X-RAY STRUCTURE ANALYSIS OF CHITINASE A FROM *SERRATIA MARCESCENS*

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SUMMARY

Chitinase A from *Serratia marcescens* was overproduced in *E. coli*, purified and crystallized from PEG. The crystals grow to space group $C222_1$. X-ray diffraction data to 2.3 Å resolution were collected using synchrotron radiation. The structure was solved by multiple isomorphous replacement. The crystallographic R factor for the final model is 16.2%. The structure consists of three domains. The N-terminal domain consists of β strands. The core domain is an 8 strand αβ barrel. The third domain has an α+β fold formed by an insertion in the barrel motif. A revised primary structure of the chitinase A protein is presented. Data about substrate binding and the mode of the enzymatic action are also presented.

INTRODUCTION

The enzymatic hydrolysis of chitin, the homopolymer of N-acetyl-D-glucosamine in β(1,4) linkage to N—acetyl-D-glucosamine, is performed by the chitinolytic system which involves two hydrolases. The first chitin hydrolase is a chitinase (E. C. 3. 2. 1. 14) which splits the chitin polymer randomly into small oligomers of N-acetyl-D-glucosamine. The second hydrolase is a β-N-acetyl-D-glucosaminidase or chitobiase (E. C. 3. 2. 1. 30) which further degrades these oligomers to N-acetyl-D-glucosamine.

Chitinases are proteins found abundantly in nature. In higher plants chitinases serve as a defense mechanism against fungal pathogens. The way in which chitinases, as well as other pathogen-related proteins, contribute to pathogen resistance is under intensive investigation. In bacteria the degradation products of chitin are used solely as a nitrogen and carbon source [1, 2].

Numerous chitinases have been detected and in most cases the corresponding gene has been isolated, cloned and the primary structure of the enzyme has been determined. Chitinases are classified into two different families. These correspond to the family 18 and 19 of glycosyl hydrolases [3].

During the course of this study we focused on determining the 3-D structure of both chitin degrading enzymes. Therefore, relatively high amounts of pure and well charac-

terized protein were necessary. Both chitinase A and chitobiase from *Serratia marcescens* have been cloned in *E. coli* as described by Jones et al. in [4, 5]. Previously we described the crystallization of chitinase A [6] and chitobiase [7] as well as purification protocols for the preparation of highly pure enzymes overexpressed in *E. coli*. Some of their biochemical and biophysical features were also presented [8].

In this paper we present the structure of chitinase A at 2.3 Å resolution. The obtained results allow us to suggest the possible enzymatic mechanism of chitin hydrolysis by chitinase A.

OVERPRODUCTION OF CHITINASE A IN *E. COLI* AND PROTEIN PURIFICATION

The construction of the *E. coli* clone A5745 which overproduces Chitinase A and the overproduction and preparative purification of the enzyme has been described previously in [9].

PRIMARY STRUCTURE OF CHITINASE A FROM *SERRATIA MARCESCENS*

The first DNA sequence determination of chitinase A gene from *Serratia marcescens* by Jones et al. is described in [4]. A second chitinase A sequence differing in eleven amino acids to the originally published sequence has also appeared [10].

While building the last 58 amino acids into the electron density map, a 20 amino acid area clearly did not matching the electron density map. The part of the gene encoding for this area was copied/amplified using appropriate primers and the PCR reaction. The resulting DNA fragment was cloned into the pCRII vector from InVitrogen. The cloned fragment was sequenced and the new open reading frame, shown in Figure 1, matched the electron density map.

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          L  N  A  r  p  g  s  r  h  r  l  h
          CTGAATGCGC-GGCCTGGAA-GCCGACACCGCTTACACC

1219      CTGAATGCGCCGGCCTGGAAACCGGACACCGCCTACACC
407       L  N  A  P  A  W  K  P  D  T  A  Y  T

          h  g  e  r  r  e  c  a  a  g  Q  G  V
          ACGGTGAACGGCGTGAATGCGCTGCTGGC-CAGGGCGTC

          ACGGTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTC   1296
           T  V  N  G  V  N  A  L  L  A  Q  G  V    432
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Figure 1. The old (upper)and the new (lower) gene fragments are presented above. Amino acids in lower case were corrected. The revised amino acid sequence for this fragment is in the lower line (upper case)

CRYSTALLIZATION OF CHITINASE A

Chitinase A was initially crystallized as described in [6]. Alternative crystallization conditions are presented in Table 1. Flat crystals of 0.5mm x 0.5mm x 0.05 mm appeared after 1-2 weeks. Crystals for the complex of chitinase A with 4-NAG were prepared by soaking the crystals in 50 mM substrate solution for 24 h.

