

Production of the refolded oligopeptide-binding protein (OppA) encoded by the citrus pathogen *Xanthomonas axonopodis* pv. *citri*

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ABSTRACT. The oligopeptide-binding protein, OppA, binds and ushers oligopeptide substrates to the membrane-associated oligopeptide permease (Opp), a multi-component ABC-type transporter involved in the uptake of oligopeptides expressed by several bacterial species. In the present study, we report the cloning, purification, refolding and conformational analysis of a recombinant OppA protein derived from *Xanthomonas axonopodis* pv. *citri* (*X. citri*), the etiological agent of citrus canker. The *oppA* gene was expressed in *Escherichia coli* BL21 (DE3) strain under optimized inducing conditions and the recombinant protein remained largely insoluble. Solubilization was achieved following refolding of the denatured protein. Circular dichroism analysis indicated that the recombinant OppA protein preserved conformational features of orthologs expressed by other bacterial species. The refolded recombinant OppA represents a useful tool for structural and functional analyses of the *X. citri* protein.

Key words: OppA; *Xanthomonas citri*; Refolding

INTRODUCTION

Bacterial oligopeptide-binding proteins (OppA) mediate the binding of oligopeptides required for nutrition in several bacterial species and participate in diverse physiological functions including recycling of cell wall muropeptides in *Escherichia coli* and *Salmonella typhimurium* (Goodell and Higgins, 1987), sporulation control in *Bacillus subtilis* (Perego et al., 1991; Perego, 1997), modulation of gene expression among different Gram-positive bacterial species (Rudner et al., 1991; Lazazzera et al., 1997), and regulation of virulence-associated factors (Perego et al., 1991; Perego, 1997; Borezee et al., 2000; Claverys et al., 2000; Gominet et al., 2001).

Xanthomonas axonopodis pv. *citri* (*X. citri*) causes citrus canker, a disease affecting most orange cultivars around the world, inflicting significant economic loss to farmers and industry (Lee et al., 2004). So far, the control of the citrus canker has relied mainly on the identification and eradication of infected trees (Brunnings and Gabriel, 2003). The recent report of the complete genomic sequences of two *Xanthomonas* species, including the *X. citri* 306 strain, created perspectives concerning the development of more efficient and rational control methods based on a better knowledge of the pathogen's genetics and physiology (Da Silva et al., 2002).

X. citri OppA belongs to the *opp* operon and is composed of two transmembrane pore-forming domains (OppBC) and two nucleotide-binding domains (OppDF) responsible for the generation of energy required for the transport process. The presence of the *opp* operon in *X. citri* but not in *X. campestris*, phytopathogens with distinct host specificities and infection mechanisms, suggests that oligopeptides may have different physiological roles for the two species. The presence of OppA, a 59.9-kDa periplasmic protein, has been demonstrated by Western and Northern blot analysis of *X. citri* cellular fractions (Moutran et al., 2004). Nonetheless, further structural and functional analyses require the availability of purified protein in a soluble and stable form. In order to generate material for such studies, we report the successful expression and purification of a recombinant protein that remained soluble following denaturation and refolding steps preserving conformational features of known bacterial orthologs.

MATERIAL AND METHODS

Strains and plasmids

The *oppA* gene was obtained from *X. citri* 306 strain, cloned into the pGEM T-Easy vector (Promega) and sub-cloned into the pET28a expression vector (Novagen). The recombinant protein was expressed in *E. coli* BL21 (DE3) (Stratagene).

Cloning of the *oppA X. citri* gene

The nucleotide sequence encoding the mature *X. citri* OppA (without the 19 encoding the amino acid long signal peptide) was amplified by PCR (forward primer, 5' CAGTCAGCTAGCACCGCT 3' and reverse primer, 5' CGCAAAGCTTTCATTTGC 3') using Platinum high-fidelity Taq polymerase (Invitrogen) and standard amplification conditions: an initial step of 5 min at 96°C (1), 2 min at 96°C (2), 1 min at 66°C (3), 2 min at 73°C (4), followed by the repetition of the steps 2 to 4 by 29 times. The final extension was performed at 73°C for 10 min (5). The forward primer included an *NheI* restriction site while the reverse primer

