

High-Performance Capillary Electrophoresis of Glycoproteins: The Use of Modifiers of Electroosmotic Flow for Analysis of Microheterogeneity

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High-performance capillary electrophoresis (HPCE) is rapidly gaining acceptance as an analytical tool for the study of biological macromolecules. In the present study, the utility of HPCE for separation of glycoproteins is highlighted using a pure ovalbumin preparation. Ovalbumin, the 43-kDa glycoprotein of avian egg white, is known to be heterogeneous in nature with at least nine different carbohydrate structures having been identified on the single Asn residue. HPCE separation in an 87-cm capillary containing borate buffer and 1 mM putrescine resolves five major protein peaks in less than 30 min under nondenaturing conditions. This effect appears to be specific to glycoproteins since analysis of the nonglycosylated protein carbonic anhydrase under the same conditions showed no enhanced separation. The sodium borate buffer is proposed to play a key role in the separation by preferentially complexing with the diols of specific carbohydrate moieties on ovalbumin. Addition of putrescine enhances resolution by slowing bulk flow through the capillary and allowing electrophoretic separation of what is deduced to be closely related glycoforms of ovalbumin. Dephosphorylation of the ovalbumin with either calf intestinal alkaline phosphatase or potato acid phosphatase results in a shift of all peaks to a more rapid migration time and is consistent with a loss of negative charge. This suggests that all major ovalbumin isoforms are phosphorylated to the same degree and that heterology among ovalbumin isoforms resides solely in the carbohydrate structure. The enhanced resolution obtained with the employment of longer capillaries and modifiers of endo-osmotic flow was not restricted to ovalbumin since partial resolution of pepsin isoforms was observed under the same conditions. © 1992 Academic Press, Inc.

High-performance capillary electrophoresis (HPCE)⁴ is a relatively new tool for the analysis of biologically

active molecules. In addition to the capability of attaining separation efficiencies superior to those with high-performance liquid chromatography (HPLC), it also has the potential to overcome several of the problems associated with slab gel electrophoresis systems (1). Electrophoretic separation is carried out in a fused-silica capillary tube having an internal diameter of less than 100 μ m and a total volume of no greater than several microliters. The simplicity of the design, that is, use of a narrow-bore capillary, allows for the efficient dissipation of Joule heat. Therefore, electrophoretic separations can be carried out in free solution under high fields (up to 30,000 V), rapidly (<1 h), under denaturing or nondenaturing conditions, with very small sample volumes (as little as 5 μ l) and with tremendous efficiencies (several hundred thousand to one million theoretical plates). The result is a technique capable of rapid, automated, reproducible, and high-resolution separation of complex mixtures of molecules of diverse size and nature (2-4).

HPCE has been shown to be useful for the separation of a diverse array of molecules including ions (5), small organic molecules (6), sugars (7,8), peptides (9), and oligonucleotides (4). The electrophoretic separation of proteins in uncoated fused silica capillaries has been more problematic due mainly to protein interactions either with the inner capillary wall or with other proteins.

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⁴ Abbreviations used: HPCE, high-performance capillary electrophoresis; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; EOF, endo-osmotic flow; ABI, Applied Biosystems, Inc.; BSA, bovine serum albumin; TFA, trifluoroacetic acid; CIP, calf intestinal alkaline phosphatase; PAP, potato acid phosphatase buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

This problem has been circumvented through the use of chemical additives that either coat the inner wall or augment the buffer to reduce protein-wall interactions (10–14). While the basic principles for HPCE were developed in the early 1900s (1), the separation of proteins with this technique is somewhat unique since it can involve the resolution of complex mixtures under non-denaturing conditions based on differences in charge-to-mass ratios in relatively short periods of time. This provides several advantages over conventional techniques such as HPLC, FPLC (fast protein liquid chromatography), and other chromatographic techniques.

Ovalbumin is a glycoprotein of avian egg white. It is 385 amino acids in length and has a molecular weight of $\sim 43,000$ and two possible asparagine-linked glycosylation sites, one at amino acid 292 and the other at amino acid 312. Early studies established that the carbohydrate moiety of chicken ovalbumin was confined to a single Asn residue (15). However, later studies demonstrated that glycosylation at a single site (Asn₂₉₃) was an *in vivo* phenomenon and that Asn₃₁₂ could be glycosylated *in vitro* in a manner that was dependent upon the polypeptide and not the first glycosylation site (16). As with the carbohydrate component of most glycoproteins, ovalbumin carbohydrate structure is very heterogeneous (17). A total of nine different oligosaccharide chains have been identified (18–22) with eight of these chains composing the major glycan component of ovalbumin (23). All oligosaccharide chains identified thus far fall into one of two categories, high mannose or hybrid structures. The presence of substantial amounts of hybrid glycans makes ovalbumin somewhat unique and, interestingly, the hybrid and high mannose forms are present at approximately a 1:1 ratio.

In this study we describe the utility of HPCE for the analysis of glycoprotein microheterogeneity. An optimized HPCE separation of ovalbumin was carried out using conditions shown to be amenable to the separation of other glycoproteins such as the enzyme pepsin. The many glycoforms of these proteins can be putatively resolved when modifiers of endo-osmotic flow (EOF) and longer capillaries are employed.

MATERIALS AND METHODS

Materials. Boric acid, borax (sodium tetraborate), ovalbumin, carbonic anhydrase, calf intestinal and potato acid phosphatase, and putrescine (1,4-diamino butane) were obtained from Sigma (St. Louis, MO). Ovalbumin was also purchased from Pierce and Pharmacia. Mesityl oxide, a neutral marker, was part of a Micro-Coat Protein Analysis Kit from Applied Biosystems, Inc. (Foster City, CA). Pepsin was purchased from Boehringer-Mannheim (Indianapolis, IN). Polybuffer 74 was purchased from Pharmacia. The ovalbumin from Pharmacia was used for the majority of these studies.

