ISSN: 1813-4890

Refolding and characterization of the extracellular loops of Occludin

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Abstract

Tight junctions (TJs) are present at the apex of the surface of the epithelial lateral membrane. The main functions of TJs are barrier function and fence function. Occludin is a main component of TJs. But the structure of two extracellular loop domains (EL) of the Occludin is not clear. To explore structure of two extracellular loop domains of the Occludin, we used the linker to fuse EL1 and EL2 of the Occludin. The fusion protein is successfully expressed as inclusion bodies in E. coli. The inclusion body was dissolved in urea under reducing conditions, refolded under redox conditions, purified by C18 RP-HPLC and structure detected by circular dichroism. The result of RP-HPLC showed the fusion protein was refolded successfully. CD analysis indicates that the fusion protein adopts β-sheet. It provides a basis for further research into the structural biology of Occludin.

Keywords

Tight Junctions, Occludin, RP-HPLC, Refold, Circular Dichroism.

1. Introduction

Tight junctions (TJs) are stable multifunctional complexes that consist of intact transmembrane proteins and peripheral membrane proteins[1,2]. The former is mainly responsible for the interaction between adjacent cells to achieve the function of TJs. The latter is primarily responsible for regulating the function and for connecting TJs with the actin backbone. There are many proteins involved in TJs, including transmembrane proteins such as Occludin, Claudins, junctional adhesion molecules, and peripheral membrane proteins such as ZO-1, ZO-2 and ZO-3[3]. TJs play a key role in the barrier function and fence function of normal cells. These two functions are well established, in addition to two other functions: regulation of signal transduction and involvement in the immune system, which are still at an ongoing stage of research[4]. Dysfunction of TJs is related to inflammatory diseases of the intestine, edema, jaundice, diarrhea, blood-borne metastasis and cancer[4,5,6,7].

Occludin was the first integral membrane protein to be identified at TJs[8]. It was a quadruple transmembrane protein encoded by the *OCLN* gene, which contains three cytoplasmic domains and two extracellular loops (EL). In the first EL, more than half of the amino acid residues are tyrosine and glycine, whereas the second EL is rich in tyrosine residues[9]. At present, the crystal structure of the C-terminal domain of the Occludin has been resolved. The 1.45-Å crystal structure of the C-terminus domain of the Occludin comprises three helices, two of which show anti-parallel coiled-coils. The structure revealed a large positively charged surface that contains the binding site for ZO-1[10].The N-terminal domain of the Occludin binds to a multidomain of the E3 ubiquitin-protein ligase Itch to mediate the ubiquitinated degradation of the Occludin[11]. The two extracellular loops of the Occludin have been shown to play an important role in maintaining and regulating tightly junction paracellular macromolecular fluxes[12].

ISSN: 1813-4890

Since Occludin is difficult to express in the soluble form, the structural functions of its two extracellular loop domains are not clear. Here, we selected two extracellular loop domains of the Occludin for structural characterization studies. Circular dichroism spectroscopy reveals that the fusion protein after oxidative folding adopt β-sheet structures. The finding provides a basis for further research into the structural biology of the Occludin.

2. Experimental procedures

2.1. Design of clones expressing fusion protein

The cDNA sequence encoding linker was applied to fuse the cDNA sequence that encoding the EL1 domain and the EL2 domain of human OCLN (UniProKB accession number Q16625), with C-terminal His6-tags, denoted as EL1_EL2. Briefly, the linker was applied to fuse EL1 and EL2 of human OCLN.

2.2. Fermentation and isolation of inclusion bodies

The genes of fusion proteins was cloned into the pET-15b vector. The fusion protein was expressed in Escherichia coli BL21 (DE3) cells, grown in Luria Bertani medium, at 37℃ for 6h after induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at OD600 reached 0.4-0.6. Cells were harvested by centrifugation, resuspended in lysis buffer containing 20 mM Tris, pH 8.0, 500 mM NaCl and homogenized using a cell disruptor (JNBIO). Inclusion bodies were collected by centrifugation and washed by suspension in wash buffer containing 2 M Urea, 20 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, incubated for 1 h at room temperature, collected by centrifugation and the wet paste stored at -80°C.

