Optimal Reagents for Neuroproteomics: Understanding the Molecular Organization and the Complexity of Brain / Nervous System

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Abstract

The interplay of synaptic proteins within the brain controls functions such as learning, memory, sensory, integration, motor coordination, and emotional responses. The functional loss or dysregulation of proteins within the brain are associated with neurological disease. The brain's unique morphology and lipid content make protein extraction and further downstream proteome analysis more difficult than other tissue types. Here we show three approaches to simplify the proteome of neuronal cells and tissue. First, a general neuronal protein extraction reagent enables total functional protein isolation from neuronal tissue to explore the whole brain proteome. Different neuronal proteins, such as the membrane receptors NMDAR2B and AMPA, as well as cytoplasmic and nuclear proteins, were extracted with high efficiency as analyzed by Western blot. In addition, we have developed reagents to further simplify the brain proteome by using differential detergents to fractionate whole brain tissue. Five protein fractions, cytoplasmic, membrane, nuclear, chromatin-bound, and cytoskeletal, are extracted from one tissue sample. Western blot analysis of proteins, such as the membrane sodium-potassium channel and cytoskeletal vimentin, shows that the proteins are fractionated with little to no cross contamination. Alternatively, we can fractionate brain tissue into cytosolic and synaptic compartments. Enrichment of synaptic proteins is shown by Western blot analysis of proteins such as synaptophysin and NMDAR2B. The preservation of phosphoprotein integrity is also indicated by Western blot analysis of proteins such as p-PSD95 and p-GluR2 of AMPA receptor, which is crucial to their function. Functional synaptosomes are isolated based on the ability to take up and release the fluorescent dye FM210. These three approaches decrease the complexity of brain tissue while allowing extraction of functional proteins that can be used for downstream proteomic analysis.

Introduction

Neurons have a unique morphology and lipid content that make total protein extraction from all cellular compartments more challenging than other cell types. Axons are coated with an insulative myelin sheath rich in glycolipid, sphingomyelin, and cholesterol, which adds to the complexity. The Thermo Scientific N-PER Protein Extraction Reagent is a specialized formulation for extracting total protein from neuronal tissue while maintaining protein function.

Subcellular protein fractionation of tissue samples enables protein localization discoveries, enhances detectability of low-abundance species, reduces sample complexity for proteomics and enables the monitoring of physiologic fluxes and redistribution under basal and stimulated/diseased conditions. The Thermo Scientific Subcellular Protein Fractionation Kit for Tissues specifically addresses the fractionation of tissue subcellular structures (Figure 1). The tissue sample is first homogenized in the cytoplasmic extraction buffer, which causes selective membrane permeabilization, releasing soluble cytoplasmic contents. This homogenate is then strained to remove excess debris. The second buffer dissolves plasma, mitochondria and ER-golgi membranes but does not solubilize the nuclear membranes. After recovering intact nuclei by centrifugation, a third reagent yields the soluble nuclear extract. An additional nuclear extraction with micrococcal nuclease releases chromatin-bound nuclear proteins. The recovered insoluble pellet is then extracted with the final buffer to isolate cytoskeletal proteins.

The interaction junction of a neuron and another cell is known as a synapse. Synaptic function is governed by complexes held together by protein-protein, protein-lipid and lipid-lipid interactions. There is evidence that the functional loss or dysregulation of various synaptic proteins is associated with neurodegenerative diseases. The ability to isolate and observe molecular changes in protein composition and function at synapses is important in understanding these disease mechanisms. An enriched fraction of synaptic proteins can be obtained from isolated nerve terminals (i.e., synaptosomes) created during nerve tissue homogenization (Figure 2). Here, we also show that the Thermo Scientific Syn-PER Synaptic Protein Isolation Reagent is effective in isolating functional synaptosomes containing active synaptic proteins from neuronal tissue. Additionally, the Syn-PER Reagent facilitates the study of labile or transient neuronal protein phosphorylation events by stabilizing or preserving these modifications during tissue disruption.

Figure 1: Schematic of the subcellular fractionation protocol.

Proteins are sequentially extracted using differential detergents and a micro-centrifuge. CEB = cytoplasmic extraction buffer; MEB = membrane extraction buffer; NEB = nuclear extraction buffer; NEB + MNase = nuclear extraction buffer plus micrococcal nuclease (chromatin-bound fraction); PEB = pellet extraction buffer.

