The advantages and disadvantages of bioorthogonal proteins

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Chapter 2

Expression and purification of bioorthogonal ovalbumin

Abstract

Ovalbumin is an oft-used model protein in immunology. In this Chapter the recombinant expression is explored, and the recombinant expression using bioorthogonal methionine substitution. It was shown that the protein solubility was substantially reduced when the protein was N-terminally ligated to a linker sequence or a purification tag. The protein was therefore expressed without a purification tag.

Introduction

Chicken ovalbumin (Ova), a member of the serine protease inhibitors (serpin) protein family, is the most abundant (~58% by weight) protein in hen egg white [1]. Due to its high abundance and the fact that Ova is antigenic to mice, it has become a stalwart in immunological research [1]. When used immunologically, it is of paramount importance to consider the structural features of ovalbumin, particularly its post-translational modifications, as these can have profound effects on the interactions with immune cells [2].

Nascent Ova may undergo a number of post-translational modifications that combined make that the protein exists in hen egg white in a variety of isoforms. For instance, the Ova polypeptide contains six cysteine (Cys) residues of which two (Cys73 and Cys120) form a disulfide bridge [3-5]. Mature Ova features one *N*-linked glycan at asparagine (Asn) 292 [6, 7], with a second potential glycosylation site at Asn311 [7-9]. In addition, the N-terminus may exist as free amine as well as in N-acylated forms and finally three serine residues within the Ova sequence may isomerize from L to D configuration over time [10].

These and other properties can vary strongly between different Ova-isolates. This is particularly troublesome in the context of the immunological use of the antigen: changes in glycosylation, for example, can change the immunological properties of the protein (by altering protein/lectin interactions) [11]. Changes in stability – such as those that occur by Ser-isomerization – can also alter the rate of proteolysis and antigenicity of antigens [12].

These problems are further compounded when traceable variants of Ova are used. Fluorophore conjugation to the protein has been used extensively to track its intracellular fate [13-16]. However, this conjugation alters the surface charge and lipophilicity by replacing the positive charges of lysine (Lys)-residues with large – often hydrophobic – groups. This results in a full loss of secondary structure of the protein [16]. The features of these reagents resulted in a significantly different T cell activation [16].

To prevent both these problems, it was hypothesized that a recombinant, bioorthogonal, form of the protein would be a powerful reagent. It would result in a reduction of the batch-to-batch variation that complicates the use of this model antigen.

Expression in a prokaryotic expression system, such as *Escherichia coli* (*E. coli*) has, for instance, been reported to yield homogeneous Ova lacking any post-translational modification [4, 5, 17-20], whilst keeping other structural features intact. It would also allow the production of the protein with non-canonical amino acids, using for example the bioorthogonal non-canonical amino acid tagging strategy (BONCAT) [21]. In this approach the detectable groups are not appended from Lys/Cys sidechains, but incorporated as amino acids isosteric to methionine [22]. It was further hypothesized that this Ova-variant would be very suitable as a detectable reagent that would be compatible with the wealth of other available immune reagents for Ova, including T cell reagents targeting specific MHC-I and MHC-II restricted regions of the protein [23-26].

Bioorthogonal Ova variants are attractive commodities, as they allow for the introduction of desirable functionalities pre-, ante- or post-experimentation, thus enabling flexibility in immunological studies, especially given the increase in specificity, selectivity and sensitivity in bioorthogonal reactions that are continuously becoming available in present times. The availability of flexible and effective expression systems is key for the use of bioorthogonal Ova in immunological studies. Recent studies [16] have addressed this issue and set the stage for the experimental work presented in this Chapter.

Thus, this Chapter presents optimized protocols for the introduction of bioorthogonal amino acids in well-defined Ova constructs. Of the various methodologies that have seen the light in the past decades for introducing such non-canonical commodities, the strategy developed initially by Tirrell and co-workers was selected. The reason for this was the relative ease of execution and the relatively large quantities of modified protein that can be obtained.

The strategy is rooted in supplementing culture media of an amino acid auxotrophic bacterial strain with an amino acid that structurally closely resembles that of the amino acid the bacteria cannot produce itself. Methionine-auxotrophic *E. coli* strains are most often used for this purpose and this determines also the nature of the substituting amino acid, which needs to resemble methionine and at the same time needs to harbor a bioorthogonal group. Ova encompasses 17 methionine residues, that in theory may all be substituted for a bioorthogonal lookalike, and the research

described here therefore also aimed to establish which methionine, and what number of methionine, could be supplanted depending on the conditions used.

Results

The introduction of non-canonical amino acids in proteins through modified expression systems can be achieved through expanding the genetic code, or by substitution in growth media of a canonical amino acid for a close structural analogue. The first strategy, originally developed by Schultz *et al.*, allows for introduction of a single bioorthogonal amino acid at a precise position within the polypeptide sequence [27]. The methodology is however laborious, fraught with failure and oftentimes yields only small quantities of the desired protein. The second strategy, originating from work by Tirrell and co- workers, is experimentally more robust and allows for the generation of large quantities of the desired protein, but has the caveat that a canonical amino acid is potentially replaced by a non-canonical one throughout the polypeptide [22, 28]. Work described in this chapter followed the Tirrell procedure, in particular the substitution of methionine for either azidohomoalanine or homopropargylglycine, both bioorthogonal amino acids and both close structural methionine analogues [22, 28].

The general workflow followed is depicted in Figure 1 and comprises selection of a suitable expression host (here: a methionine-auxotrophic *E. coli* strain), cloning of the desired plasmid into this, expression of the protein of interest using culture media in which the canonical amino acid is substituted for the desired non-canonical one (here: methionine for either azidohomoalanine or homopropargylglycine). This is followed by protein isolation, purification and finally analysis of the thus obtained protein product to establish structural integrity (have the bioorthogonal amino acids been incorporated in the predetermined positions, and if so, which of the 17 possible positions, and to what extent) and functionality (does the bioorthogonal protein act as the wild type one does). The following sections detail results on the design, cloning, expression, purification, and analysis of a number of bioorthogonal Ova constructs. The Chapter will conclude with general observations on the obtained results, will weigh advantages and disadvantages for the various constructs and their preparation and will end with recommendations

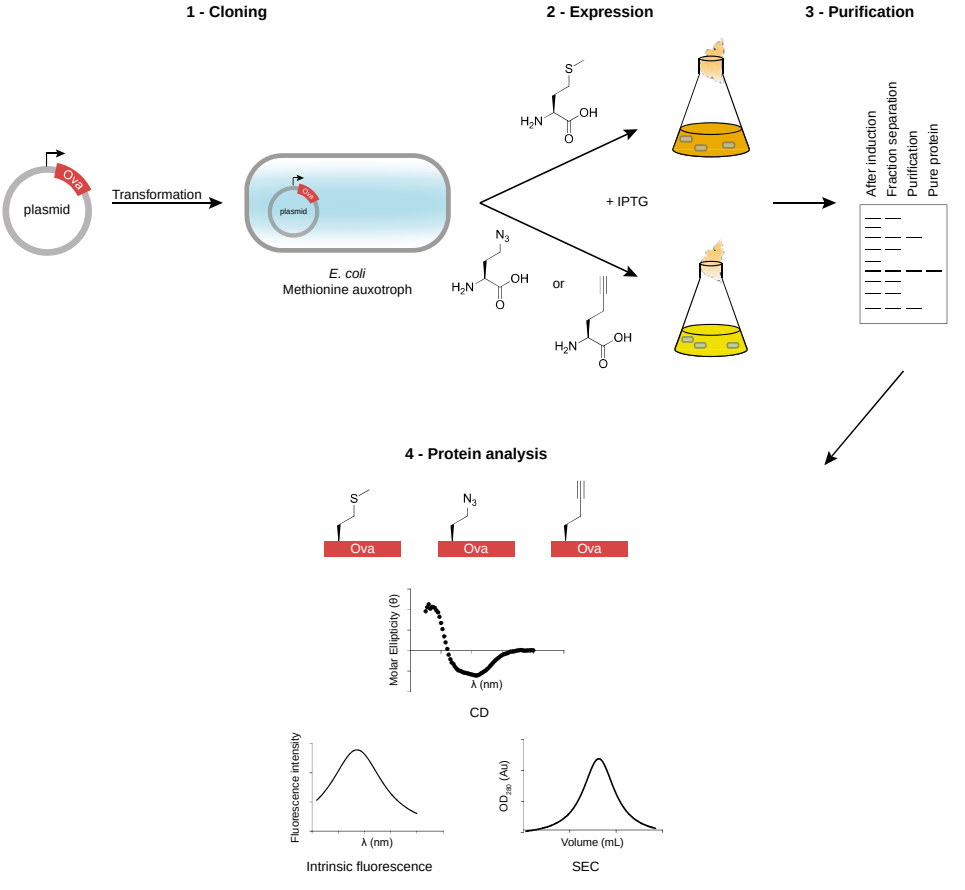


Figure 1. General strategy for the production of bioorthogonal Ova as described in this chapter. Individual steps subject of here-described studies are the design of Ova constructs and their incorporation into appropriate plasmids, which are then used to transform methionine-auxotrophic bacteria (**1**); induced expression in the presence of either methionine or analogous, bioorthogonal amino acids (**2**); protein separation and detection of (modified) Ova (**3**); and functional analysis of thus obtained (bioorthogonal) Ova (**4**).

which procedure to follow in function of the type of bioorthogonal Ova construct desired.

2.1 Expression of 6His-TEV-Ova

As a first starting point for producing bioorthogonal Ova, the previously reported expression construct bearing a six-histidine (His) tag and a Tobacco Etch Virus (TEV) cleavage site at its N-terminus (6His-TEV-Ova), was taken [29]. The expression plasmid pMSCG7 was kindly provided by N. Del Cid. The 6-His-tag allows for immobilized metal

affinity chromatography (IMAC) purification, whereas the TEV cleavage site facilitates proteolytic removal [30] of the His-tag from the target protein following nickel-nitrilotriacetic acid (Ni-NTA) purification to yield a minimally modified protein.

