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Enzyme-Assisted Extraction Enhancing the Phenolic Release from Cauliflower (*Brassica oleracea* L. var. *botrytis*) Outer Leaves

Nguyen Thai Huynh,[†,‡,§] Guy Smagghe,[‡] Gerard Bryan Gonzales,[†,‡,§] John Van Camp,[†] and Katleen Raes*[,§]

[†]Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, and [‡]Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

[§]Laboratory of Food Microbiology and Biotechnology, Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University − Campus Kortrijk, Graaf Karel de Goedelaan 5, 8500 Kortrijk, Belgium

ABSTRACT: Phenolic compounds are highly present in byproducts from the cauliflower (*Brassica oleracea* L. var. *botrytis*) harvest and are thus a valuable source for valorization toward phenolic-rich extracts. In this study, we aimed to optimize and characterize the release of individual phenolic compounds from outer leaves of cauliflower, using two commercially available polysaccharide-degrading enzymes, Viscozyme L and Rapidase. As major results, the optimal conditions for the enzyme treatment were: enzyme/substrate ratio of 0.2% for Viscozyme L and 0.5% for Rapidase, temperature 35 °C, and pH 4.0. Using a UPLC-HD-TOF-MS setup, the main phenolic compounds in the extracts were identified as kaempferol glycosides and their combinations with different hydroxycinnamic acids. The most abundant components were kaempferol-3-feruloyldiglucoside and kaempferol-3-glucoside (respectively, 37.8 and 58.4 mg rutin equiv/100 g dry weight). Incubation of the cauliflower outer leaves with the enzyme mixtures resulted in a significantly higher extraction yield of kaempferol-glucosides as compared to the control treatment.

KEYWORDS: *Brassica oleracea, cauliflower outer leaves, enzyme-assisted extraction, release of phenolic compounds, kaempferol, flavonoids*

1. INTRODUCTION

Cauliflower (*Brassica oleracea* L. var. *botrytis*) is one of the cruciferous vegetables belonging to the *Brassicaceae* family that are widely consumed all over the world. These products contain considerable amounts of health beneficial compounds, such as phenolic compounds, glucosinolates, and vitamins.[1−4] These vegetables are also characterized by their high amount of nonedible parts, such as outer leaves, stems, and pods. These nonedible parts are now valorized only as raw materials for industrial fertilizer, animal feed,[5] and fiber production,[6,7] or they are left on the fields. However, as they contain high amounts of bioactive compounds, their valorization potential can be much higher.

In the past, a number of techniques have been applied to obtain phenolic compounds from plant materials, such as cold pressing, supercritical fluid extraction, and organic solvent extraction.[8,9] Nevertheless, the drawback of these methods is the low extraction yield as the phenolic compounds are bound to plant cell wall material. In cauliflower leaves, just as in other vegetables, phenolic compounds may be classified as bound phenolics found in cell walls in which they are linked to polysaccharides by ester bonds, hydrophobic interactions, and hydrogen bonds, and as free phenolic compounds found in the vacuoles of plant cells.[10−12] As a consequence, preprocessing techniques prior to extraction may be used to reduce the loss of bioactive components and to improve the yields of the extraction process. Degradation and disruption of the cell-wall matrix have been considered as an appropriate step to improve the release of phenolic compounds, keeping their stability and antioxidant activity.[13,14] The mechanism for this treatment is based on the use of cell-wall degrading enzymes to depolymerize cell-wall polysaccharides,[15] and to hydrolyze the glycosidic linkages between phenolic compounds and cell-wall polymers.[12] In addition, enzyme systems originating from microorganisms can transglycosylate the target compounds.[16,17] As a result, not only the structure of cell walls can be weakened and broken down, whereby intracellular materials are more exposed for extraction,[13,14] but also the solubility of the target compounds in the extractant can be improved.[16]

The successful application of carbohydrate-cleaving enzymes for the extraction of phenolic compounds has been reported in several studies, mainly focusing on other plant sources, such as apple peel, citrus peel, grape pomace, *Thymus vulgaris*, *Ginkgo biloba* leaves, berries, and oat bran.[10,13−16,18−21] However, the investigation field was restricted to the factors influencing enzyme-assisted extraction of phenolic components,[14,22] and information on the impact of enzymatic treatment on the release of individual components from plant waste material is lacking. The aim of this study was to investigate the potential of using enzyme-assisted extraction and to evaluate its effect on the yield and the profile of extracted phenolic compounds from *Brassica* cauliflower outer leaves.

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2. MATERIALS AND METHODS

2.1. Materials. *Plant Material.* The outer fresh leaves, a byproduct from the cauliflower harvest, were collected from a local farm in West-Flanders, Belgium, during the harvesting of cauliflowers from one field in July 2012. The leaves were kept frozen at −18 °C until further analysis.

Chemicals and Reagents. Folin-Ciocalteu reagent and sodium carbonate (Na_2CO_3) were purchased from VWR International (Leuven, Belgium), and gallic acid, hesperetin, and rutin were from Sigma-Aldrich (Bornem, Belgium). Viscozyme L and Rapidase vegetable juice were purchased from Novozyme (Bagsvaerd, Denmark) and DSM Food Specialties (Delft, The Netherlands), respectively.

Viscozyme L, produced by *Aspergillus aculeatus*, contains different polysaccharide-degrading enzymes, including beta-glucanase, cellulase, hemicellulase, and xylanase. The specific enzyme activity of Viscozyme L, as given by the supplier, is 120 Fungal Beta-Glucanase units (FBG)/mL, in which 1 FBG is the amount of enzyme required under standard conditions (30 °C, pH 5.0, and 30 min reaction time) to degrade barley α-glucan to reducing carbohydrates with a reducing power corresponding to 1 μmol glucose/min.

Rapidase vegetable juice is an enzyme mixture produced by both *Aspergillus niger* and *Trichoderma longibrachiatum*. As stated by the supplier, it contains pectinase and hemicellulase (optimal pH 4.0−5.0, optimal temperature 10−55 °C).

