Heterologous expression and characterization of wild-type and mutant forms of a 26 kDa endochitinase from barley (*Hordeum vulgare* L.)

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To investigate structure–function relationships in plant chitinases, we have developed a heterologous expression system for the 26 kDa endochitinase from *Hordeum vulgare* L. (barley). *Escherichia coli* cells harbouring the gene in a T7 RNA polymerase-based expression vector synthesized completely insoluble recombinant protein under standard induction conditions at 37 °C. However, a concentration of soluble recombinant protein of approx. 15 mg/l was achieved by inducing bacteria at low temperature (15 °C). Recombinant endochitinase was purified to homogeneity and shown to be structurally and functionally identical to the seed protein. An average of three disulphide bonds are present in the recombinant enzyme, consistent with the number found in the natural form. The seed and recombinant proteins showed the same specific activity towards a high-molecular-mass substrate and exhibited similar anti-fungal activity towards *Tricoderma reesei*. Site-directed mutagenesis was used to replace residues that are likely to be involved in the catalytic event, based on structural similarities with lysozyme and on sequence alignments with related chitinases. The $Glu^{67} \rightarrow$ Gln mutation resulted in a protein with undetectable activity, while the $Glu^{89} \rightarrow$ Gln mutation yielded an enzyme with 0.25 % of wild-type specific activity. This suggests that two acidic residues are essential for catalytic activity, similar to the situation with many other glycosyl hydrolases. Examination of conserved residues stretching into the proposed substrate binding cleft suggests that Asn^{124} also plays an important functional role.

INTRODUCTION

Plants do not have an immune system and instead use various defence mechanisms to protect their vegetative and reproductive organs against pathogen infection. One response to pathogenic attack involves expression of pathogenesis-related proteins such as chitinases and β-1,3-glucanases. Chitinases (EC 3.2.1.14) catalyse the hydrolysis of chitin, a β-1,4-linked homopolymer of GlcNAc. Chitin is not present in plant cells, but is a major component of the cell walls of many filamentous fungi. Endochitinases randomly hydrolyse internal β-1,4-linkages of chitin, releasing oligomers of GlcNAc [1,2]. Evidence that chitinases play a role in plant defence has been obtained from both *in vitro* and *in vivo* studies. It was initially observed that purified barley and bean chitinases inhibited the growth of fungal hyphae [3,4], and later that chitinases and β-1,3-glucanases act synergistically to inhibit fungal growth [5,6]. It has also been demonstrated that transgenic plants that overexpress chitinases have increased resistance to fungal attack [7].

Five classes of chitinases have been proposed, based on amino acid sequences derived primarily from sequence analyses of cDNA clones [8,9]. Class I chitinases are two-domain proteins consisting of an N-terminal wheat-germ agglutinin domain [10] linked through a short glycine/proline-rich region to the chitinase domain. The wheat-germ agglutinin lectin domain is of importance for binding of chitin, but not for catalytic activity [11]. Class II chitinases are homologous with the class I chitinase domain, but lack the wheat-germ agglutinin domain. Class I, II and IV enzymes are of plant origin and comprise family 19 of the glycosyl hydrolases [12]. Chitinases of class III and V, which comprise glycosyl hydrolase family 18, are of diverse origin and are structurally unrelated to chitinases of family 19.

The proposed role of plant chitinases in defence has resulted in great interest in the molecular and biotechnological aspects of these enzymes. However, biotechnological application requires detailed mechanistic and structural knowledge. The determination of the three-dimensional structures of two prototypical plant chitinases, the 26 kDa *Hordeum vulgare* L. (barley) class II endochitinase (BEC) [13] and the 29 kDa class III hevamine from *Hevea brasiliensis* (rubber tree) [14], has resulted in new insight into these enzymes, which have different structures.

The BEC gene is specifically expressed at high levels in seed aleurone layers during development [15], suggesting a role for the protein in seed defence. The protein is mainly built from α-helical structural elements and contains a long cleft proposed to be the substrate binding region [13]. Database searches revealed topological similarities between the core structure of BEC and lysozyme from animals and phages [16]. A similar core structure was recently identified in *Streptomyces* N174 chitosanase, which can hydrolyse polymers of β-1,4-linked GlcN [17,18]. Based on structural criteria, this new chitinase/chitosanase/lysozyme superfamily can be divided into two archetypal families: one of prokaryotic origin, represented by bacterial chitosanase and phage T4 lysozyme, and one of eukaryotic origin, represented by BEC and goose egg-white lysozyme [18].

Similar catalytic mechanisms have been suggested previously for lysozymes and chitinases [19], based on observations that some chitinases will hydrolyse the main substrate of lysozyme

Abbreviations used: BEC, 26 kDa barley endochitinase; CM-chitin-RBV, carboxymethylchitin–Remazol Brilliant Violet 5R; HEWL, hen egg-white lysozyme; IPTG, isopropyl β-D-thiogalactopyranoside.

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(alternating β-1,4-linked GlcNAc and N-acetyl muramic acid residues) [2] and some lysozymes will hydrolyse chitin [20]. The three-dimensional position of Glu^{67} in BEC is analogous to that of the essential catalytic acidic residue in several different lysozymes. It has therefore been suggested that Glu^{67} is the catalytic residue in BEC [16,21]. Often, two acidic residues are involved in the catalytic mechanism of glycosyl hydrolases [22]. A candidate for the second acidic catalytic residue in BEC is Glu^{89}, although the role of this residue is not as obvious from structural comparisons as that of Glu^{67} [21]. In hen egg-white lysozyme (HEWL), the substrate is bound in a deep cleft with six subsites (A–F), and is cleaved between sugars D and E [23]. Based on a HEWL–substrate structure, a chitinase–substrate model was proposed [21] in which a GlcNAc hexamer forms hydrogen bonds with several highly conserved residues stretching into the proposed active-site cleft of BEC.

