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Overexpression, Purification, and Characterization of a Thermostable Chitinase (Chi40) from *Streptomyces thermoviolaceus* OPC-520

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A new procedure for the large-scale purification of the recombinant thermostable chitinase (Chi40) cloned from *Streptomyces thermoviolaceus* in various expression vectors in *Escherichia coli* is described. Chi40 was overproduced in the cytosolic and secreted forms. The cytosolic form (Chi40c) was highly overproduced and purified by metal-affinity and ion-exchange chromatography in large amounts. The protein was highly active and thermostable but not homogeneous, since a considerable proportion of the Chi40c protein was not correctly folded as determined by native polyacrylamide gel electrophoresis. The Chi40 protein secreted into the culture medium (Chi40s) was purified by hydrophobic interaction and ion-exchange chromatography and high amounts of correctly folded and active Chi40 protein could be recovered in a short time. The enzymatic activity of Chi40s on a synthetic and on its natural substrate, chitin, was studied. Thermostability measurements showed that Chi40 has a T_m of 60.7°C at neutral pH. ^{13}C–^{15}N double-labeled recombinant Chi40s was also produced and purified from the pECHChi40-9 construct introduced into BL21*trx*B(DE3) cells grown in minimal medium in the presence of the paramagnetic elements [^{13}C]glucose and $^{15}NH_4Cl$. The presented data open the possibility of an extensive structural study on Chi40s by X-ray crystallography and on enzyme–substrate interaction by NMR spectroscopy.

Chitin, the insoluble linear β-1,4-linked polymer of *N*-acetyl-β-glucosamine, is degraded by chitin hydrolases, chitinases, which have been classified into families 18 and 19 of the glycosyl hydrolase superfamily, and chitobiases, to family 20. These families are based on primary structure comparisons and it is well established that enzymes that belong to the same family share several common properties in terms of folding of the catalytic domain, substrate specificity, and stereochemistry of the reaction as well as catalytic mechanism (1, 2).

Among the known chitinolytic enzymes, chitinases (EC 3.2.1.14) have been detected in a wide range of organisms such as fungi, crustaceans, and insects as well as in organisms that do not contain chitin such as archaea, bacteria, viruses, higher plants, animals, and humans (3–6).

The gram-positive soil bacteria of the genus *Streptomyces* produce, among other enzymes, a variety of enzymes to degrade naturally occurring macromolecules to survive in their environment. Within actinomycetes, *Streptomyces* spp. are particularly efficient in the breakdown of chitin by chitinolytic enzymes that facilitate chitin degradation in the soil, which is an excellent carbon and nitrogen source for many *Streptomyces* strains (7–15). During the past decade several reports described chitinolytic activities and the corresponding genes have been isolated and characterized. At the gene regulation level, several gene control elements of the chitinolytic system of *Streptomyces* that are subject to carbon and catabolite control have been detected and studied (16–18). The chitinolytic system of the thermostable bacterium *Streptomyces thermoviolaceus* OPC-520 consists of four chitinase genes (*chi*40, *chi*35, *chi*30,

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and *chi*25) and two *N*-acetyl-β-glucosaminidase genes (*nag*A and *nag*B). Comparisons of the deduced amino acid sequence of the four distinct chitinase genes have shown that the Chi40 and Chi30 proteins belong to family 18, while Chi35 and Chi25 belong to family 19 of the glycosyl hydrolases. These enzymes have been detected as active enzymes from *S. thermoviolaceus* OPC-520, while the *chi*35 gene product was inactive for chitin. It is interesting to mention that while the product of the *nag*A gene has been classified as a family 3 glycosyl hydrolase, immunoreactivity assay shows no detectable β-glycosidase activity, although it revealed homology with microbial β-glycosidases. The product of the *nag*B gene has been clearly assigned to family 20 of the glycosyl hydrolases (7–5),

The structure and mechanism of chitin degradation by chitinase A and B and *N*-acetyl-β-glucosaminidase (chitobiase) from the mesophilic soil bacterium *Serratia marcescens* are well studied (19–24). Unfortunately, so far, no structural data on thermostable chitinases are available. We have chosen this organism because the Chi40 is a suitable model molecule, because of its size and similarity to other chitinases, to study the molecular basis of its thermostability and the interaction with the natural inhibitor allosamidine that is derived from *Streptomyces* spp.

In this study, we present the results of the production of Chi40 from *S. thermoviolaceus* in *Escherichia coli* and purification of the thermostable chitinase in quantities that are required for structural studies by X-ray crystallography. Furthermore, we were able to obtain $^{13}C-^{15}N$-labeled soluble Chi40 (Chi40s) and the preliminary NMR results are suitable for further analysis of Chi40s to study the substrate–enzyme interaction, catalytic mechanism, and inhibitor–enzyme interaction in solution.

