Isolation, characterization, sequencing and crystal structure of charybdin, a type 1 ribosome-inactivating protein from *Charybdis maritima* agg.

Eleftherios Touloupakis¹,*, Renate Gessmann²,*, Kalliopi Kavelaki¹, Emmanuil Christofakis¹, Kyriacos Petratos² and Demetrios F. Ghanotakis¹

1 Department of Chemistry, University of Crete, Greece
2 Institute of Molecular Biology and Biotechnology (IMBB), FORTH, Heraklion, Crete, Greece

**Keywords**

active site; *Charybdis maritima* agg.; ribosome-inactivating protein; sequence; structure

**Correspondence**

D. F. Ghanotakis, Department of Chemistry, University of Crete, PO Box 1470, 71409, Heraklion, Crete, Greece
Tel: +30 2810545034
Fax: +30 2810393601
E-mail: ghanotakis@chemistry.uoc.gr

*These authors contributed equally to this work

**Database**

DNA sequence data from this article have been deposited with the GenBank data library under accession number DQ323742, protein sequence data with UniProt Knowledgebase under accession number P84786, and the crystal structure with the PDB database under accession code 2B7U

(Received 3 March 2006, revised 18 April 2006, accepted 19 April 2006)


A novel, type 1 ribosome-inactivating protein designated charybdin was isolated from bulbs of *Charybdis maritima* agg. The protein, consisting of a single polypeptide chain with a molecular mass of 29 kDa, inhibited translation in rabbit reticulocytes with an IC₅₀ of 27.2 nM. Plant genomic DNA extracted from the bulb was amplified by PCR between primers based on the N-terminal and C-terminal sequence of the protein from dissolved crystals. The complete mature protein sequence was derived by partial DNA sequencing and terminal protein sequencing, and was confirmed by high-resolution crystal structure analysis. The protein contains Val at position 79 instead of the conserved Tyr residue of the ribosome-inactivating proteins known to date. To our knowledge, this is the first observation of a natural substitution of a catalytic residue at the active site of a natural ribosome-inactivating protein. This substitution in the active site may be responsible for the relatively low *in vitro* translation inhibitory effect compared with other ribosome-inactivating proteins. Single crystals were grown in the cold room from PEG6000 solutions. Diffraction data collected to 1.6 Å resolution were used to determine the protein structure by the molecular replacement method. The fold of the protein comprises two structural domains: an α + β N-terminal domain (residues 4–190) and a mainly α-helical C-terminal domain (residues 191–257). The active site is located in the interface between the two domains and comprises residues Val79, Tyr117, Glu167 and Arg170.

**Abbreviations**

RIP, ribosome-inactivating protein.

*Charybdis maritima* agg. (previously *Urginea maritima* agg.) commonly known as squill, is a poisonous plant that belongs to the family of Liliaceae. It is a large, onion-like plant that grows wild on the coast around the Mediterranean Sea.

Both varieties of squill (red and white) have fibrous roots proceeding from the base of a large and tunicated bulb. The bulb contains the pharmacologically active compounds of *Charybdis maritima* agg., which are bufadienolides and cardiac steroid glycosides. Squill has been used medicinally since ancient times. In human phytotherapy, the dried bulb of the white variety is used orally as a diuretic, emetic, expectorant and cardiotonic [1].
Ribosome-inactivating proteins (RIPs) are a heterogeneous group of enzymes, identified in plants, bacteria and fungi. They are distributed throughout the plant kingdom and are active against ribosomes from different species, although the level of activity depends on the source of the RIP and of the ribosome. There are many reports that RIPs induce apoptosis [2,3]. The main application has been focused on the construction of chimeric molecules known as immunotoxins for cancer immunotherapy [4].

RIPs are RNA N-glycosidases that inactivate ribosomes by the selective cleavage of an adenine residue at a conserved site of the 28S rRNA, arresting protein synthesis. The nature of the enzymatic modification of ribosomes was discovered by Endo & Tsurugi [5]. Interest in RIPs has arisen from their potential medical and therapeutic applications, as several of these proteins have been found to be more toxic towards tumor cells than to normal cells [6].

RIPs have been classified into three types based on their primary structures [7]. Type 1 RIPs are single-chain proteins which contain the ribosome-inactivating entity, with a molecular mass of ≈30 kDa. Type 2 RIPs are two-chain proteins which consist of an A-chain, functionally equivalent to type 1, linked through a disulfide bond to a lectin-like B-chain which promotes uptake by the cell. Type 3 RIPs are composed of a single chain containing an extended C-terminal domain with unknown function. Although type 1 and type 2 RIPs are equally effective inhibitors of protein synthesis in cell extracts, the absence of the B-chain in type 1 does not allow the protein to bind and enter cells with high efficiency. Therefore they are considerably less cytotoxic [8].