Only a few experimental studies of the catalytic mechanism of plant chitinases have been reported. It has been suggested that a highly conserved tyrosine residue is important for productive substrate binding to chitinases of family 19 [19,24], and chemical modification of a chitinase from rye (*Secale cereale*) seeds suggested that acidic residues and a tryptophan residue are important for chitinase activity [25]. Recent studies have shown that a yam (*Dioscorea opposita*) chitinase and a family 19 bean (*Phaseolus vulgaris*) chitinase are both inverting enzymes, hydrolysing substrate with ensuing inversion of configuration at the anomeric carbon [26,27]. In contrast, two plant and two bacterial chitinases from family 18 catalyse hydrolysis via a mechanism that involves retention of the anomeric configuration [27–29]. Here we report the development of a bacterial expression system for BEC and its use for initial investigations of structure–function aspects of this enzyme.

MATERIALS AND METHODS

Materials

All chemicals were analytical grade or higher and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany).

Oligonucleotide primers for PCR and sequencing reactions were purchased from Pharmacia Biotechnology (Uppsala, Sweden), the pET-11a expression vector from Novagen (Madison, WI, U.S.A.), restriction enzymes from Gibco-BRL (Gaithersburg, MD, U.S.A.), Vent® polymerase from New England Biolabs (Beverly, MA, U.S.A.), molecular mass markers from Bio-Rad Laboratories (Melville, NY, U.S.A.), and BSA and ribonuclease A from Sigma Chemical Co. BEC was purified from seeds as previously described [6], and used to raise polyclonal rabbit antiserum (Dako A/S, Copenhagen, Denmark).

DNA manipulation and bacterial strains

All DNA manipulations, including isolation of DNA from agarose gels, DNA ligation and the transformation of competent cells, were carried out according to standard procedures [30] with minor modifications as previously described [31]. The open reading frame of BEC was amplified using a 5′ *Nde*I linker-primer (5′-TATTATCATATGAGCGTGTCCTCCATCGTCT-CGCGCGCA-3′) and a 3′ *Bam*HI linker-primer (5′-TATTAT-GGATCCTTAGGCGAAGGGTCTCTGGCTGTAGCA-3′) by PCR, using Vent polymerase in reactions containing 20% glycerol, and cloned into pET-11a. The constructions were initially transformed into *Escherichia coli* strain XL1-Blue (Stratagene G.m.b.H., Heidelberg, Germany). The correct assembly of the resulting plasmid and the sequence of the amplified DNA were confirmed by sequencing with a commercial sequencing protocol and reagents (Sequenase version 2.0; United States Biochemical, Cleveland, OH, U.S.A.) and BEC specific primers.

Site-directed mutagenesis was performed by overlap extension PCR [32]. The following internal primers were used together with the 5′ *Nde*I and 3′ *Bam*HI linker-primers for generating the specific mutations (mismatched bases underlined): $Glu^{67} \rightarrow Gln$, 5′-GACCTCCCAC<u>C</u>AGACCACCGG-3′ and 5′-CCGGTGG-TCT<u>G</u>GTGGGAGGTC-3′; $Glu^{89} \rightarrow Gln$, 5′-CTTCAAGCAG-<u>C</u>AACGTGGCGC-3′ and 5′-GCGCCACGTT<u>G</u>CTGCTTGA-AG-3′; $Gln^{118} \rightarrow Ala$, 5′-CGGGCCAATC<u>GC</u>GCTCTCCCAC-3′ and 5′-GTGGGAGAGC<u>GC</u>GATTGGCCCG-3′; $Tyr^{123} \rightarrow$ Ala, 5′-CTCCCACAAC<u>GC</u>CAACTATGGA-3′ and 5′-TCCA-TAGTTG<u>GC</u>GTTGTGGGAG-3′; $Asn^{124} \rightarrow Ala$, 5′-CCACAA-CTAC<u>GC</u>CTATGGACCT-3′ and 5′-AGGTCCATAG<u>GC</u>GT-AGTTGTGG-3′. Verification of the correct mutations was checked by DNA sequencing. Before expression, the recombinant plasmids were transferred to *E. coli* strain BL21(DE3) (Novagen, Madison, WI, U.S.A.).

Expression of BEC in *E. coli* BL21(DE3)

E. coli BL21(DE3) harbouring the recombinant expression plasmid was grown to $A_{600} = 0.6$ before induction with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After induction, growth was continued either for 4 h at 37 °C or for 20 h at 15 °C. Cells were harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C and stored at −20 °C.

Protein analyses

Concentrations of protein mixtures were determined from values obtained in bicinchoninic acid assays (Pierce, Rockford, IL, U.S.A.). Purification by column chromatography was monitored by measuring the A_{226} and A_{280} of the column effluent. The concentration of purified BEC was determined by amino acid analysis. The hydrolysis of approx. 200 pmol of material was carried out at 110 °C for 20 h in pyrolysed, sealed, evacuated tubes containing 100 μl of redistilled 6 M HCl, 5 μl of 1% phenol and 5 μl of 2% dithiodipropionic acid in 0.2 M NaOH, and analysed as previously described [33]. N-terminal amino acid sequencing was carried out by Edman degradation using an Applied Biosystems 477A instrument (Perkin Elmer, Foster City, CA, U.S.A.). SDS/PAGE was performed using 15% polyacrylamide gels [34], and gels were stained with Coomassie Brilliant Blue. Samples for SDS/PAGE were heated at 100 °C for 4 min in the presence or absence of 100 mM dithiothreitol before they were incubated for 20 min at room temperature in 0.2 vol. of 0.5 M iodoacetamide dissolved in 1 M Tris/HCl, pH 7.5. For Western blotting, separated proteins were transferred to PVDF protein transfer membranes (Immobilon P; Millipore Corp., Bedford, MA, U.S.A.), which were then blocked in 10% (w/v) non-fat dry milk. The membranes were incubated for 1 h at room temperature with the primary antibody, diluted 1:500 in 10% (w/v) non-fat dry milk. After washing (3 × 10 min in non-fat dry milk), horseradish peroxidase-conjugated anti-(rabbit IgG) (Dako A/S) was used as the secondary antibody. Peroxidase activity was detected by incubation with H_2O_2 and 3-amino-9-ethylcarbazol.

