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CHROMATOFOCUSING OF SIALOGLYCOPROTEINS

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SUMMARY

Sialoglycoproteins of different sialic acid contents have been separated from each other by chromatofocusing on the ion exchanger PBE 94 using gradients of pH 4.00 down to pH 1.00. The technique is much faster than isoelectric focusing, apparently does not result in desialylation of the sialoglycoproteins and can handle with ease 10 ng to 50 mg quantities of protein on a 22 × 0.9 cm column. The technique revealed that commercial preparations of fetuin and human acid glycoprotein contained several components. Glycophorin, desialylated by controlled neuraminidase treatment, was fractionated by chromatofocusing into several components which differed in sialic acid content and in ability to inhibit haemagglutination by wheat germ agglutinin and encephalomyocarditis and influenza viruses.

INTRODUCTION

We are interested in the rôle of sialic acid in the biological function of sialoglycoproteins. In particular, we have been studying the effect of desialylation of glycophorin, the major sialoglycoprotein of the human erythrocyte surface membrane, on its ability to serve as a receptor for wheat germ agglutinin (WGA) and for encephalomyocarditis (EMC) and influenza viruses[1]. During the study, it became necessary to separate species of glycophorin of different sialic acid contents and isoelectric focusing was considered for this purpose. However, sialoglycoproteins have isoelectric points in the region of pH 4 and below[2,3] and, although isoelectric focusing has been used to separate molecules in this low pH range [4,5], difficulties were anticipated with this technique because the time taken to reach equilibrium combined with the acid lability of the sialyl bond has been reported to cause partial desialylation of sialoglycoproteins[6].

Chromatofocusing is another technique in which molecules are separated from each other on the basis of differences in their isoelectric points[7]. In chromatofocusing, amphoteric molecules, such as proteins, are adsorbed to an ion-exchange resin which is then washed with a pH gradient. When the pH of the gradient reaches that of the isoelectric point of a particular adsorbed molecule, the latter becomes electrically neutral and is released. A feature of chromatofocusing is that the pH gradient used for elution is generated "internally". The gradient arises through the buffering action

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of the ion-exchange resin and the running of a buffer initially adjusted to one pH through a column initially adjusted to a second pH[7]. The technique is usually conducted in the range pH 4–11[8] which is above the isoelectric point of sialoglycoproteins. We report here adaptation of chromatofocusing for operation in the range pH 1–4 and show that the technique is capable of separating sialoglycoproteins of different sialic acid contents without apparent desialylation.

EXPERIMENTAL

Materials

The K2 strain of EMC virus and the PR/8 strain of influenza virus were grown and purified as described previously[1]. Glycophorin was prepared by lithium 3,5-diiodosalicylate–phenol extraction of membranes from outdated type 0 human erythrocytes[9] and, where indicated, was ^{131}I-labelled using the IODO-GEN procedure[10]. Iodine-131 was purchased from New England Nuclear (Lachine, Quebec, Canada); IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was from Pierce (Rockford, IL, U.S.A.); wheat germ agglutinin (WGA) and the ion-exchange resin, PBE 94 from Pharmacia (Dorval, Quebec, Canada); *Vibrio cholerae* neuraminidase from Calbiochem-Behring (Montreal, Quebec, Canada); fetuin, type IV and lithium diiodosalicylate from Sigma (St. Louis, MO, U.S.A.) and human glycoprotein from Miles Laboratory (Kankakee, IL, U.S.A.).

Chromatofocusing

This was performed at room temperature on 22 × 0.9 cm columns of the ion-exchange resin PBE 94 at a flow-rate of about 50 ml/h; 5-ml fractions were collected. The resin was equilibrated before each run with start buffer (25 m*M* formic acid adjusted to pH 4.00 with NH_4OH). Samples were added in about 1 ml start buffer to the column which was then washed with a series of buffers: A, B or C to elute bound material.

The A series of buffers consisted of 50 ml start buffer followed successively by 50 ml 25 m*M* ammonium formate, pH 3.00 and 200 ml 25 m*M* oxalic acid adjusted to pH 1.00 with HCl. The B series of buffers consisted of 50 ml start buffer followed successively by 50 ml 25 m*M* ammonium formate, pH 3; 150 ml 25 m*M* oxalic acid adjusted to pH 1.40 and 50 ml 25 m*M* oxalic acid adjusted to pH 1.00. The C series of buffers consisted of 50 ml of each of the following in the order: 25 m*M* formic acid adjusted to pH 4.00, pH 3.00, pH 2.50 and pH 2.00, and 25 m*M* oxalic acid adjusted to pH 1.50 and 1.00.

