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# SCALE-UP FROM ANALYTICAL TO PREPARATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SOYBEAN GLYCININ

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## SUMMARY

Analytical (50 μg) reversed-phase high-performance liquid chromatography (RP-HPLC) of fractions from soy protein purifications has been scaled up to injections in the preparative (1–200 mg) range to obtain sufficient quantities of glycinin subunits for sodium dodecyl sulfate-polyacrylamide gel electrophoresis characterization. We describe here the glycinin separations obtained by using analytical, semi-preparative, and preparative columns with an analytical gradient HPLC system. Parameters such as injection volume, flow-rate, and detector sensitivity are noted. Our results suggest that analytical RP-HPLC separations of proteins can be conveniently scaled up in most HPLC systems.

## INTRODUCTION

Cereal[1–5] and soy[6,7] protein preparations have been characterized by reversed-phase high-performance liquid chromatography (RP-HPLC) in which various packings (300 Å pore size) are used with gradient systems of water and acetonitrile modified with trifluoroacetic acid. During our studies of storage proteins, we have also used RP-HPLC to characterize samples from soybean protein fractionations. Analytical RP-HPLC profiles were established initially for each fraction from the purification scheme. In order to characterize the various peaks in the chromatograms by techniques such as gel electrophoresis, scale-up was required to obtain milligram quantities of samples. Since the two major components (glycinin and β-conglycinin) of soybean protein comprise approximately 70% (ref. 8) of the seed protein, we selected glycinin to illustrate the scale-up achievable by using an analytical HPLC system and a series of wide-pore (300 Å) reversed-phase columns. The profiles indicate that analytical protein separations can be directly scaled up to preparative quantities by increasing injection volumes and desensitizing the detector to prevent apparent column overload.

## MATERIALS AND METHODS*

### *Apparatus*

The chromatographic systems were: (1) Waters Chromatography Div. (Millipore, Milford, MA, U.S.A.): 6000 A and M45 pumps; 660 solvent programmer; WISP 710A automatic injector; Isco (Lincoln, NE, U.S.A.) $V^4$ variable-wavelength detector; Houston Instrument (Austin, TX, U.S.A.) Omniscribe recorder; and a central ModComp (Modular Computer Systems, Fort Lauderdale, FL, U.S.A.) 32/85 computer system; and (2) Spectra-Physics, Autolab Div. (San Jose, CA, U.S.A.) 8100XR automated liquid chromatograph; Hewlett-Packard (Palo Alto, CA, U.S.A.) 8450A diode array UV–VIS spectrophotometer; LDC/Milton Roy (Riviera Beach, FL, U.S.A.) SpectroMonitor D variable-wavelength detector; Houston Omniscribe recorder; and ModComp 32/85 computer system. The Valco Instrument, Houston, TX, U.S.A.) injector was fitted with 20-$\mu$l, 5-ml, and 10-ml injection loops for the 100-$\mu$l, 2-ml and 8-ml injection volumes. The smaller loops could be accommodated by the column oven; however, the 10-ml loop was connected externally with 6 in. × 0.04 in. I.D. stainless-steel tubing.

### *Columns*

Separations were performed at 60°C on the following columns: Vydac (Vydac/The Separations Group, Hesperia, CA, U.S.A..) 218TP54 (250 × 4.6 mm I.D.), Vydac 218TP510 (250 × 10 mm I.D.) and a Rainin Instrument (Woburn, MA, U.S.A.) Dynamax-300 Å (250 × 21.4 mm I.D.). All columns contained reversed-phase ($C_{18}$) packings having 300 Å pores. A Brownlee Labs. (Santa Clara, CA, U.S.A.) RP-2 cartridge column (30 × 4.6 mm I.D.) served as a guard column.

### *Solvents*

HPLC-grade acetonitrile was obtained from various suppliers (Fisher Scientific, Springfield, NJ, U.S.A., J. T. Baker, Phillipsburg, NJ, U.S.A. and EM Science, Cherry Hill, NJ, U.S.A.) while trifluoroacetic acid (TFA) (Sequanal grade) was obtained from Pierce (Rockford, IL, U.S.A.) Distilled water was treated with a Barnstead (Boston, MA, U.S.A.) NANO pure system before use. Solvents were helium-sparged and held under a low (1–2 p.s.i.g.) positive helium atmosphere during chromatography.

