. marcescens* indicate possible roles for ChiA and ChiB in chitin breakdown.

**Keywords:** chitinase, *Serratia marcescens*, chitin, chitobiosidase

**INTRODUCTION**

Several bacteria and fungi are capable of enzymic degradation of chitin, the (1→4)-β-linked polymer of N-acetyl-β-D-glucosamine (GlcNAc), for autolytic, morphogenetic or nutritional purposes (Gooday, 1990). Since chitin is a major structural component of fungal cell walls, chitinolytic enzymes could in principle be employed as natural anti-fungal agents and are therefore of biotechnological importance (Shapira et al., 1989; Schickler et al., 1993; Lorito et al., 1994). The conversion of chitin to GlcNAc involves several hydrolases. Chitinases (EC 3.2.1.14), also called endochitinases, cleave glycosidic linkages randomly along the chitin chain, eventually producing short oligomers of GlcNAc (Monreal & Reese, 1969; Robbins et al., 1988; Roberts & Selitrennikoff, 1988; De la Cruz et al., 1992). Exo-N,N'-diacetylchitobiobiohydrolases, also called exochitinases or chitobiosidases, cleave off chitobiose (GlcNAc₂) from the non-reducing end of (GlcNAc)ₙ (Davis & Eveleigh, 1984; Robbins et al., 1988; Tronsmo & Harman, 1993). Finally, N-acetyl-β-glucosaminidases (EC 3.2.1.52) cleave off GlcNAc monomers from the non-reducing ends of chitobiose and higher analogues (Monreal & Reese, 1969; Robbins et al., 1988).

*Serratia marcescens*, a Gram-negative bacterium belonging to the family Enterobacteriaceae, is one of the most efficient bacteria for degradation of chitin (Monreal & Reese, 1969; Schickler et al., 1993). It may produce up to five different chitinolytic enzymes upon induction with chitin (Fuchs et al., 1986). The best known of these is the secreted chitinase A (ChiA), the three-dimensional struc-
ture of which has been described (Perrakis et al., 1994). However, knowledge of the enzymic properties of ChiA is very limited (Monreal & Reese, 1969; Roberts & Cabib, 1982; Roberts & Selitrennikoff, 1988; Vorgias et al., 1993) and most of this knowledge is derived from studies with partly purified enzyme preparations containing an incompletely defined mixture of chitinases (Monreal & Reese, 1969; Roberts & Cabib, 1982). Genes encoding ChiA have been isolated from various strains of S. marcescens, and their nucleotide sequences have been determined (Jones et al., 1986; Koo et al., 1993; Brurberg et al., 1994). Harpster & Dunsmuir (1989) and Brurberg et al. (1995) have described two almost identical S. marcescens genes encoding a periplasmic chitinolytic enzyme, called chitinase B (ChiB). Little is known about the properties of the latter enzyme and its purification has not been described. ChiA (Mr 58514) and ChiB (Mr 55469) share 32% sequence identity (Brurberg et al., 1994, 1995). Both enzymes are able to inhibit fungal growth under some conditions (Sundheim et al., 1988; Shapira et al., 1989; Schickler et al., 1993).

Apart from a few characteristics of ChiA, little is known about the properties of the individual S. marcescens chitinases. The present report describes a detailed study of ChiA and ChiB produced by S. marcescens BJL200 (Sundheim et al., 1988). This study was conducted to elucidate the individual roles of ChiA and ChiB in the degradatory pathway for chitin and, furthermore, to determine general enzyme characteristics, such as stability and catalytic properties, that could be of importance for the application of these enzymes. ChiA and ChiB were purified from recombinant Escherichia coli strains over-expressing the previously cloned cbiA (Brurberg et al., 1994) and cbiB (Brurberg et al., 1995) genes. Since the purification of ChiB had not been described previously, an efficient protocol for the purification of this enzyme was developed.

**METHODS**

**Purification of the chitinases.** ChiA and ChiB were purified from periplasmic extracts of early stationary phase cultures of E. coli harbouring plasmid pMAY20-1 (Brurberg et al., 1994) and E. coli harbouring plasmid pMAY2-10 (Brurberg et al., 1995), respectively. The extracts were prepared by osmotic shock, as described by Manoil & Beckwith (1986). The volume of the periplasmic extracts was one-tenth of the volume of the original culture.

