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Optimization of hapten–protein conjugation by high-performance capillary electrophoresis

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Abstract

HPCE has been found to be efficient for the optimization of conjugation procedures employed for coupling of the haptens *p*-nitrophenyl-α-D-galactopyranoside (PNPG) and soyasaponin I to a carrier protein, Kunitz soybean trypsin inhibitor (KSTI). The carbohydrate moieties of the haptens were oxidized with periodate followed by reaction with ε-amino groups of the carrier. For PNPG, the periodate oxidations (0.01–0.2 *M* $NaIO_4$) were followed by HPCE and 0.1 *M* periodate was chosen as the optimum concentration. The coupling of PNPG to KSTI was found to proceed at a constant rate for more than 250 min. The reaction rate for the soyasaponin conjugation to KSTI declined after 80 min of incubation. The coupling of soyasaponin to KSTI was confirmed by enzyme-linked immunosorbent assay with monoclonal antibodies directed against soyasaponin I. The method was found to be particularly powerful for the investigation of conjugates with low epitope densities that are difficult to determine otherwise.

1. Introduction

Haptens are small molecules incapable of eliciting immune responses without previous conjugation to immunogenic molecules, e.g., proteins. A wide variety of coupling reactions are available [1,2] and the choice of coupling reaction depends on the desired conjugate structure and the chemical structure of the hapten. Method adjustments and optimization are often required to obtain useful hapten–protein conjugates. The ease of method optimization is dependent on the methods available for evaluating the conjugation reaction. With haptens devoid of chromophoric groups or otherwise distinctive characteristics, method optimization may be difficult and sample consuming owing to the use of destructive methods, e.g., determination of free amino groups on the carrier protein by reaction with trinitrobenzenesulfonic acid [3]. There is, therefore, a need for generally applicable methods for analyses of hapten–carrier conjugation.

High-performance capillary electrophoresis (HPCE) offers a method with very low sample consumption (nl) and a high separation capacity, which can be utilized for separating uncoupled and conjugated carriers based on the differences in electrophoretic mobilities introduced by covalently coupling the hapten to the carrier. In addition, frequent on-line analyses from the sample vials are possible by HPCE, thus facilitating optimization of incubation times. The haptens used in the study were soyasaponin I and *p*-nitrophenyl-α-D-galactopyranoside (PNPG).

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The latter was chosen as a model hapten for soyasaponin I owing to its carbohydrate content and chromophoric group that allows traditional spectrophotometric characterization of the conjugation products.

Various leguminous plants have been shown to contain saponins [4,5], which comprise a group of glycosides. Many saponins have a bitter taste [4] and consequently saponins are considered to be important in relation to the production of high-quality legumes for food and feed and for the production of protein concentrates and isolates. The predominant saponin in extracts of pea has been shown to be soyasaponin I [6].

Current methods of analyses for soyasaponin I are hampered partly owing to the lack of an efficient detection system for soyasaponin I. Monoclonal antibodies (mAbs) against soysaponin I offer a specific detection system that can be employed in an enyme-linked immunosorbent assay (ELISA) method for analyses of large samples numbers. For this purpose, coupling of soyasaponin I to a carrier is necessary, as soysaponin is too small a molecule to elicit an immune response.

The periodate oxidation method is applicable for coupling of carbohydrate moieties containing vicinal hydroxy groups to amino groups, e.g., lysine side-chains in carrier proteins. This method was chosen for coupling of soyasaponin I and PNPG to protein carriers. The drawbacks of the periodate oxidation method is that optimization may be necessary [2].

An HPCE method for protein analyses has been presented previously [7], and this method has been shown to be applicable for the characterization of hapten–protein conjugates [8]. The aim of this work was to show that HPCE is a valuable tool in the optimization of coupling reactions for generating immunogenic hapten–carrier conjugates.

2. Experimental

2.1. Materials

Kunitz soybean trypsin inhibitor (Type I-S) (KSTI), *p*-nitrophenyl-α-D-galactopyranoside (PNPG), $NaIO_4$, taurine (2-aminoethanesulfonic acid) and cholic acid were obtained from Sigma (St. Louis, MO, USA), 3,3′,5,5′-tetramethylbenzidine (TMB) from Merck (Darmstadt, Germany), Triton X-100 from Serva (Heidelberg, Germany) and horseradish peroxidase (HRP)-labelled rabbit anti-mouse antibody from Dako (Glostrup, Denmark). Soyasaponin I and mAbs with soyasaponin I specificity were prepared as described elsewhere [8,9]. The structures of PNPG and soyasaponin I are shown in Fig. 1.

