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High temperature conjugation of proteins with carbohydrates

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A new procedure was used to conjugate lactose and dextran with BSA without using coupling or activating reagents. The method is simple, rapid and cheap. Reducing sugars covalently bind to proteins when lyophilized together and briefly heated to a high temperature.

***Keywords*: protein, conjugate, carbohydrate, lyophilization, vaccine, temperature, heating**

***Abbreviations*: BSA; bovine serum album**

Introduction

Neoglycoconjugates are synthetic compounds made of carbohydrate groups coupled to proteins, lipids, and other carriers. They are of interest as powerful reagents for isolation and assay of glycotransferases, characterization of lectins, or targeting drugs (for review see [1]). Neoglycoconjugates are used as diagnostic reagents, immunogens, and artificial vaccines. There are numerous methods for the synthesis of such conjugates (for reviews see [2, 3]). As a general rule the carbohydrate moiety, and sometimes also the carrier, have to be chemically converted into their reactive derivatives to facilitate formation of a covalent bond. In some cases, glycation can be achieved with the use of bifunctional cross-linking reagents or enzymatically by transglycosylation [4]. Here we demonstrate a novel approach to the synthesis of neoglycoconjugates. We have made and explored an observation that reducing sugars covalently bind to proteins after they are lyophilized together to dryness and then briefly heated to a high temperature. Optimization of conditions for the reaction resulted in a protocol for the simple production of highly substituted glycoproteins with a well preserved, biologically reactive structure of the original carbohydrate as well as retained activity of the model proteins. Our method allows the direct attachment of carbohydrate moieties to the protein without any prior modification or chemical activation, is simple, and well suited for large scale applications.

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Materials and methods

Reagents

All reagents were of analytical grade. Dextran T10 (average molecular weight 10 kDa) and molecular weight standards were purchased from Pharmacia (Sweden), lactose was purchased from POCh (Poland), BSA, lectin from *Ricinus communis* I, *Concanavalin* A, streptavidin-peroxidase conjugate and biotinaminocaproate *N*-hydroxysuccinimide ester were purchased from Sigma (USA), lectins were biotinylated according to [5]. *N*-benzoyl-DL-arginine-4-nitroanilide was from Fluka (Switzerland). Pure bovine pancreas trypsin [6] was obtained from the Institute of Biochemistry, Wrocław University (Poland). *Hafnia alvei* strain PCM 1192 core hexasaccharide [7] was from Institute of Immunology and Experimental Therapy, Wrocław (Poland).

Synthesis of BSA–lactose, BSA–dextran, immunoglobulin-dextran, lactose-trypsin, and BSA-*Hafnia alvei* LPS hexasaccharide glycoconjugates

Protein and sugar were dissolved in water at 1 : 1 weight ratio, typically 2 mg each in 1 ml solution. The solution was frozen in the acetone–dry ice bath in a glass test tube and lyophilized. Dry lyophilizate was then placed in an air oven at 95–125 °C for 20–40 min (for *H. alvei* hexasaccharide 105–110 °C, 15 min), with the tube left open. Care was taken not to let the tube walls touch any metal parts in the oven to prevent localized hot spot formation. The tube was allowed to cool down to room temperature, the powder was dissolved in water or PBS. Trypsin conjugates were dissolved in 5 mM calcium nitrate. Low molecular weight unbound carbohydrates were separated by multiple dialysis.

Unreacted excess of high molecular weight dextran was removed by ion exchange chromatography on a DEAE 5PW column (7.5 × 75 mm). Free dextran did not bind to the column and was eluted with 0.02 M phosphate buffer, pH 7, whereas the conjugate was eluted with 0.5 M NaCl.

Analytical methods

The overall carbohydrate content of the samples was measured colorimetrically by the phenol sulfuric acid method [8]. The glucose content of glycoconjugates with dextran or lactose was determined using the phenol–acetone reagent [9]. Briefly, 0.1 ml of sample was mixed with 0.5 ml of 5% phenol, 2% acetone in water, followed by 1.5 ml of concentrated sulfuric acid. The absorbance at 568 nm was read after a 40 min incubation at 37 °C. Free amino groups in the conjugates were determined by the reaction with trinitrosulfonic acid and a colorimetric readout at 335 nm [10]. BSA was used as a standard. Trypsin amino group determination was done in 0.012 M borate and 0.07 M sodium acetate containing 0.015 M calcium nitrate and 0.1% TNBS (pH was adjusted to 8.5).

Determination of the monosaccharide composition. Glycoconjugates were hydrolized in 2 M trifluoroacetic acid at 120 °C for 2 h, reduced, and acetylated with 1 : 1 (by vol) mixture of acetic anhydride and pyridine at 100 °C for 1 h. The resulting mixtures of acetylated monosaccharides were separated and individual peaks were identified by gas chromatography–mass spectrometry (Hewlett-Packard 5971 A system using glass capillary column 0.2 mm × 12 m). Glass tubes were used for the glycoconjugate synthesis because the plastic Eppendorf tubes were found to release, at the temperatures employed, plasticizers evident in the GLC–MS analysis.

