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INTERACTION OF OXIDIZED GLUTATHIONE WITH ALLYL ISOTHIOCYANATE

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Key Word Index—*Brassica* species; Cruciferae; oxidized glutathione; disulphide cleavage; allyl isothiocyanate; glutathionyl dithiocarbamate.

Abstract—Isothiocyanates formed from glucosinolates in *Brassica* species have a strong affinity for amino acids and proteins, especially for their thiol, sulphide and terminal amino groups. To investigate the action of isothiocyanate on cystine residues in proteins and peptides, the present study on the interaction between allyl isothiocyanate and oxidized glutathione under physiological conditions was undertaken. Oxidized glutathione was oxidatively cleaved to some modified glutathiones by the attack of allyl isothiocyanate on its disulphide bond. Two new modified products were isolated from the reaction mixture by gel chromatography and HPLC, and their structures were determined by NMR and mass spectral analyses as glutathionyl *N*-allyldithiocarbamate and its allyl thiohydantoin derivative. The formation of these products indicated oxidative cleavage of the disulphide bond in the cystine residue; the electrophilic attack of the isothiocyanate on the sulphur atom must cleave the disulphide bond oxidatively to dithiocarbamate and sulphenate, as in the case of cystine.

INTRODUCTION

Glucosinolates, widely distributed in the Cruciferae, especially in *Brassica* species, are decomposed to alkyl isothiocyanates (mustard oils), glucose and sulphate by the action of myrosinase, a kind of thioglucosidase, when the plant tissues are crushed [1]. It is well known that some kinds of mustard oils and their degradation products have an anti-thyroid action [2], while many *Brassica* plants are used as vegetables and some are used as spices. Isothiocyanates generally are strong electrophilic reagents and react easily with free amino groups of amino acids and proteins to give their thiourea derivatives. This type of reaction is well known as a method for the determination of amino acid sequences in protein by the reaction of α-amino group of the *N*-terminal amino acid in protein with synthetic phenyl isothiocyanate [3, 4]. Also, benzyl isothiocyanate formed from benzyl glucosinolate inhibits the papain activity by its addition to the SH group in papain [5].

Consequently, isothiocyanates formed from *Brassica* seeds and other tissues may decrease the function of protein by their interaction. Moreover, part of the isothiocyanates is decomposed by the addition of water [6–9]. We have already reported that the disulphide bond in cystine is oxidatively degraded by the action of allyl isothiocyanate (AITC) under mild conditions [10, 11]. To clarify the action of isothiocyanate on the disulphide bond in protein, the interaction of oxidized glutathione (GSSG) and AITC was studied in detail and the oxidative cleavage of the disulphide bond in GSSG has been clearly demonstrated.

RESULTS AND DISCUSSION

Aqueous suspensions of AITC (4 mmol) and GSSG (0.2 mmol) were incubated at 40°, 60° and 80° with stirring. The reaction was followed by colour development with the DTNB reagent (see Experimental) (Fig. 1). The positive products for the DTNB in the reaction mixtures were formed rapidly with increase in reaction temperature and reached their maxima after 40 hr at 40°, 2 hr at 60° and 0.5 hr at 80°. The colour development with DTNB detected the formation of SH or SOH compounds during the reaction, suggesting the cleavage of the disulphide bond in GSSG, as in the case of cystine [11]. TLC

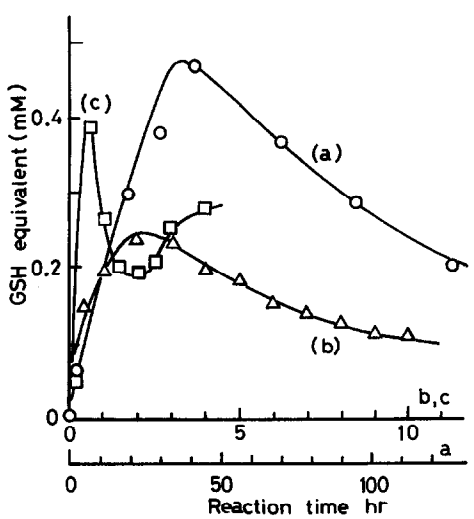


Fig. 1. Time course changes in the formation of the DTNB positive products from the reaction mixtures composed of 0.2 mmol GSSG and 4 mmol AITC in 50 ml of phosphate buffer (pH 6) at various temperatures: (○) 40°; (△) 60° and (□) 80°. *A* at 412 nm was determined.

analyses also showed the formation of new ninhydrin positive products during decomposition.

