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Plant-Derived Biomolecules in Fermented Cabbage

MARJA TOLONEN, MARIANNE TAIPALE, BRITTA VIANDER, JUHA-MATTI PIHLAVA, HANNU KORHONEN, AND EEVA-LIISA RYHÄNEN*

MTT Agrifood Research Finland, Food Research, FIN-31600 Jokioinen, Finland

The formation of plant-derived biomolecules during sauerkraut fermentation was studied. Cabbage was fermented with a starter culture, and the results were compared to the results of spontaneous fermentation. The concentration of flavonoids and glucosinolates was analyzed by HPLC, and that of the glucosinolate breakdown products, by GC-MS. Of the 20 different flavonoids tested, only kaempferol was found (0.9 mg/ kg FW, fresh weight). The content of kaempferol remained constant in the cabbage fiber matrix over the fermentation process. The nitrite concentration was below the detection limit in both fermentations. The total glucosinolate content in the raw material was 3.71 μmol/g DW, dry weight. Glucosinolates were totally decomposed in both fermentations during two weeks, and different types of breakdown products were formed. Isothiocyanates, indole-3-carbinol, goitrin, allyl cyanide, and nitriles were determined in the fermented cabbage. Isothiocyanates and allyl cyanide were the predominant breakdown products in both fermentations. Sulforaphane nitrile and goitrin were found only in small quantities in the end products.

KEYWORDS: sauerkraut; glucosinolates; glucosinolate breakdown products; flavonoids; cabbage; fermentation

INTRODUCTION

Cabbage is a widely consumed cruciferous vegetable in the human diet. The most common among the fermented *Brassica* products is sauerkraut. Some animal studies (*1, 2*) have shown that *Brassica* vegetables have beneficial effects on health, which may be due to phytochemicals such as flavonoids, glucosinolates, and their breakdown products. Although *Brassica* vegetables have been studied widely, the effect of processing on their bioactive compounds is still not well-known.

Glucosinolates are sulfur-containing glucosides, which are classified into three different classes: aliphatic glucosinolates (sinigrin, glucoiberin, glucoraphanin) derived from methionine, indole glucosinolates (glucobrassicin, 4-OH-glucobrassicin, 4-MeO-glucobrassicin) derived from tryptophan, and aromatic glucosinolates (glucotropaeolin, gluconasturtin, sinalbin) derived from phenylalanine and tyrosine (*3*). Glucosinolates are decomposed enzymatically by the enzyme myrosinase when the plant cells are disturbed by cutting or chewing, and several breakdown products can be formed. Some hydrolysis products (isothiocyanates and indole-3-carbinol) are considered capable of modulating biotransformation enzyme activity and so, acting as anticarcinogens, capable of preventing certain cancers (*1, 4, 5*). Glucosinolate breakdown products also contribute significantly to the typical flavor of *Brassica* vegetables (*6*). Further, cabbage juice has been documented in some studies to have antibacterial activity (*7, 8*). The antibacterial activity of fresh cabbage juice, moreover, has been reported to be heat-labile and pH-dependent (*7*).

Flavonol and flavone glycosides occur in *Brassicaceae* plants mainly as quercetin and kaempferol (*9*). Because their formation normally depends on light, they are mainly concentrated in the outer tissues of plants. The effect of favorable growth conditions, for example, the long hours of daylight typical of Northern countries, may enhance the accumulation of secondary compounds containing potential health-promoting properties (*10*).

The glucosinolate content of fresh vegetables varies considerably with such factors as soil type, plant spacing, light, temperature, and application of sulfate and nitrogenous fertilizer (*11, 12*). Identical cultivars may differ in their total glucosinolate content up to 200% because of these effects (*13*). The glucosinolate content of white cabbage, for example, has been reported to vary from 10.9 μmol/ g DW, dry weight (*13*) to 15.4 μmol/g DW (*14*).

