Short Communication

Received: 12 February 2021 Revised: 30 August 2021 Accepted article published: 27 September 2021 Published online in Wiley Online Library: 3 November 2021

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11547

# Enzymatic hydrolysis of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), the key bitter compound of rapeseed (*Brassica napus* L.) protein isolate

**Mareike Siebert,[a] Ulrich Krings,[a] Thorben Günther,[a]* Apostolos Fragalas[b] and Ralf G Berger[a]**

## Abstract

**BACKGROUND: The use of rapeseed protein for human nutrition is primarily limited by its strong bitterness, which is why the key bitter compound, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), is enzymatically degraded.**

**RESULTS: Mass spectrometry analyses of an extract from an untreated rapeseed protein isolate gave three signals for *m/z* 815 [M-H]. The predominant compound among the three compounds was confirmed as kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside). Enzymatic hydrolysis of this key bitter compound was achieved using a sinapyl ester cleaving side activity of a ferulic acid esterase (FAE) from the basidiomycete *Schizophyllum commune* (ScoFAE). Recombinant ferulic acid esterases from *Streptomyces werraensis* (SwFAE) and from *Pleurotus eryngii* (PeFAE) possessed better cleavage activity towards methyl sinapate but did not hydrolyze the sinapyl ester linkage of the bitter kaempferol sophoroside.**

**CONCLUSION: Kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) was successfully degraded by enzymatic treatment with ScoFAE, which may provide a means to move the status of rapeseed protein from feed additive to food ingredient.**


**Keywords:** rapeseed protein; bitter off-taste; kaempferol sophoroside; ferulic acid esterase

## INTRODUCTION

Rapeseed oil was the third most consumed vegetable oil worldwide in 2018/2019, when 28.1 million tonnes was used.[1] During its production, a protein-rich press cake accrues as a by-product. The use of this protein for human nutrition is primarily limited by its strong bitterness. Kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) was recently identified as the key compound of the unpleasant bitter taste.[2] The application of enzymes to improve the quality and palatability of food is well known. For example, the stomach-irritating chlorogenic acids in coffee are rapidly and selectively removed by an immobilized esterase from the basidiomycete *Rhizoctonia solani*.[3]

Analogously, the use of fungal enzymes to degrade kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) was considered to be a promising approach. The chemical structure of the bitter compound suggested the use of a glucosidase or esterase. Glucosidases may cleave the glucoside bond of the sophoroside moiety, whereas esterases may hydrolyze the ester linkage between the sophoroside moiety and the sinapinic acid. Both options would result in non-bitter products.[2] For the latter, ferulic acid esterases (FAEs) are promising candidates.

In recent years, various esterases capable of hydrolyzing methyl sinapate were identified, produced in pure form through heterologous hosts, and characterized. In particular, a feruloyl esterase from *Streptomyces werraensis* (SwFAE)[4] and one from *Pleurotus eryngii* (PeFAE)[5] showed substrate specificity for methyl sinapate. A feruloyl esterase of *Schizophyllum commune* (ScoFAE) hydrolyzed methyl sinapate too, but not very efficiently.[6] The aim of this study was to degrade kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) enzymatically to reduce the undesirable bitter off-taste of rapeseed protein isolate using pre-selected esterases from different microorganisms.

* *Correspondence to: T Günther, Institute of Food Chemistry, Gottfried Wilhelm Leibniz University, Callinstraße 5, D-30167 Hannover, Germany. E-mail: thorben.detering@lci.uni-hannover.de*

a *Institute of Food Chemistry, Gottfried Wilhelm Leibniz University, Hannover, Germany*