To confirm the cleavage of the disulfide bond in GSSG by the addition of AITC, and to determine the structures of its modified peptides, the fractionation and isolation of the reaction products were performed using gel chromatography (Figs. 2 and 3) and HPLC. Two amorphous products, S_{1-3} and S_2, were obtained which gave a single peak on HPLC and a single spot on TLC, respectively. They were the main products in this reaction and their acid hydrolysis showed them to be glutathione derivatives. However, the spots S_{1-1} and S_{1-2} in Fig. 3 were not peptide derivatives, but were amino-acid-like compounds derived from AITC.

The UV spectra of S_{1-3} and S_2 suggested them to be dithiocarbamate or thiohydantoin derivatives (λ_{max} 250 and 270 nm for S_2, and 279 nm for S_{1-3}). To confirm the structures, their ^{1}H NMR spectra were measured and assignment of all of their protons was performed by comparison with the spectrum of GSSG and by the proton decoupling method. The chemical shifts of the respective protons in GSH, GSSG, AITC, S_{1-3} and S_2 are given in Table 1.

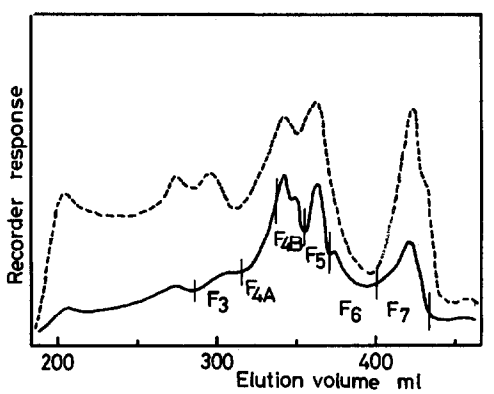


Fig. 2. Gel chromatography of reaction products formed from GSSG and AITC. The reaction mixture prepared from 0.6 mmol GSSG and 12 mmol AITC in 150 ml of distilled water (adjusted to pH 6) was incubated at 80° for 5 hr. After ether extraction, the aqueous solution was concentrated and applied to a Sephadex G-25 (fine) column (3 × 65 cm) and eluted with water. RI (——) and UV (at 280 nm, – – – – –) detectors were used.

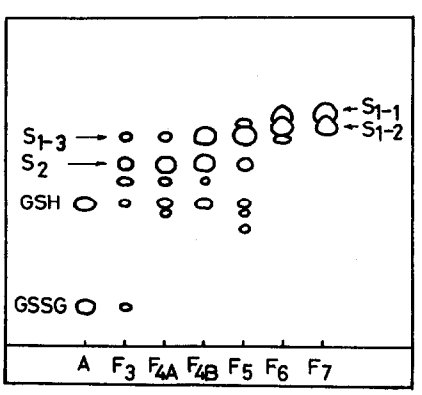
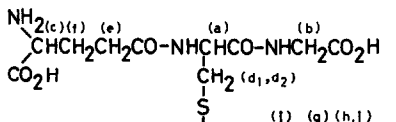


Fig. 3. TLC of each fraction in Fig. 2. Fractions F_3–F_7 were concentrated to a small volume and submitted to cellulose TLC, developed with *n*-BuOH–HOAc–H_2O (4 : 1 : 5) and detected with 0.2% ninhydrin in ethanol.