Myrosinase (EC 3.2.3.1), which hydrolyzes the glucosinolates, is a well-characterized glucoprotein and has been cloned from a number of plant sources (*15*). The action of the hydrolyzing enzyme causes the release of glucose and an unstable intermediate which further rearranges. The nature of the forming breakdown products depends on the glucosinolate substrate and the pH, as well as on the presence of other activators (e.g., ascorbic acid) and inhibitors. The breakdown products of glucosinolates proceed by way of an unstable aglucone which, at neutral pH, rearranges with a loss of sulfate to form isothiocyanates, nitriles, oxazolidienethiones, hydroxynitriles, or epithionitriles (*15*). At neutral pH, indolyl glucosinolates yield

* To whom correspondence should be addressed (telephone +358-3-41883273; fax +358-3-4188324; email eeva-liisa.ryhanen@mtt.fi).

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first an unstable indolyl isothiocyanate and further degrade with loss of S to form indole-3-carbinol (I3C), ascorbigen, and related compounds which are known to be anticarcinogens and may play a protective role against cancer in the diet (*17*). Sulforaphane, the isothiocyanate of glucoraphanin, is the most potent naturally occurring inducer of phase II enzymes (*18*). Induction of phase II enzymes has been shown to protect cells against toxic electrophiles (*1*).

At low pH, glucosinolates of *Brassicas* are decomposed to nitriles rather than to isothiocyanates (*17*). Progoitrin is hydrolyzed at low pH to yield nitrile 1-cyano-2-hydroxybut-3-ene (*19*). Glucobrassicin yields 3-indoleacetonitrile (*20*) and glucoraphanin sulforaphane nitrile upon hydrolysis by myrosinase at low pH (*13*). Daxenbichler et al. (*21*) found the nitriles 1-cyano-3-methylsulfinylpropane (derived from glucoiberin) and 1-cyano-2,3-epithiopropane (derived from sinigrin) in commercial sauerkraut.

Glucosinolates can also be degraded by enzymes of human gastrointestinal bacteria (*22*, *23*). Intestinal bacteria may possess thioglucohydrolases similar to plant myrosinases (*17*). Strains of *Lactobacillus agilis* and *L. acidophilus* have been reported to degrade sinigrin (*24*, *25*).

The purpose of this work was to study the content of flavonoids, nitrate, nitrite, glucosinolates, and their breakdown products in cabbage before and after fermentation carried out using spontaneous and starter induced fermentation.

MATERIALS AND METHODS

Raw Material and Cabbage Handling. White cabbage *Brassica oleracea* L. (var. *capitata* cv. Lennox) was applied as the raw material for the fermentation studies. The cabbage was cultivated at MTT Agrifood Research Finland, Horticulture (Piikkiö). Once harvested, the cabbage heads were stored at +0.5 °C, trimmed, and sliced into small pieces with a food cutter (Robot Coupe R20, France). The sliced cabbage was mixed with granular food-grade mineral salt (57% NaCl, 28% KCl) to obtain a total salt concentration of 0.9% (w/w). The mineral salt was successfully used in spontaneous fermentation in previous study (*26*). A starter mixture, containing lactic acid bacteria *Leuconostoc mesenteroides* and *Pediococcus dextrinicus* (mixture of pure cultures 1:1), was applied in the first fermentation trial (starter fermentation). The strains used were isolated from spontaneously fermented sauerkraut, purified, and identified in a previous work by using API50CHL test (bioMe'rieux sa, France) (Viander, unpublished results). The number of inoculated lactic acid bacteria in starter fermentation was 10^6 cfu/g. The second fermentation was spontaneous. For each processing batch, the sliced, salted, and, in the case of starter fermentation, inoculated cabbage was packed by hand into 50-L plastic fermentation vats to contain 20 kg of raw material. The salted raw material in the chambers was covered aseptically with plastic, water-locked, and closed tightly. After packing, the fermentation chambers were incubated at 20 °C throughout fermentation. Each fermentation was performed in four parallel vats. The fermentations were allowed to proceed to pH 3.9 at 20 °C. After the fermentation processes were completed, juice was pressed, and the products were stored at +4 °C.

Samples. Samples were taken from the fermented cabbage and cabbage juice during and after fermentation as follows. For microbial analysis and pH measurement, samples of fermented cabbage juice were collected throughout fermentation. The first samples were collected from the fermentation vats after the mixing of salt and, in the case of starter fermentation, after the addition of the starter culture. The last samples were collected when the juice was pressed. Cabbage samples (50 g) were taken before fermentation (sliced raw cabbage) and when fermentation was completed (end product; fermented cabbage containing solid and liquid portions, sample taken after careful mixing of the content of the fermentation vat). The concentrations of nitrate, nitrite, and isothiocyanate were analyzed both in raw cabbage and in the end products. The glucosinolate content was analyzed in the fresh white cabbage (raw material), and the glucosinolate breakdown products were analyzed in end products. The content of flavonoids was determined both in raw cabbage and the end products, separately in the liquid and solid portions. All samples were stored at −20 °C before analysis, except for microbiological analysis and pH measurement.