Determination of free thiol groups

Alkylation of cysteine by iodoacetamide was performed on samples of both native and denatured recombinant BEC. For denaturation, 25 μg of recombinant BEC was concentrated to a

final volume of 5 μl in a SpeedVac concentrator (Savant Instruments Inc., Farmingdale, NY, U.S.A.) and 50 μl of 7 M guanidinium chloride, 0.2 M Tris/HCl, 5 mM EDTA, pH 8.4, was added. The sample was flushed with argon and incubated in the dark for 2 h at 20 °C. Alkylation was performed by addition of 1 μl of a freshly prepared solution of 0.011 M iodoacetamide in 0.1 M NaOH. The solution was left for 30 min at 20 °C in the dark, diluted five times with water and transferred to a 5.000 MWCO ultrafiltration unit (Centi/Por; Spectrum Medical Industries, Houston, TX, U.S.A.) containing a PVDF membrane at the bottom. Filtration was performed at 4000 ***g*** overnight, the membrane was transferred to a pyrolysed Pyrex tube and amino acid analysis was performed. *S*-Amidomethylcysteine was determined as *S*-carboxymethylcysteine after deamidation during acid hydrolysis [35].

Enzyme assay

Chitinase activity measurements were performed using a colorimetric assay with carboxymethylchitin–Remazol Brilliant Violet 5R (CM-chitin-RBV; Loewe Biochemica G.m.b.H., Otterfing, Germany) as substrate. The conditions originally described by Wirth and Wolf [36] were modified to obtain substrate-saturated enzyme reactions and linearity with respect to incubation time and enzyme concentration (measured over an order of magnitude) with the substrate batch and proteins used in the present study. The assay was performed at pH 6.2, which was determined to be the pH optimum (results not shown). For the assay, protein was diluted with 0.1 M sodium phosphate buffer (pH 6.2) to yield a volume of 200 μl. The reaction was started by the addition of 200 μl of 2 mg/ml CM-chitin-RBV, resulting in a final incubation volume of 400 μl. The amounts of protein used were: 2.5–25 ng (6.3–63 ng/ml) recombinant and native wild-type BEC, 4.0–40 μg (10–100 μg/ml) BEC $Glu^{67} \rightarrow Gln$, 0.80–8.0 μg (2.0–20 μg/ml) $Glu^{89} \rightarrow Gln$, 2.6–26 ng (6.5–65 ng/ml) BEC $Gln^{118} \rightarrow Ala$, 7.7–77 ng (19–190 ng/ml) BEC $Tyr^{123} \rightarrow Ala$, and 0.18–2.0 μg (0.45–5.0 μg/ml) BEC $Asn^{124} \rightarrow Ala$. After 10 min of incubation at 30 °C, the reactions were stopped by the addition of 100 μl of 1.0 M HCl, and non-degraded material was precipitated on ice overnight. The samples were centrifuged at 20000 ***g*** for 5 min at 4 °C. The absorbance of the supernatant was measured photometrically at 550 nm using a Beckman DU-70 spectrophotometer, and values were corrected for background (absorbance in the absence of protein). Five independent measurements were performed and average values used. Activity, expressed as $A_{550}/\mu g$, was determined from the slope of a straight line fitted to seven data points by linear regression (FigP; Biosoft®, Cambridge, U.K.).

Fungal growth inhibition assay

The anti-fungal activities of wild-type and recombinant BEC were tested against *Tricoderma reesei* obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Spores were collected from 8-day-old cultures grown at 25 °C on 24 g/l Potato Dextrose Broth (pH 5.5) plates. The spores were harvested as described by Broekaert et al. [37] and counted in a haemocytometer. Fungal spore suspensions (10000 spores/ml) were grown for 42 h at 25 °C in 12 g/l Potato Dextrose Broth (pH 5.5) and then incubated with 0.05–2.5 μg (0.25–12.5 μg/ml) of purified BEC in a total volume of 200 μl, as outlined in the Results section. A_{540} values are correlated linearly with fungal biomass within a broad range. Therefore the inhibitory activities of purified proteins were determined from plots of A_{540} versus chitinase protein concentration. Each value represents the average of eight independent measurements. A value of 100 % fungal growth (no protein, or addition of seed BEC heated for 5 min at 100 °C) under these conditions yielded an A_{540} value of 0.22.

CD studies

The secondary structures and tertiary interactions of wild-type and mutant BEC were evaluated by CD measurements. CD spectra were recorded with a Jobin Yvon Mark V autodichrograph calibrated with (+)-10-camphosulphonic acid. Far-UV spectra were recorded between 190 and 250 nm using a cell path of 1 cm and a protein concentration of 0.02 mg/ml (corresponding to a mean residue concentration of 0.26 mM) in 5 mM potassium phosphate, pH 7. Near-UV spectra were recorded similarily, but between 250 and 300 nm with a protein concentration of 0.2 mg/ml. Results were expressed in terms of mean residue ellipticity (degrees $\cdot$ cm^2 $\cdot$ dmol^{-1}).

Purification of recombinant BEC

All steps in the purification of the recombinant protein were performed at 4 °C.

Extraction

Frozen cells were resuspended in a 7 ml/g cell paste in buffer A [50 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.1 % (v/v) Triton X-100 and 1 mM dithiothreitol] and lysed by sonication on ice with three bursts of 30 s at 25 units (Sonifier B-12 Cell Disrupter; Branson Sonic Power Company). Insoluble material was separated from the soluble fraction by centrifugation at 10000 ***g*** for 10 min.

