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INTERACTION BETWEEN PROTEINS AND GLUCOSINOLATE ISOTHIOCYANATES AND OXAZOLIDINETHIONES FROM *BRASSICA NAPUS* SEED

RUNE BJÖRKMAN

Institute of Biochemistry, University of Uppsala, Box 531, S-751 21 Uppsala, Sweden

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Abstract—The proteins from rape seed meal and serum albumin were incubated with the ^{35}S-labelled glucosinolates progoitrin, gluconapin, and glucoalyssin in a variety of reaction conditions. Intact glucosinolates and oxazolidinethiones were found to combine with the proteins to a very small extent, independently of pH; but the isothiocyanates reacted readily with the proteins at pH values higher than 6. Fractionation of the rape seed protein conjugates on Sephadex G200 showed that isothiocyanates particularly reacted with the basic low molecular weight proteins. Changes in UV-spectrum and electrophoretic mobility after reaction with isothiocyanates were also demonstrated.

INTRODUCTION

THE DEVELOPMENT of procedures for the production of protein concentrates from rape seed (*Brassica napus* L.) for human consumption is now in progress.[1,2] The utilization of these proteins has hitherto been restricted owing to the presence of glucosinolates which are hydrolyzed to anti-nutritional isothiocyanates and oxazolidinethiones by myrosinase when the seed is soaked in water. Thiocyanates, nitriles, and episulphides may also be formed[3] and conjugates of such substances with the proteins may lower the nutritive value of the concentrate. Amino and sulphydryl groups react with isothiocyanates to form substituted thioureas and dithiocarbamates, particularly at high pH.[4] We thus expected the amino and sulphydryl groups of the rape seed proteins to react under certain conditions with the isothiocyanates liberated from the glucosinolates. It was also of interest to discover whether the intact glucosinolates and the goitrogenic oxazolidinethiones would interact with the proteins. The present investigations were performed on rape seed proteins and on serum albumin. The interactions were followed by using ^{35}S-labelled glucosinolates, prepared as described in a previous paper.[5]

RESULTS

Protein (HSA)—Glucosinolate Interactions

Reaction experiments with human serum albumin (HSA) and intact ^{35}S-glucosinolates (progoitrin, gluconapin, and glucoalyssin) were performed at pH 4, 7 and 9 for 1 hr at 40°. Generally about 15 000 cpm were added. After fractionation on Sephadex G50 only 100–250 cpm were found in the protein. No pH-dependence was observed.

[1] TAPE, N. W., SABRY, Z. J. and EAPEN, K. E. (1970) *J. Can. Inst. Food Technol.* **3**, 78.
[2] OHLSON, R. Karlshamns Oljefabriker AB, Sweden, Personal communication.
[3] VAN ETTEN, C. H., DAXENBICHLER, M. E. and WOLFF, J. A. (1969) *J. Agr. Food Chem.* **17**, 483.
[4] EDMAN, P. (1970) in *Protein Sequence Determination* (NEEDLEMAN, S. B., ed.), p. 211, Springer, Berlin.
[5] BJÖRKMAN, R. (1972) *Acta Chem. Scand.* **26**, 1111.

HSA—Isothiocyanate Interactions

Isothiocyanates, obtained by hydrolysis of glucosinolates with myrosinase, reacted readily with HSA. The reaction was found to be strongly pH-dependent. Figure 1 shows the incorporation of 3-butenylisothiocyanate (from gluconapin) and 5-methylsulphinylpentylisothiocyanate (from glucoalyssin) into HSA at various pHs for 75 min at 40°.

The influence of various amounts of 3-butenylisothiocyanate on the incorporation into HSA at pH 8·5 for 1 hr at 40° was investigated. When relatively small amounts were added (up to 2000 cpm) the reaction was almost quantitative (1600 cpm incorporated). On adding greater activity than this the curve indicates that the reaction velocity, although linear, is lower. For 30 000 cpm added, only 2500 cpm were incorporated. To some extent the appearance of the curve may also be influenced by the volatility of the 3-butenyl isothiocyanate. This possibility was demonstrated by performing the reaction for 4 hr in an open tube at pH 5 and 24°. The pH was adjusted to 8·5, and the reaction was allowed to proceed for another hour at 40°. Now only about 25% of the normal amount of radioactivity was found in the protein fraction.

