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► To cite this version:

Rasmus Kock Flygaard, Beatrice Malacrida, Patrick Kiely, Lasse Bohl Jenner. Purification and characterization of native human elongation factor 2. *Protein Expression and Purification*, 2019, 158, pp.15 - 19. <10.1016/j.pep.2019.02.005>. <hal-03484568>

HAL Id: hal-03484568

<https://hal.science/hal-03484568v1>

Submitted on 20 Dec 2021

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Purification and characterization of native human elongation factor 2

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Abstract

Human elongation factor 2 is the translocase that is responsible for the movement of tRNA from the A- to P and P- to E-site on the ribosome during the elongation phase of translation. Being a vital factor of protein biosynthesis, its function is highly controlled and regulated. It has been implicated in numerous diseases and pathologies, and as such it is important to have a source for isolated pure and active protein for biomedical and biochemical studies. Here we report development of a purification protocol for native human elongation factor 2 from HEK-293S cells. The resulting protein is active, pure, has an intact dipthamide and is obtainable in yields suitable for functional and structural studies.

Keywords

Human eEF2, translation

Abbreviations

eEF2, eukaryotic elongation factor 2; HEK-293F, Human embryonic kidney cells 293; mRNA, messenger RNA; tRNA, transfer RNA; GMPPCP, Guanosine-5'-[(β,γ)-methyleno]triphosphate; eEF1A, eukaryotic elongation factor 1A.

1. Introduction

Protein biosynthesis is the process whereby the ribosome translates messenger RNA (mRNA) into protein. This process is divided into three steps: Initiation, Elongation, Termination and Recycling (for recent review see (Dever, Kinzy, and Pavitt 2016)). During the initiation phase in eukaryotes a very complex series of events finally leads to formation of the 43S pre-initiation complex where initiation Met-tRNA_i is positioned at the start codon on the mRNA. In the elongation step, an aminoacylated tRNA in complex with eukaryotic elongation factor 1A (eEF1A) and GTP is brought into the ribosomal A-site depending on the codon of the mRNA. The peptidyl transferase reaction then takes place between the growing polypeptide carried by the tRNA in the P-site and the amino acid on

the tRNA in the A-site. The peptide bond thus formed results in the transfer of the growing polypeptide chain to the tRNA in the A-site. Subsequently, GTP-dependent translocation is catalysed by eukaryotic elongation factor 2 (eEF2) and advances the mRNA one codon, moving the peptidyl-tRNA from the A- to the P-site, and the deacylated tRNA from the P-site to the E (Exit) site thereby vacating the A-site so that it is ready to receive a new aminoacylated tRNA. This continues until a stop codon is encountered where release and recycling factors dissociate the ribosome and release the nascent polypeptide.

All eEF2 proteins have a uniquely modified histidine residue that has been transformed post-translationally into a dipeptidylamide (Van Ness, Howard, and Bodley 1980; Collier 2001). This residue is a well-known target for ADP-ribosylation by several bacterial toxins thereby effectively inactivating the translation factor and causing cell death (René Jørgensen et al. 2008; R. Jørgensen, Merrill, and Andersen 2006). Although the role of the dipeptidylamide modification still remains somewhat elusive, it has been implicated in fidelity of translation by prevention of frameshift (Liu et al. 2012). Furthermore, eEF2 function is highly regulated via phosphorylation by the eEF2K kinase controlled via MAP kinase signalling and the mTOR signalling pathway (Kaul, Pattan, and Rafeequi 2011).

As eEF2 is one of the most important regulators of the elongation phase of protein synthesis, it is not surprising that changes in both its level and activity are related to a number of different diseases, including cardiovascular diseases, neurological disorders and cancer (Taha et al. 2013; Heise et al. 2014; Kameshima et al. 2016). Particularly, eEF2 has been found to be upregulated in gastric, breast, lung and prostate cancer (Nakamura et al. 2009; Oji et al. 2014; Meric-Bernstam et al. 2012). In addition, eEF2 has been proposed as a potential biomarker for the diagnosis of prostate cancer because of its high correlation with circulating prostate-specific antigen (PSA), one of the most recognized clinical factors used to identify the best treatment regime in prostate cancer (Zhang et al. 2017). High expression of eEF2 is also correlated with incidence, recurrence and resistance to cisplatin in lung adenocarcinoma (Chen et al. 2011).

