Determination of the Stabilizer Sucrose in a Plasma-Derived Antithrombin Process Solution by Hydrophilic Interaction Chromatography with Evaporative Light-Scattering Detection

Göran Karlsson*, Ann-Charlotte Hinz, and **Stefan Winge**
Octapharma AB, SE-11275 Stockholm, Sweden

Abstract

Hydrophilic interaction chromatography (HILIC) is used for the quantitation of sucrose in the range of 10–100 µg/mL. A poly-2-hydroxyethylaspartamide column is eluted with 25% water–75% acetonitrile, and evaporative light scattering is utilized for detection. A process sample of antithrombin (Atenativ) from Octapharma AB (Stockholm, Sweden) containing 20% sucrose is analyzed. The precision for this high-performance liquid chromatographic method is a percent relative standard deviation (%RSD) of 4, limit of detection (s/n = 3) of 1 µg/mL, and mean recovery of spiked samples of 101% (RSD% of 3, *n* = 6). Analysis time is 10 min/sample. Glucose, fructose, sodium citrate, sodium phosphate, Triton X-100, and tri-*n*-butyl phosphate do not interfere with the method.

Introduction

The quantitation of neutral carbohydrates is often performed by high-performance liquid chromatography (HPLC) techniques. Normal-phase chromatography (NPC), using amino-bonded silica columns, are among the most used methods (1). NPC diol columns have also been used (2), but diol columns usually give a lower selectivity compared with amino columns. Other techniques used for the analysis of neutral carbohydrates include high-performance anion-exchange chromatography (HPAEC). In HPAEC, a very alkali mobile phase is used, which ionizes sugar hydroxyl groups. The carbohydrates are then bound to the anion exchange column, which is followed by elution (e.g., using a sodium acetate gradient), and a pulsed amperometric detector (PAD) is often used for detection (3). Reversed-phase chromatography (RPC) of reducing carbohydrates, after derivatization of the reducing end by attachment of a hydrophobic chromophore or a fluorophore, is a widely used technique (4,5). Cyclodextrin-bonded silica gel columns have also been used for carbohydrate analysis (6).

Hydrophilic interaction chromatography (HILIC), a variant of NPC, consists of a hydrophilic stationary phase combined with a mostly organic mobile phase, and elution is usually performed by increasing the water concentration. HILIC was introduced by Alpert in 1990 (7) and has been used for analysis of many types of carbohydrates (7,8), peptides (9), and amino acids (10).

Direct detection of carbohydrates by UV absorbance or fluorescence is usually not possible and refractive index (RI) detectors are therefore commonly used. Nevertheless, RI detectors suffer from such disadvantages as low sensitivity, noncompatibility with gradient elution, and being affected by small fluctuations in temperature and pressure. Evaporative light scattering detection (ELSD) is currently used as a semiuniversal HPLC detector because it is compatible with gradient elution and is widely used for the detection of nonvolatile compounds such as carbohydrates (1,11) and lipids (12). ELSD is based on the light scattering detection of nonvolatile compounds after nebulization and evaporation of the mobile phase.

Human antithrombin (AT) is the prime plasma serine-protease inhibitor that inhibits several proteases in the coagulation system, including thrombin and Factor Xa. It thus plays a major role in the regulation of blood coagulation. The rate of inhibition of thrombin is strongly enhanced by the binding of heparin to AT (13). The total molecular weight of AT, which is glycosylated on four amino acid residue positions, is 58 kDa (14), 15% of which consists of carbohydrates (15). AT is a recognized pharmaceutical treatment for patients with congenital AT deficiency, and has also been investigated for treatment in acquired AT deficiency conditions such as sepsis and disseminated intravascular coagulation syndrome (13,16).

Disaccharides, especially sucrose, and other polyhydroxylated compounds, such as glycerol, have been widely used as stabilizers in protein solutions (17). A combination of high concentrations of both sucrose and citrate was shown to be

* Author to whom correspondence should be addressed.

effective in stabilizing AT during the pasteurization step (18,19).

In this work, HILIC was used with ELSD detection to quantitate sucrose, which was used as a stabilizer of antithrombin during the antivirus heat treatment.