Peptide analysis. 1.2 mg of the BSA-lactose conjugate or control BSA preparations were digested with 0.05 mg of trypsin in 0.3 ml of 0.08 M Tris-HCl, 0.02 M $Ca(NO_3)_2$, pH 8.3 and 37 °C for 16 h. The peptide profiles were obtained by the reversed-phase high-performance liquid chromatography (C18 5 μm column, 0.5 × 25 cm, eluent A: 0.85% phosphoric acid, eluent B: acetonitrile, linear gradient from 0 to 30% in 10 min, flow 1 ml min^{-1}). Chromatograms were monitored at 280 nm, using a photodiode-array detector (Waters 990 +).

SDS-polyacrylamide gel electrophoresis was carried out in 8% gel under reducing conditions according to Laemmli [11]. The apparent molecular weight of BSA conjugates was assessed by gel chromatography on TSK G3000SWxl and TSK G2000SWxl HPLC columns (Beckman) in 0.1 M phosphate buffer, pH 6.7. Traces of aggregates were removed prior chromatography by filtration through a 0.45 μm membrane.

UV–VIS absorbance spectra of the water solution of BSA–lactose conjugate were obtained on a Hewlett Packard photodiode–array spectrophotometer.

Immunoenzymatic assays

Rabbit anti-*Hafnia alvei* LPS antiserum was obtained from Dr Ługowski [7]. The wells of 96-well ELISA plates were coated overnight with 50 μl of 1 μg ml^{-1} solutions of either *H. alvei* LPS, or LPS hexasaccharide-BSA glycoconjugate, or BSA in 50 mM sodium borate, pH 9.5. The wells were blocked with 0.1% BSA for 4 h, washed with 0.05% Tween − 20 in PBS and incubated with serial two-fold dilutions of the rabbit anti-*H. alvei* LPS antiserum at 25 °C for 4 h. Bound rabbit immunoglobulin was detected by incubation with goat-anti rabbit peroxidase (Sigma) with 3.7 mM *o*-phenylenediamine and 20 mM hydrogen peroxide in a 0.2 M citric buffer, pH 5.0 (0.1 ml per well) for 5 min at room temperature. Following the 0.1 ml of 0.8 M sulfuric acid was added and the absorbances were read at 490 nm.

Lectin binding assay

Ninety-six well plates were coated overnight with 1, 0.25, 0.06, and 0.01 μg per 50 μl water solutions of the BSA-lactose glycoconjugate. Plates were blocked with 0.1% BSA solution for 1 h, washed with 0.005% Tween-20 in 0.15 M NaCl, 0.01 M Tris-HCl, pH 8.3, and incubated with 0.05 μg per well of biotinylated lectin from *Ricinus communis* I, or 0.05 μg per well of biotinylated Concanavalin A in 0.005% Tween-20. After 2 h, the wells were washed with 0.05% Tween-20 in the NaCl-Tris, pH 8.3, buffer, incubated for 1 h with streptavidin–peroxidase (diluted 1 : 2000). After washing with 0.005% Tween − 20 the reaction was developed with *o*-phenylenediamine (as above).

The double diffusion test was done in 1% aqueous agarose gel. The central well contained 10 μl of 10 mg ml^{-1} *Ricinus communis* lectin which was allowed to diffuse for 24 h prior to the application of 20 μl of the 10 mg ml^{-1} glycoconjugate solutions to the sample wells.

The double diffusion test for antibody activity was done in 0.05 M barbiturate buffer, pH 8.0. All reagents (dissolved at 1 mg ml^{-1} in 0.15 M NaCl) were applied simultaneously in 10 μl volume.

Results and discussion

In the course of preliminary experiments we noticed that a brief exposure of lyophilized powdered mixtures of BSA and sugars to temperatures up to 125 °C produced adducts with specifically increased molecular weights as demonstrated by gel filtration chromatography in Figure 1A and B. Heating of BSA with lactose preserved the characteristic dimer–monomer elution profile of the native BSA and shifted both peaks towards a 5 to 15% larger apparent molecular weight. This effect was not observed when lactose was absent in the reaction mixture. Heating of the BSA–dextran lyophilizates resulted in a much larger increase in the

