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Determination of isothiocyanate-protein conjugates in milk and curd after adding garden cress (*Lepidium sativum* L.)

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ABSTRACT

Isothiocyanates (ITC) play an important role in health promotion and cancer prevention due to their anti-bacterial, anti-inflammatory, and anti-cancerogenic properties. However, ITC are highly reactive so that a reaction with further food components is very likely. For example, a reaction of ITC with nucleophilic amino acid side chains of proteins such as cysteine and lysine can occur, reducing the bioavailability of indispensable amino acids and protein functions may be altered. Therefore, it is of great interest to investigate the fate of ITC in the food matrix. Accordingly, the aim of the present study was to investigate the interaction of milk proteins and the ITC benzyl isothiocyanate (BITC) and allyl isothiocyanate (AITC) forming dithiocarbamates and thioureas in milk and curd. After incubating milk and curd with pure ITC or ITC-containing garden cress (*Lepidium sativum* L.), proteins were isolated, digested, and analyzed via LC-ESI-MS/MS as amino acid derivatives ("conjugates"). Protein conjugates of AITC and BITC were detected in all samples investigated. Further, the acidic pH value in curd favored the formation of dithiocarbamates over the formation of thioureas. Slightly acidic or neutral conditions like in fresh milk favored the formation of thioureas. The investigations also indicated that AITC shows a higher reactivity and dithiocarbamates are formed preferably, whereas incubation with BITC lead to less protein conjugates and the ratio of thioureas and dithiocarbamates was more balanced. In addition, amino acid modifications were often analyzed with indirect methods like measuring the decline of the amino acid residues. In this study, the modified amino acids were analyzed directly leading to more reliable results concerning the amount of modification.

1. Introduction

Glucosinolates are secondary plant compounds present in brassicaceous vegetables. Several epidemiological studies demonstrated a correlation between consumption of vegetables containing high amounts of glucosinolates and a reduced risk of cancer (Dinkova-Kostova & Kostov, 2012; Higdon, Delage, Williams, & Dashwood, 2007). The chemopreventive properties can be assigned to glucosinolates and specifically to their breakdown products. The latter emerge from the glucosinolates after plant tissue gets damaged by chewing, cutting or during food processing (Fenwick & Heaney, 1983). The plant-endogenous enzyme myrosinase which is physically separated from the glucosinolates in the intact plant tissue cleaves them into their breakdown products (Bourderioux et al., 2005). Depending on the conditions during cleavage, e.g., pH-value and the presence or absence of modifying proteins, different breakdown products including isothiocyanates (ITC), thiocyanates, nitriles, and epithionitriles are formed (Bones & Rossiter, 1996). Among the breakdown products, ITC seem to play the most important role in the prevention of cancer (Wu, Kassie, & Mersch-Sundermann, 2005; Zhang & Talalay, 1994). With regard to metabolism, ITC react with glutathione (GSH) in the human body after consumption of ITC-containing food, forming ITC-GSH conjugates, which are further metabolized via the mercapturic acid pathway (Shapiro, Fahey, Wade, Stephenson, & Talalay, 2001). Thereby, ITC act as strong electrophiles reacting with the nucleophilic thiol group of GSH. Due to the chemical resemblance, namely the nucleophilic properties of thiol and amino groups and the high electrophilicity of ITC, reactions of ITC with these groups occur as well depending on the further reaction

Abbreviations: AITC, allyl isothiocyanate; AITC-Cys, allyl isothiocyanate-cysteine; AITC-Lys, allyl isothiocyanate-lysine; BITC, benzyl isothiocyanate; BITC-Cys, benzyl isothiocyanate-cysteine; BITC-Lys, benzyl isothiocyanate-lysine; EtOH, ethanol; FA, formic acid; GSH, glutathione; HPLC-UV, high performance liquid chromatography-ultraviolet; ITC, isothiocyanate; LC-ESI-MS/MS, liquid chromatography tandem mass spectrometry; MeOH, methanol; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PITC, phenyl isothiocyanate; SPE, solid phase extraction; TFA, trifluoroacetic acid

