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Purification of the *Drosophila melanogaster* Proteins Inscuteable and Staufen Expressed in *Escherichia coli*

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Abstract

The proteins Inscuteable and Staufen are key components during asymmetric cell division of neuroblasts for the development of *Drosophila melanogaster*. Expression and purification of both proteins has been a difficult task for structure-function studies. Based on codon optimization for protein expression in *Escherichia coli*, we have been able to produce, in soluble form, the C-terminal domains of Inscuteable and Staufen as chimeras with N-terminal maltose binding protein tag that contains a rigid linker between them for feasible crystallization. In addition, using an optimized synthetic gene, corresponding to the amino acid region 250 - 623 of Inscuteable fused to glutathione-S-transferase, low-scale expression experiments showed production of soluble protein. Finally, eukaryotic expression of Inscuteable in the methylothropic yeast *Pichia pastoris* failed to produce the *Drosophila* protein at detectable amounts, reinforcing the fact that *E. coli* still was the microorganism of choice for high-yield protein expression.

Keywords

Inscuteable, Staufen, Protein Expression and Purification, Maltose-Binding Protein, *Escherichia coli*

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1. Introduction

Asymmetric cell division is of one nature's processes to generate cellular diversity; in *Drosophila melanogaster* neuroblasts are considered the stem cells of the central nervous system [1]. Neuroblasts undergo repeated asymmetric cell divisions to produce a lineage of ganglion mother cells (GMCs) and neurons [2].

The cell fate determinants are biomolecules that regulate differentiation between neuroblasts and GMCs, and the asymmetric localization of these determinants is the key for the proper development and lineage of GMCs [3]. Prospero and Numb are examples of these cell fate determinants in *Drosophila* central nervous system [1]. Prospero is a multidomain protein with DNA-binding motifs that is required for the expression of several genes [4]. Prospero localization during neuroblast-GMC cell division requires the function of Inscuteable and Miranda, while Inscuteable and Staufen localize *prospero*RNA [5] [6].

Inscuteable is the most upstream component for the asymmetric division of neuroblasts and GMCs [7]. It has been demonstrated that the C-terminal domain of Inscuteable (751 - 859 aa) interacts with the mRNA-binding protein Staufen (in its 769 - 1026 aa region) for the proper asymmetric localization of *prospero*RNA [6]. The Inscuteable 252 - 615 amino acid region forms a complex with Miranda and Prospero for the appropriate localization of the latter [5]; also this region is sufficient for most of Inscuteable function [8].

The understanding of asymmetric localization of cell determinants during cell division at the molecular level would be extremely useful in order to further comprehend development and cellular diversity from stem cells. Here we describe the expression and purification of the two domains of Inscuteable mentioned above and the C-terminal of Staufen for protein crystallization and structure-function studies. The task was undertaken with different methodologies in order to obtain several milligrams of protein for structural studies: from eukaryotic protein expression in the eukaryote *Pichia pastoris* to gene synthesis for codon and DNA secondary structure optimization for expression in *Escherichia coli*. We found that with the right construct *E. coli* is still the most suitable host microorganism for high-yield production of recombinant proteins.

2. Materials and Methods

2.1. DNA Constructs

Table 1 shows all the constructs made for this study. The DNA encoding proteins for expression in *Escherichia coli* with a glutathione-S-transferase (GST) tag were cloned into pGEX-4T-2 plasmid (GE Life Sciences). For those proteins expressed as chimeras with maltose binding protein (MBP), the DNA was cloned in a special pET plasmid containing the gene for MBP and a multiple cloning site to produce a rigid linker between MBP and the protein of interest. Cloning was done in pPIC3.5K (Life Technologies) for protein expression in *Pichia pastoris*. The 50 µL PCR reactions consisted of 10 ng of template DNA (DNA library or synthesized genes), 60 pmoles of each primer, 2 µL of 10 mM dNTPs mix, and 2.5 units of Pfu DNA polymerase (Stratagene). The thermocycler conditions were 95˚C for 2 minutes; 30 cycles of 95˚C-45 seconds, 56˚C-45 seconds, and 72˚C 1 minute per kb; and a final extension for 10 minutes at 72˚C. Plasmids were linearized with BamHI and EcoRI restriction enzymes (New England Biolabs) and Inscuteable or Staufen DNA ligated into them after digestion with the same enzymes; other restriction enzymes were used as necessary. All DNA constructs were confirmed by sequencing.