In this study, we describe the purification, characterization and structural analysis of charybdin, a novel 29-kDa type 1 ribosome-inactivating protein, from bulbs of the white variety of *C. maritima* agg.

**Results**

Charybdin was purified from *C. maritima* agg. bulbs by using a combination of hydrophobic and ion-exchange chromatography (see Experimental procedures). It is interesting to note that the *C. maritima* agg. bulbs contain extremely high quantities of the charybdin protein. The initial extract contained mainly charybdin and very small amounts of other proteins, which were only observed when the gel was overloaded. The main impurities were pigments and other small hydrophobic molecules. The objective of the purification protocol was not only to remove traces of other proteins, but also smaller molecules, which caused problems during the characterization and crystallization of charybdin. The yield of the purified protein was 150–200 mg protein per 100 g of bulbs. Charybdin appeared as a single band with a molecular mass of 29 kDa on SDS/PAGE (Fig. 1A). The pI was found by isoelectric focusing PAGE to be ≈7 (data not shown). The pI calculated from the derived sequence (see below) was 5.8.

**Translation inhibition of rabbit reticulocytes by charybdin**

The *in vitro* translation inhibitory effect of charybdin was analyzed. As shown in Fig. 1B, charybdin inhibits the rabbit reticulocyte translation system. The calcula-
ted IC50 of 27.2 nm for charybdin is at least 100 times higher than the value (0.25 nm) reported for saporin L1 [9]. IC50 represents the concentration of charybdin that inhibited in vitro protein synthesis by 50%.

DNA sequence and derived amino-acid sequence

The DNA sequence and the derived amino-acid sequence are shown in Fig. 2. The amino-acid sequence shows homology to RIPs and exhibits identity of 46.7–37.7% with the musarmins [10], 36.6% with the RIP of Hyacinthus orientalis (UniprotKB/TrEmbl code Q677A1), 28.4% with pulchellin [11], which is highly homologous to abrin, and 25.3% with ricin. The sequence similarities were calculated using the program BLAST [12]. There are 15 identical residues among seven sequences (charybdin, musarmins I and III, Hyacinthus, Iris holl, pulchellin and ricin), which share high sequence similarity. Three of the four key residues of the active site, Tyr123, Glu177 and Arg180 (ricin numbering [13]), are among the identical residues. Thus, it is interesting to note that the fourth residue, which is an invariant Tyr80 (ricin numbering) among more than 360 RIP sequences known to date, is replaced by Val in charybdin. To exclude the possibility of a local geographical mutation, DNA sequencing was also carried out on a plant collected from another region of Crete, and this residue substitution was confirmed. There are no N-glycosylation sites in the deduced sequence.

Quality of the model

The high quality of the collected diffraction data and the resulting refinement of the structure are shown in Table 1. A thin section of the structure with its electron-density map is shown in Fig. 3. A total of 232 out of 257 amino-acid residues fit very well in the electron-density map. Exceptions are certain regions on the surface of the molecule, which are quite flexible, as reflected in the higher thermal parameter values. These regions are the N-terminus and three turns comprising amino-acid residues 48–56, 96–102 and 183–188. Residues 1–3 and 99–101 are not included in the final refined model.

Fig. 2. Nucleotide sequence and derived amino-acid sequence (GenBank accession number DQ323742 and UniProt Knowledgebase accession number P84786). Y = T or C, R = A or G, N = A or G or C or T, W = A or T, V = G or A or C. Underlined sequences are the primers used for PCR on the genomic DNA. The N-terminal and C-terminal protein sequences were determined by N-terminal and C-terminal amino-acid sequencing; the parts of the DNA sequence outside the primers (coding for SQC and CAAG) were taken from the genetic code table.
The geometry of the model was analyzed by PROCHECK [14]. In the Ramachandran plot [15], 91.5% of the residues (glycine and proline residues excluded) lie in the core region, and 7.1% lie in the additional allowed region. Three residues, Leu48, Glu52 and Arg96, lie in less favored regions. These residues belong to the above mentioned poorly defined turns of the structure.