2.2. HPCE procedure

HPCE was performed using an $HP^{3D}CE$ system (Hewlett-Packard, Waldbronn, Germany) with a 614 mm × 0.05 mm I.D. fused-silica capillary. Detection was performed by on-column measurements of UV absorption at 200 nm at a position 530 mm from the injection end of the capillary. All measurements were made at 16 kV and 30°C. Buffer solutions of 75 m*M* Na_2HPO_4–50 m*M* taurine and with or without 35 m*M* cholic acid were prepared and filtered through a 0.2-μm membrane filter. The buffer pH was 8.0–8.1. Preconditioning of the capillary was performed prior to each run by flushing with 0.1 *M* NaOH for 1–2 min and run buffer for 1–3 min.

2.3. Hapten–carrier conjugation

PNPG (1.8 mg/ml in water; 133 μl) and 100 μl of water were incubated with $NaIO_4$ (0.1 *M* in water; 50 μl) in a buffer vial and on-line

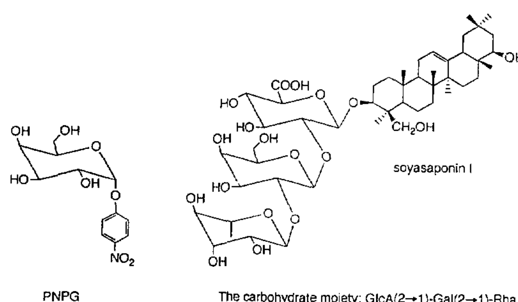

Fig. 1. Structures of PNPG and soyasaponin I. Periodate oxidation of the carbon–carbon bond at vicinal hydroxy groups on the carbohydrate moieties of the haptens results in the formation of aldehyde groups.

HPCE analyses were performed to follow the oxidation of vicinal hydroxy groups on PNPG.

For conjugation, PNPG (1.8 mg/ml in water; 133 μl) or water (133 μl; blank sample) and 100 μl of water were preincubated with $NaIO_4$ (0.1 *M* in water; 50 μl) for various periods followed by mixing with 70 μl of KSTI (10 mg/ml in 0.1 *M* $NaHCO_3$, pH 9.3). The mixture of PNPG and KSTI was analysed on-line by HPCE. Soyasaponin I (50 μl; 1.5 mg/ml in 10% MeOH) was preincubated with $NaIO_4$ (25 μl; 0.1 *M* in water) and mixed with 13 μl of KSTI (10 mg/ml in 0.1 *M* $NaHCO_3$, pH 9.3).

The conjugation products were finally reduced with $NaBH_4$ [8].

2.4. Coupling efficiency measured by ELISA

Wells of microtitre plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 100 μl of saponin–KSTI conjugate (1 mg/ml) dissolved in carbonate buffer (50 m*M*; pH 9.6). The plates were incubated for 1 h at 20°C on a shaker and then washed with washing buffer containing detergent (0.5 *M* NaCl–27 m*M* KCl–15 m*M* KH_2PO_4–100 m*M* $Na_2HPO_4 \cdot 2H_2O$–0.1% Triton X-100). Dilution rows of mAbs were incubated (150 ng/ml in the first well) for 1 h and the plates were washed four times. HRP-labelled rabbit anti-mouse antibody diluted 1:1000 in washing buffer was added to each well and incubation proceeded for 1 h. After washing, 100 μl of a substrate solution (0.2 *M* potassium citrate–3 m*M* H_2O_2–0.6 m*M* TMB) were added to each well. Colour development was stopped with addition of H_3PO_4 (100 μl per well; 2 *M*), and absorbance were read at 450 nm using an EL 312e microplate reader (BioTek Instruments, Winooski, VT, USA).

3. Results and discussion

The use of HPCE for on-line evaluation of coupling reactions was investigated. The coupling kinetics of two different molecules to carrier proteins were investigated by electrophoresis of the mixtures during conjugation.

