ELSEVIER

Journal of Chromatography A, 680 (1994) 437–445

JOURNAL OF CHROMATOGRAPHY A

High-performance capillary electrophoresis for characterization of hapten–protein conjugates used for production of antibodies against soyasaponin I

Hanne Frøkiær[a], Peter Møller[b], Hilmer Sørensen*[,b], Susanne Sørensen[a]

[a]*Department of Biochemistry and Nutrition, Technical University of Denmark, Building 224, DK-2800 Lyngby, Denmark*
[b]*Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark*

Abstract

Micellar electrokinetic capillary chromatography using sodium cholate as the micellar phase has been investigated for characterization of hapten–protein conjugates. Special focus has been placed on the hapten soyasaponin I which is a quantitatively dominating glycoside in seeds of several legumes including pea (*Pisum sativum* L.) and soybean [*Glycine max* (L.) Merr.]. Soyasaponin I has been isolated from pea and used as hapten for production of anti-saponin specific polyclonal antibodies. Soyasaponin I was coupled to Kunitz soybean trypsin inhibitor (KSTI) and bovine serum albumin. The degree of coupling was determined by high-performance capillary electrophoresis (HPCE). Capillaries dynamically coated with zwitterions were found to be efficient for reduction of interaction between the silica capillary surface and the proteins. The applicability of HPCE for determination of coupling density was confirmed by investigation of a model hapten (*p*-nitrophenyl-α-D-galactoside; PNPG) coupled to KSTI. The PNPG–KSTI conjugates were examined by both HPCE and by spectrophotometric determination of the PNPG density on KSTI. The HPCE method was shown to be efficient in studies of the formation of hapten–protein conjugates and to be more specific than alternative techniques applied for determination of coupling densities.

1. Introduction

A variety of leguminous plants has been shown to contain saponins [1,2]. Cultivars of pea (*Pisum sativum* L.) primarily contain soyasaponin I [3,4] which has been isolated from soybean and characterized by Kitagawa and coworkers [5–7]. Soyasaponins are triterpenoid aglycones linked to one or two carbohydrate chains. The presence of soyasaponin I in pea flour has been related to the bitterness and astringency sometimes found in milled, whole pea seed [4]. In addition, a variety of other biological activities can be caused by saponins, and efficient analytical methods are thus required for specific quantification of saponins in food and feed [1].

Currently used methods for quantitative analyses of soyasaponin I comprise TLC, GC and HPLC [1,2]. However, these methods require extended extraction and purification procedures to obtain a quantitative analysis of the saponin content. In addition, GC normally involves hydrolysis of the saponins and subsequent derivatization of the sapogenol (triterpenoid part) prior to GC. The results obtained by these

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00434-B

methods show large variations, partly due to low sensitivity and specificity. Limitations with respect to the HPLC methods [1] are often caused by low UV sensitivity, relatively low solubility and problems related to the critical micelle concentration (CMC) when more concentrated solutions of soyasaponins are wanted. The use of enzyme-linked immunosorbent assay (ELISA) for measuring soyasaponin I has the potential for a simple, cheap and fast method applicable for a large sample number. In addition, a high sensitivity and specificity is obtainable by ELISA thus reducing the limitations caused by the CMC. A sensitive and precise ELISA against glycoalkaloids from potato tubers has been developed [8].

Soyasaponin I is a small molecule incapable of eliciting an immune response (a hapten). Development of an ELISA against soyasaponin I requires a coupling of the saponin to a carrier to elicit an immune response. The nature of soyasaponin I does however not allow easy confirmation and characterization of the conjugate. In fact, characterization of hapten–carrier conjugates is a general problem for conjugates with haptens that do not possess special chemical characteristics that allow specific detection of the hapten. Only a very limited number of general methods for conjugate characterization are available, e.g. radioactive labelling of the hapten.

This study was aimed at investigating the use of a HPCE method [9] for characterization of hapten–protein conjugates not easily characterized otherwise. The method was employed for confirmation of the coupling of soyasaponin I to a protein carrier, and the conjugate was shown to elicit an immune response against soyasaponin I.