It is well known that eEF2 functions are mainly regulated by the eEF2 kinase, a Ca^{2+} /calmodulin dependent kinase, which is sensitive to nutrient availability and to stress affecting cells (Ryazanov et al. 1997; Hizli et al. 2013; Kenney et al. 2014). Particularly, the PI3K and MAPK pathways are able to inhibit its activity, while stress conditions lead to the activation of the kinase through a series of activating autophosphorylation events (Pyr Dit Ruys et al. 2012; Kenney et al. 2014; Kameshima et al. 2016). However to date, the precise mechanism by which eEF2 is regulated still remains elusive. For this reason, access to a purified human version of eEF2 will be very useful to facilitate biochemical assays that will advance our understanding of eEF2 properties and functions.

2. Materials and methods

2.1 HEK-293 cell production

HEK-293F cells were cultured in FreeStyle 293 medium (Gibco) until reaching a cell density of 0.2×10^6 cells/mL. Cells were then used for recombinant expression of secreted proteins for another two to four days. Subsequently, cells were harvested by centrifugation at $220 \times g$ followed by storage of the cell pellets at -20°C until further use.

2.2 eEF2 purification

Approximately 60 g of HEK-293F cell pellet (wet weight) was used per purification. Cells were resuspended in buffer L (20 mM Hepes/KOH pH 7.2, 5 mM MgCl_2 , 10% v/v glycerol, 0.5% v/v NP-40, 1 mM DTT, one tablet of protease inhibitor (Sigma) per 100 mL) using 2 mL per gram of cells. The resuspended cells were left on ice for 30 min to complete the detergent-based lysis, followed by centrifugation for 30 min, $30000 \times g$ and 4°C to clear lysate. The pH value of the cleared lysate was adjusted from 6.2 back to 7.0 using untitrated 1 M Tris base and diluted with one volume buffer S (20 mM Hepes/KOH pH 7.2, 5 mM MgCl_2 , 10% v/v glycerol, 1 mM DTT). Glycerol was used for its protein stabilizing effects and magnesium added to importance for GTP/GDP binding although it

might be left out at this step. This diluted lysate was dialyzed overnight at 4°C against 4 L buffer S.

The dialyzed solution was filtered through a 0.45 µm filter prior to loading on a 10 mL sulphopropyl (SP)-column (GE Healthcare) equilibrated in buffer S20 (buffer S with 20 mM KCl). The loaded column was washed in buffer S20 and bound protein eluted with a ten column volumes (CV) linear gradient to buffer S150 (buffer S with 150 mM KCl). Peak fractions containing eEF2 protein were pooled and diluted with four volumes buffer S followed by loading onto a 9 mL Source15S column (GE Healthcare) equilibrated in buffer S20. Bound protein was eluted with a 24 CV linear gradient to buffer S150. Fractions containing eEF2 were pooled and diluted with one volume buffer Q40 (20 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 10% v/v glycerol, 1 mM DTT, 40 mM KCl) and loaded onto a 9 mL Source15Q column (GE Healthcare) equilibrated in buffer Q40. Unbound proteins were washed off with 3 CV buffer Q40 before eluting bound proteins with a 9 CV linear gradient to buffer Q275 (as buffer Q40 but with 275 mM KCl). Fractions containing eEF2 protein were pooled and concentrated to approx. 5 mg/mL in a centrifugation concentrator tube with a molecular weight cut-off of 30000 Da. Concentrated eEF2 protein was centrifuged for ten minutes at 30000 x g and 4°C before loaded onto a Superdex 200 Increase 10/300 column equilibrated in buffer F (20 mM Hepes/KOH pH 7.5, 100 mM KCl, 1 mM DTT). Final peak fractions containing eEF2 protein were pooled and the protein concentrated to a final concentration of 3.2 mg/mL prior to flash freezing in liquid nitrogen and storage at -80°C. Total yield was approximately 4 mg of pure eEF2 protein from 60 g cell pellet. For all SDS-PAGE analysis, samples were resolved on 4-20% Bis-Tris gels (GenScript) using MOPS running buffer. Gels were stained with Instant Blue (Expedeon).