3.1. PNPG periodate oxidation

Initially, the necessity for cholate in the buffer system was evaluated for the determination of PNPG by HPCE. In the absence of cholate, PNPG co-migrated with the solvent peak whereas increased cholate concentrations gradually increased the migration time of PNPG relative to the solvent peak. A concentration of 35 m*M* cholate was found to be appropriate for the determination of PNPG and the periodate oxidation product of PNPG.

The periodate oxidation of PNPG was investigated for various incubation periods (Fig. 2) at different periodate concentrations (0.01–0.2 *M*), as optimization of the periodate concentration has been reported to be important for obtaining a suitable degree of oxidation [2]. The oxidation of PNPG at vicinal hydroxy groups does not influence the chemical structure and the molar absorptivity of the chromophoric group of PNPG. Consequently, the total normalized area (NA) can be used as a measure of the amount of the chromophoric groups present in the sample.

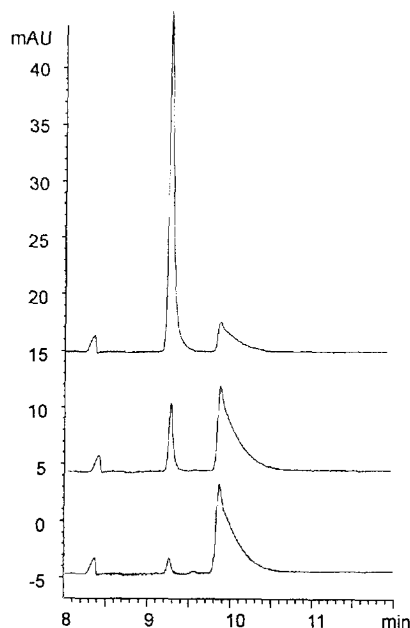

Fig. 2. Electropherograms recorded at 305 nm of PNPG incubated with $NaIO_4$ for 2 min (top), 15 min (middle) and 29 min (bottom). The peak at 9.3 min is PNPG and that at 9.9 min is the reaction product.

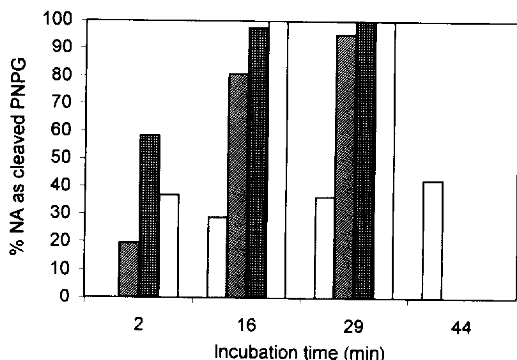

Fig. 3. Proportion of PNPG present as oxidized PNPG determined from NA at various periodate concentrations (grey, 0.01 *M*; diagonal hatched, 0.05 *M*; checked, 0.1 *M*; white, 0.2 *M*). At 44 min of incubation only the sample with 0.01 *M* $NaIO_4$ was analyzed.

In Fig. 3, the oxidation rate is illustrated as a percentage of total PNPG present as oxidized PNPG. With 0.01 *M* $NaIO_4$ the oxidation proceeds slowly, whereas $NaIO_4$ concentrations above 0.05 *M* result in complete oxidation after ca. 20 min. However, oxidation at high $NaIO_4$ concentrations and a prolonged incubation time markedly decreased the total NA measured (Fig. 4), implying that excessive oxidation destroys or changes the chromophoric group of PNPG. This was also seen by observations on the PNPG sample colour, which changed from colourless to yellow, indicating that a more conjugated system was generated from the PNPG molecules. For further studies, preincubation with 0.1 *M* $NaIO_4$ was chosen.

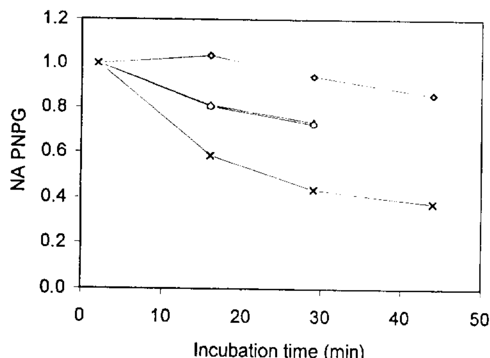

Fig. 4. Degradation of PNPG measured as total NA in relation to the total NA found after 2 min of incubation at different periodate concentrations: ◇ = 0.01; △ = 0.05; ○ = 0.1; × = 0.2 *M*. Electropherograms for the NA determinations were recorded at 305 nm.