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conditions (Hanschen et al., 2012). Proteins containing amino acids with thiol groups or free amino groups such as cysteine and lysine undergo reactions with ITC forming dithiocarbamates and thioureas, respectively (Cejpek, Valusek, & Velusek, 2000; Kroll, Rawel, Krock, Proll, & Schnaak, 1994; Rawel, Kroll, & Hohl, 2001). Studies indicated that the antiproliferative effect of ITC can be ascribed to interactions of ITC with macromolecules, especially proteins, but also DNA, in cells leading to cell cycle arrest and apoptosis (Mi & Chung, 2008; Mi, Di Pasqua, & Chung, 2011). While dithiocarbamates are not stable under basic and neutral conditions, conjugates with amino groups (e.g., the α- and ε-amino groups of lysine) form stable reaction products (Kumar & Sabbioni, 2010; Platz et al., 2013). The reactions of ITC with food proteins have been investigated in some studies. It has been shown that BITC and phenyl isothiocyanate (PITC) interact with egg white protein, myoglobin, and legumin leading to changes of the physicochemical properties of the derivatized proteins which will affect their biochemical function (Kroll, Noack, Rawel, Kroeck, & Proll, 1994; Rawel & Kroll, 1995; Rawel, Kroll, & Schroder, 1998a). Furthermore, the covalent binding of AITC to *β*-lactoglobulin showed that AITC is capable of cleaving disulfide bonds in the protein modifying the protein structure (Keppler et al., 2014). Although the reaction of ITC with model proteins has been investigated in some studies, there is only limited data about the formation of protein conjugates in different food matrices/recipes. In addition, amino acid modifications were often analyzed with indirect methods like measuring the decline of the amino acids in question, e.g. after a derivatization or fluorescence measurement (Rawel & Kroll, 1995).

From a physiological point of view, interactions of ITC with food proteins could lead to a reduced availability of either these health-promoting plant compounds or indispensable amino acids in food. Especially the transformation from reversible conjugates of ITC and thiol groups into irreversible conjugates of ITC and amino groups could lead to a reduced amount of available ITC in food and therefore to altered physiological properties. Consequently, the aim of this study was to investigate the formation of protein conjugates (Fig. 1) in milk and curd after treatment with AITC and BITC as well as in milk and curd containing garden cress (*Lepidium sativum* L.), as example for typical dishes containing glucosinolate-containing spices or herbs and protein-rich dairy products. Curd with herbs is a typical German dish ("*Kräuterquark*") for several occasions such as breakfast or dinner. Milk proteins were isolated from the reaction mixes/foods and the formation of AITC-cysteine (AITC-Cys), AITC-lysine (AITC-Lys), BITC-cysteine (BITC-Cys), and BITC-lysine (BITC-Lys) was investigated after digestion of the proteins into single amino acid derivatives ("conjugates").

2. Material and methods

2.1. Chemicals and materials

AITC (95%), BITC (98%), Boc-L-lysine (99%), methanol (ultra LC-MS grade), and pronase E (from *Streptomyces griseus*) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); L-cysteine (99%), 1,4-dioxane, ethanol (EtOH; HPLC grade), ethyl acetate (99.5%), disodium hydrogen phosphate (98%), sodium hydroxide (100%), boric acid solution (4%), hydrochloric acid (0.1 M), and dialysis membranes (regenerated cellulose, molecular weight cutoff (MWCO) 3.5 kDa) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany); formic acid (FA; 98%) and sodium chloride (99.5%) were purchased from VWR International GmbH (Darmstadt, Germany); trifluoroacetic acid (TFA; 99.5%) was obtained from AppliChem GmbH (Darmstadt, Germany), potassium chloride (99.5%), sodium sulfate (99%), selenium reaction mixture, and potassium dihydrogen phosphate (99.5%) were purchased from Merck KGaA (Darmstadt, Germany), dimethyl sulfoxide-d_6 (DMSO-d_6) was obtained from Eurisotop GmbH (Saarbrücken, Germany), sulfuric acid (95%) was purchased from Grüssing GmbH (Filsum, Germany). C_{18}ec solid phase extraction cartridges (3 mL, 200 mg) were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Water was of Milli-Q quality. Curd and milk were obtained from a local supermarket.

