

Refolding of the *Cupressus arizonica* major pollen allergen Cup a1.02 overexpressed in *Escherichia coli*

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Abstract

The cDNA encoding an isoform of the cypress major pollen allergen, Cup a1.02, has been cloned and expressed in *Escherichia coli* as a N-terminal 6× His-tagged protein. To increase recovery, Cup a1.02 was expressed at high levels exploiting the T5 strong promoter and led to accumulate as inclusion bodies. The insoluble purified aggregates were solubilized in 6 M guanidine hydrochloride, immobilized using nickel-chelating affinity chromatography, and successfully refolded by controlled removal of the chaotropic reagent. Enhanced protein refolding was observed by reducing the protein concentration at 0.6–0.8 mg/ml. SDS–PAGE and gel filtration chromatography indicated an apparent molecular mass of ≈40 kDa and the occurrence of the protein as monomers. The reconstituted fusion protein displayed the same immunological properties of the native Cup a1.02 protein as proven by IgE immunoreactivity. Immunoblotting, ELISA, and histamine release test showed that the tag did not preclude the protein functionality hence validating its correct three-dimensional folding. The protein fold was also assessed by CD spectroscopy and deconvolution of the spectrum allowed to estimate the secondary structure as a prevalence of β structures (higher than 60%) and a small contribution from α helices (less than 12%). The reported procedure has proven to be useful for the production of multi-milligrams of recombinant Cup a1.02 allergen suitable for structural biology studies and for the molecular and functional characterization of the IgE binding sites.

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Pollinosis is an IgE-mediated disease affecting, with increasing prevalence, the worldwide populations. In various geographic areas, due to the extensive planting of cypress trees, pollen of the Cupressaceae family is the major aerospore component in the period ranging from late winter to early spring, giving rise to diffused respiratory allergies [1–3].

Allergy diagnosis is currently performed by detecting specific IgE either in vivo or in vitro, and immunotherapy is regarded as an important treatment capable of strongly modifying the outcome of the disease. In both instances, whole allergenic extracts are currently used, but in the case of Cupressaceae, the extracts are of limited quality and the content of relevant allergens is not normally evaluated [4,5]. For this reason, the use of recombinant molecules, that are more and more proposed at the clinical trial level in the field of immunodiagnosis

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and immunotherapy, could represent an important issue in Cupressaceae allergy that could then be more easily diagnosed and treated [6,7].

Cup a1, the *Cupressus arizonica* major pollen allergen, has been identified and its importance for the immunodiagnosis recognized [8]. It is a 43 kDa glycoprotein, bearing both peptide and carbohydrate epitopes that are recognized by IgE from Cupressaceae allergic patients [9]. Two separate cDNAs encoding Cup a1 and the isoform Cup a1.02 have been cloned and sequence analyses indicate they share high primary structure identity with allergens of mountain cedar Jun a1 (90%), Japanese cypress Cha o1 (80%), and the Japanese cedar Cry j1 (75%) [10–13], thus giving structural support to the well-known cross-reactivity phenomena described for conifer pollens [9,10,14].

A limit for using recombinant allergens is related to difficulties in expressing large amounts properly refolded molecules, capable of interacting with IgE of the patient.

Moreover, high resolution structural studies to gain a further insight into their three-dimensional conformation are considered as highly desirable, to identify and analyse IgE epitopes and to rationally design hypoallergenic derivatives for vaccine development for immunotherapy.

To achieve this goal, the production of multi-milligrams of purified protein that is suitable for three-dimensional structural studies represents a major issue. Many different expression systems have been employed, each one with its own drawbacks, and the production of the protein in inclusion bodies followed by refolding in vitro resulted in the best approach. The reconstitution of the protein has been evaluated by circular dichroism spectroscopy and in vitro immunoreactivity assay. Overall, the proposed strategy that combines a high expression level of the protein as inclusion bodies followed by in vitro refolding of immobilized protein by affinity chromatography provides a source of highly purified Cup a1.02 protein.