2.3 Western blot analysis

Purified eEF2 was separated on 12% SDS-PAGE gels. Once separated on the gel, protein samples were transferred on a nitrocellulose membrane and then blocked for 1 hour using 5% BSA in TBS with 0.5% of Tween 20, shaking at room temperature. Diluted anti-eEF2 primary antibody (Santa Cruz, catalogue number: sc25634) was added on the membrane and incubated overnight.

Secondary antibody (LiCor, catalogue number: 926-32211) was added at room temperature.

2.4 Ribosome Binding

The activity of eEF2 was asserted based on its ability to bind to the ribosome. Human 80S ribosomes were isolated essentially as described in (Khatter et al. 2014). 80S ribosomes at a final concentration of 5.9 mg/mL were incubated with purified eEF2, tRNA^{Phe} in molecular ratios of 1 RS : 5 eEF2 : 2 tRNA^{Phe} in the presence of 25 µM fusidic acid and 180 µM GMPPCP. The reaction mixture was incubated at 37°C for 10 minutes before loaded onto 5-30% (w/V) sucrose gradients. Peak fractions were analysed by SDS-PAGE and blue-staining.

3. Results and Discussion

The purification protocol developed here for human eEF2 was adapted from a protocol for purification of native yeast eEF2 (René Jørgensen et al. 2002). The critical step is the initial capture of eEF2 protein on the SP-sepharose cation exchange resin. Despite having a theoretical pI value of 6.41 (ExpASy-ProtParam), which is below the pH value of buffer S, the protein will bind under low-salt conditions. We found that two steps of cation exchange chromatography worked the best, with the first step serving to capture the eEF2 protein on the SP-sepharose column and the second step on Source15S column to increase separation of eEF2 from other bound proteins during gradient elution (Figure 1A). eEF2 eluted at 55-60 mM salt concentration in cation exchange chromatography.

The following purification step using anion exchange chromatography was straightforward as eEF2 readily binds the resin at the pH value of the buffer. We found eEF2 protein to elute at 130 mM salt concentration with purity level in the peak greatly increased compared to the first step (Fig. 1B). A second anion exchange chromatography step using a 1 mL MonoQ column as described in (René Jørgensen et al. 2002) was tried but did not result in increased purity so was not pursued further (not shown). As a final purification step the eEF2 protein was subjected to size exclusion chromatography where it eluted with a very high purity level (Fig. 1C). The elution volume of the eEF2 peak

corresponded to a molecular weight of 94.7 kDa when compared to elution volumes of standard globular proteins (Fig. 2). This in turn corresponds well with the theoretical molecular weight of 95.3 kDa. In conclusion of purification, we found this protocol to work very well in terms of obtaining high purity eEF2 protein in a monomeric state for use in downstream experiments. The yield of 4 mg of eEF2 from 60 g of HEK-293F cell pellet compared very well with that reported for yeast eEF2, where 10-12 mg per 100 g yeast cells was obtained (René Jørgensen et al. 2002). Since the HEK293F cells used for eEF2 isolation were a waste product from expression of a secreted protein, this protocol describes a rather inexpensive method for obtaining relatively high yields of eEF2. Furthermore, this inexpensive source of human cells could possibly be used for isolation of other abundant native proteins in a similar manner as long as the expressed protein is either tagged or secreted. The purification protocol described here for human eEF2 also provides a faster way to obtain eEF2 protein as compared to other purification protocols of mammalian eEF2 protein (Merrick et al. 1975).