Microbiological Analysis and pH. The samples for microbiological analysis and pH were taken from the brine during fermentation by using a sterile pipet. To obtain representative samples, equal volumes of juice were taken from three different places in the container from a depth of approximately 5 cm. The three samples were then pooled to form one sample. The number of lactic acid bacteria (cfu/mL) was enumerated regularly throughout fermentation. Yeasts, molds, and enterobacteria were determined when fermentation had proceeded for 20 h and after juice had been pressed. Lactic acid bacteria were cultivated anaerobically on an MRS nutrition medium (Biokar Diagnostics or Difco Laboratories) containing 0.02% sodium azide and 1.5% agar for 2−3 days at 30 °C. The sample volume plated was 0.1 mL. The yeasts and molds were enumerated by cultivation on YGC (yeast extract glucose chloramphenicol) -agar (Difco Laboratories). The sample (0.1 mL) was plated on the agar, and the Petri dishes were incubated for 7 days at 25 °C. Enterobacteria were cultivated on VRB (violet red bile) -agar (Biokar Diagnostics or Difco Laboratories) with 0.1% glucose added. The sample (0.1 mL) was plated on the agar and incubated for 2−3 days at 37 °C. All the analyses were performed in duplicate, and the results were averaged. The decrease in pH of the cabbage juice was measured during fermentation by using a RadiometerPHM93 (Copenhagen, Denmark) pH meter.

Nitrate, Nitrite, and Isothiocyanate Content. Nitrate, nitrite, and isothiocyanate were extracted from the samples with water according to Lyons et al. (*27*). The anions were analyzed using Hewlett-Packard's 1090 series HPLC equipped with a diode array detector. The analytical column was Ashahipak ODP-50 125 × 4.0 mm, 5 μm (Hewlett-Packard), operated at 40 °C. The isocratic mobile phase consisted of water with a UV absorbing mobile phase additive (Hewlett-Packard) and acetonitrile (86:14 v/v, pH 8.70). The decrease in absorbency indicating the elution of anions was monitored at 360 nm with a reference wavelength of 256 nm (*27*).

Flavonoid Content. Flavonoids were analyzed according to the method of Hertog et al. (*28*). Flavonoid aglycons were liberated from their sugar conjugates with hot acid hydrolysis. The flavonoids were analyzed using Hewlett-Packard's 1100 series HPLC with Novapack C-18 (Waters, 3.9 × 150 mm, 4 μm) as the analytical column. The mobile phase was a gradient of phosphate buffer (pH 2.4) and methanol (*28*).

Glucosinolate Content. The extraction and cleanup of glucosinalates in the cabbage samples were performed according to British Standard 4325 (*29*). The desulfoglucosinolates were analyzed using Perkin-Elmer's HPLC 200 system with LiChrospher RP-18 (3.9 × 150 mm, 5 μm) as the analytical column. The mobile phase was a gradient of water and acetonitrile (*30*).

Breakdown Products of Glucosinolates. The breakdown products of the glucosinalates were analyzed according to Daxenbichler and VanEtten (*30*) with slight modifications. The breakdown products extracted in methylene chloride were analyzed by GC-MS (Hewlett-Packard's 5890 series II gas chromatograph with 5972 mass selective detector) using pulsed−splitless injection (initial pressure 25 psi, split on-time 0.20 min after which pressure down to 7 psi at 99 psi/min, pressure from 7 to 19 psi at 1 psi/min in 19 min). HP-5 MS (30 × 0.25 × 0.25 μm) was used as the analytical column. The oven temperature started from 35 °C (hold 2 min) and was heated from 35 to 90 °C at 10 °C/min and finally to 270 °C at 15 °C/min. The injector was set to 250 °C, and the GC-MS transfer, to 260 °C. MS was employed at scan mode 40−550 *m*/*e*. Because of the lack of commercial standards for buten-4-isothiocyanate, sulforaphane nitrile, 3-methylsulfinylpropyl-isothiocyanate, and sulforaphane, their quantification was performed by using the relative ion ratio.