2. Experimental

2.1. Materials

Soyasaponin I obtained from pea seeds was prepared by group separation, anion-exchange chromatography and solid-phase extraction techniques essentially as described for other anions by Bjerg and Sørensen [10] and with TLC detection of saponins in the obtained fractions by use of the methods described elsewhere [1–5]. Kunitz soybean trypsin inhibitors (Type I-S; KSTI), *p*-nitrophenyl-α-D-galactoside (PNPG), bovine serum albumin (BSA), $NaIO_4$, trinitrobenzenesulfonic acid (TNBS), taurine (2-aminoethanesulfonic acid) and cholic acid were obtained from Sigma (St. Louis, MO, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) was from Merck (Darmstadt, Germany). Freunds complete and incomplete adjuvants were from Difco Labs. (Detroit, MI, USA). Triton X-100 was purchased from Serva (Heidelberg, Germany). Horseradish peroxidase (HRP)-labelled swine–anti-rabbit antibody was obtained from Dako (Glostrup, Denmark). All chemicals and organic solvents were of analytical-reagent grade.

2.2. Hapten–protein conjugates

PNPG was coupled to KSTI whereas soyasaponin I was coupled to both KSTI and BSA after periodic cleavage of carbon–carbon bonds at vicinal hydroxyl groups on the carbohydrate moieties of the haptens according to the principles described by Butler and Chen [11] and Morgan et al. [8]. The aldehyde groups formed are then available for formation of Schiff bases by reaction with lysine amino groups on the carrier. Saponin or PNPG (1.5 m*M* in MeOH–water, 70:30) were mixed with a 6–25 molar excess of periodate (0.1 *M* in water). The reaction was allowed to proceed at room temperature for 1 h and then carrier protein (10 mg/ml in Na_2CO_3; 0.1 *M*; pH 9.3) was added to a final concentration of approximately 2 mg/ml. After reaction for 1 h at room temperature, a 6–25 molar excess of $NaBH_4$ was added, and the mixture was left at 4°C overnight. Samples were dialyzed (2 days) against ionized water (cutoff M_r 10 000).

2.3. Investigation of coupling by HPCE

HPCE was performed according to Arentoft et al. [9] using an ABI Model 270 A-HT capillary electrophoresis system (Applied Biosystems,

Foster City, CA, USA) with a 760 mm × 0.05 mm I.D. fused-silica capillary. Detection was performed by on-column measurements of UV absorption at 214 nm at a position 530 mm from the injection end of the capillary. For data processing, an IBM-compatible 486DX, 50 MHz personal computer with Turbochrom 3.3 (PE Nelson, Perkin-Elmer, Beaconsfield, UK) was used. A buffer solution (35 m*M* cholate; 100 m*M* Na_2HPO_4; 50 m*M* taurine; 2% 1-propanol; pH 8.03) was prepared and filtered through a 0.45-μm membrane filter before use. All samples were run at 20 kV, 30°C.

2.4. Investigation of coupling by TNBS method

The number of free lysine amino groups on the carrier protein was determined [12,13] in order to evaluate the coupling density. A mixture of 0.2 ml protein solution (0.1–0.5 mg/ml in water), 0.2 ml $Na_2B_4O_7$ (0.1 *M*; pH 9.4) and 0.2 ml TNBS (0.1%, w/v, in water) was incubated for 60 min at 40°C. The reactions were stopped with 0.1 ml HCl (1 *M*) after addition of 0.2 ml sodium dodecyl sulfate (SDS) (10%, w/v, in water). Absorbances were read at 335 nm against a blank.

2.5. SDS–polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed as described previously [14] and gels were silver stained [15].

2.6. Immunization

A rabbit was immunized subcutaneously with 100 μg saponin–KSTI conjugate in Freunds complete adjuvant and after 16 days immunized subcutaneously with 100 μg saponin–KSTI conjugate in Freunds incomplete adjuvant. The rabbit was bled a week after the second immunization. In addition, mice were immunized with either saponin–KSTI or saponin–BSA (10 μg per injection) according to Frøkiær et al. [14].

2.7. Measurement of saponin by ELISA

Anti-saponin antibody titers in sera were determined by ELISA. Wells of microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with 100 μl of saponin–BSA conjugate (5 μg/ml) dissolved in carbonate buffer (50 m*M*; pH 9.6). Plates were incubated 1 h, 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). Two-fold dilutions of soyasaponin I (initial concentration 10 μg/ml) were preincubated 45 min with rabbit sera diluted 100 times in washing buffer. The preincubated mixtures were transferred (100 μl/well) to the saponin–BSA coated plate and left to incubate for 1 h followed by washing. HRP-labelled swine–anti-rabbit 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/well; 2 *M*), and absorbances were read at 450 nm using a Microplate Reader EL312e (BioTek Instruments, Winooski, VT, USA).

