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Biochimica et Biophysica Acta 1622 (2003) 42–49
BIOCHIMICA ET BIOPHYSICA ACTA
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Polysialylated insulin: synthesis, characterization and biological activity in vivo

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Received 6 March 2003; received in revised form 22 May 2003; accepted 26 May 2003

Abstract

Polysialic acids (PSA) (colominic acid; CA) of 22 and 39 kDa average molecular weight were oxidized with sodium periodate at carbon 7 of the nonreducing end to form an aldehyde group. The oxidized CAs (96–99% oxidation) were then reacted with the amino groups of recombinant human insulin at various CA/insulin molar ratios (25:1 to 150:1 range) for up to 48 h in the presence of sodium cyanoborohydride (reductive amination). Polysialylated insulin conjugates were precipitated (together with intact nonreacted insulin, if any) at time intervals from the reaction mixtures with ammonium sulfate, further purified by size exclusion chromatography and/or ion exchange chromatography (IEC), and the final conjugates assayed for PSA and protein. Results showed an initial rapid conjugation rate peaking at about 12 h, to form a plateau over a period of 12–48 h. Moreover, the extent of polysialylation (CA/insulin molar ratios in the conjugate) was dependent on the PSA used, the initial CA/insulin molar ratios in the reaction mixture and the time of the coupling reaction. Thus at 48 h of incubation, CA/insulin molar ratios in the conjugates were 1.60–1.74 for the 22-kDa CA and 2.37–2.45 for the 39-kDa CA. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of intact insulin and insulin reacted with non-oxidized CA for 48 h revealed well-resolved single bands which migrated similar distances in the gel. On the other hand, polysialylated (22-kDa CA) insulin yielded multiple diffused bands suggesting heterogenicity as a result of differential polysialylation. The pharmacological activity of polysialylated insulin was compared with that of intact insulin in normal female outbred T/O mice. After subcutaneous injection of intact insulin (0.3 units per mouse), blood glucose levels were reduced to nadir values at 1 h to return to normal at 3 h. In contrast, blood glucose levels in animals injected with polysialylated insulin (0.3 units or protein equivalence for polysialylated insulin), having attained nadir values also at 1 h, returned to normal levels after 6 h (39 kDa) and 9 h (22 kDa CA-insulin). It is concluded that polysialylation offers a promising strategy for the enhancement of the therapeutic value of insulin and other pharmacologically active peptides.

Keywords: Insulin; Polysialic acid; Colominic acid; Protein and peptide delivery

1. Introduction

Diabetes mellitus is responsible for major health care problems, leading to severe complications such as kidney disease, retinopathy, neuropathy and heart disease. Since its discovery, insulin has become indispensable for the treatment of insulin-dependent diabetes. However, it faces many problems typical of protein and peptide pharmaceuticals, including poor physical and chemical stability, increased susceptibility to proteolysis, immunogenicity and antigenicity, and a relatively short-lasting biological activity [1]. Great efforts have been made to enhance the therapeutic performance of insulin using various drug delivery systems, for instance nanoparticles, microspheres, liposomes, buccal patches, stimuli-sensitive hydrogels, osmotic pumps, electrically controlled devices, pancreatic transplants and islet cell implants [2–7], applied by a variety of routes [8–14]. Moreover, new insulin forms have been obtained by genetic or chemical engineering to overcome problems such as self-aggregation and enzymatic degradation [15–26]. For instance, one of the promising approaches to enhance the therapeutic performance of

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doi:10.1016/S0304-4165(03)00116-8

insulin is its modification with water-soluble, biocompatible polymers.

It has been recently reported [27–32] that covalent coupling of the naturally occurring, highly hydrophilic, non-immunogenic, linear polymer of *N*-acetylneuraminic acid (Neu5Ac; sialic acid) to drugs, including peptides and proteins, produces constructs exhibiting improved stability and prolonged survival in the blood circulation. Polysialic acids (PSA; also referred to as colominic acids, CA) can be produced from bacteria in varying lengths, with long PSAs attaining longer circulation times after intravenous injection (e.g. up to 40 h half-life in mice) than shorter ones [28]. In the former case, a long PSA (e.g. average molecular weight of 60 kDa) has been shown to greatly prolong the circulation time of a small model drug (fluorescein), which by itself has a very short half life [28]. On the other hand, conjugation of shorter PSAs (e.g. average molecular weight of 22 kDa or less) to enzymes (e.g. catalase [30], the anticancer agent asparaginase [29]) or a cytokine (e.g. interferon α-2b [33]) not only preserves their activity, but it can also increase the half life and the area under the curve of the therapeutic (e.g. asparaginase) significantly. Similar results have been obtained with a variety of other protein therapeutics, e.g., aprotinin [34], IgG immunoglobulin [34] and IgG Fab fragments [31]. Polysialylation also helps to reduce the immunogenicity and antigenicity of proteins, for instance asparaginase [32,35]. In this respect, it is of interest that certain bacteria use their PSA coat as a means to evade recognition by the immune system of the host. Moreover, and in contrast to other hydrophilic molecules (e.g. dextran, polyethylene glycol) also proposed [1,2,26,36–39] for peptide and protein delivery, PSAs are biodegradable in biological fluids and their catabolic products are not known to be toxic [27]. Both of these properties are important in non-life-threatening situations (e.g. insulin-dependent diabetes) where chronic use is required. From a practical standpoint, PSAs are commercially available and can be easily and cheaply harvested from cultures of bacteria such as laboratory-adapted wild-type strains of *E. coli* K1.