2.2. Experimental Procedure. *Enzyme-Assisted Pretreatment.* The frozen outer leaves of cauliflower were cut into pieces (2 × 5 cm), followed by 3 min mixing using a kitchen homogenizer. Enzymes were first dissolved in water taking into account the appropriate concentrations to obtain a correct E/S (cauliflower outer leaves) ratio. The cabbage mixture (60 g) then was placed in a 500 mL glass vial, and 90 mL of aqueous enzyme solution was added. In a first series of experiments, we searched for the optimal conditions for the two enzyme pretreatments. A first set of incubations was done at 40 °C, pH 4.0 with different enzyme concentrations (0−5% E/S ratio). In the second set with the optimal enzyme concentrations (E/S ratio of 0.2% for Viscozyme L and 0.5% for Rapidase), the incubation temperature varied from 30 to 50 °C, always at pH 4.0. In a last step, the optimal enzyme concentrations and optimal temperature (35 °C) were used, while the pH value varied from 3.0 to 6.0. All incubations were performed in a temperature-controlled water bath with agitation. Samples of the treated cabbage were taken at 0, 6, and 24 h for methanol extract, and a sample of the supernatans was taken at 24 h, representing the aqueous extract. All samples were stored at −18 °C for further analysis of total phenolic content. All incubations were done in triplicate.

Using the optimal conditions for both enzymes obtained in the first experiment, a second series of experiments was performed to investigate the enzymatic kinetics of the two enzymes. All of the experimental parameters were the same as in the first series of experiments, except that samples were taken at 2 h intervals during the first 12 h and then at 30 h. All incubations were done in triplicate. Samples were stored at −18 °C for analysis of total phenolic content, and to profile the individual phenolic compounds.

Extraction of Phenolic Compounds. Samples were divided into two parts. Supernatant from the liquid phase, after 24 h of incubation, was obtained by centrifugation (13 000*g*, 10 min, 4 °C) and filtered through filter paper (VWR, grade 413) (aqueous extract, AE). The solid samples at 0, 6, 12, and 24 h of incubation were extracted by the method according to Olsen et al.[23] In brief, 15 mL of methanol was added to 5 g of sample, and the suspension was homogenized at 9500 rpm using an ultraturrax for 40 s, followed by incubation in ice water for 15 min. Supernatant was collected after centrifugation (13 000*g*, 10 min, 4 °C). The pellet obtained was then re-extracted by the same procedure, using 10 mL of methanol/water (80/20; v/v). Both extracts were pooled and filtered through filter paper (VWR, grade 413) before adding methanol to a final volume of 25 mL (methanolic extract, ME).

2.3. Analytical Methods. *Determination of Total Phenolic Content.* Total phenolic content was determined according to Folin−Ciocalteu,[24] with slight modifications. In brief, 1.8 mL of deionized water was added to 0.2 mL of sample. After thorough mixing, Folin−Ciocalteu reagent (0.5 mL) was added, and the tube was shaken vigorously. After 6 min, 1.5 mL of sodium carbonate solution (20%) was added, followed by adding deionized water to a final volume of 5 mL and mixing well again. The solution was then allowed to stand in the dark for 2 h at ambient temperature, and the absorbance was read at 760 nm. Total phenolic content (TPC) was expressed as milligrams of gallic acid equiv per 100 g of dry weight (mg GAE/100 g DW) by using a standard curve of gallic acid in the range of 10−100 mg/L. The blank was prepared with 0.2 mL of methanol instead of sample solution.

Sample Purification Using SPE. In brief, 1 mL of sample was diluted in water (0.1% formic acid) up to 20 mL of total volume, followed by adding 100 μL of 100 μg/mL hesperetin as internal standard. The mixture was slowly loaded onto a solid-phase extraction (SPE) C18 column (50 mg, 4 mL; Davison Discover Science, Deerfield, IL), which was previously preconditioned with methanol (0.1% formic acid). The column then was washed with 5 mL of water (0.1% formic acid) prior to loading 3 mL of methanol (0.1% formic acid). The eluent obtained was evaporated under nitrogen flux. After drying, the residue of the sample was redissolved in 100 μL of methanol (0.1% formic acid) prior to adding 900 μL of water (0.1% formic acid). The sample obtained was kept at −18 °C until UPLC-DAD-HDMS-TOF-MS analysis.

Quantification and Identification of Phenolic Compounds by UPLC-DAD-HDMS-TOF-MS. UPLC-DAD analysis was performed on a bride ethylene hybrid (BEH) C18 column (150 × 2.1 mm, 1.7 μm particle size) by using an Ultimate U(H)PLC (Dionex, Breda, The Netherlands). The column and autosampler temperatures were kept at 40 and 4 °C, respectively. The mobile phase was formed by two solvents, solvent A (water containing 0.1% formic acid; v/v) and solvent B (methanol containing 0.1% formic acid; v/v). A stable flow rate of 250 μL/min was applied at 0 min, 90% A; followed by linear and isocratic gradient of eluting solvents as 0−6 min, 20% B linear; 6−12 min, 20% B isocratic; 12−13 min, 30% B linear; 13−23 min, 50% B linear, 23−30 min, 90% B linear; 30−35 min, 90% isocratic; 35−40 min, 10% B linear; and 40−45 min 10% B isocratic. Quantification was achieved by comparing the peak areas to a calibration curve made using rutin. Quantities of the compounds were expressed as rutin equivalents (RE).

Identification was achieved using an ACQUITY UPLC system (Waters, Milford, MA) coupled to a Synapt HDMS-TOF mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray ionization source. The working conditions of MS analysis were: capillary voltage, 2 kV; sampling cone voltage, 40 V; extraction cone voltage, 4 V; source temperature 150 °C; desolvation temperature, 350 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 550 L/h. Collision energies for the trap and transfer were set at 6 and 4 V for low energy, while a ramp of 15−45 V was applied for the high energy collision-induced dissociation. The instrument was operated in negative and positive continuum mode, and data were acquired using Waters MassLynx software. Detailed information on the MS analysis and peak identification is described by Gonzales et al.[25]

2.4. Statistical Analysis. The data are given as mean ± standard deviation (SD) for the optimization of the enzyme treatment and as mean with the standard error of mean (SEM) for the individual phenolic compounds. One-way and two-way ANOVA were used to determine significant differences between individual means using Tukey posthoc test for P values < 0.05. Principal component analysis (PCA) was applied to visualize differences in effects between treatments as well as incubation times based on the contents of individual phenolic compounds. The SPSS software v.22 (IBM, Chicago, IL) was used for all statistical analyses.

