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High-performance size-exclusion chromatography of bovine somatotropin*

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High-performance size-exclusion chromatography (HPSEC) has been applied by numerous researchers to the separation of proteins[1–7]. Recent improvements in column packing have provided very efficient columns[8–10].

Development of a process for producing bovine somatotropin (bSt) in *E. coli* modified by recombinant DNA techniques (rbSt) prompted a need for a HPSEC method for estimation of levels of high-molecular-weight protein components in rbSt preparations. This report summarizes a survey of HPSEC conditions studied.

EXPERIMENTAL

Materials

Water was distilled and deionized. Analytical-grade reagents (Fisher Scientific, Pittsburgh, PA, U.S.A.) were used throughout. Ultrapure guanidine hydrochloride (GnHCl) (Schwartz/Mann, Cambridge, MA, U.S.A.) and sodium dodecylsulfate (SDS) (Bio-Rad, Richmond, CA, U.S.A.) were used as denaturing agents. Molecular weight standards were obtained from Sigma (St. Louis, MO, U.S.A.), rbSt samples from Upjohn sources and pituitary bSt (pbSt) from A. F. Parlow (Harbour Medical Center, UCLA, Los Angeles, CA, U.S.A.).

Chromatography

Modular HPLC systems consisting of combinations of the following components were used (see figure captions for other details). Columns included DuPont GF-250 (DuPont, Wilmington, DE, U.S.A.), TSK 3000SW (Toyo Soda, Tokyo, Japan) and Waters I-125 (Waters Assoc., Milford, MA, U.S.A.). A Varian Vista 5500 HPLC system (Varian, Walnut Creek, CA, U.S.A.) or a Perkin-Elmer Series 10 pump (Perkin Elmer, Norwalk, CT, U.S.A.) were used. Detection was accomplished using an LDC 1203 UV monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) with a 214-nm kit or 280-nm filter. Samples and standards were prepared in mobile phase typically at 1.0 mg/ml with subsequent 100-μl injection using a Rheodyne 7125 valve (Rheodyne, Cotate, CA, U.S.A.). Chromatographic data was collected and

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analyzed on an in-house VAX-based computer system (Digital Equipment, Merrimack, NH, U.S.A.).

RESULTS AND DISCUSSION

Two classes of high-molecular-weight protein components were of concern. First, oligomeric or aggregated forms of bSt may be present in bSt preparations due to covalent or non-covalent interactions of bSt with itself[7,11–12]. Secondly, proteins from the host bacteria used for rbSt production[13] or bovine proteins from the purification of pbSt[11] may be present as process impurities. We chose HPSEC columns designed to separate in the range of about 5000–150 000 molecular weight[14–16], to examine bSt [molecular weight = 21 816 (ref. 17)] for the presence of dimeric, trimeric and oligomeric bSt species in particular. Our studies focused mainly on the use of HPSEC systems which utilize mobile phases containing denaturing agents[3,7–9]. The column/mobile phase combinations were; GF-250/6 *M* GnHCl (Fig. 1), GF-250/0.1% SDS (Fig. 2), TSK 3000SW/6 *M* GnHCl (Fig. 3), and I-125/0.1% SDS (Fig. 4). Each of these systems separate dimer and higher-molecular-weight oligomers from bSt monomer. The GF-250 column proved to be the most efficient with about 4200 plates calculated for rbSt monomer using the band width at half-height method[18]. The TSK 3000SW and Waters I-125 column exhibited theoretical plate values for rbSt monomer of 3700 and 2900, respectively. We use the GF-250 column with either GnHCl or SDS mobile phases for routine studies of rbSt based on this superior peak shape and resolution. Coupling two GF-250 columns provided slightly better resolution of bSt oligomers from bSt monomer at the expense of doubling the separation time (*cf.* Figs. 1 and 2).

We also attempted to use the GF-250 column with a simple, non-denaturing buffer mobile phase in an attempt to study bSt in its native conformation[13]. Oligomers were also resolved on this system (Fig. 5), but significant portions of the rbSt samples did not dissolve in this buffer. Dilute ammonia was used to prepare samples