3. Results and discussion

HPCE was investigated for its potential as a method for characterization of hapten–carrier conjugates with proteins as carriers. Determination of the coupling density of hapten to conjugate requires separation of unconjugated and conjugated protein and that the amount of carrier protein can be quantitatively determined in the applied HPCE system. Therefore, the correlation between normalized peak area and KSTI concentration was investigated by triplicate analyses of a range of KSTI concentrations (0.4–2.0 mg/ml), and a standard curve with $r^2 = 0.98$ was obtained. KSTI was chosen as carrier molecule, as previous studies performed in our laboratories had shown KSTI to be a good immunogen. Besides, the low molecular mass of KSTI

(20 100) may increase the impact of coupled hapten on the electrophoretic properties of the carrier compared to conjugation products employing large carriers.

The coupling of soyasaponin I to carriers required investigation and optimization of suitable conjugation methods. The chosen coupling method involved periodate cleavage of carbon–carbon bonds at vicinal hydroxyl groups. However, the method development was hampered by the low CMC and difficult detection of soyasaponin I. Therefore, coupling conditions were optimized using a model hapten, PNPG. The galactoside moiety of PNPG was expected to behave similarly to the carbohydrate moiety of saponin, and PNPG had the advantage of being a compound with a chromophore having a relatively high extinction coefficient at 305 nm. Thereby, spectrophotometric evaluation of the coupling ratio was possible. The structures of PNPG and soyasaponin I are shown in Fig. 1.

3.1. Coupling of PNPG to KSTI

Various molar ratios of PNPG to KSTI were subjected to conjugation and the obtained mixtures of unconjugated KSTI and PNPG–KSTI conjugates were investigated by HPCE (Fig. 2). In Fig. 2, electropherogram 1, KSTI is represented by the large peak (a) whereas the additional small peaks next to KSTI represents impurities such as isoinhibitors and other proteins present in the commercially available sample [9]. In the presence of PNPG in the coupling mixture, a peak with larger migration time (t_m) than KSTI appeared (electropherogram 2, peak b). As the molar proportion of PNPG to KSTI in the coupling mixture was further increased, the proportion of conjugated to unconjugated KSTI increased, as illustrated by the relative heights of peaks a and b in electropherograms 3 and 4. Furthermore, additional peaks appeared (c), presumably representing KSTI with two PNPG conjugated per molecule.

The increase in t_m for PNPG–KSTI conjugates compared to KSTI could be explained by the presence of cholate micelles in the electrophoresis buffer [9,16–18]. These micelles move in the opposite direction to the electroosmotic flow, thus increasing t_m for compounds interacting with the micelles. The relatively hydrophobic *p*-nitrophenyl moiety of the amphiphilic PNPG is expected to be directed at the bulk solution as coupling takes place at the hydrophilic galactose moiety, and therefore interactions of the *p*-nitrophenyl group with the cholate micelles could explain the increase in t_m of the conjugates.

The coupling ratios of PNPG to KSTI were calculated from the obtained electropherograms by using the normalized peak areas for quantification (Table 1). The electropherograms were recorded at 214 nm. At this wavelength PNPG

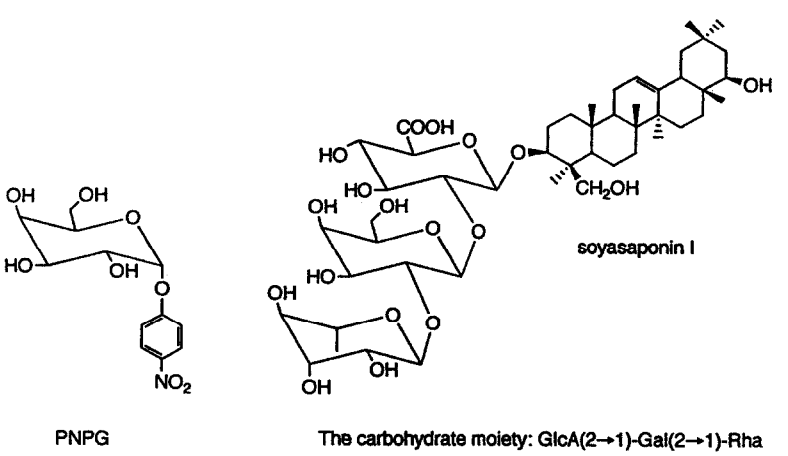

Fig. 1. Structures of soyasaponin I [7] and *p*-nitrophenyl-α-D-galactoside (PNPG). GlcA = Glucuronic acid; Gal = galactose; Rha = rhamnose.