2.2. Gene Synthesis

The primary sequences of Staufen and Inscuteable were entered into the Primo Optimum 3.4 software (Chang Bioscience), which produced DNA sequences containing the codons most commonly used by *E. coli*. The program also designed the alternating oligonucleotides needed for the PCR synthesis of the gene [9]. A two-step PCR methodology, gene assembly and gene amplification, was followed in order to obtain the full-length double-stranded DNA constructs for the Staufen C-terminal (aa 761 - 1026) and two domains of Inscuteable (aa 719 - 859 and aa 250 - 623). For the gene assembly, 10 µL of each oligo solution, at 25 mM, were mixed. 5 µL of this solution were added to a 50 µL PCR solution with the components mentioned above (except primers). The thermocycler conditions consisted of one step at 94˚C; 25 cycles of 94˚C-30 seconds, 52˚C-30 seconds and 72˚C-2 minutes. For the gene amplification, 5 µL from the gene assembly reaction were added to a second PCR mixture with 60 pmoles each of the outer primers (the two external ones used during the assembly). The thermocycler program was 94˚C-60 seconds, 25 cycles of 94˚C-45 seconds, 68˚C-45 seconds, 72˚C for 3 minutes and a final extension for 10 minutes at 72˚C. All genes were sequenced for authentication.

Table 1. DNA constructs for expression and purification of Inscuteable and Staufen.

NAME	PROTEIN	AA REGION SIZE[§]	AFFINITY TAG	HOST CELL
Insc1	Inscuteable	719 - 859 15.3 kDa	GST[*] N-terminal	*E. coli*
Insc2	Inscuteable	719 - 857 15.0 kDa	GST N-terminal	*E. coli*
Insc3	Inscuteable	1 - 859 96.4 kDa	His-tag C-terminal	*P. pastoris*
Insc4	Inscuteable	719 - 857 15.0 kDa	His-tag C-terminal	*P. pastoris*
Insc5	Inscuteable	719 - 857 15.0 kDa	GST N-terminal	*P. pastoris*
Insc6	Inscuteable	250 - 601 37.1 kDa	GST N-terminal	*P. pastoris*
Stau1	Staufen	761 - 1026 27.7 kDa	GST N-terminal	*E. coli*
Insc7	Inscuteable	719 - 857 15.0 kDa	GST N-terminal	*E. coli*
Insc8	Inscuteable	719 - 857 15.0 kDa	His-tag N-terminal	*E. coli*
Insc9	Inscuteable	250 - 623 41.6 kDa	GST N-terminal	*E. coli*
Insc10	MBP[+]/Inscuteable chimera	719 - 857 55.9 kDa	MBP N-terminal	*E. coli*
Insc11	MBP/Inscuteable chimera	719 - 859 57.2 kDa	MBP N-terminal His-tag C-terminal	*E. coli*
Stau2	MBP/Staufen chimera	761 - 1026 68.6 kDa	MBP N-terminal	*E. coli*

[§]Indicates the molecular weight without considering the removable affinity tag. For the chimeras, it includes the affinity tag(s). [*]Glutathione-S-transferase. [+]Maltose binding protein.

2.3. Protein Expression

For *E. coli* expression, the BL21(DE3) or Rosetta strains were used. For pilot protein expression experiments, 2 mL of LB medium (containing 100 μg/mL ampicillin) were inoculated with a single *E. coli* colony and incubated at 37˚C in a shaking incubator. During log-phase growth, expression was induced with 0.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG). After induction, the temperature was lowered to 25˚C and the cells were incubated overnight. Cells were harvested and resuspended in 100 μL of SDS-PAGE sample buffer and boiled for 10 minutes. After centrifugation the supernatant was analyzed by electrophoresis. Large-scale protein expression was performed in baffled fernbach flasks using LB-amp medium, cells were incubated at 37˚C until $O.D._{600}$ reached 0.6. Then the temperature was lowered to 25˚C and IPTG was added to a final concentration of 0.5 mM. Cells were further incubated for 16 hrs. and harvested by centrifugation. For *P. pastoris* expression, the GS115 strain (Life Technologies) was transformed by electroporation with 10 μg of linear DNA and selection of transformants was performed using the manufacturer's suggested protocol. For *P. pastoris* protein expression induction, cells were grown in minimal methanol medium in baffled fernbach flasks at 30˚C.

