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Effect of Boiling on the Content of Ascorbigen, Indole-3-carbinol, Indole-3-acetonitrile, and 3,3′-Diindolylmethane in Fermented Cabbage

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The aim of the study was to investigate the effect of the boiling process on the content of ascorbigen, indole-3-carbinol, indole-3-acetonitrile, and 3,3′-diindolylmethane in fermented cabbage. The cabbage was boiled for 5 to 60 min. Boiling resulted in a decrease of the total content of the compounds analysed. The changes were mainly caused by leaching of ascorbigen predominating in cabbage into cooking water and by its thermal hydrolysis. Ascorbigen losses resulting from thermal hydrolysis accounted for 30% after 10 min of boiling and for 90% after 60 min of boiling. One of the ascorbigen breakdown products was indole 3 carbinol; the decrease in ascorbigen content was accompanied by a drastic increase in the content of 3,3′-diindolylmethane, a condensation product of indole-3-carbinol. After 40 and 50 min of boiling, the total content of 3,3′-diindolylmethane in cabbage and cooking water was approximately 0.2 μmol/100 g and was 6-fold higher than that in uncooked cabbage. 3,3′-Diindolylmethane synthesis proceeded within the plant tissue. After 10 min of boiling, the content of free indole-3-carbinol and indole-3-acetonitrile stabilized at the level of about 80% as compared to the uncooked cabbage.

KEYWORDS: Fermented cabbage; glucobrassicin breakdown products; ascorbigen; indole-3-carbinol; indole-3-acetonitrile; 3,3′-diindolylmethane

INTRODUCTION

Consumption of Brassica vegetables is commonly known to reduce the risk of some carcinoma forms in humans. They contain aliphatic, indole and aralkyl thioglucosides referred to as glucosinolates (GLS). Numerous in vitro and in vivo studies have shown that the breakdown products of indole and aliphatic GLS have anticarcinogenic properties (*1*, *2*). Nowadays, a lot of attention has been paid to sulforaphane, the main isothiocyanate formed after enzymatic hydrolysis of the aliphatic GLS glucoraphanin (*3*). The hydrolysis products indole-3-acetonitrile and indole-3-carbinol are produced from glucobrassicin, the main indole GLS of vegetables (*4*, *5*), whereas ascorbigen is produced during the reaction of indole-3-carbinol with ascorbic acid (*6*). The product 3,3′-diindolylmethane is an indole-3-carbinol dimer formed as a result of acid condensation of this compound (*4*, *6*−*8*).

The anticarcinogenic properties of the above-mentioned compounds have raised considerable interest resulting in dietary supplements producers. Apart from a broccoli extract, the market offers capsules containing synthetically produced indole-3-carbinol and 3,3′-diindolylmethane. It should be emphasized, however, that the long-term effects of taking these compounds in pure form are not known yet. There are reports warning against their potential harmful effect when taken in excess (*1*). Their natural and safe source is Brassica vegetables, among which broccoli and red cabbage are rich in glucoraphanin (*9*−*11*). Glucobrassicin occurs abundantly in most vegetables of this family (*12*).

Brassica vegetables are not always consumed raw; they are often subjected to industrial processes and culinary treatments. Numerous studies have been conducted on the effects of these processes on GLS content in the final product (*13*−*19*). However, the effects of industrial or culinary processes on GLS degradation products, at a concomitant lack of native GLS have not been studied so far. An example of such processes is hydrothermal treatment of fermented cabbage. During the fermentation of white cabbage, glucosinolates undergo complete degradation already in the early stage of fermentation (*20*, *21*). The end product, i.e., cabbage after spontaneous fermentation obtained 2 weeks after the beginning of the fermentation process, was found not to contain native GLS (*20*, *22*). The main product of GLS hydrolysis in fermented cabbage is ascorbigen (*20*);

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other components are indole-3-carbinol, indole-3-acetonitrile, and isothiocyanates as well as nitriles released from aliphatic GLS breakdown (*20*−*22*). Fermented cabbage is consumed raw or cooked, usually for 5 to 40 min, depending on individual culinary preferences and the type of the final product to be achieved.

The aim of the study was to investigate the effect of the boiling process on the content of ascorbigen, indole-3-carbinol, indole-3-acetonitrile, and 3,3′-diindolylmethane in fermented white cabbage.