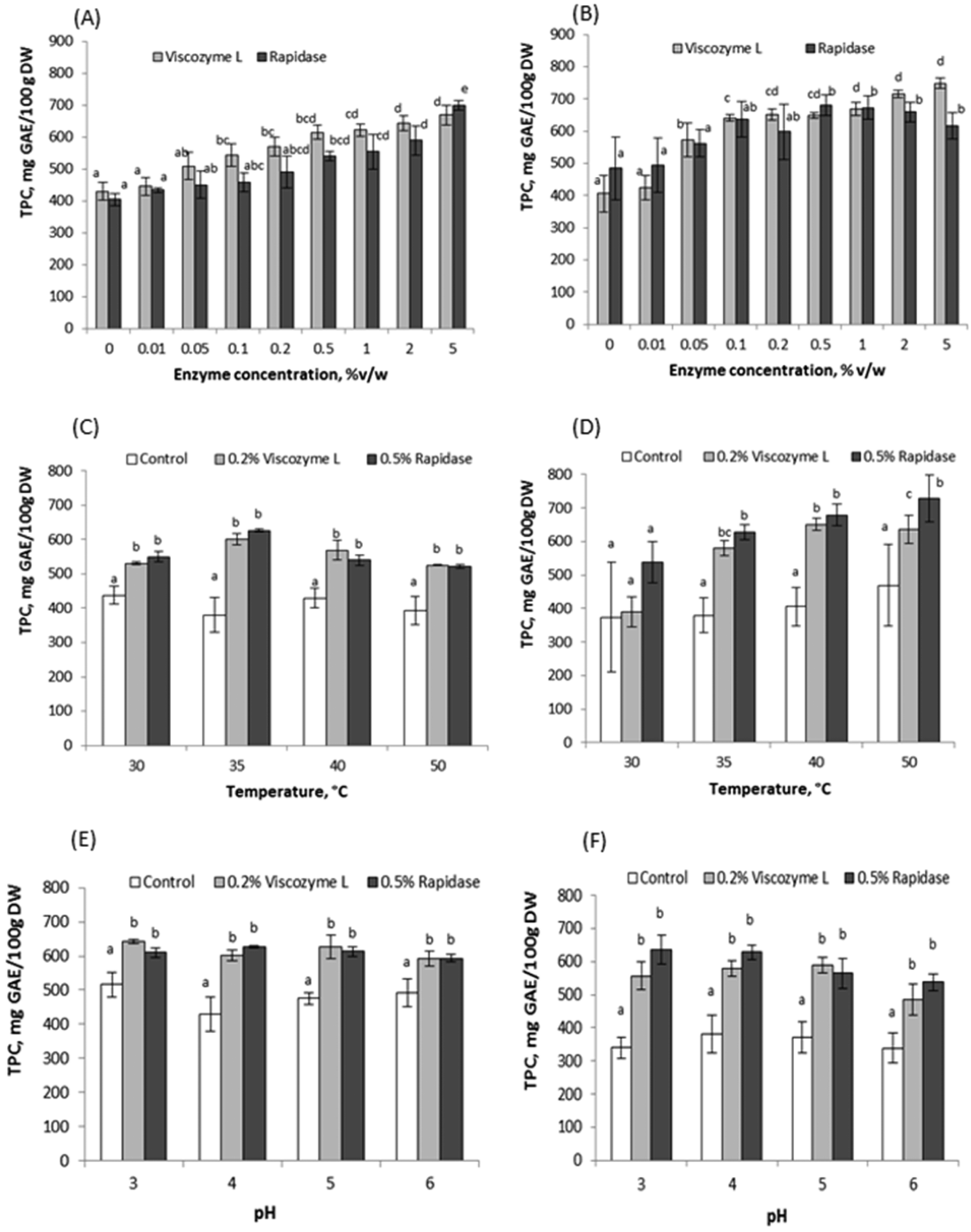

Figure 1. Effect of enzyme concentration (A,B), temperature (C,D), and pH (E,F) on the extraction of phenolic compounds from outer leaves of cauliflower after 24 h of incubation. (A,C,E) Total phenolic content in AE assisted by enzymes. (B,D,F) Total phenolic content in ME after enzymatic pretreatment. Different letters (a−e) indicate significant difference (P value < 0.05) within the same color column. The total phenolics of methanolic extract from cabbage solid residue at 0 h of incubation (control sample): 336 ± 30 mg GAE/100 g DW (n = 3).

3. RESULTS

3.1. Optimization of Enzyme-Assisted Extraction of Phenolic Compounds. Independent of the treatment (type of enzyme, concentration of enzyme, temperature, and pH), the TPC at 0 h of incubation in the ME was 336 ± 30 mg GAE/100 g DW. During 24 h of incubation, it was observed that enzyme dosages, temperature, and pH, as well as their two-way interaction term with time significantly affected the total phenolic release (P-value < 0.001), with an exception for Rapidase concentration × time.