2.3. Oxidative folding and purification of fusion protein

Inclusion bodies were solubilized (as a 10% (w/v) solution) for 60 min at room temperature, in 8 M urea, 40 mM glycine, 100 mM Tris and 5 mM dithiothreitol (DTT), pH8.0. Refolding was achieved by diluting the supernatant of reduced inclusion bodies to a final protein concentration of 0.1 mg/ml in 2 M urea, 100 mM Tris, 10 mM glycine, 0.4 mM DTT, and 1 mM 2-hydroxylethyl disulfide, pH 8.0. After slow stirring for 2 h at room temperature, the reaction was stopped by acidification to pH 3.0 with HCl. The refolding fusion proteins were monitored by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase HPLC (RP-HPLC). The refolding fusion proteins were purified by a RP-HPLC step, employing a Globalsil C18-BIO column (25 mm diameter x 10 cm long, 300 Å pore size, 10 μm bead size) and a linear gradient from 20% to 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) over 40 min at a flow rate of 1 ml/min. Collected fractions were resolved on an SDS-PAGE gel, Coomassie Brilliant Blue stained, and imaged. Final fractions were freeze-dried overnight on Christ Alpha 1-2 LDplus.

2.4. Circular dichroism spectroscopy

CD spectra was measured with a CD spectrometer (Chirascan, Applied Photophysics). Lyophilized proteins from the previous step were prepared at a concentration of 0.05mg/ml was dissolved in 10 mM HCl. Protein concentrations were determined by the UV absorbance. A quartz cell with 1-mm path length was used. The instrument parameters were set as follows: 25°C, 1nm band width, 190-260 nm scanning range, 100 nm min^{-1} scanning rate, and 1nm wavelength step. Two accumulations were recorded for each spectrum, and a blank containing the same buffer was subtracted as a reference. CD results were expressed in millidegrees (mdeg). Analysis of the experimental data was performed using the software Chirascan's Pro-data viewer. The spectrum was further analyzed using BeStSel software to determine secondary structure content[13].

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3. Results

3.1. Over-Expression of EL1_EL2

After induction with IPTG, the expressed of EL1_EL2 was detected by SDS-PAGE analysis (Fig 1). A prominent protein band was observed ~15 kDa confirming that the EL1_EL2 protein was successfully expressed in BL21. We found that most of the expressed of EL1_EL2 was insoluble body protein (Lane 4).

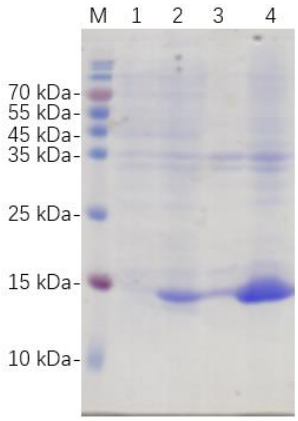

Fig 1. SDS-PAGE analysis for expression of recombinant EL1_EL2 protein. Lane M, protein marker, Lane 1, Uninduced bacterial lysate, Lane 2, IPTG-induced bacterial lysate, Lane 3, Supernatant after crushing, Lane 4, Precipitate after crushing

3.2. Oxidative folding and purification of EL1_EL2

The inclusion body of EL1_EL2 was dissolved in urea/DTT-containing buffer. The solubilized fusion protein was subject to oxidative refolding in the presence of 2-hydroxyethyldisulphide. The result of solubilized and refolded of fusion protein was analyzed by C18 RP-HPLC. Chromatograms of the solubilized inclusion bodies as well as fusion proteins after oxidative refolding is illustrated in Fig 2.A. Upon oxidative refolding, a new peak eluting 3 min earlier than the solubilized denatured peak was observed, an indication of correct refolding. The final product purity was determined by analytical SDS-PAGE. A prominent protein band was observed ~15 kDa confirming the purity of the protein. Fusion proteins from refolding were considered to be essentially successful as judged by RP-HPLC and SDS-PAGE (Fig 2).

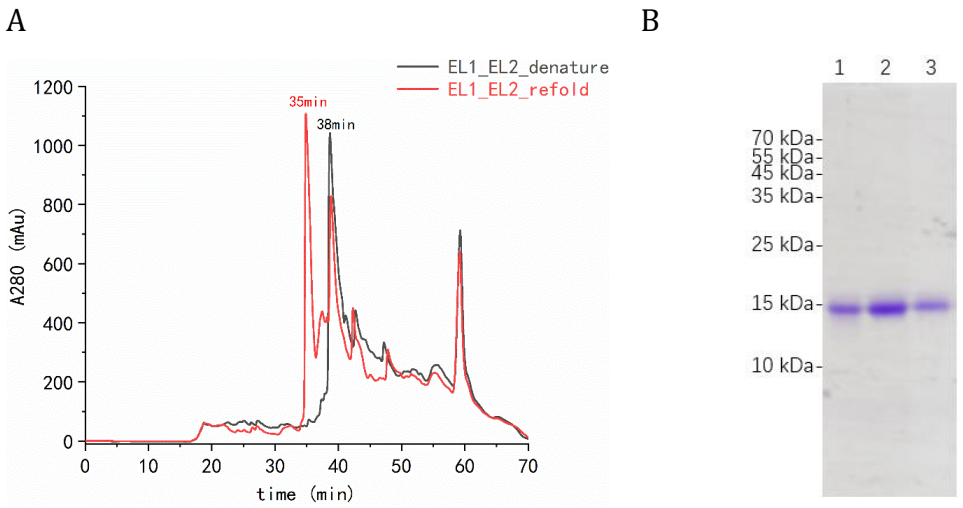

Fig 2. Analysis of fusion protein by RP-HPLC and SDS-PAGE. (A) RP-HPLC analysis of the EL1_EL2 fusion protein. (B) The peak of 35 min evaluated by SDS-PAGE.