carried an *Hind*III site (underlined). The resulting amplified fragment, with a total length of 1557 bp, was first cloned into the vector pGEM T-Easy (Promega) using standard cloning conditions. After transformation of *E. coli* DH5 α cells and screening of recombinant colonies, a recombinant plasmid named pGEMOppA was selected, amplified and cleaved with *Nhe*I and *Hind*III enzymes (Invitrogen) in order to release a 1.6-kbp fragment, which was purified from agarose gels and subsequently cloned into the expression vector pET28a (Novagen), previously treated with *Nhe*I/*Hind*III. Transformation efficiencies of approximately 10⁸ CFU/ μ g DNA were routinely achieved with chemically competent *E. coli* DH5 α cells. One recombinant colony, selected out of twenty chosen colonies, was subjected to restriction analysis and nucleotide sequencing. The recombinant plasmid, named pETOppA, was further purified and introduced into the *E. coli* BL21 (DE3) strain (Novagen). One clone chosen at random among a lawn of recombinant colonies was selected for further analysis for expression and purification of the recombinant protein. The *X. citri* OppA was expressed as a cytoplasmic protein with a His₆-tag genetically fused at the N-terminal end.

Computational analysis

The nucleotide and corresponding amino acid sequences of the *X. citri oppA* gene (GenBank accession number NC003919) were made available by the NCBI. Prediction of signal peptide and transmembrane sequences were determined with SignalP and DAS programs, respectively (<http://www.cbs.dtu.dk/services/SignalP/> and <http://www.sbc.su.se/~miklos/DAS/>). Protein parameters of the *X. citri* OppA were calculated with the application of programs available at the Expasy Molecular Biology Server (<http://www.au.expasy.org/>).

Protein expression

Cultures of the recombinant *E. coli* BL21 (DE3) strain carrying pETOppA were grown aerobically in Erlenmeyer flasks containing LB with 50 μ g/mL kanamycin until mid-log phase (OD₆₀₀ = 0.7-0.8) before addition of inducer (0.2 mM IPTG). The cultures were induced aerobically (200 rpm) either for 2 or 4 h or statically overnight at different temperatures (23 $^{\circ}$, 28 $^{\circ}$ and 37 $^{\circ}$ C). Higher expression levels were achieved after evaluating different induction parameters including growth medium, aeration and incubation temperature during the induction period. Cells were collected by centrifugation and stored at -20 $^{\circ}$ C for approximately 16 h before preparation of the cell extracts. Cell pellets from 1-L cultures were suspended in 10 mL 10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 0.5 mM PMSF, and 3 mM imidazole, and incubated with lysozyme (final concentration of 100 μ g/mL) for 1 h in an ice bath. Cells were maintained in ice and sonically disrupted after 5 pulses of 15 s in a Branson Digital Sonifier (Model 450) followed by centrifugation at 18,000 rpm for 20 min to obtain the soluble and non-soluble proteins.

Purification and refolding of *X. citri* OppA

The recombinant OppA was purified from insoluble fractions after solubilization with buffer B (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 8 M urea) and addition of nickel-charged Sepharose (ProBond, Invitrogen) slurry (1 mL resin per 15 mg total protein), previously washed with 2 volumes of water and 1 volume of buffer B, and incubated for 1 h under mild agitation. The charged resin was transferred to a plastic column and washed with 10 volumes of buffer B with 10 mM imi-

dazole added, followed by washing with 3 volumes of buffer B plus 20 mM imidazole. The bound OppA was serially eluted using buffers with increasing imidazole concentration. After purification, eluted samples were submitted to SDS-PAGE to confirm purity and protein concentration. Refolding of the denatured protein was attempted by both dialysis and dilution methods, but successful results were achieved only with the dilution method in which protein aliquots (approximately 2 mg) diluted with 100-fold volumes of refolding buffer (PBS, pH 7.4, 1 M urea, 10% glycerol, 0.005% Tween 20, 0.5 mM PMSF and 5 mM DTT) were maintained under vigorous agitation at 4°C for 8 h. The total volume was concentrated on Amicon filters centrifuged at 18,000 rpm at 4°C for 20 min, followed by dialysis with 10% glycerol and 0.5 mM PMSF in PBS, pH 7.4, followed by three dialysis steps with 20 mM Tris-Cl, pH 8.0, and 50 mM NaCl. After centrifugation, the soluble fractions were concentrated with Ultrafree MWCO 30,000 centrifugal filters (Amicon Millipore) and analyzed on 12% acrylamide gels stained with Coomassie blue. In all steps, the protein concentration was determined spectrophotometrically using the Edelhoch method (1967).