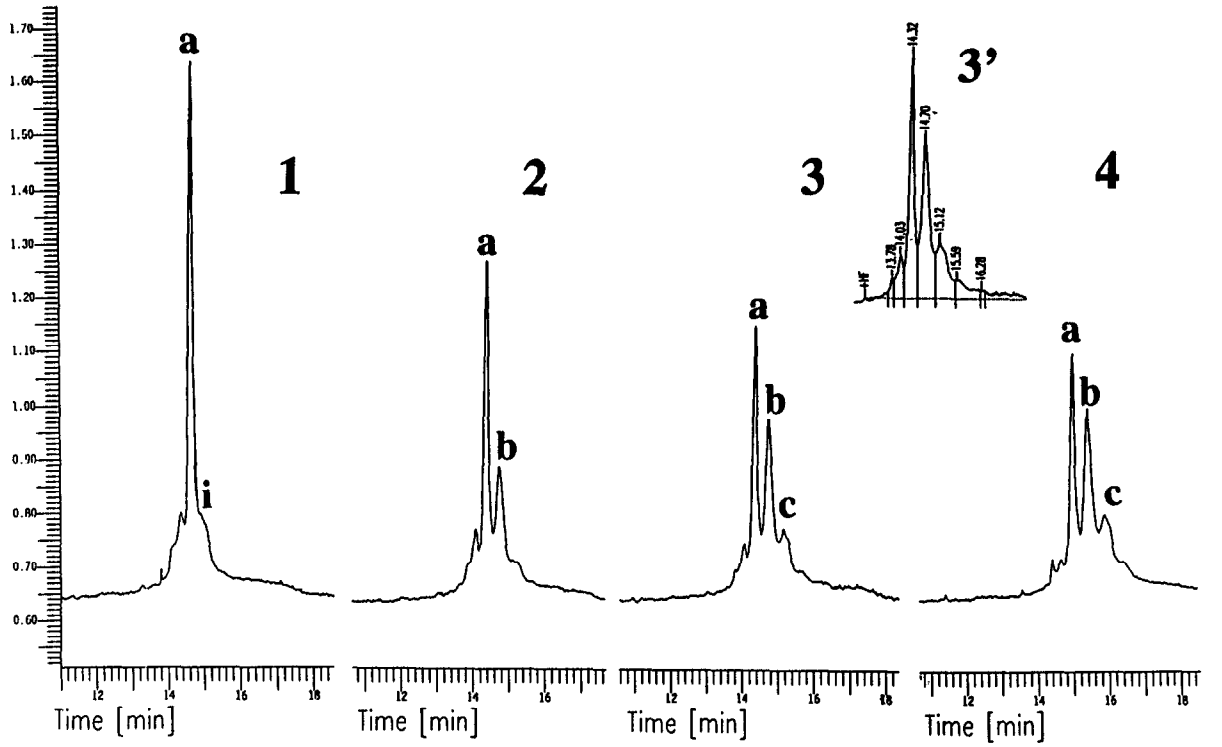

Fig. 2. Electropherograms of samples with different molar ratios of PNPG conjugated to KSTI. Separation conditions: buffer, 35 m*M* cholate–100 m*M* phosphate–50 m*M* taurine (pH 8.7); voltage 20 kV; temperature 20°C; total length of capillary, 760 mm; length from injection end to detection, 530 mm; detection wavelength, 214 nm; vacuum injection for 3 s. Molar ratios in the conjugation mixture (PNPG:KSTI): 1 = 0:1; 2 = 6:1; 3 = 12:1; 4 = 48:1. Peak a represents the dominating KSTI; peak i is an impurity; peak b and c are PNPG–KSTI conjugation products. The inserted electropherogram (3') shows the calculation mode for individual peak areas.

contributes to the absorbance, but the contribution is expected to be small compared to the absorbance at 214 nm of KSTI which contains 10 aromatic amino acids (Phe, Tyr, Trp). Therefore, the response factors are expected to be identical for KSTI and PNPG–KSTI conjugates. The PNPG–KSTI conjugate (Fig. 2, peak b) comigrated with the peak of an impurity present