Ammonium sulphate fractionation

The proteins were initially fractionated by ammonium sulphate precipitation. Solid ammonium sulphate was added slowly to the supernatant to 40 % saturation (226 mg/ml), and the solution was allowed to precipitate with slow stirring for 30 min. The precipitate was removed by centrifugation at 10000 ***g*** for 30 min. Additional ammonium sulphate was added to the resulting supernatant to 60 % saturation (120 mg/ml), and the precipitate was collected by centrifugation as above. This precipitate was dissolved in buffer B (5 mM sodium phosphate, 10 mM NaCl, pH 7.0) to a final protein concentration of 7 mg/ml and transferred to a dialysis bag (Spectra/Por, MWCO: 6000–8000; Spectrum Medical Industries, Houston, TX, U.S.A.) and dialysed against buffer B with one change of dialysis buffer.

Ion-exchange chromatography

The dialysate was clarified by centrifugation at 6000 ***g*** for 15 min, and the resulting protein solution was passed over a cation-exchange column (18 cm $\times$ 2.7 cm) of CM-cellulose (CM52; Whatman International, Maidstone, U.K.) pre-equilibrated with buffer B. The column was initially washed with buffer B until the A_{280} of the eluate dropped to a stable baseline. This wash procedure was repeated with the same buffer containing 100 mM NaCl, before bound protein was eluted with a linear gradient from 100 to 300 mM NaCl over a time period of 220 min. The flow rate was 1.5 ml/min and the fraction size was 3 ml. Fractions containing recombinant BEC were identified by SDS/PAGE and Western blotting, and dialysed into 20 mM Tris/HCl, pH 8.0 (buffer C). To remove trace contaminants, the pool was passed over a Mono Q HR 5/5 anion-exchange column (Pharmacia Biotech). The column was washed with buffer C and then eluted

with a linear gradient to 0.3 M NaCl over a time period of 30 min. Fractions of 1 ml were collected at a flow rate of 1 ml/min.

Visualization of three-dimensional structure

RasMol v2.6 (Roger Sayle, Glaxo Wellcome Research and Development, Stevenage, U.K.) was run under Microsoft Windows and used to display the three-dimensional structure of BEC. The program was obtained by anonymous FTP (ftp.dcs.ed.ac.uk), and the co-ordinate file for BEC (Protein Data Bank entry 2BAA) was obtained from the Brookhaven Protein Databank.

RESULTS

Expression of insoluble and soluble recombinant BEC

The BEC gene was cloned into the prokaryotic expression vector pET-11a [38]. The cloning strategy was designed such that a protein identical to the seed protein or a protein containing an additional N-terminal methionine residue would be produced, depending on processing events in the host. Induction of expression at 37 °C resulted in a 26 kDa protein, the identity of which was confirmed by Western blot analysis to be BEC. The expressed protein was present entirely in insoluble inclusion bodies (Figure 1). However, a fraction of soluble recombinant BEC was obtained when expression was performed at 15 °C. The amount of soluble recombinant BEC was dependent on the IPTG concentration, with the maximum yield of soluble material (approx. 15 mg/l) achieved on induction with 0.1 mM IPTG.

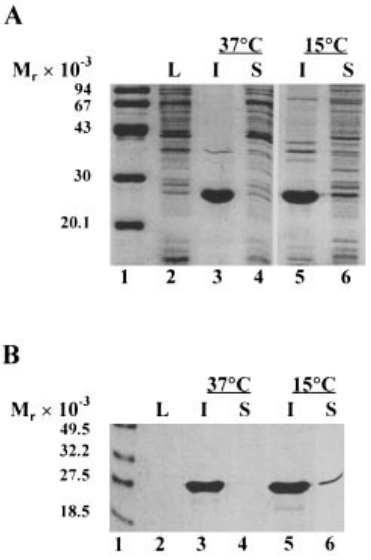

Figure 1 SDS/PAGE and Western blot analyses of the expression of recombinant BEC in *E. coli* BL21(DE3)

BL21(DE3) cells containing the expressed plasmid were grown to an A_{600} of 0.6 and then induced with 0.1 mM IPTG for either 4 h at 37 °C or 20 h at 15 °C. (**A**) SDS/PAGE analysis. Lane 1, molecular mass markers (kDa); lane 2, crude *E. coli* extract at time of induction with IPTG (L); lane 3, insoluble material from *E. coli* extract after 4 h of induction at 37 °C (I); lane 4, soluble material from *E. coli* extract after 4 h of induction at 37 °C (S); lane 5, insoluble material in *E. coli* extract after 20 h of induction at 15 °C (I); lane 6, soluble material in *E. coli* extract after 20 h of induction at 15 °C (S). Portions of 20 μg of soluble protein and an equivalent volume fraction from each sample were subjected to electrophoresis. (**B**) Western blot incubated with anti-BEC antibody. Lane 1, prestained molecular mass markers (kDa). The samples in lanes 2–6 are the same as in (**A**).

Table 1 Purification of recombinant BEC

The cell pellet (24 g) was lysed and the recombinant BEC purified as described in the Materials and methods section.

Purification step	Total protein (mg)	Total activity (A_{550} units)	Specific activity (A_{550} units/mg of protein)	Recovery of activity (%)	Purification (fold)
Supernatant	1.4×10^{3}*	1.7×10^{5}	1.2×10^{2}	100	–
Ammonium sulphate 60% pellet	6.1×10^{2}*	1.0×10^{5}	1.6×10^{2}	59	1.3
CM52	6.8*	3.5×10^{4}	5.1×10^{3}	21	43
Mono Q	1.8†	1.9×10^{4}	1.1×10^{4}	11	92

* Determined in bicinchoninic acid assay.
† Determined by amino acid hydrolysis.