With the aim to substitute methionine residues for bioorthogonal isosteres, the auxotrophic *E. coli* strain B834(DE3) [31] was transformed with the pMSCG7 plasmid bearing ovalbumin. Expressions were first attempted in (methionine-containing) lysogeny broth (LB) medium and expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) [32]. After harvesting, the cells were subjected to a freeze-thaw cycle followed by suspension in lysis buffer and subsequent lysis via French press. The soluble fraction was separated by ultracentrifugation and protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing robust overexpression of a protein at the correct size (45 kDa) (Figure 2A), further confirmed by Western blot as well as its presence in both the soluble and insoluble fractions (Figure 2B). However, some pre-induction background expression was observed in this system as revealed by Western blot analysis (Figure 2A). As this can lead to lower incorporation of the unnatural amino acids, it was attempted to suppress this using the addition of 1% glucose (w/v) to the pre-expression culture medium (Figure 2A right lanes) [32].

Next, Ova expression in methionine replacement medium was tested. This SelenoMet-medium is of slightly different nutrient composition, which may affect expression levels. Expression in SelenoMet-medium augmented with methionine (L-Met; 40 mg/L) showed robust expression of recombinant Ova (Figure 2C).

Following these successful pilot experiments, expression with bioorthogonal amino acids was attempted next with this construct. After growing *E. coli* B834-6His-TEV-Ova to the exponential phase (OD_{600} of ~0.8) in glucose-augmented LB medium, the cells were harvested by centrifugation and a medium switch was performed to SelenoMet medium supplemented with azidohomoalanine (L-Aha; 72 mg/L) or homopropargylglycine (L-Hpg; 40 mg/L). Expression was then induced with IPTG as before and continued overnight. Ensuing sample preparation and SDS-PAGE analysis as before showed overexpression of a protein band at 45 kDa, that was present in both fractions.

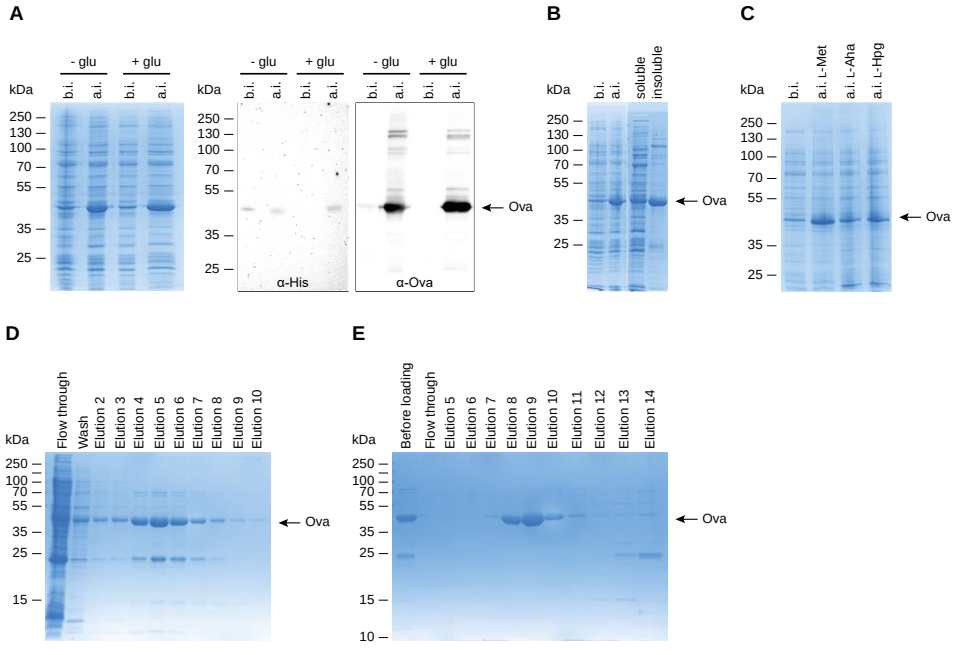


Figure 2. Expression of 6His-TEV-Ova. A) Expression of Ova with and without the suppression of glucose before induction. Protein expression was suppressed in the presence of 1% glucose, as revealed by Western blot analysis using α-His (middle) and α-Ova (right) antibodies. B) Expressed Ova separated into the soluble and insoluble protein fractions, with most of the protein ending up in the insoluble fraction. C) Expression of Ova in media supplemented with L-Met, L-Aha or L-Hpg. D) Ni-NTA purification of Ova-Aha, of which fractions 4 - 7 were further purified over E) a Q column, resulting in >95% pure protein. A-E) Proteins were resolved in a 10% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue G-250.

The recombinant 6His-Ova-Aha was then purified by IMAC using a Ni-NTA column (Figure 2D), followed by anionic ion exchange (IEX) chromatography using a Q column (Figure 2E). This resulted in >95% pure ovalbumin (as determined by SDS-PAGE analysis) in a (low) yield of 0.4 - 0.8 mg per liter expression culture.

2.2 Expression of 10His-TEV-Ova

The low recovery yield of the bioorthogonal Ova from expression experiment **2.1** could be because Ova proteins were produced in inclusion bodies; that the 6His-tag proved too short to effect complete binding to the Ni-NTA column; that the B834(DE3) strain proved insufficiently efficient in expressing the target protein; or a combination

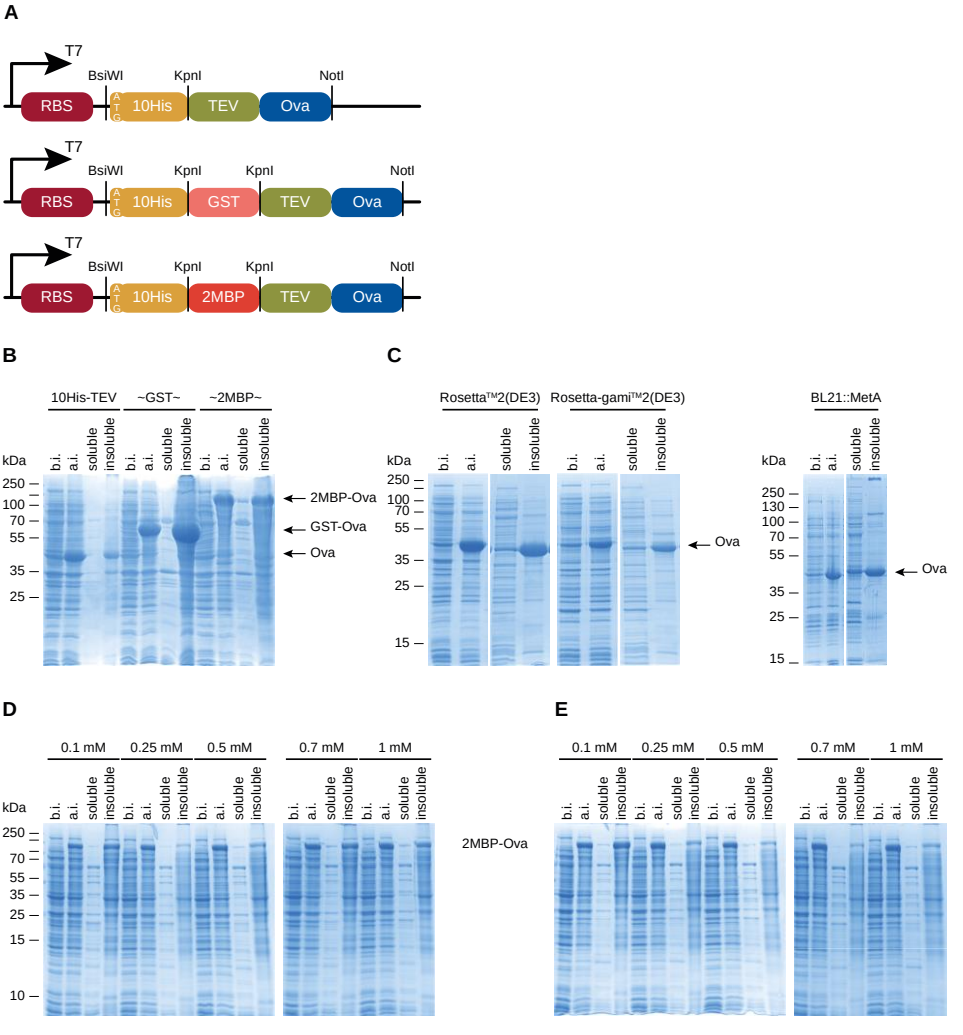


Figure 3. Comparison of soluble expression levels of 10His-x-TEV-Ova. A) Schematic overview of the used gene constructs. B) Protein expression of 10His-TEV-Ova, 10His-GST-TEV-Ova and 10His-2MBP-TEV-Ova in B834, as separated in soluble and insoluble protein fractions. C) 10His-TEV-Ova expressed in Rosetta™ 2(DE3), Rosetta-gami™ 2(DE3) and BL21::MetA as separated in soluble and insoluble fractions. D) Expression of 10His-2MBP-TEV-Ova at 18˚C or E) 30˚C with different concentrations of IPTG, as separated in soluble and insoluble protein fractions. B-E) Proteins were resolved in a 10% or a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue G-250 or R-250.

of these. Experiments discussed in this section were conducted with the aim to address these issues.

With the aim to potentially increase protein binding to the Ni-NTA column [33], a plasmid allowing the expression of Ova with a larger His-tag was constructed:

pET28a_10His-TEV-Ova. The multiple cloning site of plasmid pET28a was extended with a linker sequence containing 10, rather than 6, histidine residues, again linked to *Ova* via a TEV cleavage site. The full-length *Ova*-gene was ligated N-terminally to the 10His-TEV linker sequence into the multiple cloning site (Figure 3A). The plasmid was then transformed into the same auxotrophic expression strain B834(DE3) as used under **2.1**, but also into the non-auxotrophs Rosetta™ 2(DE3) [34], Rosetta-gami™ 2(DE3) and the in-house produced Met-auxotroph BL21::MetA [35]. After expression in LB medium, harvesting of the cells and their lysis, the soluble and insoluble fractions were separated by centrifugation [20]. Both fractions were analyzed by SDS-PAGE, which revealed overexpression of a protein at the correct size (45 kDa). Most of the protein was found in the insoluble fraction (Figure 3B and 3C). It was first attempted to increase the solubility of the Ova-constructs by creating genetic fusions to the solubility-enhancing proteins GST and MBP [36-38]. For this reason, plasmids pET28a_10His-GST-TEV-Ova and pET28a_10His-2MBP-TEV-Ova were designed. The plasmids were obtained by extending the 10His-TEV linker with either a *GST* gene [39] or a *MalE* gene [40]. Again, Ova was N-terminally ligated (Figure 3A) and the resulting plasmids were transformed to B834(DE3). The protein constructs were expressed and solubilized as described under **2.1** and the resulting fractions were analyzed by SDS-PAGE showing expression of 10His-GST-TEV- Ova (at 71 kDa) and 10His-2MBP-TEV-Ova (at 129 kDa). Again, fusion proteins proved to localize predominantly to the insoluble fraction during expression (Figure 3B).