MATERIALS AND METHODS

Materials

All enzymes used in the cloning procedures were from Boehringer Mannheim (Germany), AGS (Heidelberg, Germany), and New England Biolabs. The sequencing kit Sequenase was purchased from United States Biochemical and the TA cloning system from InVitrogen. The column chromatography media were from Pharmacia or Clontech and all the other chemicals were from Sigma or Merck, in the highest analytical grade. Synthetic oligonucleotides were prepared by MWG (Munich, Germany). The bacterial strain *S. thermoviolaceus* was supplied by DSMZ (German Collection for Microorganisms) and grows at 50°C, in a medium containing 10 g/L, yeast extract (DIFCO), 5 g/L proteose peptone (DIFCO), 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, pH 7.0.

Cloning Procedures

The *chi*40 gene from *S. thermoviolaceus* was amplified by PCR from total genomic DNA using the following primer pairs: (i) C40(*Nde*I), TCCATGGCCGCCGCCGACAACGGC, and C40(*Bam*HI), GGATCCTCACGCCAGGCCGTTGCTC, incorporated the cloning sites *Nde*I and *Bam*HI at the 5′ and 3′ ends of *chi*40, respectively, allowing the cloning of the Ala^{42}-end part of *chi*40 into pET-3a, pET-11a, pET-15b, and pET-19b; (ii) C4011a(*Nde*I), CATATGGCCACCGACCACTCC, and C4011a(*Nco*I), CCATGGCCACCGACCACTCC, incorporated the cloning sites *Nde*I and *Nco*I at the 5′ end of *chi*40 and in combination with C40(*Bam*HI) allowed the cloning of the Ala^{31}-end part of the *chi*40 gene into pET-3d, pET-11d, and pET-15b; (iii) C4011a(*Nco*I) and C40(*Bam*HI) were used in the cloning of the Ala^{31}-end part of the *chi*40 gene after the *E. coli pel*B secretion signal in the cloning sites *Nde*I and *Bam*HI of pET-20b and pET-11a expression vectors; and (iv) C40SP(*Nde*I), CATATGCGCTTCGCACACAGAGC, and C40(*Bam*HI) incorporated the cloning sites *Nde*I and *Bam*HI at the 5′ and 3′ ends of the *chi*40 gene, allowing the cloning of the entire *chi*40 gene, including its *S. thermoviolaceus* signal peptide in pET-3a and pET-11a expression vectors (25).

The gene amplification was carried out using "hot-start" conditions: denaturation at 94°C for 5 min, followed by the addition of *Taq* DNA polymerase; the next steps, 94°C for 1 min, 55–62°C for 1 min, 72°C for 1 min 30 s, were repeated for 30 cycles; and the final step was 72°C for 15 min. The resulting 1.2-kb fragment was ligated into the pCR2.1 vector and transformed into *E. coli* INVαF′ strain according to the manufacturer's instructions. The clones were verified by sequencing (26) and compared to the published sequence (AC:D14536). Plasmid DNA was isolated from a positive clone and the fragment containing *chi*40 was subcloned into the expression vectors pET11a, pET15b, pET19b, and pET22b and transformed into Novablue competent cells (25). Plasmid DNA was isolated from the host *E. coli* strain Novablue and transformed into the *E. coli* expression strains AD494(DE3), AD494(DE3)pLysS, BL21(DE3), BL21(DE3)pLysS, BL21*trx*B(DE3), BL21*trx*B(DE3)pLysS, BLR(DE3), and BLR(DE3)pLysS. The genotype of the host cells is fully described in the manual of Novagen for the pET cloning system. The cloning protocols were carried out as described in (26) or according to the manufacturer's directions. Plasmids were purified using the plasmid preparation kit from Qiagen.

Cell Growth

In all cases the antibiotics were used at the following concenration: ampicillin (amp) at 100 μg/ml, kanamycin (kan) at 15 μg/ml, and chloramphenicol (chl) at 34

μg/ml. The clones containing the *chi*40 gene were grown in LB medium (Luria–Bertani medium: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.0). Protein production from a fresh overnight culture was induced with 0.5 mM IPTG for 3 h when the cells reached an OD_{600nm} of 0.6–0.7. The bacteria were separated from the medium by centrifugation in a GSA Sorvall rotor at 4000 rpm, at 4°C, for 10 min. The bacterial pellets were washed with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4 at pH 7.4 adjusted with HCl) and either stored at −80°C or used directly.