All chromatographic media, columns and equipment were supplied by Pharmacia-LKB. The purification of ChiA has been described elsewhere (Brurberg et al., 1994). In the optimized purification procedure for ChiB, the periplasmic extract (0.65 mM MgCl2, 0.1 mM PMSF, 1 mM EDTA) was diluted 1:4-fold and adjusted to 20 mM Tris/HCl, pH 8.0 and 0.4 M ammonium sulphate. From this dilution, 2 ml was loaded onto a Phenyl-Superose HR 5/5 column (5 x 50 mm) in an FPLC system, equilibrated in buffer A (20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF) containing 0.4 M ammonium sulphate. After loading the sample, the column was washed with the starting buffer followed by a 5 ml linear gradient of 0-4 M ammonium sulphate. Subsequently, a linear gradient of 0-6% (v/v) 2-propanol was applied (see Fig. 1). The same purification procedure could also be performed successfully using a volatile buffer system (25 mM ammonium bicarbonate, pH 8.0; no addition of PMSF and EDTA).

Enzyme purity was verified by SDS-PAGE, essentially according to Laemmli (1970). The gels were stained with Coomassie brilliant blue G-250. The purified enzymes were identified by N-terminal amino acid sequencing, using an Applied Biosystems automatic sequenator, model 477A.

**Enzyme assays.** Chitinolytic activity was determined using the (GlcNAc)₄₅₅ analogue 4-methylumbelliferyl-β-d-N,N′-diacetylchitobioside [(4-MU)-(GlcNAc)₂; Sigma; Kuranda & Robbins, 1987]. Standard reactions mixtures contained 69 µM 4-MU-(GlcNAc)₅, 0.1 mg ml⁻¹ purified BSA (New England Biolabs) and the enzyme, in 50 mM citrate phosphate buffer, pH 6.1 (Stoll & Blanchard, 1990). The reaction mixture (total volume 100 µl) was incubated at 37°C for 10 min, after which the reaction was stopped by adding 1:9 ml 0.2 M Na₂CO₃. The product formation was linear with time during the reaction. The 4-methylumbelliferosine (4-MU) moiety is fluorescent when it becomes ionized above pH 8 and after excitation at 380 nm, 4-MU emits at a wavelength of 460 nm. The amount of 4-MU released was determined with a TKO 100 Mini Fluorimeter using a 4-MU solution as standard (Hoefler Scientific Instruments). The activities were determined as the mean of at least three independent measurements and expressed as the amount of 4-MU released min⁻¹. Standard deviations were less than 3% of the means in all cases.

Activity towards 4-MU-GlcNAc and 4-MU-(GlcNAc)₅ was determined using the same conditions as for 4-MU-GluNAc. Cleavage of 4-MU-(GlcNAc)₅ into (GlcNAc)₅ and 4-MU-GlcNAc (undetectable) was monitored by measuring the amount of substrate remaining after the incubation. The substrate was measured by adding excess purified ChiA, which converts 4-MU-(GlcNAc)₅ almost exclusively into 4-MU and GlcNAc mono- and oligomers (see Results).

Variation in the pH in the assays was achieved by replacing the standard pH 6.1 buffer with the following buffers (50 mM): citrate/phosphate (pH 2.6-6.6), phosphate (pH 6.2-8.0) and Tris/HCl (pH 7.6-8.8) (Stoll & Blanchard, 1990). The standard pH 6.1 citrate/phosphate buffer was replaced by 50 mM MES, pH 6.1, in reaction mixtures containing metal ions.

