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Use of ion-exchange and hydrophobic-interaction chromatography for the rapid purification of lysozyme–estrone glucuronide conjugates

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Abstract

Estrone glucuronide conjugates of hen egg white lysozyme were prepared by both the mixed-anhydride and active-ester coupling procedures. Both methods gave good yields of conjugate but the active-ester procedure gave a more diverse range of products consistent with a greater acylating ability. Unreacted lysozyme which was present in all cases was removed by a combination of cation-exchange chromatography on a Pharmacia Mono-S column and hydrophobic-interaction chromatography on an Alkyl Superose column. The conjugate families were more hydrophobic than native lysozyme. The chromatographic behaviour of the reaction mixtures on Mono S columns under non-denaturing conditions was complex as a result of hydrophobic effects and only at pH values above 7.0 did the conjugates elute in the order of their overall charges. At pH values below 6.0 the conjugates, although less charged than lysozyme, eluted last on salt gradients. In contrast when denaturing 7 *M* urea buffers were used the conjugates eluted in the order of their electrostatic charges and reproducible patterns were obtained which served as an excellent analytical system for lysozyme–steroid glucuronide conjugates. The purified conjugate material from the active-ester reaction gave over 90% inhibition of the lytic activity in the presence of an estrone glucuronide antibody. When used in a homogeneous enzyme immunoassay system the levels of urinary estrone glucuronide encountered in a normal menstrual cycle were easily measured.

1. Introduction

Homogeneous enzyme immunoassays have been used successfully for the determination of a variety of analytes in biological fluids [1,2] and form the basis of the Ovarian Monitor home test for estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) in urine [3–7]. The attraction of a homogeneous enzyme immunoassay for this purpose lies in the fact that the activity of the lysozyme conjugate (which replaces the radioactive label in radioimmunoassays) is extensively inhibited (>90%) in the immune complex. Hence determination of the amount of bound enzyme label (which depends on the hormone concentration) can be made in solution by a simple kinetic measurement [8] without the need for time consuming and technically demanding physical separation procedures.

Of the limited number of enzymes which have been employed in homogeneous enzyme immunoassays, hen egg white lysozyme (EC

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3.2.1.17) possesses an apparent specific activity with its bacterial substrate *Micrococcus lysodeikticus* which makes it ideal for the measurement of menstrual cycle levels of urinary E1G and PdG [9]. The enzyme possesses six lysine residues which can be acylated readily by a variety of acylating reagents [10] to give stable products (conjugates) which retain lytic activity providing the total number of lysine residues acylated is less than four [11]. Hence, it is necessary to use conjugation conditions which maximise the concentration of conjugates with low degrees of steroid substitution. This means that significant amounts of unreacted lysozyme will inevitably remain after completion of the conjugation reaction.

To obtain the maximum assay discrimination between similar hormone concentrations, as is required for the delineation of the fertile period by a home assay [3], it is necessary to isolate an enzyme conjugate with a high degree of inhibition. Ideally the enzyme activity of the conjugate in the immune complex should be zero since the accuracy, working range and gradient of the standard curve are directly proportional to the difference in enzyme activity between the bound and free conjugate. This requirement necessitates that after conjugation any unreacted lysozyme is completely separated from the conjugated material.

There have been few reports of chromatographic purification procedures for protein products after conjugation with small molecules apart from the use of a gel filtration step [2]. While such a step removes unreacted haptens or coupling reagents it does not remove unconjugated protein. For lysozyme conjugates the only reported purification procedure for the separation of unconjugated lysozyme involves serial dialysis of conjugated material from 7 *M* urea solutions [12]. Although this procedure produces fractions with high degrees of inhibition ($>90\%$) they are uncharacterised and the majority of the conjugated material is unsuitable for use in immunoassays either because it still contains lysozyme or the specific activities are too low.

Since conjugation of lysozyme with steroid glucuronides at the ϵ-amino groups of lysine residues decreases its charge characteristics and increases its hydrophobicity we have investigated the use of fast protein liquid chromatography as a means for the rapid removal of unreacted lysozyme. The resulting E1G–lysozyme conjugates are suitable for use in homogeneous enzyme immunoassays. Cation-exchange chromatography on Mono-S columns in 7 *M* urea also serves as a rapid analytical system for the assessment of lysozyme conjugate fractions.

2. Experimental

2.1. Apparatus

All chromatographic procedures were performed on a Pharmacia fast protein liquid chromatography (FPLC) system at room temperature. This system consisted of two P-500 pumps, an LCC-500 liquid chromatography controller, an MV-7 motor valve injector, a P-1 peristaltic pump, a Pharmacia mixer and a 280-nm single-path UV-1 monitor which was coupled to a two-channel Pharmacia chart recorder. Purifications were performed on prepacked Mono-S strong cation-exchange and Alkyl Superose hydrophobic-interaction columns HR 5/5 (50×5 mm I.D.). A Pharmacia HR 16/50 (500×16 mm I.D.) column packed with Pharmacia CM-Sepharose fast flow resin was also used.

