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Research Communications

Benzyl-isothiocyanate (BITC) decreases quality of egg white proteins in rats

Manuel Hernández-Triana, Jürgen Kroll,* Jürgen Proll,‡ Jutta Noack,‡ and Klaus J. Petzke‡

*Institute of Nutrition and Food Hygiene, Infanta 1158, Havana, Cuba; *Department of Nutritional Science, University of Potsdam, Bergholz-Rehbrücke, Germany; ‡Department of Biochemistry and Physiology of Nutrition, German Institute of Human Nutrition in Potsdam-Rehbrücke, Bergholz-Rehbrücke, Germany*

Isothiocyanates (ITC) are breakdown products of glucosinolates, which occur in some cruciferous vegetables. They have a high reactivity with the amino and sulfhydryl groups of proteins to produce thiourea derivatives and dithiocarbamate esters. Egg white proteins were treated with benzyl-ITC (BITC) and tested by protein quality rat bioassay. BITC reduced the lysine content of the treated egg white protein as well as lysine availability and also affected significantly the bioutilization of nitrogen and the deposition of energy. Supplementation of the damaged egg white protein with lysine alone enhanced the nitrogen retention almost to about 90% relative to the untreated proteins. (J. Nutr. Biochem. 7:322–326, 1996.)

Keywords: glucosinolates; isothiocyanates; dietary protein; protein quality; lysine; bioutilization

Introduction

Glucosinolates and their breakdown products (some of the most important hydrolysis products being isothiocyanates ITC) are natural toxicants. They occur in some vegetable foods (cruciferous plants, including cole crops [*Brassica oleracea*] and condiments [*B. nigra, B. hirta, B. alba*]) as well as in animal feedstuffs as rapeseed (*B. campestris* and *B. napus*) and their milled products.[1] Daily consumption of glucosinolates in humans has been estimated to be up to 300 mg per day.[2] Investigations regarding possible related health risks to have shown contradictory results as documented by Lange et al.[3]

The high reactivity of ITC is a well-known process that is largely due to their strong electrophilic nature, resulting in reactions with nucleophiles at physiological pH.[3–5] Kawakishi et al. reported that allyl-ITC react with proteins, cleaving the disulphide bond to their cysteine moieties.[6–8] According to Murthy et al., ITC react with the epsilon amino group of lysine and the phenolic group of tyrosine residues of the investigated mustard 12S protein, resulting in a notable increase in the electrophoretic mobility.[9] Comparable results have also been published by Björkman who in turn also reported that the reaction of ITC with basic low molecular weight proteins leads to changes in UV-spectrum and electrophoretic mobilities.[10]

In addition to the splitting of disulphide bonds as previously mentioned,[11–13] isothiocyanates also react with cysteine (–SH groups). The reaction with the amino groups of proteins leads to the formation of thiourea derivatives. Such reactions are characterized by a decrease in the content of free amino groups and available lysine. The reaction with the sulfhydryl side-chains of proteins leads to formation of dithiocarbamate esters and to a decrease in cysteine content.[13,14]

This paper describes the effect of these chemical amino acid modifications (derivatization of benzyl-ITC, BITC) on the nutritional quality of egg white protein in rats.

Address reprint requests to Dr. K.J. Petzke, at the Deutsches Institut für Ernährungsforschung, Arthur-Scheunert-Allee 114, D-14558 Bergholz-Rehbrücke, Germany.
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Methods and materials

Derivatization of egg white proteins

Freshly cracked hen eggs were used as starting material. Two parts of distilled water were added to one part of egg white (w/w, egg white = one complete egg white from a single egg, weighing about 40 g). The initial pH-value of egg white protein solution of about 9.0 was adjusted to 9.5 using 0.5 M NaOH (pH-Meter 691, Deutsche Metrohm GmbH, Filderstadt, Germany). Under continuous stirring at room temperature benzyl-ITC (200 mg, (Fluka Chemie AG, Buchs, Switzerland) dissolved in 80 ml ethanol/water (1:1, v/v) was added to a final concentration of 50 mg BITC per g protein. After 3 hr, the sample was dialysed (Thomapor-standard tube, Reichelt Chemietechnik GmbH & Co., Heidelberg, Germany) for 20 hr against flowing tap water. Finally the samples were lyophilized (ALPHA 1–4, Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The non-derivatized egg white (control) was prepared under the same conditions but without addition of BITC.

