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Analytical Methods

# Acidification, crushing and thermal treatments can influence the profile and stability of folate poly-γ-glutamates in broccoli (*Brassica oleracea* L. *var. italica*)

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ABSTRACT

The influence of different treatments, i.e., crushing, high temperature short time (90 °C/4 min) (HTST) and low temperature long time (60 °C/40 min) (LTLT) blanching, acidification (pH 4.3), and sequences of these treatments on the folate poly-γ-glutamate profile and stability were investigated. In this study, broccoli was used as a case study. Regarding the folate poly-γ-glutamate profile, endogenous folate poly-γ-glutamates in broccoli florets were found predominantly as hepta- and hexa-γ-glutamates. Crushing raw broccoli, acidification and LTLT blanching enhanced folate deconjugation resulting in monoglutamate, di- and tri-γ-glutamates. Compared to other treatments, HTST blanching preformed prior to crushing resulted in the highest concentration of long chain poly-γ-glutamates. Regarding folate poly-γ-glutamates stability, acidification combined with LTLT blanching decreased folate stability whereas HTST blanching combined with different sequences of blanching and crushing did not affect folate poly-γ-glutamates stability. It was concluded that crushing (prior to heating), acidification and blanching could be strategically applied to increase the folate monoglutamate content of broccoli.



## 1. Introduction

Folates have been attracting a lot of attention due to their association with reduction in the occurrence of neural tube defects, certain types of cancer and diseases, e.g., cardiovascular disease by lowering homocysteine levels (Lucock, 2002; McKillop et al., 2002; Stranger, 2002). Man cannot synthesize folates and therefore has to obtain them from the diet. Dietary sources rich in folates include yeast extracts, liver, eggs, kidney, green leafy vegetables, legumes and citrus fruits (Scott, Rébeillé, & Fletcher, 2000).

The bioavailability of folates from plant based foods has been reported to vary from 50% to 98% in comparison with that of synthetic folic acid (Brouwer et al., 1999; Hannon-Fletcher et al., 2004; Sauberlich, Kretsch, Skala, Johnson, & Taylor, 1987; Seyoum & Selhub, 1998). Low folate bioavailability could be attributed to various factors such as folate instability and existence of dietary folates as poly-γ-glutamates (Seyoum & Selhub, 1998). Due to their state of reduction at the 5–8 positions of the pteridine moiety, dietary folates are quite unstable during processing, and can easily undergo oxidation (McKillop et al., 2002; Murphy, Boyle, Weir, & Scott, 1976; Murphy, Keating, Boyle, Weir, & Scott, 1978). The stability of folates during processing is influenced by temperature, pressure, pH, oxygen, light, metal ions, antioxidants and duration of heating (Gregory, 1989; Hawkes & Villota, 1989; Indrawati et al., 2004; McKillop et al., 2002; Verlinde, Oey, Hendrickx, & Van Loey, 2008).

In fruits and vegetables, folates mainly occur as poly-γ-glutamates with typically 2–11 γ-glutamic acid residues (Eitenmiller & Landen, 1999; Gregory, 1989; Lucock, 2002; Melse-Boonstra et al., 2002; Scott et al., 2000; Seyoum & Selhub, 1998). Before absorption in the intestines, folate poly-γ-glutamates must first be deconjugated to monoglutamate by the intestinal γ-glutamyl hydrolase (GGH, EC 3.4.19.9). However, the activity of intestinal GGH is said to be susceptible to inhibition by constituents found in some foods (Bhandari & Gregory, 1990), and such inhibition may further jeopardize the bioavailability of dietary folates. Therefore, increasing folate monoglutamate concentration in foods prior to ingestion could increase the bioavailability of dietary folates. Plant GGH, the enzyme that catalyses the hydrolysis (deconjugation) of folate poly-γ-glutamates to shorter chain poly-γ-glutamates and monoglutamates (Orsomando et al., 2005), has been reported to occur in the cytosol, extracellularly and in the vacuole (Huangpu, Pak, Graham, Rickle, & Graham, 1996; Lin, Rogiers, & Cossins, 1993; Orsomando et al., 2005). Therefore, matrix disruption (e.g., cutting, crushing, processing) facilitate the GGH catalyzed hydrolysis of native folate poly-γ-glutamates to shorter poly-γ-glutamates and monoglutamate (Leichter, Landymore, & Krumdieck, 1979; Melse-Boonstra et al., 2002).

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Despite the potential of exploiting processing techniques to increase the bioavailability of dietary folates (by enhancing poly-γ-glutamate deconjugation), only limited data on the folate poly-γ-glutamate profile and the influence of processing on the poly-γ-glutamate profile of fruits and vegetables is available (Ndaw, Bergaentzlé, Aoudé-Werner, Lahély, & Hasselmann, 2001; Verlinde et al., 2008; Zheng, Lin, Lin, & Cossins, 1992). Hereto, the objectives of this research were to evaluate the native profile of folate poly-γ-glutamates; the effects of different treatments namely blanching, crushing and acidification on stability and deconjugation of folate poly-γ-glutamates and the effect of different sequences of performing these treatments on the stability and deconjugation of folate poly-γ-glutamates. In this study, broccoli was used as a case study since it is rich in folate poly-γ-glutamates.