MATERIALS AND METHODS

Fermented cabbage was obtained through spontaneous fermentation of white cabbage (*Brassica oleracea* cv. Kamienna Glowa). The fermentation method was previously described by Ciska et al. (*20*). The resultant fermented cabbage was found to possess good organoleptic properties, i.e., taste, smell, and appearance, and suitable hardness of strips. The pH value of 3.8 for fermented cabbage juice indicated a proper course of fermentation.

Fermented Cabbage Boiling. One-hundred gram samples of drained fermented cabbage were boiled for 5, 10, 20, 30, 40, 50, and 60 min at a constant 1:2 cabbage to water ratio (m/v). After boiling, the samples were immediately cooled in a water−ice mixture. Next, the cabbage was filtered with a Büchner funnel and washed twice with 100 mL of distilled water. Filtrates and cooking water were combined and transferred to a flask and filled up to 200 mL with water. The cooking process was conducted in triplicate.

Standards. Indole-3-carbinol and indole-3-acetonitrile were purchased from Merck, Darmstadt, Germany.

Synthesis of Ascorbigen. Ascorbigen was synthesized from indole-3-carbinol and ascorbic acid according to Kiss and Neukom (*23*) as described earlier (*20*). Chromatographic purity (HPLC) of the compound obtained reached 99.6%.

Synthesis of 3,3′-Diindolylmethane. 3,3′-Diindolylmethane synthesis was conducted according to Buskov et al. (*24*). 3,3′-Diindolylmethane was prepared from indole and formaldehyde; synthesis was carried out at 80 °C for 5 h in the dark. Recrystallization was performed from methanol. Chromatographic purity (HPLC) of the compound obtained reached 99.7%. The structure confirmation of 3,3′-diindolylmethane was carried out using the GC-MS method as described by Chevolleau et al. (*4*). The analysis of trimethylsilated 3,3′-diindolylmethane was performed on a Hewlett-Packard 5975 mass selective detector coupled with a HP 7890A gas chromatograph. The compounds were separated in a capillary column BGB-1 (30m × 0.25 mm, film thickness − 0.25 μm). The carrier gas was helium (1 mL/min). The injector and interface temperatures were 280 and 270 °C, respectively. The oven temperature was initially set at 130 °C for 5 min, then increased to 300 °C (4 °C/min). Mass spectra were obtained by electron ionization (EI) over the range of *m*/*e* 50−550. The ion source temperature was 280 °C and the electronic impact energy was 70 eV. The obtained mass spectra of trimethylsilated 3,3′-diindolylmethane were consistent with those reported by Chevolleau et al. (*4*).

HPLC Analysis. Ascorbigen. The analysis was carried out according to Aleksandrova et al. (*6*) with some modifications. Briefly, duplicate 25 g samples of cabbage were homogenized with 25 mL of distilled water and 2.5 g of NaCl using an Ultra Turrax homogenizer (Janke & Kunkel, Germany). Next, the samples were extracted three times with 30 mL of acetone. The acetone extracts were combined and following filtration concentrated to the volume of ca. 15 mL. The combined organic layers were dried over anhydrous sodium sulfate and evaporated in vacuo to dryness. The residue was dissolved in acetonitrile and made up with mobile phase to 10 mL. Cooking water was analyzed directly using the HPLC method.

The HPLC analysis was run with a Shimadzu LC-6A system. Ascorbigen was separated from 20 μL samples in an LiChrospher 100 RP-18 (5 μm) column (250 × 4 mm). Chromatography was carried out at a flow rate of 1 mL/min by eluting in gradient of 10% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.7) (A) and 80% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.7) (B) as follows: linear gradient from 30% to 90% B for 30 min, isocratically 90% B for 15 min, linear gradient to 30% B for 5 min, and equilibrated for 5 min. Detection was performed at $\lambda = 280$ nm.

Indole-3-carbinol. The analysis was carried out according to Anderton et al. (*25*) with some modifications. Briefly, duplicate 25 g samples of cabbage were homogenized with 25 mL of distilled water. Next, the samples were extracted three times with 30 mL of TMBE. The combined organic layers were dried over anhydrous sodium sulfate and filtered. TMBE was evaporated under nitrogen in the presence of 2 mL of DMSO. The residue was transferred to a measuring flask to which 5 mL of 50 mM HEPES at pH 7 was added and then adjusted up to 10 mL with acetonitrile. Extraction of 20-mL samples of cooking water was carried out in the same way using TMBE.