Protons g, h, i and j in S_2 were assigned to the allyl group and suggested the presence of one allyl group in S_2 from the relative strength of their signals. The protons of the glutathione residue in S_2 gave similar values to that of GSSG, except for the shift to lower fields of Hd_1 and Hd_2. From these NMR data, the structure of S_2 was proposed as glutathionyl *N*-allyl dithiocarbamate (**3**). Also, from the NMR spectrum of S_{1-3}, the protons, δ4.37 (Hj) and 3.16 (Hj'), indicated the presence of two kinds of allyl group, in which Hj was attributed to the methylene protons of the allyl group in *N*-allyl dithiocarbamate residue, as with S_2, and Hj' revealed them in the allyl thiohydantoin residue. H_b in S_{1-3} was determined to be ring methylene protons in thiohydantoin, as the two doublets of H_b in D_2O were converted to one singlet by the addition of DCl for its ring opening. Moreover, mass spectral analysis of S_{1-3} gave the molecular ion at *m/z* 487, and the fragment ion at *m/z* 156 (60%) formed from the hydantoin ring by a McLafferty rearrangement of the molecular ion. From these results, the structure of S_{1-3} was designated as **5**.

It is evident from Fig. 1 that the disulphide bond of GSSG (**1**) was cleaved by the electrophilic action of AITC, as in the case of cystine. The half of GSSG cleaved by this reaction was transformed to glutathione dithiocarbamate (**3**) through the addition of AITC. Since this reaction proceeded under oxidative conditions, the other half of the scission product of **1** must be glutathione sulphenate as shown in Scheme 1. It is considered that sulphenate GSH → O reacts with DTNB, like thiol, to release a nitrothiophenol derivative which exhibits strong absorption at 412 nm. Therefore, in the reaction mixture of GSSG and AITC, GSH→O and/or its dimer must be formed with the product **3**, but their detection and identification have not yet been carried out. When this reaction was followed by TLC with time, a trace amount of GSH (**2**) was detected immediately after the detection of **3** (S_2). Since this reaction system proceeded under oxidative conditions, **2** would not be formed directly from **1** and so a small part of **3** must be decomposed to **2** by the elimination of AITC. Parallel to the formation of **3**, excess AITC was added to the amide nitrogen atom of the glycine moiety in **3** to give the allyl thiocarbamyl derivative (**4**), which was easily cyclized to hydantoin (**5**) by intra-



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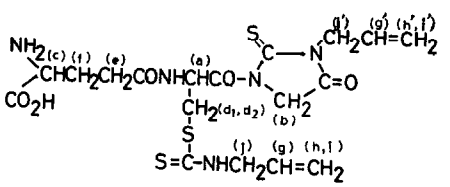

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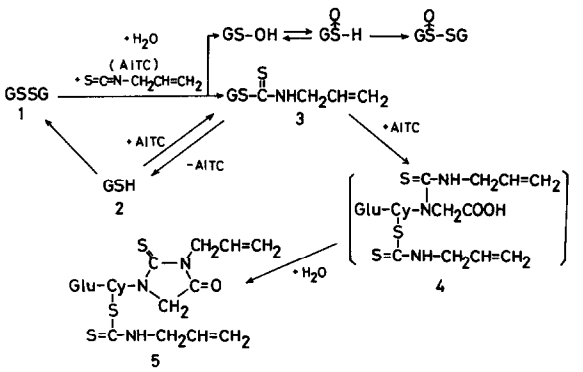
Table 1. Chemical shifts and coupling constants in ^{1}H NMR of the products S_{1-3} and S_2 compared with those of GSH, GSSG and AITC*

		GSH		GSSG		AITC	S_2		S_{1-3}	
		D_2O	D_2O+DCl†	D_2O	D_2O+DCl†	($CDCl_3$)	D_2O	D_2O+DCl†	CD_3OD	CD_3OD+DCl†
Chemical shift (ppm)	Ha	4.45 *t*	4.43	4.64 *dd*	4.64		4.50 *dd*	4.60	4.55 *dd*	4.55
	Hb	3.85 *s*	3.90	3.86 *s*	3.92		3.65 *s*	3.89	3.77 *d* 3.76 *d*	3.92
	Hc	3.70 *t*	4.03	3.71 *t*	4.03		3.56 *t*	3.99	4.28 *t*	4.27
	Hd_1	2.80 *d*	2.81	3.19 *dd*	3.18		3.79 *dd*	3.76	3.02 *dd*	2.96
	Hd_2			2.85 *dd*	2.87		3.41 *dd*	3.42	2.68 *dd*	2.68
	He	2.40 *t*	2.52	2.44 *t*	2.52		2.38 *t*	2.44	2.42 *t*	2.42
	Hf	2.00 *td*	2.14	2.06 *td*	2.18		2.02 *td*	2.08	2.14 *td*	2.12
	Hg					5.84	5.77	5.75	5.87	5.85
	Hh					5.37	5.09	5.08	5.16	5.16
	Hi					5.26				
	Hj					4.12	4.19	4.18	4.37 (Hj) 3.16 (Hj')	4.37 3.17
J (Hz)	Jad_1	6.3		4.6			5		5.6	
	Jad_2			9.2			9		8.3	
	Jd_1d_2			14.3			15		13.9	
	*J*cf	6.0		6.5			6		6.0	
	*J*ef	7.5		7.5			7		7.4	