Materials and methods

Native cup a1 isolation

The native major allergen Cup a1.02 was isolated and purified as previously described [15].

Cloning and expression of recombinant Cup a1.02

All the molecular biology protocols were performed according to procedures by Sambrook et al. [16]. The cDNA, containing the coding sequence of Cup a1.02 [9], was directionally subcloned between the *Bam*HI and *Hind*III restriction sites of the expression plasmid pQE30 (Qiagen) which contains a 6× His tag at the N-

terminus. The DNA sequence of the construct was confirmed by sequencing of either DNA strands [17]. The recombinant plasmid, named pQCup a1.02, was used to transform the host bacterial strain M15/pREP4 (Qiagen). Freshly transformed bacteria were grown overnight in 4 ml of Luria broth (LB) containing ampicillin (0.1 mg/ml) and kanamycin (0.025 mg/ml). The overnight culture was diluted into 1 L of the same media and grown to mid-logarithmic phase ($A_{600} = 0.6$) at 37°C. Protein production was induced by the addition of 1 mM isopropyl- β -D-thiogalactoside (IPTG) for 8 h. Cells were harvested by centrifugation at 5000g and stored at –80°C until used.

Isolation and purification of inclusion bodies

The frozen cell paste was suspended in 20 ml/g cells of buffer A (50 mM Tris–HCl, pH 8, 300 mM NaCl, and Complete EDTA-free) and disrupted by four cycles of sonication on ice. DNA was digested by addition of DNase I (100 μ g/g cells) in the presence of 10 mM MgCl₂ and stirring for 30 min at room temperature. The bacterial extract was centrifuged at 10,000g for 30 min to obtain the insoluble fraction. Subsequently it was dissolved in 20 ml/g cells of buffer B (50 mM Tris–HCl, pH 8, 300 mM NaCl, Complete EDTA-free, 7 mM β -MET, and 2 M urea) and sedimented as described above. The inclusion body-containing pellet was washed in 20 ml/g cells of buffer C (50 mM Tris–HCl, pH 8, 300 mM NaCl, Complete EDTA-free, 7 mM β -MET, 2 M urea, and 1% Nonidet P40) and once in buffer D (50 mM Tris–HCl, pH 8, 300 mM NaCl, and Complete EDTA-free, and 7 mM β -MET). Purified inclusion bodies were harvested by centrifugation (10,000g, 4°C, 30 min) and proteins were solubilized in 20 ml/g cells of buffer E (50 mM Tris–HCl, pH 8, 300 mM NaCl, Complete EDTA-free, 7 mM β -MET, 6 M guanidine hydrochloride, and 5 mM imidazole) by stirring overnight at room temperature. The homogenate was centrifuged (10,000g, 4°C, 15 min) to remove insoluble materials and filtered onto a 0.22 μ m filter.

Refolding of the recombinant His-Cup a1.02 allergen

The solubilized proteins were subjected to metal chelate affinity chromatography (IMAC) using a HiTrap Chelating (Amersham–Pharmacia) with a bed volume of 5 ml. It was equilibrated on an FPLC apparatus (Amersham–Pharmacia) with 5 volumes of buffer E and after loading and column washing, protein refolding was achieved by applying a linear gradient from 6 to 0 M guanidine hydrochloride at a flow rate of 0.1 ml/min in a total volume of 40 column volumes. The recombinant protein was then eluted by increasing the imidazole concentration from 60 to 500 mM.

Size-exclusion chromatography

Size-exclusion chromatography (SEC) was performed on a Superdex 75 HR 10/30 column attached to a FPLC system (Amersham–Pharmacia). The column was calibrated using a low and high molecular weight gel filtration kit (Amersham–Pharmacia) followed by equilibration with two volumes of 50 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4. The Cup a1.02 IMAC-eluted protein was dialysed overnight against the same buffer, concentrated to approx 1 mg/ml in a 10 kDa molecular weight cutoff Centricon (Amicon) run at 3000g, and injected at a flow rate of 0.5 ml/min. Elution was performed with the equilibration buffer and the collected fractions were dialysed overnight versus 5 mM sodium phosphate buffer, pH 7.4, and concentrated to ≈ 1 mg/ml.