2.2. Reagents

The following reagents were used and obtained from the sources indicated: Milli-Q water, hen egg white lysozyme, grade 1, 3 times recrystallised and lyophilised (Sigma, St. Louis, MO, USA), maleic acid (BDH, lab. grade, Poole, UK), tris(hydroxymethyl)aminomethane (Serva, Feinbiochemica, Germany), sodium chloride (May and Baker, reagent grade, Dagenham, UK), Tween 80 (Atlas, Crothall Distributors, Christchurch, New Zealand), hydrochloric acid and sodium hydroxide (BDH, Poole, UK), N-hydroxysuccinimide (United States Biochemical corporation, Cleveland, OH, USA), N,N′-dicyclohexylcarbodiimide (Merck,

synthesis grade, Darmstadt, Germany), dimethylformamide (Ajax chemicals, Sydney, Australia), sodium hydrogen carbonate (Rectapur, Prolabo, Paris, France), 2-propanol (Ajax chemicals, Sydney, Australia), sodium dihydrogen phosphate-dihydrate, urea and ammonium sulfate (Riedel-de-Haën, Seelze-Hannover, Germany). E1G antiserum was a gift from Professor J.B. Brown (Melbourne, Australia).

Ion-exchange and hydrophobic interaction chromatography buffers were prepared as described in figure legends. All buffers were filtered and degassed using 0.2-μm filters (Millipore) before use. Samples were filtered through Millipore GVWP 013 00 filters (0.22 μm). Stock Tris-maleate buffer (1.0 *M*), was prepared by mixing maleic acid (7.25 g), Tris (19.80 g), NaCl (12.75 g), Tween 80 (20 ml of 1/100 dilution in Milli-Q water) and HCl (2.8 ml of concentrated acid) in a total volume of 375 ml. The pH was adjusted to 7.0 with NaOH.

2.3. Methods

Synthesis of 17-oxoestra-1,3,5-3-β-yl-D-glucopyranosiduronic acid [E1G(H)]

17 - Oxoestra - 1,3,5 - 3 - β - yl - D - glucopyranosiduronic acid (estrone glucuronide or E1G) was synthesized essentially according to Conrow and Bernstein [13] from estrone in 39% yield. The crystalline product (m.p. = 166–168°C) was purified by XAD-2 chromatography as described by Numazawa et al. [14] with the E1G being eluted with a 75% aqueous methanol solution. The structure of the acid form [E1G(H)] was confirmed by NMR spectroscopy, the key features of which are summarised below: ^{1}H NMR (270 MHz; DMSO-d_6): 0.82 (s, 3H, 18-CH_3), 3.72 (d, 1H, $J = 8.79$ Hz, H-5′), 4.91 (d, 1H, $J = 7.96$ Hz, H-1′ anomeric proton), 6.71 (s, 1H, H-4), 6.77 (d, 1H, $J = 8.43$ Hz, H-1), 7.17 (d, $J = 8.43$ Hz, H-2).

Lysozyme purification

Commerical lysozyme (2.6 g; 179 mmol) was further purified by loading onto a CM-Sepharose fast flow column via a peristaltic pump in 10 m*M* pH 6.0 phosphate buffer (20 ml). Elution was effected with a 0.15 *M* to 0.6 *M* NaCl gradient, in the same buffer, over 600 min. The elution profile consisted of two peaks, a smaller peak which was eluted first followed by the elution of the much larger lysozyme peak. The peak fractions from the larger peak were bulked, dialysed against Milli-Q water, freeze-dried and then stored at −10°C until required for conjugation experiments.

Preparation of estrone glucuronide (E1G)–lysozyme conjugates by the mixed-anhydride procedure

Conjugation of lysozyme with E1G(H) was achieved with the mixed-anhydride procedure of Erlanger et al. [15]. The conjugation was performed with a 2:1 molar ratio of E1G to lysozyme using the following quantities: 100 mg purified lysozyme (7 μmol) dissolved in 4 ml of Milli-Q water, 10 mg E1G(H) (22.6 μmol), 220 μl dimethylformamide, 5.3 μl tri-*n*-butylamine (23.3 μmol) and 3.0 μl isobutyl chloroformate (23.3 μmol). The details of the conjugation were essentially as described elsewhere [12].

Preparation of estrone glucuronide (E1G)–lysozyme conjugates by the active-ester method

Estrone glucuronide (E1G) conjugates of lysozyme were also prepared by the N-hydroxysuccinimide/dicyclohexylcarbodiimide coupling method [16] at a 1.6:1 molar ratio of E1G to lysozyme. An active-ester reagent was prepared from E1G(H) (4.1 mg; 9.3 μmol) in dimethyl formamide (30 μl). N-hydroxysuccinimide (22.5 mg; 195 μmol) and dicyclohexylcarbodiimide (29.9 mg; 145 μmol) were each separately dissolved in dimethylformamide (100 μl). From these stock solutions N-hydroxysuccinimide (10 μl; 19.5 μmol) was added to the E1G solution followed by the addition of dicyclohexylcarbodiimide (10 μl; 14.4 μmol). After standing at room temperature for one hour the active-ester reagent was added dropwise to a stirred solution of purified lysozyme (83 mg; 5.8 μmol) dissolved in 1% aqueous sodium hydrogen carbonate (3.3 ml). After standing overnight with stirring the reaction mixture, which appeared cloudy, was

dialysed into Milli-Q water (3 × 2 l) and then stored at −10°C.