Kinetic properties were determined by initial rate measurements at various 4-MU-(GlcNAc)₅ concentrations using the standard assay conditions described above, except for a larger reaction volume. Reactions were initiated by the addition of the enzyme to a final concentration of 0.19 nM or 0.67 nM, for ChiA and ChiB, respectively. Product formation was monitored by measuring fluorescence in 100 µl samples that were transferred from the reaction mixture to 1.9 ml 0.2 M Na₂CO₃ at different time points. Under these conditions product formation was linear with time for at least 10 min, at all substrate concentrations used. Initial rates (v) were determined using at least six time points within the first 10 min after adding the enzyme to the reaction mixture. All product formation curves obtained by linear regression had correlation coefficients over 0.999. Where appropriate, the data were fitted to the Michaelis–Menten model, using a program minimizing deviations in log v (cf. Cleland, 1963).

Protein concentrations were determined by the method of Bradford (1976), using BSA as a standard.

**Analysis of hydrolysis products of GlcNAc oligomers and chitin.** ChiA (15 µg ml⁻¹) and ChiB (10 µg ml⁻¹) were incubated for various times with 2-4 µg of a GlcNAc oligomer ml⁻¹ [(GlcNAc)₅; Seikagaku Kogyo Co.; Sigma] in 50 mM citrate/
phosphate buffer (pH 6·1), at 37 °C. The hydrolysis products were separated by TLC using a silica gel 60 plate (Merck) and a solvent system of 1-butanol/ethanol/water (5:3:2, by vol.) (John et al., 1993). Sugars were detected by spraying with a diphenylamine/aniline/orthophosphoric acid reagent (Walkley & Tillman, 1977).

Colloidal chitin was prepared according to Vessey & Pegg (1973) and used as a substrate for the chitinases. The enzymes were incubated at 37 °C with 0·19 mg chitin ml⁻¹, in 50 mM citrate/phosphate buffer (pH 6·1), 0·1 mg BSA ml⁻¹. The reaction was followed by determining the turbidity of the chitin suspensions at 410 nm. The release of reducing groups was determined using the method described by Imoto & Yagishita (1971).

RESULTS

Purification of ChiA and ChiB

Several chromatographic methods were tested for the purification of ChiB from periplasmic extracts of E. coli harbouring pMAY2-10. Cation and anion exchange appeared not to be suitable, since no satisfactory binding

Fig. 1. Purification of ChiB. Hydrophobic interaction chromatography was performed as described in Methods. At the point indicated by the arrow, the sensitivity of the UV detector was increased by a factor of 2.5. Chitinase activity was only detected in the peak at 16 ml. ---, A₄₅₀; ----, ammonium sulphate concentration; -------, 2-propanol concentration.

of chitinolytic activity to SP-Sepharose (pH tested 5·0–6·9) or Q-Sepharose (pH tested 6·9–8·9) was obtained. ChiB adsorbed efficiently to Phenyl-Sepharose CL-4B at low concentrations of ammonium sulphate and therefore a hydrophobic interaction chromatography protocol was developed. Fig. 1 shows the elution profile obtained using the optimized conditions for hydrophobic interaction chromatography described in Methods. All chitinase activity adsorbed to the column and was eluted as a sharp peak at 16 ml (Fig. 1). A typical experiment yielded 70–90 µg chitinase, the recovery of enzyme activity being over 90% (Table 1). SDS-PAGE analysis (Fig. 2) showed that the peak fractions contained highly pure protein, with an Mr in good agreement with that predicted from the sequence of the cbiB gene (55469; Brurberg et al., 1995). Similar results were obtained when a volatile buffer system was used (Fig. 2, lane 3). ChiA was purified as described previously (Brurberg et al., 1994) and the

![Image](Fig. 2. SDS-PAGE analysis of purified ChiB and ChiA. Lanes 1–3, ChiB; lanes 4 and 5, ChiA. Lane 1, undiluted periplasmic extract of E. coli harbouring pMAY2-10; lane 2, purified ChiB; lane 3, fraction corresponding to that in lane 2, but obtained after hydrophobic interaction chromatography using a 25 mM ammonium bicarbonate buffer, followed by concentration by freeze-drying; lane 4, undiluted periplasmic extract of E. coli harbouring pMAY20-1; lane 5, purified ChiA. The approximate positions of marker proteins are indicated.)