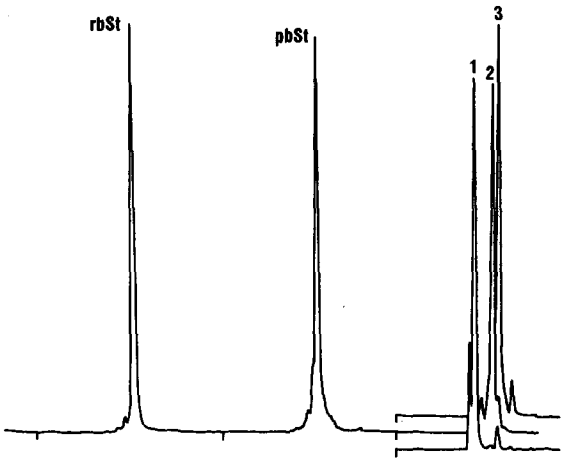

Fig. 1. Chromatograms obtained on one DuPont GF-250 column. Mobile phase, 6*M* GnHCl, 0.2 *M* sodium phosphate, pH 7.0; detection, 214 nm; flow-rate, 2.0 ml/min. 1 = Bovine serum albumin; 2 = trypsinogen; 3 = ribonuclease A. The retention times of peaks 1, 2, bSt and 3 were 3.40, 4.04, 4.32 and 4.40 min respectively.

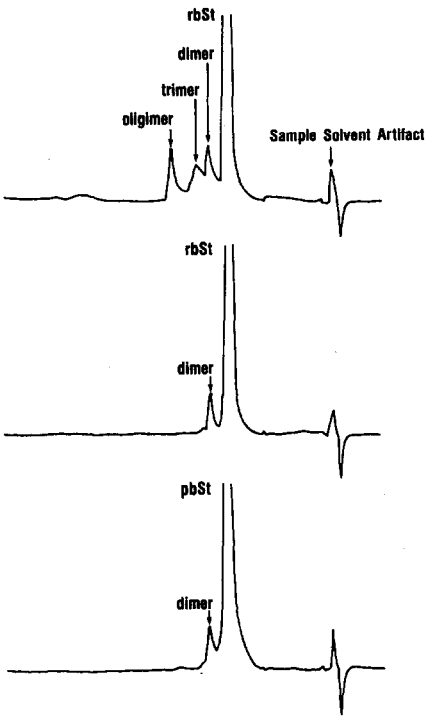

Fig. 2. Chromatograms obtained on two DuPont GF-250 columns in series. Mobile phase: 0.1% SDS, 0.1 *M* NaCl, 0.067 *M* monobasic sodium phosphate, 0.3 *M* Gly at pH 7.5; detection: 280 nm; flow-rate: 1.0 ml/min. The retention times of oligomer, trimer, olimer and bSt were 11.9, 13.9, 14.9 and 16.3 min respectively.

for injection onto the GF-250/SDS system, because the apparent dimer content doubled over 48 h when samples were prepared in ammonium bicarbonate buffers. Exposure of the GF-250 column to SDS required dedication of the column to use with this denaturing agent because SDS cannot be easily rinsed from the column[13].

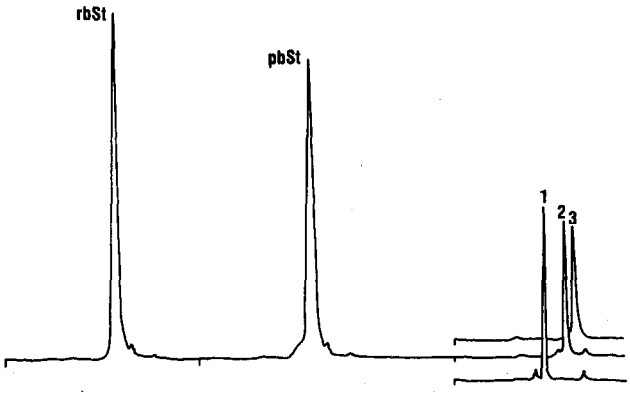

Fig. 3. Chromatograms obtained on TSK 3000SW column. Conditions as in Fig. 2. 1 = Bovine serum albumin; 2 = trypsinogen; 3 = ribonuclease A. The retention times of peaks 1, bSt, 2 and 3 were 7.45, 9.75, 9.98 and 10.88 min respectively.

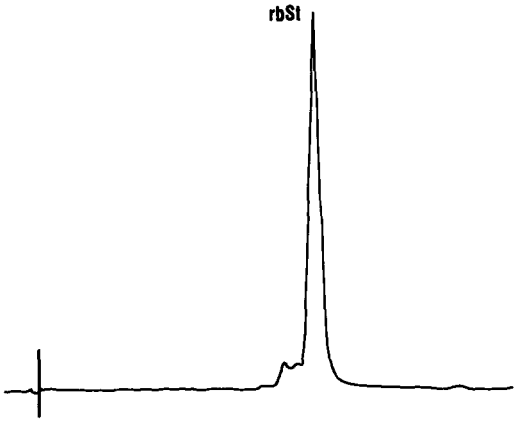

Fig. 4. Chromatograms obtained on Waters I-125 column. Conditions as in Fig. 2. The retention time of bSt was 6.5 min.