FPLC and HPLC analysis. A dual-pump FPLC with LCC-500 control (Pharmacia) was used for analysis of ovalbumin molecular size and *pI*. Ovalbumin (2 mg/ml; 100 μ l) was loaded onto an FPLC Superdex 75 (molecular sieve) or Mono P (chromatofocusing) column in starting buffer. For molecular sieve chromatography the sample was loaded and run in phosphate-buffered saline, pH 8.3, at a flow rate of 0.5 ml/min (0.9 MPa). Molecular size of the protein was determined from a standard curve derived from the retention times for proteins with known molecular weights (BSA, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa). The chromatofocusing was run at a flow rate of 1 ml/min (1.5 MPa) using 25 mM Bis-Tris, pH 6.3, as the starting buffer and a 1:10 dilution of Polybuffer 74 as the eluent buffer. A pH gradient spanning a range of 3.0–6.3 was obtained as determined from a blank run. Detection for both was at 280 nm. For HPLC analysis, ovalbumin (25 μ g) was chromatographed using an ABI 130A Microseparation System with an ABI RP-300 C8 reverse-phase 2.1×100 -mm column. The buffers were as follows: A, 5% acetonitrile in water, 0.1% TFA; B, 70% acetonitrile in water, 0.1% TFA. The flow rate was 0.2 ml/min with a gradient of 50 to 100% B in 50 min. Detection was at 220 nm.

Buffer and sample preparation. Borate buffer was made by titrating 25 mM sodium tetraborate with 100 mM boric acid, to the desired pH. Borate buffer at other pH's were made from this stock using either NaOH or HCl. A modifier of electroosmotic flow (putrescine) was added to the borate buffers from a 1 M stock solution and the pH adjusted appropriately with either HCl or NaOH. All buffers were made with Milli-Q (Millipore) water and filtered through a 0.2- μ m filter (Gelman) before use. Proteins were dissolved in the running buffer without putrescine and filtered through a 0.22- μ m microcentrifuge filter (Millipore).

Instrumentation. HPCE separation was carried out on a Beckman P/ACE System 2050 interfaced with an IBM 55SX computer utilizing System Gold software (version 6.01) for control and data collection. Peak migration times, area, and height were obtained through the System Gold software.

Capillary electrophoresis separation conditions. New capillaries were conditioned by 20-column-volume rinses each with 0.1 M NaOH, water, and running buffer. For protein analyses, the following method was typically used: a 3-column-volume rinse with running buffer, 3 s pressure injection of protein in running buffer (0.5–1.5 mg/ml), 3 s pressure injection of the neutral marker mesityl oxide (1:250 in water), separation at 25 kV (constant voltage with the inlet as the anode and the outlet as the cathode), a 3-column-volume wash with 0.1 M NaOH (or 0.1 N HCl when putrescine was used), followed by a 3-column-volume rinse with running buffer. Between buffers, the capillary was washed

for 15 column volumes with 0.1 N HCl, followed by a 3-column-volume rinse with water and a 15-column-volume rinse with the new buffer. Polyimide-coated, fused-silica capillaries were 50 μm i.d. and either 40, 50, 80, or 100 cm in length (to the detector; total length, 7 cm longer). All electrophoretic separations were carried out at 25 kV constant voltage and capillary temperature was maintained at 28°C. Detection was by absorbance was at 200 nm.

Incubation of ovalbumin with putrescine. Ovalbumin (760 μg) was dissolved in 100 mM borate buffer, pH 8.5, either lacking or containing 1 mM putrescine. After incubation overnight at 4°C, both samples were dialyzed extensively against water in dialysis tubing with a molecular weight cutoff of 1000. The dialysate was frozen on dry ice and lyophilized. The integrity of both samples was determined by HPCE analysis after each was redissolved in 100 mM borate, pH 8.3.

Dephosphorylation of ovalbumin. Ovalbumin was dissolved in calf intestinal alkaline phosphatase buffer (CIP; 100 mM Tris, 20 mM MgCl_2 , 20 mM ZnCl_2 , pH 8.0) at a concentration of 2.13 mg/ml. To 500 μl of this solution was added 10 μg (10 μl = 1 U) of calf intestinal alkaline phosphatase or 20 μl of CIP buffer as a control. Ovalbumin was also dissolved in potato acid phosphatase buffer (PAP; 100 mM acetate buffer, pH 4.5) at a concentration of 2.18 mg/ml. To 500 μl of this was added 10 μg (20 μl = 1 U) of potato acid phosphatase or 20 μl of acetate buffer as a control. Samples were incubated in a shaker at 37°C for 12 h. All samples were microdialyzed against 100 mM borate (pH 8.5) before they were subjected to separation by HPCE.

RESULTS AND DISCUSSION

Chromatographic and High-Performance Capillary Electrophoretic Analysis of Ovalbumin

FPLC analysis of the ovalbumin indicates that the protein preparation used for this study was relatively homogenous (Fig. 1). Molecular sieve analysis shows an expected molecular mass of approximately 40 kDa as indicated by elution between BSA and CA (Fig. 1A). This was confirmed by the appearance of a single Coomassie-stained band of the same weight on denaturing polyacrylamide gel electrophoresis (data not shown). Chromatofocusing of the purified preparation shows a main peak corresponding to a *pI* of approximately 4.5–5.0 and a single shoulder having a somewhat higher *pI* (Fig. 1B). Reverse-phase high-performance liquid chromatography analysis of the ovalbumin sample showed some evidence for the presence of several molecular species (Fig. 1C). HPCE analysis of the same ovalbumin preparation was initially carried out using a 50- μm $\times$ 47-cm capillary and 100 mM borate buffer at pH 8.5. Under these conditions, EOF is relatively fast as evidenced by the short migration time for the neutral

marker, mesityl oxide, which was typically observed at ≈ 1.7 min. A main peak at 2.7 min is flanked on both sides by other peaks with slightly different charge-to-mass ratios (Fig. 2A). Under these conditions, as many as 3 other peaks were detectable by integration with the System Gold software. Analysis of the same sample in a 50- μm $\times$ 87-cm capillary resulted in an increase in neutral marker migration time (6.8 min) and a moderate increase in resolution as evidenced by the separation of several components from the main peak (Fig. 2B). The addition of putrescine (250 μM) in the running buffer had the expected effect, that is, a significant slowing of the EOF and a subsequent increase in the resolution of several peaks in the ovalbumin preparation (data not shown). Increasing the concentration of putrescine in the running buffer to 1 mM was optimal for resolving the components of the ovalbumin preparation; the dramatic increase in resolution probably resulted from the decrease in EOF (indicated by the neutral marker migration time of 13.4 min) (Fig. 2C). As many as 5 major peaks are reproducibly observed with 10–15 minor peaks. Increasing the putrescine concentration in the running buffer resulted in extending the run time markedly, e.g., 3 mM putrescine, 20.6 min. migration time for the neutral marker, and no sign of glycoprotein peaks after 140 min. The effect of putrescine on the EOF (as determined from the migration of mesityl oxide) was dramatic, decreasing EOF by 4-fold at a 5 mM concentration (Table 1). Ovalbumin purchased from other sources (Sigma and Pierce) and analyzed under the same conditions was found to have identical migration patterns, although the peak heights were proportionally dissimilar to that observed with the Pharmacia preparation (data not shown).