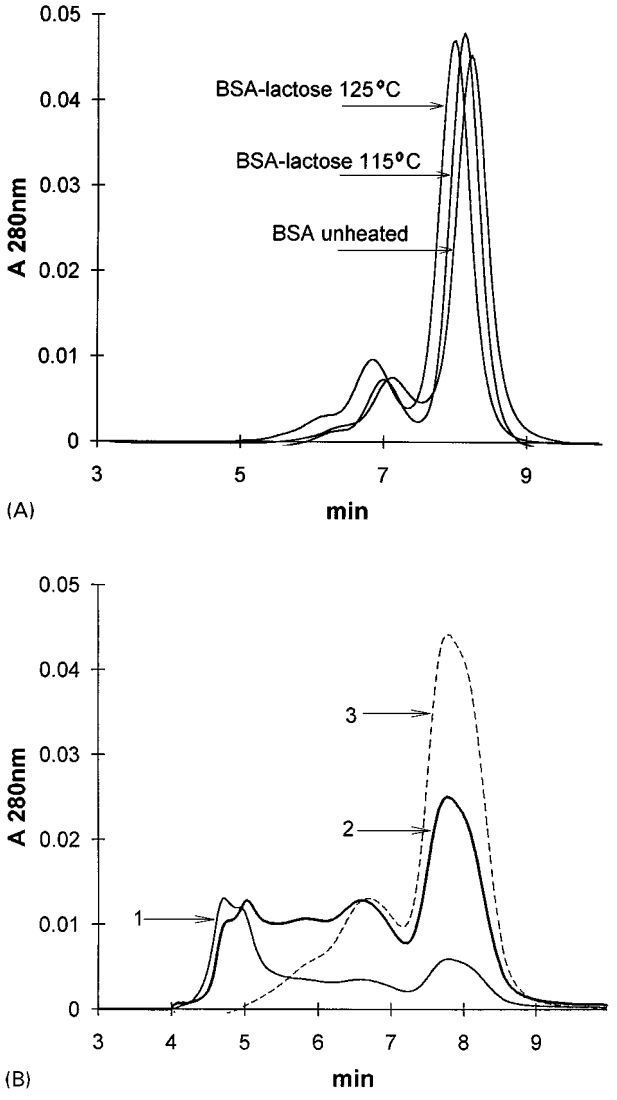


Figure 1. (A) Gel filtration on TSK G3000 SWxl TSK column of BSA–lactose (reaction mixture: 1 mg BSA and 1 mg lactose) conjugates after 15 min reaction at 115 °C, 125 °C. Eluent: 0.1 M phosphate buffer, pH 6.7, flow 1 ml min^{-1}. Molecular weight markers: JgM MOPC 104E (5.02 ml), fibrinogen 340 kDa (5.41 ml), horse JgG 150 kDa (7.56 ml), BSA 67 kDa (8.26 ml), ovalbumin 43 kDa (9.55 ml). (B) Gel filtration of BSA–lactose conjugates after 30 min reaction at 120 °C. Eluent: 0.1 M phosphate buffer, pH 6.7, flow 1 ml min^{-1}. Score 1, reaction mixture 3.15 mg BSA and 0.85 mg lactose; score 2, reaction mixture 3.15 mg BSA and 1.7 mg lactose; score 3, reaction mixture 3.15 mg BSA and 5.1 mg lactose (BSA heated and BSA unheated – 8.26 min).

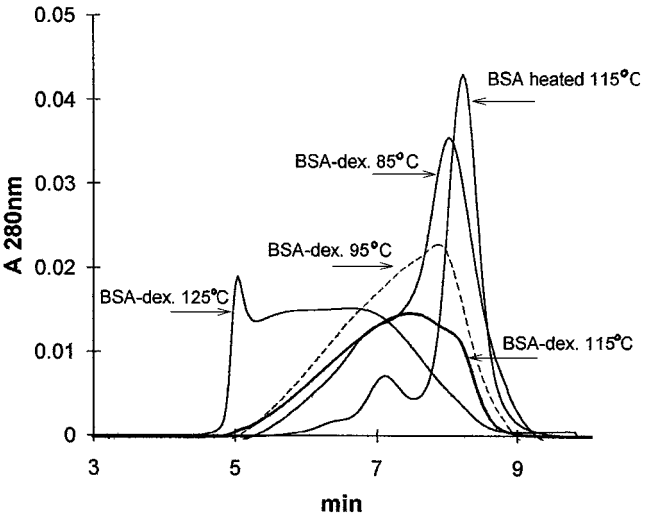


Figure 2. Gel filtration on TSK G3000 SWxl TSK column of BSA–dextran T10 (reaction mixture: 1 mg BSA and 1 mg dextran) conjugates after 30 min reaction at 85 °C, 95 °C, 115 °C, 125 °C and BSA heated at 115 °C. Eluent: 0.1 M phosphate buffer, pH 6.7, flow 1 ml min^{-1} (BSA heated and unheated – 8.26 min).

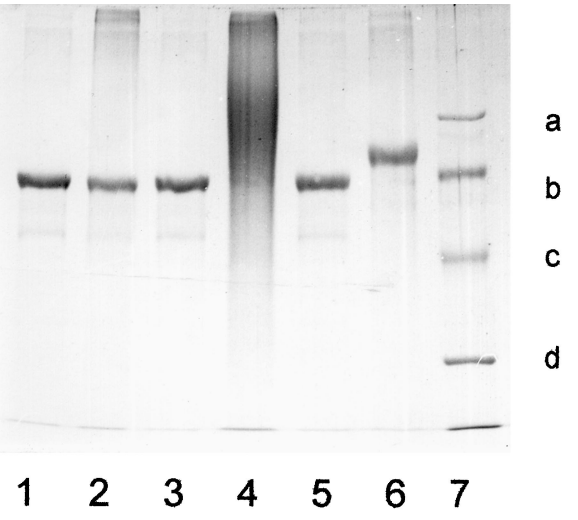


Figure 3. SDS–polyacrylamide electrophoresis in 8% gel in the presence of 1% of 2-mercaptoethanol. 1, BSA unheated; 2, BSA heated at 125 °C; 3, BSA and dextran T10 mixture; 4, BSA–dextran T10 conjugate (2.6 mol dextran per mol BSA); 5, BSA and lactose mixture; 6, BSA–lactose conjugate (17 mol lactose per BSA); 7, molecular weight markers: a: phosphorylase-b (94 kDa), b: BSA (67 kDa), c: ovalbumin (43 kDa), d: carbonic anhydrase (30 kDa). Gel was stained with Coomassie blue.