Experimental

Sucrose, sodium phosphate, and Triton X-100 were obtained from Merck (Darmstadt, Germany) and D-glucose, sodium citrate, and D-fructose (all analytical grade) were from Sigma (Munich, Germany). Acetonitrile (Far UV) and 1-propanol (HPLC grade) were obtained from Labscan (Dublin, Ireland), and analytical-grade tri-*n*-butyl phosphate (TNBP) was from Fluka (Ronkonkoma, NY). Water was taken from a MilliQ apparatus (Millipore, Molsheim, France).

Preparation of antithrombin

A process sample of the AT product (Atenativ) from Octapharma AB (Stockholm, Sweden) was used. AT was prepared from pooled plasma from human donors by a process that included heparin affinity chromatography and salt precipitation, as previously described (19,20). Virus inactivation of this AT product was performed in two steps. First, a solvent detergent (S/D) method was used to inactivate lipid-enveloped viruses (such as HIV, hepatitis B, and hepatitis C) by incubation in 1% (w/v) Triton X-100, 0.3% (w/v) TNBP, 50 mmol/L sodium phosphate (pH 7.5) according to Horowitz et al. (21). Then, after removal of the S/D chemicals by precipitation (22), soy oil extraction, and phase separation, AT was filtered and then pasteurized at 60°C for 10 h in a solution containing 20% (w/v) sucrose, 1.1 mol/L sodium citrate, and 50 mmol/L sodium phosphate (pH 7.5) (19), based on previous work (18). After a second heparin affinity chromatography step to remove small amounts of denatured AT that was generated during the pasteurization, the Atenativ product was diafiltrated and concentrated, formulated, and sterile filtered (0.22-µm pores) before being dispensed in vials and lyophilized (19). The analysis of sucrose was performed on an AT process sample and collected before the pasteurization step in the AT process described previously. The nominal content of this AT process sample was 3 mg/mL protein, approximately 20% (w/v) sucrose, 1.1 mol/L trisodium citrate, and 50 mmol/L sodium phosphate (pH 7.5).

Quantitation of sucrose by the HILIC HPLC method

A Waters Alliance 2695 HPLC system with a 996 PDA UV detector and Millennium controlling software (Waters, Milford, MA) was used. The main detection was carried out using an ELSD detector (Sedex 75) from Sedere (Vitry sur Seine, France) coupled inline after the UV detector. Nitrogen was used as the ELSD nebulizer gas (3.5 bar), at a temperature of 35°C, and the gain was set to 8. The HPLC system included a silica-based poly-2-hydroxyethylaspartamide HILIC column (polyhydroxyethyl A, 300 Å, 5 µm, 200- × 4.6-mm i.d.) (Poly LC, Columbia, MD). The column was run at room temperature (~ 22°C), and the samples were held in the autosampler at the same temperature during the analysis. The mobile phase used for elution contained 25% water–75% acetonitrile. The flow rate was 1.0 mL/min and the injection volume was 60 µL.

The AT process sample, containing approximately 200 mg/mL sucrose, was first diluted 400 times in water and then by a second dilution 10 times in 1-propanol before being centrifuged for 10 min at 7,000 *g* to remove traces of precipitated proteins and salts. The supernatant was finally injected on the HILIC column. Selectivity was investigated by injecting samples containing 100 µg/mL of glucose, fructose, trisodium citrate, sodium phosphate (pH 7.5), Triton X-100, and TNBP. Six independent analyses were performed with separately made standard curve samples (10, 20, 40, 60, 80, and 100 µg/mL sucrose, $n = 6$) and sample preparations of AT. Spiked AT, with 10% added sucrose, and two additional samples, containing 15 and 80 µg/mL sucrose, were also analyzed. All samples were injected in 10% water–90% 1-propanol (single injections) and a log–log linear plot was used for the standard curves.

Results and Discussion

The HILIC method presented here, with a total analysis time of 10 min, gave a distinctive sucrose peak at a retention time of approximately 7.5 min (Figure 1). The log–log linear fit of the standard curve (10–100 µg/mL), gave a very high squared correlation coefficient (> 0.9995, $n = 6$) (Table I). ELSD detectors give an exponential response, which is often transformed to a linear function by a double log plot (1). When the diluted AT process sample was analyzed, the sucrose peak was the only detectable peak in the chromatogram (Figure 1D).