b *Department of Food Technology, ATEI, Thessaloniki, Greece*

## MATERIAL AND METHODS

### Heterologous enzyme production

For heterologous enzyme production, *Escherichia coli* BL21(DE3) Star was chosen to produce SwFAE of prokaryotic origin. *Komagataella phaffii* GS115 was used for the PeFAE, and *Komagataella phaffii* SMD 1168 for the ScoFAE. Cultivation was conducted as described by Nieter *et al.*[7] SwFAE was produced using the cold shock expression vector pCOLD I DNA and using different chaperons (TaKaRaMobitec, Göttingen, Germany). For expression, a shaking culture was inoculated in LB medium and incubated at 37 °C. Then, 0.1 mM isopropyl-*β*-D-thiogalactopyranosid (IPTG) was added to the culture, and the cells were cultivated at 17 °C and 200 rpm for 20 h, harvested, re-suspended in 50 mM BisTris pH 7.0 and homogenized (Precellys24 bead homogenizer, Bertin Technologies SAS, Paris, France). Pre-cultures of the *Komagataella* strains were grown at 28 °C on YEPD medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone, 20 g $L^{-1}$ D-glucose). After 3 days, the medium was changed and main cultures were grown on BMMY medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone, 100 mL $L^{-1}$ potassium phosphate buffer (1 mol $L^{-1}$, pH 6), 13.4 g $L^{-1}$ yeast nitrogen base, 2 mL $L^{-1}$ biotin, 50 mg $L^{-1}$ each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucine) at 20 °C. Expression was induced with 1% methanol (*v/v*). Every 24 h, 2% methanol (*v/v*) was added to the cultures to ensure continuous expression. After 5 days, *Komagataella* cultures were harvested by centrifugation (15 000×*g*, 15 min, 4 °C) to separate the cell pellet. Afterwards, the supernatants were concentrated using ultrafiltration (Vivaspin® 10 kDa, Sartorius, Göttingen, Germany).

### Purification of SwFAE, PeFAE, and ScoFAE

The purification of the recombinant SwFAE was conducted via Ni-NTA-agarose affinity chromatography. The lysate was incubated for 1 h in binding buffer (0.5 mol $L^{-1}$ NaCl, 20 mM Tris pH 7.9, and 5 mM imidazole) at 4 °C while shaking. The sample was washed four times with increasing imidazole concentrations, eluted with 500 mM imidazole, and desalted using Sephadex G-25 Medium (GE Healthcare, Chicago, USA).

As it was not possible to purify PeFAE without losing much of its activity, the enzyme was used directly from the ultra-filtered supernatant (10 kDa cut-off, Sartorius, Düren, Germany). The recombinant feruloyl esterase of *Schizophyllum commune* was purified from *Komagataella phaffii* culture supernatant via Ni-NTA affinity chromatography. The bed volume of the self-casted column was 15 mL (column diameter 1.8 cm). Fractions of 8 mL were collected and assayed for enzymatic activity over 60 min at 37 °C as described elsewhere.[6] Binding buffer (A) consisted of 0.5 mol $L^{-1}$ NaCl, 5 mM imidazole and 20 mM Tris/HCl pH 7.9. Protein was eluted stepwise with elution buffer (B: 0.5 mol $L^{-1}$ NaCl, 500 mM imidazole and 20 mM Tris/HCl pH 7.9) with the following gradient: 56 mL 0% B, 40 mL 1% B, 24 mL 5% B, 16 mL 100% B, and re-equilibrated within 24 mL (0% B). The flow rate was 1 mL $min^{-1}$ (isocratic flow). Active fractions were pooled, concentrated to approximately 2 mL, and diluted 1/1 (v/v) in Tris buffer (50 mM, pH 7.5).

### Extraction of kaempferol 3-O-(2‴-O-sinapoyl-β-sophoroside)

Following the protocol described by Hald *et al.*,[2] 300 g rapeseed protein isolate powder (Isolexx, Teutoburger Ölmühle GmbH, Ibbenbühren, Germany) was extracted three times with 500 mL methanol/water (50/50, v/v) by stirring for 30 min at ambient temperature. Afterwards, the combined extracts were centrifuged (5 min, 8133×g, Rotina 460 R, Hettich, Kirchlengern, Germany) and filtered. The solvent was removed under vacuum at 40 °C. After lyophilization, an aliquot (1 g) of the residue was taken up in 50 mL water and applied on a Chromabond C18 cartridge (Macherey-Nagel, Düren, Germany).