After the failure of these solubility enhancing proteins, it was next attempted to enhance solubility by lowering the rate of expression. This was done in two ways: lowering the IPTG concentration [41-43], and expression at lower temperatures [44-46]. Expression of the 10His-2MBP-TEV-Ova by induction with either 0.1, 0.25, 0.5, 0.7 or 1 mM IPTG, at either 18˚C or 30˚C was attempted. Again, none of these measures improved the fraction of soluble protein (Figure 3D and 3E). Based on these results it was decided to investigate the possibility to obtain Ova constructs from the insoluble protein fraction.

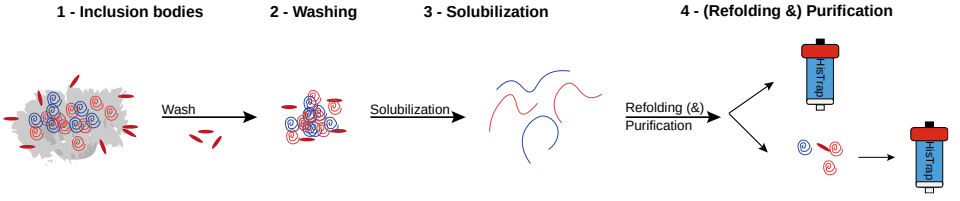


Figure 4. Workflow of inclusion body purification. The inclusion bodies were washed, solubilized and either loaded directly onto a Ni-NTA column, or refolded before loading onto a Ni-NTA column.

2.3 Isolation and purification of 10His-TEV-Ova from inclusion bodies

As all efforts to maximize expression of the protein in the soluble fraction proved fruitless (see section **2.2**), it was chosen to explore the option of obtaining Ova from the insoluble fraction. For this, protocols based on either a solubilization-refolding-purification strategy [47, 48], or a solubilization-purification-refolding strategy were investigated (Figure 4).

In both approaches the insoluble fraction after washing and harvesting is first denatured in a chaotropic buffer to solubilize the protein content (Figure 4 – step 1-3). Chaotropic buffers (buffers containing high molarity urea or guanidine) have been shown to disrupt secondary structures of proteins leading to solutions of unfolded polypeptides. The thus obtained solution of denatured proteins was either directly loaded onto the Ni-NTA column (Figure 4 – step 4 upper) or subjected to refolding using a flash-dilution protocol prior to loading onto the column (Figure 4 – step 4 lower) [20].

The latter approach was explored first. After expression and lysis, the soluble protein fractions were removed by centrifugation and the remaining insoluble fractions were washed twice with water. Next, the inclusion bodies were solubilized in 50 mM Tris-HCl pH 8.5, 8 M urea and 5 mM 1,4-dithiothreitol (DTT), with the use of sonication. The resulting soluble fraction was cleared by centrifugation and then flash diluted (10x) in 50 mM Tris-HCl pH 8.5 [20]. Aggregates were removed by centrifugation and the solute (assumed to contain the refolded Ova) was then loaded onto the Ni-NTA column. The column was then washed, and the protein eluted using increasing imidazole concentrations (Figure 5A). For the solubilization-purification-refolding route (Figure 4 – step 4 upper route), the inclusion bodies were harvested as before (see “lower route”),

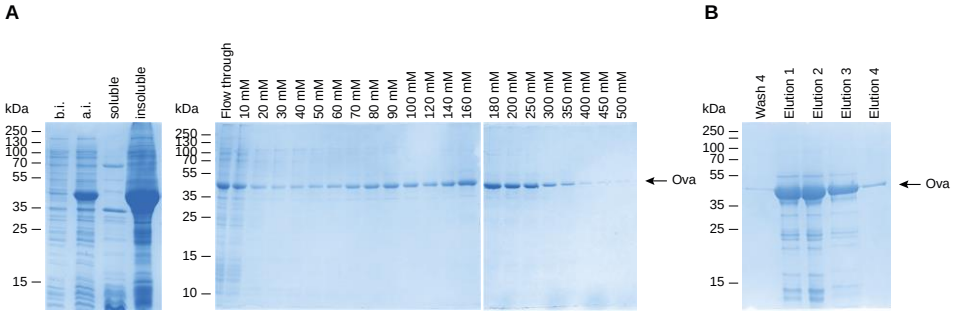


Figure 5. Ni-NTA protein purification profile of native and denatured 10His-TEV-Ova. A) Expressed 10His-TEV-Ova as separated in soluble and insoluble protein fractions were subsequently subjected to refolding conditions and loaded onto a Ni-NTA column. The protein eluted in all elution fractions. B) Purification of the same construct under denaturing conditions. A-B) Proteins were resolved in a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

with the exception that lysis was performed by French press in these particular experiments.

The insoluble fraction was then washed once with lysis buffer containing Triton X-100, with the aim to remove membrane and cell wall associated proteins [49], and twice with lysis buffer. Finally, the washed inclusion bodies were solubilized in a chaotropic buffer (6 M guanidine-HCl in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole), cleared from residual debris by centrifugation and then loaded directly onto the column in this unfolded state. The column was then washed with 2 x 5 column volumes (CV) of a wash buffer containing 6 M guanidine-HCl and twice with 5 CV of a wash buffer containing 6 M urea. The urea wash step was necessary because of the disruptive properties of guanidine in subsequent analyses. The protein was eluted with a single CV of wash buffer containing 500 mM imidazole.

SDS-PAGE of the eluted fractions obtained under native conditions showed Ova in the flow through, wash, and each of the elution fractions (Figure 5A). The same was observed for the protein isolated under denaturing conditions (Figure 5B). It was therefore concluded that Ni^{2+}-affinity chromatography was unsuitable for purifying these constructs. An alternative purification procedure was therefore investigated next (section **2.4**) with the aim to purify 10His-TEV-Ova under native conditions using ion exchange chromatography only.

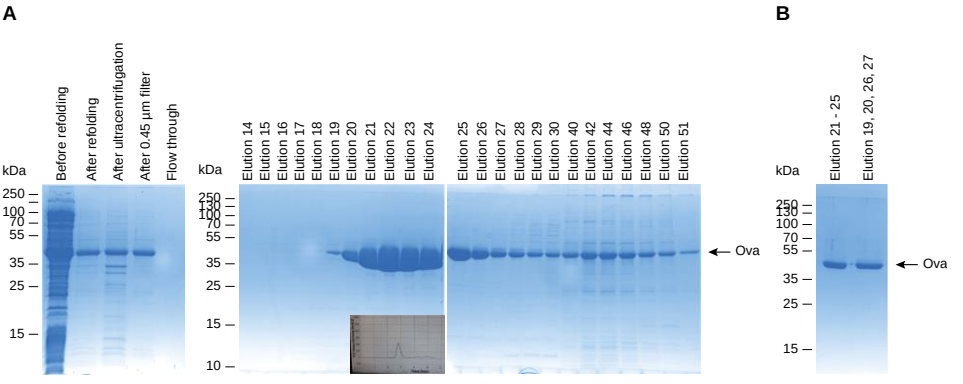


Figure 6. 10His-TEV-Ova purification using a Q column. A) Purification and elution profile of 10His-TEV-Ova, elution fractions were collected in 0.2 CV fractions. Insert shows an almost symmetrical elution pattern. B) Combined elution fractions 21 – 25; 1.5 µg and elution fractions 19, 20, 26 and 27; 1.5 µg. A-B) Proteins were resolved in a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

2.4 Isolation and purification of 10His-TEV-Ova by ion exchange chromatography

To purify Ova proteins by ion exchange, the quaternary ammonium anion exchange column as described in section **2.1** was used. However, rather than first attempting a Ni^{2+}-affinity purification, the proteins were loaded directly onto this column. For this, the inclusion bodies were denatured and flash diluted as described in section **2.3**, and then left standing for 4.5 days at 4˚C. After removal of the aggregates, the remaining solution was loaded onto a Q-column which was then washed with 10 CV of buffer (50 mM Tris-HCl pH 8.5) and eluted by increasing the salt concentration from 0 - 500 mM NaCl. SDS-PAGE analysis revealed complete binding to the column (no putative Ova observed in the flow through, see Figure 6A lane “Flow through”) and emergence of a protein at the correct molecular weight (MW) following elution at a salt concentration of 190 - 270 mM. (Figure 6A and insert). The protein obtained was >95% pure as determined by SDS-PAGE (Figure 6B) and the yield after purification was 15.1 mg/500 mL expression culture with a single purification step.

Having an optimized isolation/refolding/purification protocol in hand, this protocol was next used to investigate whether bioorthogonal Ova could be obtained. To this end, Ova-Hpg was expressed as described in section **2.2**, and the resulting insoluble fraction was subjected to the above purification protocol. Unfortunately, the protein

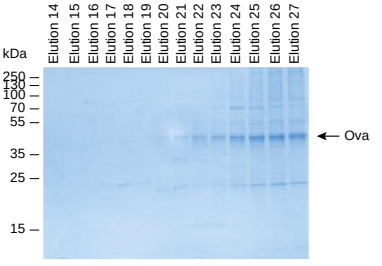


Figure 7. 10His-TEV-Ova-Hpg purification using a Q column. A) Elution fractions validated the presence of Ova only in the higher salt elution fractions. Proteins were resolved in a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

only eluted at higher salt concentrations (Figure 7). This indicated that the protein was either unfolded or aggregated, suggesting that the presence of the unnatural amino acids represents a complicating factor in refolding.

2.5 Expression of native Ova

One of the reasons for the trouble with refolding of 10His-TEV-Ova as cloned and expressed in section **2.2** could be the N-terminal 10His-TEV-appendage. As is evident from the experiments described under section **2.4**, purification protocols do not necessarily include nickel columns which would make a His-tag obsolete [50, 51] and therefore an expression plasmid for Ova lacking this sequence was constructed.