To ascertain that the peaks observed under these conditions represented protein and not simply free carbohydrate or contaminants in the purified preparation of ovalbumin, identical separations were carried out with detection at 200 and 280 nm in the absence and presence of 1 mM putrescine. Figure 3 shows that, while the absorbance at 280 nm was much lower than that at 200 nm (approximately 50-fold), the profiles at both wavelengths, either without putrescine (Figs. 3A and 3B) or with putrescine (Figs. 3C and 3D) in the buffer are virtually identical. The resolution observed in these electropherograms was comparatively poor as a result of having to overload the capillary (ovalbumin, 10 mg/ml; 6 nl injection) in order to obtain a detectable signal at 280 nm. It is unlikely that the peaks resolved in the presence of putrescine represent free carbohydrate. Sugars typically do not absorb strongly at 200 nm (and should not absorb at 280) and, therefore, the identical patterns of the electropherograms obtained with detection at both 200 and 280 nm implicate the presence of protein. Attempts were made to show, unequivocally, that the peaks observed in the presence of putrescine were indeed glycoforms of ovalbumin by deglycosylat-

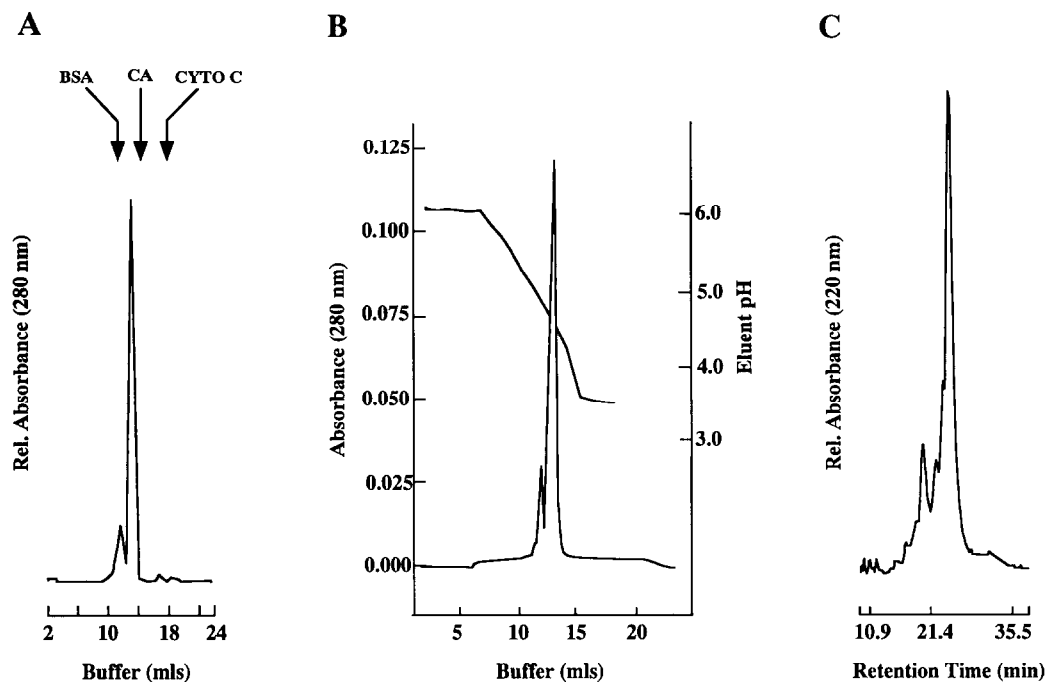


FIG. 1. Standard chromatography of purified ovalbumin. Analysis of ovalbumin by FPLC molecular sieve chromatography (A), FPLC chromatofocusing (B), and reverse-phase HPLC (C). Conditions are given under Materials and Methods. BSA (bovine serum albumin), CA (carbonic anhydrase) and cyto C (cytochrome C) have M_r of 66, 29 and 12 kDa respectively.

ing the protein. Unfortunately, chemical deglycosylation of ovalbumin with hydrofluoric acid resulted in a protein that was insoluble in borate buffer. This is not surprising since one of the *in vivo* functions of glycans on proteins is to maintain their solubility. The enzymatic (endoglycosidase F) cleavage was also not successful, most likely as a result of the very slow rate of cleavage known to accompany commercial preparations of these enzymes. The minor fraction of protein (<10%) that was deglycosylated was also suspected to be very insoluble and may have been lost during dialysis. SDS-PAGE showed that these treatments did not degrade the protein in any way.

pH Dependence of the HPCE Separation

Capillary electrophoretic separation of the components of the ovalbumin preparation was very pH-dependent. Separation in the presence of 1 mM putrescine in 100 mM borate (running buffer) over a pH range of 6–10 was carried out to optimize separation (Fig. 4). A pH of 9.0 appears to provide the optimal charge-to-mass ratio for separation of the components of the ovalbumin preparation, where the resolution of peaks 3 and 4 appears to be slightly better than that observed at pH 8.5 (Figs. 4C and 4D). Varying the borate buffer pH to less than 7 or greater than 9 resulted in a marked loss of resolution and a change in the charge on the protein. At a pH of 9.0, ovalbumin has a net charge of -18.15 , which accounts for a migration that is substantially slower

than EOF. Decreasing the running buffer pH to 6 significantly reduces the net negative charge on ovalbumin (-6.02), which appears to have a dramatic effect on the resolution and the migration time. It is interesting that the migration times for the major set of peaks follow the pattern predicted from the net charge of ovalbumin at a given pH. Ovalbumin acquires approximately four (net) negative charges per molecule with each one unit increase in pH between 6 and 9 but a substantially larger increase in charge between 10 and 11. This is vaguely reflected by the profiles in Figs. 4F–4J. However, in the presence of 1 mM putrescine, this trend tends to reverse itself, probably as a result of the putrescine-coated capillary. Altering the running buffer pH also has a predictable effect on the EOF. Table 2 outlines this effect in the absence and presence of 1 mM putrescine. The change in EOF is not surprising in light of the field strength of borate buffer at low pH. The amperage fell from $10.4 \mu\text{A}$ at pH 7 to about $5.4 \mu\text{A}$ at pH 6, which would subsequently decrease the electroosmotic flow and result in longer migration times.