The column was preconditioned with 70 mL of methanol and 70 ml of water. Stepwise elution with 75 mL water (fraction A), 75 mL methanol/water (30/70, v/v) (fraction B) and 75 mL methanol/water (50/50, v/v) (fraction C) was performed. Fractions A and B were discarded. The solvent of fraction C was removed under reduced pressure using a rotary evaporator at 40 °C. The aqueous residue was lyophilized and stored at −20 °C.

### Enzymatic hydrolysis

First, 5 mg $mL^{-1}$ of the lyophilized fraction C was dissolved in water and filtered (syringe filter Chromafil® RC-45/25, Macherey-Nagel). Then, 65 µL of this solution was incubated with 10 µL sodium acetate buffer (500 mM, pH 5.0) and 25 µL PeFAE solution, or with 10 µL Tris buffer (500 mM, pH 7.5), or 25 µL ScoFAE solution, or with 10 µL Tris buffer (500 mM, pH 7.5) and 25 µL SwFAE solution, or with sodium acetate or Tris buffer alone for 4 h at 37 °C. The enzyme activity was chosen according to the values of previous substrate-specificity assays.[4-6] The reaction was terminated by doubling the reaction volume with acetonitrile. A control sample was prepared with water instead of rapeseed protein isolate extract (fraction C).

### Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

To analyze the rapeseed bitter compounds, high-performance liquid chromatography (HPLC) coupled to a triple quadrupole mass analyzer was used (212 LC pump, 320 TQ-MS mass spectrometer, Varian, Santa Clara, USA). The analyses were conducted in negative electrospray ionization mode with a scan range of *m/z* 100 to 900. Collision-induced dissociation (CID) $MS^2$ was also

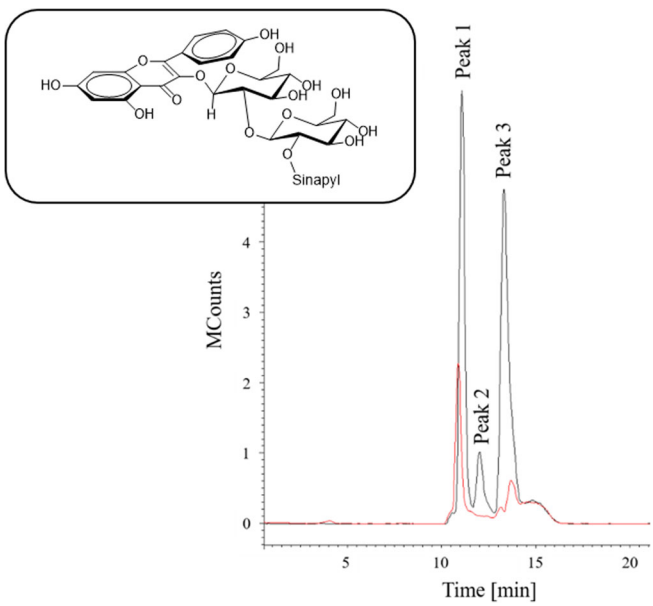


**Figure 1.** Extracted ion chromatogram (*m/z* 815). Control sample (upper curve) and enzymatically treated sample (lower curve). Peak 1 (formula inserted) was identified as the key bitter compound kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside).



  
*Journal of The Science of Food and Agriculture* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