It is proposed that covalent coupling of PSA to insulin may improve its solubility (so as to prevent aggregation) and stability, lengthen its circulatory half-life and hence biological function, thus reducing frequency of administration, and also potentially reduce immunogenicity or antigenicity, all of which could help to improve the therapeutic utility of the hormone. Here, we report on the synthesis and characterization of polysialylated insulin, and its biological activity in vivo.

2. Materials and methods

Recombinant human insulin (5.8 kDa), ammonium carbonate, ethylene glycol, polyethylene glycol (8 kDa), sodium cyanoborohydride (>98% pure), sodium meta-periodate and molecular weight markers were obtained from Sigma Chemical Laboratory, UK. The CAs used, linear α-(2 → 8) linked *E. coli* K1 PSAs marketed as 10 and 58 kDa, were from Sigma and Camida, Ireland, respectively. However, determination of their average molecular weight (nominal mass) by Viscotek Europe Ltd. (Basingstoke, UK) through measurements of refractive index, viscometry and low-angle light scattering, revealed values of 22.4 kDa (1.33) for the 10-kDa product and 38.6 kDa (1.40) for the 58-kDa product. These values (with polydispersity values in parentheses) were adopted throughout this work and are referred to in the text as 22 and 39 kDa for simplicity. Other materials included 2,4-dinitrophenyl hydrazine (Aldrich Chemical Company, UK), dialysis tubing (3.5 and 10 kDa cutoff limits; Medicell International Limited, UK), Sepharose SP HiTrap, PD-10 columns and Sephadex G50, G75 and G100 (Pharmacia, UK), Tris-glycine polyacrylamide gels (4–20%), Tris-glycine sodium dodecyl sulfate running buffer and loading buffer (Novex, UK). Deionized water was obtained from an Elgastat Option 4 water purification unit (Elga Limited, UK). All reagents used were of analytical grade. A Wallace CompuSpec UV–visible spectrophotometer connected to a PC (Wallace UK Ltd, UK), and/or a plate reader (Dynex Technologies, UK) were used for spectrophotometric determinations. Female T/O outbreed mice (8–9 weeks old; 21–24 g body weight) were purchased from Harlan UK (Bicester, Oxon, UK) and acclimatized for at least 1 week prior to their use.

2.1. Protein and CA determination

Quantitative estimation of PSAs (as sialic acid) with the resorcinol reagent was carried out by the method of Svennerholm [40] as described elsewhere [28–30]. Insulin was measured either directly by spectrophotometry (280 nm) or by the BCA colorimetric method [41].

2.2. Activation of CA

CAs (22 and 39 kDa) were oxidized with periodate as described [28–30,42]. Freshly prepared 0.1 M sodium metaperiodate ($NaIO_4$) solution was mixed with CA (10 mg CA/ml $NaIO_4$) at 20 °C and the reaction mixture was stirred magnetically for 15 min in the dark. A two-fold volume of ethylene glycol was then added to the reaction mixture to expend excess $NaIO_4$ and the mixture left to stir at 20 °C for a further 30 min. The oxidized CA was dialysed (3.5-kDa molecular weight cutoff dialysis tubing) extensively (24 h) against a 0.01% ammonium carbonate buffer (pH 7.4) at 4 °C. Reverse dialysis was used to concentrate the CA solution within the dialysis tubing by laying the tubing at the bottom of a flat tray, covered with dry polyethylene glycol (8 kDa). Following concentration to the required volume, usually within 2 h, the dialysate was lyophilized and stored at −40 °C until further required. Alternatively, the oxidized CA was precipitated with 70%

ethanol, centrifuged at 5000 × *g* for 15 min in a bench centrifuge and the pellet dissolved in a minimum quantity of water. Following a second precipitation in 70% alcohol, the pellet was lyophilized and stored at −40 °C until further required. Levels of remaining sodium metaperiodate (after oxidation of CA) assayed by a modification of Pierce's PeroXOquant™ assay [43] were below the detection limit (0.05% weight/weight).