Borate as a Requirement for Optimal Separation

The optimal resolution obtained by HPCE for ovalbumin was dependent not only upon the pH and ionic strength of the running buffer, but specifically on the buffer itself. Borate appears to be a necessary component of the buffer for attaining the high-resolution separation since substitution with phosphate buffer gave in-

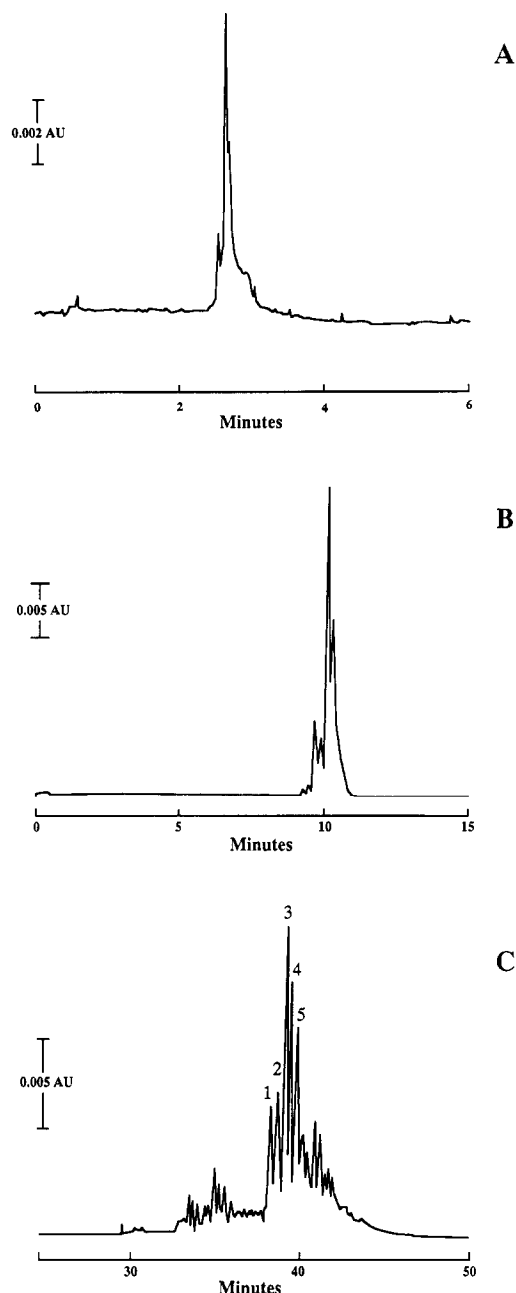


FIG. 2. High-performance capillary electrophoretic separation of ovalbumin. Purified ovalbumin (500 $\mu\text{g}/\text{ml}$) separated in an uncoated fused-silica capillary with an internal diameter of 50 μm containing 100 mM borate, pH 8.5, 3 s pressure injection in (A) a 47-cm capillary, 25 kV, 19 μA ; (B) an 87-cm capillary, 25 kV, 14.7 μA ; and (C) an 87-cm capillary, 1 mM putrescine in the running buffer, 20 kV, 10.6 μA .

ferior results. Figure 5 shows the representative electropherograms obtained in both borate and phosphate buffer systems containing 1 mM putrescine. Use of 100 mM phosphate buffer containing 1 mM putrescine resulted in high current at 25 kV ($\approx 100 \mu\text{A} = 2.87 \text{ W}/\text{m}$ compared with $20 \mu\text{A} = 0.57 \text{ W}/\text{m}$ for 100 mM borate) and necessitated the use of a 50 mM phosphate buffer for subsequent analysis. This change had a negligible

effect on resolution. The putrescine concentration in phosphate running buffer had to be increased to 5 mM before marked changes in the EOF and ovalbumin separation were observed.

Several studies have demonstrated the influence of borate on the separation of mono- or oligosaccharides by HPCE (24–26). Novotny and co-workers (7,8) have provided an explanation for the requirement of borate for optimal resolution. They found that fluorescently-labeled glucosamine and galactosamine, which differ only by orientation of the hydroxyl group on C_4 , were baseline resolved in borate but not phosphate buffer. They suggest that steric differences at this position on the sugar may result in differential borate-sugar diol complex formation. Interestingly, the electropherograms showing the separation of ovalbumin in the presence of putrescine are qualitatively similar to those obtained by Liu *et al.* (8) for a hydrolyzed oligosaccharide preparation. This suggests that the observed borate complexation-dependent separation of free carbohydrates may be relevant to the separation of borate-complexed carbohydrate linked to proteins. It is also interesting that nine different oligosaccharides have been identified on ovalbumin (18–22) and that five major and four to seven minor peaks are identified by HPCE. These data correspond very well with the results of HPCE analysis of oligosaccharides cleaved from ovalbumin (25). In that study, Honda and co-workers found five major oligosaccharides differing in size from the hepta- to the undecasaccharide. It is possible that these oligosaccharides (bound to the ovalbumin) are the constituents of the five major peaks observed in Figs. 4C and 4D.

In order to show that the effect of putrescine (in the presence of borate) was to slow endo-osmotic flow and not induce some type of protein degradative process, ovalbumin was incubated with 1 mM putrescine for an appropriate period of time and then removed by dialysis. Electropherograms of ovalbumin exposed to 1 mM putrescine for an extended period of time were qualitatively and quantitatively identical to those of freshly dissolved ovalbumin that had not been previously ex-

TABLE 1

The Effect of Putrescine on Endo-osmotic Flow (as Determined with Mesityl Oxide) in an 87-cm $\times$ 50- μm Uncoated Capillary

Concentration of putrescine (mM)	Migration time (min)
0	6.8
1	13.2
1.5	16.0
2	17.7
3	20.7
5	25.5