### *Chromatographic conditions*

A 90-min gradient of 20 to 45% acetonitrile in water (both containing 0.1% TFA) was used throughout these studies. The columns were equilibrated for 20 min at the 20% acetonitrile level prior to injection, and the 45% acetonitrile level was maintained for 20 additional min, yielding a chromatogram of 110 min duration. Flow-rates were 1, 3 and 8 ml/min for the 4.6, 10 and 21.4 mm I.D. columns, respectively. Effluents were monitored serially, first at 210 nm (0.2–2.0 a.u.f.s.), and then with the diode-array detector, which scanned 200–800 nm every 10 s. Detector

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outputs were transmitted simultaneously to the ModComp computer for subsequent graphic representations.

*Polyacrylamide gel electrophoresis*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a Hoefer Scientific Instruments (San Francisco, CA, U.S.A.) Model SE 600 unit on 1.5-mm, 12% polyacrylamide gels with an acrylamide:bis ratio of 36:1. Buffers were as described in the Fling and Gregerson[9] modification of the Laemmli[10] procedure. Gels were stained with Coomassie Brilliant Blue R-250[9] (Sigma, St. Louis, MO, U.S.A.).

*Preparation of protein fractions*

Hexane-defatted flakes of Raiden soybeans were used to prepare the various protein fractions. Water-extractable, cold-soluble, cold-insoluble, whey and acid-precipitated protein fractions were isolated as described elsewhere[11]. All preparations were dialyzed against distilled water and freeze-dried.

Glycinin and β-conglycinin were prepared by the method of Thanh and Shibasaki[12]. A pH 8.0, 0.03 *M* Tris–HCl extract (containing 10 m*M* 2-mercaptoethanol) of flakes was adjusted to pH 6.4, cooled to 3–5°C overnight and centrifuged in the cold. The resulting precipitate, crude glycinin, was washed with 0.03 *M* Tris–HCl (pH 6.4), dissolved in pH 7.6, 0.5 ionic strength potassium phosphate–sodium chloride buffer containing 0.4 *M* NaCl, 33 m*M* $K_2HPO_4$, 2.6 m*M* $KH_2PO_4$, 0.02% $NaN_3$ and 10 m*M* 2-mercaptoethanol, dialyzed against distilled water, and freeze-dried to yield glycinin. The supernatant remaining after removing crude glycinin from the pH 6.4, Tris–HCl extract was adjusted to pH 4.8 to precipitate crude β-conglycinin. The crude β-conglycinin was dispersed in 0.03 *M* Tris–HCl (pH 7.6), adjusted to pH 6.2 and centrifuged to remove polymerized β-conglycinin. The supernatant was dialyzed against water and freeze-dried to yield β-conglycinin.

For RP-HPLC separations, samples were weighed and dissolved in an appropriate volume of pH 7.6, 0.5 ionic strength potassium phosphate–sodium chloride buffer.

## RESULTS

*Analytical profiles of soybean protein fractions*

Fig. 1 shows analytical chromatograms of protein fractions obtained from Raiden soybeans. Although our primary interest was in the 40- to 90-min region of elution (depending on the chromatographic system), significant peaks also occurred in other areas, necessitating the 0.28% acetonitrile/min gradient. Steeper gradient slopes or higher initial acetonitrile concentrations shortened retention times but degraded resolution. Analyses on the Waters system showed a retention time delay of 8–10 min compared to the Spectra-Physics system, related to the injection loop and associated tubing which delayed the gradient.

*Scale-up of glycinin RP-HPLC separation*

The RP-HPLC experiments shown (in part) in Fig. 2 demonstrate chromatographic resolution achieved with increasing sample loads and column diameters. A

