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Comparative study on the distribution of ovalbumin glycoforms by capillary electrophoresis

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Abstract

Two commercial turkey egg ovalbumins (TEOs) with different quantities of mannose, were further purified by reversed-phase high-performance liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis for either of the purified glycoproteins showed one big wide band and one close small band. Capillary electrophoresis was used for the investigation of the separation of glycoforms of both glycoproteins. The best resolution of the glycoforms was obtained, reproducibly, with 100 m*M* borate, 1.8 m*M* 1,4-diaminobutane and pH 8.6 electrophoretic buffer. At least 13 glycoform peaks could be separated for either of the two glycoproteins. Their glycoform patterns were highly similar except for the conspicuous decrease in quantity of four glycoforms in the ovalbumin containing less mannose, compared to that of the other with more mannose. Coinjection electrophoresis of the two glycoproteins indicated that almost every glycoform peak of the former exactly overlapped with its corresponding glycoform peak of the latter. These results clearly indicated that the two TEOs possessed the same glycoform patterns but differed in quantity at least four glycoforms. It was found that the glycoform patterns were remarkably different between TEO and chicken egg ovalbumin.

Keywords: Ovalbumin; Proteins; Glycoproteins

1. Introduction

It is increasingly being recognized that the carbohydrate moieties of glycoproteins play many important roles in biological function. Usually, heterogeneity of the carbohydrate moieties causes heterogeneous population of glycosylation variants, which are called glycoforms. Although there is currently no strong experimental evidence to account for the reasons behind the microheterogeneity of glycoproteins, it is thought that the combinatorial biological activities of the glycoforms provide a 'composite' activity which the cell can control with high fidelity by varying the relative amount of each glycoform [1]. Recently, glycoform distributions of Prion have been applied as evidence that the agent which causes bovine spongiform encephalopathy (BSE) is also responsible for the 'new variant', Creutzfedt–Jakob disease (CJD) [2,3]. In addition, the pharmaceutical industry has realized the necessity of fast analysis of glycoform heterogeneity in order to control the cell status and culture environment so as to ensure consistency in the glycosylation pattern of a recombinant protein [4]. In the last ten

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years, capillary electrophoresis (CE) was demonstrated to be a powerful tool for the characterization of protein glycoforms [1]. Various modes of CE separation, such as, free solution capillary electrophoresis (FSCE), capillary isoelectric focusing (cIEF), micellar electrokinetic capillary chromatography (MEKC) and capillary isotachophoresis (cITP), were all exploited to resolve the heterogeneity of various glycoproteins. Excellent work on the separation of chicken egg ovablumin (CEO) glycoforms by CE was reported by Landers et al. [5]. Oda et al. [6] described the effectiveness of alkyl compounds with quaternary ammonium moieties in the separation of CEO glycoforms. Recently, Chen reported that the resolution of CEO glycoforms was improved by using a capillary coated with polyacrylamide [7].

However, the microheterogeneity of turkey egg ovalbumin (TEO) has not been examined by CE, and little work has been reported on glycoform distribution comparisons between glycoproteins with the same polypeptide chain and different carbohydrate content, or between the same kinds of glycoproteins but from different species. Here we report a comparative study on the distribution of glycoforms by CE, using preparations of two TEOs and one CEO as models.

2. Experimental

2.1. Chemicals

Two turkey egg ovalbumin (A5015, A7269, with the mannose content of 6.4 and 10.3 mol/mol protein, respectively), one chicken egg ovalbumin (A2512) and putrescine (1,4-diaminobutane) were obtained from Sigma. According to the product quality index, the purities of the two TEOs, A5015 and A7269, were both 99% as detected by electrophoresis on cellulose acetate in barbital buffer, and the purity of CEO A2512 was 99% as detected by agarose electrophoresis. Each of the three ovablumins was purified further by RP-HPLC for our study. Mesityl oxide was a part of a Micro-Coat Protein Analysis Kit from Applied Biosystems Inc. Acetonitrile and trifluoroacetic acid (TFA) were obtained from Merck. All other chemicals were of analytical reagent grade. Milli-Q water was used.

2.2. Instrumentation and method

CE was performed on an ABI 270A system from Applied Biosystems Inc. Electropherogram plotting and peak area calculating were performed by a Hewlett-Packard 3394A integrator. A HPLC system (ABI 150A) with an ABI RP-300 C_8 column (30× 2.1 mm I.D.) was used for further purification of ovalbumins. Solvent A was 0.1% TFA in water, and solvent B was 70% acetonitrile and 0.08% TFA in water. The flow-rate was set at 0.2 ml/min, and the gradient was from 20% B to 100% B in 65 min. The detection wavelength was 220 nm. Glycoproteins purified by RP-HPLC were collected, lyophilized and redissolved in water to a concentration of 1 mg/ml. Sodium dodecyl sulfate–polyacrylamid gel electrophoresis system (Bio-Rad) was used to analyze the purified ovalbumins.