Indole-3-carbinol was determined in the same HPLC system as ascorbigen using a fluorescence detector. A chromatogram was developed at a flow rate of 1 mL/min by eluting in a gradient of 10% acetonitrile (A) and 80% acetonitrile (B) as follows: linear gradient from 10% to 90% B for 20 min, isocratically 90% B for 20 min, linear gradient to 10% B for 5 min, and equilibrated for 5 min. Detection was performed at $E_x = 285$ nm and $E_m = 340$ nm.

Indole-3-acetonitrile and 3,3′-Diindolylmethane. Duplicate 25 g samples of cabbage were homogenized with 25 mL of distilled water. The homogenate was extracted three times with 30 mL of methylene chloride. The combined organic layers were dried over anhydrous sodium sulfate and filtered through a PTFE filter (0.22 μm). Next, the filtrate was concentrated to the volume of ca. 8 mL on a rotary evaporator under reduced pressure at 30 °C. The concentrate was transferred into a measuring flask and filled with dichloromethane up to 10 mL. Extraction of 20-mL cooking water samples was conducted in the same way using methylene chloride. HPLC analysis of indole-3-acetonitrile and 3,3′-diindolylmethan was conducted by applying the same separation and detection parameters as those for indole-3-carbinol.

RESULTS AND DISCUSSION

In the study, we investigated the effect of the boiling process of fermented cabbage on the contents of ascorbigen, indole-3-carbinol, indole-3-acetonitrile, and 3,3′-diindolylmethane. These compounds are products of glucobrassicin degradation formed during cabbage fermentation. The presence of ascorbigen, indole-3-carbinol, and indole-3-acetonitrile in fermented cabbage was confirmed in the earlier studies by Aleksandrova et al. (*6*) and Ciska and Pathak (*20*), but the presence of 3,3′-diindolylmethane, an indole-3-carbinol condensation product, was demonstrated here for the first time. As reported by Grose and Bjeldanes (*8*), indole-3-carbinol easily undergoes acid condensation into a number of oligomers, among others 3,3′-diindolylmethane. The presence of this compound depends most likely on the relatively low pH of fermented cabbage, which should range between 3.4 and 4.0 (*26*). The content of 3,3′-diindolylmethane in the cabbage used for cooking was 0.032 μmol/100 g cabbage and was 4-fold lower than the content of monomer, i.e., indole-3-carbinol (**Table 1**). The predominating compound was ascorbigen, a product of synthesis of indole-3-carbinol and ascorbic acid. Its content accounted for more than 8 μmol/100 g cabbage, which was 60-fold higher than the content of indole-3-carbinol.

Our earlier studies demonstrated that ascorbigen was the main product of glucobrassicin degradation in fermented cabbage and that its content did not change even during long-term storage of cabbage (*20*). Proportions between particular compounds are also in agreement with the data published by Aleksandrova et al. (*6*).

The boiling process affected the content of particular compounds in cabbage to a different extent. Ascorbigen content decreased as boiling time increased. The greatest, over 60% losses of its content in cabbage, was observed during the first 5 min of boiling; this resulted mainly from leaching of this

Table 1. Content [μmol/100g] of Particular Compounds in Cooked Fermented Cabbage and in Cooking Water

cooking time (min)	ascorbigen	indole-3-carbinol	indole-3-acetonitril	3,3'-diindolylmethane	total
			fermented cabbage		
0	8.33	0.130	0.103	0.032	8.60
5	3.01 ± 0.07[a]	0.153 ± 0.013	0.038 ± 0.007	0.060 ± 0.009	3.26 ± 0.07
10	1.97 ± 0.03	0.068 ± 0.003	0.041 ± 0.006	0.116 ± 0.003	2.19 ± 0.02
20	1.19 ± 0.09	0.075 ± 0.016	0.045 ± 0.013	0.137 ± 0.006	1.45 ± 0.09
30	1.05 ± 0.17	0.084 ± 0.011	0.040 ± 0.011	0.127 ± 0.006	1.30 ± 0.18
40	0.75 ± 0.09	0.083 ± 0.027	0.047 ± 0.006	0.171 ± 0.009	1.05 ± 0.06
50	0.34 ± 0.07	0.085 ± 0.007	0.039 ± 0.010	0.172 ± 0.009	0.64 ± 0.10
60	0.22 ± 0.07	0.091 ± 0.010	0.043 ± 0.018	0.142 ± 0.046	0.50 ± 0.13
			cooking water		
5	5.90 ± 0.19	0.028 ± 0.006	0.039 ± 0.002	0.018 ± 0.002	5.99 ± 0.19
10	3.86 ± 0.06	0.047 ± 0.016	0.047 ± 0.004	0.021 ± 0.011	3.98 ± 0.07
20	1.90 ± 0.09	0.035 ± 0.001	0.046 ± 0.008	0.028 ± 0.018	2.01 ± 0.08
30	1.54 ± 0.25	0.026 ± 0.008	0.046 ± 0.008	0.028 ± 0.016	1.64 ± 0.25
40	0.70 ± 0.07	0.013 ± 0.007	0.044 ± 0.005	0.023 ± 0.012	0.78 ± 0.07
50	0.56 ± 0.09	0.012 ± 0.006	0.044 ± 0.005	0.023 ± 0.004	0.69 ± 0.10
60	0.42 ± 0.21	0.007 ± 0.004	0.042 ± 0.008	0.024 ± 0.002	0.49 ± 0.21

