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Ion-exchange high-performance liquid chromatographic separation of protein variants and isoforms on MCI GEL ProtEx stationary phases

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Abstract

Protein variants and isoforms were successfully separated on MCI GEL ProtEx ion-exchange HPLC columns. There was no irreversible adsorption of proteins, and sample proteins were quantitatively recovered. Species variants of cytochrome *c* (bovine, horse and rabbit) were completely separated on a sulfopropyl (ProtEx-SP) stationary phase in a gradient system. Diethylaminoethyl (ProtEx-DEAE) phase was determined to be effective for the separation of human growth hormone and its deamidated isoforms. These characteristics of ProtEx stationary phases may be attributed both to particle uniformity and to hydrophillic surface coverage of the base polymeric material.

Keywords: Stationary phases, LC; Proteins

1. Introduction

In the past decade, there has been remarkable progress in analytical technology for biochemical research fields. Improvements in analytical instrumentation have brought not only better precision but also reduction of the sample amount. For example, refinements in amino acid sequencing system [1] or in X-ray diffraction equipment have reduced the required amount of sample proteins to the level of sub-μg. As a result, proteins should be separated and purified before analyses to such small quantities. High-performance liquid chromatography (HPLC) is essentially a powerful tool for these purposes, and there exists an increasing necessity for better and more precise separation of proteins by HPLC.

When a small amount of proteins is separated by ion-exchange HPLC, the hydrophobicity of the base material causes several problems. One problem is the serious decline in protein mass recovery with a decreasing amount of sample, often observed on conventional stationary phases. This decline is rarely observed for a normal amount of sample loading. Another problem is the decline in resolution when the amount of sample injection is decreased, especially when the sample load is under the sub-μg level. It is supposed that hydrophobic interaction between a protein molecule and the surface of the base material becomes significant with decreasing sample load, causing a drop in recovery and resolution. Additionally, in the case of silica-based stationary phase, surface silanol and siloxane groups affect denaturation of proteins.

To overcome these problems, surface modification of the base material is necessary for reducing the hydrophobic effect between proteins and stationary

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Table 1
Characteristics of MCI GEL ProtEx stationary phases

Stationary phase	Functionality	Ion-exchange capacity	Protein-binding capacity
MCI GEL ProtEx-DEAE	Diethylaminoethyl	ca. 0.3 mequiv./g	40 mg BSA/ml
MCI GEL ProtEx-SP	Sulfopropyl	ca. 0.3 mequiv./g	25 mg ribonuclease A/ml

phases. Polyethyleneimine coating of the surface of silica gel or poly(styrene–divinylbenzene) and subsequent crosslinking has been commonly employed [2,3]. However, such problems of hydrophobic interaction have not been completely resolved.

For the complete elimination of the hydrophobic effect between proteins and base material, another approach is adopted. This paper deals with novel stationary phases which consist of hydrophillic base material and hydrophillic crosslinked surface layers.

Table 1 shows the characteristics of MCI GEL ProtEx stationary phases used in this study. These stationary phases are made from spherical, porous and hydrophillic polymethacrylate particles with a uniform diameter of 5 μm. The pore diameter of the stationary phases has been found to be 100 nm by Hg intrusion porosimetric analysis. The surface of the base material is completely covered with a hydrophillic crosslinked surface layer. ProtEx-DEAE stationary phase has diethylaminoethyl weak anion-exchange functionality, and ProtEx-SP stationary phase has sulfopropyl strong cation-exchange functionality. These functionalities are bonded to the stationary phases via a mechanically stable crosslinked hydrophillic layer.

In this study, the resolution and recovery performance of ProtEx stationary phases with a small amount of proteins is evaluated, and the effectiveness of the structure of the ProtEx stationary phases is examined. The separation of some protein variants and isoforms is also examined as an evaluation method for separation efficiency.

2. Experimental

2.1. Materials

MCI GEL ProtEx-DEAE and ProtEx-SP packed columns (50×4.6 mm I.D.) were from Mitsubishi Chemical (Tokyo, Japan).

2.2. Proteins and chemicals

Recombinant human epidermal growth factor (EGF) was from Boehringer Mannheim (Germany). Human growth hormone (hGH) was from Chemicon International (Temecula, CA, USA). Lyphochek diabetes control levels 1 and 2 were purchased from Nippon Bio-Rad Labs. (Tokyo, Japan). Other proteins were from Sigma (St. Louis, MO, USA).