Two different techniques were used to verify that the isolated protein indeed was eEF2. First, we checked its reactivity with antibodies that recognized eEF2 (Liu et al. 2012; Irino et al. 2011). To do this, we loaded increasing amounts of purified eEF2 on denaturing gel followed by western blot (Figure 3). As expected, using an antibody recognizing eEF2, we identified a band at 95 kDa corresponding to the size of eEF2. To complement the western blot verification of human eEF2, the protein was analysed by mass spectrometry and positively identified as being human eEF2 with a peptide coverage of 90%. Only the His715 modified with diphtamide was detected in the mass spectrometry analysis, showing that all or at least the large majority of the protein is modified (See Supplementary Data). This experiment demonstrates that *in vivo* in human the vital diphtamide modification is present in all copies of eEF2.

In order to verify that the purified human eEF2 retained its biochemical functionality, we tested the ability of the eEF2 to bind human ribosomes through sedimentation in sucrose gradients. Dissociation of eEF2 from the ribosome by

turnover of the protein was prevented by the use of a non-hydrolysable GTP analogue as well as fusidic acid (Malkin and Lipmann 1969). Analysing the protein content in peak fractions from control experiments versus eEF2-ribosome binding experiments confirmed binding of the purified eEF2 protein to the ribosome (Figure 4, gel lane 3). A faint band migrating as the purified eEF2 was observed in the sedimentation control experiment (Figure 4, gel lane 4). This band represents sub-stoichiometric co-purification of human eEF2 with human ribosomes as previously observed (Anger et al. 2013). However, in the sedimentation experiments with purified eEF2 included, the protein band corresponding to eEF2 stained with an intensity comparable to the ribosomal proteins bands indicating a close to stoichiometric binding. Thus, although we have not tested directly for translocation capability, the purified human eEF2 has retained its activity towards ribosome binding.

4. Conclusions

In summary, we have purified fully intact and modified eEF2 from native human source and characterized it biophysically. The protein elutes as a single well-shaped peak on size exclusion chromatography indicating that it is correctly folded and stable. The protein was positively identified by western blot analysis and mass spectrometry. The purification protocol we have developed allows for isolation of highly pure stable and functionally active protein from human cells at a reasonably high yield that is suitable for biochemical and structure-function studies.

Acknowledgements

HEK-293S cell pellet was kindly provided by Prof. Gregers Rom Andersen, MBG, AU, Denmark. We are grateful to our colleagues in the Laboratory of Cellular and Molecular Biology (Graduate Entry Medical School, University of Limerick) for helpful discussions and critical review. Mass Spectroscopy was performed by the French Infrastructure for Integrated Structural Biology FRISBI ANR-10-INBS-05 and of Instruct-ERIC.

Financial Support

This work was supported by a Hallas-Møller Stipend from the Novo Nordic Foundation, Denmark NNF120C1015894 (to RKF and LBJ) and by grants received from Science Foundation Ireland, Grant 13/CDA/2228 (to PK) and Irish Research Council New Foundations 2015 (to BM).

Author Contributions

R.K.F and B.M purified human eEF2, B.M verified eEF2 by western blot analysis, R.K.F and L.J analysed eEF2 ribosome binding activity, eEF2 mass spec verification and drafted manuscript. All authors commented and approved final manuscript.