<p>| Table 1. One-step purification of ChiB by hydrophobic interaction chromatography |
|---------------------------------|-------------|-------------------|------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Total protein (µg)</th>
<th>Total activity* (nmol s⁻¹)</th>
<th>Recovery of activity (%)</th>
<th>Specific activity (nmol s⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted periplasmic extract</td>
<td>2000</td>
<td>955</td>
<td>11·7</td>
<td>100</td>
</tr>
<tr>
<td>Peak fractions</td>
<td>620</td>
<td>78</td>
<td>11·2</td>
<td>96</td>
</tr>
</tbody>
</table>

* Chitinase activity is expressed as the amount of 4-MU released per unit of time; standard deviations in the activity measurements did not exceed 3% of the means (n = 3).
identities of purified ChiA and ChiB were confirmed by N-terminal amino acid sequencing.

**Optimal conditions for enzyme action**

At the standard assay temperature of 37 °C, both ChiA and ChiB were active towards 4-MU-(GlcNAc)$_2$ in a broad pH range, with maximum activity occurring between pH 5.0 and pH 6.0. The enzymes retained 50% of their activity in a broad pH range (3.5–8.4 for ChiA and 3.2–8.2 for ChiB). At pH 6.1, maximum activity occurred between pH 50 and 60 °C, the optima being 54 and 58 °C, for ChiA and ChiB, respectively. At least 50% of the maximum activity of ChiA and ChiB was obtained at 39–62 °C and 46–66 °C, respectively.

Under standard assay conditions (37 °C, pH 6.1) the activity of ChiA and ChiB was not affected by the presence of 10 mM divalent metal ions (MgCl$_2$, CaCl$_2$, CoCl$_2$, CuSO$_4$, and MnCl$_2$ were tested), nor by the presence of 20 mM EDTA. The activity of both enzymes was reduced by more than 99% in the presence of 10 mM AgNO$_3$. Varying the NaCl concentration in the reaction mixtures from 0 to 0.5 M did not result in changes in activity. ChiA activity was reduced to 30% in the presence of 1:2 mM (GlcNAc)$_2$. Under the same conditions, ChiB displayed 91% activity.

**Enzyme stability**

Purified chitinases [50–200 µg ml$^{-1}$, in elution buffer: 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, 2–4% (v/v) 2-propanol] could be stored at 4 °C for several months without significant loss of activity. After dilution to working concentrations (0.1–2 µg ml$^{-1}$, pH 6.1) a rapid loss of activity was observed, which could be prevented by the addition of 0.1 mg BSA ml$^{-1}$ to the dilution buffer. Therefore, all solutions used for assaying enzyme activity in this study contained 0.1 mg BSA ml$^{-1}$.

Thermal inactivation experiments showed that ChiA and ChiB were quite stable at pH 6.1, but at temperatures over 50 °C stability was reduced considerably. ChiA was the more stable of the two chitinases, its half-life being 5–10-fold higher than that of ChiB at temperatures between 48 and 54 °C. At 37 °C, the half-lives of ChiA and ChiB were > 400 h and 280 h, respectively.

**Degradation of GlcNAc oligomers, synthetic substrates and chitin**

Analyses of the cleavage of (GlcNAc)$_n$ showed that both ChiA and ChiB convert (GlcNAc)$_3$ into (GlcNAc)$_2$ or into a mixture of (GlcNAc)$_2$ and GlcNAc, depending on the substrate (Fig. 3), but do not digest (GlcNAc)$_2$. ChiA exclusively cleaved (GlcNAc)$_2$ from the substrates, as shown by the lack of GlcNAc and (GlcNAc)$_3$ in both the partial and the complete digestion patterns obtained with (GlcNAc)$_4$ and (GlcNAc)$_5$ as substrates (Fig. 3a, c). Figs 3(b) and 3(d) show that ChiB also primarily produced (GlcNAc)$_2$ from the substrates. However, as shown by the faint spots at the (GlcNAc)$_3$ position in Fig. 3(d), lane 6, and the GlcNAc position in Fig. 3(b), lane 6, ChiB also cleaved (GlcNAc)$_3$ into (GlcNAc)$_2$ [subsequently converted into (GlcNAc)$_2$ and GlcNAc]. (GlcNAc)$_2$ and (GlcNAc)$_3$ were not detectable at any stage of the reaction of ChiB with (GlcNAc)$_4$.