Circular dichroism

Circular dichroism (CD) measurements were carried out with a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostated cell holder interfaced with a thermostatic bath. Spectra were recorded in 0.1-cm path length quartz cells at a protein concentration of 5 μ M in 20 mM Tris-HCl buffer, at pH 8.0, and containing 50 mM NaCl. Twenty consecutive scans were compiled and the average spectra stored. The data were corrected for the baseline contribution of the buffer and the observed ellipticities converted into the mean residue ellipticities $[\theta]$, based on a mean residue molecular mass of 219.2 kDa. The secondary structure was estimated from fitted far-UV CD spectra using the DICROPROT software package (Deléage and Geourjon, 1993).

RESULTS

Expression and purification of the recombinant *X. citri* OppA

X. citri OppA has 539 amino acid residues, including a putative signal peptide with 19 amino acid residues and a cleavage site located between Ala19 and Gly20. Specific primers were thus designed in order to amplify a 1.5-kbp fragment containing the *X. citri oppA* sequence. The fragment was cloned in an intermediate vector (pGEMT) before subsequent forced cloning into the *Nde*I and *Hind*III restriction sites of pET28a, which resulted in the pETOppA expression vector. The recombinant OppA of *X. citri* was expressed and purified from *E. coli* BL21 (DE3) under the control of the T7 phage promoter as a cytosolic protein genetically fused to an N-terminal His₆-tag. Attempts to express the recombinant protein with its native signal peptide and a C-terminal His₆-tag showed that *E. coli* BL21 (DE3) was unable to secrete the heterologous OppA to the periplasm (data not shown). The maximal expression yields varied from 60 to 200 mg/L, corresponding up to 30% of the total cell protein (Figure 1A). Different induction protocols based on variation of incubation temperature, aeration and induction time were tested in order to optimize the expression yields of the recombinant protein (Table 1). Under the tested conditions, maximal protein expression levels were achieved in cultures prepared in LB medium at 28°C with no aeration, which resulted in a final protein yield of 200 mg/L (Figure 1B, Table 1). Under all tested growth conditions,

only a small amount of the expressed protein (less than 10% of the total purified protein) remained soluble but readily precipitated upon storage, precluding its use for further experiments (Table 1). Extended induction periods at lower temperatures and cultivation in M9 medium with no aeration did not improve the solubilization of the recombinant protein, which remained largely insoluble as inclusion bodies. Similarly, no further improvements were achieved with different *E. coli* host strains (data not shown). Therefore, purification of the recombinant protein required a denaturation step with 6 M urea.

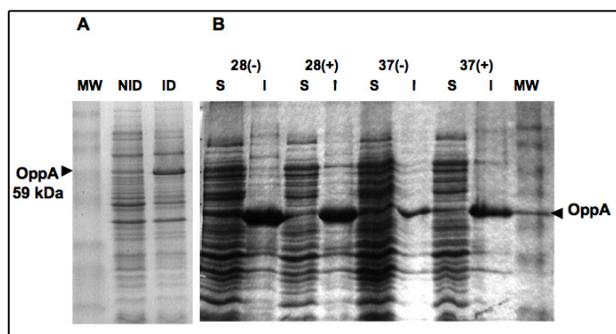


Figure 1. Expression of recombinant *Xanthomonas citri* OppA in *Escherichia coli* BL21 (DE3) cells transformed with the pETOppA vector. **A.** Whole-cell extracts of non-induced (NID) or IPTG-induced (ID) cultures were loaded on polyacrylamide gels after cultivation in LB medium at 37°C. **B.** Production of the recombinant OppA under different growth conditions. Cultures were incubated with no agitation (-) or at 200 rpm (+). Soluble (S) and insoluble fractions (I) of whole-cell lysates are indicated. MW: pre-stained molecular weight.

Table 1. Experimental conditions affecting the expression levels of soluble and insoluble recombinant *Xanthomonas citri* OppA.