Lytic assay

Lytic assays for the determination of the E1G standard curve were undertaken using an Ovarian Monitor essentially as described previously by Brown et al. [3]. The assay was performed in plastic 1-ml cuvettes (Adindas Plastics, Melbourne, Australia) designed specifically for the Ovarian Monitor. In the first step 50 μl of E1G standard (0.5 to 1083 n*M*), 50 μl of a blank urine sample diluted to 150 ml/h of collection and which contained 2.7 nmol/24 h E1G as determined by fluorescence [17] was added to each tube. Antiserum (5 μl) was then added to each tube with vortex-mixing followed by an aliquot of E1G-lysozyme conjugate (5 μl). Finally 0.04 *M* Tris-maleate buffer (pH 7.0) was added to make the volume up to 340 μl. The mixture was then equilibrated for a minimum of 10 min at 40°C. At the end of the pre-incubation period the *Micrococcus lysodeikticus* suspension (10 μl of a 7.5 mg/ml suspension in 75 m*M* Tris-maleate buffer, pH 7.0) was added with vortex-mixing and the initial transmission noted. After 20 min the final transmission was recorded automatically and the difference calculated. The rate of the reaction was therefore recorded as the change in transmission (ΔT) per 20 min. The volume of conjugate per assay tube was calculated to give a change in apparent transmission of 350 units in 20 min.

3. Results

The addition of the mixed-anhydride reagent (prepared from synthetic E1G(H) as described in section 2.3) to the purified hen egg white lysozyme solution (at a molar ratio of 2:1) resulted in conjugation of 43% of the initial amount of lysozyme with E1G. This was shown by analytical cation-exchange chromatography in 7 *M* urea buffers. After the reaction mixture had been dialysed against Milli-Q water, sufficient urea was added to an aliquot (100 μl) of the conjugate reaction mixture to give a 7 *M* urea solution which was loaded onto a Mono-S cation-exchange column for analysis (Fig. 1A). The largest peak (57% of the total) consisted of unreacted lysozyme (L) as shown by comparison with the FPLC profile of a pure sample of lysozyme. Of the conjugated lysozyme the predominant conjugate families were E3 (63%) and E1 (20%) with the remainder (17%) made up of small amounts of E4, E5 and E6. The conjugate families are numbered relative to the elution position for lysozyme, E1 being the closest to lysozyme and E6 being the first conjugate to elute from the column (see Fig. 1B).

When lysozyme was conjugated as described in section 2.3, at an E1G to lysozyme ratio of 1.6:1, using the N-hydroxysuccinimide/dicyclohexylcarbodiimide coupling method seven chromatographically distinct peaks were obtained (Fig. 1B). The largest peak (30% of the total) again consisted of unreacted lysozyme (L). The six peaks (E1–E6) which eluted earlier consisted of different conjugate fractions making up the remaining 70% of the yield. The two largest conjugate fractions E1 and E3 were present in 19% and 18% yields respectively, and together comprised 54% of the total conjugate yield.

After filtration, 700 μl of the active-ester conjugation reaction mixture was loaded onto a Mono-S column equilibrated with 50 m*M* phosphate buffer (pH 4.3) and fractions were collected as shown in Fig. 2. Five fractions (a–e) were collected and each fraction was dialysed into Milli-Q water (3 × 2 l) and stored at −10°C. The fractions were then analysed for unreacted lysozyme by removing an aliquot from each, adding sufficient urea to give a 7 *M* urea sample and then loading onto the analytical Mono-S column and eluting as described in Fig. 1B. The results are shown in Fig. 3. The chromatogram of fraction a (Fig. 3A) consisted of three main peaks; an unreacted lysozyme peak (40% of the total) and two conjugate peaks (28% and 23% respectively) while the chromatogram of fraction c (Fig. 3B) showed it to consist largely (71%) of unreacted lysozyme. However, although the chromatogram of fraction e (Fig. 3C) contained all seven chromatographically distinct conjugate

