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Interaction of Proteins with Allyl Isothiocyanate

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Interaction of allyl isothiocyanate (AITC) with proteins and the digestibility of a protein–AITC adduct were studied in detail as a model reaction of isothiocyanate and protein in crushed *Brassica* seeds. Insulin, bovine serum albumin (BSA), ovalbumin and lysozyme as model proteins reacted slowly with AITC to cleave the disulfide bond in their cystine moieties followed by polymer formation. Besides this action, AITC also attacked free amino groups of lysine and arginine residues in protein to form their thiourea-like derivatives, detected by amino acid analyses and determination of the free amino group. Proteolytic digestibility of the protein–AITC adduct was investigated on a BSA–AITC adduct using trypsin, chymotrypsin, and pepsin. Digestibilities with trypsin and chymotrypsin were decreased markedly, but not so significant with pepsin. The reasons were considered as follows: The former attacked the peptide moieties containing basic and aromatic amino acids, which were easily modified with AITC, but the latter have a wide action program compared with the former.

The seeds of the Cruciferae family contain many kinds of glucosinolates that are degraded to alkyl isothiocyanate (mustard oil), glucose, and sulfate by the action of β-thioglucosidase (myrosinase) localized in same plant cell (Ettlinger et al., 1961). This enzyme reaction usually forms thiocyanates and cyanides as minor products besides isothiocyanate.

The isothiocyanates have a strong pungent taste and easily react with some nucleophiles to give thiocarbamoyl derivatives and several kinds of degradation products (Kawakishi and Muramatsu, 1966; Kawakishi et al., 1967; Kawakishi and Namiki, 1969). Especially, isothiocyanates formed in crushed *Brassica* seeds during the processing of rapeseed oil may participate in some chemical reaction with amino acids and proteins in the seed under mild condition. Rapeseed (*Brassica nupus* or *Brassica campetris*) has been utilized as an oil seed in worldwide oil production, but its defatted meal is underestimated as an animal feed even though it contains a high-quality protein (Matsumoto, 1977). Among many reasons, there are two problems related to glucosinolates and their degradation products: one is the contents of goitrin (5-vinyloxazolidine-2-thione), which is well-known for its goitrogenic action (Kjaer et al., 1956; Greer, 1962); the other one seems to be related to the interaction between isothiocyanates and meal proteins. If such interaction arises in the processing of rapeseed oil, a part of seed protein will be transformed to the modified one with isothiocyanate, and it may change to an indigestible and/or some cytotoxic one.

From these situations, we have studied in detail the interaction of protein with isothiocyanate, and in this study allyl isothiocyanate (AITC), well-known for its formation from sinigrin and most widely distributed in the Cruciferae family, was used. The interaction of AITC with cystine (Kawakishi and Namiki, 1982; Kawakishi et al., 1983) and oxidized glutathione (Kawakishi and Kaneko, 1985) under physiological conditions has been reported, and the oxidative cleavage in their disulfide bonds with the electrophilic action of AITC was clearly demonstrated.

This paper is concerned with the interaction between some model proteins and AITC and digestibilities of protein–AITC adducts with some proteolytic enzymes.

EXPERIMENTAL SECTION

Materials. BSA (crystallized) and lysozyme (crystallized from egg white) were purchased from Seikagaku Kogyo Co., and insulin (from bovine pancreas) and ovalbumin (grade V) were purchased from Sigma Chemical Co. Trypsin (type IX), α-chymotrypsin (type I-S), and pepsin were obtained from Sigma Chemical Co. AITC was used a commercial guaranteed grade and redistilled immediately before using. DTNB, TNBS, and other reagents were obtained commercially.

Reaction Mixtures. Reaction mixtures were prepared in 25 mL of a $^1/_{30}$ M phosphate buffer, and the compositions of reaction mixtures were as follows: insulin (5 μmol)–AITC (0.5 mmol) at pH 7.5; BSA (1.25 μmol)–AITC

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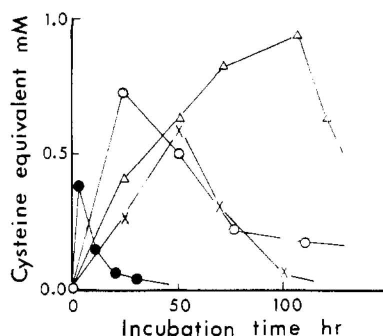


Figure 1. Cysteine equivalents in reactions of insulin (○), BSA (●), ovalbumin (△), and lysozyme (×) with AITC in time course determined by DTNB method.