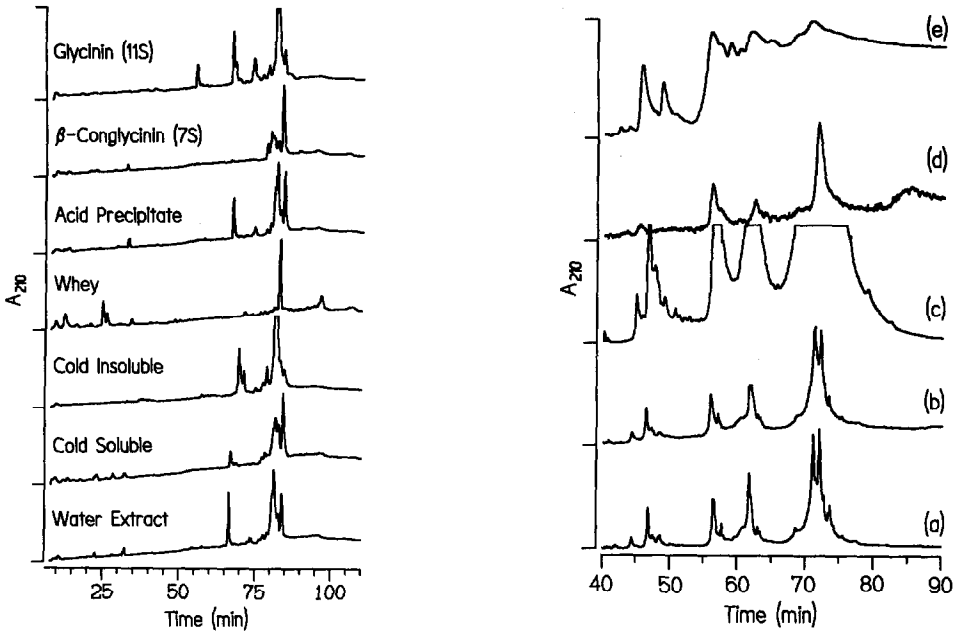


Fig. 1. RP-HPLC chromatograms of protein fractions from Raiden soybeans obtained with a Waters system. A Vydac 218TP54 column was eluted at 1 ml/min with a linear 20–45% acetonitrile gradient in water (with 0.1% TFA) for 90 min, then held for 20 min. Total time: 110 min at 60°C.

Fig. 2. Elution profiles from a Spectra-Physics-based system of soybean 11S proteins on (a) Vydac 218TP54, 10 μl (50 μg), 1 ml/min; (b) Vydac 218TP510, 10 μl (50 μg), 3 ml/min; (c) Vydac 218TP510, 2 ml (10 mg), 3 ml/min; (d) Dynamax-300 Å, 20 μl (100 g), 8 ml/min; (e) Dynamax-300 Å, 8 ml (200 mg), 8 ml/min. Other conditions as in Fig. 1.

typical 50-μg sample of glycinin, separated on an analytical column in the Spectra-Physics 8100XR apparatus, is shown in Fig. 2a. Fig. 2b shows an almost identical pattern for a sample of the same size, but with the column diameter increased to 10 mm. Effects of detector and data acquisition overload are noted in Fig. 2c (10 mg load), but peak integrity was maintained. A 100-μg load on a 21.4 mm I.D. column exhibited effects of dilution (Fig. 2d), but separation of major peaks was still acceptable, even though the doublet at 71–72 min was unresolved. When 200 mg (4000 × scale-up) was applied, detector overload was quite apparent (Fig. 2e): peaks visually overlapped from 55 to 80 min at 210 nm.

*Effect of detection wavelengths*

The output of the diode-array spectrophotometer at 210 nm (Fig. 3a) becomes saturated at 2 absorbance units during scans 350–540 (corresponding to 58.3–90 min). However, a closer look at scans 340–390 (56.7–65 min) (Fig. 4) using the ModComp 3-D program shows much more clearly the separation achieved. Changing the detection wavelength from 210 to 230 nm caused the resulting chromatogram (Fig. 3b) to approximate more closely that obtained with a 100-μg sample (Fig. 2d). The SDS-PAGE patterns (Fig. 5) of fractions 8 and 9, collected in the 340- to 390-scan

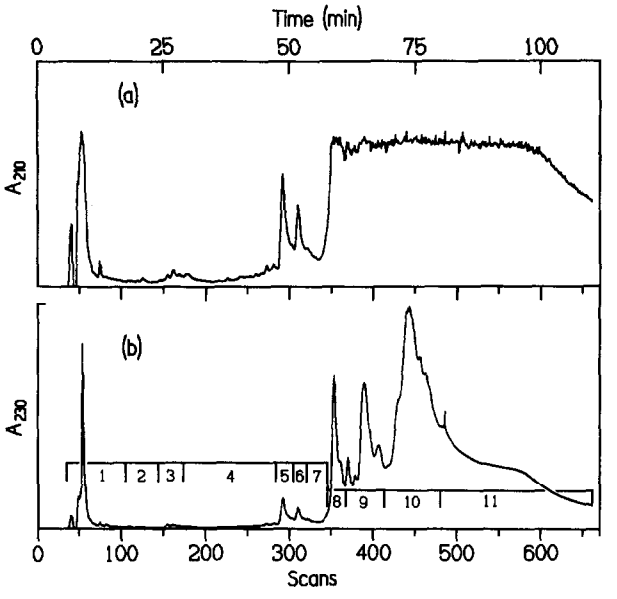


Fig. 3. Diode-array spectrometer single-wavelength chromatograms of glycinin corresponding to Fig. 2e. at (a) 210 nm, (b) 230 nm. Delineated area of scans 340–390 is shown three-dimensionally in Fig. 4. SDS-PAGE patterns of fractions 1–11 are shown in Fig. 5.

range (57–65 min), confirmed a marked difference in subunit composition. Such fractions may be re-chromatographed to provide higher purity for later studies. Fig. 5 also shows compositions of other fractions (1–7 and 10–11), obtained in the same run. Fractions 1–7 were very small but distinctly different. Fractions 10–11 were the largest ones and appeared to contain the acidic and basic polypeptides of glycinin plus the $\beta$-subunit of $\beta$-conglycinin, which is a contaminant of crude glycinin.