Effect of Enzyme Concentration. The total phenolic contents of AE (Figure 1A) and of ME (Figure 1B) after 24 h enzyme-assisted extraction at 40 °C and pH 4.0 of the cauliflower outer leaves were measured in response to the different concentrations of enzymes (Viscozyme L and Rapidase) used. The level of phenolic compounds in both extracts was significantly increased after 24 h of incubation as compared to the control incubation, when 0.1% Viscozyme L and 0.5% Rapidase were added. Viscozyme L resulted in a higher concentration of phenolic compounds in AE, ranging

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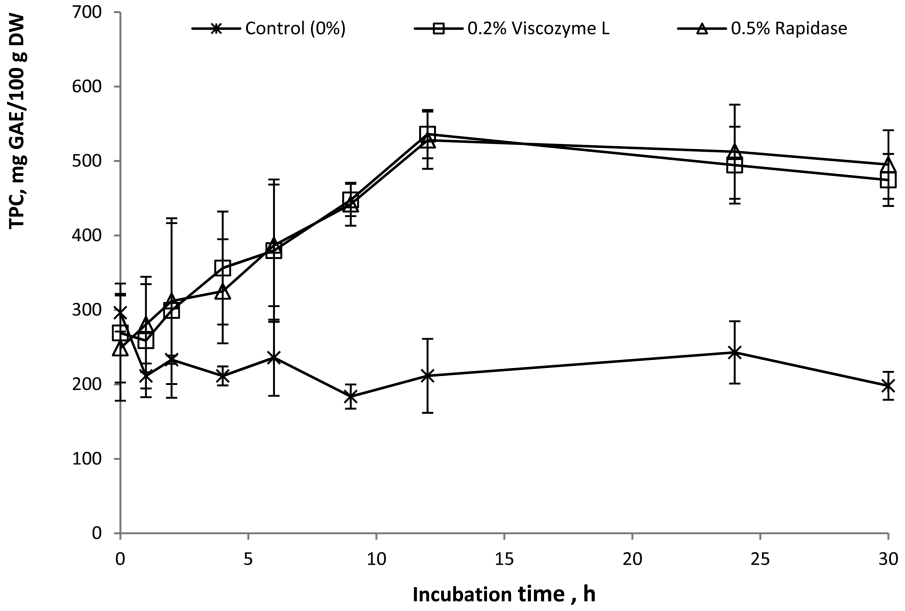

Figure 2. Changes in total phenolic content in ME from solid residue incubated with Viscozyme L and Rapidase at 35 °C, pH 4.0, during 30 h (n = 3).

Table 1. Identification of Flavonoid Glycosides from Cauliflower Byproduct Extract[a]

peak	Rt (min)	molecular ion mass	fragments	λ max (nm)	identity
1	7.8	359.07	359.07	213, 307	unknown 1
2	9.9	950.23	771, 609, 285	nd	kaempferol-C-glucoside-3-glucose units
3	10.8	963.25	963, 609, 771, 801, 284	nd	kaempferol-3-glucoside-7-hydroxyferuloyldiglucoside
4	11.11	933.21	933, 609, 285	265, 345	kaempferol-3-diglucoside-7-diglucoside
5	11.79	785	nd	nd	unknown 2
6	12.16	nd	nd	269, 318	unknown 3
7	13.26	977.35	977, 815, 609, 284, 446	268, 338	kaempferol-3-sinapoyldiglucoside-7-glucoside
8	13.59	1109.30	1109, 947, 771, 609, 447, 285	268, 331	kaempferol-3- feruloyltriglucoside-7-glucoside
9	13.92	947.24	947, 785, 609, 284	269, 322	kaempferol-3-feruloylglucoside-7-diglucoside
10	19.46	787.18	787, 625, 462, 300	254, 348	kaempferol-3- diglucoside-7-glucoside
11	20.07	609.12	609, 284	265	kaempferol-3-diglucoside
12	20.75	785.18	785, 609, 284	329	kaempferol-3-feruloyldiglucoside
13	21.08	755.18	755, 609, 284	320	kaempferol-3-coumaroyldiglucoside
14	23.39	447.07	447, 284	nd	kaempferol-3-glucoside
15	24.28		623, 447, 284	nd	kaempferol-C-feruloylglucoside/kaempferol-3-feruloylglucoside

[a]Rt, retention time; nd, not detected.

from 429 ± 28 mg GAE/100 g DW at 0% E/S (control) to 614 ± 23 mg GAE/100 g DW at 0.5% E/S, with no further significant increase in TPC at higher enzyme concentrations (Figure 1A). Similar results were found for the ME, whereby the TPC in ME significantly increased to 650 ± 18 mg GAE/100 g DW at 0.2% E/S, without further significant improvement between 0.2% and 5% E/S (Figure 1B). The Rapidase treatment with 0.5% E/S resulted in a greater release of phenolic compounds, mainly in the ME and to a lesser extent in the AE, as compared to the control sample (Figure 1A,B), whereas an increased Rapidase concentration from 0.5% to 5% did not enhance the TPC in AE and ME, except for AE from sample treated with 5% Rapidase (Figure 1B). On the basis of the results of the different enzyme concentrations, it was chosen to work further with 0.2% Viscozyme L and 0.5% Rapidase.

Temperature. The influence of incubation temperature on the extraction of phenolic compounds from cauliflower outer leaves using 0.2% Viscozyme L or 0.5% Rapidase at a pH of 4 is given in Figure 1C and D. The treatments with Viscozyme L and Rapidase resulted in a significant improvement of TPC at all temperature points examined, ranging from 30 to 50 °C, as compared to the control (no enzyme) incubation, except for ME at 30 °C, where no difference between enzymatic treatment and control sample was found (Figure 1D). Although a trend toward a higher release of phenolic compounds with higher temperatures was observed in the ME, this was not observed in AE. The TPC in ME was significantly higher at 35 °C than at 30 °C for Viscozyme L and Rapidase treatment, whereas no further increase in total phenolic release between 35 to 50 °C was observed for both ME and AE. Even the TPC at 50 °C was lower than that at 40 °C with Viscozyme L treatment. Therefore, 35 °C was chosen as the optimal incubation temperature for both enzymes.

