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Sulforaphane formation and bioaccessibility are more affected by steaming time than meal composition during *in vitro* digestion of broccoli

I. Sarvan [a,*], E. Kramer [b], H. Bouwmeester [b], M. Dekker [a], R. Verkerk [a]

[a] Food Quality and Design Group, Wageningen University, Post Office Box 17, 6700 AA Wageningen, The Netherlands
[b] Rikilt-Wageningen University & Research Center, Akkermaalsbos 2, 6708 WB Wageningen, The Netherlands

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ABSTRACT

Broccoli is a rich source of the glucosinolate glucoraphanin (GR). After hydrolysis of GR by the endogenous enzyme myrosinase, sulforaphane (SF) or sulforaphane nitrile (SFN) are produced, depending on environmental conditions. How the conversion of GR and bioaccessibility of released breakdown products are affected by steaming (raw, 1 min, 2 min and 3 min steamed) and meal composition (protein or lipid addition) was studied with an *in vitro* digestion model (mouth, stomach, intestine, but not colonic digestion).

The main formation of SF and SFN occurred during *in vitro* chewing. The contents of GR, SF and SFN did not change after further digestion, as the irreversible inactivated myrosinase under gastric conditions caused no further GR hydrolysis. SF concentrations were up to 10 times higher in raw and 1 min steamed broccoli samples after digestion compared to longer-steamed broccoli. Protein or lipid addition had no influence on the formation and bioaccessibility of SF or SFN.

1. Introduction

The isothiocyanate sulforaphane (SF) was shown by *in vitro* cell and animal studies to affect several mechanisms that could reduce the risk of developing cancer at different steps: the initiation phase, the cell proliferation and the apoptosis. (Reviewed by: Fimognari & Hrelia, 2007; Hayes, Kelleher, & Eggleston, 2008.)

In epidemiological studies the intake of cruciferous vegetables that contain precursors of isothiocyanates, namely glucosinolates (GS), is associated with a decreased risk of several cancers (Steinbrecher, Nimptsch, Hüsing, Rohrmann, & Linseisen, 2009; Traka & Mithen, 2008; Verhoeven, Goldbohm, van Poppel, Verhagen, & van den Brandt, 1996; Verkerk et al., 2009; Voorrips et al., 2000). Among cruciferous vegetables, especially broccoli (*Brassica oleracea var. italica*) is rich in the glucosinolate glucoraphanin (GR). SF can be produced by enzymatic hydrolysis of GR. The enzymatic hydrolysis can take place by the plant enzyme myrosinase (β thioglycosidase, E.C. 3.2.1.147), which is released during cutting or chewing from the so called myrosin cells in broccoli (Kissen, Rossiter, & Bones, 2009). To a lesser extent, GR can be hydrolyzed by microbiota with myrosinase-like activity that is present in the human intestine (Fahey et al., 2012). If the cell structure of the plant is disrupted, e.g. during cutting or chewing, myrosinase can make contact with GR and hydrolyze it to SF or sulforaphane nitrile (SFN), depending on environmental conditions (Verkerk et al., 2009). At neutral pH, mainly SF will be formed. At pH 4, and/or in the presence of iron-ions, e.g. from a meal, and active endogenous epithiospecifier protein, SFN formation is favoured (Matusheski, Juvik, & Jeffery, 2004).

Much is known about the mechanisms of action of SF when it has entered the gut wall, but limited research has been performed on the effect of the harsh conditions (i.e. pH dynamics and enzymatic activity) within the human digestive tract, on the formation and fate of SF (i.e. if SF is bioaccessible) before it is absorbed into the human body. Bioaccessibility is defined as the released fraction of a component from a food matrix (Marze, 2013). To be bioaccessible, the molecule needs to be either in a molecular dispersed state or, in the case of lipophilic components, part of a colloid or micellar system (Duchateau & Klaffke, 2008). It has been hypothesized by Rungapamestry, Duncan, Fuller, and Ratcliffe (2007) that the lipophilic SF might be more easily digestible in the presence of lipid food components. They hypothesized that the lipophilic isothiocyanates might interact with meal components; if isothiocyanates are formed before colonic digestion that means during preparation of the food or in the upper digestive tract.

* Corresponding author.
E-mail address: irmela.kruse@gmail.com (I. Sarvan).

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In a review by Hanschen, Lamy, Schreiner, and Rohn (2014), the reactions between isothiocyanates (due to their very electrophilic carbon atom) and amino groups to form thiourea derivatives are described. They also indicated a need to investigate the role of proteins and amino acids for understanding the glucosinolate-based bioactivity in foods.

The aim of the present study was to investigate the effects of different steaming times and the presence of other meal components (olive oil and bovine serum albumin), during *in vitro* digestion, on the formation, stability and bioaccessibility of SF.

To mimic the digestion process, a static, three stage *in vitro* digestion system (mouth, stomach, intestine, but not colonic digestion) was employed, that was used previously to assess the bioaccessibility of chemicals from toys (Brandon et al., 2006) and release of vitamins (Brandon et al., 2014). The digestion started with the oral phase (pH 6.8), followed by a gastric phase (pH 2) and the intestinal phase (pH 6.5). The *in vitro* design involves taking samples at different time points, and is thus a good non-invasive, standardized method to study the processes taking place during human digestion.

2. Material and methods

2.1. Sample preparation

Broccoli (*Brassica oleracea var. italica*) was used from one batch of 13 heads grown in Spain in winter 2012/13.