3.2. Coupling of PNPG to KSTI

PNPG preincubated with periodate for different periods of time was mixed with KSTI and the formation of coupling products was determined from the normalized peak areas. The peaks of coupling products have higher migration times (MTs) than the MT of KSTI (17.5 min, Fig. 5) owing to hydrophobic interaction of the PNPG moieties with cholate micelles [8]. At 200 nm, the molar absorptivity of PNPG is diminished compared with that of KSTI, implying similarity of response factors of the KSTI conjugate and KSTI [8], thus allowing the determination of coupling densities from NA.

In parallel with the coupling experiments, corresponding blank samples without PNPG were mixed with KSTI to study the influence of IO_4^- on the protein during incubation. The electropherograms of the blank samples showed that the electrophoresis pattern of KSTI was

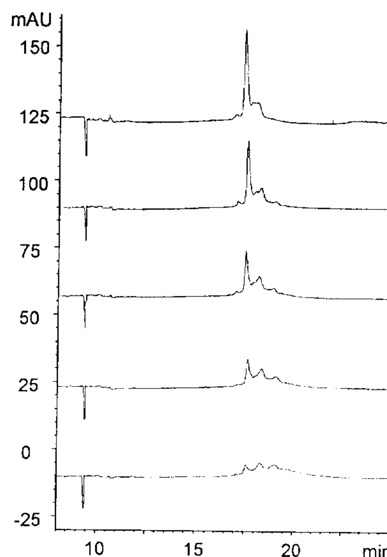

Fig. 5. Electropherograms of the reaction mixture from conjugation of PNPG with KSTI after (from top to bottom) 44, 112, 180, 247 and 382 min, recorded at 200 nm.

unchanged during the incubation periods. The additional peaks observed in the electropherogram are due to impurities present in the commercially available KSTI.

The coupling ratios of PNPG to KSTI were calculated as described previously [8] by using the NA for quantification. The control sample of KSTI without PNPG was used for correction of contributions from the unconjugated KSTI to the NA of the emerging KSTI conjugate due to co-migrating peaks.

Fig. 6 shows the coupling of PNPG to KSTI after various preincubation times of PNPG with periodate. The parameters studied did not result in substantial effects on the PNPG–KSTI coupling, implying that in the time intervals studied, activated PNPG was not a deficient component. In addition, the coupling reaction between PNPG and KSTI is apparently slow, as no coupling saturation was observed in the time interval studied.

3.3. Coupling of soyasaponin to KSTI

Soyasaponin was dissolved in a mixture of methanol and water, but the presence of methanol was found to cause denaturation of the carrier protein. Therefore, the solubility of soyasaponin I in mixtures with different ratios of methanol to water was investigated in order to minimize the use of methanol. This was evaluated from the NA obtained from HPCE of soyasaponin I samples dissolved in different mixtures of methanol and water. The methanol concentration chosen for the saponin solubilization was 10%.

The conjugation of soyasaponin I to KSTI was followed after five different preincubation periods as shown in Fig. 7. The number of saponins per KSTI molecule was calculated from the normalized peak areas of the KSTI–saponin conjugates relative to the total NA of the protein present in the sample. Examples of electropherograms after various coupling periods are shown in Fig. 8. Similarly to the PNPG–KSTI conjugates, the coupling products have higher MTs than the MT of KSTI.

Although the methanol concentration had been reduced to 10%, some changes in the migration time of the KSTI control sample was observed during incubation, probably owing to denaturation in the presence of MeOH. Therefore, the NA obtained for the conjugation products were corrected for the NA contribution from co-migrating denatured KSTI.