Protein Purification

For the purification of Chi40c, the plasmid pECH-Chi40-3 was introduced into *E. coli* BLR(DE3) and the cells were grown in LB medium in the presence of amp. The clone was induced at OD_{600nm} 0.6 by the addition of 0.5 mM IPTG. For preparative purposes 5-L culture was used and after 3 h induction the cells were collected by low-speed centrifugation. The bacterial pellet was lysed by sonication for 10 min with 30-s intervals in buffer A (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10 mM imidazole, 0.1 mM PMSF) in the presence of 0.1% Triton X-100 in an ice-water bath. The total bacterial extract was clarified by centrifugation in an SS-34 Sorvall rotor at 20,000 rpm for 20 min. The soluble supernatant was directly applied on a 5-ml Ni^{2+} column (27) equilibrated in buffer A with a flow rate of 60 ml/h. Bound Chi40c was eluted with 80 ml buffer A containing 100 mM imidazole. Fractions showing Chi40c activity were analyzed by 0.1% SDS–15% PAGE and pooled. Chi40c protein fractions were desalted by PD-10 columns and further applied to a 1-ml Q-High Performance column with a flow rate of 60 ml/h, equilibrated in buffer B (20 mM Tris–HCl, pH 8.0). Chi40c was eluted by a 45-ml gradient between 0 and 500 mM NaCl in buffer B.

For the purification of Chi40s, the plasmid pECH-Chi40-9 was introduced into *E. coli* BL21*trx*B(DE3) cells which were grown in LB medium in the presence of amp and kan. The cell cultures were divided into small batches (7 × 15 ml), inoculated from frozen glycerol culture, and grown at 37°C overnight. The cells were collected by centrifugation and stored at −80°C while the supernatant was immediately adjusted to 0.5 M ammonium sulfate in buffer B. Ten milliliters of the solution was loaded on a 1-ml phenyl–Sepharose column equilibrated with buffer C (20 mM Tris–HCl, pH 8.0, 0.5 M ammonium sulfate). The column was washed with 5 volumes buffer C and bound proteins were eluted with a descending gradient of 0.5 to 0 M ammonium sulfate (20 ml) in buffer B and finally with 5 ml buffer B. Chi40s-containing fractions were detected by activity assays using *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose (pNp-$(NAG)_2$) and verified by 0.1% SDS–15% PAGE analysis. The Chi40s fractions were pooled and applied on a 1-ml Q-High Performance column, equilibrated in buffer B. Bound Chi40 was eluted with a 20-ml 0–0.5 M NaCl gradient in buffer B and fractions containing highly pure Chi40s protein were combined and kept at 4°C.

For the purification of Chi40s labeled with paramagnetic elements, the plasmid pECHChi40-9 was introduced into *E. coli* BL21*trx*B(DE3) and kept frozen in glycerol culture. Fifteen milliliters of ^{13}C–^{15}N M9 minimal medium (0.4% [^{13}C]glucose, 1 g/L $^{15}NH_4Cl$, 1 mM $MgSO_4 \cdot 7H_2O$, 10 mM NaCl, 0.1 mM $CaCl_2$, 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4) supplemented with amp and kan was inoculated from the glycerol culture and the cells were induced with 0.5 mM IPTG after they had reached an OD_{600nm} of 0.7. The induction time was around 24 h. All further procedures were carried out exactly as described above.

Activity Assays

Activity assays using the synthetic substrate pNp-$(NAG)_2$ were carried out in a reaction volume of 1 ml in a glass cuvette equilibrated to the appropriate temperature. Activity was monitored by the release of the *p*-nitrophenyl group from the substrate, resulting in a yellow color, detected spectrophotometrically at 405 nm. The activity (μmol/min) was calculated using the molar extinction coefficient for the *p*-nitrophenyl group (E_{405nm} = 18.5 $mmol^{-1}$ cm^{-1}).

The products of the Chi40c or Chi40s reaction on colloidal chitin were detected using a modified Schales reaction, which measures the production of *N*-acetyl-β-glucosamine (NAG) quantitatively (28). The colloidal chitin was prepared as described in (29). The reaction involves the addition of 2 ml of color reagent (0.5 g/L $K_3Fe(Cn)_6$ in 0.5 M Na_2CO_3) to 1.5 ml sample, boiling for 15 min, and measuring absorbance at 420 nm. Samples were measured against a blank of water and the presence of NAG was directly proportional to the reduction in absorbance as determined using a reaction without substrate.

Protein Properties

Thermal denaturation studies were carried out using circular dichroic (CD) spectroscopy. CD spectra were recorded on a J715 JASCO Spectropolarimeter. A cuvette (Hellma 165-QS) of 0.2 mm cell length was used. The temperature of the cuvette containing the sample solution was changed at a rate of 50°C/h. The best quality spectra regarding reproducibility of the molar ellipticity were obtained at 0.2 mg/ml protein concentration. The CD spectrophotometer was interfaced to a personal computer and the collected data were processed using the sigmoidal fitting of Boltzmann's equation. In order

to collect accurate CD spectra the instrument was calibrated using an aqueous solution of (+)-10-camphosulfonic acid (Aldrich Chemicals) of 1 mg/ml in a 1-mm cell. This compound has a molar ellipticity of 2.36×10^3 at its CD maximum of 290.5 nm and a molar ellipticity of -4.9×10^3 at its CD minimum of 192.5 nm.