2.3. Determination of CA oxidation

Estimation of the degree of CA oxidation was carried out with 2,4 dinitrophenylhydrazine (2,4-DNPH), which yields sparingly soluble 2,4 dinitrophenyl-hydrazones on interaction with carbonyl compounds. For a qualitative assay, non-oxidized and oxidized CA (5 mg each) were added to the 2,4-DNPH reagent (1.0 ml), the solutions were shaken and then allowed to stand at 37 °C until a crystalline precipitate was observed [44]. A quantitative method for the estimation of CA oxidation with 2,4 DNPH was developed using propionaldehyde as a standard. To that end, a sample (10 μl) of propionaldehyde was incubated with 25 μl of 2,4-DNPH for 10 min at 37 °C, followed by the addition of 200 μl sodium hydroxide (0.5 M) and further incubation for 5 min at the same temperature. The developed color was measured at 490 nm. This method of estimation of CA oxidation was also compared with the one [45] based on the reduction of ferricyanide ions in alkaline solution to ferric ferrocyanide (Persian blue), which is then measured at 560 nm. In this instance, glucose was used as a standard.

2.4. Preparation of insulin–CA conjugates

Insulin dissolved in a minimum volume (50 μl) of 10 mM hydrochloric acid and diluted with 0.75 M dipotassium hydrogen phosphate (K_2HPO_4) was covalently linked to oxidized CA via reductive amination in the presence of sodium cyanoborohydride ($NaCNBH_3$). In order to investigate the correlation between the time of reaction of CA and insulin and the yield of the materials in the polysialylated insulin obtained (CA/insulin molar ratio), CA of different chain lengths (22 and 39 kDa) together with insulin in a variety of CA/insulin molar ratios (25:1 to 150:1 range) were reacted in 0.75 M dipotassium hydrogen phosphate (pH 8.0; 5 ml) containing sodium cyanoborohydride (4 mg/ml) in sealed vessels with magnetic stirring at 35 ± 2 °C in an oil bath. Aliquots (1 ml) of the mixtures were removed at 0, 6, 12, 24 and 48 h, pre-cooled on ice, and then subjected to ammonium sulfate ($(NH_4)_2SO_4$) precipitation by adding the salt slowly while continuously stirring, to achieve 70% w/v saturation. The samples, stirred for 1 h at 4 °C, were centrifuged for 15 min (5000 × *g*) and the pellets containing polysialylated insulin suspended in a saturated solution of $(NH_4)_2SO_4$ and centrifuged again for 15 min (5000 × *g*). $(NH_4)_2SO_4$ was chosen over other salts as a means to precipitate out the insulin–CA conjugates as it is known not to denature proteins, and also to stabilize them [46]. The precipitates recovered were redissolved in 1 ml 0.15 M Na phosphate buffer supplemented with 0.9% NaCl (pH 7.4; PBS) and dialysed extensively (24 h) at 4 °C against the same PBS. The dialysates were then assayed for sialic acid [28–30,40] and insulin [41] content and the conjugation yield was expressed in terms of CA/insulin molar ratio. Controls included subjecting the native protein to the conjugation procedure in the presence of non-oxidized CA or in the absence of CA, under the conditions described. Stirring was kept to a minimum to avoid concomitant denaturation of the protein. Polysialylated insulin was further characterized by size exclusion chromatography, ion exchange chromatography (IEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Size exclusion chromatography

Zero (control) and 48 h aliquots (1 ml) from the reaction mixtures were chromatographed on Sephadex columns (42 × 1.1 cm; flow rate 1 ml/min; eluent: 0.1 M sodium phosphate buffer, pH 5.5) using Sephadex G50 and G100 for the 22- and 39-kDa CA–insulin conjugates, respectively. Eluent fractions (1 ml) were assayed for CA and insulin content. A Sephadex G-50 column was used under the same conditions to compare the chromatographic behaviour of CA (22 kDa) with that of its oxidized derivative.

2.6. Ion exchange chromatography

Zero (control) and 48 h samples (0.5 ml) from the reaction mixtures were subjected to IEC on a Sepharose SP cation exchange column (1 ml; flow rate 1 ml/min; binding/washing buffer 0.05 M sodium phosphate, pH 4.0; elution buffer, 0.05 M sodium phosphate buffer, pH 4.0 containing 1 M sodium chloride). The columns were washed, eluted, and the eluent fractions were assayed for CA and insulin content. PD-10 columns were used for desalting samples before applying to column.