Table 1
Determination of molar coupling ratios obtained at different conjugation ratios (molar) of hapten (PNPG or soyasaponin I) to carrier (KSTI or BSA)

PNPG–KSTI conjugation				Saponin–KSTI conjugation			Saponin–BSA conjugation	
Conjugation ratio, PNPG:KSTI	Mol PNPG:KSTI ($A_{305\ nm}$)	Mol TNP–NH_2–KSTI	Mol PNPG:KSTI (HPCE)	Conjugation ratio, saponin:KSTI	Mol TNP–NH_2–KSTI	Mol saponin:KSTI (HPCE)	Conjugation ratio, saponin:BSA	Mol TNP–NH_2–BSA
0:1	0	11.9	0	0:1	13.4	0	0:1	42.3
6:1	0.19	9.5	0.27	3:1	10.9	0.33	6:1	34.6
12:1	0.59	5.5	1.1	6:1	10.5	0.56	12:1	30.4
48:1	0.70	6.8	1.6	12:1	14.2	0.21	24:1	26.2

in the blind sample of KSTI (Fig. 2, peak i). The area of the PNPG–KSTI peak b was therefore corrected for the contribution from this impurity by subtracting the relative proportion of the impurity area to the total peak area as calculated from the blind sample.

The molar coupling ratios were also determined from spectrophotometric measurements of the PNPG present as PNPG–KSTI conjugates (Table 1). For this purpose, the conjugate absorbances at 280 nm and 305 nm were measured for determination of KSTI concentration and PNPG concentration, respectively. As the protein contributes to the absorbance at 305 nm, the $A_{305\ nm}$ was corrected by subtracting the protein contribution at $A_{305\ nm}$, as calculated from the blind sample. Similarly, PNPG contributed to the absorbance at 280 nm, and the $A_{280\ nm}$ was corrected for this contribution by using the proportional absorption of PNPG at the two wavelengths as found from a spectrum of PNPG. The molar concentrations of KSTI and PNPG were calculated with the use of extinction coefficients of $2.26 \cdot 10^4\ M^{-1}\ cm^{-1}$ ($\lambda_{max} = 280$ nm) for KSTI and $1 \cdot 10^4\ M^{-1}\ cm^{-1}$ ($\lambda_{max} = 305$ nm) for PNPG. The coupling ratios obtained by spectrophotometric measurements at 305 nm were comparable to the results obtained by HPCE.

The molar coupling ratios were further evaluated by determination of the number of free amine residues present on KSTI before and after conjugation. This was performed by measuring the degree of trinitrophenylation after reaction of TNBS with the free amine residues (Table 1). The absorbance of KSTI at 280 nm corrected for the contribution of PNPG was used for calculation of the KSTI concentration. The number of trinitrophenylated amino groups was determined from $A_{335\ nm}$ corrected for the contribution from PNPG as determined from a spectrum of PNPG, and using the extinction coefficient of TNP–NH_2–protein ($1 \cdot 10^4\ M^{-1}\ cm^{-1}$). By this method, higher coupling ratios were found compared to the spectrophotometric method and calculations from HPCE data. KSTI contains 9 lysine residues, and the overestimated number of TNP–NH_2 determined are probably due to the reaction of TNBS with other groups than amino groups on the protein. Besides, a high degree of inaccuracy may be connected with the TNBS reaction when low protein concentrations are employed. This was confirmed by analysis of BSA and KSTI solutions with concentrations in the range corresponding to $A_{280\ nm}$ from 0.092 to 1.548 was performed in triplicate thus covering the range of conjugate concentrations. Relative standard deviations from 0.023 to 0.115 of the measured $A_{335\ nm}$ were found. The relative standard deviations raised markedly at protein concentrations with $A_{280\ nm}$ values less than 0.5.

3.2. Coupling of soyasaponin to protein

As the conjugation method had been shown to be efficient for the coupling of PNPG to KSTI, the coupling of soyasaponin I to BSA and KSTI was also investigated with different mixture ratios of hapten to protein carrier as indicated in Table 1. Results from HPCE analysis of soyasaponin I conjugated to KSTI are shown in Fig. 3. As with the coupling of PNPG to KSTI, a peak with an increased t_m (b) compared to KSTI (peak a) appeared. The four samples contained different concentrations of KSTI, and the coupling degree was calculated as the normalized peak area of the emerging peak relative to the normalized peak area of unconjugated KSTI. The hydrophobic triterpenoid part of the amphiphilic soyasaponin I was expected to be exposed on the surface of the protein after conjugation and consequently result in an increased interaction of protein–soyasaponin I conjugates with cholate micelles compared to unconjugated protein. As discussed for the coupling of the amphiphilic PNPG to KSTI (see above) this interaction results in an increased t_m for the saponin–protein conjugates. With use of a high conjugation ratio between saponin and KSTI (12:1; Table 1) emulsions or micelles may have been formed thus resulting in a reduced conjugation ratio.