2.3. CE separation conditions

Fused silica capillary of 72 cm (50 cm to detector window×50 μm I.D.) was used. Proteins were detected at 200 nm. Detector rise time and range were set at 0.5 s and 0.01 AU, respectively. Separation cabin temperature was controlled at 30±0.2°C. Prior to each sample injection, the capillary was sequentially washed with 0.1 *M* NaOH and water for 2 min each, then equilibrated with running buffer for 4 min.

3. Results and discussion

3.1. Purification of ovalbumin by RP-HPLC

Three impurities were detected and separated from the ovalbumins by RP-HPLC. From peak symmetry, all of the three ovalbumins appear to be homogenous on RP-HPLC. Coinjection analysis of A5015 and A7269, or of A7269 and A2512 showed they coeluated under RP-HPLC. This suggests that the three ovalbumins demonstrate the same retention behavior on RP-HPLC under the above conditions.

3.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of ovalbumins

The purified ovalbumins were then subjected to SDS–PAGE analysis. Fig. 1 indicates that both A5015 and A7269 are separated into two close coomassie-stained bands, whereas A2512 appears as a single band. Amino acid analysis of the two bands of A7269, electroblotted on polyvinylidene fluoride (PVDF) membrane, showed they have the same amino acid composition (data not given). It indicates that the purified A7269 is homogenous for the polypepetide moiety.

3.3. CE analysis of ovalbumins in phosphate buffer

Fig. 2 shows that the analysis of ovalbumins by CE in 50 m*M*, pH 2.5 phosphate buffer. The TEO peaks appears to be broader than the CEO peaks. Two slightly separated peaks for A5015 (Fig. 2A) and three for A7269 (Fig. 2B) can be observed. One small peak riding closely on the back of the major peak is also observed for A2512 (Fig. 2C). Three ovalbumins show similar migration time in low pH

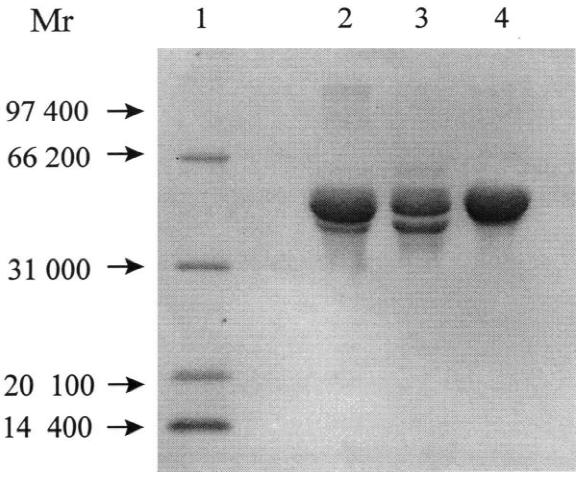

Fig. 1. Analysis of ovalbumins by a 13% SDS–PAGE (30% T, 2.7% C) with Coomassie staining [C=g *N*,*N*′-methylenebisacrylamide (Bis)/% T; T=(g acrylamide+g Bis)/100 ml solution]. Lanes 1, standard proteins; 2, A5015; 3, A7269; 4, A2512.