For investigating the dependence of the incorporation of isothiocyanate into HSA on the reaction time the non-volatile 5-methylsulphinylpentylisothiocyanate was used. The isothiocyanate was prepared in advance by incubating glucoalyssin with myrosinase for 30 min at pH 5 (pH optimum for the enzyme). Then the mixture was added to the HSA solution and the pH was raised to 8·5 (40°). For the first hour 2000 cpm were incorporated. For the following 4 hr an additional 2500 cpm were found in the HSA.

The addition of ammonia (final concentration 0·1 M, pH 9·0) to the reaction mixture reduced the incorporation of radioactivity into the protein to about 50%, compared to a control at the same pH. When glycine was added (0·35 M, pH 8, 40°, 30 min) the uptake of radioactivity was also significantly lowered.

HSA—Oxazolidinethione Interactions

Labelled L-5-vinyloxazolidine-2-thione was prepared by enzymatic hydrolysis of ^{35}S-progoitrin and purified by chromatography on a 2·5 × 42 cm column filled with an arginine derivative of Sephadex G10 (see Ref. 5). In reaction experiments with the oxazolidinethione and HSA, performed at pH 4, 5, 7 and 8·5 at 40° for 45 min only a small amount of radioactivity (200 cpm of added 10 000 cpm) was associated with the protein. The uptake did not increase when the reaction time was extended to 2 hr. Experiments in which the protein was already present during the enzymatic hydrolysis of the progoitrin were also performed. HSA, progoitrin and myrosinase were incubated at pH 5 and 9 for 45 min. Also after these experiments only about 3% were found in the protein fraction.

Experiments with Rape Seed Meal (RSM)

RSM contains about 5% of glucosinolates, of which 70–75% produce oxazolidinethiones and 25–30% produce isothiocyanates on hydrolysis with myrosinase. In the following experiments the amount of added ^{35}S-glucosinolates was usually of the same magnitude as the amount of glucosinolates naturally present. The ^{35}S-isothiocyanates from glucoalyssin and gluconapin were allowed to react with RSM at pH 9 according to 'procedure I' (see Experimental). About 36% of the amount of added isothiocyanate was found to be associated with the protein. There was no difference whether glucoalyssin or gluconapin was used. A longer reaction time did not increase the incorporation significantly. The incorporation

increases linearly with the amount of isothiocyanate added. Addition of ammonia (0·1 M final conc.) reduced the uptake to about 14% of that added. At pH 3·5 and 4·25 the reaction was almost zero.

Experiments according to 'procedure II' (see Experimental) were performed with glucoalyssin at pH 8. The addition of 50 mg glycine to the reaction mixture had no significant effect on the uptake of isothiocyanates to the protein.

When progoitrin was added to RSM according to 'procedure I', 3–4% of the radioactivity was incorporated, whether myrosinase was added or not. Neither reaction time nor pH seemed to influence the uptake (45 min and 2 hr respectively pH 4, 5, 7 and 9 were tested).

The distribution of isothiocyanate and oxazolidinethione conjugate into different protein groups in rape seed was studied by gel chromatography. The protein fraction from Sephadex G50 after ^{35}S-3-butenyl isothiocyanate incorporation ('procedure I') was chromatographed on a Sephadex G200 column as shown in Fig. 2. Fractions I and II contained neutral, high MW proteins, while fraction III consisted of basic, low MW proteins (MW 12–14 000).[6] The figure shows that proportionally more isothiocyanate was incorporated into fraction III than into fractions I and II. No radioactivity was found in the total volume (V_t), which indicates that all the isothiocyanate was covalently bound to the proteins. Figure 2 also shows the radioactivity pattern when the protein fraction from Sephadex G50 after oxazolidinethione incorporation was run on G200. In this case all the radioactivity was found in the high MW fraction I.