With the aim to investigate whether Ova constructs lacking a His-tag would better segregate into the soluble fraction or alternatively be easier to retain from the insoluble fraction in appropriately folded form, native, full-length *Ova* was cloned into the multiple cloning site of plasmid pET16b (Figure 8A) and the resulting plasmid was transformed into the methionine auxotrophs B834(DE3), and BL21::MetA. Next, the protein was expressed in LB medium and solubilized as described under section **2.1** for 6His-TEV-Ova. SDS-PAGE analysis of both soluble and insoluble fractions showed that expression was successful in both expression strains and that the Ova protein ended up predominantly in the soluble fraction in each case (Figure 8B).

Purification of soluble expressed Ova might require the introduction of an additional purification step prior to Q column loading. This because, loading the cleared lysate directly onto the column might result in the co-elution of multiple proteins together with the target protein.

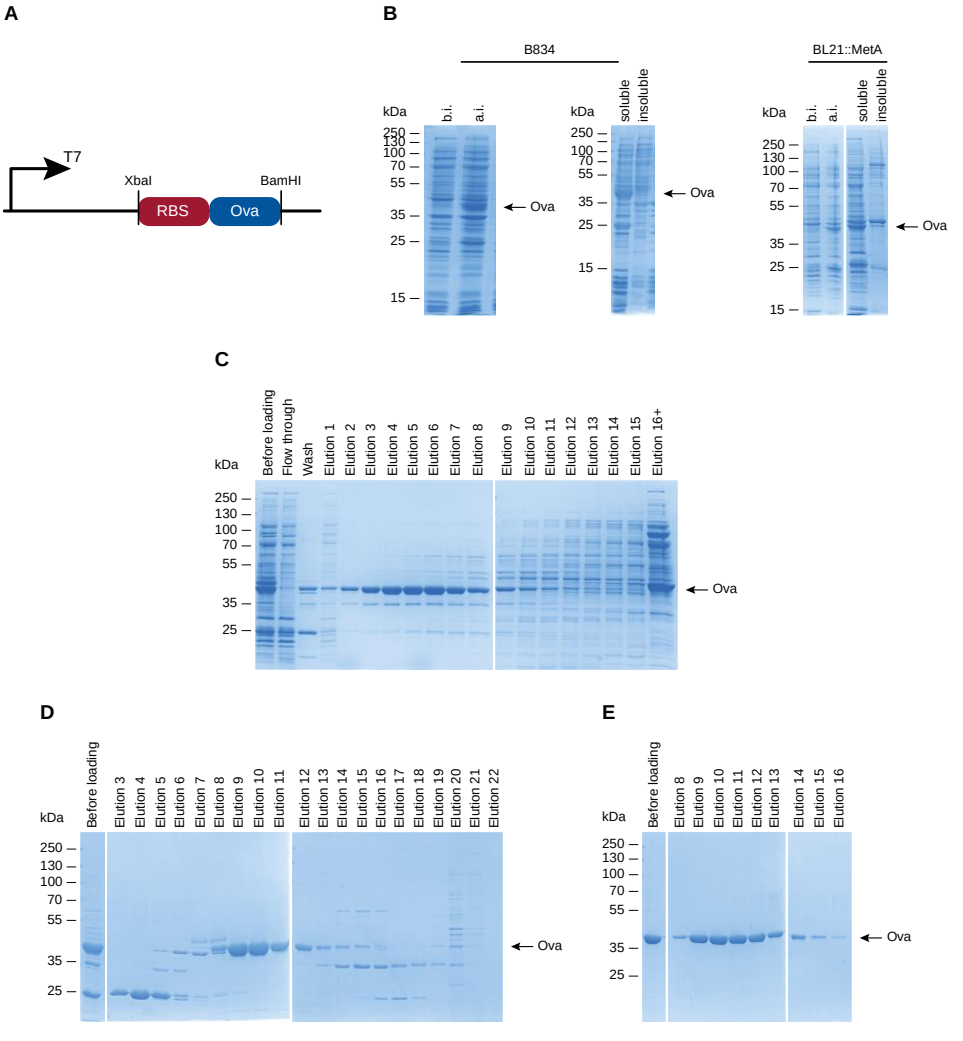


Figure 8. Expression and purification of native Ova. A) Schematic overview of the used gene construct. B) Expression of Ova in B834(DE3) and BL21::MetA and its separation in the soluble and insoluble protein fractions. C) Protein elution profile of Ova over a DEAE column in a 15 CV gradient 20 - 150 mM NaCl in 50 mM MOPS pH 7.5. D) Protein elution profile over a Q HP column in a 20 CV gradient 40 - 150 mM NaCl in 50 mM $NaHCO_3$ pH 8.0. E) Protein elution profile over a Q HP column in a 30 CV gradient 40 - 150 mM NaCl in 50 mM Tris-HCl pH 8.5. A-E) Proteins were resolved in a 10% or a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

With the aim to remove some of the co-expressed proteins before loading the soluble fraction to the column, it was decided to assess the use of osmotic stress and ammonium sulfate precipitation [52, 53]. As lysis by osmotic stress can result in the clearance of

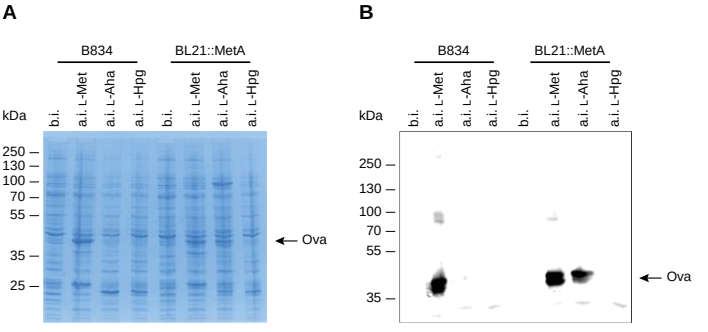


Figure 9. Expression of native Ova with the incorporation of L-Met, L-Aha and L-Hpg. A) Expression of Ova in B834(DE3) and BL21::MetA. B) Protein expression was confirmed by Western blotting against Ova. A) Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

protein without disturbing the cell wall with e.g. lysozyme or the use of French press. Additionally, ammonium sulfate precipitation after cell lysis can result in the removal of co-expressed proteins with a solubility difference as opposed to Ova in the presence of salt [53] (data not shown).

As the use of these two methods in combination with a Q column proved unsuccessful (data not shown), it was decided to perform a first purification over diethylaminoethyl (DEAE) Sepharose instead. The filtered soluble fraction was loaded onto a DEAE column and the protein was partially purified by eluting in a 10 CV gradient of 20 - 300 mM NaCl in MOPS buffer (Figure 8C). The Ova-containing fractions were next buffer exchanged to 50 mM $NaHCO_3$ pH 8.0 and loaded onto a Q column. The protein was eluted with a 20 CV gradient 40 - 150 mM NaCl in 50 mM $NaHCO_3$ pH 8.0 (Figure 8D). The proteins in elution fractions 9 - 12 were next combined, buffer exchanged to 50 mM Tris-HCl pH 8.0 and loaded again onto a Q column. Elution with a 30 CV gradient 40 - 150 mM NaCl followed by SDS-PAGE analysis of the fractions eluted from this final column showed Ova in >95% purity, in a yield of 5.2 mg/250 mL expression culture (Figure 8E).

2.6 Expression of bioorthogonal variants of native Ova

To obtain the Hpg- and Aha-containing variants of untagged Ova, which was obtained in pure form under section **2.5**, the expression with the incorporation of these

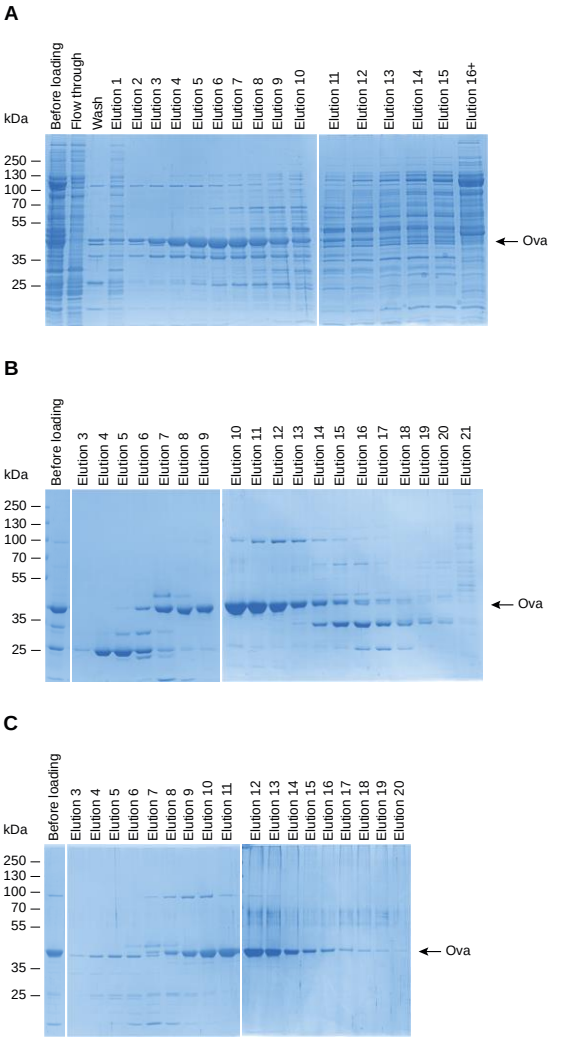


Figure 10. Purification of native Ova-Aha. A) Protein elution profile of Ova over a DEAE column in a 15 CV gradient 20 - 150 mM NaCl in 50 mM MOPS pH 7.5. B) Protein elution profile over a Q HP column in a 20 CV gradient 40 - 150 mM NaCl in 50 mM $NaHCO_3$ pH 8.0. C) Protein elution profile over a Q HP column in a 30 CV gradient 40 - 150 mM NaCl in 50 mM Tris-HCl pH 8.5. A-C) Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

unnatural amino acids in expression strains B834 and BL21::MetA in otherwise unaltered conditions was assessed next (see section **2.1**). The resulting before and after induction samples were analyzed by SDS-PAGE and Western blot. These showed that Ova-Hpg was not present in these after induction samples as derived from either of the

two expression strains (Figure 9). The same holds true for Ova-Aha in case of B834 usage. However, Ova-Aha was produced in the experiments done with BL21::MetA (Figure 9).

Next, native Ova-Aha was purified using the established (see section **2.5**) protocol. SDS-PAGE analysis of the various fractions obtained in this manner showed (Figure 10A-C) a major band corresponding to the size of Ova (and Ova-Aha) in >95% purity, in a yield of 4.3 mg/250 mL expression culture (Figure 10C).