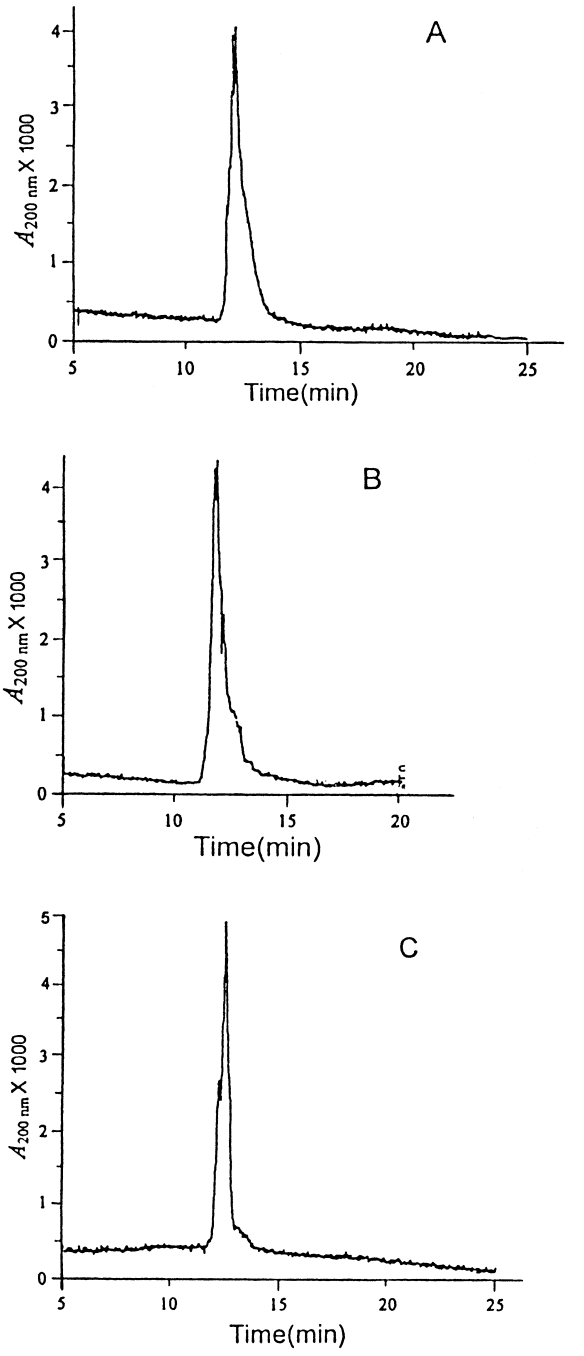

Fig. 2. Analysis of ovalbumins by CE in acidic phosphate buffer (A) A5015, (B) A7269, (C) A2512. Conditions: 50 m*M*, pH 2.5 phosphate buffer; voltage 20 kV; Current 22 μA; 1 mg/ml proteins, injection 1 s (vacuum); Other conditions are given in Section 2.2.

phosphate buffer. These results primarily demonstrate the microheterogeneity of these ovablumins.

3.4. CE of ovalbumins in disodium tetraborate buffer with high ionic strength

It was showed that a low pH phosphate buffer was obviously not suitable to resolution of ovablumin glycoforms. Borate has been shown to form stable complexes with *cis*-diols and has been used for separation of glycoforms in CE [5–8]. Because the tetrahydroxyborate ion, rather than boric acid, undergoes complexation with *cis*-diols [9], the effectiveness of borate buffer as an electrophoretic carrier for resolution of glycoforms is exhibited at alkaline pH. Separation of A5015 in 100 m*M* H_3BO_3–NaOH, pH 8.6 buffer, indicated the resolution was improved to some extent, compared to that in a phosphate, pH 2.5 buffer. However, the advantage of a borate buffer used here was still not presented sufficiently. The main reason for this is that the electroosmotic flow (EOF) is much greater in an alkaline buffer, thereby protein is swept quickly past the detector without full separation. Based on the characterization of EOF, several means to exterminate or suppress EOF have been proposed. Generally, one needs to employ, (1) a polyarylamide coated capillary; (2) use a high ionic strength buffer, or (3) the addition chemical additives, such as organic solvents and organic cations into the electrophoretic buffer.

Fig. 3 illustrates the electropherograms of the three ovalbumins in disodium tetraborate buffer with a high ionic strength. Under these conditions EOF is decreased and a better resolution is obtained. For both A5015 and A7269, five separate peak groups are observed, and A2512 is split into two peak groups. However, increasing the concentration of disodium tetraborate or potassium dihydrogen phosphate did not further improve the resolution in either case above. And a higher voltage (>150 V/cm) applied caused a decrease in the resolution of the close together peaks. This could be accounted for by the fact that higher ionic strength and higher voltage result in a large amount of Joule heat, then serious peak broadening and even collapsing. Therefore, while a high ionic strength buffer was used, the voltage applied was limited to under a certain value, which eventually hindered the resolution from being improved adequately.

3.5. CE of ovalbumins in borate buffer with putrescine as an additive

The application of putrescine as a modifier of EOF for the resolution of glycoforms, was first introduced by Landers group [5] and Taverna et al. [9], independently. After that, Watson and Yao [10] reported the separation of human recombinant erythropoietin (rhEPO) glycoforms by CE in an electric carrier contained putrescine. It has been considered that putrescine decreases the EOF by the following mechanism: the cationic amines interact with the negatively charged free silanol groups on the capillary. Because of the decrease in EOF, the migration time of the sample is increased and, thereby, the resolution of glycoforms is enhanced.

We investigated the influence of various concentrations of putrescine in 100 m*M* borate (H_3BO_3–NaOH), pH 8.6 buffer on the separation of A5015 glycoforms. Fig. 4 shows the relationship between EOF and the concentration of putrescine. The optimum concentration of putrescine is 1.8 m*M*. Fig. 5A and B show the separation of A5015 and A7269 glycoforms under optimum conditions in 100 m*M* borate, pH 8.6, 1.8 m*M* putrescine buffer and 20 kV voltage. As many as thirteen peaks are reproducibly separated in 35 min. The statistical results for the relative migration times to EOF of the glycoforms of A7269 are listed in Table 1. These indicate that good repeatabilities of the relative migration times were obtained. It is very interesting that the glycoform patterns of A5015 and A7269 are extremely similar with each other, and the relative migration times to EOF of the individual glycoform of the same peak number of the two ovalbumin, are almost the same. The electropherogram of coinjection of the two glycoproteins (1:1) demonstrates that the glycoform patterns of A5015 and A7269 overlap completely (Fig. 5C). Differences in the profiles of the glycoforms between the two glycoproteins lie mainly in the quantities of several glycoforms, peaks of 8, 9, 12 and 13. Fig. 6A and B show the comparison of the glycoform distribution by the percentage of peak integrated area of the individual glycoform of A5015 and A7269, respectively. The quantities of glycoform

