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USE OF CAPILLARY ISOTACHOPHORESIS TO MEASURE THE DEGREE OF CHEMICAL SUBSTITUTION OF DOG SERUM ALBUMIN

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SUMMARY

Analytical capillary isotachophoresis may provide a means for the rapid determination of the degree of substitution of proteins. Dog albumin conjugates with trinitrophenyl (TNP), fluorescein isothiocyanate (FITC) or monomethoxy polyethylene glycol (mPEG) have been studied as models for protein modification. An increase in the degree of covalent substitution of dog albumin with TNP, FITC or mPEG could be monitored rapidly using the UV signal or the conductivity signal as a measure of the increased protein zone width. These findings are encouraging with regard to the possible use of capillary isotachophoresis for screening the extent of protein modification.

INTRODUCTION

Chemical modification of proteins is performed for a variety of reasons, *e.g.*, protein determination, amino acid side-chain determination, sequential studies and studies of protein conformation and protein function[1]. Proteins conjugated with different chemical groups are often used as model compounds in biological systems for studying different immunological responses (*e.g.*, induction of immunological response, induction of specific tolerance). Whatever the purpose of protein modification, there is always a need for simple and rapid methods to determine the extent of the modification. Various methods have been used for this purpose, such as measurement of the decrease in biological activity, amino acid analysis, spectrophotometry, amino acid side-group reagents, electrophoresis, circular dichroism and nuclear magnetic resonance spectroscopy.

All of these methods, however, have certain limitations, rendering it necessary to seek other more rapid, high-resolution techniques. Recently, capillary isotachophoresis has been utilized to study ligand binding to serum albumin[2,3]. The high resolving power of isotachophoresis in conjunction with the requirement for a small amount of sample suggests that this technique might provide a means for the rapid analysis of hapten–protein conjugates.

In this paper, we report on the use of isotachophoresis to study the degrees of substitution of dog serum albumin conjugates. The technique offers the opportunity

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of analysing conjugates between dog serum albumin and trinitrophenyl (TNP), fluorescein isothiocyanate and monomethoxy polyethyelene glycol.

EXPERIMENTAL

Dog serum albumin (DA), fraction V powder, was bought from Sigma (St. Louis, MO, U.S.A.). Monomethoxy polyethylene glycol (mPEG) with an average molecular weight of 2000 daltons was a gift from Union Carbide, (New York, NY, U.S.A.). 2,4,6-Trinitrobenzensulphonic acid (TNBSA) was purchased from Eastman-Kodak Co. (Rochester, NY, U.S.A.). Fluorescein isothiocyanate (FITC) was prepared from aminofluorescain by established procedures[4].

DA(TNP)$_n$ conjugates

Three different DA(TNP)$_n$ conjugates were prepared by reacting 400 mg of DA with 10, 20 or 50 mg of TNBSA in 20 ml of 0.5 *M* sodium hydrogen carbonate–sodium carbonate buffer (pH 8.6) for 6 h at room temperature. The DA(TNP)$_n$ conjugates were purfied by gel filtration on Sephadex G-25 using 0.5 *M* sodium chloride solution as the eluent. The collected fraction was desalted and concentrated by ultrafiltration through a PM 30 filter (Amicon, Lexington, MA, U.S.A.) and then freeze-dried. TNBSA reacts with primarily amino groups (end groups) and ε-amino groups (lysine) in proteins. The degree of substitution was determined by amino acid analysis[5] by comparing the lysine content in DA(TNP)$_n$ conjugates with that in unmodified DA. The analysis indicated that DA(TNP)$_5$, DA(TNP)$_{10}$ and DA(TNP)$_n$ were obtained.