From the analyses of the AT process sample, a mean concentration of 46.0 µg/mL ($n = 6$) sucrose was obtained, corresponding to 18.4% in the original sample, which is in concordance with the expected value of approximately 20% sucrose (Table I). When six independent analyses were performed, the method precision was 4 RSD% for the AT process sample, and the mean recovery of the spiked AT sample (with 10% added sucrose) was 101%. Two samples with sucrose, at concentrations of 15 and 80 µg/mL, gave method precision of 4 and 3 RSD%, respectively. The repeatability was 1 RSD% ($n = 6$) at 50 µg/mL (Table I). The values for precision, recovery, and repeatability were considered to be satisfactory for the analysis of the stabilizer sucrose. In addition, the analysis time is short (10 min per sample), which is beneficial in a production environment.

Specificity was investigated to evaluate if any of the substances present in the AT process sample could possibly interfere with the analysis of sucrose. In addition, glucose and fructose were also analyzed because the disaccharide sucrose can be hydrolyzed to glucose and fructose. Triton X-100 and TNBP were also analyzed because trace amounts of these S/D chemicals could remain from the previous step in the production process of AT. Glucose, fructose, and Triton X-100, all at concentrations of 100 µg/mL, did not interfere with the method, and none of the compounds gave any peak at the retention time for sucrose (Figure 1). The monosaccharides

glucose and fructose gave less retention on the polar HILIC column than did the disaccharide sucrose, and both eluted at approximately 1–2 min earlier. Both the reducing carbohydrates glucose and fructose gave partially resolved double peaks (Figure 1), likely because of α- and β-anomers formed by mutarotation (2,7). Triton X-100 gave almost no retention on the HILIC column and eluted close to the void volume. TNBP gave no detectable peak (results not shown), which was probably attributable to its rather low molecular weight and boiling point. ELSD detection of semivolatile compounds such as