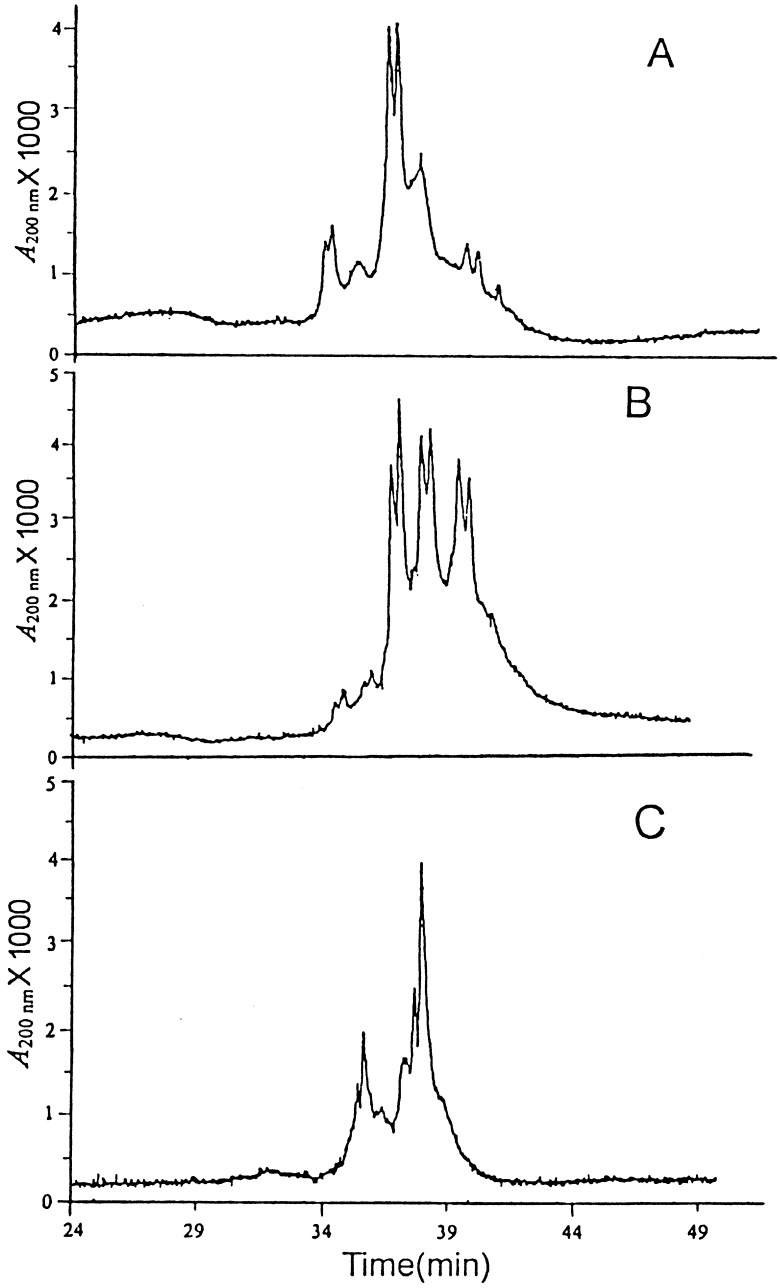

Fig. 3. Glycoform resolutions of ovalbumins by CE in high ionic strength borate buffer. (A) A5015; Buffer, 100 m*M* $Na_2B_4O_7$–100 m*M* KH_2PO_4, pH 8.2; voltage, 8 kV; current, 46 μA. (B) A7269; Buffer, 100 m*M* $Na_2B_4O_7$–40 m*M* KH_2PO_4, pH 9.0; voltage, 8 kV; current, 36 μA. (C) A2512; Buffer, 100 m*M* $Na_2B_4O_7$–60 m*M* KH_2PO_4, pH 8.8; voltage, 10 kV; current, 50 μA. Other conditions as in Fig. 2.