Tris(hydroxymethyl)aminomethane (Tris), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis–Tris) and other chemicals were of the highest quality available, and were purchased from various suppliers.

2.3. Apparatus

A gradient HPLC system consisted of Shimadzu Model LC-10AS pumps, an SIL-6B automatic sample injector, an SPD-6AV spectrophotometric detector, a C-R4A integrator and an SCL-6B system controller. The flow-rate was 0.5 ml/min, and proteins were monitored at 280 nm (except for the separation of glycated hemoglobin at 415 nm). The HPLC system was operated at 25°C.

2.4. Procedures

Standard protein mixture evaluations: Standard protein mixture evaluation for anion-exchange HPLC was performed with a gradient of 20 m*M* Tris–HCl (pH 8.2) to 20 m*M* Tris–HCl (pH 8.2) containing 0.5 *M* sodium chloride over 20 min. The proteins in the standard mixture were horse myoglobin, chicken egg conalbumin and soybean trypsin inhibitor.

In the case of cation-exchange HPLC, chromatographic evaluation was performed with a gradient of 20 m*M* potassium phosphate (pH 6.0) to 20 m*M* potassium phosphate (pH 6.0) containing 0.5 *M* sodium chloride over 20 min. The proteins in the

standard mixture were bovine ribonuclease A, bovine α-chymotrypsinogen A and bovine cytochrome *c*.

Separation of protein variants: Anion-exchange chromatographic separation of protein variants was performed with a gradient of 20 m*M* Tris–HCl (pH 8.2) to 20 m*M* Tris–HCl (pH 8.2) containing 0.5 *M* sodium chloride from 0 to 10% over 30 min. The protein variants used were human hemoglobin A_0 (Hb A_0), human hemoglobin A_2 (Hb A_2) and human hemoglobin S (Hb S).

The variants used on cation-exchange HPLC were cytochrome *c* of three species (bovine, horse and rabbit). Separation was performed with a gradient of 20 m*M* Bis–Tris–HCl (pH 7.0) and 20 m*M* Bis–Tris–HCl (pH 7.0) containing 0.5 *M* sodium chloride from 24 to 69% over 20 min.

Protein mass recoveries of DEAE stationary phase: The protein mass recovery study was performed for an anion-exchange stationary phase. Various amounts of recombinant human EGF were loaded with a gradient of 20 m*M* Tris–HCl (pH 8.2) to 20 m*M* Tris–HCl (pH 8.2) containing 0.5 *M* sodium chloride from 0 to 50% over 30 min. The relative mass recoveries were determined from the ratio of peak areas with and without the column.

Purity evaluation for recombinant protein production: Recombinant hGH was chromatographed on ProtEx-DEAE with a gradient of 20 m*M* Tris–HCl (pH 8.0) and 20 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* sodium chloride from 5 to 70% over 30 min. For observation of the hGH degradation, samples were heat-treated at 60°C for 5 h and at 70°C for 8 h.

Application for clinical diagnosis of diabetes: Lyphochek diabetes control levels 1 and 2 were used under 20 times dilution. The samples were chromatographed on ProtEx-SP with a gradient of 20 m*M* Bis–Tris–HCl (pH 6.0) and 20 m*M* Bis–Tris–HCl (pH 6.0) containing 0.5 *M* sodium chloride from 7 to 40% over 20 min.

3. Results and discussion

3.1. Standard protein mixture evaluations

The separation of proteins with different values of isoelectric point was evaluated for determination of primary performance of ProtEx stationary phases. Figs. 1 and 2 show the separation of standard protein mixture on a ProtEx-DEAE column. The excellent separation efficiency observed in Figs. 1 and 2 may be attributed both to the size effect (5.0 μm) and to the uniformity of base polymer particles. It is noticeable that the same separation as in Fig. 1 is observed in Fig. 2, in spite of one tenth of the sample loading amount.