For the coupling products with BSA as carrier protein it was not possible to distinguish any additional peaks as unconjugated BSA generated a very broad peak in the HPCE system, pre-

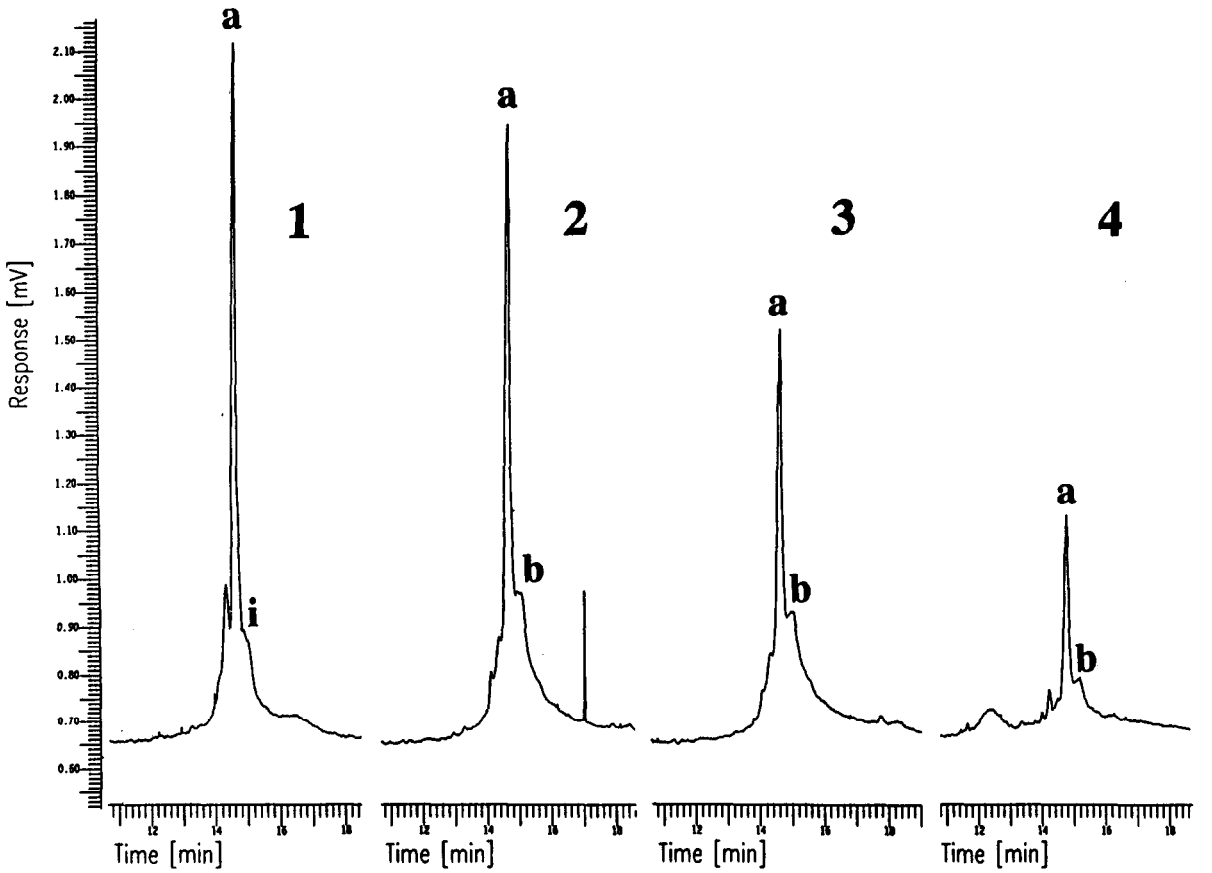

Fig. 3. Electropherograms of soyasaponin I–KSTI conjugates. Separation conditions as in Fig. 2. Molar ratios in the conjugation reaction (saponin:KSTI): 1 = 0:1; 2 = 3:1; 3 = 6:1; 4 = 12:1. Peaks a, b and i as in Fig. 2.

sumably caused by the presence of several different proteins in the commercially available BSA. However, the TNBS method indicated that coupling of saponin to the protein had occurred.