8, 9, 12 and 13 are obviously reduced in A5015, compared with those in A7269. These results are coincident with the facts that the two turkey ovalbumins are of the same polypeptide chain and A5015 contains less moles of mannose per mole of protein than A7269, according to the product quality index

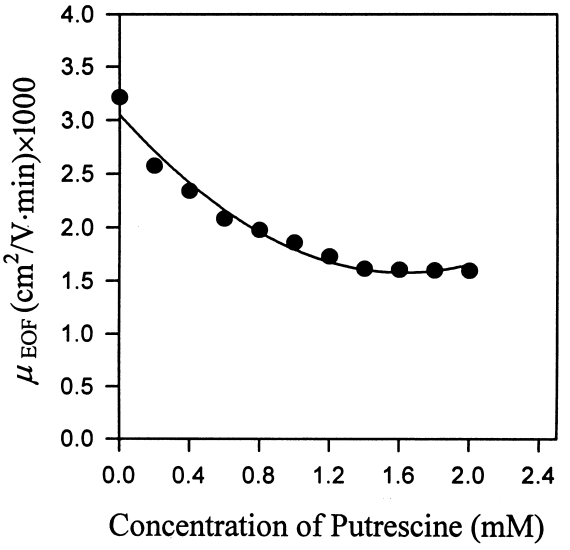

Fig. 4. The relationship between EOF and the concentration of putrescine in 100 m*M* H_3BO_3–NaOH, pH 8.6 buffer. The EOF was measured using a neutral marker of mesityl oxide.

and our monosaccharide analysis data, GlcNAC:Man are 2:4.3 and 2:8.8 for A5015 and A7269, respectively [11].

The glycoforms of CEO A2512 were also separated under the optimum conditions for TEO. The electropherogram of A2512, shown in Fig. 7, is very similar with the result obtained by Landers et al. [5]. Landers et al. [5] and Legaz et al. [12] have, separately, ruled out the possibility that putrescine or spermidine added to the electrophoretic buffer had a non-specific effect on the glycoform resolution of CEO. These facts prove that the analysis of glycoform by CE in borate buffer with putrescine additive can give out reproducible and reliable results.

Electropherograms (Figs. 5 and 7) demonstrate that TEO and CEO have, approximately, the same number of glycoforms. It may be noted, however, that the differences in glycoform migration profiles between TEO and CEO are so remarkable. Firstly, the majority of glycoforms of CEO migrate (against EOF) faster than those of TEO. A similar phenomenon was also observed previously by Fothergill et al. [13] in starch gel electrophoresis for the two ovalbumins. Secondly, differences in the distribution of glycoform migration times obviously exist between them. Factors from three aspects may be proposed to give an explanation for the fact that the mobility of CEO is faster than that of TEO. One is from polypeptide chain structures. Both CEO and TEO are 386 amino acids in length, of which 36 amino acid residues in the primary sequence are different between the two ovalbumins. Because Glu-144 and Glu-320 in CEO are changed into Gly-144 and Gly-320 in TEO, respectively, and other amino acid residue changes only take place between the neutral amino acid residues, CEO may acquire a greater amount of negative charge than TEO in the same alkaline buffer. Therefore, the former can migrate to the anode more quickly than the latter. The next one is from partial phosphorylation of the two ovalbumins. Both CEO and TEO are known as phosphorylated proteins. The results obtained by Landers et al. [5] suggest that all CEO glycoforms are almost completely phosphorylated. If TEO is partially phosphorylated, there is a possibility that this TEO, with less negative charge, migrates more slowly than completely phosphorylated CEO. The electropherograms of high voltage paper electrophoresis of trypsin digested CEO and TEO, performed by Forthergill et al. [13], demonstrated that both ovalbumins had 18 fragments. That indicates TEO is also an almost completely phosphorylated protein, otherwise it would must have produced more than 18 fragments. Hence, the factor attributed to partial phosphorylation may be negligible. The third is from different structures of carbohydrate chains linked to proteins. In an alkaline borate buffer different oligosaccharide structures may result in differential borate–sugar diol complex formation and in differential negative charges added to glycoproteins. Eventually, the mobilities of the glycoproteins are changed.

As opposed to this, the differences in the distribution of glycoform migration times between CEO and TEO indicate the differences in oligosaccharide structures between the two glycoproteins. They both have two possible asparagine-linked glycosylation sites, one at Asn-293 and the other at Asn-312. The structures of the carbohydrate chains derived from CEO have been studied intensively and at least nine oligosaccharides have been identified in detail and were found to be very heterogeneous and complicated [14–22]. They can be sorted into two types of N-linked glycans, high mannose and hybrid oligosaccharide. The sum total of each type of oligo-