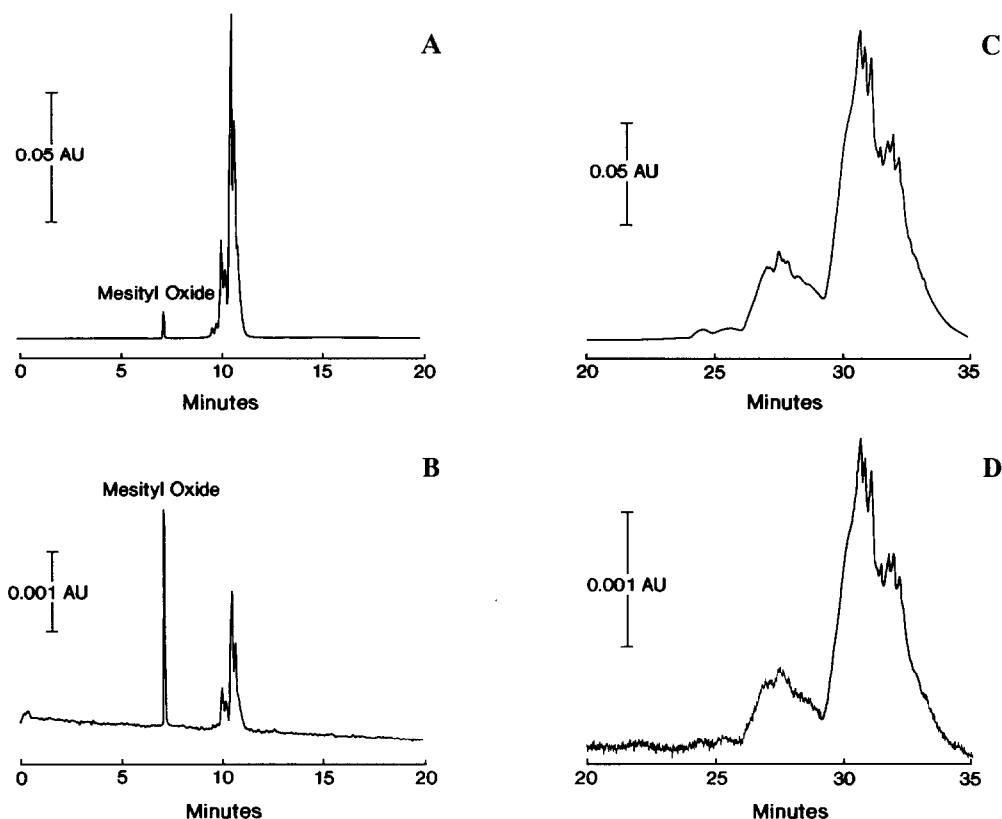


FIG. 3. Electropherograms of ovalbumin with detection at $\lambda = 200$ (A & C) and 280 nm (B & D). Electrophoretic separation was carried out in an 87-cm $\times$ 50- μ m capillary with 100 mM borate buffer, pH 8.5, 3 s injection of ovalbumin in running buffer (10 mg/ml), 25 kV, in the absence (A and B) and presence (C and D) of 1 mM putrescine.

posed to putrescine (data not shown). SDS-PAGE showed no apparent differences between fresh ovalbumin and that which had been exposed to the putrescine (data not shown).

Specificity of Glycoprotein Isoform Resolution

To rule out the possibility that the conditions described had a nonspecific effect on protein, separation of a nonglycosylated protein having roughly the same size and isoelectric point was carried out (data not shown). Carbonic anhydrase, a nonglycosylated protein of molecular weight 29,000 having a pI of 5.8, was separated in 100 mM borate containing 1 mM putrescine, pH 9.0. It migrated as a single intact peak with no indication of resolving the multitude of peaks observed with ovalbumin. Separation in borate buffer at several pH's was similar to that observed at pH 9.0.

Dephosphorylation of Ovalbumin

Two potential phosphorylation sites have been identified in ovalbumin on serine residues, one at position 68 and the other at position 344. Reported phosphate values for preparations of ovalbumin have varied from 1.75 to 1.89 (27,28) and seem to indicate that both sites are phosphorylated. Linderstrom-Lang and Ottensen (29,

30) suggested that two peaks, A_1 and A_2 , isolated by electrophoresis were the di- and monophosphate esters, respectively. This was conclusively demonstrated by Perlmann (31,32) through phosphatase digestion experiments. In the present study, incubation of ovalbumin with CIP buffer at pH 8.0 for 12 h at 37°C had little effect on the ovalbumin as indicated by the qualitative similarity of the profile in Fig. 6A with that of Fig. 4D. The presence of calf intestinal alkaline phosphatase under these same conditions (optimal for alkaline phosphatase activity) did not result in any noticeable change in the pattern but did induce a significant shift of the peaks originally appearing at 27–30 min (Fig. 6A) to the region spanning 23–25 min. (Fig. 6B). The peaks eluting at 25–26 min are unaffected by the enzymatic activity since they appear in both profiles at the same migration time. As with ovalbumin incubated in CIP buffer, ovalbumin exposed to PAP buffer at pH 4.5 for 12 h at 37°C had no detectable effect on the protein (data not shown). Addition of potato acid phosphatase to this system resulted in the same shift as that observed with the alkaline phosphatase, again with no obvious alteration of the pattern (Fig. 6C). The change in the electropherograms in response to enzymatic dephosphorylation by either calf intestinal alkaline phosphatase or potato acid phosphatase is interesting. This suggests that calf