Overall folding and the active-site region

The overall folding is similar to the known RIP structures. There are two structural domains, a large N-terminal domain (Ser1-Leu190) and a smaller C-terminal domain (Pro191-Gly257). The cleft between the two domain forms the active-site pocket (Fig. 4). The N-terminal domain is composed of a six-stranded β-sheet, which in turn contains four anti-parallel central β-strands (4–7, Fig. 4) and two parallel outer β-strands (1 and 8, Fig. 4). The β-sheet is attached to five α-helices (A, C–F, Fig. 4). In most of the RIPS there are six helices in the first structural domain. In charybdin the second helix (B) is missing. According to a structural alignment of 13 solved RIPS with charybdin (Fig. 5), this helix is a less conserved structural element. Helix B is expected to be in the region of the gap between Ser98 and Gly102. In the N-terminal domain, there is also an additional two-stranded β-sheet (strands 2 and 3, Figs 4 and 5), which lies opposite the C-terminal domain. This β-sheet is not well conserved among the known RIPs and is missing in the numbering of the structural elements of ricin [13]. The C-terminal domain consists of two consecutive α-helices (G, H, Figs 4 and 5), a third helix (I, Figs 4 and 5), which is less conserved among the RIPS, and a two-stranded β-sheet 9 and 10 (Figs 4 and 5). In charybdin there exists an additional 310 helix (J, Figs 4 and 5 close to the C-terminus of the protein). This is a unique feature of charybdin. The solved structures of this family do not exhibit a 310 helix near the C-terminus.

An intramolecular disulfide bridge (Cys217–Cys254) is formed.

The active site of the determined structure was found to be free of substrate. It is occupied by several well-ordered water molecules (Fig. 6). The four key residues for catalysis are well conserved among type 1 and type 2 RIPs [13]. In charybdin, Val79 unambiguously replaces the conserved Tyr. To our knowledge, this is the first observation of a natural substitution of a catalytic residue at the active site of an RIP.

Table 1. Data, refinement and geometry statistics. The values in parentheses refer to the highest resolution shell.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range data (Å)</td>
<td>49.6–1.60 (1.69–1.60)</td>
</tr>
<tr>
<td>Observations</td>
<td>162385 (13536)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.6 (3.2)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>6.4 (18.6)</td>
</tr>
<tr>
<td>&lt; I / σ (I)</td>
<td>18.1(6.9)</td>
</tr>
<tr>
<td>Resolution range refinement (Å)</td>
<td>20–1.60 (1.64–1.60)</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>33815 (1971)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.2 (77.6)</td>
</tr>
<tr>
<td>Rcryst (%)</td>
<td>18.1 (17.8)</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>20.8 (20.8)</td>
</tr>
<tr>
<td>Number of non-H atoms</td>
<td>2312</td>
</tr>
<tr>
<td>Protein atoms</td>
<td>2047</td>
</tr>
<tr>
<td>Water molecules</td>
<td>253</td>
</tr>
<tr>
<td>Buffer atoms (Mes)</td>
<td>12</td>
</tr>
<tr>
<td>Average B factors (Å²)</td>
<td>18.25</td>
</tr>
<tr>
<td>B factor from Wilson plot</td>
<td>18.86</td>
</tr>
<tr>
<td>Rms deviations from ideal values</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.012</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.607</td>
</tr>
<tr>
<td>Chiral volumes (Å³)</td>
<td>0.164</td>
</tr>
</tbody>
</table>

Fig. 3. Stereo view of a part of the final model in the 1.6-Å electron-density map. A section of the β-sheet in domain I is shown. The 2Fo-Fc map is contoured at 1σ.
Discussion

In this work, we describe the purification, characterization and structural determination of charybdin, a novel 29-kDa protein from bulbs of *C. maritima* agg. Charybdin was characterized by biochemical methods and its structure determined by X-ray crystallography. The DNA sequence, and derived amino acid sequence, revealed significant homology with various RIPs.

Although charybdin inhibited the rabbit reticulocyte translation system, the estimated IC₅₀ of 27 nM indicates that it is not such a strong inhibitor of protein synthesis as other RIPs. The active site of RIPs, which contains four key amino-acid residues, is highly conserved. Although three of the four key residues are present at the active site of charybdin, the fourth residue, which is an invariant Tyr₈₀ (ricin numbering) among more than 360 RIP sequences known to date, is replaced by Val. This amino-acid change at position 79 of the active site of charybdin is a striking feature of the protein and possibly explains its low inhibitory activity compared with other RIPs. In ricin A, the active-site residues were analyzed by site-directed mutagenesis to assess their role in the mechanism of action of the toxic enzyme [16,17]. It was found that replacement of Tyr (in ricin position 80) with Phe decreased activity by a factor of 15, and replacement with Ser decreased activity 170 times. It is expected that Val in this position would have an even more pronounced effect because the aliphatic side chain cannot form hydrogen bonds.