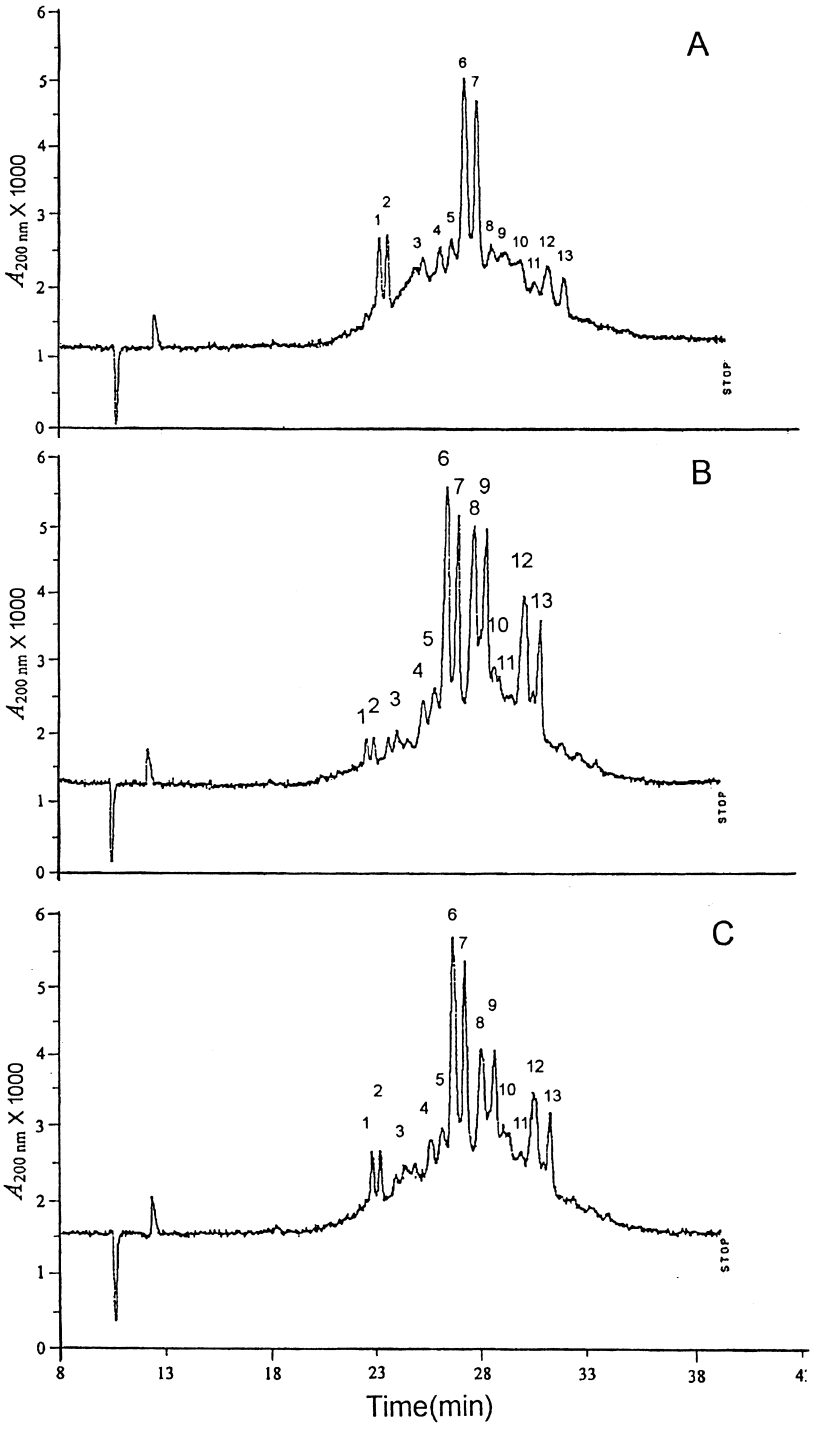

Fig. 5. Glycoform resolutions of turkey egg ovalbumins by CE in borate buffer with putrescine additive. (A) A5015. (B) A7269. (C) A5015 and A7269 (1:1). Conditions: buffer, 100 m*M* H_3BO_3–NaOH, pH 8.6, 1.8 m*M* putrescine; voltage, 20 kV; current, 10 μA; Other conditions as in Fig. 2.

Table 1
Relative migration times to EOF of the glycoforms of TEO (A7269)

No. of glycoform	$(t_m)_r$ [a]	RSD (%) (n=6)
1	2.187±0.029	1.6
2	2.222±0.031	1.7
3	2.353±0.029	1.5
4	2.456±0.040	2.0
5	2.505±0.050	2.4
6	2.568±0.045	2.1
7	2.619±0.047	2.2
8	2.690±0.053	2.4
9	2.754±0.054	2.4
10	2.809±0.053	2.3
11	2.885±0.052	2.2
12	2.939±0.063	2.6
13	3.016±0.067	2.7

[a] $(t_m)_r$ was expressed as the mean±standard error, n=6.

saccharide chain present in CEO are considered to be approximately equal. Unfortunately, the structures of oligosaccharides derived from TEO have not been reported yet. The glycoforms of CEO shown in the electropherogram are separated into two groups. For each group, the glycoforms migrate very closely. TEO glycoforms are well separated and their migration times are relatively well-distributed. This might suggest that the structures of oligosaccharide chains from TEO are not as complicated as those of CEO. Further work to identify the structures of the carbohydrate chains from TEO will benefit the further interpretation of differences in migration profiles between CEO and TEO.