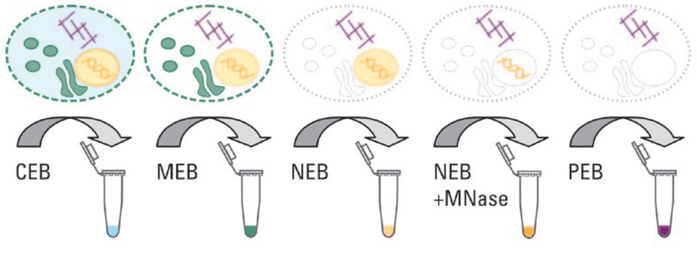


Figure 2: Synaptosome isolation.

A) Schematic of synaptosome. This structure is formed from detached nerve terminal and part of the postsynaptic membrane during mechanical homogenization. B) Protocol for isolating synaptosomes from mouse brain. The procedure requires approximately 1 hour from tissue homogenization to collection of the synaptosomal fraction.

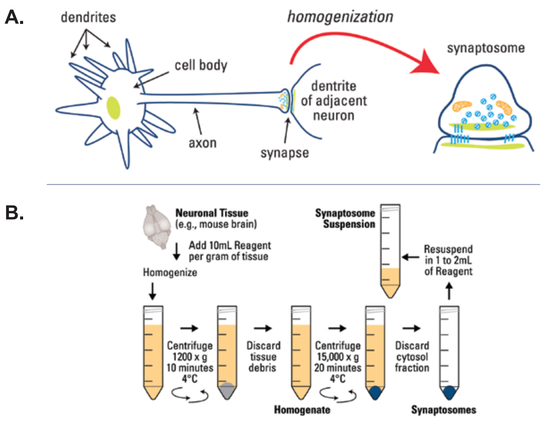


Figure 3: Efficient extraction of total neuronal proteins.

Mouse brain was harvested, divided in half, weighed, and suspended in 10 volumes of buffer per gram of tissue. Tissue was disrupted using Dounce homogenizers and incubated on ice for 10 minutes. Lysates were cleared by centrifugation, protein concentration of the supernantant was measured, and 10µl of each sample was separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis indicates that yields with N-PER Reagent were greater for membrane-bound associated neuronal proteins (NMDAR2B, AMPA, PSD-95, synaptophysin, flotillin-1). N-PER Reagent also performed well for cytoplasmic proteins (tyrosine hydroxylase, MAPK).

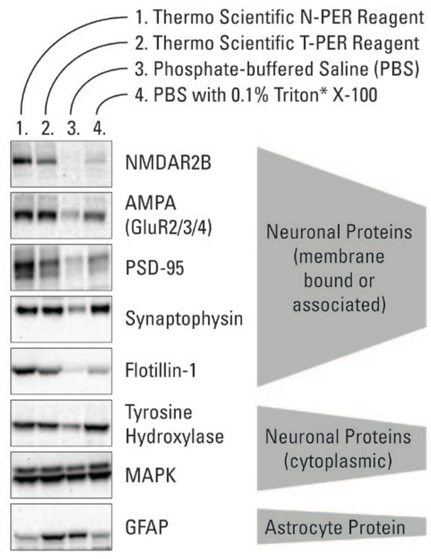
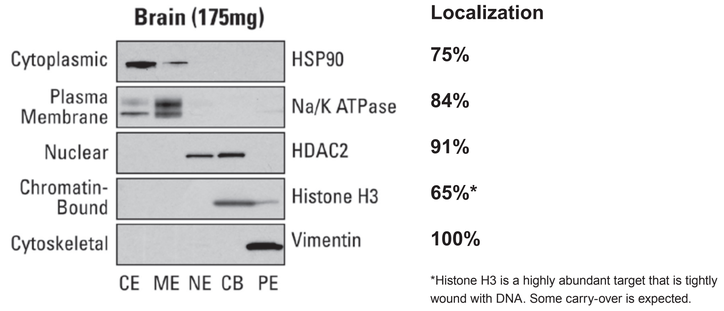


Figure 4: Subcellular fractions from brain tissue samples show minimal cross contamination of target proteins.

Mouse brain was first homogenized in the CEB using a Dounce homogenizer. Sequential fractions were extracted with MEB, NEB, micrococcal nuclease, and PEB as described in Figure 1. Extracts from each subcellular fraction (10µg) were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane. Western blots for specific cellular compartment protein markers were performed: HSP90 (cytoplasmic), Na/K ATPase (plasma membrane), HDAC2 (nuclear and chromatin bound), Histone H3 (chromatin–bound), and vimentin (cytoskeletal). Minimal cross contamination is seen between the different extracts.