Statistical Analysis. Statistical analysis involved the use of the SAS Software Release 8.1 (TS1MO). Any significant differences between the means were determined by a T-test (two independent fermentations),

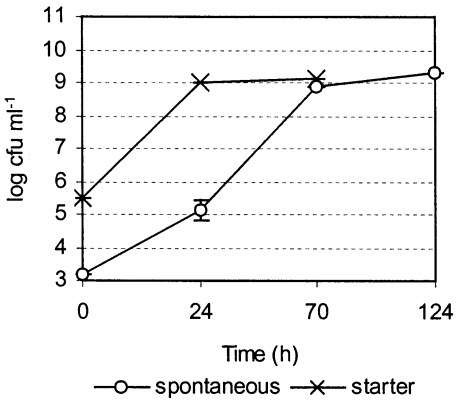

Figure 1. Number of lactic acid bacteria (cfu/mL) in starter-induced and spontaneous fermentation of sauerkraut (t = 0–124 h) (mean value and standard deviation, n = 4).

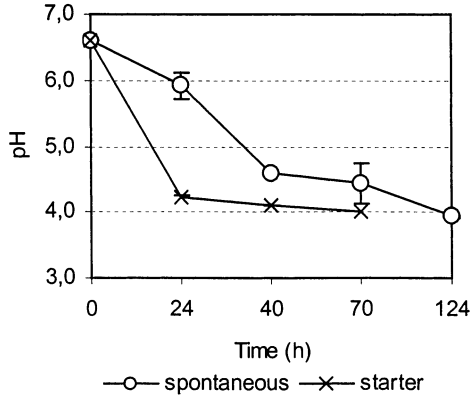

Figure 2. pH in starter-induced and spontaneous fermentation of sauerkraut (t = 0–124 h) (mean value and standard deviation, n = 4).

a Wilcoxon two-sample test (exact P-values), and a Median Two-sample test.

RESULTS AND DISCUSSION

Microbiology and Organoleptic Properties. The decrease in pH was clearly more rapid in starter fermentation compared to spontaneous fermentation (**Figure 2**). The spontaneous fermentation process was completed in 124 h whereas the starter fermentation was completed in 70 h. The number of lactic acid bacteria reached the same level (10^9 cfu/mL) in the starter and spontaneous fermentations in approximately 24 and 70 h, respectively (**Figure 1**). Low counts of yeasts and molds (9 cfu/mL) were found in the end product of the starter fermentation, whereas no yeast and molds were detected in spontaneous fermentation. No enterobacteria were detected in the end products. On this basis, the hygienic quality of the fermented products was good. The organoleptic properties of the sauerkraut and pressed juice from both fermentations were good.

Concentration of Biomolecules. The glucosinolate content in fresh white cabbage (cv. Lennox, Finland) is shown in **Table 1**. The predominant glucosinolates (3 μmol/g DW, dry weight) were sinigrin, glucoiberin, and glucobrassicin. The total content of glucosinolates was 3.71 μmol/g DW, which is significantly lower than reported in the studies by Tiedink et al. (*14*) (15 μmol/g DW) or by Kushad et al. (*13*) (11 μmol/g DW). The low glucosinolate concentrations in the present study may be due to differences between the cabbage cultivars as well as the long storage before the fermentation process. For example, Rosa (*12*) observed that the glucoiberin concentration in cabbage decreased with an increase in plant storage age. Moreover, Kushad et al. (*13*) also detected variation in the content of glucosinolates among different cabbage cultivars. They observed significant differences in the contents of sinigrin, gluconapin, and progoitrin. However, concentrations of indole glucosinolates were relatively similar among the tested cabbage cultivars (*13*). The fermenting of cabbage, as carried out in sauerkraut production, gradually decreased the glucosinolate levels to zero in this study, as was reported in earlier studies (*31*, *32*). 4-MeO-glucobrassicin was found in the final product in small quantities. Daxenbichler et al. (*21*) have reported finding no unhydrolyzed glucosinolates after two weeks' fermentation.