Chitin binding experiments were carried out using 2 mg of colloidal chitin (29) or chitin obtained from Sigma, 0.5 mg Chi40 in 20 mM Na-phosphate, pH 7.9 (buffer D), in a volume of 1 ml. The reaction was incubated shaking at 37°C for 1 h, centrifuged for 10 min, and followed by at least three wash steps with 0.5 ml buffer D until there was no protein detected in the supernatant using the Bradford protein assay (30). The protein was quantified from the polyacrylamide gel after staining with Coomassie Brilliant Blue G-250. Protein concentration was determined by the Bradford method as described in (30), using BSA as a standard. Other protein determination methods were also used for comparison (31–33).

The 0.1% SDS–15% PAGE analyses were run according to the Laemmli procedure (34). Gels were run at a constant current of 30–40 mA at room temperature and stained with Coomassie Brilliant Blue G-250. Immunological detection of Chi40s and Chi40c was carried out employing the Western blot procedure as described in (35, 36) using a polyclonal antibody raised against chitinase A from *S. marcescens* in rabbits.

Native polyacrylamide gel electrophoresis was prepared as described in (37) and run at a constant 100 V at room temperature. The samples were not boiled, and the gels were stained with Coomassie Brilliant Blue G-250.

The p*I* of the recombinant Chi40s was determined using the IEF PHAST gel system (Pharmacia). Ten micrograms of the protein was loaded onto the IEF gel (pH 3–9) and run according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Production of Chi40 in E. coli Using Different Expression Vectors and Host Strains

The chitinase gene *chi*40 (8) was cloned into five different pET expression vectors and transferred into various *E.coli* expression host strains with the purpose of finding the optimal vector/host strain combination for high-level protein production and subsequent purification. The predicted signal sequence cleavage site for Chi40 is Ala^{31} (37) while the N-terminal of the purified protein from *S. thermoviolaceus* was reported to start at Ala^{42} (7). The chitinase gene was therefore cloned in four versions: (i) Chi40 with its native signal peptide; (ii) Chi40 from the predicted cleavage site Ala^{31}, using the method of Kyte and Doolittle (37), (iii) Chi40 from amino acid Ala^{42}; and (iv) Chi40 from Ala^{31} with the *pel*B signal peptide of *E. coli*. Details of the expression constructs are listed in Table 1.

The vectors chosen contained the strong T7 or T7*lac* promoters, and two of the vectors (pET-15b and pET-19b) allowed the expression of the chitinase as a translational fusion to a histidine tag, thereby allowing the possibility of metal affinity chromatography purification. The T7*lac* promoter is more tightly regulated than the T7 promoter due to the presence of the *lac* operater sequence and *lac* repressor on the plasmid. The results describing the expression levels in the different *E.coli* hosts are summarized in Table 2 and the protein profile of the clones used for protein purification can be seen in Fig. 1. It is clearly shown that the *E. coli* host strains differed in their ability to express the recombinant chitinase to high amounts. From SDS–PAGE analysis (Fig. 1, not all data shown, and Table 1) it could be seen that 18 of the 44 expression construct/host combinations yielded high amounts of recombinant chitinase. However, the chitinase was insoluble in 14 of these 18 high-production expression construct/host combinations.

The expression of *chi*40 with its native signal resulted in either low expression levels or insolubility despite the use of two expression vectors and four *E.coli* host strains. In contrast, when fused to the *E.coli pel*B signal peptide chitinase was secreted into the culture medium at high levels from *E. coli* BL21*trx*B(DE3) (Fig. 1, lanes 1 and 2). This strain is a thioredoxin reductase mutant that allows disulfide bond formation in the *E.coli* cytoplasm in addition to being deficient in both *lon* and *omp*T proteases. This expression construct/host combination was one of two chosen for the production of chitinase for purification.

TABLE 1

Summary of Various pET-System *chi*40 Constructs Prepared for the Overproduction and Large-Scale Purification of Chi40 Protein

Construct	Host vector	Insert
pECHChi40-1	pET-3a	Ala^{42}-end of *chi*40
pECHChi40-2	pET-11a	Ala^{42}-end of *chi*40
pECHChi40-3	pET-15b	Ala^{42}-end of *chi*40
pECHChi40-4	pET-19b	Ala^{42}-end of *chi*40
pECHChi40-5	pET-3d	Ala^{31}-end of *chi*40
pECHChi40-6	pET-15b	Ala^{31}-end of *chi*40
pECHChi40-7	pET-11d	Ala^{31}-end of *chi*40
pECHChi40-8	pET-20b	Ala^{31}-end of *chi*40 cloned after the *pel*B signal peptide of *Escherichia coli*
pECHChi40-9	pET-11a	Ala^{31}-end of *chi*40 cloned after the *pel*B signal peptide of *E. coli*
pECHChi40-10	pET-3a	*chi*40 entire open reading frame
pECHChi40-11	pET-11a	*chi*40 entire open reading frame