2.7. Polyacrylamide gel electrophoresis

SDS-PAGE (MiniGel, Vertical Gel Unit, model VGT 1, power supply model Consort E132; VWR, UK) was employed to detect changes in the molecular size of insulin upon polysialylation. SDS-PAGE of insulin and its conjugates of 0 (control), 24 and 48 h samples from the reaction mixtures as well as a process control (non-oxidized CA), was carried out using a 4–20% polyacrylamide gel. The samples were calibrated against a wide range of molecular weight markers.

2.8. In vivo studies

Insulin and polysialylated insulin constructs were tested for their ability to reduce blood glucose level in normal

female T/O outbred mice. Animals were divided into groups of five, injected subcutaneously (s.c.) with insulin (0.3 units per mouse in 0.9% sodium chloride or with the same protein equivalence of polysialylated insulin) and glucose levels in blood samples were measured at time intervals using a glucose assay kit (Accu-Chek Advantage, Roche, UK).

3. Results and discussion

3.1. Activation of CA

Colominic acid, a PSA, is a linear α-(2 → 8) linked homopolymer of Neu5Ac residues (Fig. 1). The three hydroxyl groups (vicinal diols) located on adjacent carbon atoms (7, 8 and 9) at the nonreducing end of the molecule offer a unique opportunity for derivatization. Thus, controlled periodate oxidation down to carbon 7 produces an aldehyde group (Fig. 1) that may be subsequently coupled to the amino groups of peptides or proteins (ε-amino groups of lysine residue(s) and/or the terminal amino groups) via reductive amination. Periodate, however, is a powerful oxidizing agent and although selective [47] for carbohydrates containing hydroxyl groups on adjacent carbon atoms, it can cause time-dependent cleavage to the internal Neu5Ac residues [48]. Therefore, in the present work, exposure of CAs to oxidation was limited to 15 min using 100 mM periodate [48]. Moreover, as periodate decomposes on exposure to light to produce more reactive species [49], reaction mixtures were kept in the dark. The integrity of the internal α-(2 → 8) linked Neu5Ac residues post periodate treatment was tested in the case of 22-kDa CA by size exclusion chromatography and the chromatogram obtained for the oxidized material was compared with that of intact CA. It was found (Fig. 2) that both oxidized and intact CA exhibit identical elution profiles and it would thus appear that the oxidation process does not cause significant reduction of the average molecular weight of the polysaccharide.

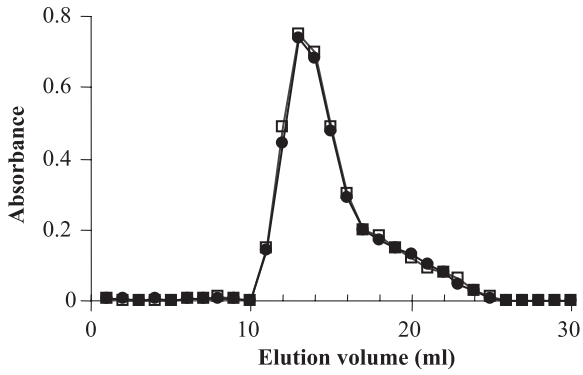

Fig. 2. Size exclusion chromatography of native (□) and oxidized (●) 22-kDa CA. Samples (0.6 ml) were chromatographed on a Sephadex G50 column and eluted fractions were assayed for CA.

3.2. Degree of oxidation

Quantification of the aldehyde moiety of the oxidized CA (i.e. aldehyde-containing CA) was carried out by reaction with 2,4-dinitrophenyl hydrazine (2,4-DNPH). In the presence of carbonyl compounds, 2,4-DNPH forms sparingly soluble phenylhydrazones that can be identified as a bright orange/yellow precipitate produced almost immediately. CA, as well as its oxidized derivative, possess carbonyl functionality at the reducing end, and could therefore potentially react with 2,4-DNPH to form an insoluble phenylhydrazone. However, it was reasoned that it would be possible to differentiate between CA and oxidized CA products by the use of 2,4-DNPH on the basis of intensity of the reaction. It was anticipated that the 'naked' reactive aldehyde group of the oxidized CA would form a dinitrophenylhydrazone precipitate more efficiently than intact CA, which possesses a 'masked' ketone functionality, in equilibrium with the more thermodynamically favoured ring structure of CA. On addition of 2,4-DNPH to a sample of intact CA, the pale yellow color of the reagent remained initially unchanged. However, a faint yellow