(1.5 mmol) at pH 6.0; ovalbumin (1.25 μmol)–AITC (1.0 mmol) at pH 6.0; lysozyme (4 μmol)–AITC (1.5 mmol) at pH 6.0.

All mixtures were incubated at 40 °C with vigorous stirring. The reaction processes were followed by the DTNB method using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959; Janatova et al., 1968).

Gel Chromatography. The reaction mixtures were submitted to a Sephadex G-25 column (2.0 × 30 cm) to remove the salts and the decomposition products of AITC, and protein fractions were lyophilized.

These protein fractions were dissolved in a small volume of water and submitted to a gel column (2.0 × 110 cm) with 0.05 M ammonium hydrogen carbonate as the eluate. Gels used: insulin and lysozyme, Sephadex G-50 fine; BSA and ovalbumin, Sephadex G-100.

Amino Acid Analyses. A protein sample (2 mg/mL of 6 N HCl) in a sealed tube was hydrolyzed for 24 h at 100 °C. The hydrolysate was concentrated, adjusted to pH 2.2, and followed by amino acid analysis using a JEOL Model JLC-6AH amino acid analyzer.

Determination of the Free Amino Group in Protein. This was done by the TNBS method (Okuyama and Satake, 1960). Proteins prepared from the reaction mixtures by desalting and freeze-drying were dissolved in a 0.1% solution with 4% $NaHCO_3$. To this solution (0.5 mL) were added 2.0 mL of 0.1 M KBO_3, 0.5 mL of 0.01 M Na_2SO_3, and 0.5 mL of 0.1% TNBS (sodium 2,4,6-trinitrobenzenesulfonate). After the reaction mixtures were incubated at 37 °C for 2 h, intensities of the developed colors were determined with their optical density at 420 nm.

Proteolytic Digestibilities of BSA–AITC Adducts. Proteolytic hydrolyses of BSA–AITC adducts were carried out by the modified procedures of Kato et al. (1983), Kimball et al. (1981), and Groninger and Miller (1979). BSA–AITC adducts and BSA were treated at 38 °C for 1, 3, and 24 h with trypsin (pH 8.0), α-chymotrypsin (pH 8.0), and pepsin (pH 1.6). These enzymes were used at enzyme to substrate ratios of 1:20, 1:50, and 1:35, respectively. After the incubation, 1-mL aliquots were taken and the reaction was stopped by the addition of 1 mL of TCA and then allowed to stand overnight at 5 °C. The precipitates obtained by centrifugation at 900*g* were dissolved in 2 mL of 0.1 N NaOH–10% Na_2CO_3 to determine protein concentration in above reaction mixtures by the Lowry method.

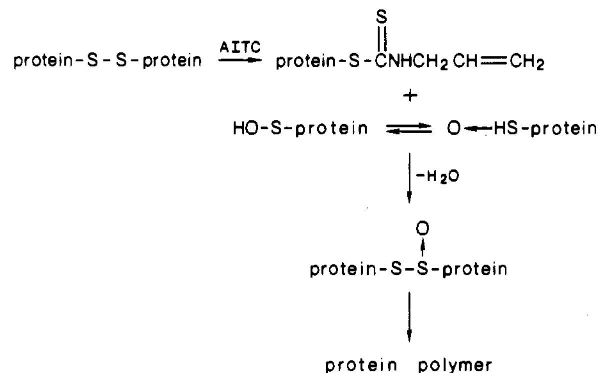
RESULTS AND DISCUSSION

Action of AITC on the Disulfide Bond of Proteins. Reaction mixtures prepared with proteins (insulin, BSA, ovalbumin, lysozyme) and AITC were incubated at 40 °C with vigorous stirring under a nitrogen atmosphere, and the cleavages of their disulfide bond were determined in time course by the DTNB method. As shown in Figure 1, the processes of cleavage were markedly different for each kind of protein. The reactions reached their maxima in 25 h for insulin, 100 h for ovalbumin, and 50 h for lysozyme, respectively, but BSA exhibited a high reactivity with AITC to reach its reaction maximum within 1 h.