Induction temperature ¹ (°C)	Aeration ²	Culture medium ³	Induction period ⁴ (h)	Total protein ⁵ (g/L)	Soluble protein ⁶ (g/L)	Insoluble protein ⁷ (g/L)
37	+	LB	2	0.120	0.020	0.100
37	-	LB	4	0.060	-	0.060
28	+	LB	2	0.200	0.010	0.190
28	-	LB	4	0.200	0.020	0.180

¹Incubation temperature following addition of inducer to the bacterial culture, as described in the text; ²Cultures kept in flasks on an orbital shaker set at 200 rpm (+) or kept static (-) during the induction period; ³LB - Luria broth; ⁴Cultures were kept under inducing conditions for 2 or 4 h; ⁵Total protein yields as obtained with 1-L cultures kept in 3.6-L Fernbach flasks; ⁶Amount of soluble protein in the cell extracts; ⁷Amount of insoluble protein obtained as inclusion bodies.

Once bound to the nickelated resin, the recombinant protein was recovered after elution with imidazole-containing buffer at concentrations ranging from 200 to 300 mM (Figure 2A, lanes 4-7). A large amount of OppA was lost during washing steps required to remove contaminant endogenous proteins. The best recovery yields were achieved after extensive washing (5- to 20-fold bed column volume) at an imidazole concentration of 20 mM. Based on this protocol, approximately 70% of the total recombinant protein was recovered with a final protein yield of 120 mg/L.

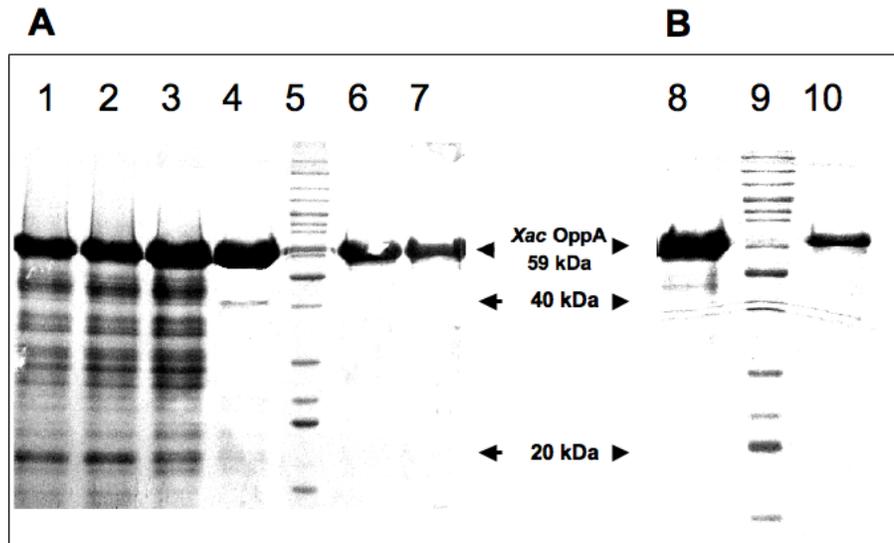


Figure 2. Purification of the recombinant *Xanthomonas citri* (*Xac*) OppA by immobilized metal affinity chromatography (A) and subsequent refolding of the denatured protein (B). Lane 1, flow through; lanes 2 and 3, fraction recovered following washing with imidazole at concentrations of 20 and 50 mM, respectively; lanes 4, 6 and 7, fractions eluted with buffers containing 100, 200 and 300 mM imidazole, respectively; lanes 5 and 9, pre-stained molecular weight markers (Invitrogen); lanes 8 and 10, purified OppA samples before and after the refolding procedure, respectively.

X. citri OppA refolding and circular dichroism analysis

The amino acid sequence analysis using bioinformatics methods revealed a protein consisting of 51.2% nonpolar, 27.3% uncharged polar, 10% acidic and 9.3% basic residues and a predicted pI of 6.5. Comparison of the primary sequence of *X. citri*, *S. typhimurium* and *B. subtilis* OppA orthologs indicated that the *X. citri* ortholog has a higher nonpolar residue content and, therefore, classified as an unstable protein (instability index of 42.33), in comparison with the orthologs expressed by *B. subtilis* and *S. typhimurium* (instability index of 23.06 and 33.14, respectively). Corroborating the unstable nature of the recombinant protein, refolding of the denatured protein using conventional dialysis method resulted in protein precipitation (data not shown). Better results were obtained after refolding of the denatured protein using a dilution method followed by dialysis steps. Based on such procedure, 20% of the total purified protein was recovered in a soluble state (Figure 2B). The purified refolded protein remained soluble (1-3 mg/mL) even when stored at 4° or -20°C.