The electropherograms obtained with saponin–protein conjugates did not result in a comparably efficient separation of the conjugates from unconjugated protein as was found with the electropherograms of PNPG–protein conjugates. This could be explained by the presence of several vicinal hydroxyl groups in the carbohydrate moiety of saponin resulting in different possible sites for conjugation and various different coupling products. This hypothesis was supported by SDS-PAGE of the conjugation products of PNPG and saponin with KSTI (Fig. 4). With the PNPG conjugates all of the samples

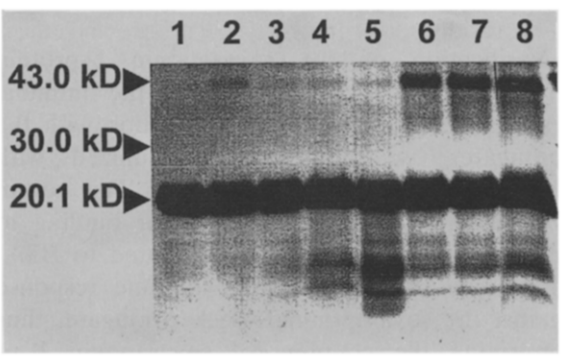

Fig. 4. PAGE of hapten–protein conjugates. Ratios in parentheses represent the molar ratios of reactants present in the conjugation reactions: 1 = PNPG:KSTI (0:1); 2 = PNPG:KSTI (6:1); 3 = PNPG:KSTI (12:1); 4 = PNPG:KSTI (48:1); 5 = saponin:KSTI (0:1); 6 = saponin:KSTI (3:1); 7 = saponin:KSTI (6:1); 8 = saponin:KSTI (12:1). kD = kilodaltons.

resulted in only one band at the molecular mass of KSTI. With saponin conjugated to KSTI, however, a small band appeared in all samples at a position of the double molecular mass of KSTI except for the blind sample. As the periodate cleavage at vicinal hydroxyl groups of the saponin carbohydrate moiety can result in several aldehyde groups, it is likely that a saponin molecule can be coupled to more than one KSTI at a time. The periodate cleavage of one carbon–carbon bond at vicinal hydroxyl groups yields two aldehyde groups available for coupling. However, steric hindrance caused by a conjugated carrier and the presence of additional available amino groups present on the same carrier reduce the possibilities for two carriers to bind at the same cleavage site. The coupling of the same carrier at two sites of the oligosaccharide part of the saponin could also explain part of the inconsistent results obtained by the TNBS method and by HPCE, as the coupling of one saponin molecule to several amino groups on the protein will result in a too high estimation of the coupling density by the TNBS method. The use of HPCE for determination of coupling ratios therefore seems to be a more promising method than the TNBS method. In addition, analyses performed by the TNBS method require far more sample compared to HPCE analyses.

3.3. Immune response against soyasaponin I

Verification of the soyasaponin I–protein conjugations was obtained from the immune response generated after immunization with the conjugates. A rabbit was immunized with soyasaponin I coupled to KSTI. The serum obtained was tested by ELISA for binding to immobilized soyasaponin I conjugated to BSA. This assay showed a strong immune response against the soyasaponin I–BSA conjugate, thus confirming the coupling of soyasaponin I to KSTI as well as to BSA. A competitive ELISA was performed in which unconjugated saponin in solution competed with immobilized soyasaponin I conjugated to BSA for the binding of antibodies. The results of this ELISA are depicted in Fig. 5, and it is seen that the antibodies present in the serum were inhibited in their binding to the immobilized soyasaponin–BSA conjugate by the free saponin present in solution. Besides, Fig. 5 demonstrates that the developed ELISA is very sensitive with a detection range from 10 to 300 ng soyasaponin I/ml.