2.7 Analysis of 6His-TEV-Ova, native Ova and the L-Aha counterparts

Having successfully expressed native Ova and native Ova-Aha, it was decided to compare these structurally to 6His-TEV-Ova, 6His-TEV-Ova-Aha and Ova extracted from chicken egg (ggOva) in order to analyze whether the modified proteins are similar and as stable as its wild type counterpart.

First, the purity of the different proteins was visualized via SDS-PAGE and Native PAGE. This showed a purity for all of the proteins of >95% (Figure 11A and 11B). However, in some of the samples a protein band at ~35 kDa was visible. Further α-Ova Western blot analysis confirmed this protein to be Ova related (Figure 11C).

In addition, for some of the protein samples a minor protein band was present. Which was especially noticeable for native Ova and native Ova-Aha at ~35 kDa, a protein band mentioned earlier. It is known that a protein in a different folding-state can run differently over SDS-PAGE, it was aimed to analyze if this was the case for these proteins. For this, native Ova-Aha was combined with Laemmli sample buffer containing different concentrations of DTT. Subsequent SDS-PAGE analysis showed that with increasing concentrations of reducing agent, an increase in the 43 kDa – ovalbumin – band was seen (Figure 11D). However, this was not the case for the complete fraction and LC-MS/MS analysis confirmed the presence of a protein of ~35 kDa in the native Ova sample.

With no major in gel differences between 6His-TEV-Ova, native Ova, and their Aha counterparts compared to ggOva, the reactivity of the proteins towards Alexa Fluor 647 (AF647) alkyne under copper-catalyzed Huisgen cycloaddition conditions was assessed, in order to test the incorporation of Aha (Figure 11E). For this each of the proteins was

combined with a copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) ligation mixture containing the fluorophore. The resulting in-gel fluorescence showed a higher intensity for 6His-Ova-TEV-Aha as compared to native Ova-Aha. This could be a first indication that not all L-Met residues were replaced by L-Aha in the latter protein.

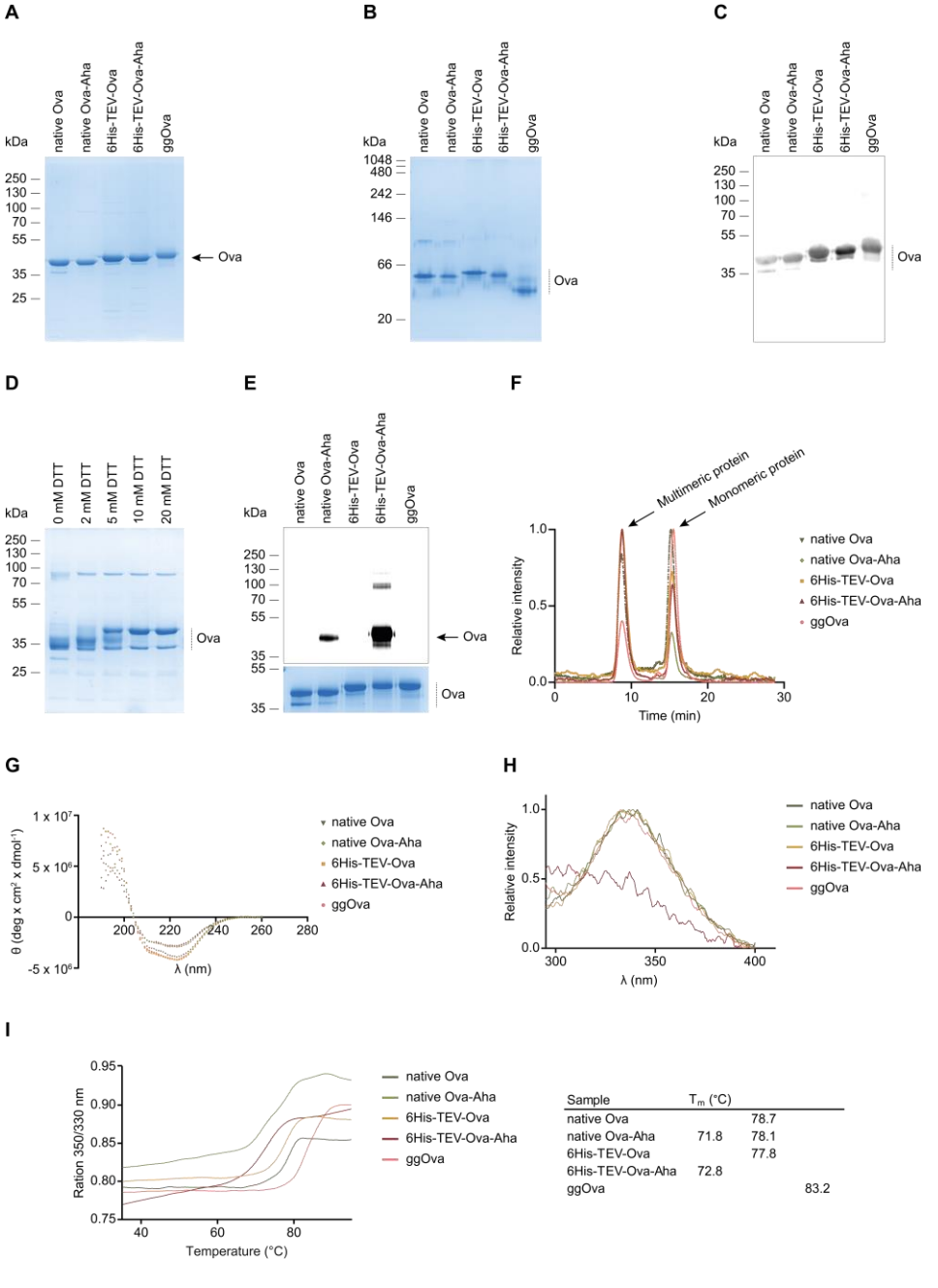
Next, the multimerization of the protein was analyzed via analytical Size Exclusion Chromatography Multi-Angle Light Scattering (SEC-MALS). The plots resulting from these measurements showed the presence of two or three different peaks, representing the monomeric and higher oligomeric states of Ova (Figure 11F).

The analysis was continued with the identification of the proteins secondary and tertiary structure. The secondary structure was measured via far-UV CD measurements (Figure 11G). The proteins were diluted to 0.2 mg/mL in 50 mM $NaHCO_3$ pH 8.0, before recording the spectra. ggOva showed a 43% α-helical and 22% β-sheet content in this buffer. The α-helical content of the protein was only different for native Ova and the β-sheet content only for 6His-TEV-Ova. Surprisingly, the azidylated versions showed to be less different from ggOva than the methionine containing proteins.

The tertiary structure was characterized via fluorescence spectroscopy [54]. For this, the proteins were diluted to 0.5 mg/mL in 50 mM $NaHCO_3$ pH 8.0 and the emission spectra of the 3 tryptophan residues were recorded after an excitation at 280 nm. An emission peak ~340 nm was measured for ggOva (Figure 11H), as reported in literature [20, 55, 56]. This peak was lacking for 6His-TEV-Ova-Aha, which indicated a folding difference not visible in other experiments [54, 57].

Another method to assess correct protein folding and stability is via thermal stability measurements. The discussed proteins showed, except for ggOva, melting temperatures (T_m) corresponding to those found in literature (Figure 11I) [19]. Both 6His-TEV-Ova and native Ova showed a T_m of ~78-79˚C and the azidylated proteins showed a T_m of ~72-73˚C. Interestingly, Ova-Aha also showed a T_m of 78.1˚C, which might be a second indication of incomplete L-Met to L-Aha replacement.

It was shown earlier that ovalbumin is resistant to trypsin when it is in its native protein conformation [20]. To test if the Ova samples are indeed in the native



Sample	T_m (°C)		
native Ova		78.7	
native Ova-Aha	71.8	78.1	
6His-TEV-Ova		77.8	
6His-TEV-Ova-Aha	72.8		
ggOva			83.2

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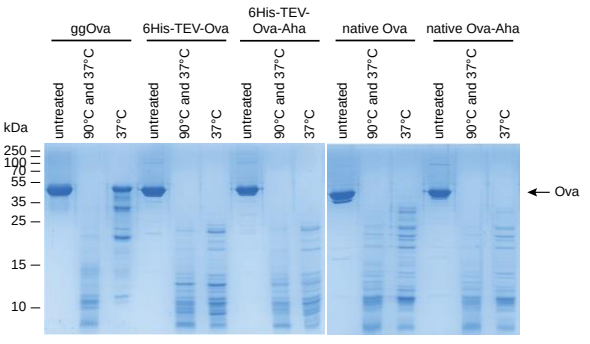

Figure 11. Complete analysis of recombinant ovalbumin as compared to ggOva. A) Protein purity of ovalbumin as assessed by SDS-PAGE. B) Protein purity as evaluated by Native PAGE. C) Western blot analysis against Ova showed the same protein purity as SDS-PAGE. D) SDS-PAGE analysis of Ova-Aha, showing the existence of different sub forms with increasing DTT concentrations. E) Protein ligation to AF647 alkyne, which resulted in fluorescence of the azidylated proteins (upper panel). Protein loading was confirmed by Coomassie staining (lower panel). F) SEC-MALS traces of the different protein samples, measured by relative intensity. G) Far-UV CD analysis of the different protein samples measured in 50 mM $NaHCO_3$ pH 8.0. H) Fluorescence emission spectra of Ova, measured by relative intensity. I) Melting temperatures of the Ova samples, measured by the 350/330 nm ratio and sorted by equal Tm. J) Trypsin resistance assay for the protein samples. With untreated samples as negative control and 90˚C treated samples as positive control. A, B, D, E and J) Proteins were resolved in a 10% or a 15% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

conformation, the protein was analyzed via a trypsin resistance assay. In this study, the protein was either heated at 90˚C before treatment with 0.25 µg of trypsin for 10 min at 37˚C or only treated with the protease at 37˚C. As a negative control, the protein was combined with buffer. Subsequently, the trypsin cleavage was analyzed via SDS-PAGE. The protein remained intact only in the negative control sample (Figure 11J). Whereas pretreatment of the sample at 90˚C or treatment at 37˚C alone, showed a complete degradation profile for each of the recombinant proteins. ggOva remained partly intact when treated with trypsin at 37˚C.

