

# Enhancement of High-Performance Liquid Chromatography of Soybean Proteins by Addition of Sodium Dodecyl Sulfate<sup>1</sup>

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Soybean proteins have been separated by reversed-phase high-performance liquid chromatography (RP-HPLC) (Ashoor and Stiles 1987; Parris and Gillespie 1988; Peterson and Wolf 1988; Buehler et al 1989a,b), primarily on wide-pore (300 Å) C<sub>18</sub> or C<sub>4</sub> packings eluted with water and acetonitrile gradients containing small percentages of trifluoroacetic acid. We have used this method on a preparative scale to separate sufficient quantities of soybean glycinin components for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) identification (Peterson and Wolf 1988). In succeeding studies, methods were investigated to increase resolution of RP-HPLC to yield more peaks for varietal determinations and to give separations at least as good as those achieved by SDS-PAGE.

Several reports (Hancock et al 1978, Takagi 1981, Bietz 1983, Wetlaufer and Koenigbauer 1986) indicate that incorporation of low levels of SDS in elution solvents has enhanced separations of proteins and peptides by ion pairing or surfactant mediation. Micellar chromatography (Dorsey 1987, Khaledi 1988) has also been explored and found to be useful for certain biological separations. We chose to incorporate low levels of SDS to eliminate difficulties of column plugging and micellar peaks while retaining the beneficial effects of higher resolution. We describe here the enhancement of soybean protein separations by SDS-RP-HPLC.

## MATERIALS AND METHODS

The chromatographic system was composed of M6000A and M45 pumps (Waters Chromatography Div., Millipore Corp., Milford, MA), a 660 solvent programmer, a WISP 710A automatic injector, a V<sup>4</sup> variable wavelength detector (Isco, Lincoln, NE), an Omniscrite recorder (Houston Instrument, Austin, TX), and a central ModComp 32/85 computer system (Modular Computer Systems, Inc., Fort Lauderdale, FL). Separations were performed at 60°C on a 218TP54 column (C<sub>18</sub>, 5-μm particle diameter, 250 × 4.6 mm i.d.) (Vydac/The Separations Group, Hesperia, CA). An RP-2 cartridge (C<sub>2</sub>), 30 × 4.6 mm i.d. (Brownlee Labs, Santa Clara, CA), served as a guard column.

HPLC-grade acetonitrile was obtained from several suppliers. Distilled water was treated with a Nanopure system (Barnstead, Boston, MA) before use. HPLC/Spectro grade trifluoroacetic acid was purchased from Pierce (Rockford, IL), Type I-S trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO), and electrophoresis purity grade SDS from Bio-Rad Laboratories (Richmond, CA).

Soybean protein fractions were processed from hexane-defatted flakes of Raiden soybeans as described by Wolf et al (1966). The procedure provided samples of water solubles, cold solubles, cold insolubles, whey, and acid-precipitated protein fractions. Also, glycinin (11S) and β-conglycinin (7S) were prepared by the method of Thanh and Shibasaki (1976) as described by Peterson and Wolf (1988). Preparations were dialyzed against

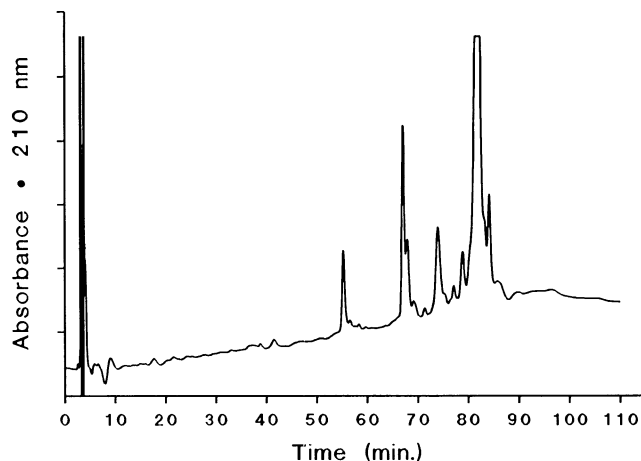
distilled water and freeze-dried before reconstitution to 5 mg/ml with potassium phosphate-sodium chloride buffer, pH 7.6, 0.5 ionic strength (Wolf and Briggs 1956).

A 90-min gradient of 20–45% acetonitrile in H<sub>2</sub>O (both containing 0.1% trifluoroacetic acid) was used as the reference method for soy protein separations. The column was equilibrated for 20 min with 20% acetonitrile prior to injection and was eluted with 45% acetonitrile for 20 min following the gradient, yielding a 110-min chromatogram. Effluent was monitored at 210 nm (0.2 AUFS) and the detector output plotted and transmitted simultaneously to the computer for data storage and later graphic representations.

