Ion-exchange (IEX) chromatography has been among the most frequently used chromatographic techniques for separation and purification of proteins, polypeptides, nucleic acids, polynucleotides and other charged biomolecules. The success of IEX is due to its widespread applicability, resolving power, high capacity and simplicity. Separation times can be reduced using HPLC, but at increased cost in materials and setup time. Alternatively, IEX can be accomplished using membrane adsorbers. We report a fast and efficient method of purification of two inositol hexakisphosphate (InsP$_6$) derivatives, one bearing a tritiated photoaffinity moiety and the other a fluorescent reporter group.

InsP$_6$, a ubiquitous constituent of plants, has been shown to be an antioxidant and possess anticancer properties (13). It occurs in mammalian tissues in substantial levels and has been reported to influence calcium flux and alter electrophysiological and cardiovascular events (1). InsP$_6$ binds synaptic vesicle proteins and may be an important regulator in synaptic vesicle docking during exocytosis and endocytosis (14).

Several affinity probes have been synthesized in our laboratory (11) to facilitate the isolation and characterization of InsP$_6$-binding proteins. These proteins mediate biological activities such as binding to synaptotagmin domains (9), assembly proteins AP-2 (7) and AP-3 (16), and Golgi coatamer complexes (5), and act as substrates for InsP$_6$ kinases (15). The probes used include photoaffinity (4,11,12) and fluorescent labels (6), caged compounds (2) and affinity resins (1,15).

Tethered benzophenone-containing photophores (4) have been successfully used as photoaffinity reagents for inositol phosphates (11,12). They have traditionally been purified on a diethyl-
Benchmarks

aminoethyl (DEAE) cellulose (HCO\textsubscript{3}\textsuperscript{2-} form) column (3,10). This method suffers from the drawback of an extremely slow rate of flow, which can lead to isomerization of the compound on the column. Application of pressure to increase the flow rate leads to poor resolution.

We report an extremely rapid and efficient purification of reporter group-tagged InsP\textsubscript{6} using IEX membranes built into units with Luer-Lok\textsuperscript{TM} fittings (Becton Dickinson Labware, Bedford, MA, USA). These units have large pores (3–5 µm) that serve as IEX channels. The large pore size and high-dynamic binding capacity (15–20 mg bovine serum albumin per D15 membrane adsorber [Sartorius, Edgewood, NY, USA]) allow an extremely efficient flow using a syringe.

Fluoresceinyl-aminohexyl InsP\textsubscript{6} (Figure 1B) was prepared as follows. A solution of 2.7-mg aminohexyl InsP\textsubscript{6} (8) in 0.25 M triethyl ammonium bicarbonate (TEAB) buffer (pH 8.5) was added to a solution of 1.6 mg (1.1 equivalents) of NHS-fluorescein in 50 µL of dimethylformamide (DMF), and the mixture was stirred at ambient temperature overnight. The solution was then concentrated in vacuo, dissolved in 50 µL of nanopure water and concentrated to dryness to remove any remaining TEAB and DMF. p-[2,3-\textsuperscript{3}H\textsubscript{2}]Benzoylhydridrocinnamyl P-2-(O-aminohexyl)-tethered InsP\textsubscript{6} ([\textsuperscript{3}H] BZDC-InsP\textsubscript{6}) (Figure 1A) was synthesized from P-2-(O-aminohexyl) InsP\textsubscript{6} as described (3,8–10).

Purification of the fluorescent probe B was performed as follows. The residue was dissolved in 0.5 mL of nanopure water and injected onto a D15 membrane adsorber weakly basic anionic exchanger. The membrane adsorber was washed with 10 mL H\textsubscript{2}O, then eluted with increasing concentrations of TEAB buffer. The fluoresceinyl-InsP\textsubscript{6} was eluted in the 0.4–0.6 M fractions, as determined by spectrophotometric monitoring at 518 nm. The unreacted and hydrolyzed fluorescein NHS ester eluted in the 0.1–0.2 M fractions. All previous attempts to purify this compound on conventional DEAE cellulose (HCO\textsubscript{3}\textsuperscript{2-}) columns had been unsuccessful.

Similarly, [\textsuperscript{3}H]BZDC-InsP\textsubscript{6} probe A

Figure 1. (A) [\textsuperscript{3}H]BZDC-InsP\textsubscript{6} and (B) fluoresceinyl-InsP\textsubscript{6}. “=” denotes that counterions may be sodium, protons or triethylammonium, depending on the isolation conditions.

Figure 2. HPLC profile of the purified high-radioactivity fractions of [\textsuperscript{3}H]BZDC-InsP\textsubscript{6} using (A) DEAE cellulose column and (B) D15 membrane adsorber. HPLC conditions are described in the text; note that a 2.5-min offset occurs between the UV and β-RAM detectors.
was purified on a D15 membrane adsorber. The high radioactivity (0.4–0.6 M) TEAB fractions were re-analyzed by reversed-phase HPLC, Aquapore RP300, by isocratic elution with 15% CH$_3$CN in 0.05 M KH$_2$PO$_4$ buffer (pH 4.4) with detection at 280 nm and with a $\beta$-RAM radiochemical detector (IN/US, Tampa, FL, USA). Figure 2 shows the HPLC profile of the [3H]BZDC-InsP$_6$-containing fraction purified on a DEAE cellulose (HCO$_3$-form) column (top) and on a D15 membrane adsorber (bottom). Use of conventional DEAE cellulose (HCO$_3$-form) leads to poor purification and low yields. Most of the high-activity fractions eluting at 0.4–0.5 M TEAB buffer were found to be contaminated with free BZDC acid. Photoreactive tritium-labeled impurities can lead to extensive nonspecific labeling of proteins, and this background interferes with the observation of ligand-specific photoaffinity labeling (4,12). The D15 membrane adsorber units eliminated this problem and gave a faster, cleaner purification as shown in Figure 2. Purification of the highly charged compound A was of higher resolution on D15 weakly basic anion exchanger membrane adsorber than on the Q15 strongly basic anion exchanger membrane adsorber (data not shown).

The purified photoaffinity probe has been successfully used in photoaffinity labeling of a large repertoire of InsP$_6$-binding proteins (9,11,12). The speed of purification and disposability of the units make membrane adsorbers extremely useful for the purification of reporter group-tagged probes. fluorescent and radioactive impurities are readily removed from both newly synthesized materials and from stored samples just prior to usage.

REFERENCES


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