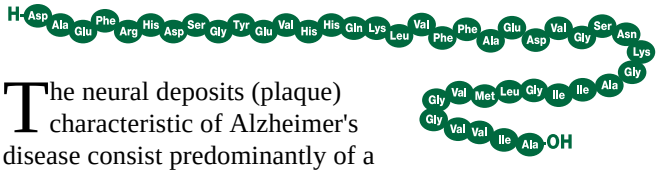
Reversed-Phase Purification of Protein from a Recombinant Fusion

Recombinant gene technology has made practical the production of working quantities of proteins that are either difficult to purify, unavailable, or present only in miniscule quantities from natural sources. In fact, it is now possible to produce proteins that have never been found in nature but are presumed to exist because their sequences have been detected in genomic DNA or messenger RNA, as well as artificial proteins that do not exist in nature. Production of polypeptides and proteins using the machinery of living organisms can be preferable to chemical synthesis because it overcomes many limitations inherent in synthetic peptide chemistry – contamination by side products, residual blocking groups, incomplete or failure sequences, and upper limits on chain length. Genetically engineered production in eucaryotic organisms can also have the advantage of mimicking post-translational processing and modifications which for various reasons may be difficult or impossible to ascertain by analysis beforehand. These powerful recombinant techniques are opening a new era in understanding natural processes, protein biochemistry, and production of useful products.

Polypeptide production in recombinant systems has its own challenges. Chief among these is the need for rapid purification of the target protein from the cellular millieu to prevent degradation. Genetic technology has also provided solutions to this problem. One of the most promising strategies involves production of the target protein as a component of a longer polypeptide called a “fusion protein” (or simply “fusion”). Typically the fusion consists of an N-terminal leader sequence, one or more intermediate engineered sequences, and the target protein at the C-terminus. The leader sequence may be a protein native to the host with adjacent control sequences that permit production to be induced to a high level so that the fusion constitutes a significant percentage of the cellular protein at time of harvesting. Intermediate sequences may include a region designed to facilitate single-step purification, for example a hapten that binds to a specific antibody on an affinity column or a polyhistidine sequence for purification by metal-chelating chromatography. When it is desired to separate the target protein from the fusion and obtain it in purified form, the intermediate sequence will also include a target sequence for a protease of known specificity to facilitate cleavage. Many variations on these principles are possible, almost as diverse as human imagination will allow.

Once a purified fusion protein has been cleaved, a second purification step is necessary to separate the target protein or polypeptide from contaminants including the protease or cleavage reagents, truncated remnants of the fusion, residual uncleaved fusion, and degradation products incidental to the cleavage. This purification can be accomplished conveniently by chromatography on a wide-pore reversed-phase column, as illustrated by the following example.

Purification of Recombinant Alzheimer’s Protein



The neural deposits (plaque) characteristic of Alzheimer's disease consist predominantly of a 42-amino-acid-residue protein, Aβ(1-42), and to a lesser extent a 40-residue truncated version, Aβ(1-40). Production of these proteins in purified form would be useful for studying Alzheimer's plaque formation. Both have been synthesized chemically, but batch-to-batch reproducibility, presence of blocking groups, and variable peptide lengths make routine production by chemical synthesis undesirable. Also, Aβ(1-42) has an exceptional tendency to aggregate and is therefore difficult to purify by traditional methods.

At the Prep '99 Meeting in San Francisco in May, 1999, researchers from the University of Georgia reported using a recombinant system to produce Aβ(1-42) and Aβ(1-40) as soluble fusion proteins in *E. coli*. In this case the fusion consists of a leader protein, a histidine tag sequence, a Factor Xa cleavage site and the Aβ(1-42) or Aβ(1-40) peptide sequence. The fusions are expressed by IPTG induction of the T7/lac promoter of the pET-24a vector (Novagen). Fusion levels attained by this method average 40 mg/L of culture.

After harvest, cells are frozen at -20°C, then thawed as desired, resuspended, homogenized, and centrifuged. Fusion protein in the supernatant is purified by a single chromatographic step on a high-performance metal-chelating sepharose column (Hi-Trap™; Pharmacia) charged with nickel and eluted with buffers containing increasing levels of imidazole.

For cleavage, the purified fusion is combined with Factor Xa enzyme (Boehringer Mannheim) at a ratio of 250:1 and incubated at 25°C for 5-7 hours. The digestion yields the desired protein plus a lesser amount of a truncated protein, Aβ(6-42) or Aβ(6-40), produced by nonspecific activity of the protease, and some residual fusion.

A single purification step by reversed-phase chromatography is sufficient for isolation of highly purified product from the cleavage mixture. For Aβ(1-40), reversed-phase chromatography is performed under acidic conditions at 60°C on a Vydac LC/MS silica-based C4 or C18 column (Fig. 1).

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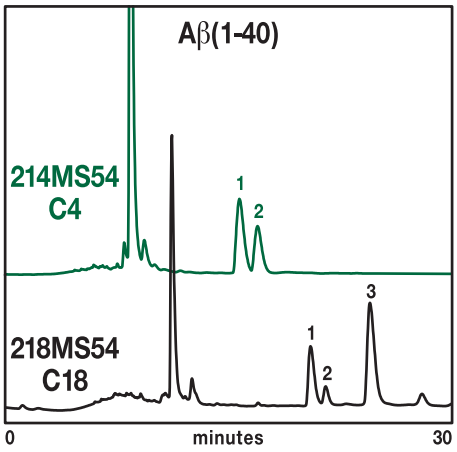

Figure 1. Separation of digested Aβ(1-40) fusion on silica-based low-TFA reversed phase.
Columns: Vydac 214MS54 C4 and 218MS54 C18. Both 5µm, 300Å, 4.6mmID x 250mmL. Conditions: 60°C, 1.0 mL/min. C4 mobile phase: A = 0.05% TFA in 5% MeCN. B = 0.05% TFA in 95% MeCN. Gradient: Linear 0 to 20% B over 8 minutes. Then 20 to 24% B over 24 minutes. C18 mobile phase: A = 0.075% TFA in 5% MeCN. B = 0.075% TFA in 95% MeCN. Gradient: Linear 0 to 25% B over 8 minutes. Then to 29% B over 24 minutes. Peaks: 1. Aβ(1-40); 2. Aβ(6-40); 3. uncut fusion.