Studies with SDS-enhanced RP-HPLC separations were conducted with solvents modified by addition of 0.05% SDS. SDS was added to each solvent in an aqueous 10% solution; therefore, the acetonitrile also contained 0.5% water. Solvents were vigorously helium-sparged for 5 min prior to addition of SDS and then were held under a low (1–2 psig) positive helium atmosphere during chromatography. The 90-min, 20–45% acetonitrile gradient used in the absence of SDS was changed to 20–60% when SDS was present, with equilibration and final hold times as previously noted. All components were eluted during the 110-min runs, as evidenced by the lack of additional peaks eluted by a further 10-min 80% acetonitrile column wash after each run.

## RESULTS AND DISCUSSION

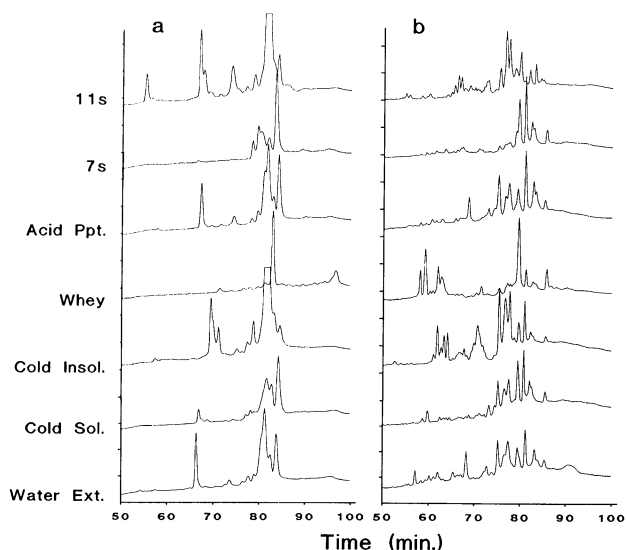
Our reference method, as noted above, routinely yielded chromatograms of soybean proteins as shown in Figure 1. This procedure was successfully scaled up to injections of 200 mg on larger C<sub>18</sub> columns to provide sufficient quantities of individual components for characterization (Peterson and Wolf 1988). SDS-PAGE electropherograms of glycinin and fractions chromatographically derived from it exhibited many bands not satisfactorily resolved with the original RP-HPLC non-SDS solvent-gradient combination (Peterson and Wolf 1988).



**Fig. 1.** Reversed-phase high-performance liquid chromatogram of glycinin from Raiden soybeans. A Vydac 218TP54 column was eluted at 60°C and 1 ml/min with a linear 20–45% aqueous acetonitrile (with 0.1% trifluoroacetic acid) gradient for 90 min, then held for 20 min at 45% acetonitrile.

<sup>1</sup>Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or products not mentioned.

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**Fig. 2.** Reversed-phase high-performance liquid chromatograms of protein fractions from Raiden soybeans. **a**, Vydac 218TP54 column eluted at 1 ml/min with a linear 20–45% aqueous acetonitrile (with 0.1% trifluoroacetic acid) gradient for 90 min, then held for 20 min.; total time, 110 min at 60°C. **b**, Same conditions, except that a 20–60% aqueous acetonitrile (with 0.1% trifluoroacetic acid and 0.05% sodium dodecyl sulfate) gradient was used.

Soybean protein chromatograms of 100- $\mu$ g injections obtained without and with SDS-containing solvents are compared in Figure 2. Although some minor components elute in the 0–50-min region, only retention times of 50–95 min are shown since most significant peaks of these preparations occur here. Individual chromatograms are normalized to provide full-scale deflection for the largest peak. Particularly noteworthy results are apparent in the two chromatograms of water solubles ( $H_2O$  extract) from defatted soybean flakes. We found more than twice the number of component peaks in solvents with SDS than without SDS, which is most important, since all other fractions originate from this water extract. Similar improvements in resolution were evident in all other preparations. This should be helpful in varietal differentiation or preparative isolation of individual peaks.

The increase in number of peaks in the whey protein chromatogram with SDS is partially the result of disaggregation of some of the protein components. A solution of whey proteins in buffer was turbid but cleared when 0.05% SDS was added. Also, in preparative non-SDS RP-HPLC of the whey proteins (*unpublished results*), we occasionally encountered plugging of the columns, suggesting that some of the proteins bound irreversibly to the column and caused stoppage. Presumably, SDS prevents such binding and gives rise to some of the additional peaks noted in the chromatogram.