Competing Interests

The authors declare no competing interests

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Figures

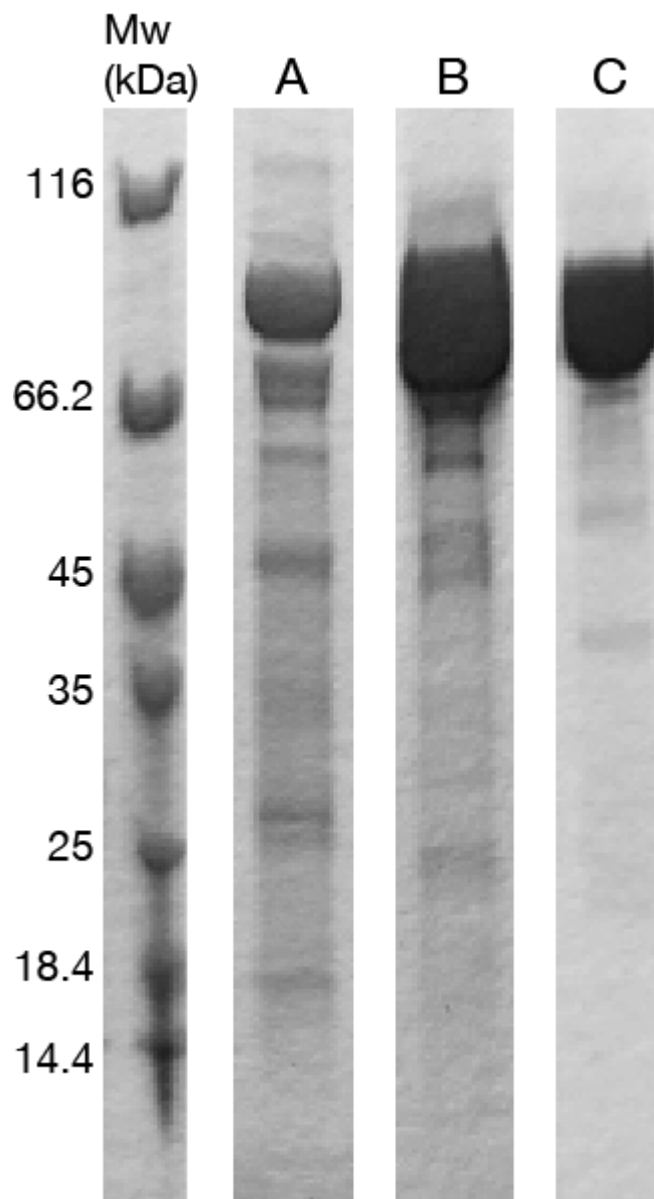


Figure 1. SDS gel analysis of the eEF2 protein at various steps in the purification. A) Peak fraction from SourceS purification step. B) Peak fraction from SourceQ purification step. C) Peak fraction from Superdex200 Increase gel filtration purification step. Mw lane on the left shows molecular weight markers.

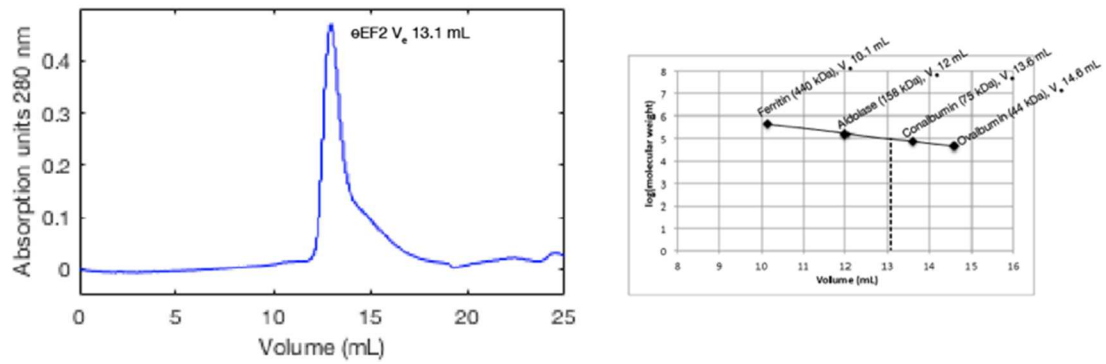


Figure 2. Gel filtration chromatogram of eEF2 from Superdex 200 Increase. On the left is shown the chromatogram with peak elution volume for eEF2 indicated. The graph on the right shows the eEF2 elution volume (dashed line) compared to calibrated elution volumes.