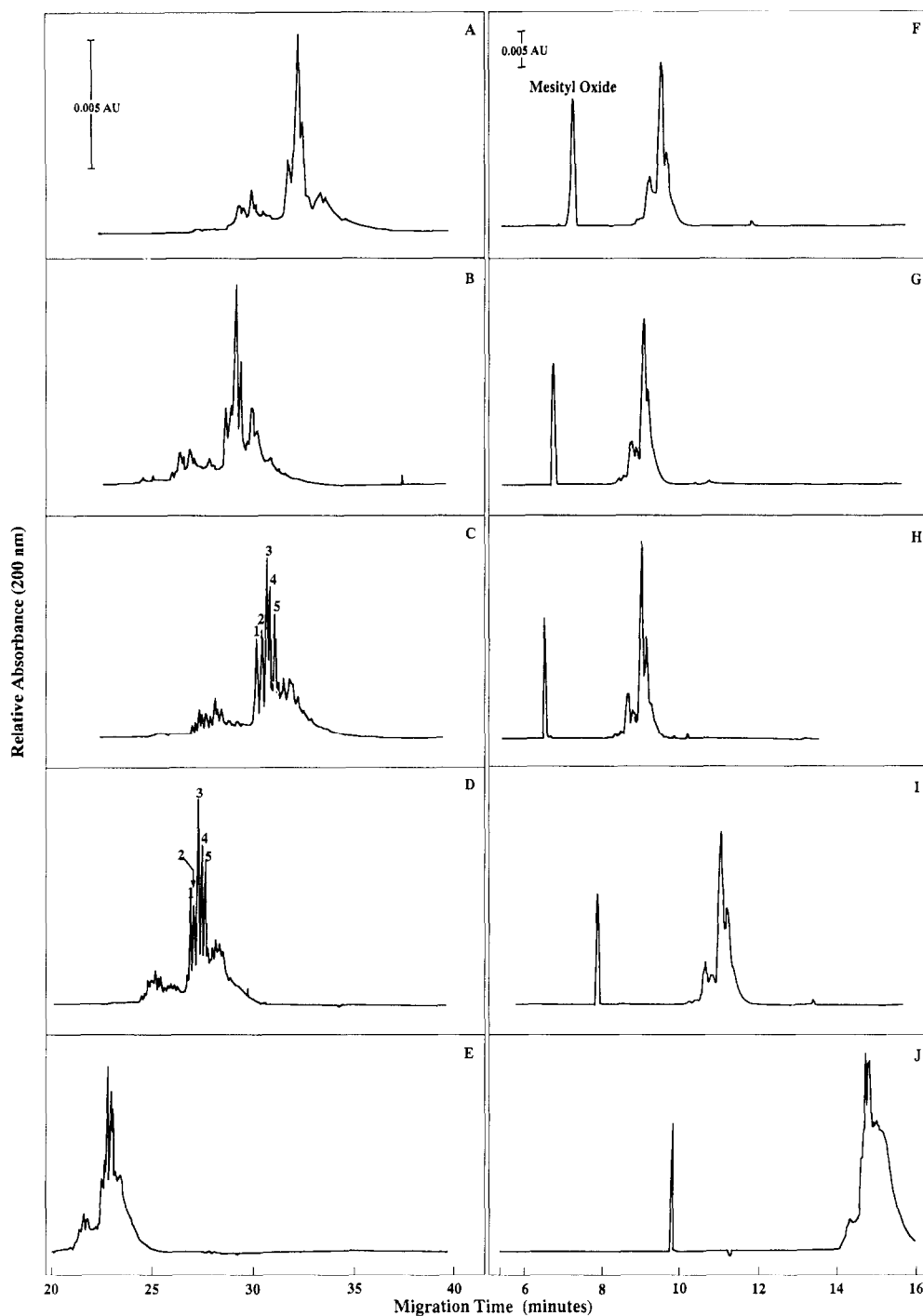


FIG. 4. pH dependence of electrophoretic separation of ovalbumin. Ovalbumin (1 mg/ml) in 100 mM borate buffer, pH 8.5, was pressure injected in an 87-cm $\times$ 50- μ m capillary equilibrated with the appropriate buffer and separated at 25 kV in the presence (A-E) and absence (F-J) of 1 mM putrescine. Running buffer pH's for A-E and F-J were 6, 7, 8, 9, and 10, respectively.

intestinal alkaline phosphatase and potato acid phosphatase convert most of the components of the ovalbumin preparation to molecular species with faster migration times. The resulting shift to an earlier migration time is consistent with the removal of (negative) phosphate groups (and hence a more rapid migration toward the cathode) and suggests that all ovalbumin glyco-

forms are phosphorylated. While we did not observe any conversion to an intermediate form (such as A_1 and A_2) nor three peaks consistent with A_1 , A_2 , and A_3 , it has been deduced that dephospho-ovalbumin is a minor (negligible) component of this preparation and the conversion of the monophosphate ester to the dephospho-protein is much slower than the dephosphorylation of

TABLE 2

Variation of EOF (Migration of Mesityl Oxide) as a Function of pH in the Presence or Absence of Putrescine

pH	Migration time (min)	
	+Putrescine	-Putrescine
6	16.6	6.9
7	14.4	6.4
8	13.9	6.2
9	13.5	7.5
10	12.3	9.3

A₁ to A₂ (31). Hence, assuming that complete dephosphorylation has occurred with both phosphatases, the results indicate that none of the peaks in the electropherogram represent ovalbumin glycoforms that are differentially phosphorylated (i.e., all ovalbumin molecules appear to be phosphorylated equally). This suggests that the microheterogeneous nature of ovalbumin may be due solely to the variation in the carbohydrate structure and, hence, the term "glycoforms" may more accurately represent ovalbumin microheterogeneity.

HPCE Separation of Other Glycoproteins

The enhanced resolution obtained through the use of putrescine as a modifier of electroosmotic flow in borate buffer appears to be an effect specific to glycoproteins. Analysis of the digestive enzyme pepsin gave results similar to those obtained with the ovalbumin in 100 mM borate buffer in a 50- μ m $\times$ 87-cm capillary with and without the addition of 1 mM putrescine (Fig. 7). The separation of this glycosylated protein is enhanced under the modified conditions while carbonic anhydrase (a nonglycoprotein) appears homogenous under the same conditions. A lower running buffer pH was required (pH 7 for pepsin vs 9 for ovalbumin) and a putrescine concentration of 1 mM was not adequate for the resolution of pepsin. Increasing the putrescine concentration in the running buffer to 2 mM had both positive and negative effects. The components of peak 2 begin to resolve while the resolution of peaks 3 and 4 appears to have decreased. While these results indicate that the described conditions are probably not universal for separation of all glycoproteins, they provide strong evidence that the combination of decreased EOF (via putrescine or like compounds), increased capillary length, and the presence of borate in the buffer may optimize the separation.

CONCLUSIONS

The results of this study have shown that through the employment of longer capillaries and putrescine as a buffer additive to reduce endo-osmotic flow, enhanced

resolution of proteins can be obtained in borate buffer.