From Fig. 7, it seems that the conjugation rate declined after 60–90 min of incubation of activated soyasaponin I with KSTI, although continued conjugation seemed to take place at a lower reaction rate. Preincubation for 17 h resulted in a lower epitope density, which may be due to excessive oxidation as proposed for the PNPG oxidation. Soyasaponin I was present in a twelvefold molar excess over KSTI, and during the conjugation the NA of saponin did not

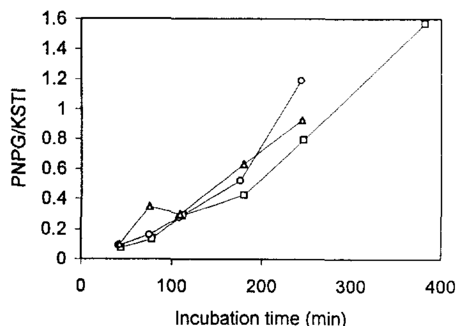

Fig. 6. Coupling density of PNPG to KSTI following preincubation with 0.1 M $NaIO_4$ for (□) 10, (○) 30 and (△) 60 min.

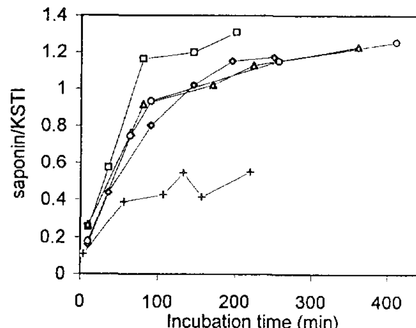

Fig. 7. Conjugation of soyasaponin I to KSTI following preincubation with 0.1 M IO_4^- for (□) 10, (◇) 30, (△) 50 and (○) 310 min and (+) 17 h.

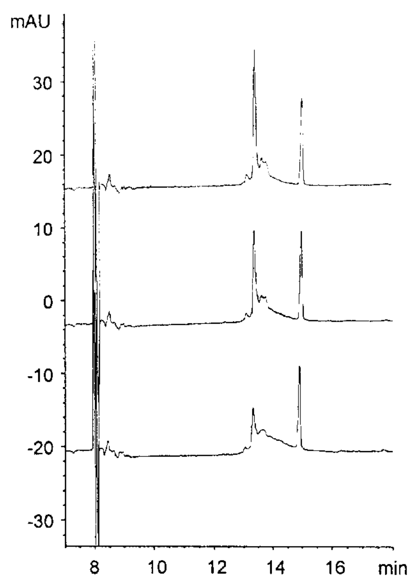

Fig. 8. Electropherograms of soyasaponin I conjugation to KSTI after 60 min of preincubation with 0.1 *M* $NaIO_4$. Coupling after 10 (top), 90 (middle) and 140 min (bottom). The MT of soyasaponin I is 14.8 min and that of KSTI is 13.5 min. Electropherograms were recorded at 200 nm.

decrease noticeably, thus supporting the contention that the epitope density is low.

Experiments were performed in duplicate in which oxidized saponin was reacted with KSTI for various lengths of time and then exposed to $NaBH_4$ and desalted [8]. Determination of degrees of conjugation from electropherograms of the reaction mixture and the reduced, desalted sample gave similar results, showing that the conjugation optimization can be performed directly on the reaction mixture.

The desalted samples were also investigated for their ability to bind monoclonal antibodies with specificity for soyasaponin I. For both experiments, samples incubated for 60, 90 and 150 min showed an increasing antibody binding as a function of reaction time. However, the sample incubated for 10 min showed an antibody binding capacity similar to that observed with 150 min of incubation. However, a correspondingly high epitope density was not found from the HPCE data of the samples, indicating that the high antibody binding capacity may be due to higher accessibility of the epitopes instead of epitope density. This could be explained by the coupling reaction, an prolonged incubation may cause the coupling of the same saponin molecule at multiple sites, thereby reducing the epitope accessibility.

A sample incubated for 27 h showed a higher antibody binding capacity than found for samples incubated for 150 min, indicating that a prolonged incubation time results in an increase in coupling density. On HPCE this conjugation product showed a broad peak with an increased migration time.

In conclusion, HPCE has been shown to be a valuable tool for the investigation and optimization of the coupling of PNPG and soyasaponin I to KSTI. The HPCE method presented has several advantages over alternative methods and particularly important is the ability to measure low coupling densities, in contrast to spectrophotometric methods, e.g., TNBS conjugation to ϵ-amino groups on lysine. In addition, on-line HPCE of the conjugation mixture allows the optimization of the incubation time and concentrations of reactants with a minimum consumption of reactants owing to the low sample volumes necessary.

Acknowledgements

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