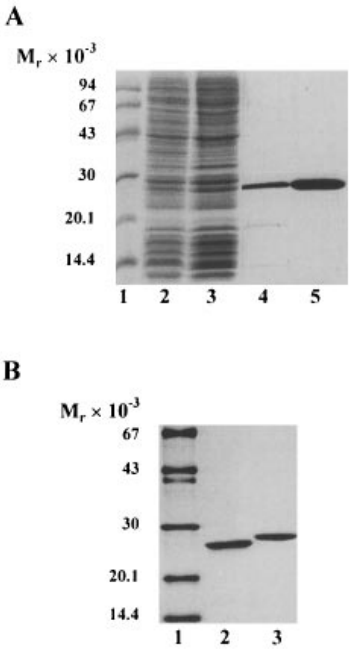

Figure 2 SDS/PAGE showing purification of recombinant BEC and the different electrophoretic mobilities of reduced and non-reduced recombinant BEC

(**A**) Purification of recombinant BEC. Lane 1, molecular mass markers (kDa); lane 2, 25 μg of soluble protein from *E. coli* extract after 20 h of induction at 15 °C; lane 3, 40 μg of ammonium sulphate pellet from 60% precipitation; lane 4, 1.5 μg of pooled fractions after CM52 chromatography; lane 5, 4 μg of pooled fractions from Mono Q column. (**B**) Effect of reduction on electrophoretic mobility. Lane 1, molecular mass markers (kDa); lane 2, 1.7 μg of non-reduced recombinant BEC; lane 3, 1.7 μg of reduced recombinant BEC.

Purification and protein chemical characterization of recombinant BEC

The results described for the purification and protein chemical characterization of recombinant BEC are those obtained for the wild-type form, but identical results were obtained for the mutant proteins. Recombinant BEC was purified using a three-step procedure to homogeneity, as judged by SDS/PAGE, with approx. 11 % recovery and a 92-fold increase in specific activity. The progress of purification is summarized in Table 1 and Figure

Table 2 Amino acid composition of BEC deduced from its cDNA, recombinant BEC (rBEC), carboxymethylated recombinant BEC (CM-rBEC) and denatured carboxymethylated recombinant BEC (dCM-rBEC)

The cDNA for BEC is described in [6]. The values for recombinant BEC represent the averages of two independent determinations after 20 h of hydrolysis. Tryptophan was not determined. The number of residues for each amino acid was calculated assuming a total number of nine leucines. The values for threonine and serine were corrected for hydrolysis losses of 5 and 10% respectively.

	No. of residues			
Amino acid	BEC cDNA	rBEC	CM-rBEC	dCM-rBEC
Asp + Asn	22	22.2	22.3	22.0
Thr	13	13.2	13.0	13.1
Ser	16	16.4	16.2	16.2
Glu + Gln	15	14.5	14.9	14.5
Pro	14	14.0	14.0	14.2
Gly	30	29.3	29.6	28.6
Ala	35	36.0	36.1	34.9
TP-Cys*	7	6.6	5.9	5.9
CM-Cys†	–	–	0.46	0.98
Val	12	11.7‡	10.7	10.4
Met	2	1.8	1.9	2.2
Ile	11	10.2‡	8.8	8.1
Leu	9	9.0	9.0	9.0
Tyr	12	11.3	11.3	11.4
Phe	13	12.7	12.6	12.3
His	5	4.9	4.8	4.8
Lys	7	6.8	7.0	7.1
Arg	14	13.7	13.9	13.5

* Thiopropionic cysteine [34].
† *S*-Carboxymethylated cysteine.
‡ Determined after hydrolysis for 72 h.

2(A). The soluble material from a crude *E. coli* lysate was subjected to ammonium sulphate fractionation followed by two steps of ion-exchange chromatography using CM52 and Mono Q resins. Ammonium sulphate fractionation did not result in any significant degree of purification, but aggregated BEC was selectively removed by this procedure. SDS/PAGE showed that recombinant BEC became the major protein after purification by CM52 chromatography (Figure 2A), and that the impurities remaining after this step were completely removed by Mono Q chromatography. The major fraction of BEC was eluted from the CM52 column at a NaCl concentration of approx. 150 mM. Recombinant BEC present in fractions eluted earlier showed no or low specific activity, and may represent misfolded forms of the recombinant protein.

The amino acid composition of purified recombinant BEC agreed with that derived from the cDNA sequence (Table 2) [6]. A total of 2 mol of methionine was present per mol of recombinant BEC, indicating that the N-terminal methionine residue was removed. This was confirmed by sequence analysis of the N-terminus, which gave the sequence Ser-Val-Ser-Ser-Ile-Val-Ser-Arg-Ala-Gln in a yield corresponding to the amount applied to the sequenator.

BEC from barley seeds contains three disulphide bonds and a single free thiol group [13]. Disulphide bond formation in the recombinant form was initially suggested from a comparison of the mobilities of reduced and non-reduced BEC on SDS/PAGE (Figure 2B). The non-reduced sample contained only monomeric BEC and migrated faster, suggesting a more compact structure resulting from disulphide bond formation. To examine the extent of disulphide bond formation, the number of free thiol groups was determined. Cysteine residues were alkylated using iodoacetamide, hydrolysed, and subsequently quantified as *S*-carboxymethylcysteine after acid hydrolysis (Table 2). The average numbers of free thiol groups in native and denatured recombinant BEC were determined to be 0.46 and 0.98 respectively, in accordance with the structure of the natural protein. The cysteine residue in BEC is partly buried in the interior of the protein and, therefore, probably is not as reactive in the native, folded, protein as in the denatured protein.