Drastic attenuation of protein synthesis was also observed with two mutations in the Shiga-like toxin I A-chain [18]. Replacement of the active-site Tyr (position 77 in this case) with Phe resulted in 10–20-fold less activity, and replacement with Ser made the protein completely inactive.

As charybdin is the main protein constituent of the bulb of *Charybdis*, one may speculate that protein translation inhibition is not its major (or only) function; it may, for example, act as a special storage protein [19].

Although charybdin was isolated by a series of purification steps, we cannot exclude the possibility that it exists in various isoforms (as is the case with other monocots such as *Muscaria*, *Hyacinthus* and *Iris* [10]), some of them highly active and others inactive. If this is the case, the protein that we isolated and studied may be an inactive isoform, and the observed activity may be due to ‘impurities’ of another highly active isoform. A definitive answer to this question will be given by cDNA cloning, which is one of our objectives. We are also planning to carry out site-specific mutagenesis experiments to replace the active-site Val with Tyr and study the effects on the activity and structure of charybdin.

Experimental procedures

Fresh *C. maritima* agg. bulbs were collected from a hill near Agia Galini (N35.06°-E24.41° Crete-Greece), and for DNA sequencing also from the hamlet of Samaria (N35.17°-E23.58°).

Preliminary sequencing experiments after tryptic digestion of the denatured protein provided small fragments and an 87-amino-acid sequence (F. Lottspeich, unpublished data). This allowed us to identify charybdin as a putative RIP.

Protein purification

Fresh bulbs of *C. maritima* agg. (100 g) were homogenized in a blender at 4 °C with 300 mL extraction buffer containing 60 mM sodium phosphate, pH 7.2, 100 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethanesulfONYl fluoride and 1.5% (w/v) polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth, and
the filtrate was centrifuged at 34 000 g for 30 min at 4 °C. The supernatant was passed through filtration paper. The yellowish crude protein solution was first dialyzed against a solution containing 60 mM sodium phosphate, pH 7.2 and 0.75 mM ammonium sulfate, and was subsequently loaded on to a column packed with a matrix substituted with hydrophobic ligands. The column (dimensions 1 × 10 cm) was packed with phenyl-Sepharose CL-4B (Pharmacia, Upsala, Sweden) and equilibrated with 10 column volumes of 60 mM sodium phosphate, and 0.75 mM ammonium sulfate at 10 °C. The sample was applied to the column at a flow rate of 0.75 mL min⁻¹. A fraction eluted with 60 mM sodium phosphate and 0.3 mM ammonium sulfate contained the protein of interest. The eluted protein was dialysed in 50 mM Heps, pH 7.7, and then loaded on a Q-Sepharose anion-exchange column pre-equilibrated with the same buffer. The purified protein was eluted with 0.3 M NaCl.

For crystallization experiments, the protein isolated by the chromatographic procedure described above, was further purified by an additional sucrose density gradient step. More specifically, a continuous sucrose density gradient (10–40% sucrose in 60 mM sodium phosphate buffer, pH 7.2) was used. Centrifuge tubes were put in a swing-out rotor and ultracentrifuged at 150 000 g for 22 h at 6 °C in a Sorvall Ultra 80 centrifuge. This sucrose density gradient step resulted in the removal of pigments, which were copurified with the protein, and it was necessary for the crystallization of charybdin. Protein concentration was determined by the method of Bradford, using BSA as standard.

Fig. 5. Alignment of 14 crystal structures based on secondary-structure elements assigned by the program SPDBVIEW [23]. The structures are: cha, title compound (2B7U); abr, abrin (1ABR); ebu, ebulin (1HWM); mob, momordin (1MOM); lec, mistletoe lectin (1TFM); tri, trichosanthin (1MRJ); ric, ricin (1J1M); bry, broydin (1BRY); pa3, pokeweed pap-III (1LLN); agg, agglutinin (1RZO); luf, luffin (1NIO); dia, dianthin (1LP8; sap, saporin (1QI7); pok, pokeweed antiviral protein (1QCG). Secondary-structural elements are colored as in Fig. 4. The key residues of the active site are marked with arrows; asterisks denote identical residues. The respective Protein Data Bank codes are given in parentheses.
Electrophoresis
Preparations were analyzed by SDS/PAGE by the method of Laemmli.