Effect of pH. During enzymatic treatment, the pH values were varied from 3 to 6 to investigate its effect on extraction

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Table 2. Effect of Different Treatments on the Profile of Phenolic Release in ME from Cauliflower Outer Leaves (n = 3)[a]

methanol extraction							
			treatment				
peak	individual compound (mg RE/100 g DW)	Rt (min)	control	viscozyme	rapidase	SEM	P value
1	unknown 1	7.8	10.1 a	16.0 b	12.2 ab	1.1	0.003
2	kaempferol-C-glucoside-3-glucose units	9.9	10.0	19.4	15.0	3.9	0.250
3	kaempferol-3-glucoside-7-hydroxyferuloyldiglucoside	10.8	6.1 a	10.8 b	8.1 ab	1.2	0.034
4	kaempferol-3-diglucoside-7-diglucoside	11.11	5.7 a	8.1 b	6.0 a	0.5	0.007
5	unknown 2	11.79	5.0 a	6.8 b	5.1 ab	0.5	0.030
6	unknown 3	12.16	5.0	6.4	4.8	0.6	0.125
7	kaempferol-3-sinapoyldiglucoside-7-glucoside	13.26	6.5 a	11.5 b	8.2 ab	1.2	0.026
8	kaempferol-3-feruloyltriglucoside-7-glucoside	13.59	9.0 a	21.0 b	14.8 ab	3.2	0.046
9	kaempferol-3-feruloylglucoside-7-diglucoside	13.92	5.8 a	10.0 b	7.3 ab	0.8	0.007
10	kaempferol-3-diglucoside-7-glucoside	19.46	7.1 a	12.5 b	11.8 ab	1.5	0.033
11	kaempferol-3-diglucoside	20.07	48.0	52.8	43.6	4.5	0.369
12	kaempferol-3-feruloyldiglucoside	20.75	17.2 a	37.8 b	28.7 ab	3.5	0.002
13	kaempferol-3-coumaroyldiglucoside	21.08	10.0 a	16.7 b	13.1 ab	1.3	0.004
14	kaempferol-3-glucoside	23.39	8.1 a	58.4 b	29.9 c	5.4	<0.001
15	kaempferol-C-feruloylglucoside/kaempferol-3-feruloylglucoside	24.28	7.1 a	12.2 b	10.7 a	1.1	0.012
	total compounds		160.3 a	300.0 b	219.2 a	19.8	<0.001

[a]Different letters (a, b, c) indicate significant difference (P value < 0.05) within a row. SEM, standard error of mean.

Table 3. Effect of Incubation Time on the Profile of Phenolic Release in ME from Cauliflower Outer Leaves (n = 3)[a]

methanol extraction								
			incubation time (h)					
peak	individual compound (mg RE/100 g DW)	Rt (min)	0	6	12	24	SEM	P value
1	unknown 1	7.8	13.0	12.7	13.7	11.6	1.3	0.732
2	kaempferol-C-glucoside-3-glucose units	9.9	32.8 b	10.9 a	8.6 a	6.9 a	4.5	0.001
3	kaempferol-3-glucoside-7-hydroxyferuloyldiglucoside	10.8	12.3 b	8.8 ab	6.6 a	5.7 a	1.4	0.011
4	kaempferol-3-diglucoside-7-diglucoside	11.11	6.1	7.7	7.0	6.1	0.6	0.441
5	unknown 2	11.79	5.0	6.2	6.2	5.2	0.6	0.364
6	unknown 3	12.16	4.4	5.9	6.1	5.1	0.7	0.276
7	kaempferol-3-sinapoyldiglucoside-7-glucoside	13.26	9.2	9.7	8.1	7.8	1.4	0.757
8	kaempferol-3-feruloyltriglucoside-7-glucoside	13.59	23.2 b	15.7 ab	12.6 ab	8.1 a	3.7	0.051
9	kaempferol-3-feruloylglucoside-7-diglucoside	13.92	9.6 b	8.5 ab	7.1 ab	5.7 a	1.0	0.047
10	kaempferol-3-diglucoside-7-glucoside	19.46	8.0	12.8	9.4	11.6	1.7	0.230
11	kaempferol-3-diglucoside	20.07	31.6 b	61.2 a	59.6 a	39.9 b	5.2	0.001
12	kaempferol-3-feruloyldiglucoside	20.75	12.0 b	26.2 ab	33.7 a	39.7 a	4.1	<0.001
13	kaempferol-3-coumaroyldiglucoside	21.08	6.6 b	15.3 a	16 a	15 a	1.5	<0.001
14	kaempferol-3-glucoside	23.39	6.1 c	26.3 bc	37.2 ab	59.0 a	6.3	<0.001
15	kaempferol-C-feruloylglucoside/kaempferol-3-feruloylglucoside	24.28	5.7 b	10.7 a	11.6 a	11.6 a	1.3	0.008
	total compounds		185.6	237.9	243.6	239.1	22.9	0.258

[a]Different letters (a, b, c) indicate significant difference (P value < 0.05) within a row. SEM, standard error of mean.

yields of total phenolic compounds (Figure 1E and F). The results obtained showed that the TPC did not significantly change in response to the difference in pH values during extraction for both AE and ME, within one enzymatic treatment.

3.2. Identification and Quantification of Phenolic Compounds. Using the optimal conditions for the enzyme treatment, the individual phenolic compounds released from the cauliflower outer leaves were identified and quantified. The shift in the phenolic profile during time of incubation and type of enzyme was studied.

Kinetics of the Release of TPC at Optimal Conditions. Selecting the optimal conditions obtained for enzyme treatment (0.2% Viscozyme L or 0.5% Rapidase, temperature 35 °C, and pH 4), we evaluated the effects of incubation time on the phenolic extraction from outer leaves of cauliflower (Figure 2). The TPC in ME of the control incubation remained stable. A significant increase in the TPC in the ME after enzyme treatment was observed, with a more than 100% increase after 12 h of incubation for the enzyme-treated incubations as compared to the control ones. The TPC of ME increased as a function of incubation time. No difference in TPC in the ME extract was observed between the two enzyme treatments. After 12 h of incubation, the TPC in the ME remained constant, and this trend was observed for both enzymes. The enzymatic treatments also caused significant effects on released phenolic compounds in AE after 30 h of incubation, increasing to 581 ± 16 mg GAE/100 g DW (with Viscozyme L) and to 604 ± 9 mg GAE/100 g DW (with Rapidase) as compared to 457 ± 23 mg GAE/100 g DW for the control without enzyme treatment.