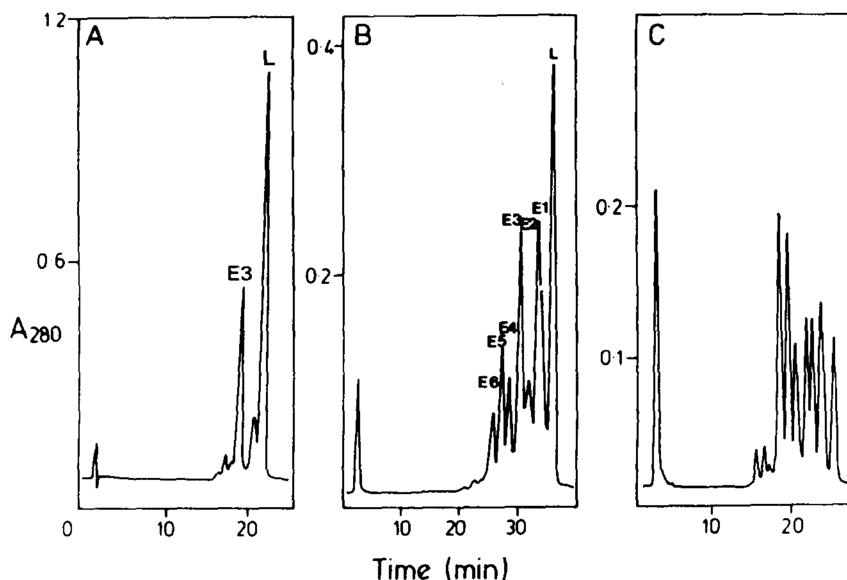

Fig. 1. E1G–lysozyme conjugate reaction mixture [mixed-anhydride method (A), active-ester method (B) and (C)] in 7 M urea on a Mono-S cation-exchange HR 5/5 column. Conditions: buffer A, 7 M urea + 50 mM NaH_2PO_4 buffer titrated to pH 6.0 with 1 M NaOH and buffer B, buffer A + 1 M NaCl titrated to pH 6.0 with 1 M NaOH; gradient, 0% B for 5 min, (A) 0–40% B in 30 min, (B) 0–30% B in 40 min, (C) 0–40% B in 40 min at 0.5 ml/min; chart, 0.2 cm/min.

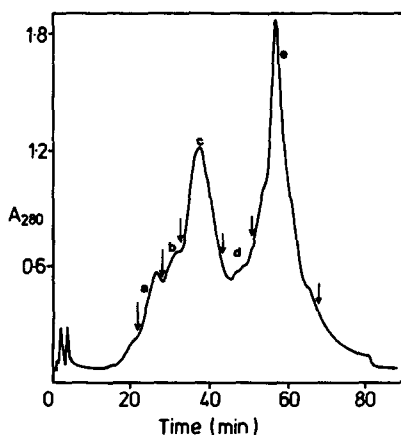

Fig. 2. E1G–lysozyme active-ester conjugate reaction mixture on a Mono-S cation-exchange HR 5/5 column. Conditions: buffer A, 50 mM NaH_2PO_4 and buffer B, 50 mM NaH_2PO_4 + 1 M NaCl; gradient, 0–35% B in 5 min, 35% B for 35 min, 35–37% B in 2 min, 37% B for 3 min, 37–40% B in 1 min, 40–45% B in 4 min, 45–75% B in 2 min, 75% B for 8 min, 75–100% B in 1 min, 100% B for 14 min at 0.5 ml/min; chart, 0.2 cm/min.

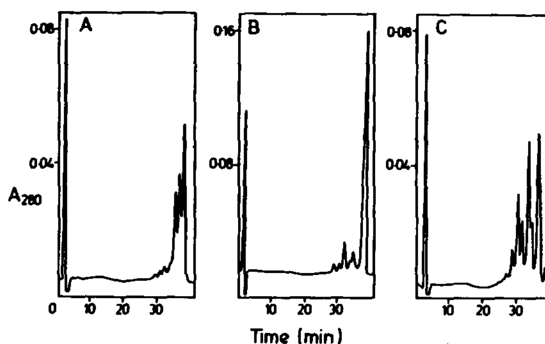

Fig. 3. Fractions a (A), c (B), and e (C) from Fig. 2 in 7 M urea on a Mono-S cation-exchange HR 5/5 column. Conditions: buffers, gradient and chart as in Fig. 1(B).

peaks (as seen in Fig. 1) there was only a small unreacted lysozyme peak (4%). The six conjugate peaks were present in about the same relative ratios as prior to the purification step (cf. Fig. 1B) with fractions E1 and E3 constituting 52% of the total conjugate yield. Fraction b was almost identical to fraction c, and fraction d, as expected, consisted of relatively more unreacted lysozyme and less of the conjugate peaks (E1–

E6) than fraction e. The lytic activity of conjugate fraction e was inhibited by 78% when excess E1G antibody was present in the assay mixture.

The order of elution of the bulk conjugate fraction e and the unreacted lysozyme fraction c changed greatly with pH, under non-denaturing conditions, on the Mono-S column. Table 1 shows the retention times for the bulk conjugate (fraction e) relative to the retention time for unreacted lysozyme (fraction c) as a function of pH. At pH 4.3 the bulk conjugate (fraction e) eluted after the unreacted lysozyme fraction c as shown in Fig. 2. However, at pH 6.0, the two peaks co-eluted and at pH 7.6 the elution positions of the unreacted lysozyme fraction c and the bulk conjugate fraction e had reversed so that the conjugate fraction e now eluted slightly before the unreacted lysozyme. As the buffer pH was further increased, the conjugate containing fraction e continued to elute earlier than the unreacted lysozyme fraction c. Although the resolution between the two peaks increased with increasing pH, even at pH 10 the two peaks were not completely resolved. As the pH of the buffer system increased the ionic strength required to elute the conjugate fraction decreased as shown in Table 1. Addition of 1.5% 2-propanol to the buffer system at pH 4.3 resulted in a greatly reduced retention time for the bulk conjugate fraction e as shown in Fig. 4. However the retention time for the unreacted lysozyme fraction c did not change significantly and the two fractions (c and e) co-eluted.

