Design and *Escherichia coli* expression of a natively folded multi-disulfide bonded influenza H1N1-PR8 Receptor Binding Domain (RBD)

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Abstract

Refolding multi-disulfide bonded proteins expressed in *Escherichia coli* into their native structure is notoriously challenging. However, due to its cost-effectiveness, simplicity, and versatility, *E. coli* expression of viral envelope proteins holds significant promise for advancing research on viral infections. For influenza A virus, the glycoprotein hemagglutinin (HA) on its surface is of great importance. This protein plays a crucial role in the early stage of virus infection by binding with the host cell and mediating the membrane fusion. The HA protein binds to sialic acid-containing receptors on the host cell through its globular receptor-binding domain (RBD), which is located on the HA1 fragment and contains the antigenic sites recognized by the host's immune system. Therefore, the RBD of the influenza A virus has the potential to serve as a subunit protein vaccine candidate, and the efficient production of the native RBD might address challenges posed by the virus's rapid mutation and the limitations of conventional egg-based vaccine production.

In this study, we report the successful *E. coli* production and refolding of the H1N1-PR8-RBD, a 27 kDa protein containing four cysteines forming two disulfide bonds. The protein was expressed as inclusion bodies and refolded, yielding a significant amount of 40 mg/L after purification using nickel affinity chromatography and reversed-phase HPLC. We confirmed the molecular weight (MW) of the purified H1N1-PR8-RBD protein by MALDI-TOF mass spectroscopy, which had a relative error of 0.086% compared to the MW calculated from its amino acid sequence. The protein conserved its native structure, as assessed with several biophysical and biochemical techniques. Dynamic light scattering and analytical ultracentrifugation were utilized to determine the monomeric state of the H1N1-PR8-RBD, revealing a hydrodynamic radius of around 2.5 nm. This was an indication that the protein was not aggregating, which was further corroborated by static light scattering data. The secondary structure contents of the *E. coli*-expressed H1N1-PR8-RBD assessed with circular dichroism (CD) were close to the secondary structure contents calculated from the crystal structure of this fragment in the hemagglutinin protein. We further employed differential scanning calorimetry (DSC) and CD spectroscopy to assess the thermal stability of this protein. The thermal denaturation profile, monitored via DSC and CD at a wavelength of 222 nm, exhibited cooperative unfolding with a midpoint temperature around 55°C, strongly suggesting that the protein was natively folded. Tryptophan fluorescence spectroscopy showed a strong intensity at 25°C and later showed a 10-nm red shift of emission maximum as the temperature increased, indicating the typical unfolding of a native protein at high temperatures. The ^{1}H-NMR spectrum measured at 20°C showed well-dispersed and sharp peaks, suggesting a natively folded structure that was later confirmed by several ^{1}H-^{15}N resonances in the high-resolution ^{15}N-HSQC NMR spectrum. In addition, H1N1-PR8-RBD exhibited good biochemical stability against trypsin digestion. The recombinant H1N1-PR8-RBD was not completely digested by trypsin after 120 min incubation, with an undigested fraction of 42%, which was 7% less than that of the well-folded RNase A used as a control. By comparing the undigested fraction of the

oxidized and the reduced H1N1-PR8-RBD, we could conclude that the biochemical stability of this protein stems from its well-folded tertiary structure.

In conclusion, the recombinant receptor-binding domain of H1N1-PR8 (strain A/Puerto Rico/8/1934 H1N1) was successfully produced in *Escherichia coli* with a high yield. The purified protein maintained its native structure and biophysical properties, demonstrating the effectiveness of producing a multi-disulfide bonded protein in *E. coli*. Immunization experiments will be performed to evaluate the protein immunogenicity, and the recombinant H1N1-PR8-RBD will hopefully be valuable for developing influenza subunit vaccine antigens in the future.