Some early experimentation indicated that approaching the critical micellar concentration (0.0081 M) impaired the separations achieved here and retarded peak elution. In fact, even a 20–80% acetonitrile gradient, when enhanced with 0.25% SDS, failed to elute the soybean proteins. The acetonitrile concentration was then adjusted to 20–60% in solvents containing 0.05% SDS so that peaks would elute in the same area as those from non-SDS RP-HPLC of the same preparations. Higher levels of SDS also may preclude the use of helium sparge during chromatography because of severe foaming. In experiments with glycinin, 0.025% SDS gave slightly better resolution than 0.05%; however, the latter concentration was selected to increase the probability of solubilization of various soybean protein samples. Though the level of SDS used in these experiments is approximately 21% of the critical micelle concentration, the observed enhancements in HPLC resolution are unlikely to be caused by micelle formation and subsequent interaction with protein and stationary phase. Separation improvements with these conditions may be attributed

to the ion-pairing properties of SDS and the increased affinity of the SDS-soybean protein moiety for the  $C_{18}$  chain. This would explain the need for an increase in the percentage of acetonitrile necessary to completely elute the proteins.

With regard to the observation by Ameer (1987) of a short-term (six injections) retention time reduction in SDS-RP-HPLC determinations of bumetanide, our times remained relatively constant during more than 200 runs. Some variation in retention time was apparent during initial runs with new columns; however, after three to six injections, retention times became reproducible for a given solvent delivery system. Solvent age, as well as differences between pumping and autosampler systems, can also cause retention-time variability. A solution of trypsin inhibitor was injected periodically for estimation of a column's health. This trypsin inhibitor standard gave a mean retention time of 83.6 min (SD, 1.8;  $n$ , 11). Eventually, column degradation was apparent, as indicated by a loss of resolution coupled with increased retention times, and columns were replaced when chromatographic results were no longer acceptable. Since an exact match in phase selectivity to the Vydac column could not be assured with the Brownlee guard column system, a  $C_2$  (instead of  $C_{18}$ ) bonded phase was selected to appear transparent to the separations yet provide some degree of filtration.

This SDS-HPLC system has been used extensively and successfully in succeeding studies of extraction methods for protein solubilization and trypsin inhibitor characterization and detection.

#### LITERATURE CITED

- AMEER, B. 1987. Micellar chromatography usefulness may be limited. *Chromatography* 2:37.
- ASHOOR, S. H., and STILES, P. G. 1987. Determination of soy protein, whey protein, and casein in unheated meats by high-performance liquid chromatography. *J. Chromatogr.* 393:321-328.
- BIETZ, J. A. 1983. Separation of cereal proteins by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 255:219-238.
- BUEHLER, R. E., McDONALD, M. B., VAN TOAI, T. T., and ST. MARTIN, S. K. 1989a. Soybean cultivar identification using high performance liquid chromatography of seed proteins. *Crop Sci.* 29:32-37.
- BUEHLER, R. E., VAN TOAI, T. T., and McDONALD, M. B. 1989b. The optimization of elution and extraction conditions for the separation of soybean proteins using RP-HPLC. *Seed Sci. Technol.* 17:193-204.
- DORSEY, J. G. 1987. Micellar mobile phases for liquid chromatography: Academic research curiosity or useful alternatives? *Chromatography* 2:13-20.
- HANCOCK, W. S., BISHOP, C. A., MEYER, L. J., HARDING, D. R. K., and HEARN, M. T. W. 1978. High-pressure liquid chromatography of peptides and proteins. VI. Rapid analysis of peptides by high-pressure liquid chromatography with hydrophobic ion-pairing of amino groups. *J. Chromatogr.* 161:291-298.
- KHALEDI, M. 1988. Micellar reversed phase liquid chromatography. *BioChromatography*. 3:20-35.
- PARRIS, N., and GILLESPIE, P. J. 1988. HPLC separation of soy and beef protein isolates. Pages 142-155 in: *Methods for Protein Analysis*. J. P. Cherry and R. A. Barford, eds. American Oil Chemists Society: Champaign, IL.
- PETERSON, R. E., and WOLF, W. J. 1988. Scale-up from analytical to preparative reversed-phase high-performance liquid chromatography of soybean glycinin. *J. Chromatogr.* 444:263-268.
- TAKAGI, T. 1981. High-performance liquid chromatography of protein polypeptides on porous silica gel columns (TSK-GEL SW) in the presence of sodium dodecyl sulphate: Comparison with SDS-polyacrylamide gel electrophoresis. *J. Chromatogr.* 219:123-127.
- THANH, V. H., and SHIBASAKI, K. 1976. Major proteins of soybean seeds. A straightforward fractionation and their characterization. *J. Agric. Food Chem.* 24:1117-1121.
- WETLAUFER, D. B., and KOENIGBAUER, M. R. 1986. Surfactant-mediated protein hydrophobic-interaction chromatography. *J. Chromatogr.* 359:55-60.
- WOLF, W. J., and BRIGGS, D. R. 1956. Ultracentrifugal investigation of the effect of neutral salts on the extraction of soybean proteins. *Arch. Biochem. Biophys.* 63:40-49.
- WOLF, W. J., SLY, D. A., and KWOLEK, W. F. 1966. Carbohydrate content of soybean proteins. *Cereal Chem.* 43:80-94.

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