Physico-chemical Effects on the Protein

The reaction between an isothiocyanate and a protein involves a change in the physicochemical properties of the protein. The change in electrical charge was demonstrated by electrophoretic experiments. Samples of HSA reacted with different amounts of 3-butenylisothiocyanate were subjected to electrophoresis in a Gelman apparatus. After staining the strips, the migration distances of the bands were measured. Relative to the unconjugated protein, incorporation of 3000 cpm isothiocyanate increased the mobility by 20%. Up to this value the migration rate was proportional to the amount of radioactivity incorporated. The change in the UV-spectrum of HSA on conjugation with 3-butenylisothiocyanate was also studied. The maximum at 278 nm was not altered, but the minimum at 250–270 nm was considerably reduced. The increase in absorbance at 254 nm was linearly proportional to the amount of isothiocyanate (cpm).

DISCUSSION

3-Butenyl- and 5-methylsulphinylpentylisothiocyanate were found to react readily with proteins at high pH. As is shown in Fig. 1 the reaction rate increased rapidly at pH values higher than 6. The incorporation of radioactivity (isothiocyanate) in the protein as a function of the amount of isothiocyanate added, shows a change in slope at about 1700 cpm incorporated. Besides the effect of the volatility of 3-butenylisothiocyanate this change might reflect the point where the most reactive and accessible sulphydryl, ϵ-amino, and terminal α-amino groups were saturated. Reactions with lower rates, e.g. with available ϵ-amino groups in lysine, brought about by larger amounts of isothiocyanate will also influence the appearance of the curve. This is also supported by the corresponding change in slope

[6] LÖNNERDAL, B. and JANSON, J.-C. (1972) *Biochim. Biophys. Acta* **278**, 175.

found in the incorporation of isothiocyanate with time. It is noteworthy that even after 5 hr of reaction the uptake of isothiocyanate increases linearly.

Substances such as ammonia and glycine which might compete with the protein amino and sulphydryl groups caused a reduction in the incorporation of the isothiocyanate to the protein which was less than expected. Although they were added in large excess, the reduction by ammonia was only about 50% at pH 9 when HSA was used. In RSM the uptake was reduced to 14%. Glycine had no effect on the RSM.

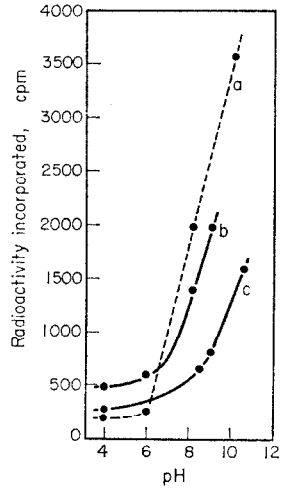

FIG. 1.

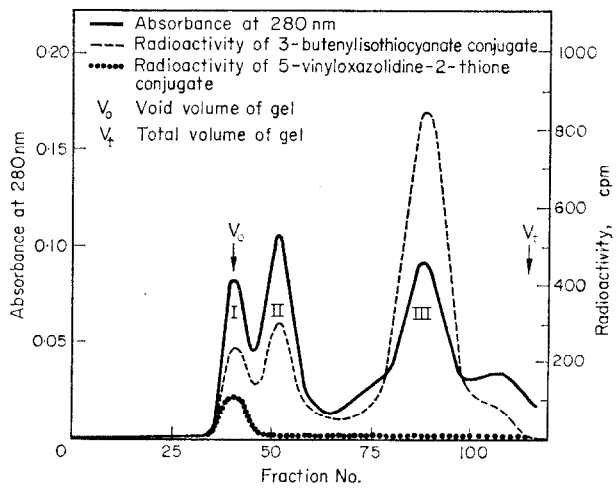

FIG. 2.