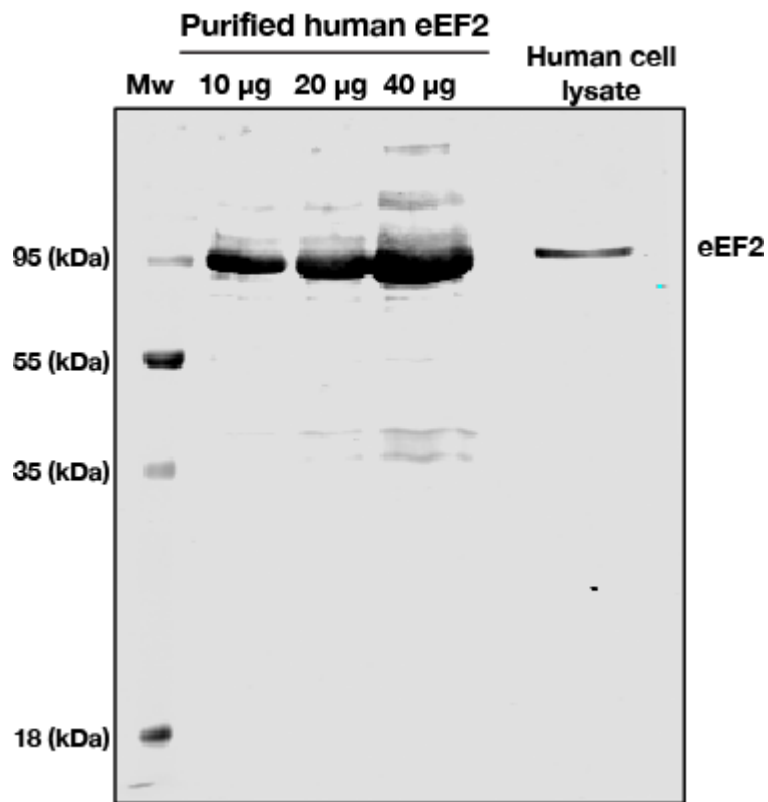


Figure 3. Western blot verification of purified human eEF2 protein. Increasing concentrations of purified eEF2 and 80 ug of total HCT116 cell lysate used as positive control were run on a 12% SDS-PAGE gel and probed with eEF2 antibody to confirm the identity of eEF2. Mw, molecular weight marker bands.

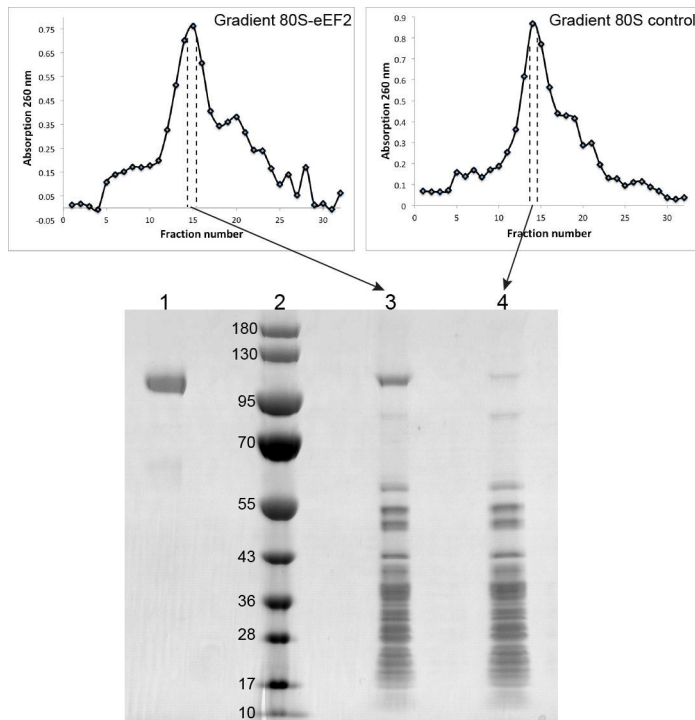


Figure 4. Ribosome binding activity of native purified human eEF2. Top inserts show 5-30% w/V sucrose-density gradient profiles of samples with (left) human 80S ribosome, human eEF2, tRNA^{Phe}, GMPPCP and fusidic acid and (right) a similar experiment with human eEF2 omitted. Peak fractions from density gradients were analysed on the SDS gel shown under the profiles. Lane 1; control with purified human eEF2, lane 2; molecular weight marker, lane 3; peak fraction from sucrose gradient with human eEF2, lane 4; peak fraction from sucrose gradient without human eEF2 added.