molecular weight (Figure 2). The heating of BSA or trypsin did not influence the elution profile of heated and unheated protein (Figures 1A, 2 and 8). Traces of aggregates were removed prior to chromatography. To test whether the observed effect was accompanied by the formation of covalent sugar-protein bonds, the BSA–lactose and BSA–dextran conjugates were boiled in 5% SDS, 1% 2-mercaptoethanol and analysed by SDS polyacrylamide gel electrophoresis. The BSA–lactose conjugate migrated as a sharp band of 75 kDa whereas BSA (either heated without lactose or lyophilized with lactose but without thermal processing) was indistinguishable from the control 67 kDa BSA band. Similarly, the BSA–dextran thermal conjugate (but not the unheated mixture of BSA with dextran) migrated as a high molecular weight diffuse band spanning a 75–200 kDa range (Figure 3).

Table 1A. Effect of reaction time on lactose substitution of BSA.

Reaction time (min)	*mol lactose per mol BSA*
0	0.12
10	4.4
20	17.8
30	17.3
40	18.3
60	17.9

The reaction was run at 1 : 1 BSA to lactose weight ratio, at 120 °C, concentration of BSA and lactose before the lyophilization was 10 mg/ml in 0.2 ml volume.

Table 1B. Proportion of reagents versus level of substitution in BSA–lactose conjugates (40 min reaction at 120 °C).

mg BSA per mg lactose in reaction mixture	*mol lactose/mol BSA*
3.3/0.34	11.9[a]
3.3/0.85	9.8
3.3/3.3	19.8
3.3/6.4	20.2
3.3/21	21.4

[a] The sample contained aggregated material.

To optimize the conditions of the reaction, lyophilized mixtures of BSA and lactose were exposed to a temperature of 120 °C for different time intervals. The resulting powder was dissolved in water and the unbound lactose was removed by multiple dialysis. Determination of the total sugar content demonstrated that the reaction reached the plateau after 20 min (Table 1A). Thermal treatment of longer than 60 min or at temperatures higher than 125 °C resulted in a visible yellowing of the samples. Comparison of UV–visible absorbance spectra of identical weight concentrations of lactose–BSA and native BSA revealed a small increase in absorbance at 300 nm (Figure 4). The accompanying decrease in absorbance at 280 nm was in agreement with the lower protein content of the conjugate. The degree of substitution depended on the proportion of reagents in the reaction mixture (Table 1B, Figure 1B). The influence of the lactose : BSA weight ratio on the reaction product is very important. At a low concentration of lactose the aggregated material was formed. We have found that there was no aggregating effect of dextran when the dextran : BSA weight ratio ranged below 1 (0.1 to 1). The sugar to protein weight ratio of 1 : 1 was adopted for all subsequent experiments.

Characterization of the conjugates

Tryptic digestion of BSA or heated BSA showed similarity between these macromolecules. However, there was a significant difference between digested BSA–lactose conjugate and both albumins (Figure 5). To explain why a larger amount of peptides formed in the course of trypsin digestion, it can be anticipated that conjugates constitute a heterogeneous population of macromolecules with incidental lactose substitution. Each heterogeneous population will yield a wider spectrum of digested products. This may mean that the glycation of lysyl residues results in change in the conformation and susceptibility of the molecule, thus allowing a more efficient hydrolysis at the arginine residue.

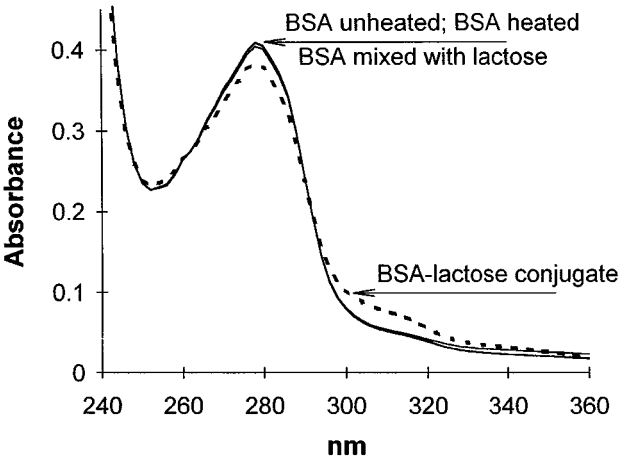


Figure 4. Spectrum of BSA–lactose conjugate (20 mol lactose per mol BSA, dashed line), BSA heated at 120 °C for 20 min and BSA unheated. The spectrum was obtained for 0.06% conjugate in water.