The apparent molecular weights of bSt monomer and other components were determined based on a protein standard calibration plot as shown in Fig. 6. Seven lots of bSt were examined using the GF-250/6 *M* GnHCl system and found to have apparent molecular weights ranging from 24 600 to 26 100. Precision of 0.2% relative standard deviation (R.S.D.) for molecular weight determination on five replicate injections of the same lot of material was obtained.

Recovery of bSt from the GF-250/6 *M* GnHCl system was determined to be 100% based on a comparison of 220-nm absorbance of samples before and after elution. The peak area response for bSt monomer at 214 nm for GF-250/6 *M* GnHCl and at 280 nm for GF-250/SDS was shown to be linear and to have no significant bias across the range of 0.1–1.0 mg bSt/ml in the sample preparation. Multiple in-

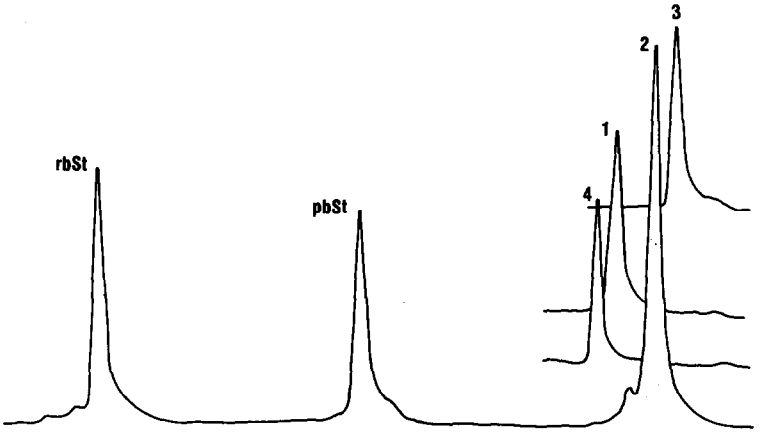

Fig. 5. Chromatograms obtained on one DuPont GF-250 column. Mobile phase: 0.2 *M* sodium phosphate, pH 6.8; detection: 214 nm; flow-rate: 1.0 ml/min. 1 = Bovine serum albumin; 2 = trypsinogen; 3 = ribonuclease A; 4 = phosphorylase A. Note: portions of bSt preparations are *not* soluble in this mobile phase. The retention times of peaks 4, 1, bSt, 2 and 3 were 6.6, 7.8, 10.1, 10.3 and 11.9 min respectively.

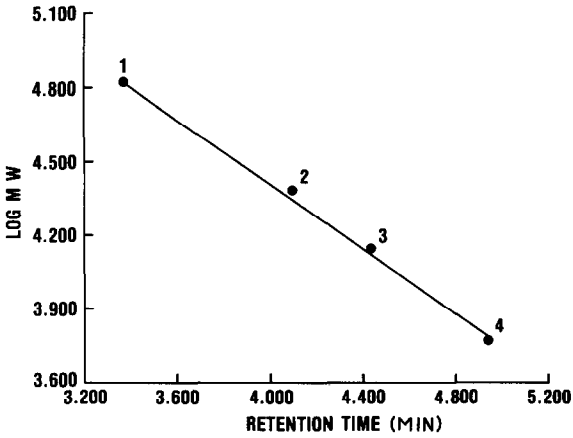

Fig. 6. Molecular weight (MW) calibration plot for DuPont GF-250 column with pH 7.0/6 *M* GnHCl mobile phase. *y*-Intercept = 7.15; slope = 0.685; correlation coefficient = 0.998. Standards: 1 = albumin (MW 66 000); 2 = trypsinogen (MW 24 000); 3 = ribonuclease A (MW 13 700); 4 = insulin (MW 5700).

jections of preparations demonstrated that the precision of quantitation of monomer was 0.5–2% R.S.D. for monomer levels of 80% (w/w) or higher and about 1.5–10% R.S.D. for dimer, trimer or oligomer levels below 20% (w/w).

CONCLUSION

HPSEC using a denaturing mobile phase and the GF-250 column provided a method for determining levels of impurities, *i.e.* non-bSt monomer, and estimating apparent molecular weights of monomer and bSt oligomers in bSt preparations.

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