It has been made conclusively clear that almost all glycoproteins are characterized by microheterogeneity. However, whether every peak from certain 'pure' glycoprotein separated by CE, for example in the ovalbumins case, arises from the microheterogeneity of the carbohydrate chains attached to the protein or not, is currently and strictly not clear in general. Microheterogeneity of a protein may be categorized in more than one way [14], genetic variation, glycosylation, partial phosphorylation, partial acylation of free amino groups, disulfide interchange, even differential borate-glycoprotein, etc. To rule out all of such possibilities other than glycosylation is obviously difficult and labor-intensive. A more powerful and promising technique, capillary electrophoresis–mass spectrometry (CE–MS), has been developed for the characterization of the glycoforms of an intact glycoprotein [23]. Theoretically,

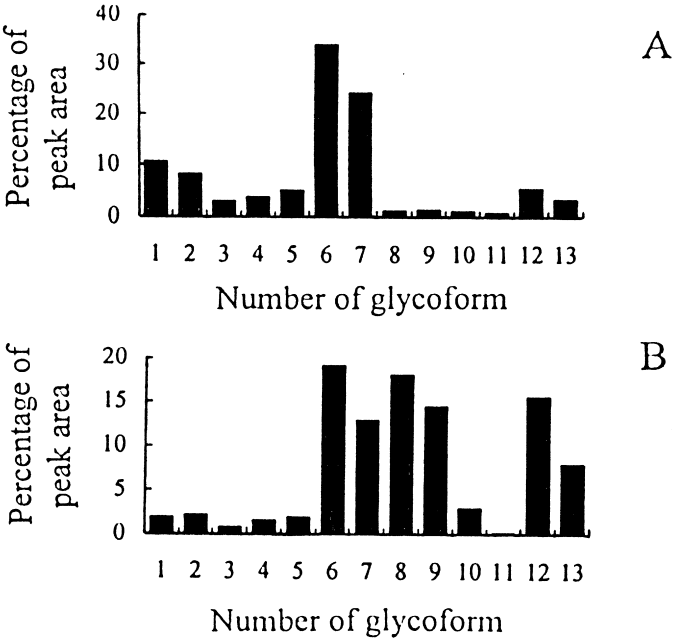

Fig. 6. Comparison of the glycoform distribution between two turkey egg ovalbumins A5015 (A) and A7269 (B).

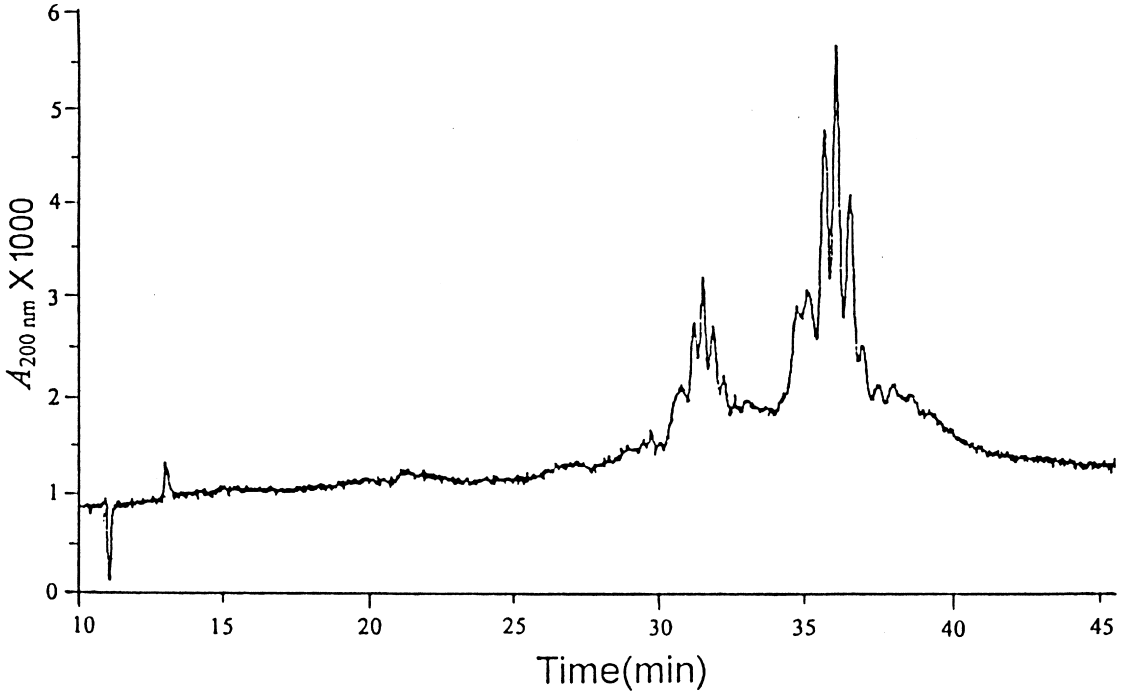

Fig. 7. Glycoform resolution of chicken egg ovalbumin A2512 by CE in borate buffer with putrescine additive. Conditions as in Fig. 5.