2.4. Protein Purification

After cell disruption (sonication for *E. coli* and treatment with YeastBuster (Novagen) for *P. pastoris*) and clarification of lysates by centrifugation, proteins were purified by affinity column chromatography. For GST-tagged proteins, lysates were incubated with glutathione sepharose 4B in PBS buffer at room temperature for one hour. The resin was loaded into a column and washed several times with PBS buffer; proteins were eluted in three column-volumes using elution buffer: 50 mM TRIS-HCl, 10 mM reduced glutathione, pH 8.0. Purification of proteins with a His-tag was performed using the His-Pur Ni-NTA resin from Thermo Scientific following manufacturer's instructions. Insc10 and Insc11 were further purified by anion exchange and size exclusion chroma-

tography using the ÄKTA PrimePlus FPLC system from GE Life Sciences. After affinity chromatography, proteins were loaded into a MonoQ anionic exchange column in 50 mM TRIS-HCl buffer at pH 7.8. The column was washed extensively with the same buffer; a 0 - 250 mM NaCl gradient was applied in 40 column-volumes, the fractions that showed the higher protein concentration were analyzed by SDS-PAGE. The purest fractions were pooled and concentrated up to 240 µL, then loaded in a Superose 12 column for size-exclusion separation. Final protein purity was determined by SDS-PAGE.

3. Results and Discussion

3.1. Prokaryotic Expression of Inscuteable

The C-terminal domain of Inscuteable that interacts with Staufen for *prospero* RNA localization is reported to be the amino acid region 751 to 859 [6]. Two C-terminal Inscuteable DNA constructs that include this region, Insc1 and Insc2 (see **Table 1**), were tested using the glutathione-S-transferase expression system. Both constructs were transformed for protein expression in *E. coli* strains BL21(DE3) and Rosetta. **Figure 1** shows a typical result after expression, in any of the two strains, and purification of Insc1 and Insc2; mostly just GST protein (approximately 26 kDa) was obtained after affinity purification. An expected band for GST-Insc2 at approximately 41 kDa was not observed. Purification was also done under denaturing conditions to see if the protein was being expressed as inclusion bodies but no protein was detected.

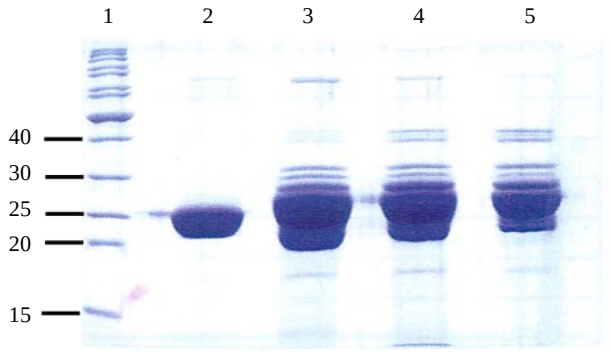

Figure 1. 12% SDS-PAGE showing expression of Insc2 in the *E. coli* strain Rosetta. Lanes 1: protein marker, 2: GST, 3. elution fraction 1, 4: elution fraction 2, 5: elution fraction 3. Sizes of the protein marker bands are shown in the left (in kDa).

This kind of result is not uncommon for eukaryotic proteins expressed in *E. coli* [10], as each unique protein has certain features that allow its proper expression in bacteria. Even though the Rosetta strain, which contains all codons for translation of eukaryotic proteins, was used, *E. coli* failed to express the C-terminal of Inscuteable.