FIG. 1. THE INFLUENCE OF pH ON THE INCORPORATION OF ISOTHIOCYANATES INTO PROTEIN (HSA). 5-Methylsulphinylisothiocyanate from 50 μl of glucoalyssin (a) and 3-butenylisothiocyanate from 50 μl (b) and 10 μl (c) of gluconapin were incubated with HSA for 75 min at various pH (40°). About 10 000, 6000, and 3000 cpm resp. were added.

FIG. 2. MOLECULAR SIEVING CHROMATOGRAPHY ON SEPHADEX G200 OF RAPE SEED PROTEIN-ISOTHIOCYANATE AND OXAZOLIDINE-THIONE CONJUGATES IN 0·1 M $NaHCO_3$ (pH 8·7).

Neither intact glucosinolates nor the oxazolidinethiones seemed to react with the protein. However, small but significant amounts of radioactivity, independent on pH and reaction time, were always associated with the protein fraction, probably as a result of non-specific adsorption rather than chemical incorporation.

Figure 2 shows that the reaction mechanism for protein-isothiocyanate and protein-oxazolidinethione is quite different. Isothiocyanates mainly reacted with the low MW basic proteins (their isoelectric points are about 11) rich in lysine and terminal amino acids. The oxazolidinethione on the other hand, was exclusively associated with the high MW protein aggregates in the void fraction.

When progoitrin is hydrolyzed with myrosinase, 2-hydroxy-3-butenyl isothiocyanate is first formed, which spontaneously cyclizes to L-5-vinyloxazolidine-2-thione.[3,7] If the cyclization step is slow, the intermediate presumably would be able to react with the protein at high pH. However, experiments where HSA was already present during the enzymatic hydrolysis showed no increased radioactivity incorporation into the protein.

[7] ETTLINGER, M. G. and LUNDEEN, A. J. (1956) *J. Am. Chem. Soc.* **78**, 4172.

Reaction experiments with RSM were performed according to two different procedures. In 'procedure I' the labelled glucosinolates were hydrolyzed to isothiocyanates with myrosinase before they were added to a fresh RSM-extract. In this way principally the labelled isothiocyanates react with the proteins since the glucosinolates naturally present were mainly intact at the start of reaction. About 36% of the amount of added isothiocyanate was incorporated by the protein at pH 9 in 30 min. Experiments performed with longer reaction times gave similar results. This may be explained by competition from isothiocyanates from the naturally present glucosinolates which are not labelled. In 'procedure II' the labelled and native isothiocyanates reacted under the same conditions with the protein, since the labelled glucosinolate was added direct to the meal. No extra myrosinase was added. The differences between the previous experiment conducted at pH 9·0, and this experiment in which radioactivity was incorporated at pH 8·0 depend both on pH and differences in the procedures. In 'procedure II' the curve had a concave tendency. Some radioactivity is also lost in the solid residue after the meal was extracted ('procedure II').

EXPERIMENTAL

^{35}S-labelled glucosinolates from rape seed were prepared biosynthetically by cultivating summer rape (*Brassica napus* L.) on soil containing ^{35}S-sulphate. The preparation and isolation was performed as described previously.[5] The ^{35}S-glucosinolates progoitrin, gluconapin, and glucoalyssin were used. The sp. act. of the isolated glucosinolates was at the start of the experiments about 40 μCi/mmol. The radioactive decay was corrected for. The cpm values given only refer to the isothiocyanate sulphur. This implies that the contributing sulphate and sulphoxy sulphur have been subtracted. It was therefore assumed that the specific radioactivity was equal for all the sulphur atoms in the glucosinolates. Water solutions of the glucosinolates with a content of about 100 μmol/ml were kept frozen at $-20°$.