Discussion and conclusion

In this Chapter the expression and purification of different Ova constructs was described. In sector **2.1** it was shown that 6His-TEV-Ova could be expressed even with the incorporation of the unnatural amino acids L-Aha and L-Hpg. The purification of this protein was exemplified by 6His-TEV-Ova-Aha, but was the same for 6His-TEV-Ova and 6His-TEV-Ova-Hpg.

In sector 2.2-2.4 the expression and purification of 10His-TEV-Ova was described. And though protein expression was successful when the protein was either grown in LB medium or with the incorporation of L-Hpg, purification of this protein construct was more troublesome. For each purification, the same protocol was followed. Nonetheless, refolding and Q column purification showed varying amounts of protein aggregates in different purifications. Moreover, it was so far impossible to refold and purify 10His-TEV-Ova-Hpg. Expression and purification of 10His-TEV-Ova with the incorporation of L-Aha was not assessed, but due to the mentioned issues it is not suggested to try this approach.

The expression and purification of native Ova was successful in the preferred methionine auxotroph *E. coli* strain B834(DE3). However, incorporation of the unnatural amino acids was troublesome and therefore it was necessary to use the in-house produced BL21::MetA strain to express Ova-Aha. One of the explanations for the expression differences between B834(DE3) and BL21::MetA could be a discrepancy in expression rates, which was observed earlier by van Elsland *et al.* [58], which could contribute to premature protein degradation. The irreproducible protein expression when L-Hpg was incorporated in native ova, which was sometimes observed after 1 or 2 hours of expression, supported the idea that higher expression levels of the protein triggered protein degradation. A second explanation for this could be that BL21::MetA was not a complete methionine auxotroph, which means unnatural amino acids compete with methionine to be incorporated [59]. This consequently lowers the amount of unnatural amino acids in the protein and might therefore result in a heterogeneous protein product bearing these two amino acid residues. This idea was supported by genomic DNA extraction and subsequent sequencing of the MetA region, which showed to be fully intact. However, as it is not known how the strain was designed, this data remains inconclusive.

The only conclusive way to confirm the incorporation of L-Aha residues in native Ova (and 6His-TEV-Ova), is via LC-MS/MS analysis. Comparison of the LC-MS/MS spectra of both native Ova and native Ova-Aha showed no differences. This was most

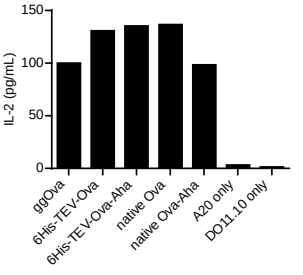

Figure 12: Antigen presentation of different Ova proteins by A20 to DO11.10 T cell hybridomas. DO11.10 activation after antigen presentation of ggOva, 6His-TEV-Ova, 6His-TEV-Ova-Aha, native Ova or native Ova-Aha via A20. IL-2 read out was measured by an ELISA assay.

likely due to insufficient ionization capacities of azidylated ovalbumin. In future experiments, the protein should be digested with trypsin and subsequent peptide analysis should be performed.

All these proteins were expressed and purified with the aim to subsequently use them in T cell assays. For this reason, 0.25 mg/mL (final concentration) of the five different proteins as described in section **2.7** were given to A20 B cells and after 4 hours of initial pulse, the antigen was presented for 20 hours to DO11.10 T cell hybridomas and their activation was subsequently measured via an IL-2 ELISA assay. The preliminary data, of this single replicate, hinted at equal processing of the antigens (Figure 12), but a proper full scale experiment will be conducted in future.

In conclusion, this Chapter showed the successful expression and purification of 6His-TEV-Ova and 6His-TEV-Ova-Aha, moreover it showed the successful optimization of these for native Ova and native Ova-Aha. Which methionine residues were replaced remains inconclusive. Nevertheless, initial results suggest the successful use of the proteins in T cell assays.

Acknowledgements

Hans den Dulk for his help with the design of the pET28a_10His-TEV construct. Nora Goossen, Geri Moolenaar and Anneloes Cramer-Blok for general advice regarding protein expression and purification. Patrick Voskamp for general advice regarding protein purification and help with protein analysis. Robbert Q. Kim of the LUMC Protein Facility for his help with SEC-MALS and thermostability measurements. Can Araman for

general advise and help with CD measurements. Koen Pelsma for his work on MBP and GST fusion proteins and initial purifications. Thomas Bakkum and Mikkel Marqvorsen for the synthesis of (D/)L-Hpg.

Materials and Methods

General

All chemicals and reagents were purchased at Sigma-Aldrich, Alfa Aesar, Acros, Merck or VWR, unless stated otherwise. SDS-PAGE, Native PAGE and Western blot materials were purchased at Bio-Rad. Cloning reagents were ordered at Thermo Fisher Scientific. DNA primers were ordered at Sigma-Aldrich or Integrated DNA Technologies. Protease inhibitors were obtained from Roche or Amresco. Cell culture disposables were from Greiner or Sarstedt.

Solutions

PBS contained 5 mM KH_2PO_4, 15 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 and PBST was PBS supplemented with 0.05% Tween-20. TBS contained 50 mM Tris-HCl, 150 mM NaCl and TBST was TBS supplemented with 0.05% Tween-20. Laemmli sample buffer 4* contained 60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.01% (v/v) bromophenol blue.

Strains and plasmids

E. coli strains XL10, B834(DE3), Rosetta-gami™ 2(DE3), Rosetta™ 2(DE3) and BL21::MetA were used as cloning and expression strains. Plasmid pMSCG7 was used for the expression of 6His-Ova, pET28a was modified accordingly and used for the expression of 10His-TEV-Ova, 10His-2MBP-TEV-Ova and 10His-GST-TEV-Ova, and pET16b was used for the expression of native Ova. All plasmids contain the IPTG-inducible T7 promoter.

Cloning

Plasmid design

pMSCG7 containing full-length chicken Ova was a kind gift from the M. Raghavan lab. For bacterial expression constructs, pET28a was modified with a 10His-TEV linker sequence, which was ligated into the vector using the BsiWI and NheI restriction sites. This vector was extended with a *GST* or a 2 *MalE* gene, using the KpnI restriction site, resulting in pET28a_10His-GST-TEV and pET28a_10His-2MBP-TEV respectively. To obtain 10His-TEV-OVA, 10His-2MBP-TEV-Ova or 10His-GST-TEV-Ova the DNA

fragment encoding Ova was amplified by PCR from the pMSCG7 plasmid containing full-length Ova, and ligated into pET28a_10His-TEV vector using the NcoI and NotI restriction sites. To obtain native Ova, Ova was ligated into the pET16b vector using the XbaI and BamHI restriction sites. All sequences were verified by Sanger sequencing (Macrogen).

Genomic DNA extraction

An overnight culture of BL21::MetA in LB medium was sedimented (500 µL) and the pellet was flash frozen in liquid N_2. The pellet was thawed at rt and resuspended in 500 µL NT1 buffer, further purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Genomic PCR reactions were performed on 2 µL isolated genomic DNA using Phusion High-Fidelity DNA Polymerase in Phusion GC buffer in a final volume of 50 µL. These products were gel purified and sequences were verified by Sanger sequencing.

Table 1: List of primer sequences

#	primer name	sequence 5' -> 3'
p1	pET28a_His-TEV linker_fwd	CATGTGGTACCATGCATCACCATCATCACCATCACCACCATCACTGTACAGAGAACCTGTACTTCCAAGGCCCATGGG
p2	pET28a_His-TEV linker_rev	CTAGCCCATGGGCCTTGGAAGTACAGGTTCTCTGTACAGTGATGGTGGTGATGGTGATGATGGTGATGCATGGTACCACATG
p3	10HisTEV_Ova_fwd	GGCGGCGGTCTCCCATGCGGCTCCATCGGCGCAGC
p4	10HisTEV_Ova_rev	GGCGGCGCGGCCGCTTAAGGGGAAACACATCTGCC
p5	10HisTEV_MBP_fwd	CAGGGTACCAAAATCGAAGA
p6	10HisTEV_MBP_rev	CGTGGTACCGTTGTTGTTGTTATT
p7	10HisTEV_GST_fwd	CAGGGTACCAGCCCTATACTAGG
p8	10HisTEV_GST_rev	CGTGGTACCTTTTGGAGGATGG
p9	pET16b_Ova_fwd	GGCGGCGGATCCTTAAGGGGAAACACATCTGC
p10	pET16b_Ova_rev	GGCGGTTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCTCCATCGGCGCAGC
p11	MetA_genomic DNA_fwd	CATGCAGGCTCGACATTGGC
p12	MetA_genomic DNA_rev	CACCTGCTGAGGTACGTTTC

Protein expression

Expression of 6His-TEV-Ova, 10His-TEV-Ova, 10His-GST-TEV-Ova, 10His-2MBP-TEV-Ova and native Ova

An overnight culture of B834(DE3) containing pMSCG7_Ova, pET28a_10His-TEV-Ova, pET28a_10His-GST-TEV-Ova or pET28a_10His-2MBP-TEV-Ova or pET16b_Ova or BL21::MetA containing pET16b_Ova was diluted 1:100 in LB medium containing 50 µg/mL ampicillin (pMSCG7 and pET16b) or 25 µg/mL kanamycin (pET28a) in the presence or absence of 1% glucose. Cells were grown at 37˚C, 180 rpm to an OD_{600} of ~0.7-1.2 and sedimented (3220 rcf, 20 min, 4˚C) before being resuspended in LB medium containing either 50 µg/mL ampicillin or 25 µg/mL kanamycin. The culture was induced

with either 1 mM or indicated concentrations of IPTG (final concentration) and expression took place for 4 h at 18˚C or 30˚C. After expression, cells were sedimented and washed once with PBS. Pellets were stored at -80˚C until protein purification. The before and after induction samples were suspended in 1* Laemmli buffer and resolved in either a 10% or a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250 or G-250) was used for protein analysis.