Electrophoresis and Western blotting

Protein samples were resolved using SDS–PAGE in 12% (w/v) polyacrylamide under reducing conditions. The gels were stained with Coomassie brilliant blue R-250 or transferred onto nitrocellulose membrane for Western blot analyses. For Western blots, non-specific proteins were blocked by incubating for one hour with 3% BSA in Tris-buffered saline. The membrane was incubated with the monoclonal antibody 2D5 [18] (1:1000 dilution) followed by a horseradish peroxidase-labeled secondary antibody and detection was performed using 4-chloro-1-naphthol (Sigma) and H_2O_2 as substrates, according to the manufacturer's instruction.

CD spectroscopy

CD spectra were measured with a Jasco J-715 spectropolarimeter (Jasco, Japan). Protein solutions of about 1 mg/mL in 5 mM phosphate buffer, pH 7.0, were placed in a strain-free 1-mm quartz cuvette, and spectra were measured between 260 and 178 nm with the following setup: bandwidth 2 nm, time constant 2 s, scan rate 20 nm/min, and N_2 purging rate 25 L/min. Eight spectra corrected to background (phosphate buffer) were averaged and successively smoothed with an 8-point Savitsky–Golay smoothing procedure. The CD spectrum was normalized to $\Delta\epsilon$ units using the molar extinction coefficient at 280 nm, $\epsilon_{280} = 28,400 \text{ M}^{-1} \text{ cm}^{-1}$.

Secondary Structure Analysis. Analysis of CD data for secondary structure determination was carried out according to the Locally Linearized [19] and Random Variable Selection [20,21] methods.

Human sera and direct ELISA

Human sera were obtained from patients allergic to Cupressaceae pollen, as proven by clinical history, skin

prick test (SPT) with commercial extracts, and specific IgE in vitro detection (CAP System, Pharmacia and Upjohn, Uppsala, Sweden). The subjects were free from allergen-specific immunotherapy, corticosteroids and antihistaminic therapy. Their informed consent to participate in the study was obtained.

Serum samples from non-atopic donors were collected to be used as controls. Sera were stored at -20°C until use.

ELISA experiments were carried out as previously reported [22,23]. The amount of nCup a1 and rCup a1.02 coated on the ELISA plates (polystyrene microtitre 96-well, Greiner, Frickenhausen, Germany) was $5 \mu\text{g/ml}$. The amount of antigens to be used in plates coating was determined on the basis of preliminary titration experiments. Human sera were diluted 1:10 in phosphate-buffered saline, pH 7.2 (PBS) plus 0.05% Tween (PBS-T).

Basophil histamine release assay

Heparinized blood from a non-atopic donor was collected and mixed with 0.25 volumes of 6% w/v dextran. Leucocytes were isolated as described by Okayama et al. [24] and washed in Ca^{2+} , Mg^{2+} -free PBS. Passive sensitization of basophils with specific IgE from allergic sera was performed after non-specific IgE stripping according to the method of Pruzansky et al. [25]. Two representative sera from Cupressaceae allergic patients and two control sera (one from a non-atopic subject and one from a house dust mite allergic patient) diluted 1:2 were used in this test. The samples passively sensitized were diluted 1:7 in the buffer provided by the kit utilized to measure histamine release (Histamine kit: Immunotech Pharmaceuticals, San Diego, CA, USA). The procedure was carried out according to kit instructions as described by Batanero et al. [26].

Results

Production, refolding, and purification of Cup a1.02

An efficient method has been developed to recover high protein levels based on the expression vector pQE30 under control of the T5 promoter (Fig. 1A).