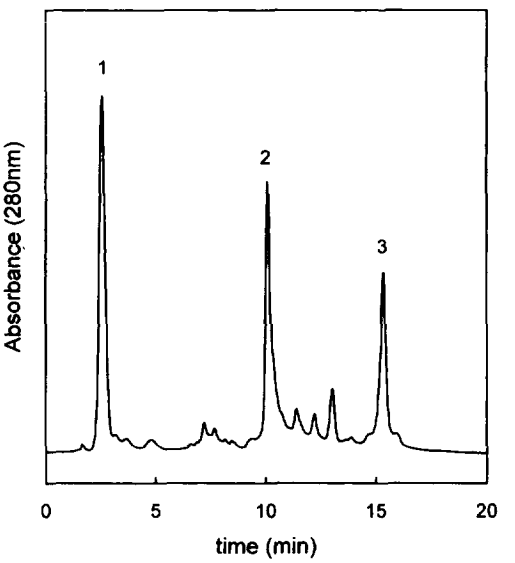

Fig. 1. Separation of standard protein mixture on ProtEx-DEAE stationary phase. Conditions: eluent A, 20 m*M* Tris–HCl, pH 8.2; eluent B, A+0.5 *M* NaCl; gradient, 0–100% B over 20 min; flow-rate, 0.5 ml/min. Samples: 1=myoglobin, 10 μg; 2=conalbumin, 10 μg; 3=trypsin inhibitor, 20 μg.

A chromatogram of standard protein mixture on a ProtEx-SP column is shown in Fig. 3. It also shows high column efficiency at μg loading, as in the case of ProtEx-DEAE.

In the case of conventional stationary phases, the resolution of proteins declines with decreasing amount of injection, especially with sample loads of less than 1 μg. It is supposed that the surface of the conventional stationary phases has hydrophobic regions, and ion-exchange functionalities are directly coupled to the surface. When a protein molecule interacts with the ion-exchange functionalities of the conventional stationary phases, hydrophobic interaction between the protein molecule and the surface of the conventional stationary phases is unavoidable;

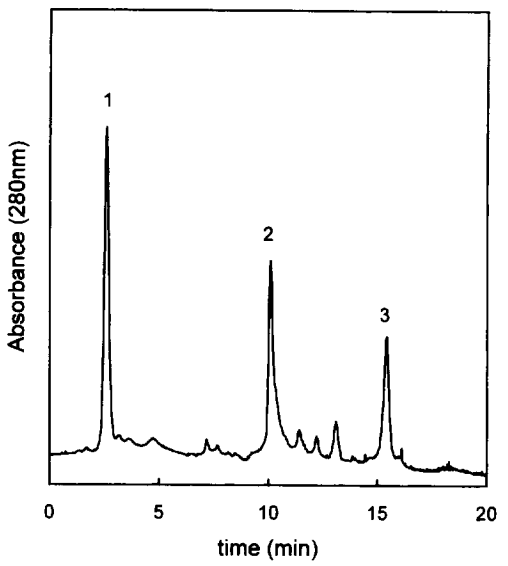

Fig. 2. Separation of standard protein mixture on ProtEx-DEAE stationary phase. Conditions: eluent A, 20 m*M* Tris–HCl, pH 8.2; eluent B, A+0.5 *M* NaCl; gradient, 0–100% B over 20 min; flow-rate, 0.5 ml/min. Samples: 1=myoglobin, 1 μg; 2=conalbumin, 1 μg; 3=trypsin inhibitor, 2 μg.

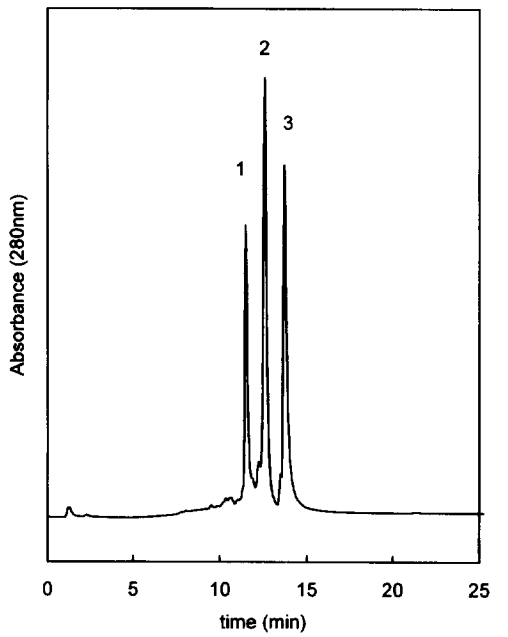

Fig. 3. Separation of standard protein mixture on ProtEx-SP stationary phase. Conditions: eluent A, 20 m*M* potassium phosphate, pH 6.0; eluent B, A+0.5 *M* NaCl; gradient, 0–100% B over 20 min; flow-rate, 0.5 ml/min. Samples: 1=ribonuclease A, 10 μg; 2=α-chymotrypsinogen A, 5 μg; 3=cytochrome *c*, 5 μg.

this kind of hydrophobicity causes the drop of resolution with a decreasing amount of injection. The surface of ProtEx base materials is completely covered with a hydrophillic layer, which eliminates hydrophobic interaction, and enables the high resolution of proteins even with a small amount of sample injection.