For Aβ(1-42), purification on silica-based reversed-phase columns is problematic due to low solubility, strong retention, and a tendency to aggregate. These problems can be overcome by raising the temperature to 80°C in the mildly acid mobile phase, but high temperature makes the method difficult to scale up for preparative separations. Alternatively, pH above neutrality reduces retention and helps prevent aggregation, but high-pH mobile phases are detrimental to silica-based columns.

A Vydac 259VHP54 polymer-based reversed-phase column provides the answer, by virtue of its resistance to high pH. Separation with good yields can be performed at pH 8 and either 60°C or 40°C (Fig. 2). Polymer-based 259VHP columns also have the advantage that they can be cleaned by more aggressive washing solutions.

Finally, the reversed-phase separation on 259VHP can be scaled up to a 22mmID preparative column. A sample load of 15 mg total protein on that column resulted in a yield of 2 mg of purified Aβ(1-42) protein (Fig. 3). Purity of final product was verified by MALDI/TOF mass spectrometry with each product yielding a single peak of the correct molecular weight.

Purified Aβ proteins contain a methionine at position 35 that can become oxidized under certain conditions to yield a sulfoxide form which bears one additional negative charge. The Vydac 259VHP54 column is also able to separate the sulfoxide form from the native form of Aβ(1-42), as shown in Figure 4. (Peak identities confirmed by MS.)

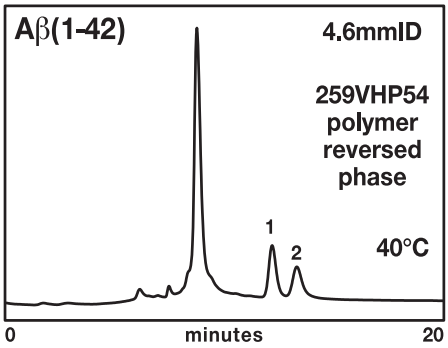

Figure 2. Separation of purified digested Aβ(1-42) fusion on polymer-based analytical reversed phase. Column: Vydac 259VHP54 5µm, 300Å, 4.6mmID x 250mmL. Conditions: 40°C, 1.0 mL/min. Mobile phase: A = 5mM potassium acetate, pH 8.0 in 5% MeCN. B = 5mM potassium acetate, pH 8.0 in 90% MeCN. Gradient: Linear 0 to 20% B over 8 minutes. Then to 26% B over 12 minutes. Peaks: 1. Aβ(1-42); 2. Aβ(6-42).

Information and chromatograms courtesy of

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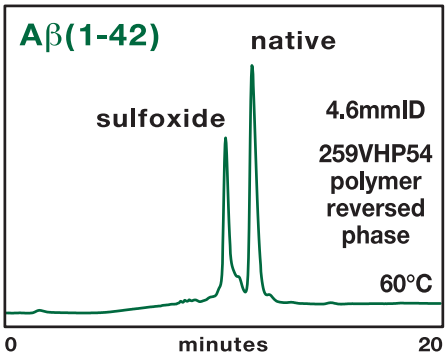

Figure 4. Separation of pure Aβ(1-42) peptide, native form and sulfoxide form on polymer-based reversed-phase. Column: Vydac 259VHP54 5µm, 300Å, 4.6mmID x 250mmL. Conditions: 60°C, 1.0 mL/min. Mobile phase: A = 0.05% TFA in 5% MeCN. B = 0.05% TFA in 90% MeCN. Gradient: Linear 0 to 28% B over 8 minutes. Then to 40% B over 24 minutes.

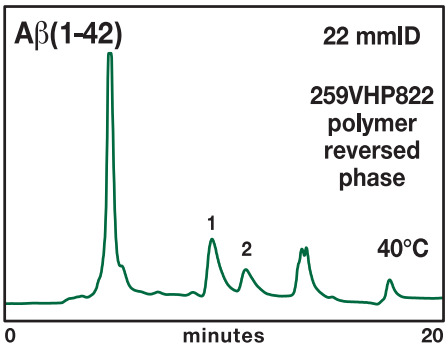

Figure 3. Separation of purified digested Aβ(1-42) fusion on polymer-based preparative reversed phase. Column: Vydac 259VHP822 8µm, 300Å, 22mmID x 250mmL. Conditions: 40°C, 15 mL/min. Mobile phase: A = 5mM potassium acetate, pH 8.0 in 5% MeCN. B = 5mM potassium acetate, pH 8.0 in 90% MeCN. Gradient: Linear 0 to 20% B over 7 minutes. Then to 25% B in 8 minutes. Peaks: 1. Aβ(1-42); 2. Aβ(6-42).

Ordering Information Call 800.347.6378

Cat.No.	Description
214MS54	Column, LC/MS, C4 Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
218MS54	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
259VHP54	Column, Polymer Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
259VHP5415	Column, Polymer Reversed Phase, 300Å, 5µm, 4.6mm ID x 150mm L
259VHP822	Column, Polymer Reversed Phase, 300Å, 8µm, 22mm ID x 250mm L

Other column sizes are available for analytical and preparative applications.