DA(FITC)$_n$ conjugates

Four different DA(FITC)$_n$ conjugates were prepared by reacting 400 mg of DA with 12, 40, 160 or 320 mg of FITC. The first three reactions were run in 20 ml of 0.13 *M* borate buffer (9.7) for 3 h at 8°c and the fourth in 10 ml of 0.5 *M* borate buffer (pH 9.8) for 3 h at 20°C. FITC was dissolved in a minimum amount of dimethyl sulphoxide (0.2–1.2 ml) before addition to the protein–buffer solution. Hydroxylammonium chloride (1.08 g) sodium hydroxide (0.62 g) were added to the reaction solution in such a way that pH was kept above 8.8. Hydroxylamine reacts with the excess of FITC and decouples it from unstable bonds with the protein.

The DA(FTIC)$_n$ conjugates were purified by gel filtration on Sephadex G-25 using 0.5 *M* sodium chloride solution as eluent. The collected fraction was desalted and concentrated by ultrafiltration through a YM-5 filter (Amicon). The degree of substitution was determined by measuring the UV absorption at 493 nm and pH 9.2 of the DA(FITC)$_n$ conjugates and fluorescein thiocarbamate[6]. The analysis indicated that DA(FITC)$_3$, DA(FITC)$_9$, DA(FITC)$_{17}$ and DA(FITC)$_{40}$ were obtained.

DA(mPEG)$_n$ conjugates

Three different DA(mPEG)$_n$ conjugates were prepared by reacting 400 mg of DA with 1.0, 1.4 or 5.0 mg of monomethoxypolyethylene glycol succinate of molecular weight 2000 daltons using the mixed anhydride method[7]. The product was purified by gel chromatography on Sepharose 6B using water as the eluent and then freeze-dried. The degree of substitution was determined by measuring the protein