TABLE 2

Summary of Chi40 Constructs Introduced into Various *Escherichia coli* Hosts for Protein Overproduction

Construct	AD494 (DE3)	AD494(DE3) pLysS	BL21 (DE3)	BL21(DE3) pLysS	BL21*trx*B (DE3)	BL21*trx*B (DE3)pLysS	BLR (DE3)	BLR(DE3) pLysS
pECHChi40-1	—	—	Low	Low	Low	Low	—	—
pECHChi40-2	—	—	Low	Low	Low	Low	—	—
pECHChi40-3	High–IS	Medium	High–Sol	Low	—	—	High–Sol	Low
pECHChi40-4	—	—	High–Sol	Low	—	—	—	—
pECHChi40-5	Medium	High–IS	—	—	Medium	High=nIS	—	—
pECHChi40-6	Low	Low	Low	High–IS	—	—	—	—
pECHChi40-7	High–IS	Medium	—	—	High–IS	Low	—	—
pECHChi40-8	Low	High–IS	—	—	Medium	Medium	—	—
pECHChi40-9	High–IS	High–IS	—	—	High–Secr	Low	—	—
pECHChi40-10	Low–IS	High–IS	—	—	High–IS	High–IS	—	—
pECHChi40-11	High–IS	Low–IS	—	—	High–IS	Low–Sol	—	—

Note. Low, low protein production; High, high protein production; Medium, medium protein production; IS, insoluble; Secr, secreted; Sol, soluble.

In addition to expression of the chitinase with different signal peptides the predicted mature Chi40 from Ala^{31} and Chi40 from Ala^{42} were expressed in the cytoplasm of *E. coli*. Expression levels of chitinase-Ala^{31} were high in some *E.coli* hosts but the protein was insoluble. In contrast, chitinase-Ala^{42} was highly expressed in a soluble form from *E.coli* BL21(DE3) and BLR(DE3) with a histidine tag, which should allow a one-step purification. The expression construct/host combination pECHChi40-3/BLR(DE3) was therefore also chosen for the production of chitinase for purification (Fig. 1, Lane 3).

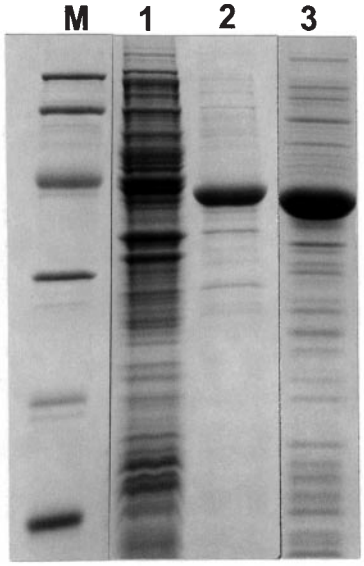

FIG. 1. 0.1% SDS–15% PAGE of total *E. coli* protein extract of the *chi*40 constructs used to produce the Chi40s and Chi40c proteins. The pET-derived constructs are described in Tables 2 and 3. Lane M, molecular weight markers of 94, 67, 43, 30, 20.1, and 14.4 kDa from the top to the bottom. Lane 1, total bacterial extract of pECHChi40-9 construct in BL21 *trx*(DE3) grown in LB medium supplemented with amp and kan after overnight growth, and lane 2, the protein profile of the cell culture medium, Lane 3, total bacterial extract of pECHChi40-3 construct in BLR(DE3) grown in LB medium supplemented with amp after 3 h induction with IPTG.

The results from our gene expression experiments show that it is beneficial to test several vector/host combinations when producing a new protein since expression levels and ease of purification differ drastically.

Purification of Chi40c from E. coli Cell Pellets Using Metal-Affinity Chromatography

Production of the Ala^{42}-chitinase or Chi40c (cytoplasmic) of Chi40 in pECHChi40-3/BLR(DE3) was induced by the addition of IPTG (Fig. 1, lane 3). We developed a two-step chromatographic purification procedure for Chi40c using metal-affinity chromatography followed by ion-exchange chromatography. The procedure involves sonication of the bacterial cell pellet in Buffer A (Ni^{2+}-column binding buffer) and application of the solution to a Ni^{2+}-affinity column. Chi40c was obtained as almost 90% pure (Fig. 2) by one-step elution with 100 mM imidazole in buffer A. To further increase the purity of Chi40c to over 95% an ion exchange step on a Q-High Performance column at pH 8.0 was necessary (Fig. 3). The highly pure Chi40c was eluted as the first peak at 200 mM NaCl while the second peak contained Chi40c with major impurities due to aggregation. The summary of the purification steps with a total yield of 74% and a purification factor of 12.7 is presented in Table 3. A total of 110 mg pure chitinase was purified from 5.1 g cell pellet (wet weight).