Chemical determinations

Nitrogen analysis. Nitrogen analysis of diets, urine, and fecal samples were performed by semi-micro Kjeldahl analysis using a digestion apparatus (Kjeldatherm System KT 40, Gerhardt Laboratory Instruments, Bonn, Germany) and a titration system (T110-TR160-TA10-TM120, Schott-Geräte GmbH, Hofheim, Germany). The crude protein content was calculated by using the factor 6.25.

Amino acid analysis. The amino acid composition of egg white protein and its BITC-derivatives were determined as previously described[14] by ion-exchange-chromatography with post-column ninhydrin detection according to a modified procedure of Moore et al.[15] using the amino acid analyzer LC 3000 (Biotronik, Maintal, Germany). The different protein samples were hydrolysed with 6 M HCl (10 mg protein per 150 ml HCl, 24 h) by reflux, evaporated to dryness (40°C), washed twice with distilled water for removal of residual HCl and dried once more. To prevent destruction of sulphur-containing amino acids, cysteine and methionine, during acid hydrolysis, conversion to acid-stable derivatives (cysteinic acid and methioinesulphone, respectively) was accomplished by performic acid oxidation.[16] The oxidized samples were then hydrolysed in 6 M HCl as described above.

Determination of available lysine. Available lysine (fluorodinitrobenzene-reactive lysine) was estimated by the method of Carpenter[17] modified by Booth.[18]

Protein quality assay

The protein quality assay was performed according to the UNU/WHO guidelines.[19] Thirty Wistar male rats weighing 70 to 73 g were divided into five groups, so that the mean group weights differed by no more than ±0.5 g. They were housed in individual metabolic cages at 24.6 ± 1.3°C with a dark-light cycle of 12 hr. Water was provided ad libitum. The test diets were prepared at 8% protein level (*Table 1*). Group I was maintained on a casein diet, fortified with 1% DL-methionine (relative to caseine), which was used as an internal standard. Untreated and treated egg proteins were also included at the 8% protein level in the diets of groups II and III. Group IV was supplemented with lysine up to the level calculated for group II. Metabolic and endogenous nitrogen losses per gram dry matter intake were determined in a group fed a diet containing 4% freeze-dried chloroform-methanol-extracted whole egg protein and then adjusted for each animal. After a preliminary feeding period of 4 days, subsequently, diets were provided during a nitrogen balance period of 5 days. Body weight was recorded at the end of the preliminary and balance period, after access to feed and water was refused 3 hrs before weighing procedures.

The food intake was monitored daily and any remaining feed were taken into account in the calculations. Biological value (BV), true digestibility (D), and net protein utilization (NPU) were expressed on a percentage scale and calculated according to the UNU/WHU guidelines[19] using the following equations:

Table 1 Composition of experimental diets (g/kg diet)

Component	(I) Casein + Met	(0) Whole egg 4%	(II) Egg white	(III) Egg white + BITC	(IV) Egg white + BITC
Casein	85.7	—	—	—	—
DL-Methionine	0.8	—	—	—	—
Whole egg	—	48.2	—	—	—
Egg white	—	—	91.4	—	—
Egg white + BITC[1]	—	—	—	91.7	91.7
L-Lysine-HCl	—	—	—	—	3.2
Sunflower seed oil	80.0	78.1	80.0	80.0	80.0
Mineral mixture[2]	50.0	50.0	50.0	50.0	50.0
Vitamin mixture[3]	20.0	20.0	20.0	20.0	20.0
Cellulose	50.0	50.0	50.0	50.0	50.0
Wheat starch	714.0	753.7	708.6	708.3	705.1
Dry matter	910.0	915.0	913.0	915.0	898.0
Crude protein (N × 6.25)	87.3	46.9	85.8	81.0	82.0
Gross energy (kJoule/g)	18.56	18.23	18.48	18.47	18.81

[1]Egg white treated with benzyl-isothiocyanate (BITC, for treatment conditions see Methods and materials).
[2]Mineral mixture per 100 g diet: Ca, 950 mg; P, 750 mg; Mg, 75 mg; Na, 250 mg; K, 700 mg; S, 280 mg; Cl, 360 mg; Fe, 18 mg; Mn, 10 mg; Zn, 3 mg; Cu, 1.2 mg; J, 45 µg; F, 400 µg; Se, 30 µg; Co, 13 µg.
[3]Vitamin content in 100 g diet: A, 0.5 mg; D_3, 1.3 µg; K_3, 1 mg; B_1, 2 mg; B_2, 2 mg; B_6, 1.5 mg; B_{12}, 4 µg; niacine, 5 mg; pantothenoic acid, 5 mg; folic acid, 1 mg; biotin, 20 µg choline chloride, 100 mg; p-aminobenzoic acid, 10 mg; inositol, 11 mg.