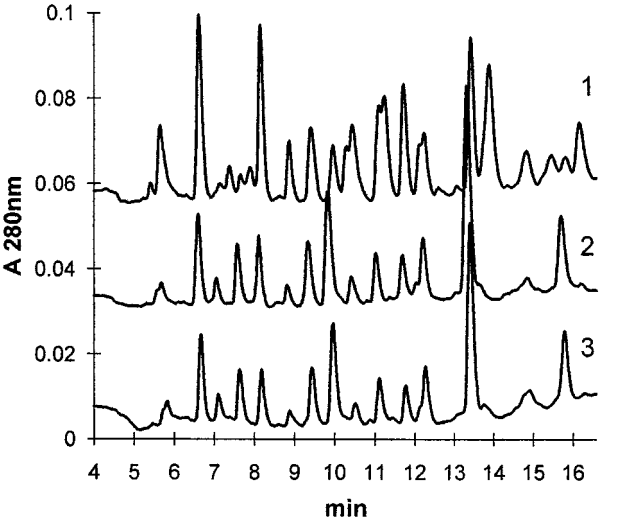


Figure 5. Chromatography of tryptic digest of BSA–lactose conjugate (score 1), BSA heated at 120 °C for 30 min (score 2) and native BSA (score 3) obtained by reversed-phase high-performance liquid chromatography (C18 5 μm column, 0.5 × 25 cm, eluent A: 0.85% phosphoric acid, eluent B: acetonitrile, linear gradient from 0 to 30% in 10 min, flow 1 ml min^{-1}).

Table 2. Stability of lactose–BSA conjugate after 24 h storage in guanidine hydrochloride, basic and acidic solution.

	mol lactose per mol BSA
water (control)	18 ± 1.7
6 M guanidine hydrochloride	19.6 ± 10.6[a]
0.1 M sodium carbonate, pH 10.5	16.1 ± 2.2
0.05 M sodium borate, pH 9.2	17 ± 1.7
0.1 M sodium bicarbonate, pH 8.3	18 ± 1.4
0.1 M phosphate buffer, pH 5.0	18.2 ± 1.9
0.05 M potassium acid phthalate, pH 4.0	17.9 ± 1.3
0.2% trifluoroacetic acid	17.9 ± 1.8

[a] Guanidine hydrochloride releases carbohydrates from dialysis tubing.

Stability of the glycoconjugates was tested after 24 h incubation at pH ranging between that for 0.2% trifluoroacetic acid and 10.5, as well as in the presence of guanidine hydrochloride as a chaotropic agent (Table 2). Samples were dialysed and sugar content was determined. No appreciable decomposition was noticed.

Immunological activity of the BSA-LPS hexasaccharide conjugate

Dextrans and lactose are relatively simple saccharides. Their conjugation to protein carriers bears low potential significance in biological applications. We therefore, wanted to find out if a more complicated carbohydrate would withstand the seemingly harsh conditions of dry thermal glycation. We made use of our method to conjugate BSA and hexasaccharide which is a constituent of the *H. alvei* LPS molecule [8]. The conjugate contained about eight hexasaccharide chains per mol BSA after reaction at 105–110 °C for 15 min. Specific anti-LPS antiserum was obtained by immunization of rabbits with hexasaccharide coupled to tetanus toxoid. We wanted to know if the anti-LPS antibodies would cross react with the BSA–hexasaccharide. The results of Figure 6 demonstrate very good specific recognition of the BSA–hexasaccharide conjugate by rabbit antiserum raised against conventionally synthesized TTx–hexasaccharide immunogen. Thus, the conditions of thermal glycation did not appear to adversely affect the biological integrity of a complex carbohydrate. Neoglycoconjugates containing bacterial or tissue carbohydrates synthesized by this method could be used as antigens for immunization or as components in diagnostic assays.

Integrity of carrier proteins

To determine how the reaction conditions affect the carrier protein we thermally conjugated dextran T10 to affinity purified goat immunoglobulin anti-rabbit IgG and tested it for the presence of immunological activity. As shown in Figure 7, the conjugate retained its specificity in the immunodiffusion assay. The most important finding is that lyophilized antibodies can be heated up to very high temperatures without loss of activity. This makes the method applicable to the preparation of immunotoxins and conjugates for use in diagnostics.