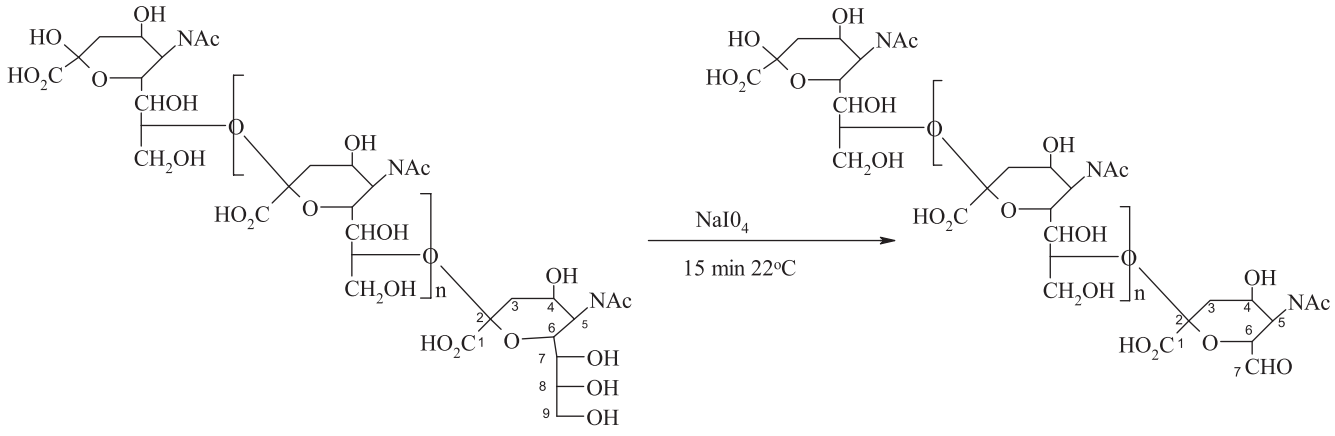

Fig. 1. Structures of intact and oxidized CA. Neu5Ac units are linked via α-(2 → 8) glycosidic bounds. Arrow indicates the carbon atom at the nonreducing end of CA (left) where periodate oxidation introduces an aldehyde group (right).

precipitate was formed within 15 min. When the 2,4-DNPH reagent was also added to a sample of the oxidized CA, the reagent turned an intense orange instantly. At 10 min, an intense orange precipitate was formed, which continued to develop further. Measurement of CA oxidation in the presence of 2,4-DNPH, carried out with propionaldehyde as a standard, showed that 96–99% of the CA was oxidized. Similar results were obtained when CA oxidation was measured on the basis of ferricyanide ion reduction in alkaline solution to ferrocyanide (Persian blue) [44] using glucose as a standard.

3.3. Preparation of insulin–CA conjugates

It was noted that on addition of intact CA or oxidized CA (both soluble in 0.75 K_2HPO_4, pH 8.0) to solutions of insulin, they turned slightly cloudy, suggesting partial aggregation of the protein, possibly due to ionic interaction with CA even though the ionic strength of the solution was high. A similar observation has been made with other polymers, for instance PEG [50]. However, after 48 h of reaction, the turbidity of the solutions was much less pronounced with oxidized CA (which was linked to the insulin) than with intact CA, presumably because of an increase in the solubility of insulin as a result of the highly polar nature of the polysaccharide. A similar increase in the solubility of insulin was seen after a 12-h reaction with dextran aldehyde [51].

In preliminary experiments, it was found that optimal reaction conditions for best CA/insulin molar conjugation yields required a temperature of 35 ± 2 °C in 0.75 M K_2HPO_4 buffer at pH 6–9 for 48 h. The imine (Schiff base) formed under these conditions was successfully reduced with $NaCNBH_3$ to form a stable secondary amine [29,30]. As already reported with the polysialylation of catalase [30], after polysialylation in the absence of $NaCNBH_3$, the imine generated from oxidized CA was stable to some extent so as to exist as a Schiff base conjugate. Fig. 3 shows the reaction

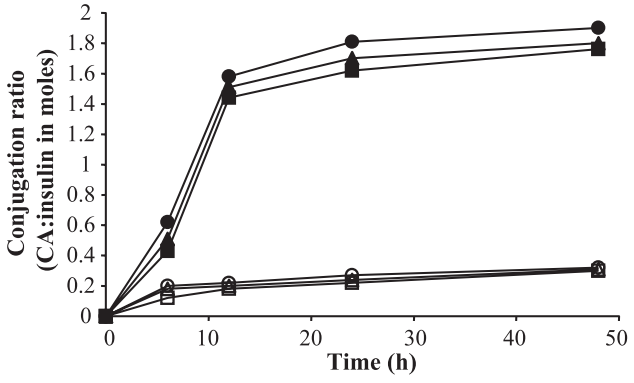

Fig. 3. Effect of reaction time on the yield of conjugation of 22-kDa CA to insulin. Intact (open symbols) and oxidized (filled symbols) CA was conjugated to insulin as described in Materials and methods. Molar ratios of CA and insulin in the conjugation mixture were 25:1 (squares), 50:1 (triangles) and 100:1 (circles).