[a] Values are means ± SD (n = 3).

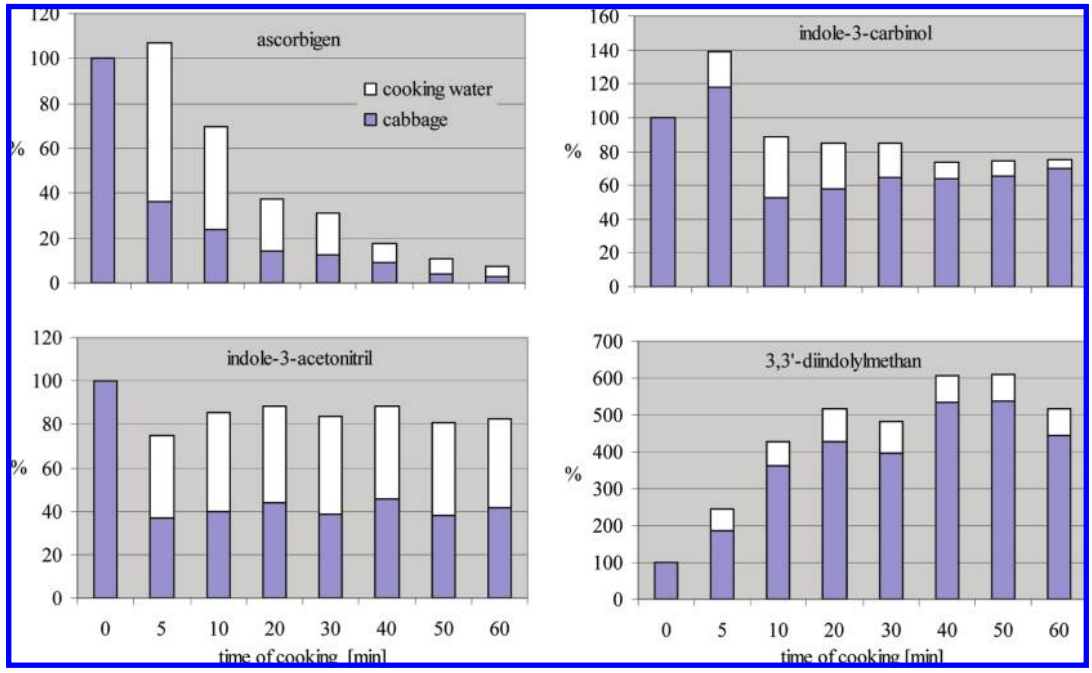

Figure 1. Relative content of particular compounds in cooked fermented cabbage and cooking water expressed as percentage of their content in uncooked cabbage.

compound into cooking water (**Figure 1**). The mean total content of ascorbigen in cabbage and cooking water after 5 min was approximately 7% higher as compared to the uncooked material. That tendency for a negligible increase in ascorbigen content observed during those first minutes was probably due to the improved extractability of the system. Boiling the plant material resulted in loosening of tissue structures and consequently more effective ascorbigen leaching. Such an effect, related to hydrothermal treatment, was also observed in the case of other compounds, including glucosinolates (*16*, *17*, *19*). Extending the boiling time resulted in thermal hydrolysis of ascorbigen, which was manifested by a gradual decrease in its content both in cabbage and in cooking water. Ascorbigen losses caused by thermal hydrolysis were about 30% after 10 min and over 90% after 60 min of boiling. The content of indole-3-acetonitrile and indole-3-carbinol was affected by boiling to a lesser extent; during the first 5 min, the total content of indole-3-acetonitrile decreased both in cabbage and in cooking water and that of indole-3-carbinol increased by 20 and 40%, respectively. After 10 min of boiling, the total content of these compounds in cabbage and in cooking water reached about 80% irrespective of further cooking time. The distribution of indole-3-acetonitrile in cabbage and in cooking water was approximately equal; however, the prevailing part of indole-3-

carbinol remained in cabbage, and the disproportion between cabbage and cooking water grew along with extension of the boiling time. The changes in the content of 3,3′-diindolylmethane, an indole-3-carbinol dimer, were different. From the very first minutes of boiling, drastic increase in its content was observed. After 40 and 50 min of boiling, the total content of 3,3′-diindolylmethane in cabbage and in cooking water was ca. 0.2 μmol/100 g and was 6-fold higher than that in the uncooked cabbage.