Sufficient ammonium sulfate was added to an aliquot of fraction e (from Fig. 2) to bring it to 1.4 M and the resulting solution was loaded onto

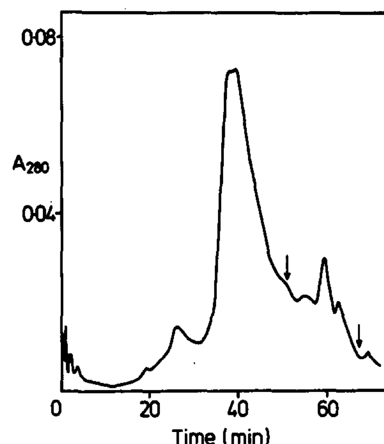

Fig. 4. E1G–lysozyme conjugate reaction mixture on an Mono-S cation-exchange HR 5/5 column. Conditions: buffers as in Fig. 2 + 1.5% 2-propanol in both buffers; gradient and chart as in Fig. 2. The position in which fraction e elutes in the absence of 2-propanol is indicated by arrows (cf. Fig. 2).

an Alkyl Superose 5/5 column and eluted as shown in Fig. 5. The chromatogram consisted of an initial broad peak (f), (which passed straight through the column) followed later in the gradient by a series of peaks (fraction g) as indicated in Fig. 5. The two fractions were then dialysed into Milli-Q water (3 × 2 l) and stored at −10°C. Both fractions were analysed by adding the appropriate amount of urea and loading onto a Mono-S column as previously described. The resulting analytical chromatographs are shown in Fig. 6. Fraction f (Fig. 6A) consisted mainly of a single peak whose lytic activity was not significantly inhibited when excess E1G antibody was present, while fraction

Table 1
Retention of conjugate fraction e on the Mono-S column relative to unreacted lysozyme and the corresponding ionic strength at elution

Buffer pH	[NaCl] at elution (mol/l)	R_f value of fraction e
4.3	0.45–0.75	1.52
6.0	0.20	1.0
7.6	0.17	0.90
9.5	0.16	0.88
10.0	0.11	0.76
4.3 + 1.5% 2-propanol	0.35	1.0

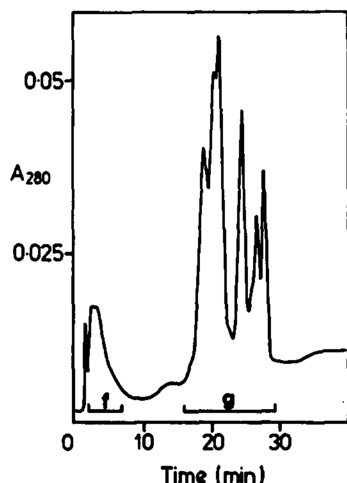

Fig. 5. Fraction e from Fig. 2 in 1.4 M ammonium sulfate on an Alkyl Superose hydrophobic interaction HR 5/5 column. Conditions: buffer A, 50 mM NaH_2PO_4 + 1.4 M $(NH_4)_2SO_4$ titrated to pH 6.6 with 1 M NaOH and buffer B, 50 mM NaH_2PO_4 titrated to pH 6.6 with 1 M NaOH; gradient, 0% B for 10 min, 0–100% B in 20 min, 100% B for 15 min; chart, 0.2 cm/min.

g (Fig. 6B) consisted mainly of conjugate peaks E1–E5 and a small amount of material (5%) which eluted at the unreacted lysozyme position (L). The lytic activity of fraction g was now inhibited by 93% when an excess of E1G antibody was present.

A standard curve suitable for the determination of urinary E1G levels was established in pH 7.0 Tris-maleate buffer by utilising the inhibition of the lytic activity of the E1G-lysozyme conjugates contained in fraction g by an E1G antibody as described in section 2.3. Assays carried out with variable amounts of E1G standards produced the sigmoidal standard curve shown in Fig. 7. The working range of the curve was 20 nM to 290 nM which covered the range of E1G concentrations found in urine during a normal menstrual cycle. A typical menstrual cycle was then analysed using this system whereby the steroid standard in the assay was replaced by 50 μl of urine (diluted to 150 ml/h of collection). The resulting daily profile until the end of the first E1G peak is shown in Fig. 8.

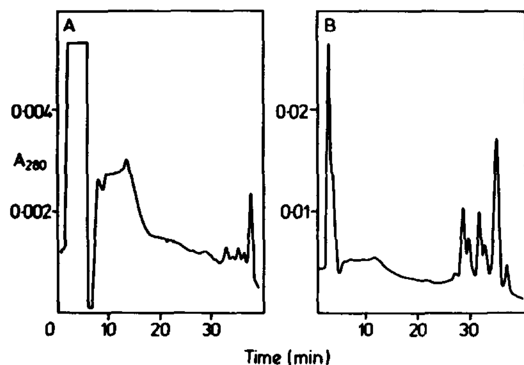

Fig. 6. Fractions f (A) and g (B) from Fig. 5 in 7 M urea on a Mono-S cation exchange HR 5/5 column. Conditions: buffers, gradient and chart as in Fig. 1(B).