3.2. Separation of protein variants

For the evaluation of the separation capability of various stationary phases, it is an effective method to separate protein variants or protein isoforms. Protein variants have differences in amino acid sequence from different codes of gene. Lots of protein variants exist not only between the species, but also between internal organs which synthesize the proteins, and, in some cases, genetic diseases are caused by substitution of only several amino acids. Protein isoforms have only little differences in post-translational changes such as glycosylation and phosphorylation. The degradation of proteins which involves deamidation of asparagine and oxidation of methionine also provides isoforms.

Despite the little differences in the chemical structure, protein variants and protein isoforms often affect physiological properties, and so on. Especially, differences in enzymatic activities of protein isoforms have been widely studied [4,5]. Thus, the separation of variants and isoforms has become increasingly important. Chromatographic separation of protein variants and protein isoforms is essentially difficult because there are only subtle differences in the chemical structure, and it depends on the separation capability of a stationary phase.

Fig. 4 shows the separation of human hemoglobin variants on ProtEx-DEAE anion exchanger. The structural difference between Hb A_0 and Hb S is only one amino acid substitution in the sequence of the β chain, and ten amino acid substitutions between the β chain of Hb A_0 and the δ chain of Hb A_2 [6,7]. From Fig. 4, it is found that ProtEx-DEAE is highly effective for the separation of protein variants of slight structural difference even with a small amount of sample injection.

Cation-exchange HPLC separation of cytochrome *c* species variants shown in Fig. 5 also demonstrates the excellent separation efficiency of ProtEx-SP

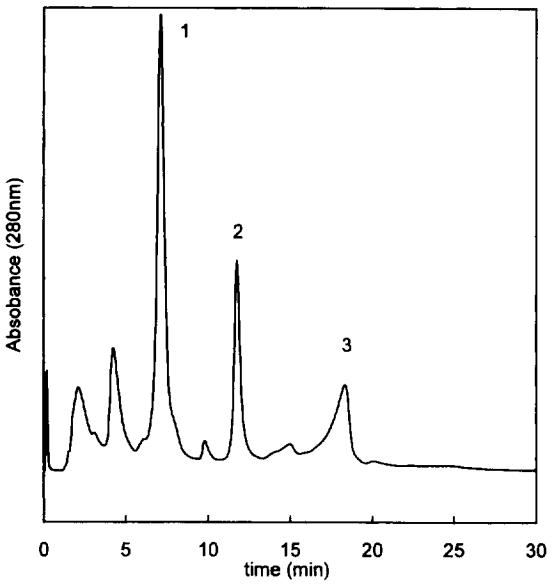

Fig. 4. Separation of human hemoglobin variants on ProtEx-DEAE stationary phase. Conditions: eluent A, 20 m*M* Tris–HCl, pH 8.2; eluent B, A+0.5 *M* NaCl; gradient, 0–10% B over 30 min; flow-rate, 0.5 ml/min. Samples: 1=Hb A_2; 2=Hb S; 3=Hb A_0; 10 μg each.

