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Research article

Cloning, purification and characterization of a 90 kDa heat shock protein from *Citrus sinensis* (sweet orange)

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ABSTRACT

Protein misfolding is stimulated by stress, such as heat, and heat shock proteins (Hsps) are the first line of defense against these undesirable situations. Plants, which are naturally sessile, are perhaps more exposed to stress factors than some other organisms, and consequently, the role of Hsps is crucial to maintain homeostasis. Hsp90, because of its key role in infection and other stresses, is targeted in therapies that improve plant production by increasing resistance to both biotic and abiotic stress. In addition, Hsp90 is a primary factor in the maintenance of homeostasis in plants. Therefore, we cloned and purified Hsp90 from *Citrus sinensis* (sweet orange). Recombinant *C. sinensis* Hsp90 (rCsHsp90) was produced and measured by circular dichroism (CD), intrinsic fluorescence spectroscopy and dynamic light scattering. rCsHsp90 formed a dimer in solution with a Stokes radius of approximately 62 Å. In addition, it was resistant to thermal unfolding, was able to protect citrate synthase from aggregation, and Western blot analysis demonstrated that CsHsp90 was constitutively expressed in *C. sinensis* cells. Our analysis indicated that CsHsp90 is conformationally similar to that of yeast Hsp90, for which structural information is available. Therefore, we showed that *C. sinensis* expresses an Hsp90 chaperone that has a conformation and function similar to other Hsp90s.

1. Introduction

Molecular chaperones consist of several protein families that assist protein folding and prevent nonproductive interactions [1,2]. Chaperones of the Hsp90 family are present in a wide range of organisms, from prokaryotes to eukaryotes, are ubiquitously expressed under non stress situations (up to 2% of all cellular protein), and have increased expression under situations of biotic stress [3–6]. Hsp90 interacts with a broad and well-defined group of proteins, termed clients, which are associated with vital cell functions, such as signal transduction pathways, transcription factors, protein kinases that control cellular homeostasis, proteins involved in DNA repair and synthesis, cell division, proliferation, differentiation and apoptosis [3,6–11].

Hsp90 is a homodimer of approximately 160 kDa, and each monomer is divided into three structural domains [7–9]. The C-terminal domain (residues 600–709 in yeast) contains the dimerization interface and the MEEVD motif that interacts with co-chaperones containing the tetratricopeptide repeat (TPR) domain [6,7,12]. The middle domain (residues 255–599 in yeast) is important for interaction with protein clients and is connected to the N-terminal domain by a highly charged linker. Finally, the N-terminal domain (residues 1–220 in yeast) has a nucleotide-binding site with weak ATPase activity. Few high-resolution structural studies for the full-length molecule are available, however, information from truncated constructs exists [7–11].

Mechanisms that facilitate developmental stability are likely to be of greater importance to plants in comparison with other organisms since plants are more vulnerable to environmental stress [4]. Hsp90s are important for plant development and are involved in mechanisms of resistance to pathogens [13–16]. However, information regarding the structure and function of Hsp90 in plants is scarce, whereas a large amount of information is available for the yeast and mammalian homologs. Thus, we cloned and expressed Hsp90 from *Citrus sinensis* (CsHsp90) (nucleotide and amino acid sequences are available in Fig. S1) and characterized

Abbreviations: CD, circular dichroism; SEC-MALS, size exclusion chromatography coupled to multi-angle light scattering.

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this protein using a biophysical analysis. In addition, we demonstrated that CsHsp90 was constitutively expressed in *C. sinensis* cells using Western blot analysis. Furthermore, we showed that the recombinant protein was functional by measuring its ability to prevent citrate synthase aggregation.

2. Results and discussion

2.1. Purification of CsHsp90

Hsp90 is a particular molecular chaperone involved in the conformation activation of a large number of client proteins that plays an important role in both biotic and abiotic stress in plants [4–11]. The cloning of a candidate Hsp90 gene from *C. sinensis* was motivated by the yet scarce information on Hsp90 in plants. From sequence alignment, CsHsp90 was 63% identical to yeast cytosolic Hsp90 (Fig. 1) and about 90% identical to a cytosolic Hsp90 from *Arabidopsis thaliana* (data not shown) indicating that the cloned Hsp90 from *C. sinensis* was also cytosolic. Cells were transformed with the pET-28aCsHsp90 expression vector and SDS-PAGE analysis was performed on lysate from non-induced cells and cells induced for 5 h (Fig. 2A). Recombinant CsHsp90 (rCsHsp90) was observed as a band of approximately 80 kDa, which is similar to the predicted protein mass of 82 kDa (see arrow in Fig. 2A). Recombinant CsHsp90 (rCsHsp90) was the main protein observed in the induced cells, and at least half of the yield was present in the soluble fraction (compare lanes 7 and 8 in Fig. 2A), which was submitted to a nickel affinity chromatographic step followed by gel-filtration chromatographic step (Fig. 2B). Table 1 shows the yield and purity for every step of the purification process showing that CsHsp90 was more than 95% pure after the two step chromatographic process.