Table 1. Crystallization conditions and physical properties of chitinase A crystal

Protein	Chitinase A
Space group	$C222_1$
Unit cell size	a=203.1, b=133.9, c=59.9 Å
Precipitant	PEG 20%
Additives	AS/isopropanol
Buffer	0.1M acetate pH 5.5
V_m (kDa/Å^3)	3.2
Solvent content (%)	54%

DATA COLLECTION

All data were collected at EMBL beamline X11 located at HASYLAB (DESY), Hamburg. Native data were collected to 2.3 Å and complex with 4-NAG data to 2.5 A resolution. All data were indexed and scaled by the DENZO and SCALEPACK programs, respectively [11]. Table 2 shows data collection statistics for the native crystal.

Table 2. Quality of the native data of chitinase A

Resolution (Å)	2.3
Rmerge (%)	7.7
Rmerge (last shell) (%)	24.6
$I/\sigma I$	12.5
$I/\sigma I$ (last shell)	12.5
Unique reflexions	38115
Redundancy (meas/unique reflexion)	5.2
Completeness (%)	97.2

PHASES DETERMINATION

All scaling of native and derivative data, Patterson map calculation, heavy atom refinement, solvent flattening and map calculations were performed by the programs included in the PHASES package [12]. The final figure of merit was 0.66. The MIR map was solvent flattened and the resulting map was of good quality. Statistics for the phasing are shown in Table 3.

Table 3. Heavy atom derivative data of chitinase A

	Resolution (Å)	Rmerge	Compl.(%)	Phas.power
1. Trimethyl-lead-acetate (2 days)	3.2	6.5	59.6	2.08
2. Trimethyl-lead-acetate (7 days)	2.4	3.4	96.2	1.99
3. Platinum tetrachloride (6 hours)	4.5	5.9	99.0	1.38
4. Platinum(II) 2,2'-6',2''-tetrapyridin (12 hours)	4.5	8.5	96.4	1.31

MODEL BUILDING

Model building was performed by the "0" program [13]. After editing the skeletal model (bones), Cα positions were assigned by the "baton" option. A polyalanine model was built by the "lego" commands which perform a data base search to obtain the best fitting penta-peptide for the given Cα conformation. The sequence was then assigned using the "slider guess" and "slider combine" options. Side chains were put in the most common rotamer position and then manually inspected and fitted into the electron density using only the most common rotamers. Using the initial MIR map we were able to built 481 amino acids this way. Refinement was carried out utilizing the standard XPLOR simulated annealing protocol [14]. The remaining amino acids were built into the resulting $3F_o$-$2F_c$ map. Further refinement was carried out with the PROLSQ program. Addition of solvent molecules was performed by the ARP package [15]. The final crystallographic R factor, included in the model 312 water molecules, is 0.162. Standard deviation in bond lengths is 0.016 Å.