Table 1
Conjugation of colominic acid to insulin

Initial CA/insulin molar ratio		CA/insulin molar ratio in the conjugate	
		Oxidized CA	Intact CA
22-kDa CA	25:1	1.60 ± 0.14	0.20 ± 0.02
	50:1	1.65 ± 0.14	0.21 ± 0.04
	100:1	1.74 ± 0.12	0.24 ± 0.06
39-kDa CA	37.5:1	2.37 ± 0.16	0.32 ± 0.03
	75:1	2.40 ± 0.01	0.35 ± 0.04
	150:1	2.45 ± 0.19	0.40 ± 0.03

Oxidized and non-oxidized (intact) CA (22 or 39 kDa) were conjugated to insulin at the initial molar ratios shown. CA/insulin molar ratio values in the conjugates are mean ± S.D. (n = 3).

kinetics over 48 h of incubation at 37 °C when insulin was polysialylated using the oxidized and non-oxidized 22-kDa PSA at three different CA/insulin molar ratios (25:1, 50:1 and 100:1). Polysialylated insulin was isolated from aliquots taken at time intervals by ammonium sulfate precipitation (as described in Materials and methods) and the results expressed in terms of CA/insulin molar ratios in the conjugates. The data reveal an initial rapid reaction, peaking at about 12 h to form a plateau over a period of 12–48 h. In order to study the effect of CA size on the polysialylation yield, two different sizes of CA (22 and 39 kDa) were used under the conditions described in Fig. 3, except that the initial molar ratios of CA/insulin used for the 39-kDa CA were 37.5, 75:1 and 150:1. Results in Table 1 show that conjugation yield values (CA/insulin molar ratios) were

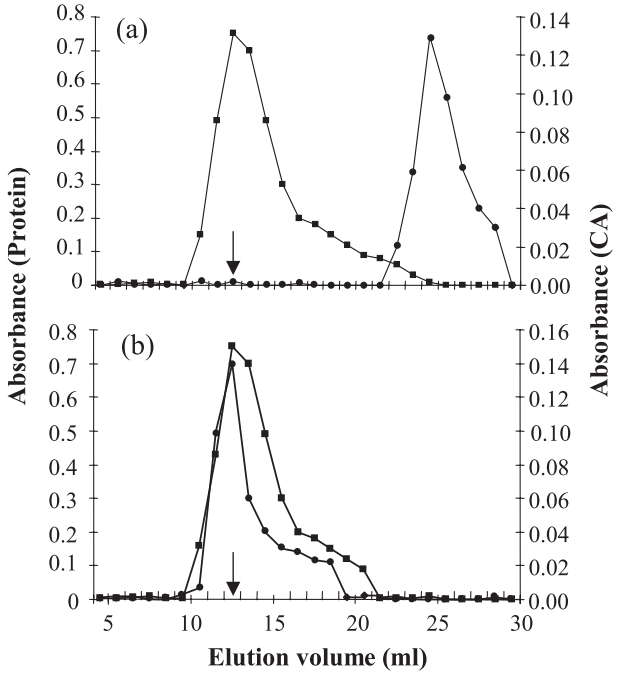

Fig. 4. Size exclusion chromatography of CA and insulin after 0 h (a) and 24 h (b) of reaction. Initial molar ratio of CA/insulin was 25:1. Samples (0.6 ml) were chromatographed on a Sephadex G-50 column and eluant fractions assayed for protein (■) and CA (●). Arrow denotes the void volume (fraction 13) of the column.

lower for the 22-kDa CA (1.60–1.74) than for the 39-kDa CA (2.37–2.45). Table 1 also reveals that when non-oxidized CA (in the presence of cyanoborohydride) was used, some conjugation did occur with both CAs (0.20–0.41, CA/insulin molar ratios), presumably via the hemiacetal group (C=O) of CA at its reducing end. With regard to the sites of conjugation on the insulin molecule, it is likely that in addition to the ε-amino group of the lysine, either or both of the two N-terminal amino groups (PheB1 and GlyA1) also participated in the reaction. This is supported by work on insulin pegylation [50] in which PEG was conjugated to the N-terminal amino groups. However, the extent of participation of the two terminal amino groups and the lysine ε-amino group of the insulin molecule in polysialylation remains at present unknown.