Formation of the same number of disulphide bonds in recombinant and seed BEC is consistent with the hypothesis that the two forms display the same folding. This was further supported by their essentially identical far-UV CD spectra (Figure 3a). The features of these spectra are determined by α-helical strutural elements [39], and are in accordance with the three-dimensional structure of seed BEC [21]. CD experiments were also done in the near-UV region to obtain information about the tertiary structures, monitored as specific orientations of aromatic groups (Figure 3b). These spectra were also almost identical for the two forms, further confirming their structural identity.

Activities of wild-type and mutant recombinant BEC

The abilities of wild-type seed BEC, wild-type recombinant BEC and mutant BEC to hydrolyse the soluble high-molecular-mass substrate CM-chitin-RBV were investigated. The colorimetric

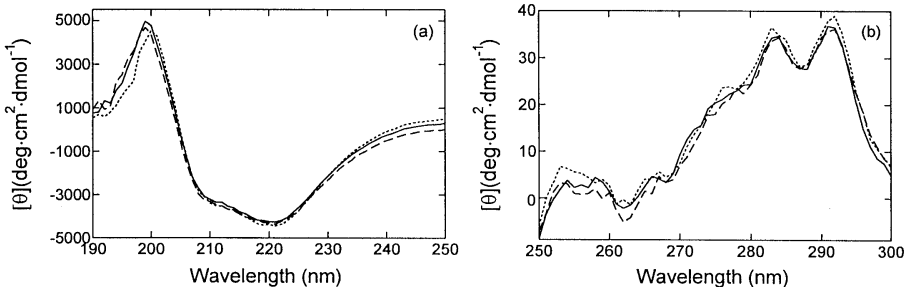

Figure 3 UV CD spectra of wild-type and mutant BEC

(**a**) Far-UV CD spectra of 0.02 mg/ml wild-type recombinant BEC (solid line), wild-type seed BEC (dashed line) and $Glu^{67} \rightarrow Gln$ BEC (dotted line). Essentially identical spectra were obtained for $Glu^{89} \rightarrow Gln$, $Tyr^{123} \rightarrow Ala$ and $Asn^{124} \rightarrow Ala$ BECs. (**b**) Near-UV spectra of samples containing 0.2 mg/ml protein. Samples were the same as in (**a**). Essentially identical spectra were obtained for $Glu^{89} \rightarrow Gln$, $Tyr^{123} \rightarrow Ala$ and $Asn^{124} \rightarrow Ala$ BECs.

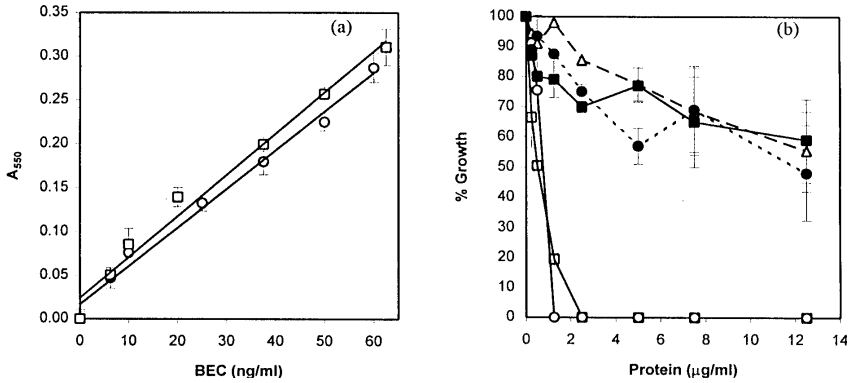

Figure 4 Hydrolysis of CM-chitin-RBV by wild-type BEC and anti-fungal activities of wild-type and mutant BECs

(**a**) CM-chitin-RBV (1 mg/ml) was incubated at 30 °C in 50 mM sodium phosphate, pH 6.2, for 10 min with increasing amounts of chitinase. The total assay volume was 400 μl. Values for absorbance at 550 nm are average values from five independent determinations and are shown with S.D.s. The slopes of the linear regression curves were used to determine activities, expressed as A_{550}/μg. Concentrations between 6.3 and 63 ng/ml wild-type recombinant BEC (○) and wild-type seed BEC (□) were used. (**b**) Spores of *Tricoderma reesei* were grown for 42 h at 25 °C in the presence of 0.25–12.5 μg/ml wild-type recombinant (○), wild-type seed (□), $Glu^{67} \rightarrow Gln$ (△), $Glu^{89} \rightarrow Gln$ (●) or $Asn^{124} \rightarrow Ala$ (■) BEC. Fungal growth is expressed as a percentage of that in the absence of BEC. Each point represents the average of eight independent measurements, with relative S.D.s as indicated.

Table 3 Specific hydrolysing activities of seed BEC and of wild-type and mutant recombinant BEC (rBEC)

The soluble dye-labelled substrate CM-chitin-RBV was used [37]. Activity (A_{550} units/μg) was determined from the slope of a linear regression curve. Five independent measurements were performed; results are means ± S.E.M. N.D.A., no detectable activity.

Protein	Activity (A_{550}/μg)	Relative specific activity (%)
Seed BEC	12 ± 0.7	109
rBEC, wild-type	11 ± 0.5	100
rBEC, $Glu^{67} \rightarrow Gln$	N.D.A.	0
rBEC, $Glu^{89} \rightarrow Gln$	$(2.7 \pm 0.2) \times 10^{-2}$	0.25
rBEC, $Gln^{118} \rightarrow Ala$	8.4 ± 0.2	76
rBEC, $Tyr^{123} \rightarrow Ala$	1.6 ± 0.08	15
rBEC, $Asn^{124} \rightarrow Ala$	$(9.0 \pm 0.6) \times 10^{-2}$	0.82