Analysis of column eluates

The pH of each fraction was measured with a combination electrode using a Beckman Expandomatic SS-2 pH meter. For columns on which fetuin and acid glycoprotein were run, the optical density of each fraction was measured at 280 nm. Samples containing optical density were scanned between wavelengths of 230 nm and 360 nm to check for the presence of a maximum at about 280 nm indicating protein. The points of elution of species of [^{131}I]glycophorin were detected by analysis of fractions in a Beckman gamma counter 300.

Fractions containing material of interest were combined, brought to pH 7,

dialysed against water and lyophilized. These samples were assayed for protein by the Lowry method[11], for sialic acid by the thiobarbituric acid method[12] and, in the case of the glycophorin samples, for their ability to inhibit EMC virus, influenza virus or WGA haemagglutination as described previously[1].

Desialylation

Sialic acid was released from sialoglycoproteins either (i) by incubation at 80°C for 1 h with 0.1 *N* H_2SO_4 or (ii) by incubation at 37°C with neuraminidase (5 μg protein per unit of enzyme) in phosphate buffer, pH 8 for the times indicated in the figures, after which the enzyme was destroyed by boiling for 10 min.

RESULTS

pH gradients generated during chromatofocusing

It is recommended that for optimum resolution the pH gradient generated internally during chromatofocusing should be linear[13]. When a PBE 94 column initially equilibrated with 25 m*M* formate, pH 4.00, was washed with 25 m*M* oxalate, pH 1.00, the pH gradient generated was not linear (Fig. 1) When the column initially adjusted to pH 4.00 was washed with an externally produced pH 4 to pH 1 linear gradient, the pH gradient of the eluate was again far from linear (Fig. 1). Washing the column with combinations of several substances with p*K* values of 4.00 and below including formate, citrate, phosphate and oxalate failed to produce an acceptable linear gradient. However, by following start buffer at pH 4.00 with successive buffers of lower pH, a reasonably linear gradient was generated (Fig. 1).

Chromatofocusing of sialoglycoproteins

Fetuin, for which a variety of isoelectric points in the range pH 3.3–4.1 have been reported[2], was chromatofocused to determine if the pH gradient produced by stepwise addition of buffers could elute a typical sialoglycoprotein. Two different prepa-

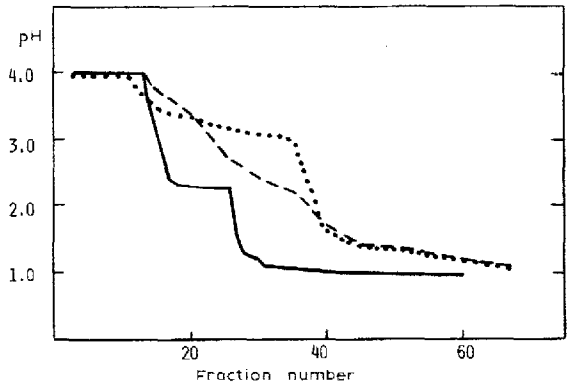

Fig. 1. pH gradients produced on a PBE 94 column. The ion exchanger was pre-equilibrated with 25 m*M* ammonium formate, pH 4.00 then washed with 50 ml of the same buffer followed by: (a) 300 ml 25 m*M* oxalic acid adjusted to pH 1.00 (———); (b) the B series of buffers (– – –) or (c) a linear pH gradient produced externally by mixing 150 ml 25 m*M* formate, pH 4.00 with 150 ml 25 m*M* oxalate, pH 1.00 (· · ·).