This effect appears to be specific to glycoproteins, at least for the case of ovalbumin and pepsin. Based on the fact that (a) ovalbumin is known to be microhetero-

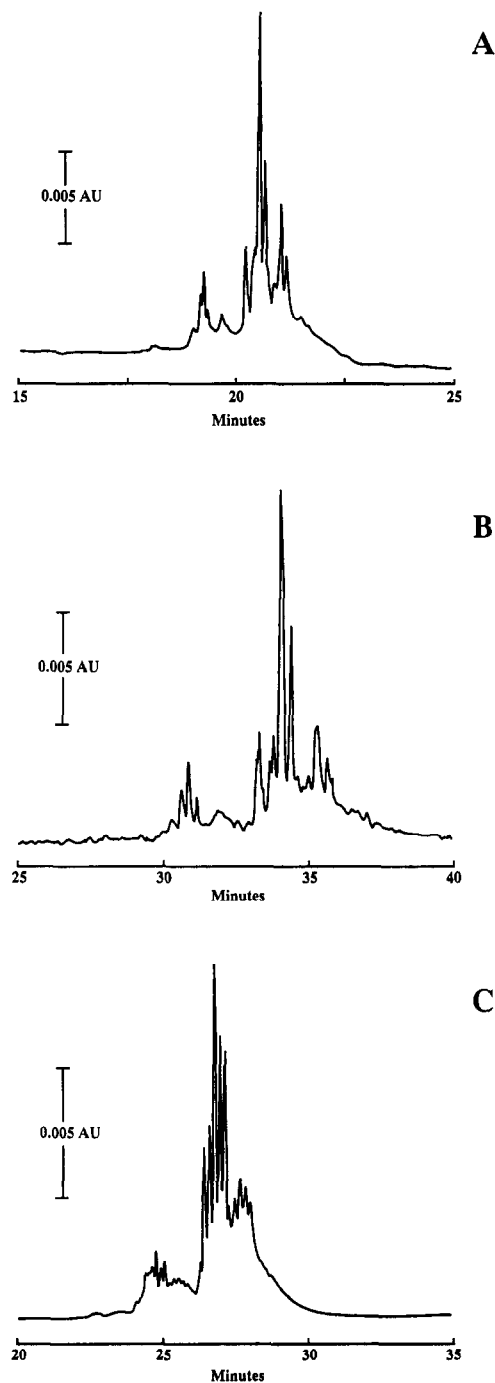


FIG. 5. Borate as a buffer requirement for ovalbumin separation. Ovalbumin (500 μ g/ml) in running buffer, pH 9.0, was pressure injected into an 87-cm $\times$ 50- μ m capillary equilibrated with the appropriate buffer and separated at 25 kV. The running buffers were (A) 50 mM phosphate + 1 mM putrescine (52.3 μ A); (B) 50 mM phosphate + 5 mM putrescine (54.2 μ A); and (C) 100 mM borate + 1 mM putrescine (13.6 μ A).

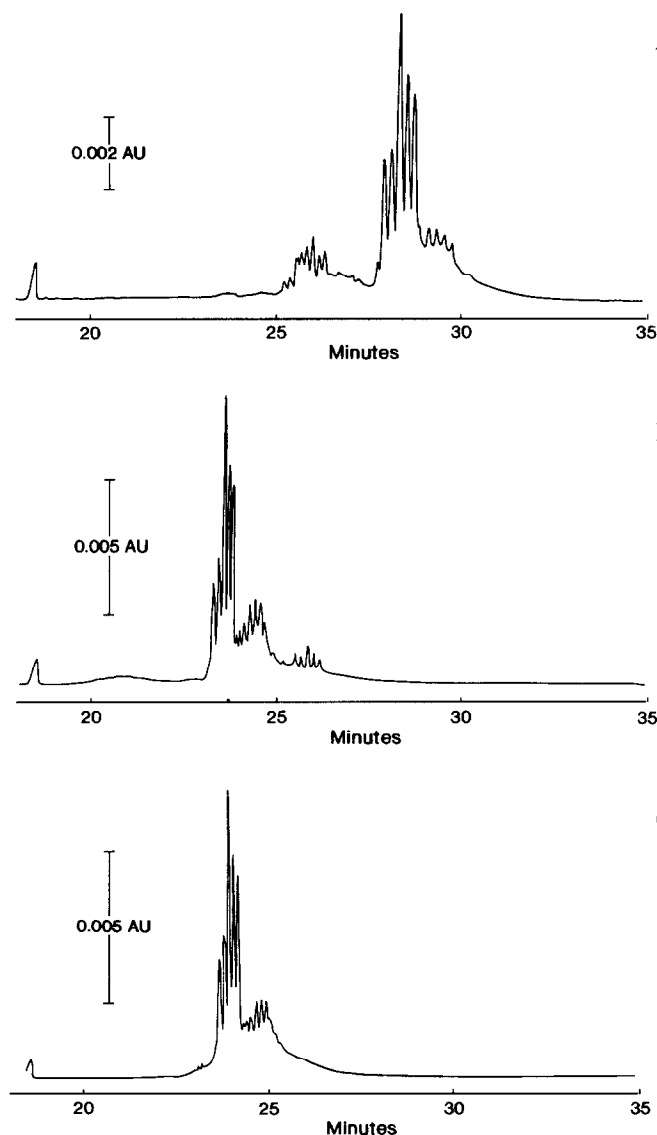


FIG. 6. Enzymatic dephosphorylation of ovalbumin. Ovalbumin that was either untreated (A) or treated with calf intestinal alkaline phosphatase (B) or potato acid phosphatase (C) was dialyzed into 100 mM borate, pH 8.5, 3 s pressure injected in an 87-cm $\times$ 50- μ m capillary equilibrated with the 100 mM borate buffer, pH 9.0, containing 1 mM putrescine and separated at 25 kV.

geneous, (b) oligosaccharides cleaved from ovalbumin are observed as five major peaks by HPCE analysis (26), (c) separation of a similar nonglycosylated protein is not enhanced under the same conditions, (d) borate is crucial to the enhancement of resolution, and (e) borate has been shown to have a similar effect on oligosaccharides, it is highly suggestive that the peaks observed represent isoforms of ovalbumin. Since no mechanism presently exists to explain how borate may enhance the separation of protein isoforms of ovalbumin, it is possible that the peaks represent specific glycoforms of ovalbumin. This is supported by the dephosphorylation studies described above, although differential borate-

glycoprotein complexation states cannot be ruled out. HPCE carried out under the described conditions may present a new approach for obtaining unprecedented resolution in the analysis of glycoprotein microheterogeneity.