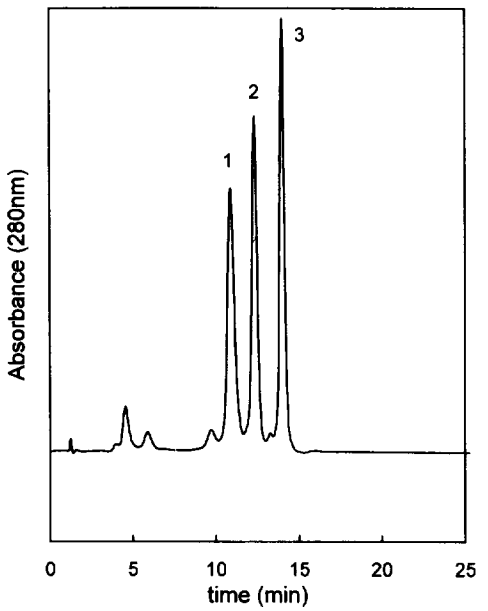

Fig. 5. Resolution of cytochrome *c* species variants on ProtEx-SP stationary phase. Conditions: eluent A, 20 m*M* Bis–Tris–HCl, pH 7.0; eluent B, A+0.5 *M* NaCl; flow-rate, 0.5 ml/min; gradient, 24–69% B over 20 min. Samples: 1=bovine; 2=horse; 3=rabbit; 10 μg each.

stationary phase. The amino acid sequence of cytochrome *c* variants differs in different species, but there are only three substitutions between bovine and horse, and only four substitutions between bovine and rabbit cytochrome *c* [8–10]. This result also confirms the excellent separation capability of ProtEx stationary phases.

3.3. Protein mass recoveries of DEAE stationary phase

In case of protein purification of a small amount, mass recovery is one of the most important factors. Relative mass recovery of EGF on ProtEx-DEAE stationary phase is shown in Fig. 6. Almost quantitative mass recovery of EGF is observed even for 1 μg of injection. The complete coverage of the surface of ProtEx stationary phases provides quantitative recovery of proteins for a wide range of loading. Fig. 6 demonstrates that ProtEx stationary phases are effective for microseparation of proteins.

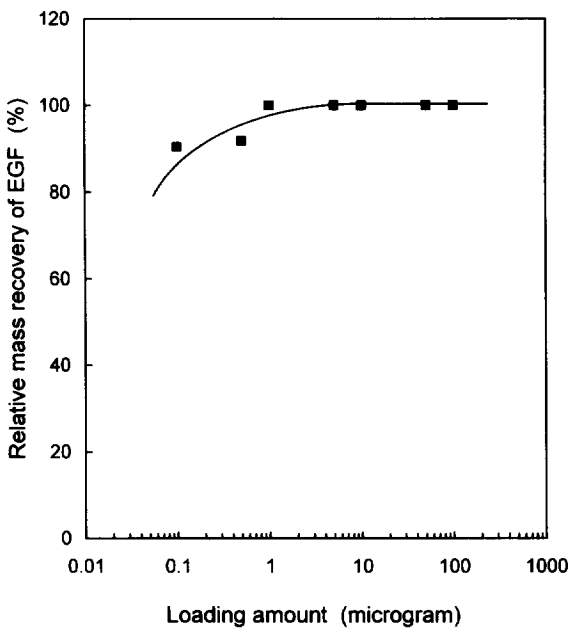

Fig. 6. Relationship between loading amount and relative mass recovery of recombinant human EGF on ProtEx-DEAE stationary phase. Conditions; eluent A, 20 m*M* Tris–HCl, pH 8.2; eluent B, A+0.5 *M* NaCl; gradient, 0–50% B over 30 min; flow-rate, 0.5 ml/min.

3.4. Purity evaluation for recombinant protein production

In the case of the production of proteinaceous pharmaceuticals, purification is a key process and the accuracy of purity evaluation is a primary concern. When a proteinaceous pharmaceutical is produced by means of recombinant technology, the impurities often involve isoforms of the objective made by post-translational modification or degradation such as glycosylation, phosphorylation, deamidation, etc. Fig. 7 shows the separation of hGH and its deamidated isoforms on ProtEx-DEAE. Minor peaks eluted after hGH are desamido derivatives [11–13], and the first minor peak is presumed to be mono-desamido hGH and the second to be di-desamido hGH, respectively [11,13]. It is remarkable that the second minor peak is clearly eluted on ProtEx-DEAE. It should be noted that the amount of deamidated hGH increased with increasing heat treatment. Thus, ProtEx stationary phases are thought to be suitable for purity evaluation or impurity identification of recombinant proteins.