Preliminary experiments indicated that the recombinant Cup a1.02 can be overexpressed mainly in inclusion bodies. Therefore, methods for further increasing protein concentration in inclusion bodies and protocols to reconstitute the active molecule from the insoluble aggregates of misfolded peptides were developed, following the general guidelines indicated by Rehm et al. [27]. Anyway, the induction temperature was shifted from 28 to 37°C and the induction time prolonged from 3 to 8 h, thus allowing the His-Cup a1.02 levels to reach $\approx 25\%$ of total cell proteins (Fig. 1B, lane 3). These con-

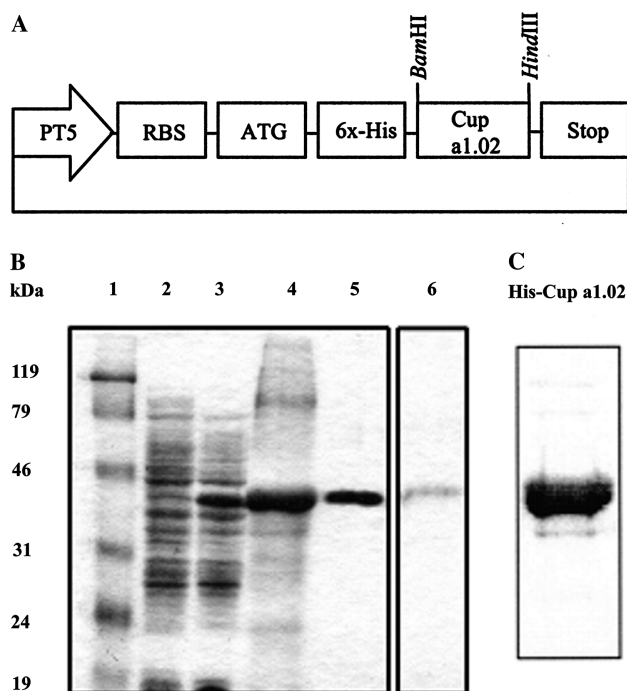


Fig. 1. (A) Schematic representation of the pQCup a1.02 expression vector. The DNA fragment carrying the Cup a1.02 coding sequence was subcloned into the plasmid pQE30 using *Bam*HI and *Hind*III restriction sites, yielding a sequence encoding the N-terminal His-tagged Cup a1.02 fusion protein. The plasmid contains a promoter region (PT5), a ribosome binding site (RBS), a sequence coding for six histidine residues (6x His), and a stop codon. The restriction site positions are also indicated. (B) SDS-PAGE analysis of His-Cup a1.02 protein at various stages of purification. Lane 1, MW markers (Fermentas); lane 2, pre-induction bacterial lysate; lane 3, IPTG-induced bacterial lysate; lane 4, His-Cup a1.02 purified inclusion bodies; lane 5, His-Cup a1.02 following refolding by nickel-chelating chromatography; and lane 6, His-Cup a1.02 after size exclusion chromatography. The His-Cup a1.02 is ≈ 40 kDa. (C) Western blot analysis carried out on purified inclusion bodies as described in Materials and methods.

ditions allowed the maximum protein accumulation levels.

Inclusion bodies from *E. coli* M15 harbouring pQCup a1.02 from a 1 L culture were isolated and purified as described in Materials and methods. After the purification of the inclusion bodies, a Cup a1.02 fusion protein with a purity of $\approx 80\%$ was obtained (Fig. 1B, lane 4; Table 1). The purified inclusion bodies were dis-

solved in 6 M guanidine hydrochloride o/n at room temperature and after removal of the insoluble particle, the denatured proteins were applied to a metal chelating affinity column. The matrix-bound fusion protein was subjected to a refolding process using a gradient from 6 to 0 M guanidine. A flat gradient was used to avoid protein aggregation. The fusion protein was finally eluted employing imidazole. The peak fraction at an imidazole concentration of ≈ 200 mM was collected and subjected to SDS-PAGE analysis. The latter showed that the recovery of refolded His-Cup a1.02 was about 19% of the inclusion body proteins (Table 1; Fig. 1B, lane 5) and Western blot analysis confirmed the identity of the refolded protein (Fig. 1C).