Neither ChiA nor ChiB released 4-MU from the (GlcNAc)$_2$ analogue 4-MU-GlcNAc, confirming the conclusion from the TLC analyses above that neither of the enzymes was capable of converting (GlcNAc)$_2$ into monomers. Upon incubation of ChiA with the (GlcNAc)$_4$ analogue 4-MU-(GlcNAc)$_2$, more than 98% of the substrate was converted into 4-MU plus GlcNAc monomers. 4-MU was released at a rate comparable to that of the conversion of 4-MU-(GlcNAc)$_2$. In contrast to ChiA, ChiB did not release 4-MU from 4-MU-(GlcNAc)$_3$ but converted it into (GlcNAc)$_2$ plus the non-fluorogenic 4-MU-GlcNAc, at a rate that was approximately 10-fold higher than its rate of conversion of 4-MU-(GlcNAc)$_3$.

TLC analysis of the digestion products of colloidal chitin showed that both ChiA and ChiB degrade chitin to (GlcNAc)$_2$ and a minor fraction of GlcNAc (results not shown). Differences between ChiA and ChiB in terms of reaction products seen in TLC analysis were not detectable at any stage of the reactions. Furthermore the ratio between the decrease in turbidity of the chitin suspension and the release of reducing groups was approximately the
Table 2. Degradation of chitin by ChiA and ChiB

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Degradation (%)</th>
</tr>
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<tbody>
<tr>
<td>ChiA (1.8 µg)</td>
<td>26 ± 2.2</td>
</tr>
<tr>
<td>ChiA (3.6 µg)</td>
<td>36 ± 2.3</td>
</tr>
<tr>
<td>ChiB (1.8 µg)</td>
<td>16 ± 3.6</td>
</tr>
<tr>
<td>ChiB (3.6 µg)</td>
<td>21 ± 1.9</td>
</tr>
<tr>
<td>ChiA (1.8 µg) + ChiB (1.8 µg)</td>
<td>65 ± 3.7</td>
</tr>
</tbody>
</table>

The indicated amounts of enzyme were incubated with colloidal chitin in a total volume of 1 ml for 24 h at 37 ºC, as described in Methods. Chitin degradation is expressed as the percentage reduction in turbidity of the reaction mixture. Values are means ± SD for three determinations.

same for both enzymes (differences in this ratio could indicate differences between the exo/endo character of the enzymes; Tronsmo & Harman, 1993). In contrast with this apparent similarity of the two enzymes, clear synergistic effects on the rate of degradation of chitin were observed when ChiA and ChiB were combined (Table 2). Consequently, the two chitinases must differ in the way they contribute to the degradation of chitin, as already indicated by the differences observed with synthetic substrates and GlcNAc oligomers (see above). Another difference between the two enzymes is illustrated by the fact that ChiA has a higher specific activity towards chitin than has ChiB (Table 2).

Kinetic properties

The assay for chitinolytic activity with the (GlcNAc)₄ analogue 4-MU-(GlcNAc)₄ is sensitive and reliable, and permitted detailed kinetic analyses. As illustrated by Fig. 4, both chitinases were inhibited at high concentrations of the substrate. At the lower substrate concentrations, ChiA exhibited sigmoid kinetics, indicating cooperativity (Fig. 4a), whereas ChiB displayed a normal hyperbolic relationship between the rate of catalysis, v, and substrate concentration, [S] (Fig. 4b). Kinetic parameters for ChiB were approximated using eight points at low substrate concentration (1.5–8 µM), resulting in a Kₘ of 34±1±4 µM and a kₗ of 19±1±7 s⁻¹.