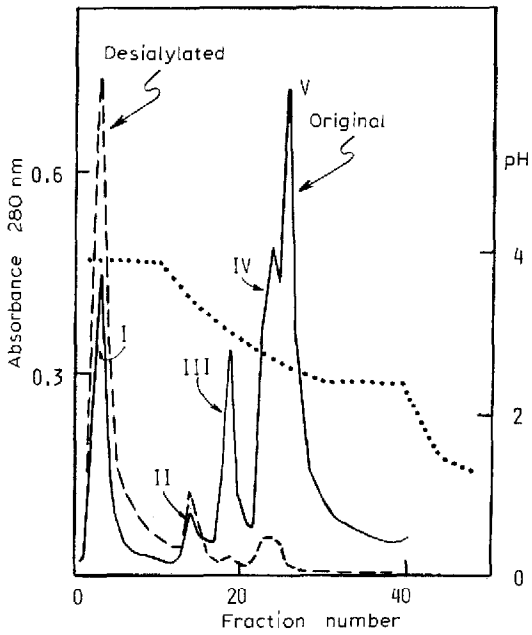

Fig. 2. Chromatofocusing of fetuin before (———) and after (– – –) acid desialylation. The PBE 94 ion exchanger was pre-equilibrated with 25 m*M* ammonium formate, pH 4.00. The pH gradient (· · ·) was generated by elution with the B series of buffers. Approximately 50 mg protein were used in each run.

rations of fetuin were each found to contain at least five components (Fig. 2) which eluted respectively at pH 4 (in the void volume; component I), pH 3.74 (II), pH 3.12 (III), pH 2.70 (IV) and at pH 2.60 (V): the values are the averages for the two fetuin preparations. The original material contained 64 μg sialic acid per mg protein whereas components I, IV and V contained 1, 50 and 66 μg sialic acid per mg protein; components II and III were not analysed.

When acid-desialylated fetuin was chromatofocused, most of the material emerged from the column in the void volume with start buffer at pH 4.00 (Fig. 2).

Human acid glycoprotein has been reported to have isoelectric points ranging from pH 1.8 to 2.7[3]. When examined by chromatofocusing, the major component in human acid glycoprotein eluted at about pH 1.90 (Fig. 3, component VI) while minor components eluted at pH 4.00 (I), pH 3.80 (II), pH 3.30 (III), pH 2.52 (IV) and pH 2.4 (V). The original material contained 122 μg sialic acid per mg protein compared with 34, 11, 112 and 159 μg sialic acid per mg protein for components I, IV, V and VI, respectively. The value of 34 μg sialic acid per mg protein for component I is unreliable because of the inaccuracy of determining sialic acid and protein in trace amounts.

As with desialylated fetuin, most of the material in acid-desialylated human acid glycoprotein emerged in the void volume in start buffer, pH 4.0 (Fig. 3).

Glycophorin can contain as much as 560 μg sialic acid per mg protein[1] and would therefore be expected to have a low isoelectric point. This was apparently so

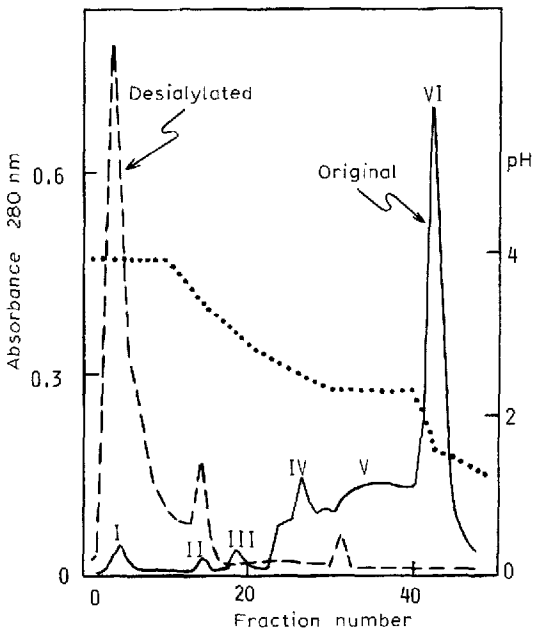

Fig. 3. Chromatofocusing of human acid glycoprotein before (———) and after (- - -) acid desialylation. The conditions were as in Fig. 2.

because when [^{131}I]glycophorin was chromatofocused, close to 100% of the radioactivity eluted in a single peak at about pH 1.7 (Fig. 4). When the fractions comprising this peak were combined after storing at 4°C overnight, brought to pH 4.0 with NH_4OH and re-run under the same conditions as the first run, an identical result was obtained with 100% of the material eluting as a single peak at about pH 1.7 (result not shown).