Initial evidence that insulin was covalently linked to PSA to form a neoglycoprotein was obtained by size exclusion chromatography (Fig. 4). Thus, elution profiles after chromatography of insulin and CA at zero time of the reaction showed that the two moieties eluted separately (Fig. 4a), whereas they eluted together (Fig. 4b) after 48 h of reaction. Formation of the conjugate was further confirmed by the co-precipitation of the two moieties on addition of $(NH_4)_2SO_4$ in the eluted samples (CA as such does not precipitate in the presence of the salt). Evidence of conjugation was also provided by IEC and SDS-PAGE. In the former case,

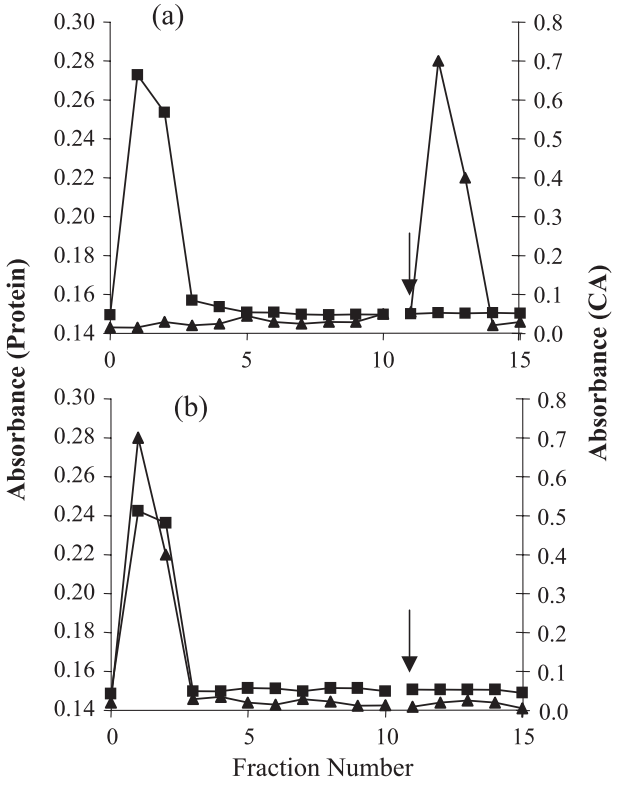

Fig. 5. Cation exchange chromatography of a mixture of CA (22 kDa) and insulin (a) and the CA-insulin conjugate (b). Arrow indicates the addition of 1 M NaCl to elute the bound protein. Eluted fractions were assayed for protein (■) and colominic acid (▲).

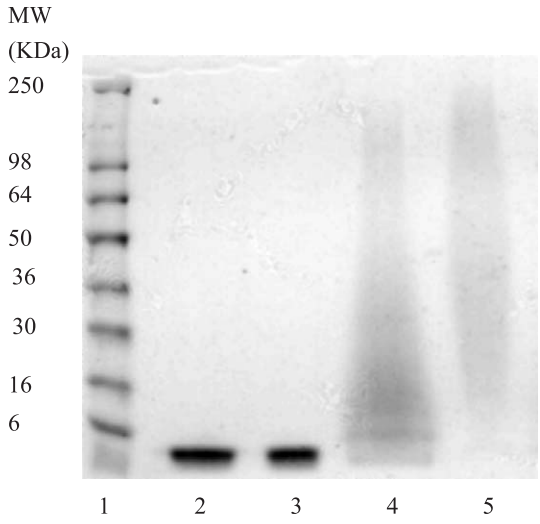

Fig. 6. SDS-PAGE of intact insulin, insulin polysialylated with 22- or 39-kDa CA and molecular weight markers. Lane 1, molecular weight markers; lane 2, intact insulin; lane 3, insulin incubated for 48 h in the presence of non-oxidized CA (process control); lanes 4 and 5, reaction mixtures of insulin and CA (22 or 39 kDa, respectively).