Refolding conditions were optimized by testing the effect of the tag position and the immobilized protein concentration. The highest recovery yield was obtained when the allergen was tagged at the N-terminus end and the loaded protein concentration ranged from 0.6 to 0.8 mg/ml. Above this threshold the recovery rapidly decreased (data not shown).

Size-exclusion chromatography was performed on the recombinant Cup a1.02 protein after dialysis and concentration, to further purify the protein. The major peak eluted at approximately 40 kDa, indicating that the majority of refolded protein ($>95\%$) was present in monomeric form (Table 1; Fig. 2). Analysis of the chromatogram indicated that some aggregates were present and removed with this procedure. Size-exclusion fractions containing refolded and purified Cup a1.02 protein (Fig. 1B, lane 6) were combined, dialysed o/n into 5 mM phosphate buffer, pH 7.4, and concentrated down to approximately

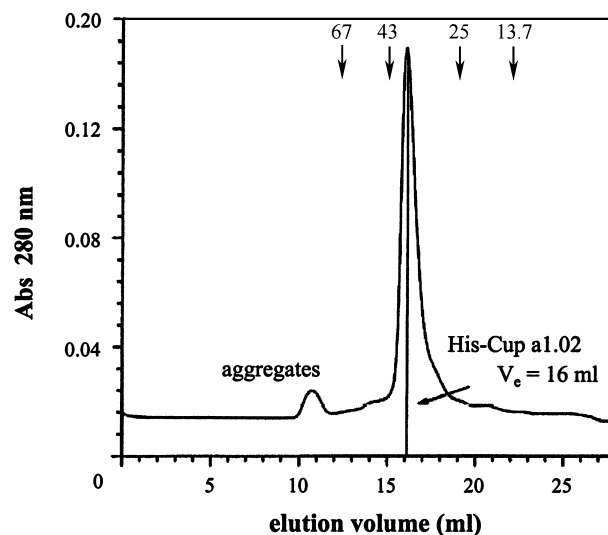


Fig. 2. Size-exclusion chromatography of soluble His-Cup a1.02 following refolding. Cup a1.02 fractions from IMAC were loaded on a properly calibrated Superdex 75 column-FPLC at a flow rate of 0.5 ml/min. Fractions were collected every minute, and the absorbance was measured at 280 nm. Arrows indicate the molecular weight markers used and the elution volume of His-Cup a1.02.

Table 1

Recovery of recombinant Cup a1.02 during refolding and purification steps

Purification steps	Total protein (mg)	Protein yield (%)	Cup a1.02 enrichment (%) ^a
Solubilized inclusion bodies	96	100	80
IMAC	18	19	>95
SEC	15	16	>95

^a The enrichment refers to total bacterial lysate.

1–1.5 mg/ml. The final recovery of soluble protein was about the 16% of solubilized proteins (Table 1).

Immunological properties of reconstituted Cup a1.02

To evaluate the property of the refolded protein to bind IgE from allergic patients, four sera (S1–S4) were tested in ELISA experiments. These sera were chosen among those previously characterized on the native and recombinant rCup a1.02 [9]. As shown in Table 2, the four sera were able to recognize the native protein as well as the refolded recombinant one.

The presence of the 6× His-tag had no effect on the refolded Cup a1.02 immunoreactivity as assessed by IgE immunoblotting and ELISA experiments. Controls performed with a recombinant 6× His-tag fusion irrelevant protein confirmed that the tag is not recognized by IgE in the cypress allergic-patients' sera.