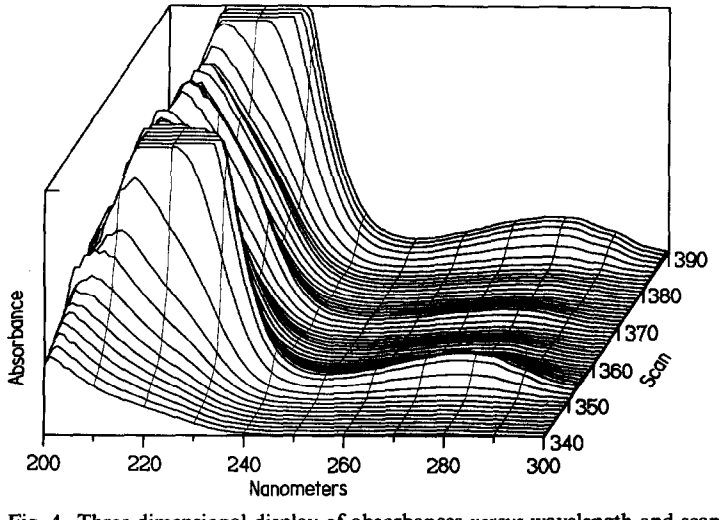


Fig. 4. Three-dimensional display of absorbances *versus* wavelength and scan (time) of glycinin. Conditions are as in Fig. 2e.

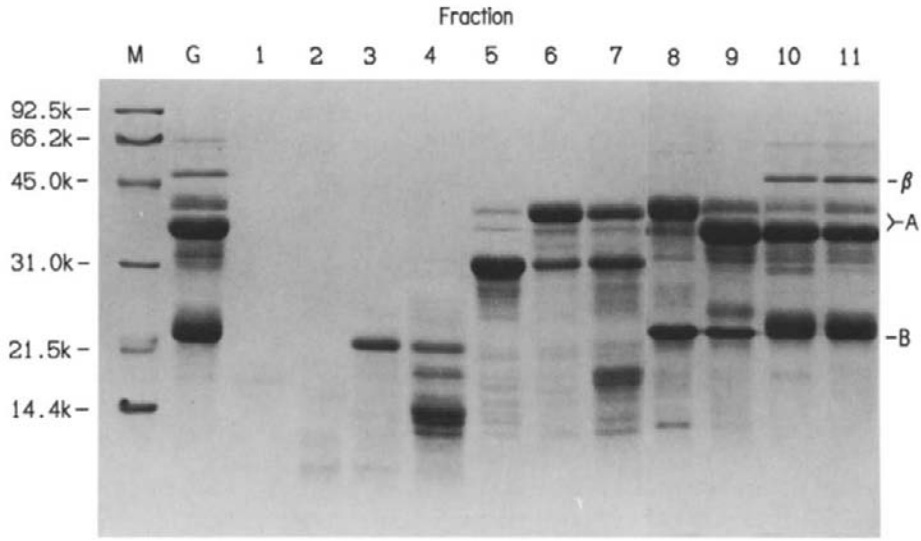


Fig. 5. SDS-PAGE (12% T and 2.7% C) of fractions 1–11 from RP-HPLC of glycinin (from Fig. 3b). M is a molecular weight standard mixture, G is glycinin before RP-HPLC fractionation, $\beta$ is the $\beta$-subunit of $\beta$-conglycinin, and A and B are, respectively, acidic and basic polypeptide chains of glycinin.

## CONCLUSION

We have demonstrated the feasibility of using an analytical chromatograph to provide quantities of specific peaks from a rather complex glycinin matrix needed for SDS-PAGE elucidation. Since most characterization studies benefit from having sufficient amounts of material to allow duplicate or multiple procedures, availability of larger samples increases the probability of success.

## ACKNOWLEDGEMENT

M. L. Schaer conducted the SDS-PAGE analysis.

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