As an alternative test we chose glycation of trypsin with lactose and assessed its enzymatic activity in relation to the

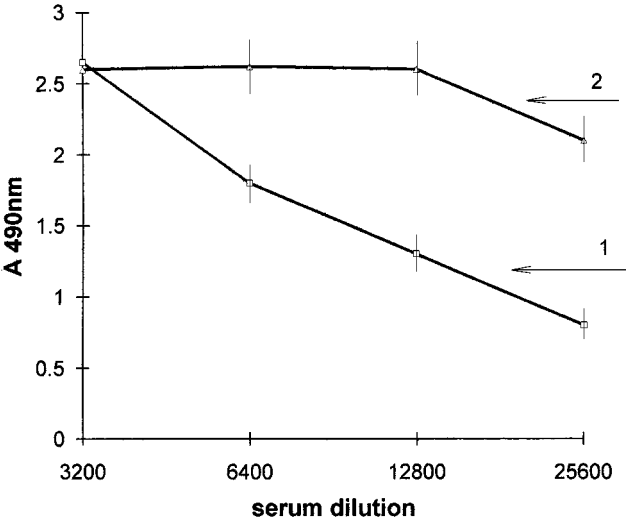


Figure 6. Titration of BSA-*Hafnia alvei* hexasaccharide conjugate in the enzyme-linked immunosorbent assay ELISA against serum anti-LPS *H. alvei* hexasaccharide-tetanus toxoid conjugate. 1, BSA-*Hafnia alvei* hexasaccharide conjugate (molecular weight of conjugate was 79 kDa); 2, *Hafnia alvei* lipopolysaccharide. Absorbance for BSA was 0.01–0.03 and for pure core hexasaccharide was 0.03–0.06, respectively.

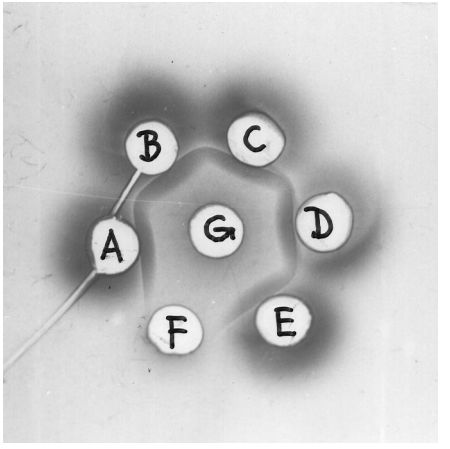


Figure 7. Precipitation of affinity purified goat immunoglobulin anti-rabbit IgG heated in the presence of dextran T10 at 115 °C for 30 min. Wells: A-lyophilized antibody; B-lyophilized antibody heated for 10 min; C-lyophilized antibody heated for 25 min; D- and E-lyophilized antibody and dextran mixture heated for 10 min and 25 min, respectively. Well F-antibody and dextran mixture heated at 95 °C in aqueous solution for 1 min. G-rabbit JgG. The concentration of each protein was 1 mg ml^{-1} (10 µl per well). Diffusion was done in 1.5% agarose gel in 0.05 M barbiturate buffer, pH 8.0.

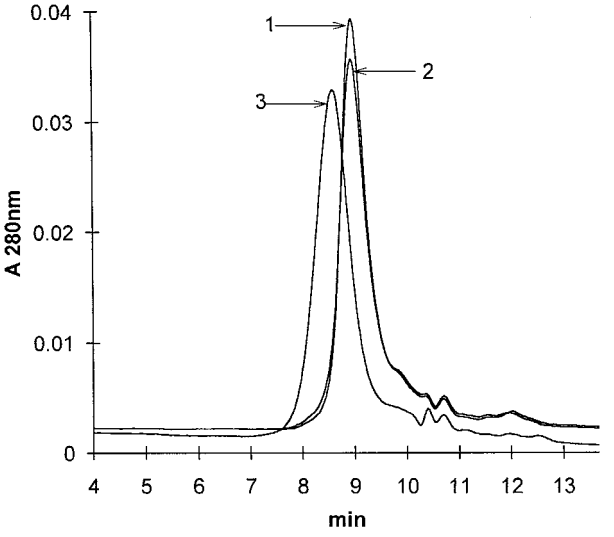


Figure 8. Gel filtration on TSK G2000 SWxl column of trypsin–lactose conjugates. Eluent: 0.1 M phosphate buffer, pH 6.7, flow 1 ml min^{-1}. Score 1: 0.5 mg trypsin heated at 120 °C; score 2: unheated mixture 0.5 mg trypsin and 7.5 mg lactose; score 3: reaction mixture (120 °C for 30 min) 0.5 mg trypsin and 7.5 mg lactose. Molecular weight markers: BSA (67 kDa) 6.73 ml, ovalbumin (43 kDa) 7.38 ml, trypsinogen A (24 kDa) 8.67 ml, cytochrome C (12.4 kDa) 9.23 ml.

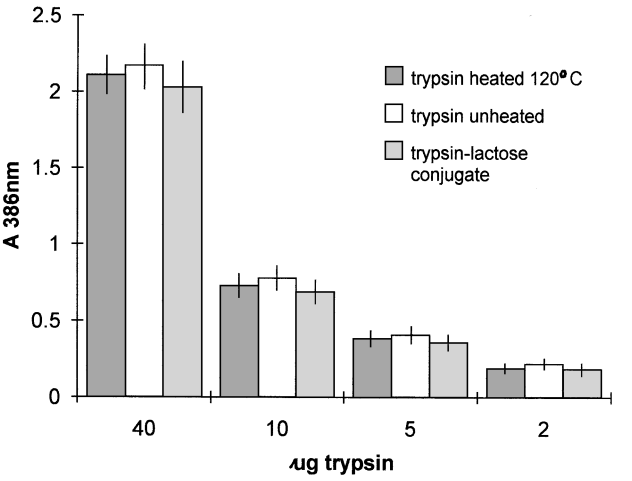


Figure 9. Enzymatic activity of trypsin–lactose conjugate after reaction at 120 °C for 30 min (1), native trypsin (2) and trypsin heated at 120 °C for 30 min (3) determined by *N*-benzoyl-DL-arginine-4-nitro anilide.