However, native polyacrylamide gel electrophoresis revealed that the purified Chi40c protein was not homogeneous but existed in two forms (upper and lower) that differed in their electropohoretic mobility (Fig. 3, inset b). Several experiments showed that the lower form was identical to the wild type while the upper form was

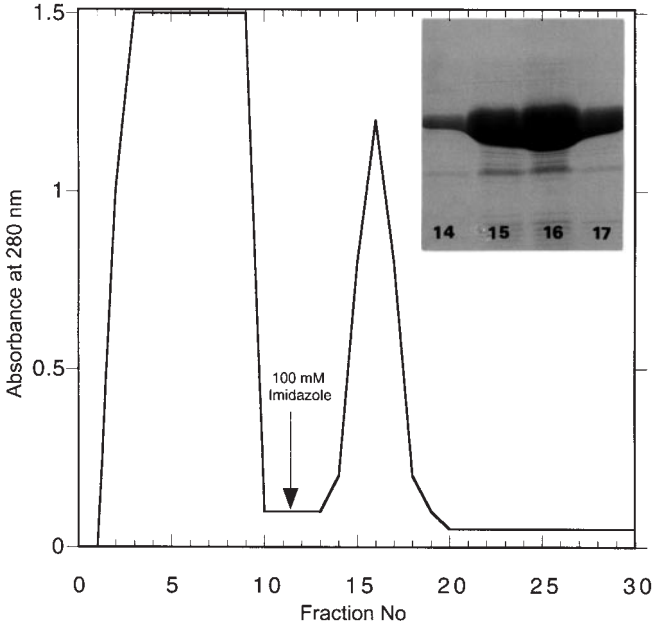

FIG. 2. Metal-affinity column chromatography profile of the extract of BLR(DE3) cells harboring the plasmid pECHChi40-3. The Chi40c was eluted with 100 mM imidazole in buffer A as a major symmetrical peak. Inset: A 0.1% SDS–15% PAGE of the Chi40c peak fractions (for details see Materials and Methods).

incorrectly folded, very likely due to disulfide bridge formation in different ways. We succeeded in separating both forms of Chi40c using a MonoQ column and their specificities and specific activities were found to be identical while the stabilities were slightly different. Both Chi40c measured by CD spectroscopy have shown identical secondary structure profiles while the determined T_m were slightly different. The Chi40c lower form has a T_m of 61.0°C, while the Chi40c upper form has T_m of 60.0°C. The fact that both Chi40c forms have the same molecular weight as determined by mass spectroscopy as well as the same mobility in native gel electrophoresis after reduction and blocking of the resulting cysteine sulfonyl residues supports our interpretation that the two forms of Chi40c protein were made due to differential formation of disulfide bridges. Unfortunately crystals of both forms of Chi40 for X-ray structural analysis were not obtained. The correct sequence of both forms was confirmed by protein microsequencing.

Purification of Chi40s Secreted into E. coli Growth Medium

Production of the Ala[31]-chitinase or Chi40s in pECH-Chi40-9/BL21*trx*B(DE3) in the culture medium did not require the addition of IPTG (Fig. 1, lanes 1 and 2). This was not surprising since we have incorporated in the expression vector, which contains the T7*lac* promoter, its *lac* repressor. The clone expressed chitinase in considerable amounts into the culture medium but only when the culture volume was very small (15 ml) and only in 50-ml conical tubes and after overnight culture without IPTG induction. Attempts to scale up the production from this clone were unsuccessful, since the yield was considerably low.

A two-step chromatographic procedure for the purification of the secreted Chi40s was established. The procedure involves the addition of ammonium sulfate to the cell-free bacterial medium supernatant (Fig. 1, lane 2), adjustment of the pH to 8.0 (buffer B), and direct application of the solution on a phenyl–Sepharose column at pH 8.0 Chi40s eluted from this column was very pure as shown in Fig. 4, inset a. The next step, a Q-High Performance column at pH 8.0, was included to remove some minor impurities and to concentrate the protein. In contrast to Chi40c isolated from the bacterial cell pellet described above, Chi40s purified from the culture medium is homogeneous and a single band can be resolved in native polyacrylamide gel electrophoresis (Fig. 4, inset b). The summary of the purification steps with a total yield of 71.7% and a purification factor of 7.3 is presented in Table 4. A total of 7 mg of purified chitinase was obtained from 100 ml culture supernatant.