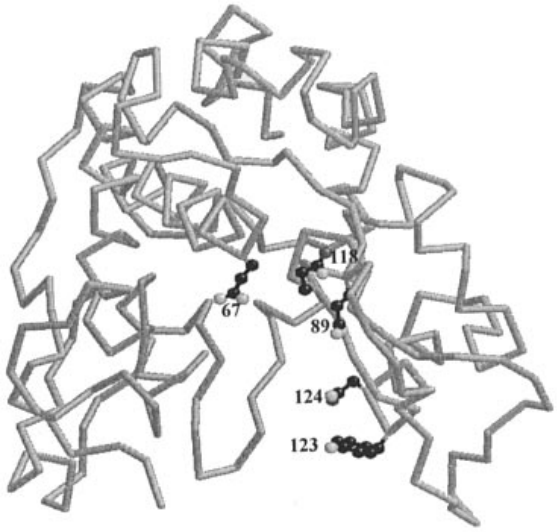

Figure 5 Schematic representation of the backbone structure of BEC, with catalytic and proposed substrate-interacting residues highlighted

Rasmol drawing showing a representation of the backbone of BEC (Protein Data Bank entry 2BAA). Catalytic residues Glu^{67} and Glu^{89} and highly conserved residues Gln^{118}, Tyr^{123} and Asn^{124} are shown in ball-and-stick representation, with carbon atoms shown in black, oxygen atoms in light grey, and nitrogen atoms in dark grey.

assay [36] was used because it is easy to handle and because of the considerable difficulties of performing detailed kinetic analyses of family 19 chitinases. Recombinant and seed BECs showed the same specific activities (Figure 4a; Table 3), indicating that recombinant BEC is a fully active protein. Furthermore, the two forms inhibited the growth of the fungus *Tricoderma reesei* to the same extent. In both cases, 2.5 μg/ml purified protein resulted in complete growth inhibition (Figure 4b).

Recombinant proteins with specific mutations were expressed, purified and characterized. Glu^{67} and Glu^{89}, previously suggested to be catalytic residues, were each altered to glutamine residues (Figure 5). The $Glu^{67} \rightarrow Gln$ mutant protein had no detectable activity towards CM-chitin-RBV, and very low activity (0.25 %) was measured for the $Glu^{89} \rightarrow Gln$ mutant protein (Table 3). The $Glu^{67} \rightarrow Gln$ and $Glu^{89} \rightarrow Gln$ mutant proteins were also less potent inhibitors of *Tricoderma reesei* growth; 2.5 μg/ml wild-type protein resulted in complete inhibition of growth, whereas the same concentrations of the $Glu^{67} \rightarrow Gln$ and $Glu^{89} \rightarrow Gln$ mutant proteins resulted in 15 % and 25 % growth inhibition respectively (Figure 4b). In contrast, heat-treated seed BEC did not result in any growth inhibition (results not shown). The decrease in the activity of these mutant proteins is unlikely to be due to structural changes caused by the amino acid substitutions. In both cases a glutamic acid residue, thought to project freely into the enzyme cleft [21], was replaced by the corresponding amide, glutamine, a change not expected to perturb the structure. Wild-type and mutant $Glu^{67} \rightarrow Gln$ and $Glu^{89} \rightarrow Gln$ recombinant BECs also showed identical behaviour during ion-exchange chromatography, indicative of identical folding. Finally, strong evidence for the structural integrity of the mutant proteins comes from analysis of their far-UV and near-UV CD

spectra (Figure 3). In both cases, spectra essentially identical to those of wild-type recombinant and seed BECs were obtained.

Mutations were also introduced at Gln^{118}, Tyr^{123} and Asn^{124}, three highly conserved residues present on the same side of the proposed substrate binding cleft as Glu^{89} (Figure 5). These residues have previously been postulated to be important for productive substrate binding [19,21,24]. When these residues were altered to alanine residues, proteins with 76 %, 15 % and 0.82 % activity respectively were obtained (Table 3). The activity of the $Asn^{124} \rightarrow Ala$ mutant protein was unexpectedly low if it is assumed that this residue does not participate in catalysis. Therefore this mutant protein was examined further in the anti-fungal assay, which revealed that its ability to inhibit the growth of fungal hyphae was comparable with those of the $Glu^{67} \rightarrow Gln$ and $Glu^{89} \rightarrow Gln$ mutant proteins (Figure 4b). The altered activity of these mutant proteins is most likely due to altered interactions with the substrate rather than structural changes. Substitution of Gln^{118} resulted in only a small decrease in activity, indicating that the structure remains intact. Tyr^{123} and Asn^{124} form part of the same side of α-helix D at the edge of the substrate binding cleft, and their side chains are apparently not fixed in position by interactions with other residues [21] (Figure 5). These mutant proteins show the same chromatographic patterns and CD spectra as wild-type BEC (Figure 3). Therefore the identities of the residues at positions 123 and 124 are not essential for secondary structural elements and tertiary interactions in BEC.

DISCUSSION

A large number of cDNA clones encoding plant chitinases have been isolated and characterized. These studies have only recently been followed by structural studies. We have established a heterologous expression system for BEC, for which the 1.8 Å three-dimensional structure is known [21]. BEC is of modest size, and no post-translational modifications have been reported for the protein [6]. We, therefore, chose *E. coli* as a host for heterologous expression and used a pragmatic approach, that of reduced fermentation temperature, to obtain soluble, active, recombinant protein. Although not universally successful, this strategy has worked with a number of proteins [40], and is not as complicated as a procedure involving refolding of an insoluble recombinant protein. It is thought that a lowered temperature slows the rate of protein synthesis, and may allow more time for productive folding. This is a further demonstration that entirely empirical approaches can be used to obtain soluble, active, eukaryotic recombinant proteins in *E. coli*.