DESCRIPTION OF THE CHITINASE A STRUCTURE

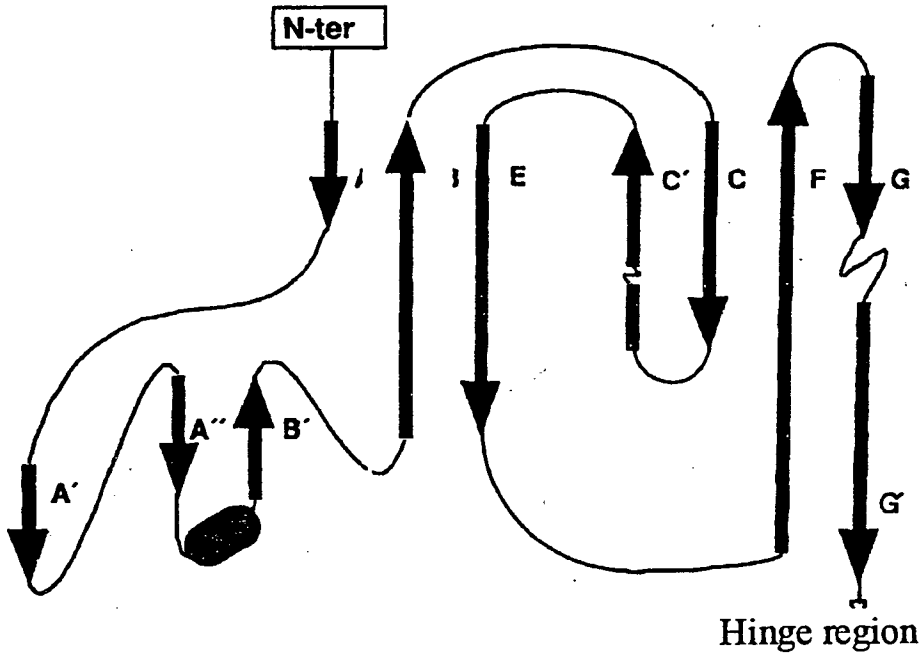

Figure 2. Topology diagram of the N-terminal domain of chitinase A

The chitinase A structure consists of three domains. The N-terminal domain which is all β-sheet is connected to the main body of the protein via a hinge region. The main body is one αβ barrel. The third domain is formed by an insertion in the main body and has an α+β fold.

The N-terminal domain has a fold comprised of only β strands (bold arrows in the topology diagram) which resembles the fold of the Fibronectin III (FnIII) module domain [16]. The sequence of this domain was identified in two more chitinases (i.e *Aeromonas caviae* and *Altreromonas sp.*). There is no biochemical data available for its function. Sequences similar to the typical sequence for FnIII type domains were identified in three other bacterial chitinases and also found in some cellulases [17]. It can be assumed that this domain facilitates either chitinase-protein(s) interaction and/or interaction with the substrate.

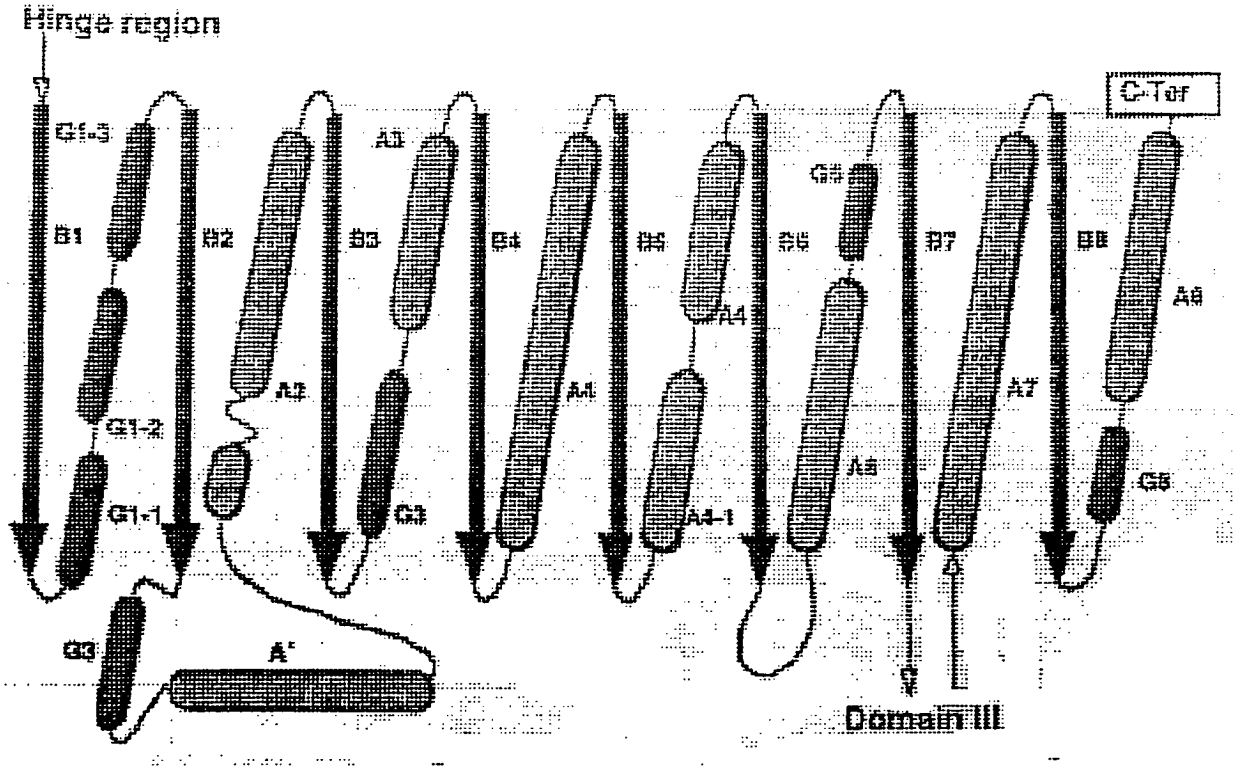

Figure 3. Topology diagram of the 8 stranded αβ barrel which is the catalytic domain of chitinase A (bold arrow indicates β-sheet and shadowed cylinder α helix)