3.2. Eukaryotic Expression of Inscuteable

Several DNA constructs were made for expression of Inscuteable in the yeast *Pichia pastoris*. Full-length Inscuteable DNA Insc3 and its C-terminal region Insc4, both with a C-terminal his-tag, were cloned into pPIC3.5K plasmid vector. **Figure 2** shows the expression and purification of Insc3. After high-imidazole elution the stronger band indicates a molecular weight of approximately 100 kDa, near the calculated size of 96.4 kDa, although this band also appears in lane 5, during the first wash step with low concentration of imidazole. Further experiments will confirm the expression of Insc3; larger protein preparations and characterization, like mass spectrometry, need to be performed. **Figure 3** shows the same experiment but for Insc4, in which an expected protein band of approximately 15 kDa was not observed. Expression of proteins in *P. pastoris* is not as consistent as in *E. coli*; each colony can produce different amounts of protein, and that is the reason why *Pichia* needs an extensive screening for heterologous protein expression.

3.3. Gene Synthesis of Staufen and Inscuteable

Because of unsuccessful attempts to amplify Staufen DNA from a cDNA library, a different approach was needed

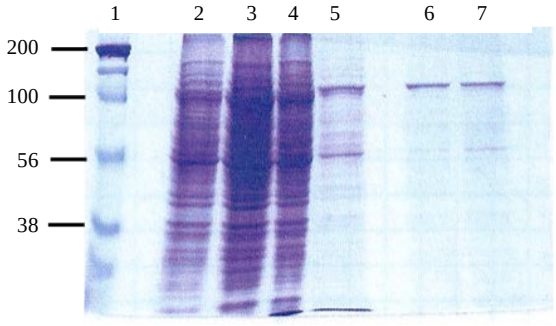

Figure 2. 10% SDS-PAGE from small-scale preparation of Insc3 using the Ni-NTA chromatography columns. Lanes 1: protein marker, 2: 5 µL lysate, 3: 10 µL lysate, 4: lysate after spin column, 5: first wash, 6: first elution step, 7: second elution step.

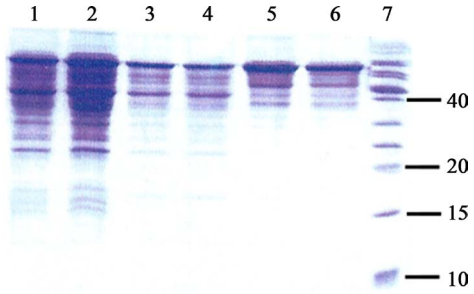

Figure 3. 15% SDS-PAGE showing the expression and purification from cultures of two different colonies of *P. pastoris* expressing Insc4. Lanes 1: lysate 1, 2: lysate 2, 3: first wash 1, 4: first wash 2, 5: elution 1, 6: elution 2, 7: protein marker.

to make the constructs for protein expression. PCR-based DNA synthesis is a method that allows rapid production of a nucleotide sequence optimized for expression in a certain system [9]. **Figure 4** and **Figure 5** show the results of the two-step PCR method for the gene synthesis of the C-terminal domain of Staufen, and two domains of Inscuteable, aa 250 - 623 and aa 719 - 859. After gel-purification of the amplification reactions, genes were cloned and then sequenced. The sequencing indicated some mistakes, though everyone was fixed by site-directed mutagenesis until the desired gene sequence was obtained for protein expression.

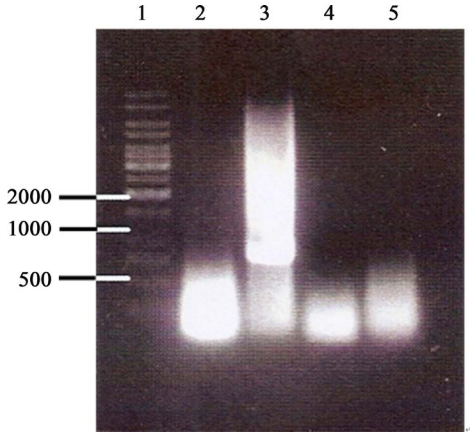

Figure 4. 0.7% agarose gel electrophoresis showing the 798 bp gene synthesis of C-terminal Staufen. Lanes 1: GeneRuler 1kb DNA ladder, 2: gene assembly reaction, 3: gene amplification reaction, 4: no-template negative control for the amplification reaction, 5: no-enzyme negative control. Length, in base pairs, of some of the DNA marker bands is shown.