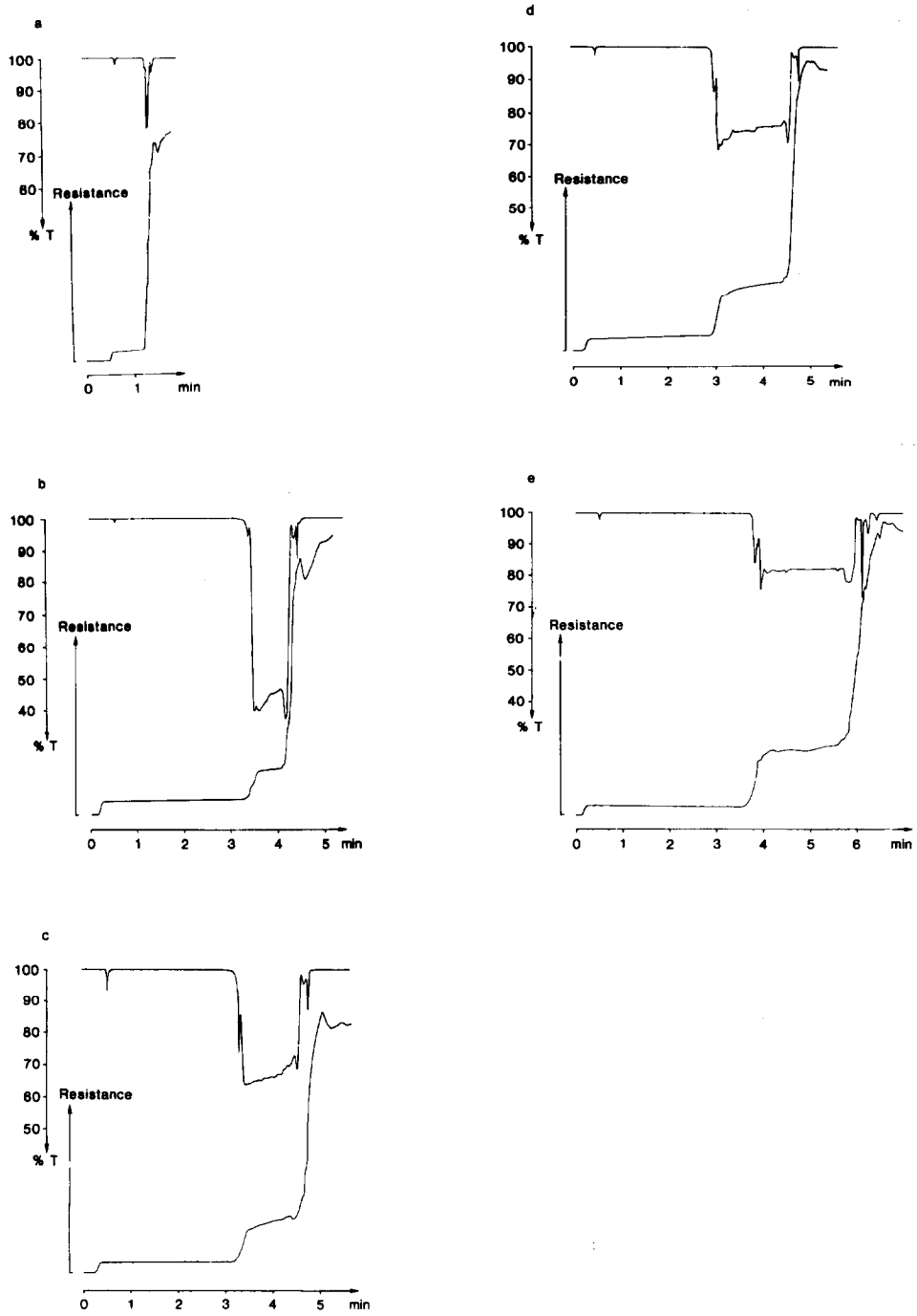


Fig. 1. Analytical isotachophoretic patterns of mPEG–DA conjugates. (a) Electrolyte system; (b) DA; (c) $DA(mPEG)_{12}$; (d) $DA(mPEG)_{22}$; (e) $DA(mPEG)_{36}$. T_{280} = transmission at 280 nm. R = increasing resistance (conductivity signal).

content by amino acid analysis and the mPEG content by NMR spectroscopy. The analysis indicate that $DA(mPEG)_{12}$, $DA(mPeg)_{22}$ and $DA(mPEG)_{36}$ were formed.

The isotachophoretic analyses were performed with an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a 23-cm capillary tube and with UV and conductivity detectors. The leading electrolyte was 10 m*M* adjusted to pH 8.0 with Tris and contained 0.08% (w/v) of hydroxypropylmethylcellulose (HPMC, grade E4M, centipoises; Colorcon, Orpington, U.K.) to reduce electroendosmosis. The terminating electrolyte was 10 m*M* β-alanine adjusted to pH 9.0 with fresly made barium hydroxide solution. The electrolytes were filtered through a 0.45-μm Millipore filter (Millex HA) prior to use. The analyses were carried out at a constant current of 200 μA during separation, with detection at 50 μA. The transmission at 280 nm and the conductivity of the sample were monitored and recorded with a chart speed of 30 mm/min.

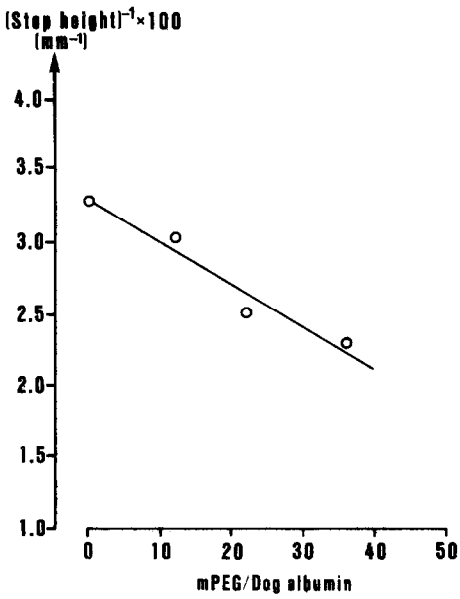
RESULTS AND DISCUSSION

Fig. 1a–e show typical isotachopherograms derived from analyses of (a) the electrolytes, (b) dog albumin and (c–e) dog albumin conjugated to varying extents with mPEG. In each experiment approximately 30 μg of dog albumin were injected. From the UV signals (percentage transmission) at 280 nm it is apparent that dog albumin appears as a broad, not completely homogeneous zone. The UV profiles of mPEG conjugated dog albumins are characterized by a markedly increased zone width with an increase in the number of mPEG groups covalently attached to the protein molecules. The conductivity signal reveals a continuing decrease in the mobility (increasing resistance) of mPEG conjugated dog albumin as the degree of substitution is increased.