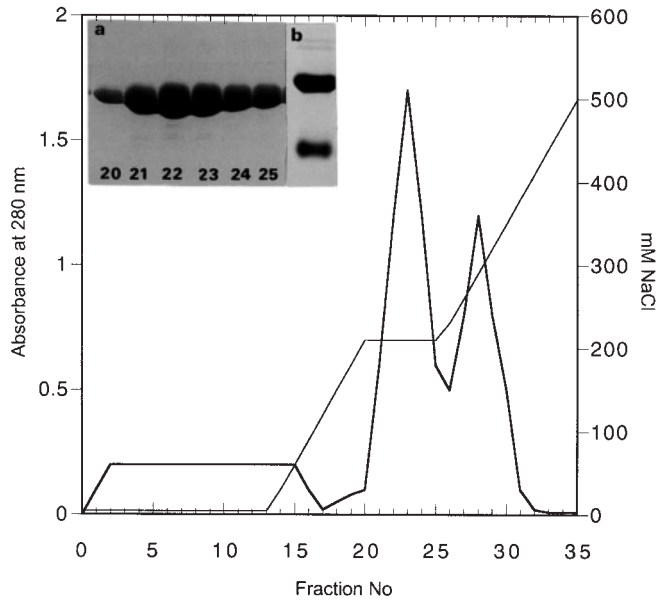

FIG. 3. Ion-exchange column chromatography profile of the Chi40c peak fractions from the Ni^{2+} column on a Q-High Performance column at pH 8.0 in buffer B. The Chi40c was eluted at 200 mM NaCl as a major symmetrical peak. Inset: (a) 0.1% SDS–15% PAGE and (b) native PAGE of the Chi40 peak fractions (for details see Materials and Methods).

TABLE 3

Purification Scheme of Chi40c from 5.1 g *Escherichia coli* BLR(DE3) Cells Harboring the Plasmid pECHChi40-3

Step	Volume (ml)	Concentration (mg/ml)	Total amount (mg)	Sp act (U/mg)	Total activity (U)	Yield (%)	Purification factor (fold)
E. coli extract after sonication	40	35.2	1408	16	22,528	100	1
Metal-affinity chromatography (Ni^{2+}) at pH 8.0, elution via 100 mM imidazole step	30	4.3	129	142	18,318	81.3	10.9
Q-High Performance column chromatography at pH 8.0, elution via 0–0.5 M NaCl gradient	12	9.2	110.4	151	16,670	74.0	12.7

Properties of Purified Chi40s

The biochemical properties of the purified Chi40s were studied. In a standard enzymatic assay the optimum temperature for activity of the recombinant Chi40s was determined to be in the range of 60–65°C and the optimum pH for activity was 6.0. There is a discrepancy with the data described in (7) in which the pH optimum of Chi40 was 9.0 and the optimum temperature 80°C at pH 9.0. The properties of the recombinant Chi40 are comparable to those described for the wild-type isolated enzyme (7). We have determined a pH of 6.0 for optimum enzymatic activity while the pH optimum for the wild type was determined to be above pH 8.0. The low pH optimum is closer to the expected value for a glycosyl hydrolase but this point needs further investigation. The p*I* of the recombinant Chi40s, in the absence of its signal peptide, determined by Phast Gel isoelectric focusing was determined to be around 3.5, which is close to the value of 3.8 described in (7). In chitin–enzyme interaction experiments at 20°C for 1 h, the binding of purified Chi40s on colloidal chitin and untreated chitin was 100 and 70%, respectively. Furthermore the enzyme hydrolyzed chitooligosaccharides from a trimer to an octamer to produce $(NAG)_2$ as the main product that did not further hydrolyze, as revealed by analysis of the degradation products of Chi40s on colloidal chitin by thin-layer chromatography (38).

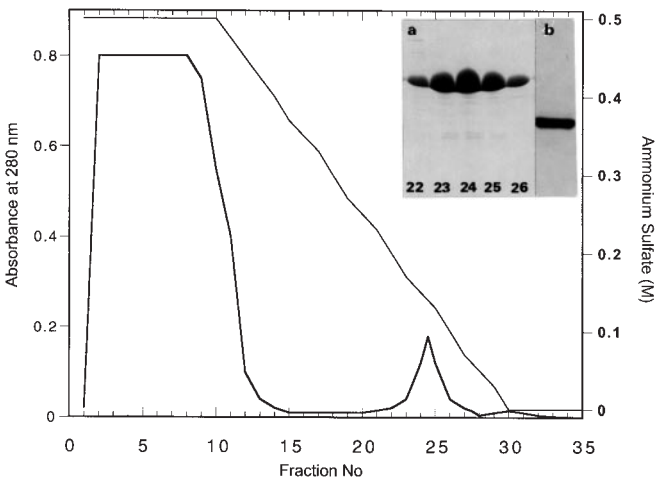

FIG. 4. Hydrophobic-interaction (phenyl–Sepharose) column chromatography profile of the Chi40s overproduced in BL21*trx*(DE3) cells harboring the pECHChi40-9 construct and secreted in the growth medium. The bacterial supernatant was adjusted to 0.5 M ammonium sulfate and buffer B. Chi40s was eluted between 150 and 100 mM ammonium sulfate as the only symmetrical peak. Inset: (a) 0.1% SDS–15% PAGE and (b) native PAGE of the Chi40s peak fractions (for details see Materials and Methods).