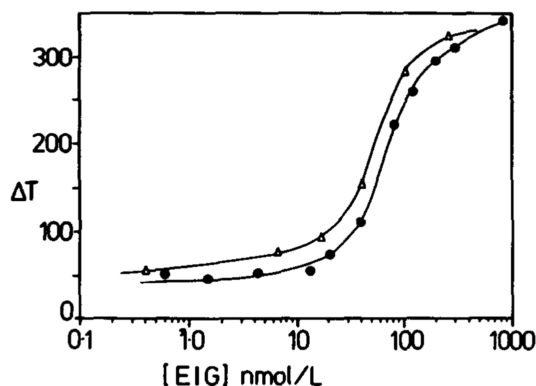

Fig. 7. An E1G standard curve using conjugate fraction g from Fig. 5 (●) and a standard curve using mixed anhydride conjugate from the Ovarian Monitor (△).

4. Discussion

The NMR spectrum of the E1G(H) product was as expected for the structure and confirmed that it had the correct stereochemistry at the anomeric carbon atom. The presence of the sugar ring in the correct orientation and the β orientation of the glucuronide moiety with respect to the steroid skeleton were clearly shown by the large coupling constants for the sugar protons C-1′ and C-5′ (7–9 Hz) indicating a trans configuration at these two positions. Hence it was identical with the naturally occurring material.

The synthetic estrone glucuronide was effi-

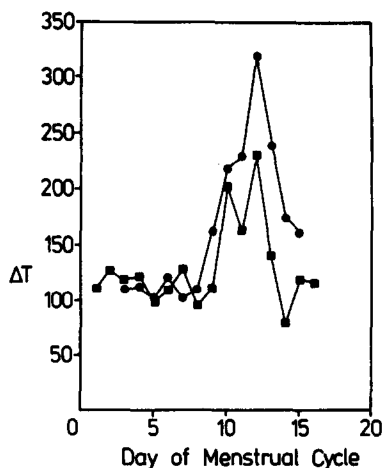

Fig. 8. A typical example of a menstrual cycle obtained using the standard curve described in Fig. 7 (see text). (●) woman's data using Ovarian Monitor and (■) data using conjugate fraction g.

ciently coupled to hen egg white lysozyme by both the mixed-anhydride and the active-ester coupling methods resulting in the formation of stable E1G–lysozyme conjugates. The mixed-anhydride method was clearly much more selective giving mainly E3 (Fig. 1A) although significant amounts of E1, E4, E5 and E6 were also present. Surprisingly E2 was absent. The reason for the selectivity is not clear but it may be a result of differential reactivity of the lysine residues towards acylating reagents [18]. The active-ester reagent appeared to be more reactive than the mixed-anhydride reagent since it gave a more diverse range of conjugate products at a lower molar ratio (Fig. 1B) but again E3 and E1 predominated.

The elution patterns for the conjugates on the Mono-S cation exchange column were highly reproducible even after extensive dialysis and storage for several weeks at $-10°C$. In the stable conjugates (E1–E6), the steroid glucuronide moieties are presumably attached to the protein by means of amide linkages via the ϵ-lysine residues in the protein since the elution pattern was not changed even after treatment with hydroxylamine. The patterns obtained on Mono-S columns in 7 *M* urea (Figs. 1A, B and C) therefore serve as a simple, rapid analytical system for lysozyme conjugation. The success of purification steps and the purity of fractions can be analysed rapidly and in this way the removal of unreacted lysozyme and subsequent changes in conjugate composition of a fraction can be followed easily throughout a purification procedure.

The order of elution of each steroid glucuronide–lysozyme conjugate on a cation exchange column in 7 *M* urea when the enzyme is partially denatured would be expected to be determined by the number of free lysine residues. Every free lysine residue in the enzyme is positively charged at the buffer pH of 6.0 and this positive charge is lost upon acylation. The order of elution of the steroid glucuronide–lysozyme conjugates should be therefore inversely related to the number of free lysine residues. Evidence that this is so was provided by titration of the lysine amino groups in the individual conjugate populations (E1–E6) [19]. The results obtained using the trinitrobenzenesulfonic acid method previously described [20] showed that E3 was more highly substituted than E1. Hence the extent of acylation of amino groups on the protein was correlated directly with the elution position of the individual conjugate populations in 7 *M* urea on Mono-S columns as expected. The most highly substituted conjugates elute first and unsubstituted, unreacted lysozyme (L in Fig. 1A and 1B) always eluted last with a characteristic elution time. Hence, the presence of lysozyme could be recognised easily in the chromatograms.