Fig. 2 is a plot of the inverse of the step height (step height for dog serum albumin minus step height for the leading electrolyte) against the degree of substitution of DA with mPEG. The data were taken from the traces of the conductivity signal in Fig. 1. The plot of the inverse conductivity step height against increasing degree of substitution of DA with mPEG shows a linear decrease, which is indicative of a decrease in the mobility of the dog serum albumin conjugates. By monitoring the change in conductivity of the various protein conjugates after isotachophoretic separation, the degree of substitution can be determined directly.

Fig. 3 demonstrates the increase in zone width after the isotachophoretic separation of DA conjugated with increasing numbers of mPEG, TNP and FITC groups. Chemical modification causes a broadening of the dog albumin zone width with these reagents, and this appears to be directly proportional to the degree of substitution. Modification with FITC groups gives a higher effective anionic mobility of $DA(FITC)_n$ conjugates (FITC contains negatively charged groups) compared with $DA(mPEG)_n$ and $DA(TNP)_n$ conjugates. This effect is also reflected in the significantly steeper slope of the curve.

The covalent attachment of mPEG and TNP to the amino groups of dog serum albumin renders the albumin molecule more negatively charged at high pH in comparison with unconjugated native DA. However, there is a difference in the broadening of the zone widths for $DA(mPEG)_n$ and $DA(TNP)_n$ conjugates, demonstrated as a difference in the slopes of the curves (Fig. 3). The mobility of the molecules in



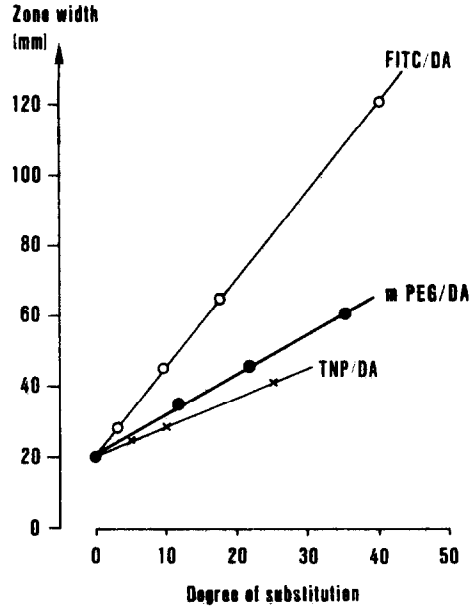


Fig. 2. Plot the inverse step height of the conductivity signal of DA against different mPEG–DA conjugates (data from Fig. 1).

Fig. 3. Plots of zone width of TNP–DA, mPEG–DA and FITC–DA conjugates.

the capillary depends substantially on size, shape and charge. Chemical conjugation with low-molecular-weight components such as TNP only blocks amino groups, without significantly affecting the size and shape of the native molecule. On the other hand, conjugation of DA with mPEG causes the DA molecule to increase the size, change the shape and significantly increase the polarity as a result of water binding. A combination of these effects might be the reason for the wider zone width of DA(mPEG)$_n$ conjugates. The net numbers of negative charges on the DA(mPEG)$_n$ and DA(TNP)$_n$ conjugates will be similar with the same degree of substitution.

Therefore, the use of capillary isotachophoresis to measure the zone width of an unknown conjugated samples gives an accurate estimation of the extent of substitution, provided that a correct calibration graph has been constructed based on protein samples with known degrees of substitution.

In conclusion, we have demonstrated that analytical capillary isotachophoresis can be applied directly to measure the degree of substitution of dog albumin after chemical modification. This rapid technique might be applied as a general analytical tool for screening protein modification with different components.

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