ISSN: 1813-4890

3.3. Conformational analysis of fusion protein using CD Spectroscopy

In this paper, the effect of the deleted sequences on the conformation of fusion protein was studied by far-UV CD analysis at 25°C (Fig 3). In the far-UV regions, the CD spectrum of EL1_EL2 featured negative bands at around 218 nm, a characteristic of a structure rich in β-sheet.

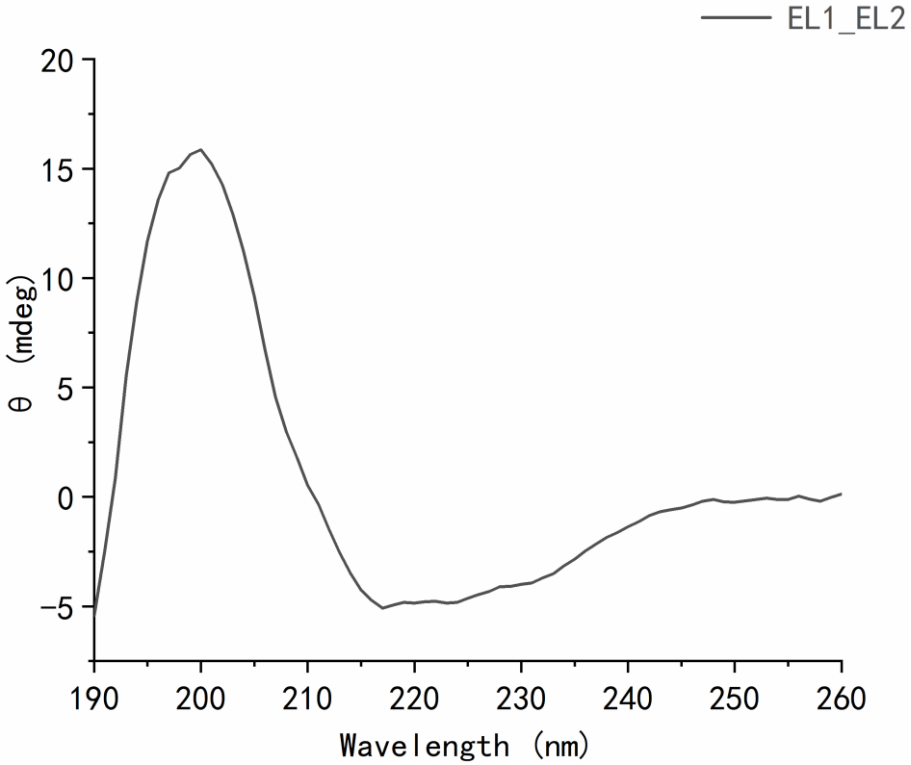

Fig 3. CD profile of fusion protein

The CD spectra of EL1_EL2 was analyzed by BeStSel in order to determine its secondary structure compositions (Table 1). The normalized root mean square deviation (NRMSD) values was widely used to evaluate the quality of CD data. Ideally, NRMSD should be lower than 0.05. The NRMSD value of EL1_EL2 in our measurement is lower than 0.05, indicating good fits. The result showed that EL1_EL2 contained 10.3% α-helical, 40.1% β-sheet, 10.4% turns and 39.2% unordered.

Table 1. The secondary structure compositions of fusion protein estimated from CD spectrum by BeStSel

fusion protein	Helix (%)	Antiparallel (%)	Parallel (%)	Turns (%)	Unordered (%)	RMSD	NRMSD
EL1_EL2	10.3	24.6	15.5	10.4	39.2	0.2232	0.02642

4. Conclusion

In this study, *E.coli* was selected as the expression host of the fusion protein, we chose inclusion body expression and then refold. By dilution oxidative refolding, the fusion protein in soluble form was finally obtained. CD analysis found that the secondary structure of the fusion protein in solution was mainly β-sheet. The finding provides a basis for further research into the structural biology of Occludin.

Acknowledgments

The author is grateful for the help from members of our lab. The author declares no conflict of interest.

ISSN: 1813-4890

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