A strong cross-reactivity between the chitinase from *S. marcescens* and Chi40c or Chi40s purified in this report was seen by Western blot analysis using a purified rabbit polyclonal antibody raised against chitinase A from *S. marcescens*.

CD spectroscopy was carried out on Chi40s in order to establish the melting temperature curve of the purified Chi40s. The best signal-to-noise ratio was obtained at a protein concentration of 0.2 mg/ml (5 μM) in 20 mM Mops, pH 7.0. The wavelength scan between 180 and 250 nm resulted in an absorbance maximum at 224 nm for Chi40s and the unfolding of the enzyme with increasing temperature was observed at this wavelength. The temperature was increased between 20 and 90°C at a rate of 50°C/h. Full CD spectra of Chi40 at various temperatures and a melting curve in 20 mM Mops, pH 7.0, are presented in Fig. 5. The obtained results showed that the T_m of Chi40s is 60.9°C at pH 7.0 while at pH 4.5 the T_m was substantially reduced to 53.5°C. Using protein unfolding gel electrophoresis in the presence of urea, Chi40s is 50% denatured at 3 M urea (data not shown) (39).

Our extensive cloning and overexpression experiments could be used to draw the conclusion that it is very likely that the proper vector/host system for a certain protein will be found provided that various combinations and several growth conditions (among others, temperatures, media, IPTG concentrations, culture volumes) have been tried.

The aim of this study was to develop a rapid and efficient purification scheme of Chi40s protein for crystallization and NMR studies. Although Chi40s is a large protein for NMR studies, preliminary experiments with labeled protein have shown very promising results of

TABLE 4

Purification Scheme of Chi40s from 100 ml *Escherichia coli* BL21*trx*B(DE3) Cells Harboring the Plasmid pECH40-9

Step	Volume (ml)	Concentration (mg/ml)	Total amount (mg)	Sp act (U/mg)	Total activity (U)	Yield (%)	Purification factor (fold)
Medium supernatant	100	0.51	51.0	28.7	1463.7	100	1
Hydrophobic-interaction column chromatography using phenyl–Sepharose at pH 8.0, elution via descending ammonium sulfate gradient	40	0.20	8.0	130.0	1060.0	72.4	6.4
Q-High Performance column chromatography at pH 8.0, elution via 0–0.5 M NaCl gradient	1.6	4.4	7.0	150.0	1050.0	71.7	7.3

such quality as to be able to determine the structure (preliminary results in cooperation with Dr. M. Casarotto). However, it is too early to provide more details. Current experiments on the mechanism of catalysis of chitooligosaccharides by chitinase A from *S. marcescens* have revealed several difficult questions which cannot be solved by X-ray crystallography (unpublished data). The production of Chi40s suitable for NMR studies will certainly support our efforts to elucidate the enzymatic mechanism of the action of chitinase A, since it is assumed that both proteins employ the same enzymatic mechanism. Finally, the produced Chi40s is suitable for crystallization and currently extensive crystallization trials are in progress. We hope to be able soon to describe the 3D structure and the interaction of the enzyme with its substrate and inhibitor allosamidin, as well as structural properties related to its thermostability.

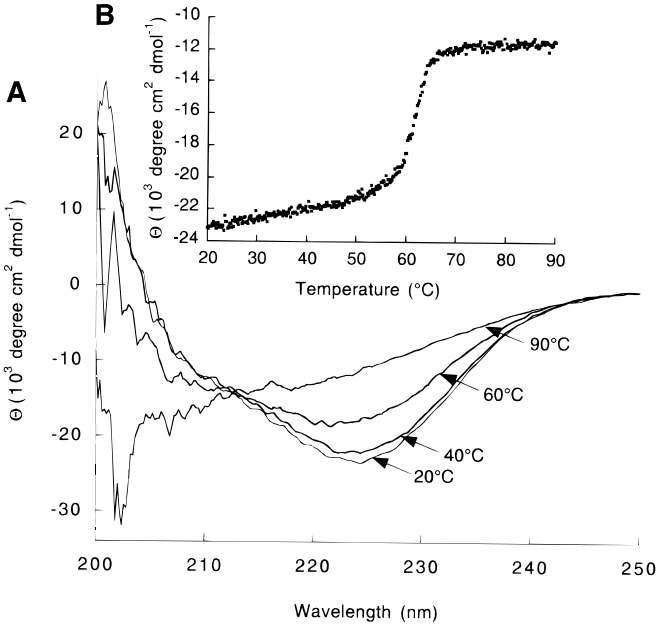

FIG. 5. (a) CD spectra of Chi40s at various temperatures as indicated. (b) Thermal melting curve of Chi40s as determined by CD spectroscopy at pH 7.0.

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