3.4. *E. coli* Expression and Purification of Inscuteable and Staufen with Optimized DNA

The newly synthesized Staufen DNA was cloned into pGEX-4T-2 vector for the Stau1 construct. After GST af-

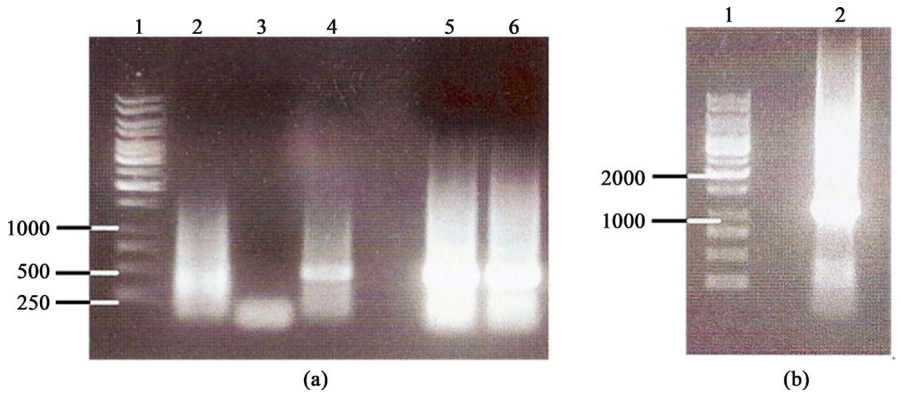

Figure 5. 0.7% agarose gel electrophoresis of synthetic gene products of two domains of Inscuteable. (a) C-terminal domain, 423 bp, Lanes 1: GeneRuler 1kb ladder, Lane 2: gene assembly mixture, Lane 3: no-template control for the amplification reaction, Lane 4: no-enzyme control, Lanes 5 and 6: amplification reaction showing the 423 bp product. (b) Main Inscuteable domain, 1122 bp, Lanes 1: GeneRuler 1kb, Lane 2: amplification reaction.

finity chromatography the construct yielded several milligrams of protein. It was observed that the band appeared as a higher molecular weight protein than expected, so the protein was further characterized by mass spectrometry and sequencing. The mass spectrum showed a value of 54,287.06 *m/z* that is in good agreement with the calculated value of 54,051.90 Daltons. **Figure 6** shows the results for Stau1 purification; after affinity chromatography the protein was treated with thrombin to remove the GST tag. Stau1 was transferred on to a PVDF membrane, stained with Coomassie blue and N-terminal sequenced. The first ten amino acids obtained from the sequencing were: GSGSNSKKLAK. The first two amino acids, Gly and Ser, are remnants after the thrombin digestion (from its recognition site LVPR/GS), the rest of the amino acids correspond to the C-terminal domain of Staufen. The C-terminal Inscuteable DNA was cloned into pGEX-4T-2 as well, Insc7, but even with the optimized DNA we found the same expression pattern as Insc1 or Insc2: just GST was present. The DNA was also cloned into pET28b vector for a N-terminal his-tag (Insc8) but pilot expression experiments did not show presence of the protein. The main Inscuteable domain DNA was cloned for GST-based expression, Insc9. Pilot expression experiments showed a new protein band appearing at approximately 68 kDa corresponding to GST-Insc9 (**Figure 7**).

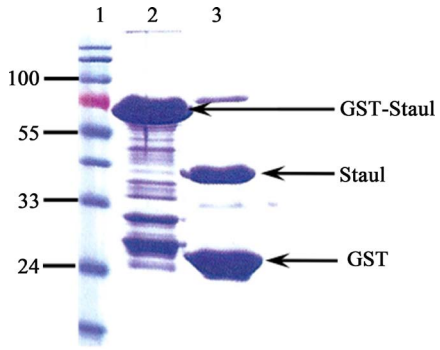

Figure 6. Expression and purification results for Stau1.12% SDS-PAGE showing thrombin digestion for GST removal for Stau1 purification. Lanes 1: protein marker, 2: elution from GST affinity chromatography, 3: protein mixture after 3 hour-treatment with thrombin.