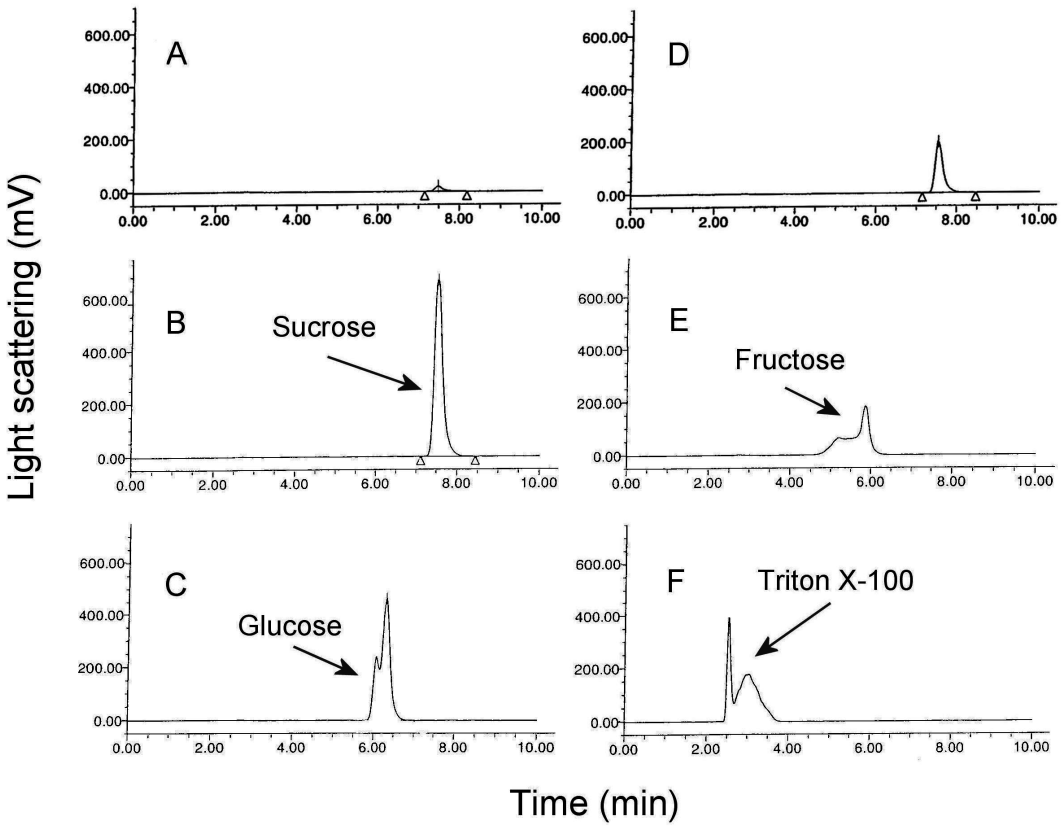

Figure 1. Hydrophilic interaction chromatography (HILIC) of sucrose. A poly-2-hydroxyethylaspartamide column was used at a flow rate of 1.0 mL/min. Elution was isocratically performed by 25% water–75% acetonitrile. The injection volume was 60 µL, and detection was performed by ELSD. (A) sucrose, 10 µg/mL; (B) sucrose, 100 µg/mL; (C) glucose, 100 µg/mL; (D) AT process sample, diluted 4,000 times; (E) fructose, 100 µg/mL; and (F) Triton X-100, 100 µg/mL. The compounds are indicated in the chromatograms. See the Experimental section for further details.

Table I. HILIC Anaysis of Sucrose*

Analysis No.	Linear correlation (r^2)	Precision Sample 1 (µg/mL)	Precision Sample 2 (µg/mL)	Precision AT (µg/mL)	Recovery Spiked AT (µg/mL)	Recovery t_R (min)	Recovery (%)	Repeatability† (µg/mL)
1	0.9998	15.4	82.2	44.0	73.1	7.6	105.9	50.0
2	1.0000	14.8	79.6	46.1	70.2	7.5	98.7	50.7
3	1.0000	15.1	79.7	45.6	72.2	7.5	102.3	50.9
4	0.9998	15.9	80.6	47.1	69.6	7.5	96.5	51.0
5	0.9996	14.2	85.2	48.6	76.0	7.5	103.3	49.9
6	0.9999	14.8	79.7	44.7	70.0	7.5	100.4	50.7
	Mean	15	81	46	72	7.5	101	51
	RSD%	4	3	4	3	0.5	3	0.9

* Linear correlation of the log–log standard curve (10, 20, 40, 60, 80, and 100 µg/mL, $n = 6$) expressed as the squared correlation coefficient (r^2). The AT process sample was spiked with 10% sucrose, which corresponded to an addition of 25 µg/mL after dilution. Recovery was calculated from the obtained difference between spiked and not spiked AT samples in respective analysis. The retention times (t_R) are also indicated for the spiked samples. The nominal concentrations of sample 1, sample 2, and the repeatability sample were 15, 80, and 50 µg/mL, respectively. See the Experimental section for further details.

† Consecutive injections from the same vial, performed in analysis 1.

TNBP is difficult to accomplish because of the vaporization of the analyte. Moreover, neither citrate nor phosphate gave any visible peaks (results not shown), probably partly caused by the precipitation of the salts by 90% 1-propanol, as used in the sample pretreatment. The lowest concentration of sucrose in the standard curve (10 µg/mL) gave a signal-to-noise ratio (s/n) of approximately 90, and the limit of detection was estimated to 1 µg/mL, giving a s/n of approximately 3 (Figure 2). During method development, samples were first prepared in 25% water–75% acetonitrile (i.e., the same concentration as that used in the HPLC method) however, this gave an unacceptable high repeatability value (~ 4 RSD%, n = 6). This may have been caused by a small precipitation of sucrose in the sample vials so that when the samples were injected in 10% water–90% 1-propanol an improved repeatability was obtained (1 RSD%, see Table I). Propanol is a more polar solvent, and therefore should give a higher solubility of sucrose compared with acetonitrile. The obtained specificity for sucrose in this HPLC method was considered to be sufficient, at least for the AT process sample.

HPLC of carbohydrates has mainly been performed by NPC on silica columns with an aminopropylsilane coating and often in combination with a mobile phase consisting of approximately 80% acetonitrile in water. Thus, the organic/aqueous property of the mobile phase is essentially the same as that used in HILIC. One disadvantage with HILIC is that the sample is usually injected in approximately 70–90% organics, which would precipitate some salts and proteins. We used a quick centrifugation step to remove trace amounts of precipitated proteins and salts from the AT sample that contained 10% water and 90% 1-propanol after the final dilution. Besides the poly-2-hydroxyethylaspartamide column, other types of commercially available HILIC columns include polysuccinimide-coated columns from Poly LC Inc. (PolyGLYCOPLEX) (8), amide columns (Amide-80, Tosohaas, Stuttgart, Germany) (10), and zwitterionic stationary phases (ZIC HILIC, SeQuant AB, Umeå, Sweden).