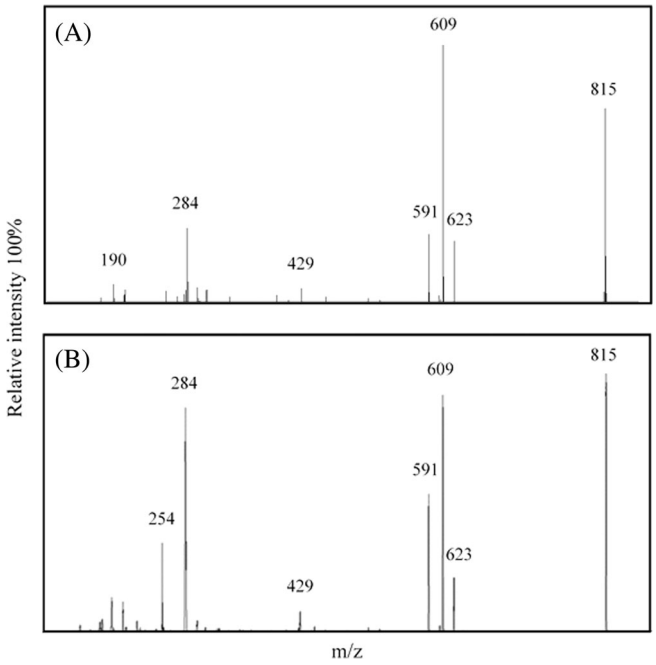


**Figure 2.** CID-MS/MS spectrum of peak 1 (A) in comparison with reference spectrum (B) adopted from Hald *et al.*[2] Copyright 2021 American Chemical Society.

performed for parent ions at *m/z* 815 (daughter ion scan range *m/z* 815 to 100) and *m/z* 223 (daughter ion scan range *m/z* 223 to 100) with the following parameters (optimized using the MS breakdown tool): capillary voltage −50 V, collision energy 30 eV, spray chamber temperature 50 °C, drying gas temperature 300 °C, drying gas pressure 124 kPa, CID gas (argon) pressure 200 mPa, needle voltage −4500 V. For HPLC, water and acetonitrile (MS-grade), both containing 0.1% formic acid, were used as the mobile phase.

Gradient elution was used for separation: 100% solvent A (0.1% formic acid in water) and 0% solvent B (0.1% formic acid in acetonitrile) (held for 2 min), 100 to 70% solvent A in 1 min (held for 5 min), 70 to 0% solvent A in 3 min (held for 25 min), 0 to 100% solvent A in 2 min (held for 2 min). A six-port valve equipped with a 20 µL sample loop was used for manual injection and the flow rate was 0.4 mL $min^{-1}$. The MS system was coupled to an UV-visible detector (ProStar 325, Varian). Ultraviolet-visible-spectra were recorded at $\lambda = 220$ nm and $\lambda = 320$ nm.

### Nano liquid chromatography-quadrupole time-of-flight-mass spectrometry (nLC-qTof-MS)

Accurate masses of compounds were determined using the nano-liquid chromatography system EASY-nLC II (Bruker Daltronik, Bremen, Germany) equipped with a 20 mm pre-column (C18-A1 3PCS, ThermoFisher Scientific, Waltham, USA) and a capillary column (0.15 × 250 mm) packed with Grace MS C18 (3 µm particles, 300 Å pore, Grace Discovery Sciences, Deerfield, USA). The analytes were elated by a linear gradient (300 nL $min^{-1}$) of water and acetonitrile (each with 0.1% formic acid (*v/v*)) from 95% water to 95% acetonitrile within 25 min and subsequent hold at 95% acetonitrile for 15 min. The nano-LC system was connected to a maXis impact QTOF mass spectrometer (Bruker Daltronik) equipped with a captive nanospray ion source for electrospray ionization in the positive mode. The orthogonal time-of-flight mass analyzer was calibrated prior to analysis using Na-formiate clusters and operated with a mass resolution >30 000.