Table 2 Amino acid composition of egg white protein before and after treatment with benzyl-isothiocyanate (BITC) (expressed as mg amino acid/g protein)

Amino acid	Egg white	Egg white + BITC
Lysine	78.7	46.7
Histidine	26.0	23.9
Arginine	63.5	57.8
Aspartic acid	107.0	97.2
Threonine	56.0	45.7
Serine	66.6	63.1
Glutamic acid	129.5	119.9
Proline	34.0	34.4
Glycine	38.6	35.8
Alanine	63.5	57.7
Cysteine	17.1	17.6
Valine	63.0	57.2
Methionine	32.6	26.7
Isoleucine	54.9	52.0
Leucine	91.8	84.9
Tyrosine	50.1	46.0
Phenylalanine	62.4	58.6
Tryptophan	nd	nd
Total	1035.3	927.2
Available Lysine	63.3	38.9

nd, not determined.

$$BV = [I - (F - F_K) - (U - U_K)]/[I - (F - F_K)],$$

$$D = I - (F - F_K)/I,$$

$$NPU = BV \times D,$$

where I is the N intake, F is the fecal N, F_K is the metabolic N (endogenous fecal), U is the urinary N, and U_K is the endogenous urinary N. The protein digestibility-corrected amino acid score (PDCAAS) was calculated according to the recommendations of the Joint FAO/WHO Expert Consultation[27] using the following equation:

PDCAAS = (Amino acid content in food protein) × (true digestibility)/(Amino acid content in 1985 FAO/WHO/UNU pattern for ages 2 to 5 years).

Statistical analysis

Differences between means of protein quality parameters were analyzed by the Kruskal and Wallis test and then compared by pairs with Wilcoxon, and Mann-Whitney tests[20] (SPSS for Windows™, SPSS GmbH Software, Munich, Germany). The significance level was 5%.

Results and discussion

Treatment with BITC reduced the total and available lysine content of egg white protein to 60% relative to the initial value, and apparently did not have any influence on cysteine concentration. However, methionine concentration in BITC-treated egg white was only 80% of the control (*Table 2*). Oxidation previous by BITC-derivatized thiol groups with performic acid is quantitatively similar to the oxidation of cysteine to cysteinic acid.[14] Hence, the measured content of cysteine remains essentially the same in the untreated control and the treated sample, although in the latter a considerable part of it had been previously derivatized by BITC.[12] However, that we were unable to detect a difference in cystein content does not mean that there is no biological effect due to a reduced availability of sulphur-containing amino acids generated by BITC-treatment.

Derivatizing egg white protein with BITC significantly reduced protein (*Table 3*) and dry matter intake (*Table 4*). The mean final body weight was about 13 g lower compared to groups I and II. The weight gain per gram protein consumed in the BITC-group was only 48% of the value in the group fed the non-modified protein. A supplementation of BITC-treated egg white protein with lysine enhanced the weight gain by up to 90% relative to the value found by the group receiving the non-derivatized protein, and the differences between those groups (II and IV) were not statistically significant. Similar results have been reported from feeding experiments with lysine-deficient or heat-damaged proteins.[21–25]

The derivatization of egg white with BITC had a direct effect on dietary and nitrogen intake of the animals receiving that diet during the nitrogen balance period (*Table 4*). In spite of the lower nitrogen intake of those animals, the relative nitrogen excretion via feces and urine was 1.4 and 3 times higher respectively, versus the group fed non-derivatized protein. A significant reduction of urinary nitrogen excretion in the animals receiving the lysine supplement (Group IV) was observed but no effect on the fecal nitrogen excretion was seen. Urinary nitrogen excretion in Group IV was similar to group II fed the non-derivatized protein diet. The true protein digestibility of the treated egg white protein revealed only a 4% reduction in comparison to the natural egg white. However only 77% of this true digested derivatized protein was retained in the organism. The correction, with reference to increasing lysine content, enhanced the BV and the NPU of the derivatized protein. Finally, derivatizing egg white protein with BITC reduced the protein digestibility-corrected lysine score to 77% (*Table 4*).