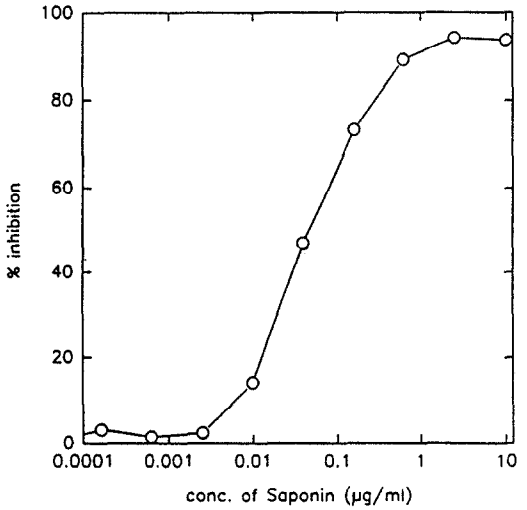

Fig. 5. Investigation of the specificity for soyasaponin I in solution of a rabbit serum produced against a soyasaponin I–KSTI conjugate. The experiment was performed by ELISA, with immobilization of soyasaponin I–BSA conjugate followed by incubation with preincubated mixtures of rabbit serum and soysaponin I at different concentrations.

4. Conclusions

The electrophoretic properties of the carrier protein in HPCE and the homogeneity of the carrier preparation are important for the use of HPCE as a method for determination of the coupling ratio. The presence of protein impurities in the carrier preparation may interfere with the interpretation of the data, as the protein impurities also may act as carriers. However, the HPCE technique now developed for studies of hapten–protein conjugates is found to be an efficient supplement to the limited number of methods available for determination of coupling ratios. The applicability of the HPCE method is independent of the site of conjugation in contrast

to the TNBS method that can only be employed after conjugation at amino groups.

Acknowledgements

The authors gratefully acknowledge support from the Danish Agricultural and Veterinary Research Council and the Danish Ministry of Agriculture.

References

[1] S.B. Mahato, S.P. Sarkar and G. Poddar, *Phytochemistry*, 27 (1988) 3037.
[2] K.R. Price, I.T. Johnson and G.R. Fenwick, *CRC Crit. Rev. Food Sci. Nutrition*, 26 (1987) 27.
[3] K.R. Price, J. Eagles and G.R. Fenwick, *J. Sci. Food Agric.*, 42 (1988) 183.
[4] K.R. Price, N.M. Griffiths, C.L. Curl and G.R. Fenwick, *Food Chem.*, 17 (1985) 105.
[5] I. Kitagawa, M. Yoshikawa, H.K. Wang, M. Saito, V. Tosirusuk, T. Fujiwara and K.-I. Tomita, *Chem. Pharm. Bull.*, 30 (1982) 2294.
[6] I. Kitagawa, M. Yoshikawa and I. Yosioka, *Chem. Pharm. Bull.*, 22 (1974) 3010.
[7] I. Kitagawa, H.K. Wang, T. Taniyama and M. Yoshikawa, *Chem. Pharm. Bull.*, 36 (1988) 153.
[8] M.R.A. Morgan, R. McNerney, J.A. Matthew, D.T.C. Coxon and H.W.-S. Chan, *J. Sci. Food Agric.*, 34 (1983) 593
[9] A.M. Arentoft, H. Frøkiær, S. Michaelsen, H. Sørensen and S. Sørensen, *J. Chromatogr. A*, 652 (1993) 189.
[10] B. Bjerg and H. Sørensen, in J.-P. Wathelet (Editor), *World Crops: Production, Utilization, Description. Glucosinolates in Rapeseed: Analytical Aspects*, Vol. 13, Martinus Nijhoff (Kluwer), Dordrecht, 1987, p. 125.
[11] V.P. Butler, Jr. and J.P. Chen, *Proc. Natl. Acad. Sci. U.S.A.*, 57 (1967) 71.
[12] A.F.S.A. Habeeb, *Anal. Biochem.*, 14 (1966) 328.
[13] L.C. Mokrasch, *Anal. Biochem.*, 18 (1967) 64.
[14] H. Frøkiær, L. Hørlyck, H. Sørensen and S. Sørensen, *J. Sci. Food Agric.*, in press.
[15] H. Blum, H. Beier and H.J. Gross, *Electrophoresis*, 8 (1987) 93.
[16] C. Bjergegaard, S. Michaelsen, K. Mortensen and H. Sørensen, *J. Chromatogr. A*, 652 (1993) 477.
[17] C. Bjergegaard, S. Michaelsen and H. Sørensen, *J. Chromatogr.*, 608 (1992) 403.
[18] C. Bjergegaard, L. Ingvardsen, and H. Sørensen, *J. Chromatogr.*, 608 (1993) 99.