The occurrence of substrate inhibition by an unknown mechanism prevented quantitative analysis of the sigmoid kinetics observed for ChiA. In an attempt to approximate kinetic parameters for ChiA, possible values for the apparent Hill coefficient (b) were tested by analysing the linearity and the distribution of residual errors in adapted Lineweaver–Burk plots (1/v versus 1/[S]ⁿ), for [S] < 12 µM; Segel, 1975). This approach indicated an b in the range 1.1–1.2. If b is known, [S]ₚₜ (S at which v = 0.5 Vₚₜ and Vₚₜ can be derived from the v and [S] values belonging to the maximum in the Eadie–Scatchard plot (Segel, 1975). The maximum in the Eadie–Scatchard plot (Fig. 4a) occurs at [S] = 26 µM, v = 13.5 ± 9 s⁻¹, v/[S] = 523 s⁻¹ nM⁻¹, which, assuming an b of 1.15, results in [S]ₚₜ = 135 µM and kₑₗ = 104 s⁻¹. The values for [S]ₚₜ and kₑₗ derived from the Eadie–Scatchard plot are an underestimation of the real values, since substrate inhibition lowers the v and [S] values at which the maximum in this plot occurs.

Sequence comparisons

Several authors have pointed out that prokaryotic chitinases are built up of regions (domains) that are recognizable by sequence similarities. Such regions are the signal sequence, the catalytic domain, and the so-called fibronectin type III-like domain (Bork & Doolittle, 1992; Watanabe et al., 1993, 1994; Blaal et al., 1993). The catalytic domain and the fibronectin type III-like domains are important for activity against chitin (Watanabe et al., 1994). Blaal et al. (1993) concluded that ChiB consists of a catalytic domain and a C-terminal domain of approximately 60 residues that
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Enzymic properties of ChiA

Roberts & Selitrennikoff (1988) showed that ChiA converts chitin into (GlcNAc)_3 only. The enzyme also converted β-nitrophenyl-β-α-N,N'-diacetylchitobioside [comparable to 4-MU-(GlcNAc)_3] into the nitrophenyl group and (GlcNAc)_3, which is considered to be characteristic for exo-N,N'-diacetylchitobiodyrolases (Robbins et al., 1988; Tronsmo & Harman, 1993). In the present study, these observations were only partly confirmed. In accordance with previous results, ChiA converted (GlcNAc)_4 and (GlcNAc)_6 into (GlcNAc)_3 only. However, noticeable amounts of GlcNAc were produced upon incubation of ChiA with colloidal chitin, indicating that ChiA possesses a second activity, for example that of an endochitinase. Endochitinolytic cleavages would result in the production of shorter GlcNAc oligomers that can be converted into mixtures of (GlcNAc)_2 and GlcNAc. Alternatively, the production of GlcNAc from chitin might indicate the capability to cleave off (GlcNAc)_3 [subsequently converted into (GlcNAc)_2 and GlcNAc] from the chitin chains by an exo-type action (exo-N,N',N''-triacetylchitotrihydrolase). Activities similar to the latter have been suggested for a Clostridium stercorarium exoglucanase (Bronnenmeier et al., 1991) and for a human chitinase (Renkema et al., 1995).

The second activity (endochitinase or exo-N,N',N''-triacetylchitotrihydrolase) of ChiA is not reflected by the presence of detectable levels of trimers or monomers in the partial and full digests of (GlcNAc)_4. An explanation for this might be that the substrate-binding site of ChiA can bind up to six GlcNAc moieties and that the catalytic centre is positioned between the fourth and the fifth GlcNAc binding-site (counted from the reducing end of a hexamer substrate; Perrakis et al., 1994). Thus, substrates containing six or less sugar units can bind to the active site with all their GlcNAc moieties, meaning that it is unlikely that oligomers longer than (GlcNAc)_3 are cleaved off from the non-reducing end. The extended active site in ChiA (which is a monomeric enzyme; Perrakis et al., 1994) might permit the simultaneous binding of two or more 4-MU-(GlcNAc)_3 molecules, which is a possible explanation for the cooperative kinetics observed with this substrate.