## 2. Materials and methods

### 2.1. Reagents and folate standards

All chemicals used were of analytical or HPLC grade. Unless otherwise stated, all reagents were prepared using double demineralized water (18 MΩ cm at 25 °C) produced with a water purification system (Simplicity 185, Millipore Massachusetts, USA). The following folate poly-γ-glutamate standards were obtained from Schircks Laboratories (Switzerland); (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid poly-γ-glutamates (5-$CH_3$–$H_4$PteGlu$_{2-5}$) trihydrochloride salts, pteroylhexa-γ-L-glutamic acid (PteGluγ$_6$) and pteroylhepta-γ-L-glutamic acid (PteGluγ$_7$). 5-$CH_3$–$H_4$PteGluγ$_6$ and 5-$CH_3$–$H_4$PteGluγ$_7$ were obtained by chemical conversion of PteGluγ$_6$ and PteGluγ$_7$ as outlined by Ndaw et al. (2001). (6S)-5-Methyl-5,6,7,8-tetrahydrofolic acid calcium salt (5-$CH_3$–$H_4$PteGlu$_1$) was obtained as a gift from Merck Eprova AG (Schaffhausen, Switzerland).

### 2.2. Sample preparation

To determine the native folate poly-γ-glutamate profile, one batch of each of two broccoli varieties (Belstar (BS) from Belgium, and unknown variety from Holland (HK)) was purchased from a local greengrocer. The broccoli was stored at 4 °C for maximum five days before use. For each of the two varieties the florets (including 1 cm of stalk) were cut from the broccoli heads, mixed to obtain a homogenous sample and then divided into two portions. One portion of intact florets was vacuum-packed in plastic bags (50 g per bag) and given a heat shock treatment by boiling in water for 10 min to inactivate endogenous GGH and other enzymes. The heat shocked florets were then cooled in ice water, frozen in liquid nitrogen and stored at −80 °C until folate extraction. The other portion of intact florets was vacuum-packed and directly frozen in liquid nitrogen without heat shock treatment (no inactivation of GGH and other enzymes) and stored at −80 °C until extraction. Prior to folate extraction, the frozen samples were ground (Grindomix, GM 200, Germany) to a frozen powder.

To determine the influence of different treatments on the stability and profile of folate poly-γ-glutamates, one batch of broccoli (20 kg, variety Grammel, Spain) was purchased from a local supplier. During the study, the broccoli was stored at 4 °C for a period not longer than 5 days. Both the florets and the stalks were used for the treatments. Broccoli heads were obtained by cutting the main stalk at the point slightly below the emergence of the smaller stalks. The florets, together with about 1 cm of stalk, were cut off from the rest of the stalk and these were used as broccoli floret samples. The rest of the stalk was cut into pieces of about 1 cm and used as broccoli stalk samples. The bottom main stalk was discarded.

### 2.3. Treatments

Two different crushing-blanching sequences were carried out as illustrated in Fig. 1. Mechanical (i.e., crushing) and thermal (blanching) treatments were conducted at the natural pH of broccoli around 6.5 and at acidic pH of 4.3. To obtain pH 6.5 and 4.3, broccoli florets or stalks were crushed (Buchi mixer, B-400, Switzerland) in the presence of distilled water or acetate buffer (0.2 M, pH 3.7) respectively, at a ratio of 1:1 (w/v). A crushing time of 20 s was standardized.

For thermal treatments, the samples were vacuum-packed (Multivac A300/16, Wolfertschwenden, Germany) in plastic bags (50 g per bag) and heated in a water bath (Memmert, WBU 45, Germany) either before or after crushing. High temperature short time (HTST) blanching was carried out at 90 °C for 4 min and low temperature long time (LTLT) blanching at 60 °C for 40 min. For the samples crushed before blanching, a 30 min period (including the time for vacuum-packing) after crushing and before blanching was standardized. For the samples crushed after the thermal treatment, a 15 min period after crushing (including the time for vacuum-packing) was standardized. After the various treatments, samples were cooled in ice water, frozen in liquid nitrogen and then freeze dried. The samples were then packed in opaque plastic containers and stored in a desiccator containing phosphorous pentoxide ($P_2O_5$) at room temperature for maximum 21 days.

### 2.4. Folate analysis

Folate analysis including extraction, chemical conversion of broccoli folates to 5-$CH_3$–$H_4$PteGlu$_n$, purification, and quantification was performed according to the method of Ndaw et al. (2001) optimized for broccoli (Verlinde et al., 2008) with a few modifications. Regarding folate extraction, 20 g heat shocked or 20 g non-heat shocked samples was respectively mixed with 50 ml cold or 50 ml boiling extraction buffer (phosphate buffer (0.1 M, pH 7, containing ascorbic acid)). Afterwards, the mixtures were homogenized (Ultra Turrax T25, IKA Labortechnik, Staufen, Germany) for 30 s. The non-heat shocked samples were hot extracted by immersion of the sample-containing tubes in boiling water for 10 min and subsequently cooled in ice water. The heat shocked samples did not receive a hot extraction to avoid folate degradation due to excessive heating and so that both categories of samples received approximately the same total heating intensity.

To extract folates from treated samples, 2 g freeze-dried broccoli was mixed with 50 ml boiling extraction buffer (phosphate buffer (0.1 M, pH 7, containing ascorbic acid) and 17.8 ml of double distilled water. The subsequent extraction steps were performed as described by Verlinde et al. (2008). The heat shocked and non-heat shocked samples were analyzed in triplicate while the treated samples were analyzed in duplicate. The dry matter content of broccoli folate and stalks was determined by oven drying broccoli at 105 °C for 16 h.

Quantification of the folates from broccoli was done using an external calibration with folate standards (5-$CH_3$–$H_4$PteGlu$_{1-7}$) and was based on peak height. The detection limit for the various poly-γ-glutamates and monoglutamates ranged from 0.015 to 0.100 pmol on column. The recovery of different folate derivatives as determined by spiking broccoli with standard (5-$CH_3$–$H_4$PteGlu$_{1-7}$) was taken into account in the quantification.

### 2.5. Method validation and recovery determination

The efficiency of the chemical conversion used in the current study had been previously demonstrated to be high (Verlinde et al., 2008). However, since the quality of the folate binding proteins (in the affinity chromatography columns) degrade with