CE–MS can be used to identify a certain peak of a glycoprotein separated by CE as a unique glycoform peak or not. But CE–MS suffers from poor concentration detection limits and an incompatibility with some electrophoresis buffers which are beneficial to the resolution of glycoforms. More endeavors are needed to circumvent these limitations.

4. Conclusions

This paper reports the resolution of glycoforms of two TEOs (A5015 and A7269) and one CEO (A2512) by capillary electrophoresis. A comparative study on the glycoform pattern was performed among the three ovalbumins. Resolution in high ionic strength electrophoretic borate buffers of 100 m*M* disodium tetraborate containing 40–100 m*M* potassium dihydrogen-phosphate, pH 8.2–9.0, is better than the low ionic strength borate buffer of 100 m*M* H_3BO_3–NaOH, pH 8.6. However, the optimum resolution of ovalbumin glycoforms was achieved in a buffer of 100 m*M* H_3BO_3–NaOH, pH 8.6, 1.8 m*M* putrescine. The key to the successful separation of the three ovalbumin is a suitable concentration of putrescine in borate buffer to reduce the rate of EOF.

Two TEOs, A5015 and A7269, have the same polypeptide chain, but contain different quantity of mannose per mole of protein. CEO and TEO are the phylogenetically more closely related proteins. Our results show that, (1) as expected, A5015 and A7269 have identical glycoform patterns, but the quantities of four glycoforms in A5015 are obviously less than those in A7269; (2) differences in the migration profiles between TEO (A7269 or A5015) and CEO (A2512) are observed. It is highly suggested that these differences might result from both a difference in polypeptide structures and a difference in carbohydrate chain structures between TEO and CEO.

Acknowledgements

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References

[1] R.P. Oda, B.J. Madden, J.P. Landers, Adv. Chromatogr. 26 (1996) 163.
[2] J. Collinge, K.C.L. Sidle, J. Meads, J. Ironside, A.F. Hill, Nature 383 (1996) 685.
[3] A.F. Hill, M. Desbruslais, S. Joiner, K.C.L. Sidle, I. Gowland, J. Collinge, L.J. Doey, P. Lantos, Nature 389 (1997) 448.
[4] P. Tanswell, E. Seifried, E. Stang, J. Krause, J. Arzneim, Forsch 41 (1991) 1310.
[5] J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelsberg, Anal. Biochem. 205 (1992) 115.
[6] R.P. Oda, B.J. Maddem, T.C. Spelsberg, J.P. Landers, J. Chromatogr. A 680 (1994) 85.
[7] Y. Chen, Sci. China (Ser. B) 41 (1998) 71.
[8] M. Taverna, A. Balliet, D. Biou, M. Schulter, R. Werner, D. Ferrier, Electrophoresis 13 (1992) 359.
[9] Z.E. Rassi, Adv. Chromatogr. 23 (1994) 177.
[10] E. Watson, F. Yao, Anal. Biochem. 210 (1993) 389.
[11] F.Y. Che, Z.Y. Liu, K.Y. Wang, Q.C. Xia, Acta Biochim. Biophys. Sinica 30 (1998) 251.
[12] M.E. Legaz, M.M. Pedrosa, J. Chromatogr. A 719 (1996) 159.
[13] L.A. Fothergill, J.E. Forthergill, Eur. J. Biochem. 17 (1970) 529.
[14] C.C. Hung, H.E. Mayer, R. Montgomery, Carbohydr. Res. 13 (1970) 127.
[15] T. Tai, K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inoue, A. Kobata, J. Biol. Chem. 250 (1975) 8569.
[16] T. Tai, K. Yamashita, S. Ito, A. Kobata, J. Biol. Chem. 252 (1977) 6687.
[17] K. Yamashita, Y. Tachibana, A. Kobata, J. Biol. Chem. 253 (1978) 3862.
[18] B.T. Sheares, P.W. Robbins, Proc. Natl. Acad. Sci. USA 83 (1986) 1993.
[19] Y. Kato, H. Iwase, K. Hotta, Arch. Biochem. Biophys. 244 (1986) 408.
[20] L.M. Chen, M.G. Yet, M.C. Shao, FASEB J. 2 (1988) 2819.
[21] A.S. Welskopf, P. Vouros, D.J. Harvey, Rapid Commun. Mass Spectrom. 11 (1997) 1493.
[22] A.S. Welskopf, P. Vouros, D.J. Harvey, Anal. Chem. 70 (1998) 4441.
[23] J.F. Kelly, S.J. Locke, L. Ramaley, P. Thibault, J. Chromatogr. A 720 (1996) 409.