HPAEC also has a good selectivity for carbohydrates, as does HILIC, but is not compatible with the direct detection by an ELSD or a mass spectrometry instrument because of the nonvolatile nature of the HPAEC mobile phases.

In the production of the pharmaceutical antithrombin product (Atenativ), a relatively high concentration of sucrose [20% (w/v)] is present together with trisodium citrate (1.1 mol/L). They are used as stabilizing agents during the pasteurization, which is performed at 60°C for 10 h. This method has been shown to inactivate several types of viruses and also generate a small amount of inactive AT (19). The thermodynamic stabilization of proteins by sucrose has been shown to be attributable to a preferential exclusion of the sugar from the surface of the protein, which causes a shift in the equilibrium between protein states towards the most compact and stable conformation (17,23). In addition, sucrose may decrease the oxidation of methionine residues in proteins (24). Citrate inhibits aggregation of antithrombin at higher temperatures, which is believed to be caused by its lyotropic effect. This effect influences the hydrophobic interactions within the proteins (25), and thus citrate is often used as a stabilizer during the pasteurization in the virus reduction procedure. Glycerol has also been widely used as a protein stabilizer.

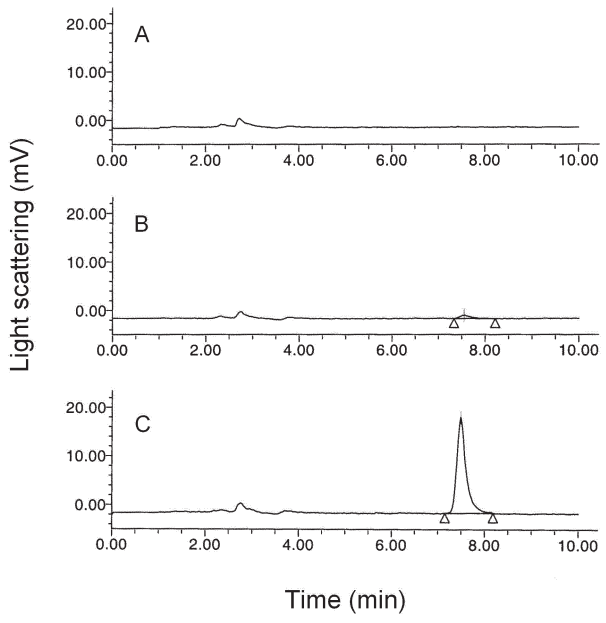

Figure 2. Detection limit for the HILIC method. (A) sample diluent (10% water–90% 1-propanol); (B) sucrose, 1 µg/mL, showing s/n of approximately 3; and (C) sucrose, 10 µg/mL, showing s/n of approximately 90. The interval 4.5–6.5 min was used to determine the maximum noise. The baseline disturbance at approximately 3 min is caused by injection of the sample.

Conclusion

A rapid and quantitative HPLC analytical method has been developed using HILIC with ELSD detection to analyze sucrose in pharmaceutical AT solutions. The method has a sufficient specificity and also gives satisfactory precision and recovery.

References

1. Y. Wei and M. Ding. Analysis of carbohydrates in drinks by high-performance liquid chromatography with a dynamically modified amino column and evaporative light scattering detection. *J. Chromatogr. A.* **904:** 113–17 (2000).
2. B. Herbreteau, M. Lafosse, L. Morin-Allory, and M. Dreux. Analysis of sugars by supercritical fluid chromatography using polar packed columns and light-scattering detection. *J. Chromatogr.* **505:** 299–305 (1990).
3. M.R. Hardy and R.R. Townsend. Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *Proc. Natl. Acad. Sci. U.S.A.* **85:** 3289–93 (1988).
4. N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata, and N. Takahashi. Analyses of N-linked oligosaccharides using a two-dimensional mapping technique. *Anal. Biochem.* **171:** 73–90 (1988).
5. Y.C. Lee, B.I. Lee, N. Tomiya, and N. Takahashi. Parameterization of contribution of sugar units to elution volumes in reverse-phase