Of the 20 different flavonoids tested in the present study, only kaempferol was found (0.9 mg/kg FW, fresh weight). The result is in agreement with the studies of Hertog et al. (*33*), who found the content of quercetin in white cabbage and sauerkraut to be below 1 mg/kg FW and that of kaempferol to be below 2 mg/kg FW. In the present study, the kaempferol concentration remained constant in the cabbage (solid portion) throughout fermentation. No flavonoids were detected in the juice. This indicates that flavonoids remain in the cabbage fiber matrix over the fermentation process. *Brassicaceae* plants are reported to be a weak source of flavonols and flavonoids, which are mainly found in free-standing leaves (*34*). The concentration of flavonoids in the leaves is generally much higher than in other tissues of the same plant. Where heads are formed from leaves, as in cabbage, there is a significant decrease in the flavonol concentration from the outer to the inner leaves (*34*).

Breakdown Products of Glucosinolates. The quantified breakdown products of glucosinolates in the two sauerkraut fermentations are shown in **Figures 3** and **4**. The results suggest that the decomposition of glucosinolates may be affected by the starter bacteria. However, significant differences could not be concluded by a statistical analysis because of the small number of parallel samples (four parallel vats). Relatively high standard deviation in the content of the breakdown products in this study may be due to variation of glucosinolate content in raw cabbage. Several factors, such as soil type, plant spacing, light, temperature, and fertilizer, have been reported to affect the glucosinolate content of fresh vegetables (*11*, *12*). Sinigrin may be hydrolyzed to allyl isothiocyanate, allyl cyanide, or nitrile 1-cyano-2,3-epithiopropane (*21*). In the present study, the breakdown products of sinigrin were predominant. This result was expected, as sinigrin was also the predominant glucosinolate. The indole-3-carbinol (derived from glucobrassicin) concentration found in the sauerkraut fermented by using a starter culture was relatively low (12 μg/100 g FW), and in the end product of spontaneous fermentation, the concentration was under the detection limit. The poor stability of I3C in acidic solution could be one explanation of the low concentration (*35*). Further, ascorbigen may be formed from the reaction between I3C and ascorbic acid (*36*), and some authors suggest that ascorbigen can be the dominant end product of indole glucosinolates (*36*, *37*). Ascorbigen was not determined in present study. Dietary I3C has been shown to cause bifunctional induction of detoxifying enzymes in concentrations of 50−1000 ppm in the diet (*38*, *39*), thus acting as an anticarcinogen. Moreover, Staack et al. (*40*) reported that a mixture of glucosinolate breakdown products (similar to that found in this study) in a crucifer diet greatly enhances the synthesis of detoxification enzymes, and that the induction of detoxification enzymes is dose-dependent. Glucoraphanin was decomposed to sulforaphane and sulforaphane nitrile which was the only nitrile found in this study. The concentration of sulforaphane nitrile was markedly lower than sulforaphane despite the low

Table 1. Glucosinolate Content in Fresh Cabbage (μmol/g DW)

	present work		Tiedink et al., 1988		Kushad et al., 1999	
glucosinolate	mean ($n = 4$)	range	mean	range[b]	mean	range
sinigrin	1.43	0.86–2.03	4.2	nd	7.8	7.6–7.9
glucoiberin	1.06	0.73–1.29	6.8	nd	0.0	0.0
glucobrassicin	0.49	0.29–0.79	3.4	nd	0.9	0.8–1.0
progoitrin	0.18	0–0.37	0.2	nd	0.2	0–0.4
glucoraphanin	0.20	0–0.31	0.2	nd	0.1	0–0.7
gluconapin	0.2	0–2.24		nd	0.7	0.3–1.1
4-OH-glucobrassicin	0.035	0–0.08		nd	0.3	0.0
4MeO-glucobrassicin	0.11	0.07–0.16	0.4	nd	0.3	0.2–0.4
total	3.71[a]		15.2		10.9	

[a] Sum of individual glucosinolates. [b] Not determined.