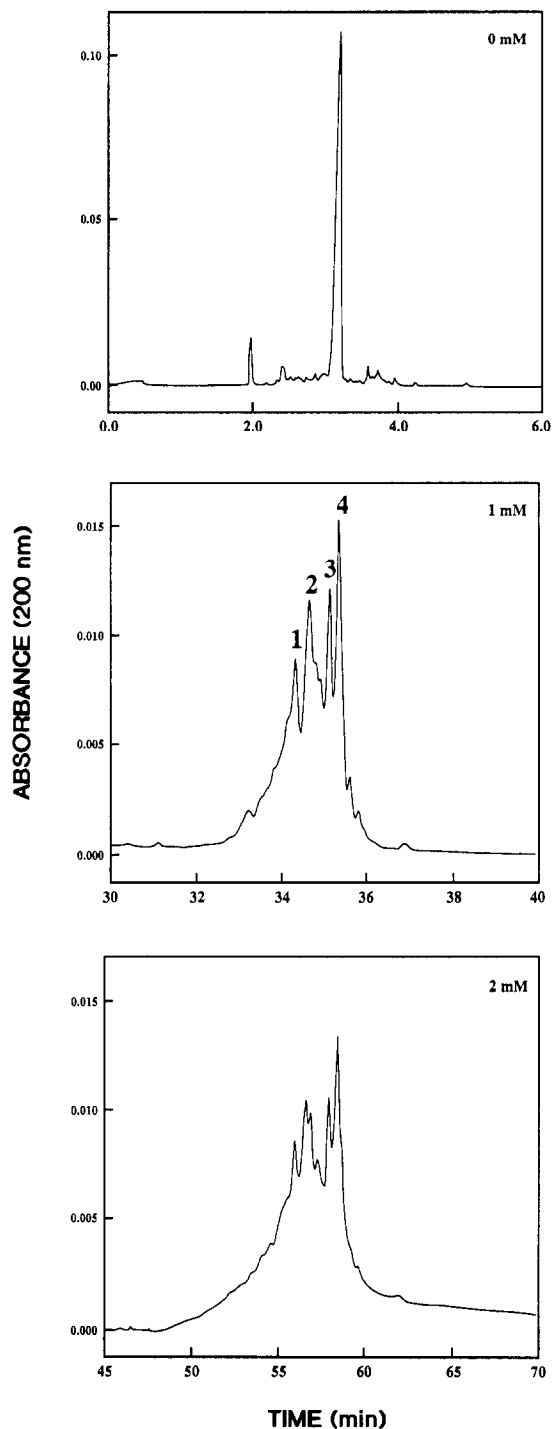


FIG. 7. HPCE separation of pepsin. Pepsin (0.5 mg/ml in borate buffer, pH 8.3) was separated in an 87-cm $\times$ 50- μ m capillary in the presence of 0, 1, and 2 mM putrescine in 100 mM borate buffer, pH 9.0, and separated at 25 kV.

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REFERENCES

1. Landers, J. P. (1991) *BioEssays* **13**, 253–258.
2. Grossman, P. D., Colburn, J. C., and Lauer, H. H. (1989) *Anal. Biochem.* **179**, 28–33.
3. Gordon, M. J., Huang, X., Pentoney, S. L., Jr., and Zare, R. N. (1988) *Science* **242**, 224.
4. Karger, B. L., Cohen, A. S., and Guttman, A. (1989) *J. Chromatogr.* **585**, 492.
5. Jones, W. R., Jandik, P., and Pfeifer, R. (1991) *Am. Laboratory*, 40–46.
6. Carney, S. L., and Osborne, D. J. (1991) *Anal. Biochem.* **195**, 132–140.
7. Liu, J., Shirota, O., Wiesler, D., and Novotny, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2302–2306.
8. Liu, J., Shirota, O., and Novotny, M. (1991) *Anal. Chem.* **63**, 413–417.
9. Grossman, P. D., Colburn, J. C., Lauer, H. H., Nielsen, R. G., Riggin, R. M., Sittampalam, G. S., and Rickard, E. C. (1989) *Anal. Chem.* **61**, 1186.
10. Gordon, M. J., Lee, K.-J., Arias, A. A., and Zare, R. N. (1991) *Anal. Chem.* **63**, 69–72.
11. Wiktorowicz, J. E., and Colburn, J. C. (1990) *Electrophoresis* **11**, 769.
12. Towns, J. K., and Regnier, F. E. (1991) *Anal. Chem.* **63**, 1126–1132.
13. Mazzeo, J. R., and Krull, I. S. (1991) *Biotechniques* **10**, 638–645.
14. Bolger, C. A., Zhu, M., Rodriguez, R., and Wehr, T. (1991) *J. Liq. Chromatogr.* **14**(5), 895–906.
15. Cunningham, L. W., Nuenke, B. J., and Nuenke, R. H. (1957) *Biochim. Biophys. Acta* **26**, 660.
16. Glabe, C. G., Hanover, J. A., and Lennarz, W. J. (1980) *J. Biol. Chem.* **255**, 9236–9242.
17. Huang, C.-C., Mayer, H. E., and Montgomery, R. (1970) *Carbohydr. Res.* **13**, 127–137.
18. Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975) *J. Biol. Chem.* **250**, 8569–8575.
19. Tai, T., Yamashita, K., Ito, S., and Kobata, A. (1977) *J. Biol. Chem.* **252**, 6687–6694.
20. Yamashita, K., Tachibana, Y., and Kobata, A. (1978) *J. Biol. Chem.* **253**, 3862–3869.
21. Sheares, B. T., and Robbins, P. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1993–1997.
22. Kato, Y., Iwase, H., and Hotta, K. (1986) *Arch. Biochem. Biophys.* **244**(2), 408–412.
23. Chen, L.-M., Yet, M.-G., and Shao, M.-C. (1988) *FASEB J.* **2**, 2819–2824.
24. Hoffstetter-Kuhn, S., Paulus, A., Gassmann, E., and Widmer, E. M. (1991) *Anal. Chem.* **63**, 1541–1547.
25. Honda, S., Iwase, S., Makino, A., and Fujiwara, S. (1989) *Anal. Biochem.* **176**, 72–77.
26. Honda, S., Makino, A., Suzuki, S., and Kakehi, K. (1990) *Anal. Biochem.* **191**, 228–234.
27. Osborne, T. B., and Campbell, G. F. (1900) *J. Am. Chem. Soc.* **22**, 422.
28. Machesboeuf, M., Sørensen, M., and Sørensen, S. P. L. (1925–27) *C. R. Trav. Lab. Carlsberg* **16**(12).
29. Linderstrom-Lang, K., and Ottensen, M. (1947) *Nature* **159**, 807.
30. Linderstrom-Lang, K., and Ottensen, M. (1949) *C. R. Trav. Lab. Carlsberg* **26**, 403.
31. Perlmann, G. E. (1952) *J. Gen. Physiol.* **35**, 711.
32. Perlmann, G. E. (1955) *Adv. Protein Chem.* **10**, 1.