The fact that ChiA is capable of cleaving off 4-MU from 4-MU-(GlcNAc)_3 could be interpreted as a confirmation of its endochitinase or exo-N,N',N''-triacetylchitotrihydrolase activity (Robbins et al., 1988; Tronsmo & Harman, 1993). It is puzzling, however, that almost no cleavage into 4-MU-GlcNAc + (GlcNAc)_3 occurs, where-

**DISCUSSION**

**General characteristics**

Purified ChiA and ChiB share several characteristics such as a broad pH optimum, a temperature optimum between 50 and 60 °C, and high stability. The less stable enzyme (ChiB) had the higher optimum temperature for activity. The optimum temperature for ChiB is probably underestimated since the enzyme is expected to undergo significant thermal inactivation at temperatures near 60 °C within the time-scale of the activity assay. The activity of both ChiA and ChiB was unaffected by the presence of divalent metal ions (10 mM) and by the presence of NaCl (0-05 M). In summary, the results concerning activity and stability indicate that both enzymes are reasonably robust and active under a great variety of conditions. Inhibition of ChiA by its product [(GlcNAc)_3] at concentrations around 1 mM might limit the industrial applicability of this enzyme in some cases.
as the natural substrate \((\text{GlcNAc}_2)\) is converted into \((\text{GlcNAc}_3)\) only. Furthermore, 4-MU-\((\text{GlcNAc}_2)\) is produced at approximately the same rate as when 4-MU-\((\text{GlcNAc}_3)\) is used as a substrate, which contrasts with the observation that ChiA primarily produces \((\text{GlcNAc}_3)\) and not \((\text{GlcNAc}_2)\). These conflicting observations indicate that the cleavage rates and patterns obtained with the artificial 4-MU-chito-oligosaccharides do not (or only partly) reflect the real action of the chitinases on chitin and \((\text{GlcNAc}_n)\) (see also Robbins et al., 1988; Watanabe et al., 1990a; Takayanagi et al., 1991). Nevertheless, comparisons of the cleavage rates of various 4-MU substrates are still used to characterize the catalytic activity of chitinases (Tronsmo & Harman, 1993; Renkema et al., 1995).

The presence of an extended substrate binding site suggests that ChiA is optimized for the degradation of longer chito-oligosaccharides and chitin. This is supported by the presence of the N-terminal fibronectin type III-like domain in ChiA that is known to have a positive effect on the activity of chitinases towards chitin (Watanabe et al., 1994). Furthermore, ChiA possesses a region sharing some similarity with part of the chitin-binding domain identified in \(B.\ circulans\) chitinase A1 (Watanabe et al., 1994; Fig. 5).

### Enzymic properties of ChiB and comparison with ChiA

ChiB was found to be similar to ChiA in that its main product was \((\text{GlcNAc}_3)\) for all natural substrates tested. In the case of ChiB, in contrast to ChiA, the endochitinase or exo-\(N,N',N''\)-triacetylchitotriohydrolase activity that causes the formation of monomers in the reaction with chitin was confirmed by the detection of \((\text{GlcNAc}_3)\) and \(\text{GlcNAc}\) as products of the reaction with \((\text{GlcNAc}_3)\). It is tempting to speculate that the substrate-binding site of ChiB is designed to bind less than six \text{GlcNAc} moieties. This could explain why the endochitinase or exo-\(N,N',N''\)-triacetylchitotriohydrolase activity of ChiB is detectable in the reaction with \((\text{GlcNAc}_3)\). Several other results support the notion that ChiB possesses a less extended substrate-binding site than ChiA and, consequently, that ChiB may be optimized for cleavage of relatively short chito-oligosaccharides. First, ChiB has a lower specific activity towards chitin than ChiA and it does not contain a fibronectin type III-like domain as does ChiA. Second, ChiB does not exhibit the cooperative kinetics that were observed for ChiA. Finally, ChiB seems to have a higher affinity for the \((\text{GlcNAc}_3)\) analogue than ChiA: whereas ChiB has a \(K_m\) of 34-1 \(\mu M\) for 4-MU-\((\text{GlcNAc}_2)\), the \([S]_{50}\) of ChiA for 4-MU-\((\text{GlcNAc}_2)\) was about 100 \(\mu M\). Vorgias et al. (1993), determined the \(K_m\) of ChiA for another \((\text{GlcNAc}_3)\) analogue to be as high as 500 \(\mu M\). However, in the latter study, the problem of substrate inhibition (which is expected to occur: see above and Krishnan et al., 1994) was not addressed.