Expression of 6His-TEV-Ova, 10His-TEV-Ova and native Ova with the incorporation of L-Met, L-Aha or L-Hpg

An overnight culture of B834(DE3; 6His-TEV-Ova and 10His-TEV-Ova) or BL21::MetA (native Ova) was diluted 1:100 - 1:143 in LB medium containing 50 µg/mL ampicillin (pMSCG7) and 1% glucose. Cells were grown at 37˚C, 180 rpm to an OD_{600} of ~0.8-0.9 and sedimented (3428 rcf, 15 min, 4˚C) before being washed twice with SelenoMet medium (Molecular Dimensions). Cells were resuspended in SelenoMet medium containing 50 µg/mL ampicillin (pMSCG7) and depleted for 30 min at 37˚C, 180 rpm before 30-50 min depletion at 30˚C, 130 rpm. After this either L-Methionine (L-Met; 40 mg/L; Ajinomoto) L-azidohomoalanine (L-Aha TFA salt; 72 mg/L) or L-Homopropargylglycine (D/L-Hpg; 80 mg/L) was added to the culture and 15 min later the culture was induced with IPTG (1 mM final concentration), expression took place overnight. After expression, cells were sedimented and washed once with PBS. Pellets were stored at -80˚C until protein purification. The before and after induction samples were suspended in 1* Laemmli buffer and resolved in either a 10% or a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250 or G-250) was used for protein analysis.

Protein solubilization

6His-TEV-Ova expressed in B834; 10His-TEV-Ova expressed in Rosetta™ 2(DE3), Rosetta-gami™ 2(DE3) and BL21::MetA; native Ova expressed in BL21::MetA

The bacterial pellets (25 mL or 50 mL culture) were resuspended in 10 mL lysis buffer containing 50 mM Tris-HCl pH 8.5, 100 mM NaCl, 1 mM PMSF, 5 mM EDTA. The bacterial cells were disrupted by sonication on ice, 30% amplitude 10 sec on, 10 sec off

for 6 cycles or by French Press, before separating the soluble from the insoluble protein (15000 rcf, 30 min, 4˚C). The resulting supernatant (soluble protein) and pellet (insoluble protein) were separated and the pellet was resuspended in 10 mL lysis buffer. Or the pellet was washed 2 times with MQ water (1 mL) and pelleted by centrifugation (15000 rcf, 20 min, 4˚C). The pellet was resuspended in 5 mL resuspension buffer containing 50 mM Tris-HCl pH 8.5, 100 mM NaCl, 1 mM PMSF, 5 mM EDTA and incubated for 2 hours on the rollerbench. Pellet and supernatant were separated by centrifugation (15000 rcf, 20 min, 20˚C). The pellet was resuspended in 250 µL or 1 mL solubilization buffer containing 50 mM Tris-HCl pH 8.5, 8 M urea, 5 mM DTT (insoluble fraction). Protein fractions were combined with 4* Laemmli buffer and all resolved in a 10% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250 or G-250) was used for protein analysis.

10His-TEV-Ova, 10His-GST-TEV-Ova and 10His-2MBP-TEV-Ova expressed in B834

The bacterial pellets (10 mL culture) were resuspended in 200 µL resuspension buffer containing 50 mM Tris-HCl pH 8.5, 100 mM NaCl, 1 mM PMSF, 5 mM EDTA. The bacterial cells were disrupted by sonication on ice, 30% amplitude 9 sec on, 6 sec off for 3 min, before separating the soluble from the insoluble protein (30130 rcf, 20 min, 4˚C). The pellet was washed 2 times with MQ water (1 mL) and pelleted by centrifugation (30130 rcf, 20 min, 4˚C). The inclusion bodies were solubilized in 40 µL MQ water and 250 µL solubilization buffer containing 50 mM Tris-HCl pH 8.5, 8 M urea, 5 mM DTT and disrupted by sonication as described before. The solubilized fraction was separated by centrifugation (17500 rpm, 30 min, 25˚C). The protein fractions were combined with 4* Laemmli buffer and all resolved in a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250 or G-250) was used for protein analysis.

Native Ova expressed in B834

The bacterial pellet (30 mL culture) was resuspended in 5 mL lysis buffer containing 50 mM Tris-HCl pH 8.5, 100 mM NaCl, 1 mM PMSF, 5 mM EDTA and the bacterial cells were disrupted by sonication on ice, 30% amplitude 10 sec on, 10 sec off for 6 cycles, before separating the soluble from the insoluble protein (15000 rcf, 20 min, 4˚C). The

insoluble protein fraction was suspended in 5 mL lysis buffer for analysis. Subsequently, the protein fractions were combined with 4* Laemmli buffer and all resolved in a 10% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis.

Large scale solubilization of 10His-TEV-Ova and 10His-TEV-Ova-Hpg

As described previously with the following adjustments: The bacterial pellet (500 mL) was resuspended in 10 mL lysis buffer. The bacterial cells were disrupted by sonication on ice 30% amplitude 9 sec on, 1 sec off for 15 min (L-Hpg), before separating the soluble from the insoluble protein fraction (15000 rcf, 20 min, 4˚C). The pellet was washed 2 times with MQ water (1 mL) and the washed inclusion bodies were solubilized in 12.5 mL solubilization buffer for 1 - 2 hours rolling at RT.

Guanidine-HCl solubilization of 10His-TEV-Ova

The bacterial pellet (250 mL culture) was resuspended in 10 mL lysis buffer containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mg/mL lysozyme, 5 mM β-ME, 250 U benzonase. The bacterial cells were disrupted by French press at 1.9 kbar or by sonication on ice, 30% amplitude 9 sec on, 6 sec off for 3 minutes, before separating the soluble from the insoluble protein (15000 rcf, 30 min, 4˚C). The pellet was washed 1x with lysis buffer containing 1% Triton X-100 and 2x with lysis buffer (1 mL each) and pelleted by centrifugation (30130 rcf, 20 min, 4˚C). The inclusion bodies were solubilized in 2.5 mL solubilization buffer containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 6 M guanidine-HCl, 10 mM imidazole overnight at 4˚C.

Refolding

The solubilized protein as described under “large scale solubilization of 10His-TEV-Ova and 10His-TEV-Ova-Hpg” was flash refolded by 10x dilution in refolding buffer (50 mM Tris-HCl pH 8.5) and either used directly or left for 4.5-5.5 days at 4˚C. Aggregates were removed by centrifugation (17500 rpm, 30 min, 4˚C or 25000 rpm, 30 min, 4˚C; Ti70 rotor, Beckman Coulter) and the supernatant was filtered over 0.2 or 0.45 µm (Filtropur S, Sarstedt).

Purification

6His-TEV-Ova; 6His-TEV-Ova-Aha and 6His-TEV-Ova-Hpg

The bacterial pellets (2 L culture) were suspended in 20 mL lysis buffer containing 50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, protease inhibitor, 250 U benzonase. The bacterial cells were disrupted by sonication on ice, 25% amplitude 4 sec on, 9 sec off for 30 cycles, before separating the soluble from the insoluble protein (15000 rcf, 30 min, 4˚C). The soluble fraction was loaded on a prewashed (50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 10 mM imidazole) 1 mL Ni-NTA agarose column, connected to an ÄKTA start FPLC system equipped with UV, pH and conductance detectors at a flow rate of 1 CV/min. Next, the column was washed with 50 CV wash buffer (50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 20 mM imidazole), before being eluted with a 20 CV gradient of wash buffer to elution buffer (50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 500 mM imidazole). Ova containing fractions were dialyzed extensively against 10 mM $NaHCO_3$ and loaded onto a 1 mL Q HP column. The protein was eluted with a 15 CV gradient 0 – 500 mM NaCl in 10 mM $NaHCO_3$. Protein concentrations were determined using the mass extinction coefficient (ε is 36900/ (M cm) and MW 43063 g/mol, Eppendorf BioPhotometer). The protein fractions were combined with 4* Laemmli buffer and all resolved in a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250) was used for protein analysis.

10His-TEV-Ova - Ni-NTA agarose

The refolded protein as described under “refolding” was loaded onto a 250 µL Ni-NTA agarose column and the protein was eluted in 2 CV fractions with increasing concentrations of imidazole (0 – 500 mM) in a buffer containing 50 mM Tris-HCl, 500 mM NaCl. The protein fractions were combined with 4* Laemmli buffer and all resolved in a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250) was used for protein analysis.

The solubilized inclusion bodies as described under “large scale solubilization of 10His-TEV-Ova and 10His-TEV-Ova-Hpg” were loaded onto a 1 mL Ni-NTA agarose column and washed 2x with 5 CV wash buffer A containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 6 M guanidine-HCl, 10 mM imidazole and 2x with 5 CV wash buffer B

containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 6 M urea, 20 mM imidazole. The protein was eluted with 4x 1 CV elution buffer containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 6 M urea, 500 mM imidazole. The urea containing protein fractions were combined with 4* Laemmli buffer and resolved in a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250) was used for protein analysis.

10His-TEV-Ova and 10His-TEV-OVA-Hpg - Q Sepharose HP IEX

The filtered (Filtropur S 0.2 or 0.45 µm; Sarstedt) refolded protein fraction was loaded on a prewashed (5 CV MQ water, 5 CV high salt buffer (50 mM Tris-HCl pH 8.5, 500 mM NaCl) and 10 CV low salt buffer (50 mM Tris-HCl)) 5 mL Q Sepharose column (HiTrap™ Q HP; GE Healthcare), connected to an ÄKTA start FPLC system equipped with UV, pH and conductance detectors at a flow rate of 1 CV/min. The column was washed with 10 CV of 50 mM Tris-HCl and bound proteins were eluted with a 10 CV gradient of the low salt buffer to the high salt buffer and 1 mL fractions were collected. The protein fractions were combined with 4* Laemmli buffer and all resolved in a 12.5% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue R-250) was used for protein analysis. Fractions were combined according to purity and extensively dialysed (6 - 8 kDa MWCO, 3.3 mL/cm, FisherBrand or 12 - 14 kDa MWCO, 2 mL/cm, Spectra/Por) against 50 mM Tris-HCl, pH 8.5. Protein concentrations were measured using the mass extinction coefficient (ε = 32890; MW = 45493 Da) and protein fractions were combined by concentration.

Native Ova and native Ova-Aha - DEAE Sepharose FF IEX

The bacterial pellet (250 mL) was resuspended in 50 mL lysis buffer containing 50 mM MOPS pH 7.5, 10% glycerol, 10 mM DTT, 500 U benzonase, 1 mM PMSF, 1 mM EDTA and the protocol was continued as described above. The bacterial cells were disrupted by French Press, before separating the soluble from the insoluble protein (15000 rcf, 30 min, 4˚C). The filtered (Filtropur S 0.2 µm; Sarstedt) protein fraction was loaded on a prewashed (5 CV MQ water, 5 CV high salt 50 mM MOPS buffer and 10 CV low salt 50 mM MOPS buffer) 5 mL DEAE Sepharose column (HiTrap™ DEAE FF; GE Healthcare), connected to an ÄKTA start FPLC system equipped with UV, pH and

conductance detectors at a flow rate of 1 CV/min. The column was washed with 5 - 10 CV of 20 mM NaCl in the corresponding buffer and bound proteins were eluted with a 10 CV salt gradient of 20 - 300 mM NaCl in 50 mM MOPS buffer containing 10 mM DTT. The eluates were collected in 1 CV fractions and combined with 4* Laemmli buffer. All were resolved in a 10% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis. Fractions were combined according to purity and concentrated via an Amicon Ultra spin filter with a 10 kDa cutoff (Merck). Next, the protein was diluted in 50 mM $NaHCO_3$ pH 8.0, 10 mM DTT.