Translation inhibition of rabbit reticulocytes by charybdin

Charybdin was tested for in vitro protein synthesis inhibition activity by using a Flexi rabbit reticulocytes system (Promega, Madison, WI, USA). The translation was performed according to the manufacturer’s protocol in the presence of [35S]Met to label the products. Rabbit reticulocytes were incubated with increasing amounts of charybdin (13.8–552 nM) for 30 min at 30°C before initiation of translation. Untreated rabbit reticulocytes were used as the negative control, while the RIP saporin (Fluka, Chemie Buchs, Switzerland) was used as the positive control. The reaction was initiated by adding luciferase control mRNA to the charybdin-treated reticulocytes. The reaction was carried out at 30°C for 60 min and was terminated by centrifugation at 100 000 g for 15 min a 4°C. The labeled products were analyzed by autoradiography. For autoradiography, the following instruments were used: Hypercassette™ (Amersham, Chalfont St Giles, UK) autoradiography cassettes, the Imaging Plate (Fujiﬁlm, Tokyo, Japan) and the Storm 840 imaging system (Molecular Dynamics, Sunnyvale, CA, USA). ImageQuant software was used for quantiﬁcation comparing the relative darkness of the different bands on the ﬁlm. Activity was expressed as a percentage of the control in which no charybdin was added. The IC_{50} was calculated by linear regression analysis.

DNA sequencing

Total plant DNA was extracted from the bulb. Approximately 0.1 g of material cut from the inner part of the bulb was frozen and ground to powder in liquid nitrogen. Genomic DNA was further isolated by using the plant DNeasy Mini Kit (Qiagen, Hilden, Germany).

Crystals obtained as described below were dissolved in water, yielding 8 µg protein, which was used for N-terminal and C-terminal sequencing by the Protein Analysis Center at the Karolinska Institutet in Stockholm, Sweden. This was necessary in order to design primers suitable for the PCR experiments.

Based on the N-terminal sequence (SQXKAMTVKFT-VELXI), the degenerate oligonucleotide primer (5’-AA RGCNATGACGGTGAAGTTCACAGTNGA-3’; where, R = A or G; N = A, C, G, T) was used as the upper primer. In this primer, several degenerate sites were converted into single nucleotides that were derived from the DNA sequences of homologous proteins.

From the crystallographic results, the C-terminal amino-acid sequence EQHPDTRSPPCAAG was found. C-Terminal sequencing of the protein conﬁrmed the last four amino-acid residues. The seven underlined amino-acid residues were also deduced from sequencing after tryptic digestion. The highly degenerate primer (5’-GGNGGAGAN CGNGTRTCNGGRTGYTGYTC-3’ where, Y = T or C) was used as the lower primer. As there are no homologous protein sequences for this part, the only assumption for lowering the degeneracy of the primer was made for Ser (genetic code assumed to be TCT) in analogy with the musarin sequences, thus risking a maximum of three mismatches. Weak PCR-product bands with the expected molecular size of ≈ 800 nucleotides were obtained only with the ‘Expand long template PCR system’ (Roche, Basel, Switzerland) at an annealing temperature of 45°C. The product was used as template for re-PCR (Deep Vent polymerase; New England Biolabs) after purification from a gel. Again the product of the re-PCR was puriﬁed from a gel and directly used for sequencing in an ABI-377 sequencer using the big determinator kit v.3.1 in the sequencing facility of IMBB. Sequencing was performed for both strands of DNA from two plants collected from different geographical environments in Crete, resulting in six sequences.

Crystallization

The protein was crystallized by the vapor-diﬀusion method. Crystals were grown during a several-day period by equilibrating a hanging drop of equal volumes of the protein...
solution (5 mg mL\(^{-1}\) in 25 mM Heps, pH 7.0) and reservoir solution (0.1 \(\text{M} \) Mes, pH 6.0, 16% PEG6000) at 10 °C. Crystals were first characterized ‘in house’ using as X-ray source a RU H3R rotating anode generator (Rigaku/MSC, Woodlands, TX, USA) and a Mar300 imaging plate detector system (MarResearch, Hamburg, Germany). Before data collection, crystals were flash-frozen in liquid nitrogen in the presence of 25% glycerol as a cryoprotectant. The crystals belong to space group C2 with unit cell parameters, \(a = 99.24 \text{Å}, \quad b = 57.24 \text{Å}, \quad c = 51.09 \text{Å} \) and \(\beta = 104.08^\circ\). The Matthews ratio \(V_M = 2.41 \text{Å}^3/\text{Da}\), which corresponds to 49% (v/v) solvent content. The asymmetric unit of the crystals contains one protein molecule. The crystals diffract synchrotron X-rays to 1.37 Å resolution.