corresponding native protease. Trypsin and lactose were heated to 120 °C for 30 min. After reaction we observed a 20.5% decrease in the free amino group content (2.8 mol of lactose per mol trypsin). The trypsin–lactose reaction mixture was chromatographed on a G2000 SWxl column (Figure 8). The conjugate fractions were found to be shifted towards high molecular weights. The shift of the trypsin–lactose conjugate peak might have been produced not only by the increment in the molecular weight (by about 4%), but also by the decrease in the isoelectric point of the macromolecule. Heating was found to be without effect on the trypsin elution profile (as in the case of BSA). Serial dilutions of glycated trypsin showed only marginally lowered protease activity determined by means of *N*-benzoyl-DL-arginine p-nitroanilide (Figure 9).

The chemical status of the attached sugars was first tested in the BSA–lactose conjugates. While the conjugate gave a positive colour reaction with the phenol reagent for the presence of sugars, it tested negative with the acetone–phenol reagent [9] for the presence of the glucose moiety. This may suggest that the glucose residue is chemically bound to protein. The BSA–lactose conjugate was hydrolyzed, reduced and acetylated. Monosaccharide derivatives were analysed by gas chromatography–mass spectrometry (GLC–MS). Only one monosaccharide was detected and was identified as galactose (Figure 10). Both chemical and the GLC–MS analyses demonstrated that lactose was bound to BSA through the glucose residue which was destroyed in the process.

To confirm this we made use of a bioassay designed to test the binding of a galactose-specific lectin from *Ricinus communis* to the BSA–lactose conjugate. Only the BSA–lactose conjugate (not the control BSA solutions) gave a positive reaction with *Ricinus communis. Con* A lectin served as a non-specific control and did not bind the conjugate. This result was confirmed by the agarose diffusion test. BSA solutions, even at high concentration gave a negative reaction with lectin (Figure 11). These results suggested that the reducing part of oligosaccharide might be crucial for the dry thermal glycation. Reduced carbohydrates, mannitol or sorbitol did not glycate BSA under identical conditions (data not shown). The BSA–lactose showed a quantitative decrease in the number of free amino groups as measured by the reaction with trinitrobenzenesulfonic acid (Table 3). The fall in the number of free amino groups corresponded to the increae in attached galactose residues, which allows an assumption that no other group on the protein surface is modified by the carbohydrate. The lower detection of free amino groups in the conjugate may have been caused by conformational changes in the macromolecule.

The chemistry of the reaction is not clear at present, although we would like to speculate that the dry thermal glycation might follow a general mechanism of spontaneous nonenzymatic glycosylation. We anticipate that at the first stage the Schiff base is formed and undergoes the Amadori rearrangement at the second stage [12]. Recently, a seemingly analogous reaction between simple amines and xylose or glucose, running at an elevated temperature (70 °C) in a solution, was demonstrated by Büttner *et al.* [13].

The method is limited by a relatively large excess of carbohydrate over protein required for conjugation although it is possible to recover precious non-reacted low molecular weight carbohydrates by gel filtration. With regard to lactose the recovery of untouched carbohydrate was