*Internal standards used were acetone in D_2O and TMS in CD_3OD.

†Addition of a drop of DCl gave a good separation of the Ha signal, especially in S_2, from that of H_2O in D_2O.



Scheme 1. Proposed mechanism of the oxidative scission in the disulphide bond of GSSG and the formation of the modified glutathione with AITC.

molecular dehydration. These reaction processes are summarized in Scheme 1.

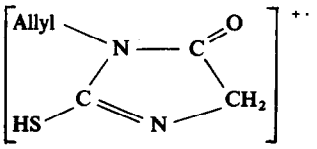
It is obvious from the above results that AITC also oxidatively cleaved the disulfide bond of GSSG under physiological conditions, as in the case of cystine [10, 11]. Consequently, if proteins interact with some alkyl isothiocyanate, for instance when *Brassica* seeds are crushed for oil extraction, a special cystine moiety in proteins will be slowly cleaved to dithiocarbamate and sulphenic acid moieties in the same protein molecule.

EXPERIMENTAL

Reaction mixtures. To 0.2 mmol of GSSG in 50 ml NaPi buffer (M/15, pH 6), 4 mmol AITC was added dropwise with vigorous stirring, and the suspensions were incubated at 40°, 60° and 80°. The reaction was followed by the colouring method [12, 13] using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman reagent) which easily exchanges its nitrothiophenyl group with SH or SHO compounds. The yellowish colour was measured at 412 nm.

Isolation of the reaction products between GSSG and AITC. The reaction mixtures for the isolation of products were prepared with 0.6 mmol (368 mg) GSSG and 12 mmol (1.19 g) AITC in 150 ml H_2O adjusted to pH 6 and incubated at 80° for 5 hr. The reaction was followed by TLC analyses on cellulose with a solvent system of *n*-BuOH–HOAc–H_2O (4:1:5) and colour development was performed with 0.2% ninhydrin in EtOH. After incubation, the reaction mixture was extracted with Et_2O to remove residual AITC and its decomposition products and the aq. soln was concd under red. pres. The dry matter (590 mg) was then dissolved in 5 ml H_2O and separated on a column (3 × 65 cm) packed with Sephadex G-25 Fine gel, eluted with H_2O, monitoring *A* at 280 nm and the refractive index (RI) of the eluate, which was collected in 5 ml aliquots. The chromatogram from the RI detector (Fig. 2) and TLC (Fig. 3) showed that F_{4A}, F_{4B}, F_5 and F_7 were the main fractions containing glutathione derivatives in this reaction mixture. They were concd to dryness under red. pres. below 40° to give 60.3, 58.8, 69.8 and 39.2 mg, respectively. Two products, S_{1-3} and S_2, were glutathione derivatives and were purified by repeated HPLC from the concentrates of F_{4A}, F_{4B} and F_5 using a column of Develosil 10 ODS (18 × 250 mm) with H_2O–MeOH (2:1) at 3 ml/min, to give pure amorphous S_{1-3} (8.5 mg) and S_2 (11.5 mg), which showed a single spot on TLC.

^{1}H NMR spectra were recorded at 100 MHz (Table 1). EIMS of S_{1-3}, *m/z* (rel. int.): 487 $[M]^+$ (22), 388 $[M-AITC]^+$ (10), 185 $[M-allyl-NHC(=S)S-CH_2CH=N-C(=O)-CH_2CH_2CH(NH_2)COOH]^{+\cdot}$ (100) and 156



(60).

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