The property of refolded recombinant Cup a1.02 to trigger basophil degranulation was investigated in a histamine release test, using basophils from a non-atopic donor passively sensitized with IgE from allergic patients S3 and S4 (Table 3). The release values obtained after stimulation with 1 µg/ml of nCup a1 or rCup a1.02 are shown in Table 3. Specific IgE from sera S3 and S4 were able to induce histamine release at the native and recombinant antigen concentration of 1 µg/ml. The percent of release values obtained with the two molecules was essentially the same for each serum, showing that the refolded molecule retained the IgE epitopes.

Sera from one non-atopic subject and one house dust mite allergic patient were used as negative controls. They did not induce histamine release when tested with any allergen amount, whereas IgE from the mite allergic patient were able to elicit histamine release when incubated with a mite extract (data not shown).

Table 2
IgE reactivity by direct ELISA (OD₄₉₀)

Sera code	ELISA nCup a1	ELISA His-Cup a1.02
S1	0.84	1.02
S2	0.26	0.23
S3	0.54	0.55
S4	0.37	0.23

The sera were tested with the nCup a1 and the His-Cup a1.02. Negativity cutoff values have been calculated on the basis of the mean OD₄₉₀ values plus 3 SDEV, obtained with six sera from non-atopic donors (OD₄₉₀ 0.04 and 0.08 for nCup a1 and His-Cup a1.02, respectively).

Table 3
Basophil histamine release test performed with IgE from Cupressaceae allergic patients

Sera code	nCup a1 (%)	His-Cup a1.02 (%)
S3	37	40
S4	53	42

The table shows the release values obtained after stimulation with 1 µg/ml of allergen.

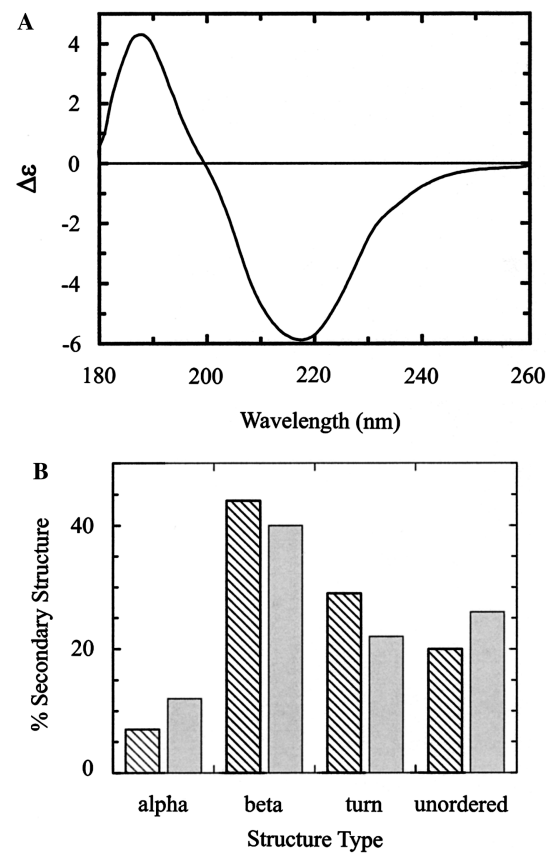


Fig. 3. CD spectroscopic analysis of the refolded His-Cup a1.02 protein. (A) CD spectrum of His-Cup a1.02 in 5 mM phosphate buffer, pH 7.4. Protein concentration was 40 µM. (B) Secondary structure determination based on locally linearized (grey bars) and random variable selection (striped bars) methods.

CD spectroscopy and secondary structure determination of His-Cup a1.02

The secondary structure content of the His-Cup a1.02 refolded protein, determined by far-UV CD spectroscopy, is shown in Fig. 3. The CD spectrum displays a pronounced negative ellipticity around 217 nm, pointing to the presence of extensive β structures. A quantitative analysis, carried out by locally linearized (LL) and random variable selection (RVS) methods, yielded the results shown in Fig. 3B. It must be pointed out that the analysis has been carried out on the uncleaved, His-tagged protein and hence the secondary structure determination contains the contribution from the tag region (most certainly unstructured).

