Christopher Southan and Patrick Lavery. Synergistic Scale-Down of Three Protein Micropreparation Techniques. (SmithKline Beecham Pharmaceuticals, Research and Development, the Frythe, Welwyn, Herts, AL6 9AR, U.K.)

Isolating proteins for sequencing increasingly involves working with small samples. Although a range of micropreparative procedures have been developed (for review, see Simpson *et al.*, 1989) we have achieved a further scale-down of three key techniques: size-exclusion chromatography (SEC), ultrafiltration (UF) and high-performance liquid chromatography (HPLC).

Solvent-exchange by SEC can be a low-sensitivity technique on conventional desalting columns. We packed SEC columns in glass tubes of 2 or 3 mm internal diameter (ID). These were run at flow rates of 50–300 μl/min with HPLC equipment. Reducing Tris buffer concentrations to 20 mM allowed absorbance to be measured at 214 nm, thereby lowering the protein detection limit to the nanogram range. This was useful to check solubility, salt separation, and proportional recovery before micropreparative runs at 280 nm. These were completed in a few minutes with a collected volume of 200–400 μl. The small total column volume facilitated rapid reequilibration with any buffer. Easy repacking with small amounts of soft gels meant that denaturants or detergents could be tested with a variety of packings. Proteins with precarious solubility were eluted in acidic/organic solvents for direct N-terminal sequencing and/or chemical cleavage.

After solvent exchange, protein solutions may require concentration for the next analytical or preparative step. We evaluated new commercial types of microcentrifuge UF concentrators with a membrane diameter of 10 mm to minimize protein adsorption. Their maximum volume of 400 μl is well matched to the volumes of the micro-SEC peaks collected as described above. A 30 kD cut-off polysulphone membrane unit was tested with a 55 kD enzyme and a 68 kD standard protein. Activity assay and micro-HPLC peak measurements (see below) showed recoveries of 90% with volume reduction down to 50 μl. Standard peptide solutions passed through this membrane quantitatively. These units could also be used for concentration and/or partial solvent exchange with protein collected from RP-HPLC solvents.

The third major scale-down involved the use of home-packed glass HPLC columns with IDs of 0.96 down to 0.45 mm (Southan, 1989). In addition to conventional HPLC packings, these were packed with a range of new large-pore material (Poros), which allows high flow velocities (Afeyan *et al.*, 1990). The glass microcolumns could be prepared in minutes. They combined the advantages of high detection sensitivity (1–2 pmol of a 68 kD protein), flow rates compatible with conventional HPLC instruments (>100 μl/min), short gradient times (1–10 min), disposability, and improved protein recoveries from the nonsilica packing. After loading 70 μg of an enzyme preparation on a 0.65 × 10 mm Poros Q-type anion exchanger, 80% of the activity was recovered in a 10-min gradient run. An orthogonal analysis of protein quantity and purity, using only 10% of each active fraction, was rapidly completed using a 0.65 mm ID RP-type Poros column. The HPLC peak areas at 205, 214, or 280 nm were used for comparative protein assay at any stage in sample handling. Calibrating any column with a known standard provided reliable estimates of total protein.

An example of synergy between the methods described is their combined use at the low microgram level for protease screening trials. Using the 3 mm ID SEC column, substrate protein was equilibrated into the digestion buffer. The total amount was estimated by subsequent injection of a standard protein, and recoveries were checked by micro-RP-HPLC of the injected and collected samples. If concentration was necessary, the microcentifuge UF unit was used and recoveries were monitored by micro-RP-HPLC analysis of both filtrate and eluate. The same analysis was informative on the amount and purity of commercial proteases (e.g., we consistently observed two major RP-HPLC peaks in a commercial Arg-C preparation). The proteases were also solvent-exchanged on the SEC column to cross-check their amounts and to remove storage buffer salts or low MW contaminants before mixing with substrate protein. Peak heights and dilution factors were used to produce final enzyme/substrate ratios and concentrations with greater consistency than using classical protein assays and/or manufacturers' nominal estimates of enzyme amounts. Small-scale control digests of standard proteins could be rapidly prepared to check the efficacy of any protease/buffer combination before, or in parallel with, the unknown sample. Using Poros RP-HPLC columns of 0.65 or 0.45 mm ID and detection at 205 nm, the cleavage of substrate into peptides was monitored with nanogram amounts of protein. The short analysis times allowed multiple digests, together with protease controls, to be screened and peptide collection runs to be completed within 1 day, thereby avoiding overnight incubations.

Combining the scaled-down methods described with other techniques, such as preparative capillary electrophoresis (Camilleri *et al.*, 1991), should offer increased flexibility and lower sample handling limits for all types of micropreparative protein chemistry.

References

Afeyan, N. B., Gordon, N. F., Maszaroff, L., Varady, L., Fulton, S. P., Yang, Y. B., and Regnier, F. E. (1990). *J. Chromatogr.* **519**, 1–29.

Camilleri, P., Okafo, G. N., Southan, C., and Brown, R. (1991). *Anal. Biochem.* **198**, 36–42.

Simpson, R. J., Moritz, R. L., Begg, G. S., Rubira, M. R., and Nice, E. C. (1989). *Anal. Biochem.* **177**, 221–236.

Southan, C. (1989). *In* Hugli, T. E. (ed), *Techniques in Protein Chemistry* (Academic Press, San Diego), pp. 392–398.