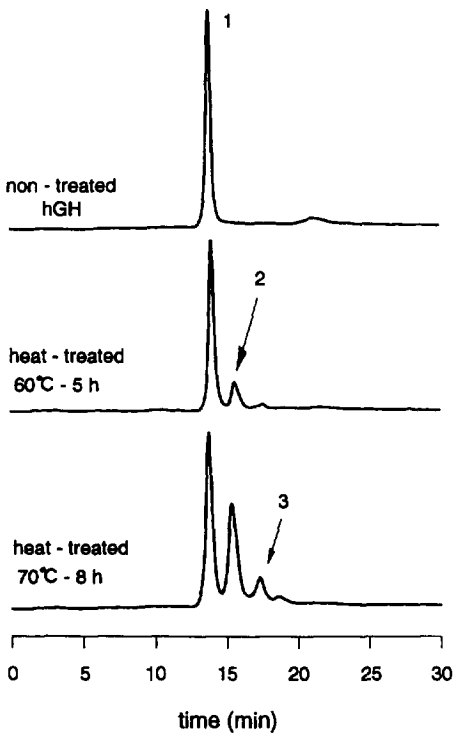

Fig. 7. Anion-exchange chromatographic separation of recombinant hGH and deamidated isoforms on ProtEx-DEAE stationary phase. Conditions: eluent A, 20 m*M* Tris–HCl, pH 8.0; eluent B, A+0.5 *M* NaCl; gradient, 5–70% B over 30 min; flow-rate, 0.5 ml/min. Samples: hGH; 10 μg. Peaks: 1=hGH; 2, 3=desamido derivatives.

3.5. Application for clinical diagnosis of diabetes

HPLC is a useful method for analyses of abnormal proteins in the field of clinical chemistry. For instance, ion-exchange HPLC quantitation of glycated hemoglobin (GHb) is established and used widely [14]. GHb separation was examined to show the applicability of ProtEx-SP stationary phase.

Separation of Lyphochek diabetes control level 2 is shown in Fig. 8. Excellent separation of glycated hemoglobin isoforms and hemoglobin A_0 was observed. The two peaks eluted at 13.1 min and 13.7 min are GHb A_1c. When various amounts of glucose was added to the Lyphochek diabetes control level 2, the peak area eluted at 13.1 min increased, and the

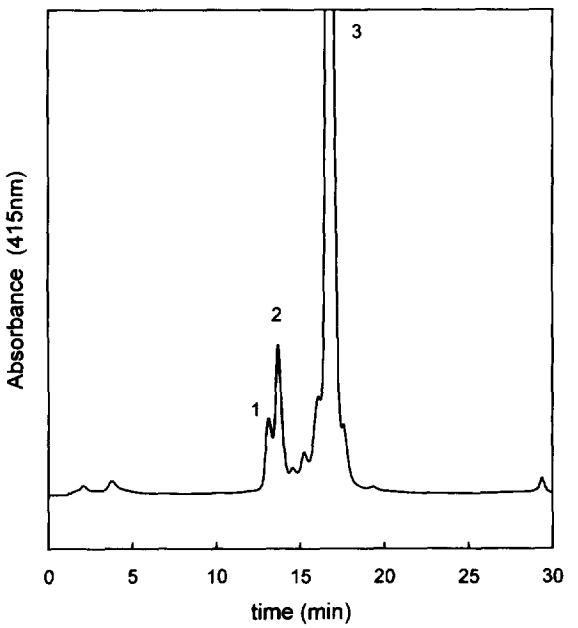

Fig. 8. Cation-exchange chromatographic separation of glycated hemoglobin isoforms and hemoglobin A_0 on ProtEx-SP stationary phase. Conditions: eluent A, 20 m*M* Bis–Tris–HCl, pH 6.0; eluent B, A 0.5 *M* NaCl; gradient, 7–40% B over 20 min; flow-rate, 0.5 ml/min. Detection: 415 nm. Samples: Lyphochek diabetes control level 2 (20 times dilution); 5 μl. Peaks: 1, 2=GHb A_{1c}; 3=Hb A_0.

results certified that the 13.1 min peak was labile GHb A_1c (data not shown). It is apparent from these facts that ProtEx-SP stationary phase can match the performance of stationary phases specially designed for the quantitation of GHb [15].

4. Conclusion

It is concluded that ProtEx stationary phases are suitable for the separation of proteins with subtle differences, such as variants and isoforms, even with a small amount of sample loading. These characteristics of ProtEx stationary phases may be attributed both to particle uniformity and to the hydrophillic surface coverage of the base polymeric material. ProtEx stationary phases would be applicable not only to biochemistry but also to product control of proteinaceous pharmaceuticals, clinical chemistry and so on.

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