Although boiling the cabbage affected the content of individual compounds to a different extent, their total content both in cabbage and in cooking water decreased with boiling time extension. After 60 min, the losses were about 90%; they were determined by the decreasing content of the predominating ascorbigen (**Table 1**). During boiling, the proportions between the compounds varied. In uncooked cabbage, ascorbigen constituted 97% of the total content of the analyzed compounds. With the progression of boiling time, the relative content of ascorbigen decreased while that of the remaining compounds increased. In the cabbage cooked for 60 min, the relative content of ascorbigen was 22% and that of indole-3-carbinol, indole-3-acetonitrile and 3,3′-diindolylmethane accounted for 9.2, 4.3 and 14.4%, respectively.

A lack of literature data on the effects of vegetable processing, hydrothermal treatment in particular, on the content of glucobrassicin degradation products makes it impossible to compare the results of our study to any other. On the basis of the results obtained it may be speculated that one of the products of ascorbigen breakdown is indole-3-carbinol. Moreover, during hydrothermal processes it may condense to 3,3′-diindolylmethane. Such an assumption would be confirmed by our results concerning changes in the content of indole-3-carbinol and its dimer. During boiling for 5 to 50 min, the amount of 3,3′-diindolylmethane in cabbage increased drastically, while the content of indole-3-carbinol was relatively stable after 10 min (**Figure 1**). Moreover, in cabbage boiled for 10 to 60 min the absolute content of 3,3′-diindolylmethane was 3−4-fold higher than that of indole-3-carbinol (**Table 1**). Thus, the losses of indole-3-carbinol as a result of condensation were compensated by the release of this compound as a result of ascorbigen thermal degradation. The conclusion seems to be that condensation of indole-3-carbinol to 3,3′-diindolylmethane occurred within the plant tissue as both compounds were present mainly in cabbage but not in the cooking water.

Most likely, thermal hydrolysis of ascorbigen occurred already during the first minutes of boiling. After 5 min, the total content of indole-3-carbinol and 3,3′-diindolylmethane in cabbage and in cooking water increased by 40 and 150%, respectively. This corresponded to the increase in the relative content of each compound by ca. 0.05 μmol. Taking into consideration the stoicheiometric relationships, this would mean that at most approximately 0.15 μmol of ascorbigen became thermally degraded. Actually, this amount may be lower because the increase in the content of indole-3-carbinol and 3,3′-diindolylmethane might have been partially related to more effective extraction after 5 min of boiling. The value of 0.15 μmol makes as little as 2% of the initial ascorbigen content. Thus, the small losses in ascorbigen content might have been covered by the increase in its content during the first minutes of boiling.

On the basis of the results obtained, it can be stated that boiling caused a decrease in the total content of the analyzed derivatives of glucobrassicin in cooked fermented cabbage. The changes resulted mainly from extraction into cooking water and thermal hydrolysis of ascorbigen predominating in the uncooked cabbage. One of the ascorbigen degradation products was indole-3-carbinol; the decrease in ascorbigen content was accompanied by a drastic increase of the content of 3,3′-diindolylmethane, a product of indole-3-carbinol condensation. 3,3′-Diindolylmethane synthesis occurred within the plant tissue. The content of indole-3-carbinol and indole-3-acetonitrile after 10 min of boiling stabilized at the level of ca. 80% as compared to raw material.

Because of the anticarcinogenic properties of ascorbigen, indole-3-carbinol, indole-3-acetonitrile, and 3,3′-diindolylmethane, special attention should be paid to the practical aspect of the results presented. Although the total content of glucobrassicin derivatives decreased during cooking, cooked fermented cabbage can still be an additional natural source of these compounds in the human diet. As the content of glucobrassicin degradation products in cooked fermented cabbage depends on the content of this GLS in raw cabbage, we recommend the use of cabbage rich in glucosinolates for fermentation.

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