3.5. Expression and Purification of Inscuteable and Staufen as Chimeras with Maltose Binding Protein

Maltose binding protein is a well-known fusion protein that produces high quantity of protein in a soluble form in *E. coli*. A different study has shown that MBP can be a powerful tool for protein crystallization and structure determination [11]. Based on these previous studies, construct Insc10 was design to create a chimera of MBP and the C-terminal domain of Inscuteable. A rigid linker (composed of amino acids AAAEF) was engineered in

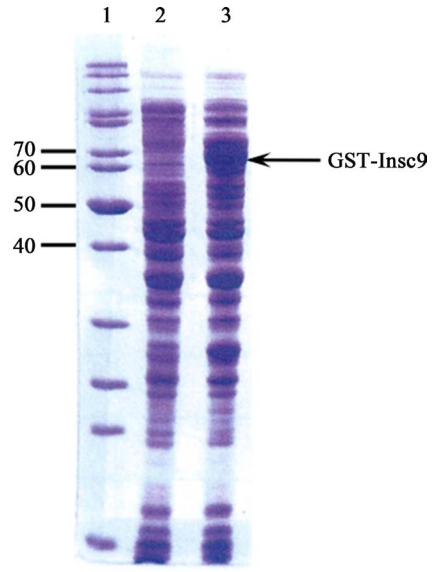

Figure 7. 12% SDS-PAGE showing pilot expression of Insc9 in BL21(DE3). Lanes 1: protein marker, 2: lysate of non-induced cells, 3: lysate of IPTG-induced cells.

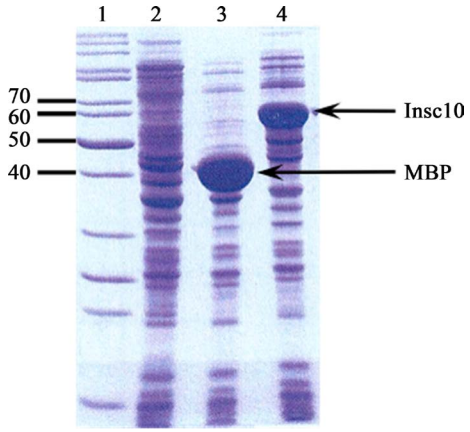

Figure 8. 12% SDS-PAGE showing pilot expression of Insc10 in BL21(DE3). Lanes 1: protein marker, 2: lysate of non-induced cells, 3: lysate of IPTG-induced cells expressing just MBP, 4: lysate of IPTG-induced cells expressing the MBP/C-terminal chimera Insc10.

order to minimize the flexibility between the two domains of the final protein [12]. **Figure 8** shows the pilot expression of this construct in *E. coli* BL21(DE3) cells induced with IPTG.

Insc10 was purified by affinity chromatography with amylose resin. The pooled fractions containing Insc10 were dialyzed and loaded into a MonoQ anionic exchange column. After several column-volume washes, the protein was eluted with a NaCl gradient. The two purest fractions were pooled, the protein concentrated and then applied to Superose 12 column for size-exclusion chromatography. This was a final purification step and it also helped to determine the oligomeric state of Insc10. Based on the elution volume (11.8 mL), it appeared that Insc10 is a monomer; it is worth mentioning that MBP is a monomer and it has been observed that its presence does not affect the quaternary structure of the other protein in the chimera [12]. **Figure 9** shows the electrophoresis analysis of the peak fractions and indicated that Insc10 is a mixture of truncated protein (although it contains more than 90% of the full protein). In order to avoid this problem, Insc11 was designed with a C-terminal His-tag. **Figure 10** shows the expression and purification results for this construct.

Fractions 3 and 4 after size-exclusion chromatography of Insc10 and pure Insc11 were concentrated up to 15 mg/mL for protein crystallization trials, and showed good solubility at that concentration. Crystallization could be feasible due to the designed linker between MBP and the C-terminal of Inscuteable [10]. Finally, since con-

structs with N-terminal MBP showed good expression levels, it was decided to apply it for Staufen as well (Stau2). **Figure 11** shows the pilot expression analysis, which indicates a very obvious new protein band at the expected size. A similar purification protocol used for Insc10 could be applied. It is worth mentioning that the quaternary structure of proteins is not disrupted by the presence of MBP, so, it may be possible to study Inscuteable-Staufen interactions both as chimeras with MBP [12].