Identification and Quantification of Individual Compounds by UPLC-DAD-HDMS-TOF-MS. The phenolic com-

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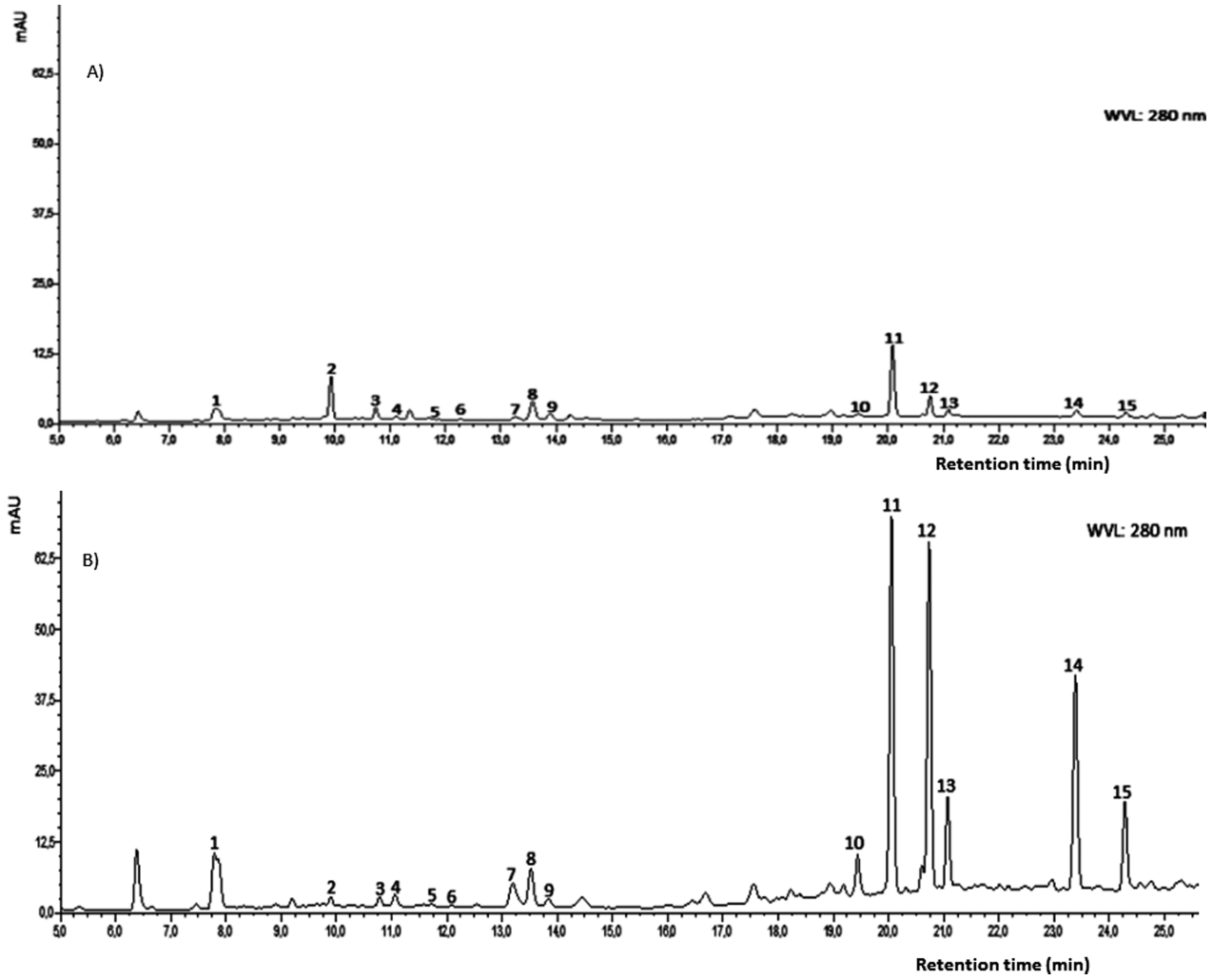

Figure 3. U(H)PLC profile of phenolic compounds from cauliflower byproduct extracts obtained by an incubation of 0 h (A) and 12 h (B) with Rapidase, recorded at 280 nm (see Table 1 for identification of peaks).

pounds in cauliflower outer leaves extracts were quantified by UPLC-DAD and identified by the UPLC-MS/MS method. As there was no interaction effect between treatment and time of incubation on the content of the individual phenolic compounds, results are presented for the main factors separately, that is, treatment and time of incubation in Tables 2 and 3, respectively.

As shown in Figure 3 and Table 1, 15 individual compounds were identified, of which three components in the extracts could not be identified by our protocol (unknown 1, 2, and 3). All individual phenolic compounds present in the extracts and identified by our analysis belonged to different derivatives of kaempferol. The results also indicated that the treatment with Rapidase did not enhance the amount of all identified compounds released as compared to the control with an exception for kaempferol-3-glucoside. In contrast, there was a clear increase in identified compounds by Viscozyme L treatment. The release of kaempferol-3-feruloyltriglucoside-7-glucoside (compound 8; 21.0 mg RE/100 g DW), kaempferol-3-feruloyldiglucoside (compound 12; 37.8 mg RE/100 g DW), and kaempferol-3-glucoside (compound 14; 58.4 mg RE/100 g DW) was sharply improved by Viscozyme L treatment, as compared to the control sample (9.0, 17.2, and 8.1 mg RE/100 g DW, respectively) (Table 1). As shown in Table 2, in general the release of the total phenolic compounds increased during time of incubation, although the increase was not significant. However, the amounts of individual components including kaempferol-3-feruloyldiglucoside (compound 12), kaempferol-3-coumaroyldiglucoside (compound 13), kaempferol-3-glucoside (compound 14), and kaempferol-*C*-feruloylglucoside/kaempferol-3-feruloylglucoside (compound 15) were significantly increased after 12 or 24 h of incubation in comparison with nontreatment samples (0 h).