Unlike fetuin and human acid glycoprotein when desialylated, not all of acid-desialylated glycophorin eluted in the void volume at pH 4.0, some eluting at pH 3.42 and at pH 2.90 with a minor peak at pH 1.7 coincident with the fully sialylated parent molecule. Whether these components arose through incomplete desialylation or through some other cause such as microheterogeneity in amino acid sequence was not determined. Glycophorin preparations, which had been subjected to freezing and thawing on an unrecorded number of occasions, were sometimes found to contain several components (result not shown) of undetermined composition.

Effect of neuraminidase treatment on glycophorin chromatofocusing profile

Since chromatofocusing appeared to separate sialoglycoproteins of different sialic acid contents, attempts were made to separate species of different sialic acid contents generated by controlled neuraminidase treatment of glycophorin. For this purpose, samples of a [^{131}I]glycophorin preparation were incubated at 37°C in the presence or absence of neuraminidase for various lengths of time, boiled for 10 min to destroy the enzyme then chromatofocused. Representative profiles are shown in Fig. 5. Most of the material in the untreated preparation eluted as a single peak at

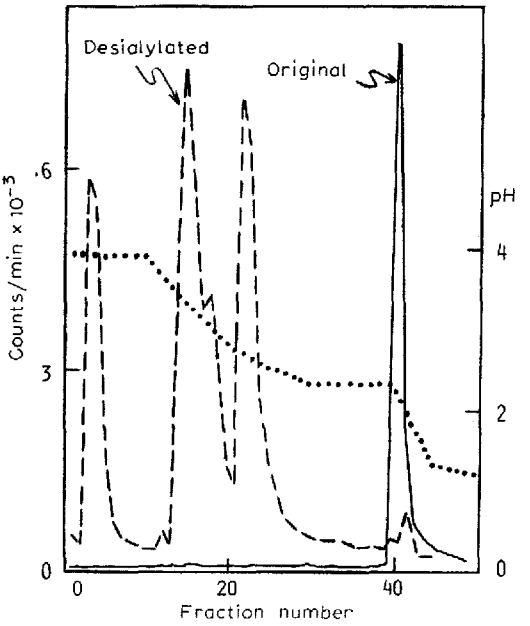

Fig. 4. Chromatofocusing of [^{131}I]glycophorin before (———) and after (– – –) acid-desialylation. The conditions were as in Fig. 2 except that only 10 ng glycophorin were used per run.

pH 1.70. In contrast, neuraminidase digests of glycophorin contained several components, the longer the digestion time the earlier or the higher the pH the peaks were eluted (Fig. 5) until at 30-min digestion, most of the material eluted in a single peak at pH 2.70 (Fig. 5). Incubation for 30 min under these conditions would possibly result in about 50% loss of glycophorin sialic acid[1].

Effect of eluate composition on chromatofocusing profile

To improve resolution of the components in neuraminidase digests of glycophorin during chromatofocusing, the effect of the composition of the eluting buffers on the profile was investigated (Fig. 6). As the elution profile became less steep (Fig. 6b) and in fact formed almost a plateau (Fig. 6c), the resolution became greater. This figure illustrates how easy it is to manipulate the gradient profile in chromatofocusing.

Properties of glycophorin species separated by chromatofocusing

The peaks in neuraminidase-digests of glycophorin (Fig. 6c) were examined for pH of elution, sialic acid content and for ability to inhibit lectin and virus haemagglutination (Table I). The peaks were seen to fall into two groups, the minor and major peaks.

The sialic acid content of the minor components (peaks I–IV and X) decreased as the pH for elution also decreased. This was unexpected since it was anticipated that the molecules which eluted early and therefore of high isoelectric point would contain less sialic acid. In contrast, the major components (peaks V–IX) behaved as expected and generally eluted in order of increasing sialic acid content (Table I).