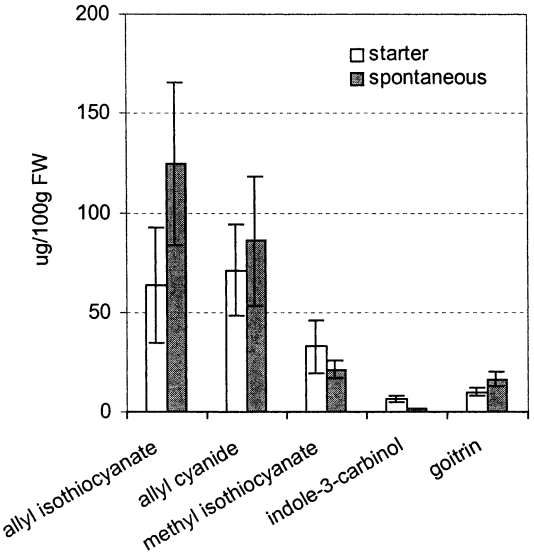

Figure 3. Quantified breakdown products of glucosinolates (μg/100 g FW) in starter-induced ($t = 70$ h) and spontaneous fermentation ($t = 124$ h) of fermented cabbage end product (mean value and standard deviation, $n = 4$).

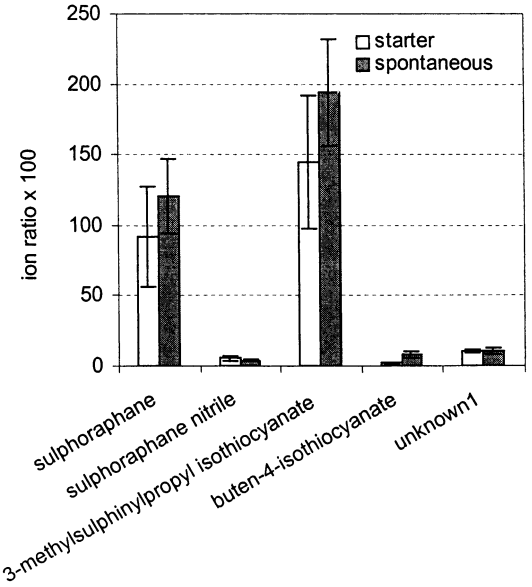

Figure 4. Relative ion ratios (×100) of glucosinolate breakdown products in starter-induced ($t = 70$ h) and spontaneous fermentation ($t = 124$ h) of fermented cabbage end product (mean value and standard deviation, $n = 4$).

pH of the sauerkraut. Acidic pH tends to enhance nitrile formation, and a basic pH and high temperature favor the formation of sulforaphane (*13, 17*).

The thiocyanate ion concentration was below the detection limit in all the samples. Daxenbichler et al. (*21*) found the concentration of thiocyanate ions in canned sauerkraut to be 0.9−1.7 mg/100 g. That is about half the theoretical value, which can be calculated from the glucoiberin content. The content of glucoiberin in fresh cabbage in the cited study (*21*) was about 13.0 mg/100 g (average).

We suggest the use of a cultivar high in glucosinolates to maximize the content of glucosinolate breakdown products in sauerkraut. The relatively low concentrations of the breakdown products in this study may be due to the low concentration of glucosinolates in the raw material, about 20−30% of the level reported by others (*13, 14*). Possible volatilization of some breakdown products during freeze storage and the long delay prior to analysis can also lower the yield.

Nitrate and Nitrite Content. Fertilizing the cultivation area has a strong influence on the nitrate and nitrite concentrations in cabbage. Nitrate and nitrite may be precursors of nitroso compounds, which are reported to have carcinogenic properties (*41*). Acceptable daily intake (ADI) is given (by WHO) as 3.65 mg nitrate per kilogram body weight (*42, 43*), equivalent to 219 mg nitrate per day for a person weighing 60 kg. In the present study, the content of nitrate in fermented cabbage was 100−200 mg/kg FW, and in raw material, 90−190 mg/kg FW. The levels of nitrite in all the samples were below the detection limit. On the basis of the available data (*42, 43*), neither the nitrate nor nitrite concentrations found in the present study represent a risk to human health.

It can be concluded that glucosinolates are decomposed during the cabbage fermentation process to form several potentially beneficial breakdown products such as isothiocyanates, indole-3-carbinol, and sulforaphane. The concentrations of goitrin and sulforaphane nitrile remained low as did nitrate and nitrite levels, thus representing no risk to human health. Further studies are still needed to determine the effects of different starter cultures and combinations of lactic acid bacteria in the decomposition of glucosinolates.

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