The changes in profiles of phenolic compounds affected by different treatments were visualized by using PCA (Figure 4). The PC 1 (86.69%) described the distinct difference between the samples without adding enzymes (control-AE) and the samples treated by enzymes, which are mostly localized on the positive axis of PC1, except for control-ME. All of the phenolic components released were positively related to the Viscozyme L and Rapidase used. The PC2 (13.20%) can be expressed as a factor associated with the profile of individual compounds in ME, as compared to AE. The observation from the graph indicated that the high values of compounds 7, 8, 12, 13, 14, and 15 are associated with AE (cluster of Viscozyme-AE, Rapidase-AE), while the phenolic profile in ME exhibited higher content of compounds 2, 4, 5, 6, 9, and 11.

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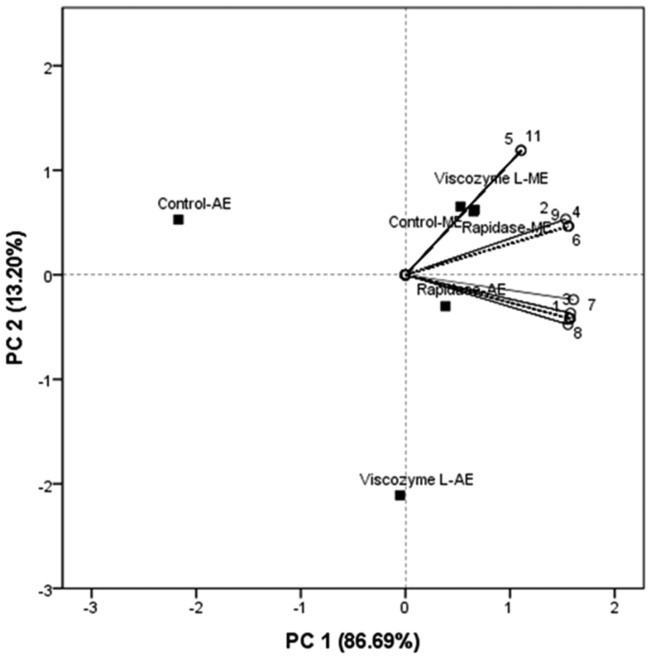

Figure 4. Principal component analysis of the individual phenolic compounds in AE and ME by different treatments (n = 3). The numbers in the PCA plot represent the different phenolic compounds as described in Table 1. Control-AE, aqueous extract obtained by an incubation of 30 h without enzyme; Viscozyme L-AE, aqueous extract obtained by an incubation of 30 h with Viscozyme L; Rapidase-AE, aqueous extract obtained by an incubation of 30 h with Rapidase; Control-ME, methanolic extract obtained by an incubation of 12 h without enzyme; Viscozyme L-ME, methanolic extract obtained by an incubation of 12 h with Viscozyme L; Rapidase-MA, methanolic extract obtained by an incubation of 12 h with Rapidase.

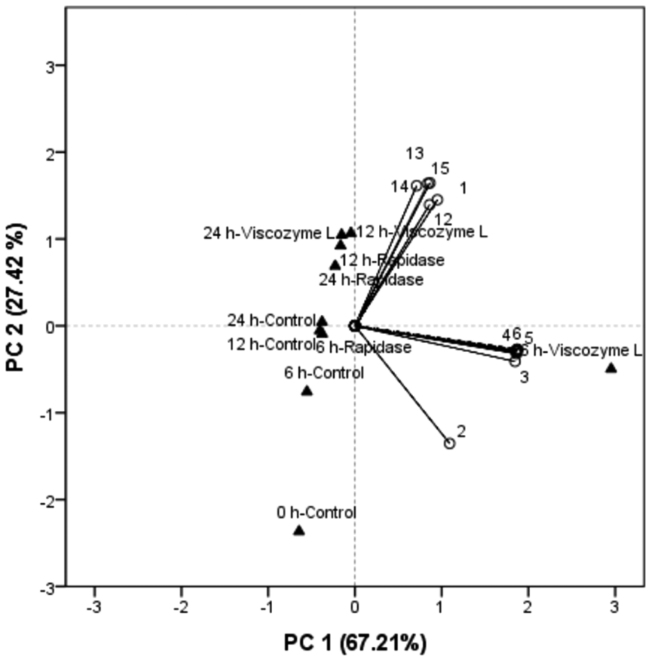

Figure 5. Principal component analysis of the individual phenolic compounds in ME from different time of treatments (n = 3). The numbers in the PCA plot represent the different phenolic compounds as described in Table 1. 0 h-Control, 6 h-Control, 12 h-Control, 24 h-Control represent methanolic extracts obtained by an incubation of 0, 6, 12, and 24 h without enzyme, respectively; 0 h-Viscozyme L, 6 h-Viscozyme L, 12 h-Viscozyme L, 24 h-Viscozyme L represent methanolic extracts obtained by an incubation of 0, 6, 12, and 24 h with Viscozyme L, respectively; 0 h-Rapidase, 6 h-Rapidase, 12 h-Rapidase, 24 h-Rapidase represent methanolic extracts obtained by an incubation of 0, 6, 12, and 24 h with Rapidase, respectively.

Figure 5 shows the effect of incubation time on the profile of phenolic release. Two PCs are responsible for 94.63% of the total variance of data. An incubation of 6 h with Viscozyme L was characterized for the high amount of compounds 2, 3, 4, 5, and 6, while the longer period of Viscozyme L and Rapidase treatments (12 h, 24 h) positively contributed to the high content of compounds 1, 12, 13, 14, and 15.