## RESULTS

The extracted ion chromatogram (EIC) of *m/z* 815 (M-H) showed three peaks in the untreated rapeseed protein extract (Fig. 1). The accurate mass measurement of the peaks gave results of 815.2340, 815.2041, and 815.2052, respectively. These masses correspond to an empirical formula of $C_{38}H_{39}O_{20}$ (815.2029). By comparing the mass spectra of the LC–MS/MS analysis with published reference spectra, peak 1 was identified as the key bitter compound kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (Fig. 2;[2]). Conclusive identification of peak 2 was not possible, but based on accurate mass and MS/MS spectrum (no daughter ion at *m/z* 653) it is suggested that this was most likely a sophoroside derivative of kaempferol. Peak 3 was identified as kaempferol-3-*O*-sinapylglucoside-7-*O*-glucoside. The daughter ion at *m/z* 653 resulted from the cleavage of the sugar moiety in position 7 of kaempferol, indicating that this compound was not a sophoroside. This compound had been found in rapeseed previously.[8]

Enzymatic hydrolysis of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) using SwFAE or PeFAE was not successful. Considering the proven activity against the artificial analogue methyl sinapate, these results were unexpected (Table 1).

Different incubation temperatures and times did not lead to the aspired result (data not shown). Treatment of the rapeseed protein extract with ScoFAE led to a decrease of 65% of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) under the conditions applied

**Table 1.** Hydrolysis of methyl sinapate and of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) by enzymes with different origins

| Enzyme | Highest activity against | Relative activity against methyl sinapate [%] | Activity against kaempferol sophoroside |
|---|---|---|---|
| SwFAE | Feruloyl arabinose | 47.13 ± 1.04[a] | n.d. |
| PeFAE | *β*-D-xylopyranosyl-(1 → 2)-5-*O*-*trans*-feruloyl-L-arabinofuranose | 4.6 ± 0.13[b] | n.d. |
| ScoFAE | Methyl *p*-coumarate | 3.0 ± 0.07[c] | High (Fig. 1) |

n.d., not detected.
[a] Schulz *et al.*[4]
[b] Nieter *et al.*[5]
[c] Nieter *et al.*[6]



  
*Journal of The Science of Food and Agriculture* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

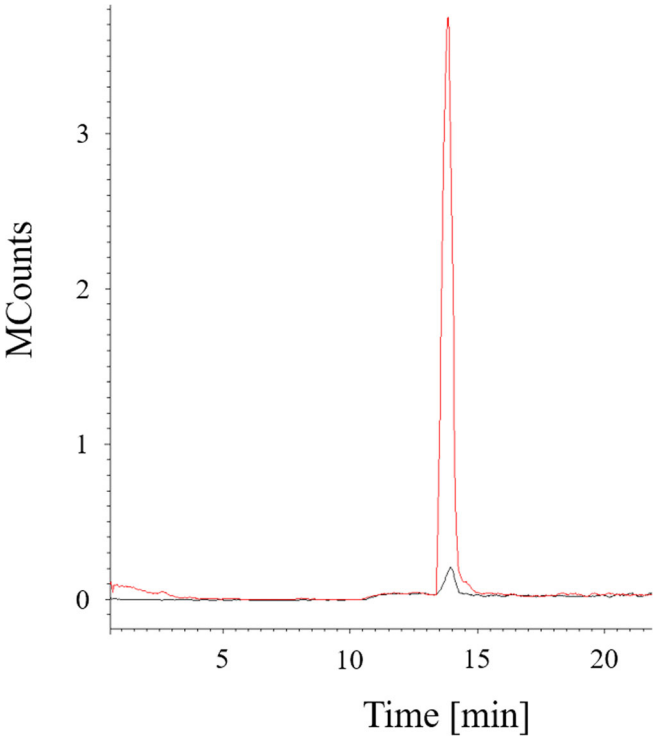


**Figure 3.** Extracted ion chromatogram (*m/z* 223). Sinapinic acid concentration of the enzymatically treated sample (upper curve) and the untreated control sample (lower curve).

(Fig. 1, peak 1). The EIC of *m/z* 223 (M-H) showed that the sinapinic acid concentration increased simultaneously (Fig. 3).

The two other substances were nearly completely hydrolyzed (peak 2: -99%, peak 3: -93% of the peak area of the control sample without enzyme addition, respectively). The hydrolysis of the ester bond of the kaempferol glycosides was also proven by the detection of two peaks showing accurate masses of 609.1468 and 609.1476, respectively, which corresponded to the kaempferol glycoside moieties of peak 1 and 3 ($C_{27}H_{29}O_{16}$, calc. 609.1455).