The CD spectrum of the soluble refolded recombinant *X. citri* OppA was characterized by a relative maximum at 195 nm and two relative minima at 208 and 222 nm, suggesting that the protein preserved its native structure (Figure 3). The correct refolding of *X. citri* OppA was confirmed by comparison with the CD spectra of the *E. coli* OppA ortholog (very high sequence identity with *S. typhimurium* protein).

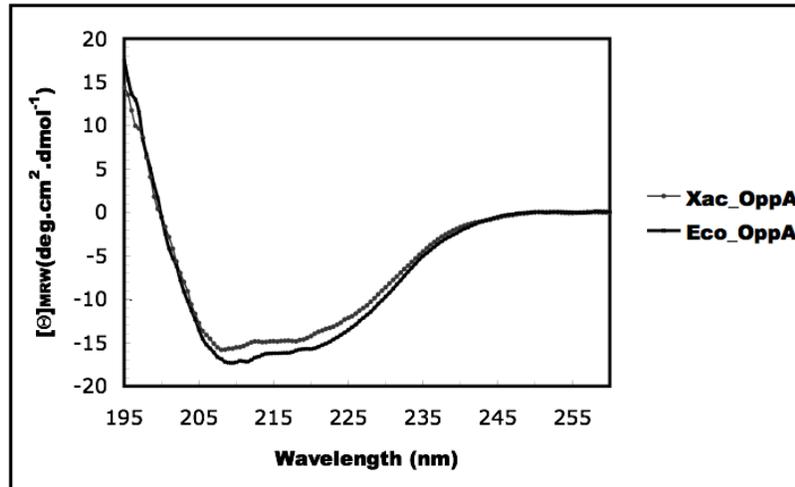


Figure 3. Circular dichroism spectra of the recombinant *Xanthomonas citri* (Xac) OppA protein expressed by *Escherichia coli* cells and the *E. coli* (Eco) OppA purified from periplasmic fractions (Nakamatsu et al., 2007). Purified protein samples were prepared to a final concentration of 10 μ M in 20 mM Tris-Cl, pH 8.0, and 50 mM NaCl. Circular dichroism spectrum of the recombinant OppA is characteristic of an α/β protein. MRW = mean residue molecular mass.

The predicted secondary structure of the recombinant protein, carried out with the DICROPROT program, indicated that the overall OppA structure encompassed 35% α -helices and 22% β -strands while turns and random coils represented 43% of the predicted protein secondary structure. Similar secondary structures were reported for the *S. typhimurium* and *B. subtilis* orthologs with solved three-dimensional structures (Tame et al., 1996; Levdikov et al., 2005), as indicated in Table 2.

Table 2. Major features of the primary sequence and secondary structures of OppAs expressed by *Xanthomonas citri* (Xac), *Salmonella typhimurium* (Sty) and *Bacillus subtilis* (Bsu).

Bacterium	Total number ³	OppA amino acid composition ¹			Isoleucine/Proline ⁴	Conformation ²	
		Charged amino acid	Polar amino acid	Nonpolar amino acid		α -helix	β -sheet
<i>Xac</i>	519	19.3	26.7	45.3	2.6/5.9	35	22
<i>Sty</i>	518	24.7	29.2	35.6	4.4/6.0	36	18
<i>Bsu</i>	520	29.0	27.7	34.0	5.2/4.0	37	26

¹Relative composition of charged, polar and nonpolar residues expressed as percentages of total residues of the respective mature proteins expressed by *X. citri*, *S. typhimurium* and *B. subtilis*; ²Major secondary structures as determined from sequence details of the PDB pages of *B. subtilis* (PDB entry 1XOC) and *S. typhimurium* OppA (PDB entry 1OLA) orthologs. ³Total number of amino acid residues of each protein; ⁴Proline and isoleucine content of the OppA proteins expressed as percentages of the total amino acid residues of the mature protein.