2.2. Plant material

Lepidium sativum L. (garden cress) seeds were obtained from Carl Pabst Samen und Saaten GmbH (Großbeeren, Germany). Plants were grown for 10 days at 20 °C on cotton wool and with natural light. After harvest of the sprouts, the plant material was homogenized using a ball mill (M400, Retsch Technology GmbH, Haan, Germany) and directly applied to analyses. The content of benzyl glucosinolate was determined from freeze-dried material using the method described by Wiesner et al. (Wiesner, Zrenner, Krumbein, Glatt, & Schreiner, 2013) and resulted in an amount of 183 ± 7 μmol/g plant material on a dry weight basis. The dry weight was determined as 8.8% leading to a benzyl glucosinolate content of 16 ± 0.6 μmol/g plant material on a fresh weight basis.

2.3. Determination of protein content and amounts of cysteine and lysine in milk and curd

The protein content of milk and curd was determined using the Kjeldahl method. Therefore, 2 g selenium reagent mixture and 20 mL sulfuric acid (95%) were added to 1 g of curd and 3 g of milk and heated until a clear solution was obtained. Distillation was performed using a Büchi distillation unit (Büchi Labortechnik GmbH, Essen, Germany). After adding 75 mL of water and 75 mL aqueous sodium hydroxide (33%), the samples were steam distilled for 5 min at 75% water vapor pressure into 50 mL aqueous boric acid (4%). The distillate was titrated with 0.1 M hydrochloric acid until pH 4.65 was reached. Protein content was calculated from nitrogen content using the protein factor 6.38.

From the analyzed protein content the amounts of cysteine and lysine were calculated based on the amino acid sequence of the proteins contained in milk and curd (obtained from UniProtKB). Cysteine represents 0.3% and 0.8% of the total protein for curd and milk, respectively. Lysine results in 8.2% and 8.3% of the total protein for curd and milk, respectively.

2.4. Chemical synthesis and characterization of the analytical standards

2.4.1. Synthesis of AITC-Cys

AITC-Cys was synthesized as described by Brüsewitz et al. with minor modifications (Brüsewitz et al., 1977). In brief, L-cysteine (4 mmol) was dissolved in water (30 mL). AITC (4 mmol), dissolved in 10 mL of EtOH-water (80:20, v/v) was added dropwise to the solution of L-cysteine. After stirring for 72 h at 20 °C, the precipitate was filtered, first washed with water (10 mL) and then with EtOH (10 mL), and recrystallized from ethyl acetate. A white powder was obtained with a purity of 98 ± 2% according to HPLC-UV analysis. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.41 (s, 3H, SCNH, NH_2), 5.95 (m, 1H, CH(δ)), 5.26 (m, 2H, CH_2 (ε)), 4.22 (m, 2H, CH_2 (γ)), 3.67 (dd, 1H, CH (α)), 3.57 (m, 1H, CH_2 (β)), 3.33 (dd, 1H, CH_2 (β)).

2.4.2. Synthesis of BITC-Cys

For the synthesis of BITC-Cys, the same protocol as described for the synthesis of AITC-Cys was followed. Instead of AITC, BITC was used. A white powder with a purity of 99 ± 3% according to HPLC-UV analysis was obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 7.31 (m, 5H, aromatic), 4.83 (m, 2H, $C_6H_5CH_2$), 3.69 (dd, 1H, CH (α)), 3.58 (dd, 1H, CH (β)), 3.33 (dd, 1H, CH (β)).

2.4.3. Synthesis of AITC-Lys

The synthesis of AITC-Lys was performed according to Kumar et al. with slight modifications (Kumar & Sabbioni, 2010). In brief, AITC (4 mmol) in 1,4-dioxane (2 mL) was added dropwise to Boc-Lys