## DISCUSSION

The results led to the safe conclusion that the ScoFAE cleaved the ester linkage between the sophoroside moiety and the sinapinic acid of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside). Surprisingly, neither SwFAE, with a much better relative activity toward methyl sinapate than ScoFAE, nor PeFAE showed hydrolytic activity – an indication of the high substrate specificity required for this very bulky substrate (Fig. 1). Glycosides of the kaempferol occur in ferns, conifers, and flowering plants, including many fruits and vegetables.[9] Rapeseed contains 39 kaempferol derivatives, mostly glycosides.[8] Comparing overlapping habitats it is obvious that an alkaliphilic soil bacterium like *S. werraensis* would have less contact with flowering plants, while xerophilic, saprobiontic Basidiomycota, such as *Schizophyllum* and *Pleurotus*, thrive on dead and living plant tissues, such as leaves, litter, and lignified materials generally. However, such a consideration does not explain why the FAE from one basidiomycete degraded the natural flavonol glycoside rapidly (Sco), and the other (Per) did not.

Sensory studies with a trained panel would have to be performed to determine the enzyme activity required to actually decrease the concentration of the bitter compound in the protein matrix below its low taste threshold of 3.4 μmol $L^{-1}$.[2]

## CONCLUSIONS

Kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) was successfully degraded by enzymatic treatment with ScoFAE, which has many advantages compared with an alternative chemical hydrolysis with harmful chemicals under harsh reaction conditions. A food-grade and highly selective and specific enzymatic hydrolysis on the other hand may provide a means to move the status of rapeseed protein from feed additive to food ingredient.

## REFERENCES

1 US Department of Agriculture and USDA Foreign Agricultural Service, Consumption of vegetable oils worldwide from 2013/14 to 2017/2018, by oil type (in million metric tons), Ed (2019).
2 Hald C, Dawid C, Tressel R and Hofmann T, Kaempferol 3-*O*-(2‴-*O*-Sinapoyl-*β*-sophoroside) causes the undesired bitter taste of canola/rapeseed protein isolates. *J Agric Food Chem* **67**:372–378 (2019).
3 Siebert M, Detering T and Berger RG, An immobilized fungal chlorogenase rapidly degrades chlorogenic acid in a coffee beverage without altering its sensory properties. *LWT* **115**:108426 (2019).
4 Schulz K, Nieter A, Scheu A-K, Copa-Patiño JL, Thiesing D, Popper L *et al.*, A type D ferulic acid esterase from *Streptomyces werraensis* affects the volume of wheat dough pastries. *Appl Microbiol Biotechnol* **102**:1269–1279 (2018).
5 Nieter A, Haase-Aschoff P, Linke D, Nimtz M and Berger RG, A halotolerant type a feruloyl esterase from *Pleurotus eryngii*. *Fungal Biol* **118**: 348–357 (2014).
6 Nieter A, Kelle S, Linke D and Berger RG, Feruloyl esterases from *Schizophyllum commune* to treat food industry side-streams. *Bioresour Technol* **220**:38–46 (2016).
7 Nieter A, Kelle S, Takenberg M, Linke D, Bunzel M, Popper L *et al.*, Heterologous production and characterization of a chlorogenic acid esterase from *Ustilago maydis* with a potential use in baking. *Food Chem* **209**:1–9 (2016).
8 Shao Y, Jiang J, Ran L, Lu C, Wei C and Wang Y, Analysis of flavonoids and Hydroxycinnamic acid derivatives in rapeseeds (*Brassica napus L. var. napus*) by HPLC-PDA–ESI(−)-MSn/HRMS. *J Agric Food Chem* **62**:2935–2945 (2014).
9 Calderón-Montaño JM, Burgos-Morón E, Pérez-Guerrero C and López-Lázaro M, A review on the dietary flavonoid kaempferol. *Mini Rev Med Chem* **11**:298–344 (2011).



  