DISCUSSION

The ATP-binding cassette (ABC) transporters comprise one of the largest paralogous protein families comprising as much as 5% of bacterial genomes and controlling the traffic of essential nutrients (Higgins, 2001). The oligopeptide (Opp) permeases, one of the most comprehensively studied ABC transporter systems, encompass a set of membrane-associated proteins responsible for the binding and translocation of peptides ranging from 3 to at least 18 amino acid residues. Moreover, besides its primary role in peptide uptake, the Opp system, and particularly OppA orthologs, has been implicated in various physiological processes required for survival in different environmental conditions including recycling of cell wall peptides, inter-cellular communication, sporulation, binding to host tissues and proteins as well as survival at low temperatures or in intracellular environments (Perego et al., 1991; Cundell et al., 1995; Claverys et al., 2000; Borezee et al., 2000; Monnet, 2003). However, little is known about the role of the Opp system in plant bacterial pathogens, particularly those belonging to the *Xanthomonas* genus, which includes several species affecting economically important cultivars. In the present report, we described for the first time the expression and purification of a soluble OppA encoded by *X. citri*, the etiological agent of citrus canker, one of the most relevant diseases affecting the orange juice industry and citrus farmers.

The *oppA*-encoding gene has been amplified from the genomic DNA of the *X. citri* 306 strain, which has been shown to carry a single copy of the *opp* operon (Da Silva et al., 2002). Genes encoding the Opp system have been found in many but not all bacterial species. The discovery that two closely related *Xanthomonas* species, *X. citri* and *X. campestris*, differ in the presence of the *opp* genes suggests that the uptake of peptides plays distinct physiological roles in these species. For example, *X. campestris* has been shown to carry a set of genes involved in the reduction of nitrate and nitrite to ammonium. Such genes, not found in the *X. citri* genome, would allow the bacteria to grow in nutrient-poor environments such as the xylem of host plants where nitrate would represent the most readily available nitrogen source. On the other hand, *X. citri* has been shown to encode several specific proteases, which generate peptides to be used as the main nitrogen source, thus allowing bacterial cells to thrive in necrotic lesions generated during infection (Da Silva et al., 2002).

Previous attempts to express *X. citri* proteins carrying the native signal sequences did not result in accumulation of the encoded peptides in the periplasm of recombinant *E. coli* strains, suggesting that differences in signal sequence length and charged residue composition negatively affect recognition of the nascent peptide by the membrane-associated protein secretion apparatus (Balan et al., 2005). Removal of the signal peptide and fusion of the His₆-tag to the N-terminal region has been the best experimental approach to express and purify *X. citri* nutrient-binding proteins, resulting in soluble proteins at nearly homogeneous and stable states (Evdokimov et al., 2001; Balan et al., 2005, 2006). Expression of the recombinant OppA in *E. coli* strains systematically resulted in the accumulation of an insoluble protein, and only a small fraction of the encoded peptide remained soluble, which readily precipitated. The higher nonpolar amino acid residue content of the *X. citri* OppA mature protein (45%), with regard to the orthologs expressed by *E. coli* and *S. typhimurium* (approximately 35%), has probably contributed to the observed behavior of the recombinant protein expressed in *E. coli* strains. The reduced isoleucine content of the *X. citri* OppA, with regard to the orthologs from *E. coli* and *B. subtilis*, may also contribute to the comparatively lower solubility of the recombinant

protein (Table 2). Indeed, as demonstrated by Evdokimov et al. (2001), the proline and isoleucine contents, as well as their presence at specific positions, confer a remarkable stability to a recombinant *Pyrococcus furiosus* maltose-binding protein.

Attempts to employ different *E. coli* host strains, cultivated under various growth conditions and using diverse gene expression inducing protocols did not enhance the solubility of the recombinant protein. Since one of our interests was the generation of soluble protein adequate for conformational and structural studies, an alternative purification procedure involving denaturation and refolding of the recombinant protein was established. Although, different methods of refolding are described in the literature (Tsumoto et al., 2003; Bondos and Bicknell, 2003), the recombinant *X. citri* OppA was successfully refolded only after dilution of the protein at low concentrations in the presence of glycerol that facilitated the proper folding and stability of the protein. Curiously, the protein refolding using the same buffer but on dialysis resulted in protein precipitation. The success of the high dilution refolding protocol may be ascribed to the vigorous stirring of the sample during the whole process. Indeed the same refolding procedure has been successfully used in other *X. citri* nutrient-binding proteins (Balan A and Perez-Santacruz C, unpublished observations).

The availability of purified OppA protein represents an important step toward the definition of functional and structural features of the *X. citri* Opp system. Although the native protein proved to be particularly difficult to obtain in a soluble state, the dilution method established in this study is more convenient in terms of handling, time and cost than the dialysis method and will be useful for large-scale production of the protein for crystallization trials and structural studies.

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