Fresh broccoli heads were cut into florets with approximately 2 cm stem length and mixed thoroughly. The florets were divided into portions of 100 g. Two portions of raw broccoli were set aside and two portions were each steamed in an electrical steam oven (Miele, Steam oven DG 1050) for 1 min, 2 min and 3 min in a non-randomized manner. The oven was preheated to 100 °C before the material was added. A thermocouple was introduced in the core of the stem of one of the florets to measure the temperature profile during steaming. After steaming, the broccoli florets were placed in aluminium dishes on ice to cool to room temperature. The temperature profile in the broccoli core was measured for 10 min in total, irrespective of the steaming time. After 10 min the core temperatures in the broccoli florets were between 34.3 °C and 17.6 °C.

Samples of two batches of raw broccoli and of each steaming time were taken to determine the myrosinase activity after steaming and cooling in duplicate. The other samples were frozen in liquid nitrogen, crushed in a blender (Waring Commercial, Torrington, USA) and stored at −20 °C for further GR, SF and SFN analysis and for the *in vitro* digestion.

2.2. Pilot study

To determine the stability of the indigenous myrosinase at different pH, buffers with various pH (2, 3, 4, 5, 7 and at pH 2 for 30 min, then adjusted to pH 5) were added to 0.5 g of crushed broccoli in duplicate, incubated at room temperature overnight and the myrosinase activity determined as described in Section 2.4.

2.3. Gastro intestinal-tract set up in vitro

Portions (4.5 g) of frozen and blended broccoli (raw and steamed for 1, 2 and 3 min) were added to the *in vitro* digestion system. To samples of each steaming time, either 1 g of bovine serum albumin (BSA) or 0.72 ml of olive oil were added or they were placed in the digestion model without further additions. For each treatment, two batches were used in the digestion system. The individual amounts of BSA and olive oil were chosen to mimic the amounts of protein and fat present in a meal of lean minced meat and broccoli (same amounts of broccoli and minced meat, calculated from food tables (Aign, Muskat, Elmadfa, & Fritzsche, 2010)). The *in vitro* digestion was performed in three subsequent steps, namely an oral phase, a gastric phase and an intestinal phase, according to Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips (2005), based on Oomen et al. (2003). For all digestion phases, the tubes were placed in a head-over-heel rotation machine that was placed in an incubator (37 °C). For the oral phase, 6 ml of artificial saliva solution at 37 °C were added to the frozen broccoli sample and rotated gently for 5 min (pH 6.8). To mimic chewing in the present research, ground frozen broccoli samples were placed in plastic tubes and warm artificial saliva (6 ml at 37 °C) was added; the tubes were closed and rotated gently for 5 min (pH 6.8). At the end of this stage, the sample was at room temperature. This treatment had shown hydrolysis rates similar to *in vivo* chewing (results not shown). Mincing of broccoli, as advised by Minekus et al. (2014), resulted in total hydrolysis of GR (results not shown) and was therefore considered inappropriate to mimic *in vitro* chewing in this study. For the gastric phase 12 ml of gastric juice were added to the digest of the previous phase and adjusted to pH 2 with HCl (37%). After incubation for 2 h, the gastric phase was completed. For the intestinal phase 12 ml of duodenal juice, 6 ml of bile solution and 2 ml of sodium bicarbonate solution (all at 37 °C) were added to the digests of the previous steps, the pH adjusted to 6.5 with NaOH (1 M) and the whole incubated for 2 h. Samples were taken after the mouth phase, after the gastric phase and after the intestinal phase, with samples in separate tubes for each extraction. Samples were frozen at −20 °C prior to further analysis of GR, SF and SFN.

2.4. Myrosinase activity

The myrosinase activity was determined by the method described by Oliviero, Verkerk, Van Boekel, and Dekker (2014). To extract the myrosinase, 0.5 g of ground broccoli sample was added to 140 ml of potassium phosphate buffer (pH 7, 50 mM) and incubated overnight at 15 °C. After incubation the samples were centrifuged at 2670*g* for 10 min and filtered through filter paper. Three ml of this solution were placed in a centrifugal filter tube (Millipore, Ultracel 30kD) and centrifuged at 4000*g* for 10 min to remove dissolved components smaller than myrosinase. The myrosinase in the filter was dissolved in 470 µl phosphate buffer (pH 7, 50 mM) and stored on ice.

A D-glucose enzyme kit (Biocontrol Europe, Enzyplus EZS 781+) was used to determine the glucose released during hydrolysis of sinigrin by myrosinase at wavelength 340 nm for 7 min in the spectrophotometer. Activity was determined, based on the slope of the linear part of the curve of absorbance versus reaction time. To quantify the myrosinase activity, a calibration curve was established with an external standard myrosinase (Sigma-Aldrich, thioglucosidase from sinapis alba seeds in buffer solution; with dilutions of 0.2–1.2 U/ml, $R^2 = 0.985$) by following the same procedure as for the sample analysis. Activity was expressed as U/mg dry weight, where one unit produces 1 µmol glucose/min from sinigrin at pH 6 and 25 °C and with a detection limit of 0.01 U.

2.5. GR extraction and determination

GR was extracted with hot methanol (70%) and analyzed by high performance liquid chromatography (HPLC), following on-column desulphation, as described by Verkerk, Dekker, and Jongen (2001). Glucotropaeolin (3 M, in water) was added as internal standard since it is not present in the explored vegetables. The column used was from Merck (Darmstadt, Germany) LiChroCART (RP-18 125 × 4 mm, 5 µm) with an attached LiChroCART guard