HPLC of 2-pyridylaminated oligosaccharides. *Anal. Biochem.* **188:** 259–66 (1990).
6. A. Berthod, S.S.C. Chang, J.P.S. Kullman, and D.W. Armstrong. Practice and mechanism of HPLC oligosaccharide separation with a cyclodextrin bonded phase. *Talanta.* **47:** 1001–12 (1998).
7. A.J. Alpert. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **499:** 177–96 (1990).
8. A.J. Alpert, M. Shukla, A.K. Shukla, L.R. Zieske, S.W Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly, and R. Orlando. Hydrophilic-interaction chromatography of complex carbohydrates. *J. Chromatogr. A* **676:** 191–202 (1994).
9. B. Zhu, C.T. Mant, and R.S Hodges. Mixed-mode hydrophilic and ionic interaction chromatography rivals reversed-phase liquid chromatography for the separation of peptides. *J. Chromatogr.* **594:** 75–86 (1992).
10. V.V. Tolstikov and O. Fiehn. Analysis of highly polar compounds of plant origin: Combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. *Anal. Biochem.* **301:** 298–307 (2002).
11. L. Morin-Allory and B. Herbreteau. High-performance liquid chromatography and supercritical fluid chromatography of monosaccharides and polyols using light-scattering detection. *J. Chromatogr.* **590:** 203–13 (1992).
12. W.W. Christie and W.R. Morrison. Separation of complex lipids of cereals by high-performance liquid chromatography with mass detection. *J. Chromatogr.* **436:** 510–13 (1988).
13. I. Björk and S.T. Olson. Antithrombin: A bloody important serpin. *Adv. Exp. Med. Biol.* **425:** 17–33 (1997).
14. W.R. Lebing, D.J. Hammond, J.E. Wydick, and G.A. Baumbach. A highly purified antithrombin III concentrate prepared from human plasma fraction IV-1 by affinity chromatography. *Vox Sang.* **67:** 117–24 (1994).
15. C.B. Peterson and M.N. Blackburn. Isolation and characterization of an antithrombin III variant with reduced carbohydrate content and enhanced heparin binding. *J. Biol. Chem.* **260:** 610–15 (1985).
16. A. Messori, F. Vacca, M. Vaiani, and S. Trippoli. Antithrombin III in patients admitted to intensive care units: A multicenter observational study. *Crit. Care.* **6:** 447–51 (2002).
17. M. Sola-Penna and J.R. Meyer-Fernandes. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: why is trehalose more effective than other sugars. *Arch. Biochem. Biophys.* **360:** 10–14 (1998).
18. L. Tengborn, B. Frohm, L.E. Nilsson, L. Johansson, and L.O. Andersson. Properties and catabolism of heat treated antithrombin III concentrate. *Thromb. Res.* **48:** 701–11 (1987).
19. S. Winge. Patent application. TITLE. PCT WO 00/78792-A1 (2000).
20. M. Miller-Andersson, H. Borg, and L.O. Andersson. Purification of antithrombin III by affinity chromatography. *Thromb. Res.* **5:** 439–52 (1974).
21. B. Horowitz, R. Bonomo, A.M. Prince, S.N. Chin, B. Brotman, and R.W. Shulman. Solvent/detergent-treated plasma: A virus-inactivated substitute for fresh frozen plasma. *Blood.* **79:** 826–31 (1992).
22. S. Isaksson and S. Winge. Patent application. PCT WO 94/26287-A1 (1994).
23. J.C. Lee and S.N. Timasheff. The stabilization of proteins by sucrose. *J. Biol. Chem.* **256:** 7193–7201 (1981).
24. R.A. DePaz, C.C. Barnett, D.A. Dale, J.F. Carpenter, A.L. Gaertner, and T.W. Randolph. The excluding effects of sucrose on a protein chemical degradation pathway: Methionine oxidation in subtilisin. *Arch. Biochem. Biophys.* **384:** 123–32 (2000).
25. T.F. Busby, D.H. Atha, and K.C. Ingham. Thermal denaturation of antithrombin III: Stabilization by heparin and lyotropic anions. *J. Biol. Chem.* **256:** 12140–47 (1981).

Manuscript accepted June 18, 2004.