Myrosinase (thioglucoside glucohydrolase, E.C. 3.2.3.1) was prepared from white mustard (*Sinapis alba* L.) seed according to a modified version of the method described by Björkman and Janson:[8] After defatting the crushed seed (25 g) with Et_2O the proteins were extracted with 0·01 M imidazole–HCl (pH 6·0, 100 ml). The crude extract was fractionated by molecular sieving on a 5 × 50 cm column packed with Sephadex G50, equilibrated and run in the same buffer. The material with myrosinase activity was further fractionated by ion-exchange chromatography. The sample was applied to a 3·2 × 7·0 cm column packed with DEAE-cellulose powder (Whatman DE-52), equilibrated in 0·01 M imidazole–HCl (pH 6·0). The enzyme, which was adsorbed to the ion-exchanger, was eluted by a linear gradient of 0·01 to 0·1 M imidazole-HCl (pH 6·0) (700 + 700 ml) at a flow rate of 50 ml/hr. The fractions containing myrosinase activity were combined and kept cold. The sp. act. of this preparation was about 40 μmol/min per mg.

Protein. Human serum albumin (HSA) was obtained from AB Kabi, Stockholm, Sweden. Rape seed meal (RSM) was prepared by extracting finely ground seeds of winter rape (*Brassica napus* L., cv. Panter) with Et_2O at room temp.

Reaction experiments with HSA. 500 μl of a 2% soln of HSA were placed in a 5 ml test tube with 100 μl of a 1 M buffer solution (phosphate or carbonate). 10 to 250 μl of radioactive glucosinolate solution, and usually also 100 μl of myrosinase solution were added. In some experiments glycine or ammonia were also added. The total vol. was made up to 1 ml with H_2O. The mixture was kept at 40° for various time intervals. To avoid oxidation the experiments were carried out under N_2.

Reaction experiments with RSM were performed according to either of two procedures: (I) 0·5 g of RSM was rapidly shaken with 2 ml of 0·1 M buffer (acetate pH 3·5–4·5, carbonate pH 8–9, and H_2O at neutral pH). The extract was immediately centrifuged. The total time for the process was 6 min. The supernatant was combined with a glucosinolate or an isothiocyanate solution, prepared by hydrolysis of 250 μl of the corresponding glucosinolate with 50 μl of myrosinase for 30 min at 40°. After adjusting the pH with NaOH the mixture was incubated at 40° for various periods. (II) 0·5 g of RSM was mixed with 250 μl o fa radioactive glucosinolate solution. 2 ml of buffer solution were added. After incubation at 40° for various periods the reaction mixture was centrifuged and the supernatant was collected.

Fractionation of the reaction mixture. Immediately after the reaction experiments the sample was fractionated on a 1·4 × 50 cm column of Sephadex G50, equilibrated and run in 0·1 M $NaHCO_3$ (pH 8·7) at a flow rate of 10 ml/hr. The UV absorption of the eluate was continuously measured at 254 nm with a Uvicord I (LKB-Produkter). Fractions of 3·3 ml were collected.

Radioactive measurements. 0·5 ml of each 3·3 ml fraction from the Sephadex G50 column was pipetted into

[8] BJÖRKMAN, R. and JANSON, J.-C. (1972) *Biochim. Biophys. Acta* **276**, 508.

steel planchettes. After addition of a drop of wetting agent (triethylamine) the samples were dried under an IR lamp and counted.

Fractionation of rape seed proteins. The protein fractions from the Sephadex G50 run were combined and concentrated to 1·5 ml by ultrafiltration (Diaflo PM-10, Amicon Corp.). The sample was applied to a 2 × 60 cm column of Sephadex G200, equilibrated and run in 0·1 M $NaHCO_3$ (pH 8·7) at a flow rate of 10 ml/hr. The UV absorption at 280 nm and the radioactivity of the fractions were measured.

Electrophoresis of protein conjugates. Electrophoretic experiments on cellulose polyacetate strips in a Sepraphore electrophoresis equipment (Gelman) were performed on some isothiocyanate protein conjugates, in 0·1 M Tris-HCl (pH 8·5) at 120 V (1 mA/strip) at 5° for 100 min. The sample vol. was 4 μl.

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