The folded state of purified recombinant CsHsp90, rCsHsp90, was investigated by spectroscopic tools. Firstly, circular dichroism (CD) analysis indicated that rCsHsp90 was an α-helical protein, as indicated by its far-UV spectrum with minima at approximately 208 nm and 222 nm (Fig. 3A). Analysis of the CD signal at 222 nm [17] predicted approximately 35% of α-helical content. This prediction is in good agreement with the α-helical content of other Hsp90 homologs for which there is structural information [1,6–8]. Additionally, the position and extension of α-helices and β-sheets predicted for CsHsp90 from sequence (see Material and Methods) are in excellent agreement with those of yeast Hsp90 (Fig. 1 and Table 2), from which structure is available. Hsp90s are formed by three well-defined domains that are rich in secondary structure and well-packed [6–8]. These domains are connected by linkers and there also several loops and turns but that correspond to a small portion of all residues in the protein, which is then considered well-folded. Also, the interfaces of the C-termini are in close contact as they are responsible for the dimer formation, whereas the dimerization of the N-terminal domains are dependent on conformational changes caused by binding to nucleotides, client proteins or co-chaperones [1,7]. There are few contacts between the middle domains although each makes contacts with the N-terminal domain of the other protomer when the N-termini dimerize [8]. The domains can be produced a part from each other and are well compact and stable at this condition. The structural features aforementioned contribute to the high stability of Hsp90 proteins and, in accord to that, the secondary structure of rCsHsp90 was stable at temperatures up to 90 °C, as there was no change in the CD signal at 222 nm from 20 °C to 90 °C (data not shown). As discussed, the thermal stability of Hsp90 is a consequence of its structural arrangement and interaction with modulators and thus reflects its importance for the protection of client proteins [18–20].

CsHsp90 has six tryptophans, which are distributed throughout the protein (Fig. 1). The fluorescence emitted from the Trp residues was analyzed in the presence and absence of 6 M guanidinium chloride (Gdm-Cl) (Fig. 3B). The center of spectral mass and maximum wavelength for native rCsHsp90 were 339 ± 1 nm and 336 nm, respectively (Fig. 3B). Since the spectrum represents the collective sum of the emitted fluorescence from the Trp residues, we conclude that one or more Trps are buried in the protein interior. Under denaturing conditions, all Trps were exposed to the solvent, and the spectrum had center of spectral mass and maximum wavelength of 345 ± 1 nm and 352 nm, respectively

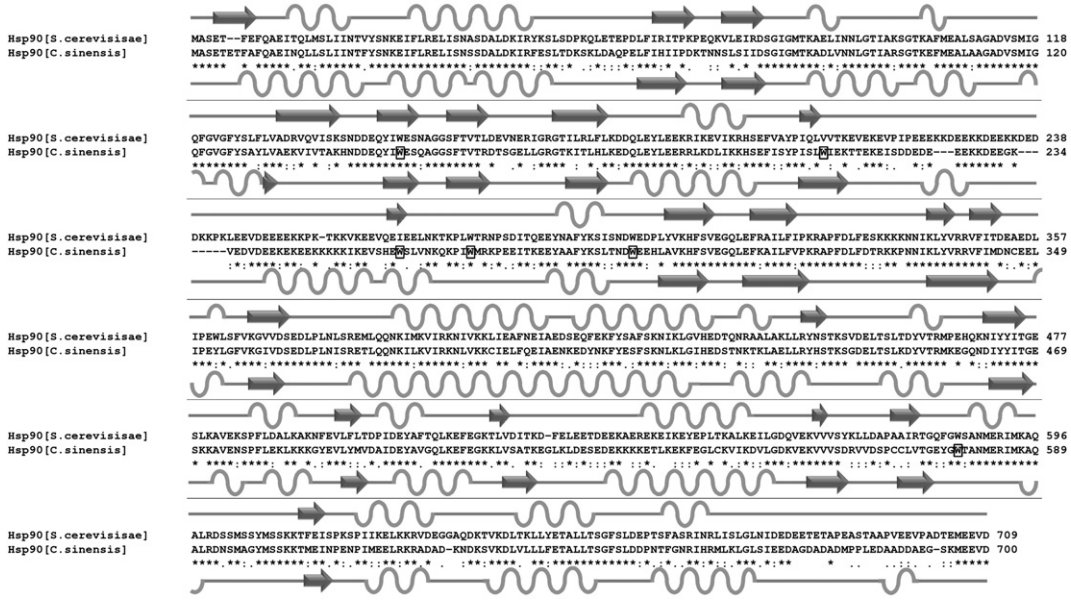

Fig. 1. Sequence alignment of *Citrus sinensis* Hsp90 and yeast Hsp90. The amino acid sequences of yeast Hsp90 (PDB 2CG9_A) and CsHsp90 are aligned indicating that they are 63% identical. Tryptophans in the CsHsp90 sequence are inside black squares. Secondary structural elements (waves for α-helices and arrow for β-sheets) originated from the structural information from yeast Hsp90 [33] or from prediction for CsHsp90 as provided by five different softwares (GOR IV, Jpred 3, NetSurfP v 1.1, SSpro 4.5 and SSpro v 8; [34–37]), are shown. The predicted secondary structure arrangement of CsHsp90 is highly similar to that of yeast Hsp90.