Analysis of the breakdown products of chitin did not reveal differences between ChiA and ChiB. This is likely to be due to the fact that only minor, undetectable amounts of products other than \text{GlcNAc} and \((\text{GlcNAc}_2)\) accumulate during chitin degradation. Likewise, and possibly for the same reason, no differences in the ratio between reducing sugar formation and degradation rate were found. Furthermore, the reducing sugar assay may provide artificial results since the signal obtained per reducing group increases with the length of the chito-oligosaccharides (Imoto & Yagishita, 1971; M. B. Brurberg, unpublished observations). The observation that combining ChiA and ChiB results in synergistic effects on the rate of chitin degradation clearly shows that the enzymes not only differ in terms of their specific activity but also in terms of the mechanism by which they degrade the substrate. An obvious explanation for the synergy would be that one of the enzymes, by exerting an endochitinase activity, increases the substrate availability for the other enzyme, which has an exo-\(N,N',N''\)-triacetylchitotriohydrolase activity.

To permit a more detailed analysis of the differences between ChiA and ChiB, we are currently conducting X-ray crystallographic studies of ChiB prepared using the purification protocol described in this report. The availability of the ChiB structure would permit a structural comparison which ChiA (Perrakis et al., 1994) which might shed new light on the function of these enzymes and could provide a structural explanation for the differences between them [see Davies & Henrissat (1995) for a review of existing structures of glycosyl hydrolases].

### The role of ChiA and ChiB in chitin breakdown

The analyses described above show both similarities and differences in the enzymic properties of ChiA and ChiB. Both enzymes seem to have exo-\(N,N',N''\)-diacetylchitobiohydrolase activity, combined with either an endochitinase or an exo-\(N,N',N''\)-triacetylchitotriohydrolase activity. In \(S.\ marcescens\) ChiA is located extracellularly (Brurberg et al., 1994) and therefore is likely to be optimized for degrading chitin and the longer \text{GlcNAc} oligomers, as suggested above. Considering its localization, the presence of domains known to be important for chitin degradation, its extended substrate-binding site, and its high specific activity towards chitin (as compared to ChiB), ChiA is likely to possess an endochitinase activity in addition to its exo-\(N,N',N''\)-diacetylchitobiohydrolase activity. Consequently, ChiB's second activity is expected to be that of an exo-\(N,N',N''\)-triacetylchitotriohydrolase. ChiB has been found in the supernatant of late-stationary cultures of \(S.\ marcescens\) (Roberts & Cabib, 1982; Fuchs et al., 1986), but analysis of the localization of ChiB in exponential and early-stationary cultures of \(S.\ marcescens\) has shown that this enzyme is almost exclusively directed towards the periplasm (without being processed; Brurberg et al., 1995). Thus it would seem that the role of ChiB \textit{in vivo} is to digest the shorter \text{GlcNAc} oligomers capable of entering the periplasm, in accordance with the suggestions described above. The deduction that ChiA and ChiB possess an endochitinase and an exo-\(N,N',N''\)-triacetylchitotriohydrolase activity,
respectively, is supported by preliminary results of studies on the degradation of chitin by mixtures of ChiA or ChiB and an endochitinase isolated from the fungus Trichoderma harzianum (Tronsmo & Harman, 1993; M. B. Brurberg & A. Tronsmo, unpublished results). These studies showed no synergistic effects on chitin breakdown upon combining ChiA with the endochitinase, whereas clear synergy was observed upon combining ChiB with the endochitinase.

A third chitinolytic enzyme of S. marcescens, an N-acetyl-β-glucosaminidase, has been described by Kless et al. (1989) and Vorgias et al. (1993). This enzyme is probably located in the periplasm (Kless et al., 1989) where its obvious role would be to convert the chitobiase produced by ChiA and ChiB into GlcNAc monomers. From the present study it seems that an enzymatic apparatus consisting of ChiA, ChiB and an N-acetyl-β-glucosaminidase could be enough for effective in vivo degradation of chitin.

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