The N-terminal domain is connected to the rest of the structure via a 20 amino acids long hinge region. It is comprised of 4 prolines, 5 arginines, 5 other charged amino acids, 4 leucines, 1 tyrosine and 1 serine.

The 8 stranded αβ barrel is the catalytic domain of the enzyme. It has a number of irregularities, compared to a typical αβ barrel fold such as triose phosphate isomerase or pyruvate kinase.

(i) There is no α helix connecting strands B1 and B2. Instead there are 3 short 3_{10} helices (G1-1,G1-2,G1-3, each 5 residues long) making the connection.

(ii) Between strands B2 and B3 2 α helices are located (A',A2) as well as 1 more 310 helix (G2) 7 residues long. (iii) Between strands B3 and B4 in addition to the α helix (A3) a 310 helix (G3) 7 residues long is located. (iv) The biggest insertion is between strands B7 and B8 which forms the third domain. (v) Another relatively large insertion is found between B6 and A6 which forms two loops which "protect" one side of the β sheet of αβ domain. (vi) After A6 comes one short (4 residue) 310 helix (G6). (vii) Finally, before the last helix (A8) a small 5 residue long 310 helix (G8) is formed.

The α+β domain is formed by the insertion between strands B7 and B8. It is comprised of 5 β strands (of which one is interrupted), forming an all anti-parallel β sheet. The α helix protects the first hydrophobic surface of the sheet. The other surface of the sheet is protected by a 310 helix together with some coil structure.

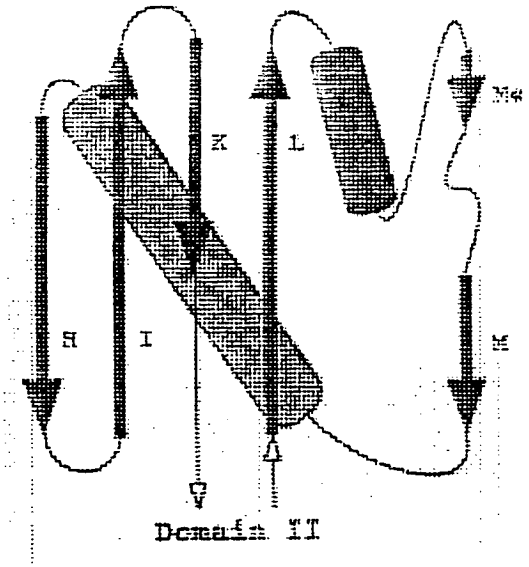

Figure 4. Topology diagram of the α+β domain of chitinase A (bold arrow indicates β-sheet and shadowed cylinder α helix)

THE ACTIVE SITE

The substrate binding site of chitinase A is formed by a long groove positioned at the C-terminal side of the β-strands involved in the αβ barrel formation. Along this groove there are many aromatic and charged amino acids residues that form the subsites for substrate binding.

Glu315 and Asp391 are completely conserved in family 19 chitinases and are considered to be the residues directly involved in the enzymatic hydrolysis of chitin. The Glu315 and Asp391 are positioned on the two sites of the groove in distances favorable for the typical acid-base catalysis mechanism with local geometry almost identical to lysozyme [18].

A point mutation of Glu204 made in the *Bacillus circulars* chitinase (aligns with Glu 315 of *Serratia marcescens*) almost abolished its catalytic activity [19]. Glu315 is sur-

rounded by hydrophobic amino acids (i.e. Phe 191, Phe 316, Met 388 and Trp 275) and in acidic pH it would be expected to be protonated and function as the proton donor in the acid-base catalytic mechanism. Asp391 is in a more hydrophilic enviroment surrounded by Tyr 444 and Arg 446 and is likely to have a negative charge at acidic pH allowing the stabilization of the carboxyanion intermediate.