Discussion

Allergies affect approximately a third of the world population and are a major cause of chronic ill health in developed countries [28]. The present work is a part of a project aiming at the investigation on Cypress

pollinosis and it was carried out to achieve high levels of a cypress protein, Cup a1.02, known to be involved in a worldwide late-winter allergy, for immunological and structural studies. As IgE antibodies of sensitized patients may recognize continuous as well as conformational epitopes, structural analysis of the proteins can have an impact on diagnosis and immunotherapy in the field of allergology. Structural information and immunological analysis of the recombinant allergen molecules have demonstrated that these proteins resemble the epitope complexity of natural allergen extracts and may replace these extracts for diagnostic and therapeutic purposes. Using single allergen components it is possible to delineate the reactivity profile of a given allergic patient and thus to identify the disease-causing allergen molecules (component-resolved diagnosis, CRD) and to use them for a specific immunotherapy [6].

Recombinant allergen mimicking the natural allergen could be used for immunotherapy. They would allow a treatment based on the individual patient sensitization profile (component-resolved immunotherapy; CRIT), which would minimize the possibility of therapy-induced de novo sensitizations. Therefore, structural data may be of outstanding relevance to engineering hypoallergenic isoforms for the development of more effective and safe vaccines for allergic subjects [6]. In any case, independently from the intended use of the molecule (either diagnosis or therapy), it is sometimes difficult to obtain sufficient amounts of functional protein. Recently, the Cup a1.02 allergen has been produced exploiting a GST fusion protein strategy [9], allowing the purification of 2 mg/L bacterial culture of soluble protein. Unfortunately, this system has revealed to be inadequate to get high levels of pure protein, because the protein yield and purity were often decreased by the procedures needed to remove the fusion partner.

In an attempt to increase recovery and improve homogeneity and purity of the recombinant Cup a1.02 protein, we have expressed the protein as inclusion bodies and we have applied a specific refolding strategy. We hence prepared a new construct by substituting the GST fusion protein with an N-terminal 6× histidine extension which allows purification procedures in denaturing conditions. Protein production as inactive aggregates of misfolded peptides can be advantageous over the soluble form, due to the high product concentration, lower levels of proteolytic degradation and reduced toxicity to the host cells. Inclusion bodies were then transformed into soluble bioactive products. After inclusion bodies purification and solubilization, a chromatographic refolding procedure by a reverse linear gradient of guanidine concentration was developed, which minimizes spatial interactions of refolding intermediates and prevents protein aggregation. Optimization of this in vitro method was achieved by a fivefold reduction of the loading protein concentration. The final yield af-

ter refolding and molecular exclusion chromatography was about 15 mg/L bacterial culture (overall yield 16%). This value corresponds to a sevenfold increase in protein yield compared to that obtained exploiting the GST strategy.

CD data confirmed the presence of extensive β -sheet and β -turn structures. Nevertheless, the spectral line-shape of the CD spectrum showed little resemblance with the spectra contained in the currently available databases [29]. Accordingly, the use of LL method alone yielded poorer results with respect to the RVS method. Both methods, however, agreed in predicting high β -sheet and β -turn contributions (60–65%) but differed in the estimation of α helical structure (12 ± 3 and $7 \pm 1\%$ for LL and RVS methods, respectively, see Fig. 3B).

The subsequent step taken was to demonstrate that the recombinant properly folded protein, assessed on the basis of the physico-chemical data (CD), was also capable of an IgE binding comparable with the binding of the natural molecule. Thus, we have demonstrated that the refolded recombinant molecule is recognized by the IgE from Cupressaceae allergic patients. Such reactivity was functionally confirmed by basophil degranulation experiments, demonstrating that IgE epitopes are properly retained and biologically active. Both approaches validated our refolding procedures.

Since the Cup a1 allergens share high primary sequence similarity with members of the pectate lyase family [30], it is likely that this refolding protocol may be successfully extended to other family members.

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