Application of a Novel Scientific Discovery to the Detection and Destruction of Pathogens

NAOMI S.HACHIYA
Tokyo Medical University

Millions of people worldwide suffer from neurodegenerative disorders. Most of these illnesses manifest themselves later in life. Correlated to the increase in life expectancy, the number of people affected by these diseases will grow. However, because of the limited sample availability and the excessive aggregation property of disease related inclusion bodies, most of the diseases causing proteins have not been identified, and their functions are mostly unknown. To investigate the protein aggregates of neurodegenerative disorders, both methods of immunohistochemistry and immunoblot are mutually complementary, in general. Concurrently, recent development of a laser-microdissection allows us to manipulate microstructures at the microscopic regions of interest in situ. Due to the highly aggregated structure, however, neuronal inclusion bodies isolated with the laser-microdissection and often disturb the immunoblot assay. For example, the major structural components of Pick bodies partially purified from total brain homogenates with Pick's disease, a type of presenile dementia with intraneuronal lesions, are known to be an abnormally phospholyrated tau, but no investigation has been reported with further purified Pick bodies isolated with the laser-microdissection method yet. Even worse, any conventional procedures including sample pretreatment with chemical denaturing agents or detergents were not effective for such highly aggregated structures. Five-hundred Pick bodies isolated with laser-microdissection were initially applied onto SDS-PAGE according to the routine immunoblotting procedure, but only blurred signals were obtained with anti-phosphorylated tau antibodies. Further increases in the number of Pick bodies applied up to 2,000 could not improve the signal intensities. We next set to utilize the chemical denaturants or detergent, however, these treatment procedures did not improve the immunoblot signals at all. While obtaining a lot of negative results with chemical denaturants or detergents examined above, we have found and purified a robust ATP-dependent protein-unfolding activity from *S.cerevisiae*. The purified factor in monomeric form is identical to the actin interacting protein 2 (Aip2p), but it has a novel oligomeric grapple-like structure of 10-12 subunits with an ATP-dependent opening. ATP regulates the opening and closing of the "gate" that forms the opening within oligomeric Aip2p, where binding to the substrate occurs while in the open form with broad specificity *in vitro*. In the presence of ATP (open state), the oligomeric Aip2p bounds the substrate within the opening, whereas in the absence of ATP (closed state), no binding was observed. Of note, the robust protein conformation unfolding activity could modify even the conformation of pathogenic highly aggregated polypeptides, such as recombinant prion protein in beta-sheet form, alpha-synuclein, and A-beta (1-42) in the presence of ATP *in vitro*. Therefore, to overcome the analysis problem of isolated Pick bodies, we utilized oligomeric Aip2p as a non-chemical denaturant. Five hundred Pick bodies were pretreated with the oligomeric Aip2p for 60 min at 30℃ before loading onto SDS-PAGE. Surprisingly, immunoblot analyses of isolated Pick bodies demonstrated discrete bands stained with anti-phospholyrated tau antibodies after the pretreatment with oligomeric Aip2p. In a serial dilution, 1/500 of 500 Pick bodies which is equivalent to a single Pick body, were detected. Only one Pick body directly pretreated with oligomeric Aip2p was sufficient to illustrate an immunoblot signal, indicating that the pretreatment with the oligoemeric Aip2p enhanced the immunoblot signal over a hundredfold. Negative staining and electron microscopy observation of the laser-

microdissected Pick bodies also revealed that they were untangled. Our novel combinatorial method targets proteins in the specific regions of interest at the micrometer order, which was separated by SDS-PAGE and immunoblotted, exclusively enables us to gather information on the molecular profile (i.e. molecular weight) of target proteins under the microscope *in situ*. Although the protein quantification of highly aggregated proteins such as Pick bodies has been quite problematic, the pretreatment with oligomeric Aip2p also allowed us to quantify the protein contents. Taken together, oligomeric Aip2p with our combinatorial method adds a great potential for investigating normal or abnormal microstructures in various conditions and disorders possibly with an extremely enhanced sensitivity. Such unprecedented property of the oligomeric Aip2p may imply further potential applications. For example, a number of proteomic strategies rely on liquid chromatography-tandem mass spectrometry (LC-MS/MS), but sample preparation methods typically involve the use of detergents and chaotropes that often interfere with chromatographic separation and/or electrospray ionization. The oligomeric Aip2p, however, does not interfere with the LC-MS/MS procedures, and it could be an ideal pretreatment. Overall, oligomeric Aip2p may greatly facilitate the nano-scale analyses, which are often hindered from the aggregation property of target proteins in various protein analytical procedures, especially in minor quantities.

Keywords:

neurodegenerative disorders: A varied assortment of central nervous system disorders characterized by the progressive loss of neural tissue or nerve cells.

immunoblots: An analytical method that involves the immobilization and detection of proteins (antigen) on membranes before detection using antibody specific to that proteins.

immunochistochemistry: Combines anatomical and immunological techniques for the identification of specific tissue components by means of a specific antigen-antibody reaction. This method visualizes the distribution and localization of specific cellular components within a cell or tissue.