A possible method of reducing the unreacted lysozyme concentration is to increase the ratio of the active-ester reagent to lysozyme. However, conjugation with a 3:1 molar ratio of active-ester reagent failed to achieve this (Fig. 1C). After completion of the reaction an insoluble precipitate was formed which was presumably due to the increased amounts of the early eluting conjugate peaks (E4–E6), with higher E1G to lysozyme ratios. This was at the expense of conjugates with lower E1G to lysozyme ratios which decreased in relative amount and unreacted lysozyme was still present. It appears that the increased reagent concentration merely results in acylation of all species present rather

than preferentially attacking the free lysozyme. This increased yield of more highly substituted conjugates is not desirable as their specific activities were low (36% of a lysozyme control) which makes them less suitable for use in homogeneous enzyme immunoassays. Hence, increasing the ratio of active-ester reagent to lysozyme was not a viable method for the complete removal of unreacted lysozyme. It is therefore essential to separate unreacted lysozyme by physical procedures before the conjugates can be used in homogeneous enzyme immunoassays.

The use of ion-exchange chromatography in 7 *M* urea would appear to be the ideal method for the removal of unreacted lysozyme from the reaction mixture. Not only are the conjugation products well separated from the unreacted lysozyme peak (see Fig. 1) allowing its removal, they are also well separated from each other allowing the purification of individual conjugate families if desired. However, conjugates purified in this way always gave lower inhibitions (<90%) than required for homogeneous enzyme immunoassays even though they appeared to contain very little unreacted lysozyme (4%) by analytical FPLC (see Fig. 3C for example). In the development of a viable purification procedure, with potential for scaling up, the ultimate test for the purity of a conjugate has to be the degree of lytic inhibition observed when excess antihapten antibody is present. If the observed degree of inhibition is not greater than 90% of the uninhibited rate the conjugate is unsuitable for use in homogeneous enzyme immunoassays irrespective of the appearance of the FPLC profiles.

It is difficult to explain the inadequate inhibition after exposure to denaturation in 7 *M* urea since it does not correlate with the amount of free lysozyme present. Monocarbamylation of lysine residues of lysozyme by cyanate ions present in the urea solution to produce enzyme forms with less positive charges is a possible source of contamination. For example, a monocarbamylated lysozyme molecule would be eluted in the same position as a di-substituted E1G–lysozyme conjugate. While the monocarbamylated enzyme would possess lytic activity, it would show no immune response towards antihapten antibodies resulting in a lowering of the degree of inhibition and its presence would not be apparent in the FPLC profile. The exposure of the E1G–lysozyme conjugates to urea results in their denaturation which must be reversed by dialysis before use in immunoassays. Hence, an incomplete or inaccurate refolding could explain the lower than expected inhibition of conjugates purified by ion-exchange chromatography on Mono-S columns in 7 *M* urea. However, lysozyme conjugates have been separated from unconjugated lysozyme using serial dialysis from 7 *M* urea solutions [12] to give highly inhibited conjugates upon their removal from urea solutions thus ruling out denaturation–renaturation as the source of the inadequate inhibition.

The chromatographic behaviour of the conjugate fractions (a–e; Fig. 2) on the Mono-S column under non-denaturing conditions was surprising if only charge interactions with the column matrix were operating. The elution order at pH 4.3 (Fig. 2) clearly did not follow that predicted by charge interactions alone. Indeed, the bulk of the conjugate was found in fraction e which was eluted after the bulk of the unreacted lysozyme (fraction c). The fact that the relative positions of the conjugate products and unreacted lysozyme changed with a change in pH of the eluting buffers shows that non-electrostactic factors also contribute to the binding of the conjugates to the Mono-S column.

The variation of relative elution position with pH can be explained on the basis of two opposing effects [21]. As the pH of a buffer system was decreased, the retention time of lysozyme on a Mono-S cation-exchange column increased [22] because of the increasing charge on the protein requiring a higher ionic strength to elute the lysozyme. However, accompanying the increasing ionic strength is an increasing surface tension which would be expected to increase hydrophobic interactions between the protein and the column matrix [23]. It has been reported previously [22] that the Mono-S Pharmacia cation-exchange column has very little, if any, hydrophobicity. This conclusion was based on the observation that the retention time for lysozyme

decreased by less than 5% in the presence of buffers spiked with 1% 2-propanol. However, no work has been reported for lysozymes acylated with hydrophobic steroids. Our results agree with Kopaciewicz et al. [22] in that addition of 1.5% 2-propanol to the buffer system (described in Fig. 4) resulted in very little decrease in the retention time for native lysozyme. However, there was a very significant decrease (ca. 25%) in the retention time for the major conjugate containing peak (fraction e). The exact magnitude of the decrease was difficult to determine as the unreacted lysozyme and the bulk of the E1G–lysozyme conjugate products eluted in virtually the same position (Fig. 4). However, this decrease in retention time for the bulk of the E1G–lysozyme conjugate products in their native conformations clearly indicates that there is an important hydrophobic interaction occurring between the hydrophobic steroid moieties of the E1G–lysozyme conjugates and the Mono-S matrix.