4. DISCUSSION

The enzyme-assisted extraction yield of phenolic compounds depends on multiple variables including type of enzyme and concentration, incubation temperature, pH, and incubation time. In this experiment, the optimization of Viscozyme L and Rapidase-assisted enzymatic pretreatment was studied on cauliflower outer leaves, with the focus on the identification of the released phenolic compounds. The significantly improved release of total phenolic compounds in both AE and ME for Viscozyme L (0.2%) and Rapidase (0.5%) treatment as compared to the control treatment clearly indicated that the hydrolysis of cell-wall components of cabbage improves the extraction yields of phenolic compounds. This observation could be explained by an increased degradation of the cell-wall structure as a result of the hydrolysis of cell-wall components, especially glycosidic bonds/linkages between phenolic compounds and cell-wall polysaccharides.[12,26] In addition, it is known that the permeability and porosity of plant cells increase by the breakdown of cell-wall polymers, improving the solubility of cell internal components, and in turn this results in an increase in concentration of phenolic compounds present in the extracts.[10,16] The treatments with higher enzyme doses did not increase the release of total phenolic compounds (p > 0.05), which could be due to competitive adsorption to the cell-wall polysaccharide when a higher enzyme concentration is applied. This leads to steric hindrance of binding positions of enzymes to the substrate, which negatively influences the breakdown of cell-wall components.[27,28] The lack of improvement at higher enzyme concentrations is in accordance with previous studies, for example, on black currant pomace,[27] or on unripened apples.[22]

The increased phenolic concentration in both AE and ME at all temperature incubations in comparison with the control sample demonstrates the importance of enzymatic catalysis to release phenolic compounds from cauliflower leaves. The significant enhancement of TPC in ME by treatments with 0.2% Viscozyme L and 0.5% Rapidase at higher temperature (35, 40, and 50 °C) could be caused by an increase of the hydrolysis activity and overall porosity of cell-wall structure, improving hydrolysis of the linkages between bound-phenolics and cell-wall compositions.[12,29] However, at 50 °C for Viscozyme L, there was a significant decrease in TPC in ME, as compared to 40 °C. This finding might be explained by a decreased activity of one of the enzymes in Viscozyme L, which is lower at 50 °C. Also, a possible loss of phenolic compounds has been observed due to thermal degradation,[26] which is mainly attributed to an increased oxidation.[30] This observation is consistent with results of Kapasakalidis et al.,[27] who reported a reduction of phenolic concentration by cellulase-assisted

extraction at 50 °C as compared to 40 °C. As the different pH values tested did not affect the level of TPC in both AE and ME, it can be concluded that the commercial enzyme mixture has a broad pH activity range.

The kinetic study of phenolic release at optimal conditions (Figure 2) indicates that the TPC in ME greatly increased during the first 12 h of enzymatic treatment with the maximum level reached at 12 h of incubation. This finding can be attributed to the catalytic action of carbohydrate-cleaving enzymes[31] as a pretreatment process. It can also be concluded that an incubation of 12 h is sufficient for Viscozyme L and Rapidase to hydrolyze the substrate. The stable level of total phenolic content in ME between 12 and 30 h incubation can be attributed to deactivation, even inactivation, of cellulase, hemicellulase, and pectinase by bioactive inhibitors, such as phenolic compounds and lignin, released from the substrate.[32] Indeed, Berlin et al.[33] and Tejirian et al.[34] reported that phenolics, flavonoids, and polymeric lignin could strongly adsorb onto cellulase and pectinase, limiting the enzyme activity and/or precipitation of enzyme. Our results confirm the possibility of enzymes as an effective method to improve the yield of phenolic compounds released from waste material. However, it is also important to know if the enzyme treatment resulted in the release of specific phenolic compounds or in a change in their structure, as compared to the nonenzymatic treated outer leaves. As far as we know, the effect of enzymatic treatment on the release of individual phenolic compounds from vegetable waste material has not been reported before.

The LC−MS analysis revealed that the main components found in ME and AE from controls (no-enzyme treatment) were kaempferol glycosides. This indicates that most phenolic compounds, naturally produced by cauliflower plants, are present in conjugated form, as was also reported on cauliflower byproducts by Llorach et al.[35,36] and Gonzales et al.[37] Similar results were also observed after the enzymatic treatment with Viscozyme L and Rapidase, meaning that no aglycones were formed. This is probably due to the insufficiency or absence of β-glycosidase, which is known for its capacity of deglycosylation by hydrolyzing the β-1,4 glycosidic bonds in aryl and alkyl β-D-glucosides as well as glycosides containing disaccharides and oligosaccharides.[38,39] The presence of the β-glycosidase was not specified by the supplier for the enzyme mixtures used. Viscozyme L was more efficient in hydrolyzing the substrate to obtain higher amounts of kaempferol-3-feruloyldiglucoside (compound 12) and kaempferol-3-glucoside (compound 14). The large increase in kaempferol-3-feruloyldiglucoside (compound 12) can be the result of the deglycosylation of two glucose molecules from kaempferol-3-feruloyltriglucoside-7-glucoside (compound 8). The large increase of kaempferol-3-glucoside (compound 14) by both enzymatic treatments is probably the result of removing one glucose molecule from kaempferol-3-diglucoside (compound 11). These cleavages may be caused by the presence of cellobiase activity or other unknown enzymatic actions in the commercial enzymes Viscozyme L and Rapidase. However, a full characterization of these commercial enzyme mixtures and their activity is not available. This behavior has also been confirmed by the results on time of incubation in the profile of phenolic release. The longer the samples were treated, the lower was the concentration of compounds 2, 8, and the higher was the amount of compounds 11, 12, and 14. However, no difference in the individual components of AE was observed as a function of the treatment, although a substantial increase in TPC was measured by Folin−Ciocalteu assay for the enzymatic treatments as compared to the control. This apparent difference can be attributed to the reaction of Folin−Ciocalteau reagent with other interfering components such as protein, enzymes, sugars, as well as ascorbic acid present in AE.[24,27,40]

Although no phenolic aglycones were found in comparison with extract from native cauliflower byproduct, the study showed that the use of Viscozyme L or Rapidase enzyme mixture results in a higher yield of kaempferol-glucosides extracted from the cauliflower outer leaves. Further treatment with β-glycosidase, either with or without Viscozyme L or Rapidase, could result in the release of kaempferol as aglycone form, a flavonoid that is characterized by several bioactivities.[41,42]

AUTHOR INFORMATION

Corresponding Author

*Tel.: +3256241255. Fax: +3256241224. E-mail: katleen.raes@ugent.be.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AE, aqueous extract; ME, methanolic extract; TPC, total phenolic content; SPE, solid-phase extraction; DW, dry weight; GAE, gallic acid equivalent; RE, rutin equivalent; E/S, enzyme/substrate; PCA, principal component analysis

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