While BSA contains 17 mol of cystine, the DTNB value is not so high level. It seemed that only a few moles of disulfide bond in the protein surface reacted with AITC. The large difference between the reactivities of BSA and ovalbumin may be attributed to the contents of cystine residue in both proteins. From UV spectra of the reaction mixtures, the increases of absorption near 280 nm were observed in all proteins, with decreases in 240 nm based on the absorption of AITC. Those changes in their absorptions suggested to be due to dithiocarbamate derivatives induced from disulfide bonds of proteins, similar to the case of oxidized glutathione (Kawakishi and Kaneko, 1985).

However, the decomposition of AITC in the reaction mixture with BSA was much faster than in that of other proteins. These results suggested that AITC reacted not only with cystine but with other amino acid residues in protein. To examine the changes in protein molecules, four kinds of reaction mixtures dialyzed to remove all salts and other decomposition products related to AITC were concentrated by a freeze-drying method and submitted to gel columns.

The changes in gel chromatography are shown in Figure 2. No change in chromatographic pattern of lysozyme was observed, but there were some changes in the other three proteins; a polymerization of protein molecule was observed as a common phenomenon in three proteins, which seemed to be derived through protein sulfenic acid formed by the scission of the disulfide bond as follows:



However, a minor part of insulin was also depolymerized to a lower molecular peptide that seemed to be insulin A or B chain derivative.

Changes in Amino Acid Residues of Proteins by the Action of AITC. In its interactions with cystine, cystine-containing peptides, and proteins, AITC has been clearly demonstrated to induce oxidized cleavage of all or part of their disulfide bonds.

Moreover, there was some possibility to modify the other amino acid residues by this action of AITC. And so the changes in amino acid composition of the reaction products of proteins and AITC were examined.

After dialysis of the reaction mixtures overnight, they were lyophilized and hydrolyzed by usual methods and amino acid analyses of these hydrolysates were carried out. As shown in Table I cystine and lysine considerably decreased in all the model proteins; decreases of lysine were about 15–20% in all proteins and those of cystine were about 10% in BSA and lysozyme, with a 50% decrease

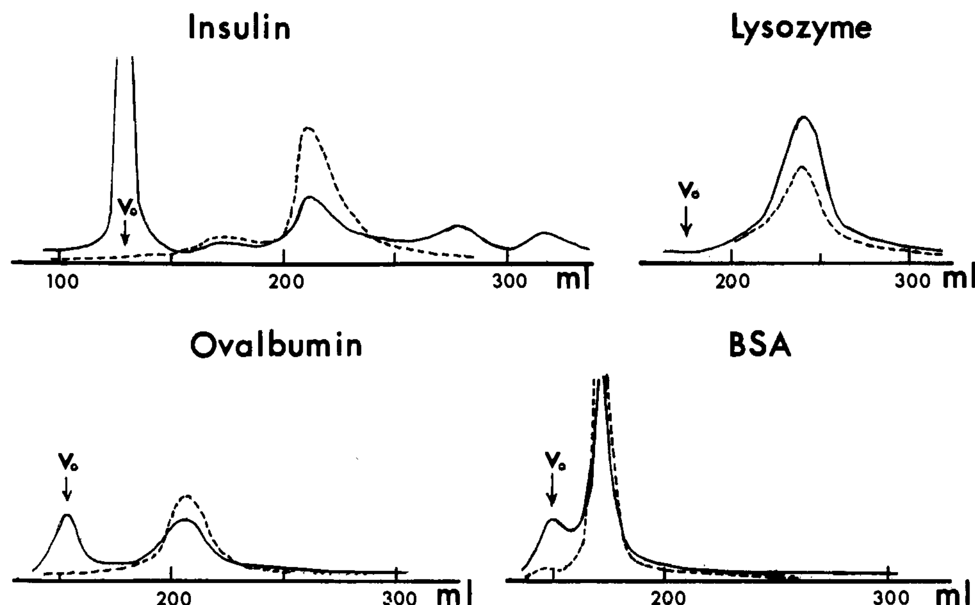


Figure 2. Gel filtration patterns for protein–AITC adducts on Sephadex G-50 (insulin and lysozyme) and G-100 (BSA and ovalbumin) expressed as absorbance at 280 nm: —, protein–AITC adduct; - - -, native protein.