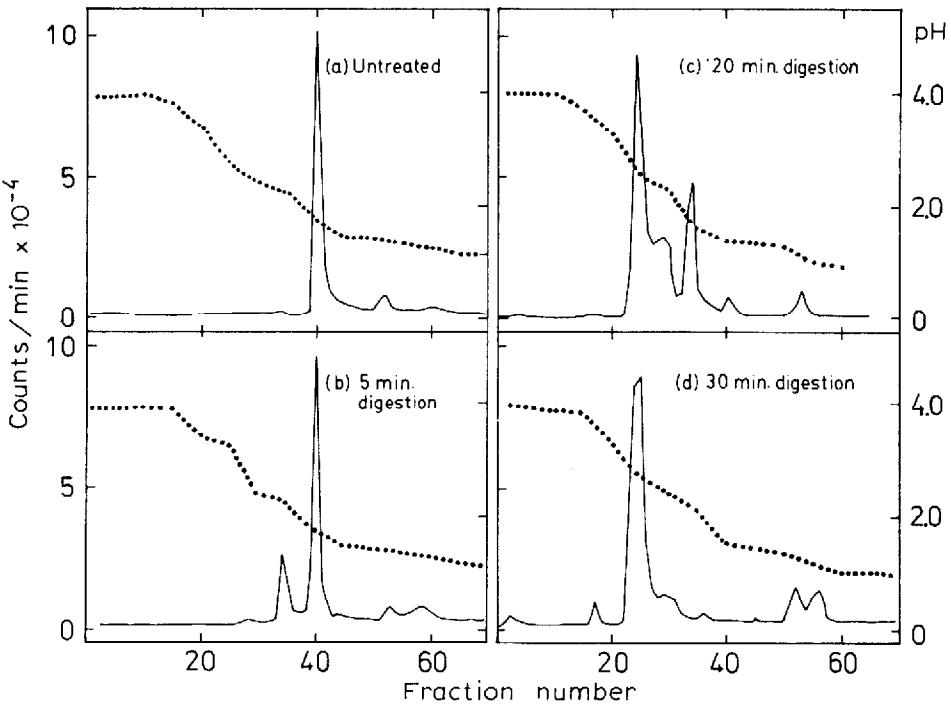

Fig. 5. Effect of neuraminidase on the chromatofocusing profile of glycophorin. [^{131}I]Glycophorin was incubated with neuraminidase for the time indicated, boiled for 10 min to destroy the enzyme then chromatofocused using the conditions described in Fig. 2.

None of the minor peaks had biological activity against WGA or against the two viruses. Of the major components, peaks V–VII had slight activity against WGA but none against the viruses, peak VIII was reasonably active against WGA but had low activity against viruses and peak IX was the most active against all three but this was not as high as for the original preparation.

DISCUSSION

In this report we have shown that proteins with isoelectric points below pH 4 can be separated from each other by chromatofocusing using stepwise application of simple buffers. In conventional chromatofocusing, the pH gradient is developed internally by elution with a single buffer containing a mixture of ampholytes, rather than externally by elution with a gradient. We regard the stepwise method we describe here as chromatofocusing in which a series of "mini"-gradients are internally produced, one after the other on the same column. Application of an external gradient certainly did not produce the equivalent desired result as the stepwise method (Fig. 1). Whether the procedure we describe here is regarded as true chromatofocusing, the result obtained was the same in that the major components in preparations of fetuin, acid glycoprotein and partially desialylated glycophorin were eluted in order of increasing sialic acid content which it is assumed is equivalent to decreasing isoelec-