Thus, we propose that the relative elution positions of the E1G–lysozyme conjugates and lysozyme on a Mono-S cation-exchange column are determined by both the electrostatic and hydrophobic interactions. In buffers of high pH hydrophobic interactions between the E1G moiety and the column matrix are minimised and thus play little part in the separation. Although, as a result the lower net positively charged E1G-lysozyme was eluted prior to the unreacted lysozyme, the two peaks were not well resolved. This lack of resolution means that, in the native state, the E1G–lysozyme conjugates cannot be separated easily from the unreacted lysozyme at pH values of 6.0 or above. However, in buffers of low pH the stronger binding of lysozyme to the Mono-S column (Table 1) and the higher ionic strength required for elution results in significant hydrophobic interactions of the E1G steroid moiety with the column. This additional factor causes the E1G conjugates to be eluted later than lysozyme which does not have the same effect. Hence, at pH 4.3, the hydrophobic interactions can be utilised to give a separation between unreacted lysozyme and the E1G–lysozyme conjugates (Fig. 2). Kopaciewicz and Regnier [24] have reported that a similar hydrophobic–ionic interaction property may be responsible for variation in retention times found on a weak glutaric anhydride cation-exchanger.

The conjugate fraction (e) prepared by cation-exchange chromatography at pH 4.3 (Fig. 2), consisted almost completely of E1G–lysozyme conjugates (peaks E1–E6) and very little unreacted lysozyme compared with the original reaction mixture (cf. Fig. 1B). Furthermore, the relative peak heights remained almost the same as found in Fig. 1B suggesting that the cation-exchange procedure had simply separated the acylated lysozyme products from unreacted lysozyme. Hence, under these conditions there does not seem to be any evidence for associative interactions between the conjugates and lysozyme. Surprisingly, despite the fact that fraction e contained the bulk of the conjugate products and only a low level (4%) of unreacted lysozyme, the lytic activity was still inadequate being inhibited by only 78% when excess antihapten antibody was present. The reason for this is not apparent but it may be the result of an interaction between the packing material and the lysozyme conjugates. If such an interaction exists it would also explain the inadequate inhibition encountered using 7 *M* urea solutions on Mono-S columns as discussed above. Rechromatography of the fraction through the Mono-S column at pH 4.3, resulted in only a small increase in the degree of inhibition (2–3%) hence a second chromatographic step was clearly required to give the desired degree of inhibition even under non-denaturing conditions.

The strong retention of the E1G–lysozyme conjugates on the Alkyl Superose column (Fig. 5) was consistent with interaction with the Alkyl Superose column matrix by hydrophobic interactions. It might be expected that passage of a conjugate reaction mixture through an Alkyl Superose column as a first step would remove the unreacted lysozyme and hence avoid the difficulties associated with ion-exchange chromatography. However, despite the fact that chromatograms obtained after loading of a conjugate reaction mixture directly onto an Alkyl Superose column looked similar to Fig. 5 (although with a

much larger peak f) the conjugate fractions all contained lysozyme. A second passage through the Alkyl Superose column did not significantly improve the extent of inhibition of the conjugate fraction showing that much of the lysozyme still remained. This result indicates an association between E1G–lysozyme conjugates and free lysozyme at high concentrations of both substances. Hence, it is necessary to utilise a first step which reduces the unreacted lysozyme concentration as in the present work otherwise all fractions are contaminated by lysozyme.

Analysis of the conjugate (fraction g) after Alkyl Superose chromatography showed that it consisted mainly of the lower substituted E1G–lysozyme conjugates but there were also significant amounts of the more highly substituted E1G–lysozyme conjugates present. The lytic activity of the conjugate was now well inhibited (by 93%) when excess antihapten antibody was present, hence it was used in homogeneous enzyme immunoassays for E1G. Thus, a combination of Mono S cation-exchange chromatography at pH 4.3 followed by hydrophobic interaction chromatography on an Alkyl Superose column rapidly produced conjugate of sufficient quantity and immune reactivity for use in enzyme immunoassays. These procedures have the potential for scaling up and represent the best procedures yet reported for purification of lysozyme–steroid conjugates.

The standard curve obtained for the determination of E1G, utilising the active-ester conjugate (Fig. 7) was found to be highly reproducible. It was in the correct working range and of suitable gradient for the accurate measurement of estrone glucuronide. A menstrual cycle obtained using this system (Fig. 8) was compared with the results obtained for the same cycle by a woman using the Ovarian Monitor at home. The beginning of the fertile period was clearly defined by the first rise in urinary E1G and the day of the peak excretion rate of E1G was cycle day 12 in both cases. The response of the assay system to a given level of urinary E1G (Fig. 8) was greater for the Ovarian Monitor assay system which uses a conjugate preparation consisting mainly of E3 (Fig. 1A). The difference in response was also apparent in the standard curves (Fig. 7) and may reflect the fact that the active-ester conjugate contains the more highly substituted E3, E4 and E5 in greater amounts. Thus, higher levels of urinary E1G are required to give the maximum response for these latter conjugates which will continue to be inhibited while the less substituted conjugates are no longer bound by the antibody. Hence, the mixed-anhydride acylation procedure may be preferable for good quality conjugate despite the fact that it is more difficult to perform and gives more variable results than the active-ester method. Irrespective of which coupling method is preferred the purification procedures described here can be applied. It is also possible that with further refinement the conjugate families E4, E5 and E6 can be removed during chromatography thus making the active-ester conjugation procedure the method of choice.

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