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Chia_Serma   VDIDWEFP--GGKG:::::::--NSMDHIFLMSYDFYGAFD
Chit_Alter   VDIDWEFP--GGDG:::::::--QYMDYIFAMTYDFYGAWN
Chit_Apalb   IDIDWEYP--ADAT:::::::LGKVLDYINLMAYDYAGSWS
Chi1_Bacci   VDLDWEYP--VSGGL::::::IAAIVDWINIMTYDFNGAWQ
Chib_Serma   VDIDWEYP--QAAEV::::::IVAPLDYINLMTYDLAGPWE
Chit_Bruma   FDLDWEYP--VGV--:::::::LGKNFDLLFLMSYDLHGSWE
Chit_Strpl   IDLDWE-----YPN::::::ASKYIDWYNVMTYDFFGAWA
Chit_Sacer   IDVDIEATEFENDASE:::::G----------DMAADTI
                    *                    *
                   315                  391
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Figure 5. Amino sequence alignment of few chitinases from family 19 of glycosyl hydrolases. The Glu315 and Asp391 completely conserved are indicated by *

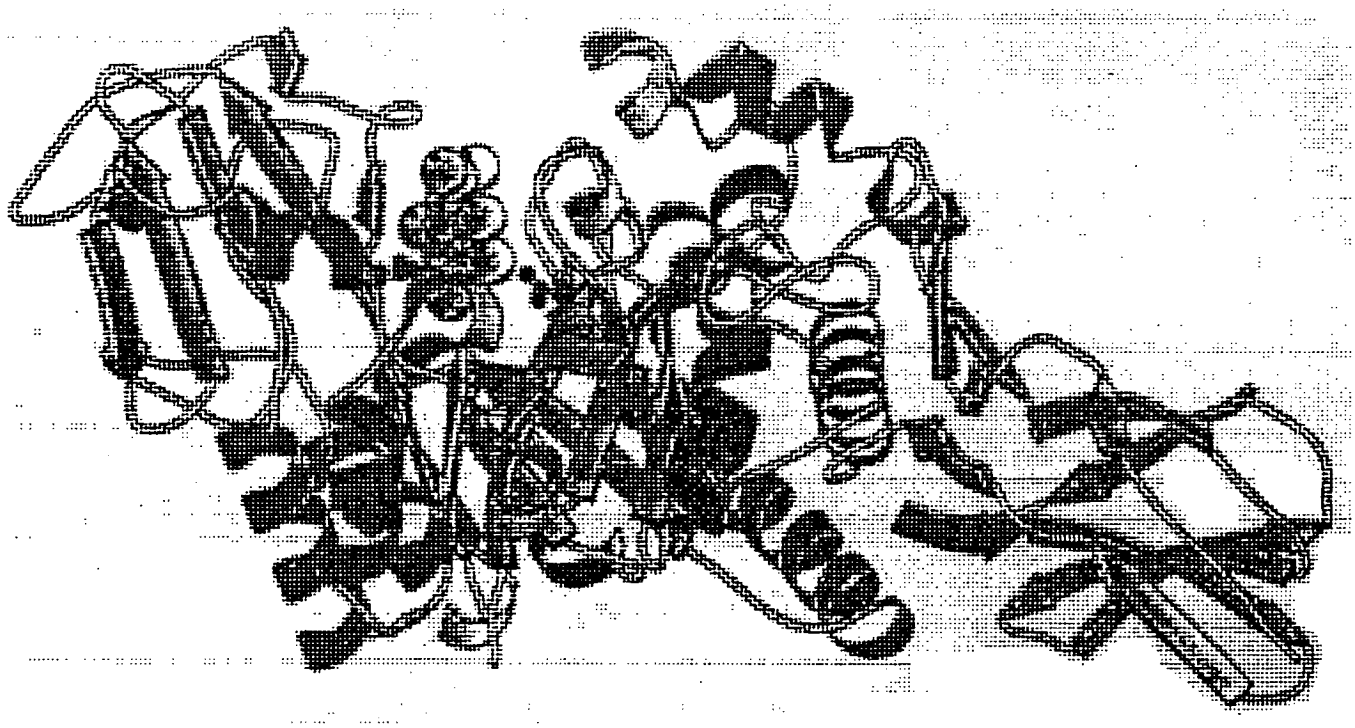

Figure 6. Schematic diagram illustrating the structure of chitinase A and the binding of the NAG

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