*Histone H3 is a highly abundant target that is tightly wound with DNA. Some carry-over is expected.

Figure 5: Enrichment of synaptic proteins.

Mouse brain was homogenized in 10 volumes of Syn-PER Reagent or a homebrew reagent using a Dounce homogenizer. Synaptosomes were extracted according to the Syn-PER Reagent instructions and as depicted in Figure 2. Final pellets were either resuspended in Syn-PER Reagent or homebrew reagent. Total protein (10µg) from homogenates (H), cytosol (C) fraction, and synaptosome suspension (Syn) were analyzed by Western blot. The pre- and post-synaptic protein markers evaluated include synaptophysin, post-synaptic density protein 95 (PSD95), NMDA receptor 2B subunit, and AMPA receptors (GluR2/3/4). Calcineurin, Cdk5, and HDAC2 were purity controls and β-actin served as a loading control.

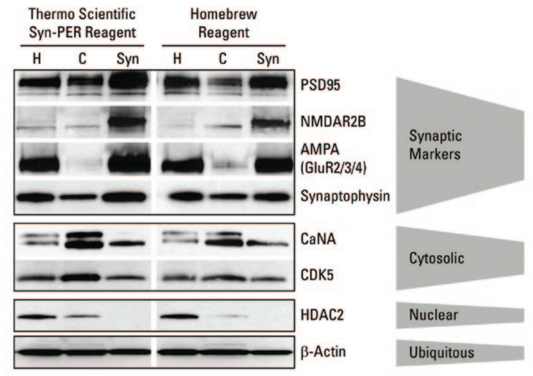
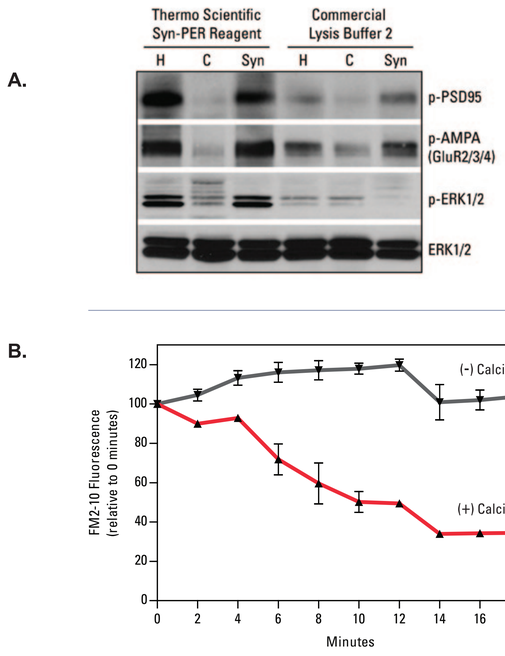


Figure 6: Synaptosomal extracts retain phosphorylation and function.

A) Syn-PER Reagent preserves phosphorylation. Mouse brain synaptosome fractions were prepped as mentioned in Figure 5. Western blot analysis of the phosphoproteins p-PSD95, p-GluR2 of AMPA receptor, and p-ERK1/2 was performed on mouse brain homogenates (H), cytosolic fractions (C) and synaptosome suspensions (Syn). B) Isolated synaptosomes retain biological function. Synaptosome suspensions prepared using the Syn-PER Reagent were incubated with FM 2-10 Dye, a styryl fluorescent dye along with KCl and and calcium. In neurons that are actively releasing neurotransmitters, this dye becomes internalized and leads to high fluorescence. In the presence of 1.2mM calcium, fluorescence (Ex506/Em620nm) slowly decreased for 18 minutes after adding 30mM KCl, indicating phosphorylation-mediated exocytosis. In the sample where no calcium is added, no release of the dye is seen because calcium is needed for exocytosis. Each point is the mean ± SD of two samples.



Conclusions

- The N-PER Reagent enables total protein isolation, including membrane proteins, from neuronal tissue or primary cultured cells, without adversely affecting protein function.
- The Subcellular Protein Fractionation Kit for Tissues effectively extracts five cellular compartments from a single tissue sample.
- The Syn-PER Reagent allows preparation of functional synaptosomes from fresh neuronal tissue while retaining phosphorprotein integrity.
- Complexity of the brain proteome can be decreased by fractionating protein samples by functional compartment upstream of proteomic analysis.