Table I. Amino Acid Analyses in Protein–AITC Adducts

	insulin		BSA		ovalbumin		lysozyme	
amino acid	native	ins–AITC[a]	native	BSA–AITC[b]	native	ov–AITC[a]	native	lyz–AITC[a]
Asp	6.4	6.6	10.8	10.9	9.5	10.0	17.7	18.0
Thr	2.1	2.5	6.9	7.2	4.5	4.8	6.0	6.0
Ser	5.6	5.9	5.7	5.8	8.9	9.0	7.9	7.8
Glu	14.8	15.4	12.8	13.1	12.6	12.8	4.4	4.5
Pro	4.9	5.3	5.5	5.3	6.0	5.2	2.1	2.3
Gly	8.6	7.8	2.8	2.7	5.7	5.9	10.3	10.5
Ala	6.6	7.2	8.0	8.2	9.1	9.1	10.3	10.4
Cys	5.7	2.8	2.5	2.2	0.9	0.6	2.3	2.1
Val	8.4	8.6	7.3	7.8	7.4	7.6	4.7	4.9
Met			1.0	1.1	3.8	3.9	1.9	2.0
Ile	1.0	1.0	2.4	2.5	5.0	5.0	4.6	4.7
Leu	12.8	13.4	10.4	10.8	8.0	8.1	6.8	6.9
Tyr	8.3	8.8	3.1	2.9	2.6	2.6	2.9	2.8
Phe	6.2	5.4	4.6	4.8	5.0	4.9	3.0	3.1
His	4.3	4.8	2.9	2.9	1.9	1.9	1.0	1.0
Lys	2.3	2.1	8.9	7.7	5.5	4.7	5.0	4.1
Arg	2.1	2.5	4.2	4.3	3.7	3.9	9.2	9.0

[a] Protein–AITC adducts were prepared from the reaction matters for 5 days. [b] BSA–AITC adducts was prepared from the reaction matter for 1.5 days. All amino acid values were expressed by the molar ratio %: molar concn ea amino acid/molar concn total amino acids × 100.

shown in insulin. However, the contents of cystine in ovalbumin and lysine in insulin are only one molecule, respectively, and therefore their percent decreases in this reaction were not so significant.

Generally, isothiocyanate forms an adduct with a free amino group, and the lysine residue located in the outer part of the globular protein molecule would react rapidly on its ϵ-amino group with AITC to give an ϵ-allylthiourea derivative.

Such a reaction would also occur with an arginine residue, but its decrease in amino acid analysis was not observed, because arginine was regenerated from the arginine–AITC adduct by acid hydrolysis. The adduct formation of the ϵ-amino group in the lysine residue with AITC must be corresponding to the decrease of free amino groups in the whole protein molecule. To prove this view, free amino groups of protein–AITC adducts were determined by the TNBS method.

Results (Table II) in BSA- and ovalbumin–AITC adducts showed the decreases of their free amino groups with reaction time, and this tendency corresponded well with the amounts of residual lysine on amino acid analyses.

Table II. Changes of Free Amino Group during BSA- and Ovalbumin–AITC Reaction

protein	reacn time, days	resid free amino gp	resid Lys[a]
BSA	native BSA	100	100
	1.5	87	89
	5	68	87
ovalbumin	native ov	100	100
	1	95	–
	2	83	–
	3	75	85

[a] The ratios were calculated from the data in Table I.

Moreover, the difference between free amino group and residual lysine seemed to be suggested by the adduct formation of arginine and AITC.