Table 3 Protein intake and growth response of rats fed for 9 days with egg white proteins and its benzyl-isothiocyanate (BITC) derivative[1]

Group/diet[2]	Protein intake (g)	Weight gain (g)	Weight gain/ g protein consumed
Casein + Met (I)	7.37 ± 0.03^{AB}	23.1 ± 1.2^{A}	3.13 ± 0.16^{A}
Egg white (II)	7.38 ± 0.03^{A}	23.1 ± 0.9^{A}	3.13 ± 0.13^{A}
Egg white + BITC (III)	6.14 ± 0.24^{C}	9.4 ± 1.5^{B}	1.51 ± 0.21^{B}
Egg white + BITC + Lys (IV)	6.95 ± 0.19^{B}	19.6 ± 1.9^{A}	2.81 ± 0.25^{A}

[1]Values are means ± SEM ($n = 6$). Means in each column having different superscript letters are significantly different ($P < 0.05$).
[2]For composition of the diets see Methods and materials.

Table 4 Protein quality evaluation of egg white protein and its benzyl-isothiocyanate (BITC) derivative[1]

Group Diet[2]	(I) Casein + Met	(II) Egg white protein	(III) Egg white protein + BITC	(IV) Egg white protein + BITC + Lys
Dry matter intake (g)	87.5 ± 0.4^{A}	87.6 ± 0.4^{A}	74.9 ± 2.9^{B}	81.3 ± 2.3^{B}
Nitrogen intake (mg)	658 ± 2.5^{A}	666 ± 1.4^{B}	607 ± 19.9^{C}	654 ± 9.9^{AB}
Fecal N (mg/g N)	116 ± 3.6^{A}	112 ± 6.7^{A}	156 ± 3.3^{B}	139 ± 4.4^{B}
Urinary N (mg/g N)	127 ± 6.8^{A}	129 ± 8.4^{A}	351 ± 21.5^{B}	141 ± 5.2^{A}
True digestibility (%)	99 ± 0.3^{A}	99 ± 0.4^{A}	96 ± 0.3^{B}	97 ± 0.4^{C}
Biological value BV (%)[3]	100 ± 0.91^{A}	99.8 ± 0.81^{A}	77.4 ± 2.02^{B}	98.0 ± 0.82^{A}
NPU (%)[3,4]	100 ± 0.91^{A}	100.3 ± 1.33^{A}	72.8 ± 1.40^{B}	96.1 ± 0.69^{C}
PDCAAS (%)[5]	100	100	77	100

[1]Values are means ± SEM ($n = 6$). Means in each line having different superscript letters are significantly different ($P < 0.05$).
[2]For composition of the diets see Methods and materials.
[3]BV and NPU values are relative to group I (Casein + Met = 100).
[4]Metabolic N (endogenous fecal) = 1.492 mg N/g dry matter intake, Endogenous urinary N = 2.027 mg N/g dry matter intake.
[5]Protein Digestibility Corrected Amino Acid Score (PDCAAS) based on lysine content, suggested scoring pattern for lysine requirement [FAO/WHO/UNU, 1985][27] and true digestibility.

One could argue that a concentration of 50 mg BITC/g protein is about 10 times the estimated dietary glucosinolate intake in humans.[2] However, to our knowledge there is no report showing the in vivo effects of a decreased protein quality due to isothiocyanates in a controlled experiment. Therefore, the purpose of this study was to investigate the potential of BITC-modified proteins to deteriorate bioutilization of dietary protein in principle. We used BITC-modified egg white protein as a model substance. Therefore, it was necessary to ensure derivatization conditions (pH, ethanol addition) allowing reaction of BITC with the epsilon amino- and SH-groups of the protein. This has been accomplished by adjusting to a pH of 9.5 because there is a known reduction of reaction rate at pH of 7.0 to 7.5. Besides the natural pH of egg white is in the range of 9.0 to 9.5. Furthermore, ethanol has been used to provide solubilization of BITC and its complete dispersion in the protein solution. After the derivatization procedure has been completed, ethanol was removed by dialysis.

In conclusion, these results show a pronounced interference of BITC with the bioutilization of dietary lysine and probably methionine. Lysine is one of the most limiting amino acids in human diets and is consistently at a much lower concentration in major plant food protein groups (except some legumes such as soybeans) than in animal foods.[26]

Although this model experiment required non-physiological derivatization conditions, we have demonstrated a deteriorated protein bioavailability in vivo caused by BITC-modified proteins. However, it remains to be elucidated in humans, whether a high intake of cruciferous vegetables along with a low intake of the indispensable amino acid lysine could lead to conditions where the metabolically available lysine is compromised to meet physiological requirement.

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