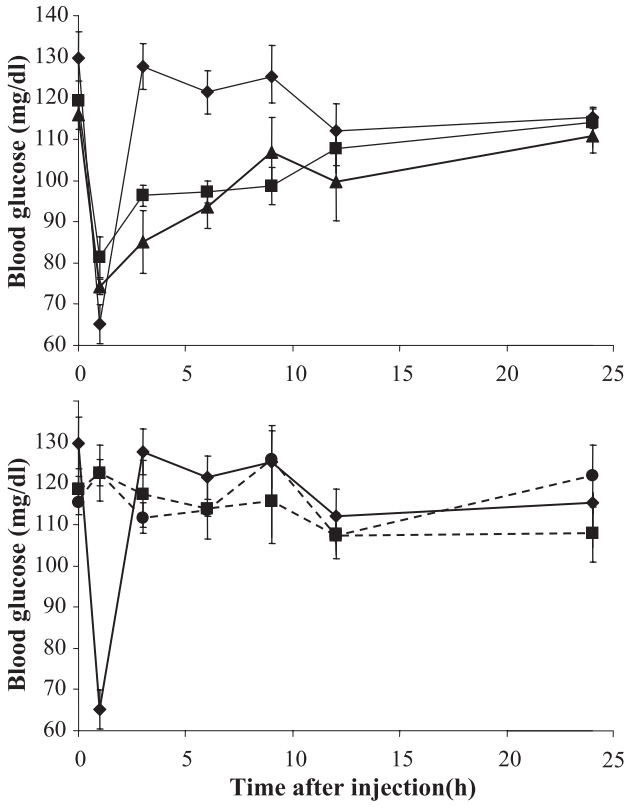

Fig. 7. Blood glucose levels in T/O mice after subcutaneous injection of intact insulin (♦), polysialylated insulin using 22-kDa (■) and 39-kDa (▲) CA (upper panel), and of equivalent doses of 22-kDa (■) and 39-kDa (●) CA alone (lower panel). Values with intact insulin in the lower panel (also shown in the upper panel) are plotted for comparison. When appropriate, animals received 0.3 units of insulin based on protein content. Values are mean ± S.D. ($n=5$).

polysialylated insulin obtained by $(NH_4)_2SO_4$ precipitation was redissolved in Na phosphate buffer (50 mM, pH 4.4) and subjected to IEC using a Sepharose SP HiTrap column (cation exchange) with zero time samples serving as a control. In contrast with results (Fig. 5) indicating complete resolution of CA (in the wash) and insulin (in eluted fractions), both CA and insulin from the 48-h reaction samples co-eluted in the wash fractions (Fig. 5). Finally, when SDS-PAGE was applied to detect changes in the molecular weight of insulin as a result of polysialylation with 22-kDa and 39-kDa CA, examination of the gels (Fig. 6) revealed well-resolved bands for both controls, i.e. intact insulin (Fig. 5, lane 2) and insulin reacted with non-oxidized CA for 48 h (process control) (Fig. 6, lane 3), which migrated approximately the same distance in the gel. On the other hand, the 48 h (Fig. 6, lane 4 for 22-kDa CA and lane 5 for 39-kDa CA) reaction samples yielded multiple diffused bands suggesting heterogeneity, presumably due to differential polysialylation of the insulin molecules (i.e. differing numbers of chains, probably of differing length per molecule). No attempts were made here to estimate the molecular weight of the conjugates. Similar findings of protein heterogeneity have been observed with polysialylated catalase [30] and asparaginase [29], and with pegylated proteins [52].

3.4. In vivo studies

The pharmacological activity of polysialylated insulin constructs was compared with that of intact insulin in normal mice injected subcutaneously and bled at time intervals. Results in Fig. 7 clearly show that insulin polysialylated with the 22-kDa CA exerted a more prolonged reduction of blood glucose levels. Thus, whereas glucose levels attained nadir values at 1 h to return to normal levels 3 h after treatment with intact insulin, glucose levels in mice treated with the polysialylated peptide, although also lowest at 1 h, returned to normal values after 9 h. While these results indicate that polysialylation under the present conditions does not prevent interaction of insulin with its receptor, it is conceivable that some masking of the active site of insulin does occur, curtailing a potentially longer duration of pharmacological activity than that seen in Fig. 7 (upper panel). Indeed, the reduced period (6 h; Fig. 7, upper panel) of insulin activity when the longer (39 kDa) CA was used for polysialylation suggests that CA may partially mask the active site, depending on the length of the polymer. On the other hand, it is also feasible that the greater size of the insulin conjugate obtained with the 39-kDa CA contributes to longer retention at the injection site and greater exposure to local proteases, leading to some loss of activity.

In conclusion, polysialylation technology offers a promising strategy for the enhancement of the therapeutic value of insulin and other pharmacologically active peptides. The results reported here show that polysialylation allows for the preparation of novel insulin constructs with improved pharmacological properties, potentially contributing to easier and more accurate long-term control of blood glucose levels. The judicial choice of the number of PSA chains and their length, conjugated at appropriate sites on the insulin molecule, are critical steps in obtaining constructs with optimal activity. Such studies, together with work on the immunogenicity and antigenicity of polysialylated insulin, are now in progress.

Acknowledgements

We thank Mrs. Concha Perring for excellent secretarial assistance.

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