Digestibilities of the BSA–AITC Adduct with Proteolytic Enzymes. In processing of *Brassica* seeds for oil extraction, the interaction between seed protein and isothiocyanate (ITC) described above may induce the formation of some protein–ITC adducts that would affect their proteolytic digestibility. These posed an important

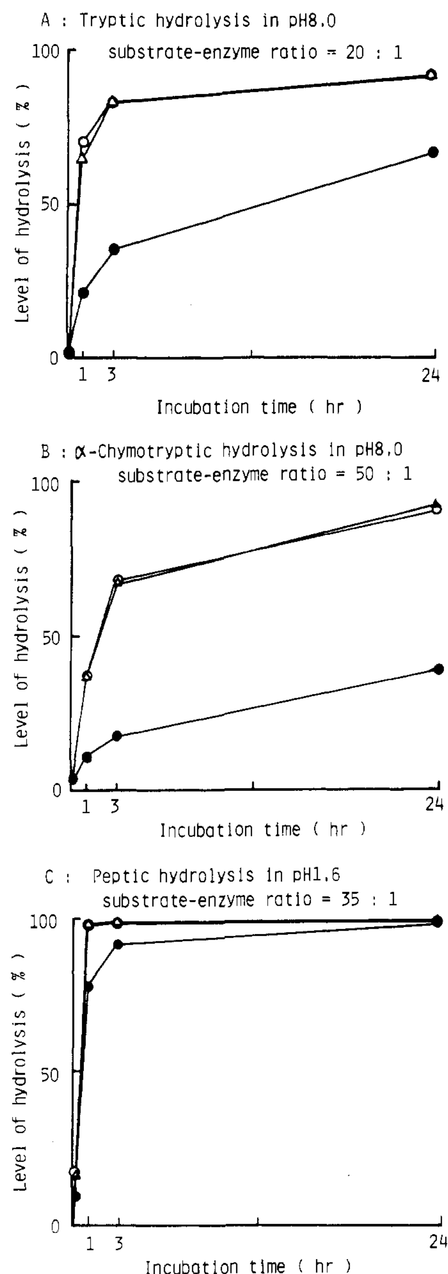


Figure 3. Proteolytic digestibilities of BSA–AITC adducts: incubated BSA in buffer solution for 1.5 days (○); BSA–AITC adduct prepared from reaction mixture incubated for 1.5 days (●); native BSA (△). Level of hydrolysis (%) = (total protein − protein precipitated with TCA)/total protein × 100. Protein concentration was determined by the Lowry method.

problem on utilization of *Brassica* seed meal as animal feed.

Hence, the digestibility of BSA–AITC adducts was examined by using typical proteolytic enzymes: trypsin, α-chymotrypsin, pepsin. The results are shown in Figure 3A–C. From these experiments, the decrease in digestibility of the BSA–AITC adduct was observed in these three enzymes.

Especially, the digestion with trypsin and chymotrypsin was remarkably reduced by comparison with that of pepsin which exhibited a little decrease. While action specificity of pepsin was lower, those of trypsin and α-chymotrypsin were very high. It is well-known that trypsin hydrolyzes peptide bonds at neighboring positions of lysine and arginine resides and α-chymotrypsin at the position of phenylalanine, tyrosine, and tryptophan.

Since isothiocyanates may form their adducts with other basic amino acid and a kind of aromatic amino acids besides cysteine, cystine, and lysine, the digestibility of the BSA–AITC adduct with both proteolytic enzymes seemed to be down significantly as described here. Recently, Kishore Kumar Murthy and Narasinga Rao (1986) reported that the adduct of mustard 12S protein with AITC was poorly hydrolyzed by the tryptic action, but its hydrolysis was not affected by α-chymotrypsin and papain. There is good agreement on the tryptic digestion but a large difference on α-chymotrypsin between their and our data. In relation to these results, the inhibiting effects of these enzymes with the BSA–AITC adduct and free AITC were also examined. Trypsin and chymotrypsin were inhibited little by addition of the BSA–AITC adduct, but free AITC exhibited some inhibiting effect on trypsin.

However, since AITC is unstable in aqueous solution and decomposes easily, the possibility of an inhibitory effect of AITC on trypsin seemed to be very low practically.

Registry No. AITC, 57-06-7; ITC, 71048-69-6; L-Cys, 56-89-3; L-Lys, 56-87-1; L-Arg, 74-79-3; trypsin, 9002-07-7; chymotrypsin, 9004-07-3; pepsin, 9001-75-6.

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