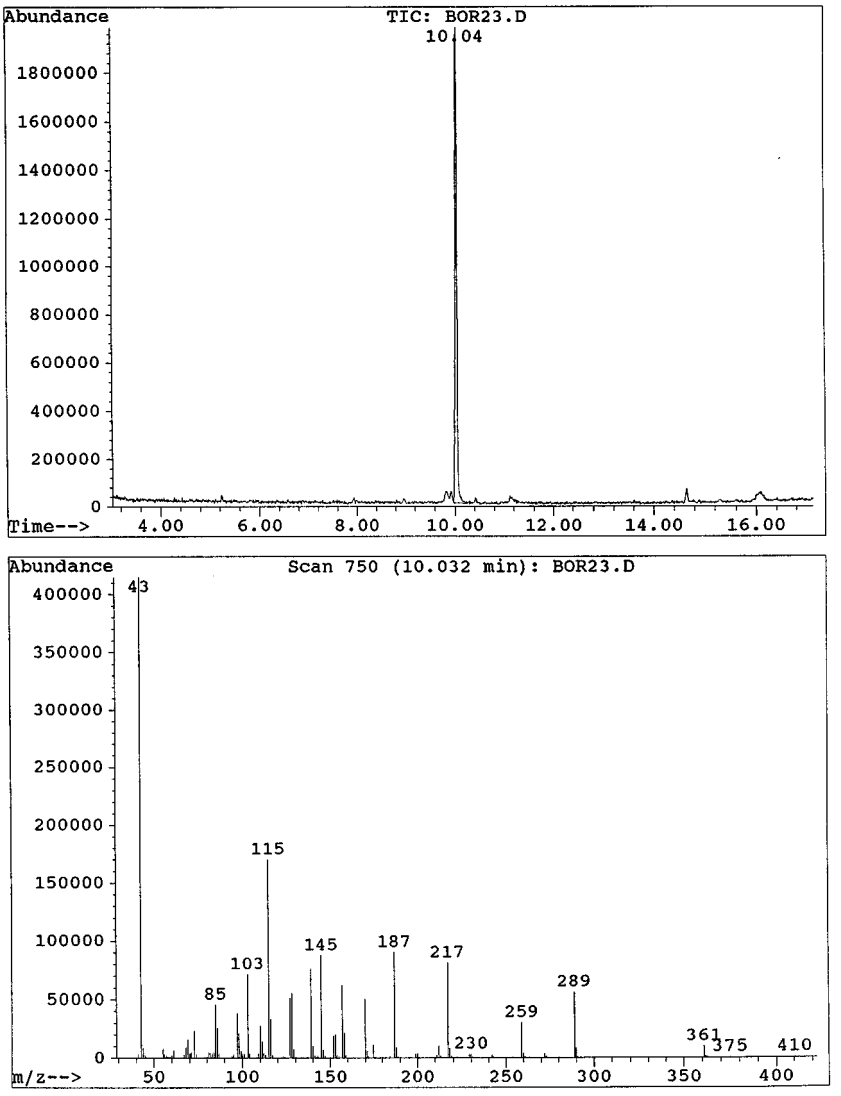
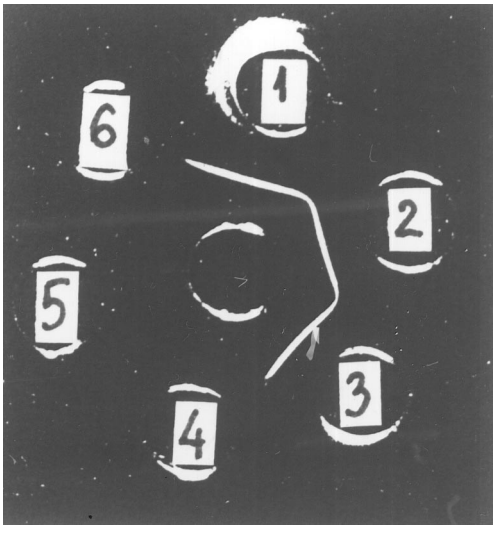


Figure 10. Gas chromatography and mass spectroscopy of acetylated carbohydrate components of BSA–lactose conjugate. HP-1 glass capiliary column (0.2 mm × 12 m) and temperature programme of 150 to 270 °C 8 °C per mim.



highly effective. After reaction of 1 mg BSA with 1 mg lactose at 125 °C for 50 min we recovered 83% of untouched lactose, about 3–5% of the lactose was decomposed (glucose residue) and about 10% was reacted with protein. The data are from a GLC–MS experiment.

But this still does not explain how the present procedure would affect the native structure of other, perhaps more labile, proteins. The good resistance of a model immunoglobulin and a protease has already opened a wide range of

Figure 11. Precipitation of BSA–lactose conjugates with *Ricinus communis* in 1% aqueous agarose gel. Lectin (10 µl, 10 mg ml^{-1}) was loaded in the central well. Wells 1, 2 and 3 contained lactose–BSA conjugates (20 µl, 10 mg ml^{-1}) 17, 13 and 9 mol lactose per mol BSA, respectively, wells 4 (20 µl, 10 mg ml^{-1}) and 5(20 µl, 90 mg ml^{-1}) contained BSA, well 6 contained BSA (20 µl, 10 mg ml^{-1}) heated at 120 °C for 20 min.

Table 3. Determination of BSA–lactose conjugate substitution by means of trinitrobenzenosulfonic acid, gas chromatography, Dubois colorimetric method and SDS electrophoresis.

	BSA	*BSA–lactose*	*mol lactose per mol BSA*
Determination of amino groups by trinitrobenzenosulfonic acid	61 ± 1.4	34 ± 2.2	27
Determination of galactose by GLC–MS chromatography	0	5.1% w/w	19
Determination of galactose by Dubois method	0	4.85% w/w	18
SDS electrophoresis	67 kDa	75 kDa	22

possible applications for our method of dry thermal protein glycation.

Summing up, the existence of a stable protein–saccharide bond has been confirmed by the following findings:

1. The conjugates are stable over a wide range of pH and in the presence of dissociating substances.
2. The presence of a reducing carbohydrate is a prerequisite to provide conjugation.
3. The increment in the molecular weight of the conjugate has been substantiated by various, widely accepted methods.
4. Lectins have the ability to recognize the saccharides of the conjugate.
5. Carbohydrate antigens retain their properties after conjugation.
6. The covalent character of the carbohydrate/carrier bond has been confirmed by GLC–MS and spectrophotometrically.
7. It is interesting to note that lyophilized antibodies and enzymes may retain their biological activity following heating to high temperatures.

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