Recombinant BEC and seed BEC were identical, based on both structural and functional criteria. Incomplete or incorrect disulphide bond formation has often been reported for foreign proteins expressed in *E. coli*. It has been suggested that thioredoxin reductase may keep cysteine residues in the reduced state in *E. coli* [41], and that the different redox environments in mammalian cells and *E. coli* may result in formation of incorrect disulphide bonds in proteins heterologously synthesized in *E. coli*. [42]. Protein chemical characterization of recombinant BEC showed that the protein contained the same number and, therefore, probably the same pattern of disulphide bonds as the natural protein. Finally, the two forms gave identical CD spectra and showed the same specific activity and ability to inhibit fungal growth, indicative of similar folding in the active-site region (Figures 3 and 4).

Using lysozyme as a paradigm, site-directed mutagenesis of Glu^{67} and Glu^{89} was performed. The results obtained by analyses of mutant proteins with sterically conservative substitution of glutamine for Glu^{67} or Glu^{89} (Table 3, Figure 4b) were in accordance with the conclusion drawn from mechanistic studies of a number of different glycosyl hydrolases. It has generally been observed that replacement of the catalytic general acid results in total inactivation, while low activity often remains after mutation of other residues involved in catalysis [22]. The low level of anti-fungal activity of the two mutant BEC proteins may be explained by their ability to bind chitin and, thereby, inhibit the growth of fungal hyphae to some degree.

Glu^{67} probably acts as the general acid catalyst, by analogy with other glycosyl hydrolases, whereas the exact role of Glu^{89} is less clear. Based on recent studies, it can be assumed that chitinases of family 19 employ an inverting mechanism to hydrolyse their substrates [26,27]. The structurally related bacterial chitosanase and phage T4 lysozyme also proceed with inversion of product anomer [18,43,44]. According to the classical mechanism for inverting glycosyl hydrolases, one of the two catalytic (carboxylic acid) residues acts as a general acid and the other as a general base [45]. Glu^{89} of BEC may thus function as a base, promoting a nucleophilic attack by water on the anomeric carbon. This suggestion is supported by recent evidence that the base form of Glu^{89} is required for enzymic activity (M. D. Andersen and K. Skriver, unpublished work), and by the three-dimensional structure of BEC, which allows space for a water molecule between Glu^{89} and the substrate [21]. In contrast, HEWL acts with retention of the configuration at the anomeric carbon [46]. This is in accordance with previous observations that the mechanism of glycosyl hydrolases is determined by specific and detailed structural aspects rather than just the general folding of the enzyme [47].

Family 18 chitinases are retaining enzymes and thus use a different catalytic mechanism than family 19 chitinases [27–29]. It has been generally accepted that retaining enzymes also utilize two acidic catalytic residues [45]. One donates a proton to the glycosidic bond, facilitating bond cleavage. The other is thought either to make a nucleophilic attack generating a covalent glycosyl–enzyme intermediate, or to electrostatically stabilize an oxycarbonium ion intermediate. The apparent lack of a negatively charged residue to stabilize the reaction intermediate in a number of glycosyl hydrolases and transglycosylases, such as the family 18 chitinases hevamine [14] and chitinase A [48], the soluble lytic transglycosylase [49], goose lysozyme [50] and chitobiase [51], has led to reconsideration of the proposed catalytic mechanism. Recent structural data on complexes between hevamine and its inhibitor allosamidin [29], and between chitobiase and its $(GlcNAc)_2$ substrate [51], have provided key mechanistic information supporting previous suggestions from studies of HEWL [52,53] that the intermediate is stabilized by the substrate itself. In such substrate assistance of catalysis, the C-2 acetamido group of the substrate would function by analogy with a catalytic base. The carbonyl oxygen of this group would either form a covalent bond with the substrate, resulting in an oxazoline intermediate, or stabilize an oxycarbonium ion electrostatically. Structural analyses will be useful to establish how common such substrate-assisted catalysis is among glycosyl hydrolases. Even in enzymes such as HEWL, where two carboxylic acids are likely to participate in catalysis, some substrate-assisted stabilization may take place [52].

One glycosidase, β-galactosidase, utilizes a tyrosine residue as general acid catalyst [54]. A conserved tyrosine residue was suggested to be of functional importance in family 19 plant chitinases. Substitution of this residue with an alanine residue in an *Arabidopsis thaliana* chitinase resulted in a mutant protein with approx. 40 % activity [19]. The corresponding tyrosine residue in BEC, Tyr^{123}, may form a hydrogen bond with the substrate [21]. To exclude the possibility that Tyr^{123} is essential

for catalysis, the $Tyr^{123} \rightarrow Ala$ mutant protein was analysed and shown to retain 15% of residual activity. This supports previous conclusions that this tyrosine residue is not essential for activity, but may be of some functional importance, e.g. in substrate binding [19].

Gln^{118} and Asn^{124} were also selected for site-directed mutagenesis in this initial screen for catalytic residues. These residues constitute part of a highly conserved region along one side of the proposed substrate binding cleft, and may also hydrogen-bond to substrates [21] (Figure 5). The very low specific activity of $Asn^{124} \rightarrow Ala$ BEC (0.82%) cannot be explained at present. According to the hypothetical substrate–BEC model, Asn^{124} is important for substrate binding, and functions as both a hydrogen bond donor and acceptor [21]. It was recently shown that an asparagine residue in HEWL (Asn^{46}) contributes synergistically with Asp^{52} to the catalytic mechanism, and that hydrogen-bond formation between these residues is likely to be essential for this synergism [55]. The distance between Glu^{89} and Asn^{124} in BEC is too large (approx. 12 Å) for a hydrogen bond to be formed, but it may change when substrate is bound. The functional importance of an asparagine residue in HEWL, together with the results obtained in the present study, makes Asn^{124} an interesting candidate for further functional studies. In contrast, substitution of alanine for Gln^{118} had a minimal effect on the catalytic efficiency. This residue may be involved in substrate interactions, but it is unlikely that it is involved directly in catalysis. Further functional studies are necessary to elucidate the details of the roles played by residues of unknown functional importance, such as Asn^{124}, in the catalytic mechanism of BEC.

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