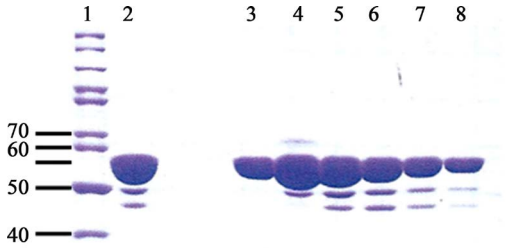

Figure 9. 12% SDS-PAGE electrophoresis analysis of Insc10 after Superose 12 size-exclusion chromatography. Lanes 1: protein marker, 2: Insc10 before chromatography, 3-8: main fractions from size exclusion chromatography.

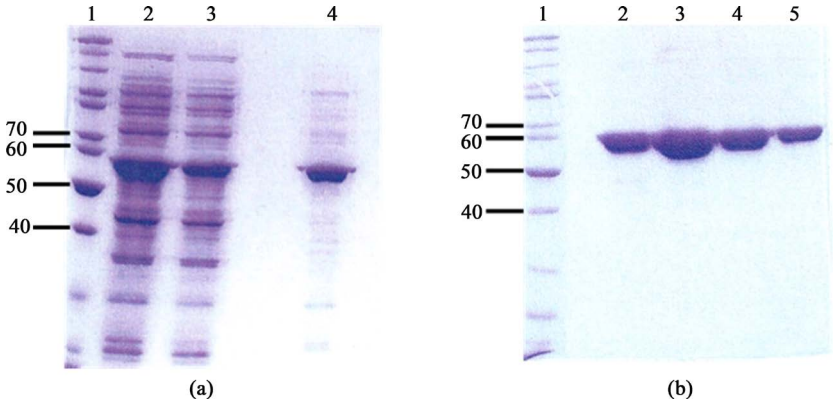

Figure 10. 12% SDS-PAGE electrophoresis analysis of Insc11 purification using metal-affinity and anion exchange chromatography.A Affinity chromatography, Lanes 1: protein marker, 2: Ins11 lysate, 3: lysate after incubation with the metal-affinity resin, 4: elution from the metal-affinity chromatography. B MonoQ anion exchange chromatography, Lanes 1: protein marker, 2-5: main fractions from elution.

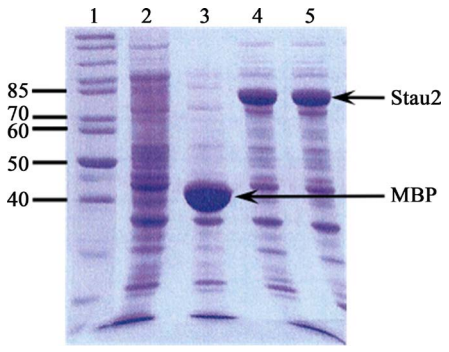

Figure 11. 12% SDS-PAGE electrophoresis analysis of the pilot expression of Stau2. Lanes 1: protein marker, 2: lysate of non-induced cells, 3: lysate of IPTG-induced cells expressing just MBP, 4-5: lysate of IPTG-induced cells expressing the MBP/Staufen chimera Stau2.

4. Conclusion

The generation of cellular diversity in developing organisms requires the synchronization of several molecular mechanisms; in *Drosophila* central nervous system, Inscuteable and Staufen play an important role for the asymmetric localization of cell fate determinants. This work provides a simple process for the production of large quan-

tities of these proteins in *E. coli* for future structure-function studies. Gene synthesis was demonstrated to be a powerful tool for the expression of the *Drosophila* proteins Inscuteable and Staufen. The chimera design with maltose binding protein helped considerably to express high amounts of the C-terminal of both proteins, while the fusion protein glutathione-S-transferase allowed the soluble production of the main functional domain of Inscuteable.

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