Data collection, structure solution and refinement

The final diffraction data were collected using the ID14-1 beamline (ESRF, Grenoble, France) at 100 K on an ADSC detector. Data extending to 1.6 Å resolution were processed using MOSFLM 6.2.3 [20]. A high-resolution dataset with overloaded reflections was scaled together with a low-resolution dataset with limited overloaded reflections, using SCALA [21]. The structure was solved by the molecular replacement method by AMoRe [22], using 5452 reflections between 10 and 3 Å resolution. At the time of the structure solution, most of the protein sequence was unknown. This made necessary a careful inspection of the crystal structures of 12 RIPs in order to choose a suitable model for molecular replacement. In the N-terminal domain, a section of five strands of the \(\beta\)-sheet and one flanking \(\alpha\)-helix was found to be relatively invariant on the basis of structural alignments using the program Swiss-PdbViewer [23]. This section was used as part A of the search model, whereas the residues were assumed to be alanine. Several flexible turns, e.g. not spatially conserved among the different RIPs, were omitted. The 87-amino-acid residue sequence deduced from a tryptic fragment was superimposed on the structures of the 12 RIPs, and a swiss model [23] was derived and used as part B of the search model. Both models were positioned on the consensus skeleton of the 12 RIPs by least-square fits. The molecular replacement search model comprised 271 atoms in 55 Ala residues (part A) and 722 atoms in 87 residues (part B), i.e. only 993 atoms, out of 2047 atoms (48.5%) of the final protein model. The rotation function with the correlation coefficient based on intensities and with the highest Patterson correlation coefficient was chosen to be the correct solution, in spite of the fact that the correlation coefficient based on F and R factor (56.2%) were not the best among the proposed solutions. The correctness of the solution was verified by building the symmetry related neighbors in the crystal lattice. No bad contacts were detected. Ninety two residues were built into electron density, which was derived from several runs of the program ARP/wARP 6.1 [24], whereby input parameters were varied. At this stage, the protein consisted of five peptide fragments, the longest comprising 69 residues. Refinement was carried out using REFMAC v.5.2 [25] followed by manual modeling using XFIT [26]. TLS [27] refinement was also used for several cycles. One cocryrstallized Mes molecule as well as all included water molecules were identified by manual model building. The final model comprises 251 out of 257 residues. The three N-terminal residues and residues 99–101 are not fitted in the final electron-density maps. The graphic illustrations of the protein were obtained using PyMOL [28].

Acknowledgements

We thank Dr F. Lottspeich for providing the sequence of various tryptic fragments, and Dr M. Aivaliotis and C. Karapidaki for their contributions during the isolation and characterization of the protein. RG would like to thank M. Providaki, A. Deli and L. Spanos for their contributions to the DNA sequencing. We thank the EMBL Grenoble Outstation, in particular, Dr Cusack and Dr Muziol, for providing support for measurements at the ESRF under the European Community – Access to Research Infrastructure Action FP6 program.

References


17 Kim YS & Robertus JD (1992) Analysis of several key active site residues of ricin A chain by mutagenesis and X-ray crystallography. Protein Eng 5, 775–779.


Supplementary material

The following supplementary material is available online:

Fig. S1. Comparison of the derived amino-acid sequence of charybdin with other known RIPs. MusI (Q8L5M2), MusIII (Q8L5M4), Hyacinthus (Q677A1), Iris holl (O04356), pulchellin (Q5CA3) and ricin (P02879). The putative signal peptide of musarmins are underlined; key residues of the active site are marked with arrows. Asterisks and double points denote identical and conserved residues, respectively. The respective Swiss/TrEMBL accession codes are given in parentheses.

Fig. S2. Possible DNA sequences of charybdin and homologous proteins coding for the N-terminal and C-terminal region of charybdin after alignment of the protein sequences. Underlined nucleotides denote different bases at the same position in different proteins; the colored sequence is the deduced primer. Other sequences: musarmin 1–4, Iris holl 1,2,3, Iris holl 4,5 (GenBank AF256085, AF256084).

This material is part of the online article from http://www.blackwell-synergy.com