Native Ova and native Ova-Aha - Q Sepharose HP IEX

The buffer exchanged eluate from the described DEAE Sepharose FF IEX column was loaded onto a prewashed (5 CV MQ water, 5 CV high salt 50 mM $NaHCO_3$ buffer and 10 CV low salt 50 mM $NaHCO_3$ buffer) 5 mL Q Sepharose column (HiTrap™ Q HP; GE Healthcare), connected to an ÄKTA start FPLC system equipped with UV, pH and conductance detectors at a flow rate of 1 CV/min. The column was washed with 5-10 CV of 20 mM NaCl in the corresponding buffer and bound proteins were eluted with a 20 CV gradient 40 - 150 mM NaCl in 50 mM $NaHCO_3$ pH 8.0 containing 10 mM DTT. The eluates were collected in 1 CV fractions and subsequently treated as described for “Native Ova and native Ova-Aha - DEAE Sepharose FF IEX”. With the exception that the protein fraction was diluted in 50 mM Tris-HCl pH 8.0, 10 mM DTT.

Subsequently, the protein was loaded as described for the purification in 50 mM $NaHCO_3$ buffer. With the exception that the protein was eluted with a 30 CV gradient 40 - 150 mM NaCl in a 50 mM Tris-HCl pH 8.0 containing 10 mM DTT. The protein was buffer exchanged to 50 mM $NaHCO_3$ buffer, pH 8.0 for subsequent analysis.

Protein concentrations were measured using the mass extinction coefficient (ε = 31775; MW = 42911 Da) and protein fractions were combined by concentration.

Native Ova - Short overview of other tested conditions

The purification conditions as described in the following table resulted in a maximal purity of 80% as analyzed by SDS-PAGE. Some of the purifications were started by osmotic lysis in a low salt buffer, subsequently followed by the addition of lysozyme

to break down the bacterial cell wall. Next, an ammonium sulfate precipitation was performed at a concentration of 40%. Other purifications were performed by loading the lysed cells immediately onto the described protein column.

Table 2: Summary of other tested conditions for native Ova purification

Buffer used for column purification	$(NH_4)_2(SO_4)$	Column
50 mM Tris-HCl pH 7.5	x	Q
50 mM Tris-HCl pH 8.0	x	Q
50 mM $NaHCO_3$ pH 7.1	x	Q
50 mM $NaHCO_3$ pH 8.0	x	Q
50 mM $NaHCO_3$ pH 8.0, 10% glycerol, 2 mM DTT, 50 U benzonase, 1 mM PMSF, 1 mM EDTA		DEAE
50 mM HEPES pH 8.0, 10% glycerol, 2 mM DTT, 50 U benzonase, 1 mM PMSF, 1 mM EDTA		DEAE

Protein analysis

Western blot analysis

Samples were resolved in a 10 or a 12.5% SDS-PAGE (proteins at indicated concentrations) along with PageRuler™ Plus Protein Marker (Thermo Scientific) and transferred onto a PVDF membrane by Trans-Blot Turbo™ Transfer system directly after scanning. Membranes were washed with TBS and TBST and blocked with 5% nonfat dry milk (Elk; Campina) in TBST at rt for 1.5 h or at 4˚C ON. Membranes containing protein were then incubated with primary antibody in 5% milk in TBST (1 h at rt) Membranes were washed 3x with TBST and incubated with matching secondary antibody in 5% milk in TBST (1 h at rt). Subsequently washed three times with TBST and once with TBS. Membranes were developed with luminol (10 mL of 1.4 mM luminol in 100 mM Tris, pH 8.8 + 100 µL of 6.7 mM p-coumaric acid in DMSO + 3 µL of 30% (v/v) H_2O_2) [60] and chemiluminescence was detected on the ChemiDoc™ MP System in the chemiluminescence channel and the protein marker was visualized with Cy3 and Cy5 settings.

Primary antibodies: polyclonal rabbit anti-Ova (1:2500, 1:5000 or 1:10000, LifeSpan BioSciences, LS-C59287), polyclonal rabbit anti-6x His epitope tag (1:1000, Rockland, 600-401-382)

Secondary antibody: mouse anti-rabbit IgG-HRP (1:5000, Santa Cruz, sc-2357).

Change of protein folding

Ova-Aha was diluted to 3.33 mg/mL and combined with 4* Laemmli sample buffer without β-mercaptoethanol, containing either 0, 2, 5, 10 or 20 mM DTT. The protein

fractions were resolved uncooked in a 10% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Subsequent Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis.

Fluorescent analysis of 6His-Ova-Aha and native Ova-Aha

6His-Ova-Aha and native Ova-Aha were characterized by ligating Alexa Fluor 647 (AF647; Invitrogen) alkyne to the azide click handle. This was done via CuAAC reaction, the protein was combined 2:1 (v/v) with click mix (containing 3 mM copper sulfate, 30 mM sodium ascorbate, 3 mM THPTA ligand, 30 mM aminoguanidine-HCl and 14 µM AF647-alkyne in 88 mM HEPES pH 7.2, final concentration in click mix) and the reaction took place for 1 hour at rt in the dark. The reaction was quenched by the addition of 4* Laemmli buffer and subsequently samples were resolved in a 10% SDS-PAGE gel along with PageRuler™ Plus Protein Marker (Thermo Scientific), before scanning Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively; ChemiDoc™ MP System, Bio-Rad). Coomassie staining (Coomassie Brilliant Blue G-250) was used for the confirmation of protein loading.

SEC-MALS

Purified ovalbumin variants were assessed for their molecular weight and multimerisation states using multi-angle light scattering (MALS) using the µDAWN detector and OptiLab RI detector (both Wyatt Instruments) in line with a Superdex200 10/300 gel filtration column (GE Healthcare). Molecular weights for the appropriate peaks were determined with the refractive index by using ASTRA software (Wyatt Instruments).

Circular Dichroism (CD) analysis

The secondary structure of ovalbumin was assessed using CD spectroscopy. Far UV-CD spectra were recorded using a Jasco J815 CD spectrometer equipped with a Jasco PTC 123 Peltier temperature controller (Easton, MD) between 190-260 nm. A minimum of five spectra with an acquisition time of 70 seconds (s) for each scan in a 1 mm quartz cuvette at 1 nm resolution were acquired at rt and averaged. Protein concentration used

for Ova was 0.2 mg/mL diluted in 50 mM $NaHCO_3$ pH 8.0. Analysis of the secondary structure was performed by BeStSel test version [61].

Intrinsic tryptophan fluorescence

Intrinsic tryptophan fluorescence emission spectra were recorded using a LS-55 Luminescence Spectrophotometer (Perkin Elmer). Protein samples were diluted to 50 µg/mL in 100 µL Tris-HCl, pH 8.5 or to 0.5 mg/mL in 75 µL $NaHCO_3$ pH 8.0. The protein was excited at 280 nm and spectra were recorded in the wavelength range of 290 - 400 nm with a 5 nm slit.

Thermostability measurements

Sample quality was assessed using Tycho (NanoTemper Technologies) in which intrinsic fluorescence was detected during a thermal ramp. The ratio between 350 nm and 330 nm was plotted and the inflection point (Ti) was determined as an indication of thermal stability.

Tryptic digest

Protein samples were diluted to 12.5 µg in 25 µL 50 mM $NaHCO_3$ pH 8.0. Each of the samples was supplemented with 1 mM $CaCl_2$. One of the series was heated for 15 minutes at 90˚C to obtain denatured Ova. Each of the samples was subsequently treated with 0.25 µg (0.5 µL) trypsin or with the same amount of buffer and the samples were incubated for 10 min at 37˚C, before adding a final concentration of 0.5 mM PMSF to quench trypsin activity. Samples were combined with 4* Laemmli sample buffer and resolved in a 15% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis.

Cell culture

General

A20 B cell was a kind gift of C. Watts and was tested on regular basis for mycoplasma contamination. Cultures were discarded after 2 months of use. The cells were cultured at 37˚C under 5% CO_2 in RPMI 1640 (containing 25 mM HEPES) supplemented with

stable glutamine (2 mM), heat inactivated fetal calf serum (10% v/v; Biowest), β-mercaptoethanol (50 µM), penicillin (200 IU/mL; Duchefa) and streptomycin (200 µg/mL; Duchefa). Cells were passaged every 2 - 3 days.

DO11.10 was a kind gift of F. Ossendorp and was tested on regular basis for mycoplasma contamination. Cultures were discarded after 2 months of use. The cells were cultured at 37˚C under 5% CO_2 in IMDM supplemented with stable glutamine (2 mM), heat inactivated fetal calf serum (10% v/v; Biowest), β-mercaptoethanol (50 µM), penicillin (200 IU/mL; Duchefa) and streptomycin (200 µg/mL; Duchefa). Cells were passaged every 2 - 3 days.

Antigen presentation assay

A20s (50000 cells/well) were seeded in a 96-well tissue-culture treated microtiter plate. Adherence was allowed at 37˚C under 5% CO_2 for at least 1 h prior to the addition of the proteins at 0.25 mg/mL final concentration. The cells were incubated with the antigens for 4 hours, followed by the addition of DO11.10 T cell hybridoma (50000 cells/well) were added to the pulsed A20s and co-cultured for 20 h for antigen recognition and IL-2 production by the T cells at 37˚C under 5% CO_2. After overnight incubation, cells were sedimented by centrifugation (360 rcf, 5 min, rt) and supernatant was transferred to a new 96-wells plate. Stimulation of the T cell hybridoma was measured by IL-2 readout using an ELISA assay according to manufacturer's protocol (Invitrogen).

Graphical analysis

All analysis was determined using GraphPad Prism® 6 or 8 or Microsoft Excel 2016.

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