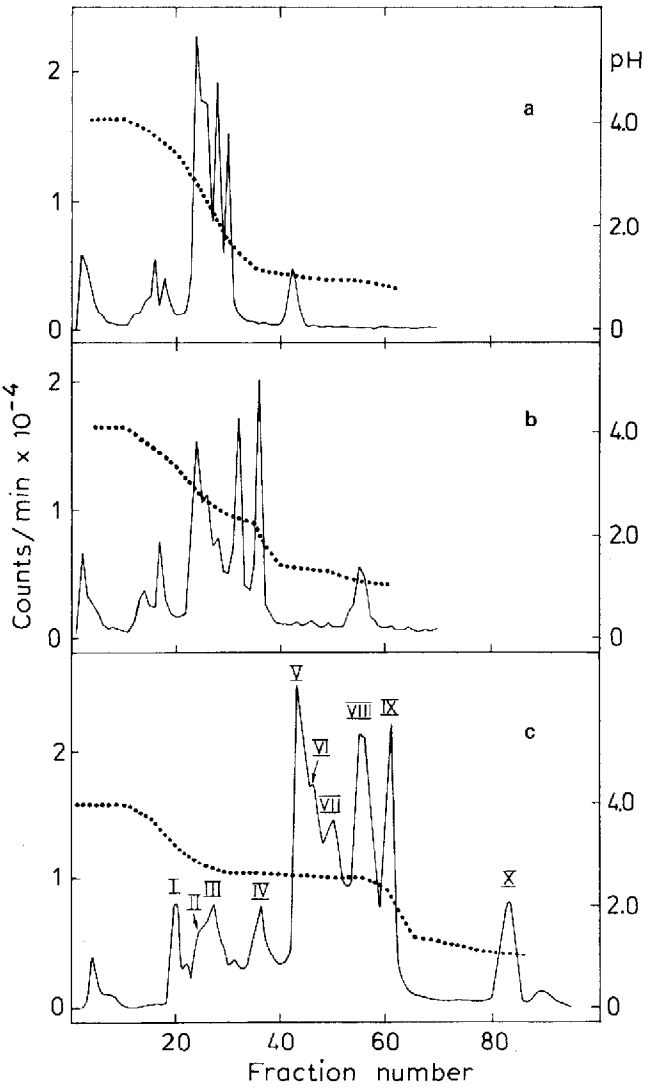

Fig. 6. Effect of eluate composition on chromatofocusing profile. [^{131}I]Glycophorin was incubated with neuraminidase for 15 min, boiled for 10 min to destroy the enzyme then chromatofocused using (a) A series; (b) B series or (c) C series of buffers. Properties of the components shown in (c) are given in Table I.

tric point. The behaviour of the minor components in partially desialylated glycophorin preparations remains to be explained.

The procedure we describe can almost certainly be improved upon. For instance, there is a requirement for an ion exchanger which has a higher buffering capacity than PBE 94 in the range pH 1–4 since we found that below pH 2, the pH dropped rapidly with loss of resolution. Another requirement is for multicomponent buffers which form a linear gradient in the range pH 1–4. The simple buffers we

TABLE I

PROPERTIES OF GLYCOPHORIN SPECIES OF DIFFERENT DEGREES OF SIALYLATION SEPARATED BY CHROMATOFOCUSING

ND = Not determined.

Sample	*pH of elution*	*μg sialic acid per mg protein*	*% HA inhibition activity against:*		
			WGA	*EMC*	*Influenza*
Original	–	516	100	100	100
Peak I*	3.20	512	0	0	0
II	2.80	456	0	0	0
III	2.67	348	0	0	0
IV	2.64	209	0	0	0
V	2.61	171	9	0	0
VI	2.59	164	9	0	0
VII	2.55	188	9	0	0
VIII	2.47	236	32	4	15
IX	2.03	373	58	67	50
X	1.07	162	ND	ND	ND

* See Fig. 6c.

describe are far from ideal, not because of their buffering capacity but because a peak of non-protein optical density at 280 nm is eluted in the pH 1.7 region of the gradient. This is only a problem if the elution of protein is being monitored by UV absorption rather than colorimetrically (*e.g.*, Lowry procedure) or by radioactivity measurements.

One reason for reporting our findings at this time is that once the need is perceived, reagents may be developed to operate in the pH 1–4 region. Another reason for describing our results now is that even though the procedure has not been perfected, the results probably could not have been obtained by any other method. Isoelectric focusing which has been extremely successful for protein separations has disadvantages compared with chromatofocusing for separating sialoglycoproteins. The time to reach equilibrium in isoelectric focusing is usually one or more days which together with the possibility of local heating and the low pH required could lead to cleavage of the acid labile ketosidic linkage joining sialic acid to oligosaccharide chains. The chromatofocusing technique we describe takes 4–8 h depending upon the flow-rate and buffer system used and the protein is eluted within about 15–20 min of being exposed to buffer of pH equal to its isoelectric point. Even though we ran the columns at room temperature, desialylation did not seem to be a problem. The evidence for this is that rechromatographed glycophorin eluted in the same same place as in the first run. More convincingly, glycophorin had the same haemagglutination inhibition titres before and after chromatofocusing.

Another advantage of chromatofocusing is that it can be used preparatively; about 50 mg fetuin and human acid glycoprotein were run in experiments described here (Figs. 2 and 3) with no problems. However, proteins are often poorly soluble at their isoelectric point and this could cause problems. For instance, we were not able to keep 50-mg quantities of bovine mucin in solution. Nervertheless, the simplicity of the technique, the ability to generate gradients of different shapes, to im-

prove resolution in the area of interest, the speed of the procedure and the amount of material that can be handled suggest that chromatofocusing should take place alongside isoelectric focusing as a powerful method for separating polyelectrolytes.

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