

Biocompounds from rapeseed oil industry co-stream as active ingredients for skin care applications

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Abstract:

OBJECTIVE: Despite the great number of substances produced by the skin care industry, very few of them seem to truly have an effect on the skin. Therefore, given the social implications surrounding physical appearance, the search for new bioactive compounds to prevent or attenuate skin aging and enhance self-image is a priority of current research. In this context, being rich in valuable compounds such as proteins, phenolics, lipids and vitamins, this study is focused in the potential activity of rapeseed press cake hydrolyzates to be used as raw materials for skin care applications.

METHODS: In this study, the protein-rich press residue from the rapeseed oil industry was converted enzymatically into short-chain biologically active peptides using four protease products with varying substrate specificity - Alcalase 2.4L FG, Protex 6L, Protamex and Corolase 7089. The antioxidant, anti-wrinkle and anti-inflammatory activity of the obtained hydrolyzates were evaluated in vitro while their biocompatibility with human skin fibroblasts was tested.

RESULTS: All hydrolyzates were biocompatible with skin fibroblasts after 24 h of exposure, while the non-hydrolyzed extract induced cell toxicity. Alcalase 2,4L FG and Protex 6L - obtained hydrolyzates were the most promising extracts showing improved bioactivities suitable for skin anti-ageing formulations, namely anti-oxidant activity, inhibiting approximately 80% cellular reactive oxidative species, anti-inflammatory and anti-wrinkle properties, inhibiting around 36% of myeloperoxidase activity and over 83% of elastase activity.

CONCLUSION: The enzymatic technology applied to the rapeseed oil industry co-stream results in the release of bioactive compounds suitable for skin care applications.

Keywords: Chemical analysis, Spectroscopy, Enzymatic hydrolysis, Cell culture, Bioactive peptides, Skin care.

INTRODUCTION

Rapeseed is one of the leading oilseed crops, ranking the first with respect to oil production in the EU, and third after soybean and palm worldwide [14]. Processing rapeseeds into vegetable oil generates a residue which accounts for 60 - 70% of the total seed mass, reaching around 13,1 million tons of rapeseed press cake per year [15]. Currently, this co-product is mainly used as an ingredient in animal feed and fertilizers [5,22,31,47]. In order to ensure an increased economic revenue in the production of rapeseed oil, the full potential of its co-streams should be investigated [11,33]. Being rich in valuable compounds such as proteins, phenolics, essential amino acids, vitamins and lipids, rapeseed press cake is an excellent raw material for skin care ingredients. A common industrially used strategy to obtain value-added ingredients from protein-based co-streams is their enzymatic conversion into smaller, biologically active peptides [34]. Peptides obtained from the hydrolysis of rapeseed press cake proteins have shown high solubility, emulsifying and foaming capacity [43] as well as biological activities, including anti-hypertensive [47] and anti-oxidant [30] properties, exploited mainly in food applications [33]. Up to our knowledge, rapeseed hydrolyzates have never been used as ingredients for skin care formulations. The only report found on their anti-ageing capacity relates to the application of a non-hydrolyzed rapeseed meal extract [27].

One of the most important issues related to skin ageing and other conditions such as skin cancer is the exposure to UV radiation. Reactive oxygen species (ROS) and lipid peroxides formed under UV radiation damage cellular lipids, proteins and DNA, which finally leads to alteration of skin structure. Neutrophils production is also stimulated, increasing the activity of neutrophil-derived myeloperoxidase (MPO) - a ROS-generating enzyme, and proteases such as elastase and collagenase [26]. A chronic state of oxidative stress is thus caused by the elevated levels of these deleterious skin enzymes and ROS [4,37,41]. As a consequence, the regular skin

function can be inhibited, reducing collagen and elastin production, decreasing fibroblasts activity, and inducing skin atrophy in both epidermis and dermis, as well as skin thinning [35]. These phenomena may cause different skin conditions: from the simple wrinkle formation and premature skin ageing to other complicated skin diseases such as pruritus, psoriasis and skin cancer [16]. Thus, anti-ageing ingredients should necessarily counteract ROS species and inhibit overexpressed MPO and elastase activity.

In this study, the protein-rich press residue from the rapeseed oil industry was converted enzymatically into short-chain biologically active peptides with potential for skin care applications. Proteolysis has potential to liberate bioactive peptides, otherwise inactive within the sequence of the parent protein. Furthermore, the peptides can be water-extracted to partially separate them from undesirable press cake components [28,38]. The efficiency of the obtained hydrolyzates to target specific mechanisms of the premature skin ageing were evaluated in vitro.

MATERIALS AND METHODS

Materials

Cold-pressed rapeseed (*Brassica rapa* ssp *Oleifera*) press cake was obtained from Kankaisten Öljykasvit Oy (Turenki, Finland). Four commercial endoproteases were used for proteolysis of the raw material - Protex 6L (from Genencor), Corolase 7089 (from AB Enzymes GmbH), Protamex and Alcalase 2.4L FG (from Novozymes). Commercial rapeseed protein isolate, Isolexx, was obtained from BioExx Proteins of Saskatoon Inc., SA (Canada). DCTM (detergent compatible) Protein Assay Kit was purchased from BioRad AlamarBlue[®]. Cell Viability Reagent and EnzChek[®] Gelatinase/Collagenase Assay kit were purchased from Invitrogen, Life Technologies Corp. (Spain). Human foreskin fibroblasts cell line BJ-5ta (ATCC[®] CRL-4001TM) and Hanks' Balanced Salt Solution (HBSS) were provided by the American Type Culture Collection (LGC Standards S.L.U, Spain). All other reagents were from Sigma-Aldrich (Spain).

Production of a Protein-Enriched Fraction (PEF) from Rapeseed Press Cake

A protein-enriched fraction (PEF) of rapeseed press cake was used as a raw material for the production of protein hydrolyzates. For PEF production, the press cake was ground at 1000 rpm using an SM 300 cutting mill (Retsch GmbH, Germany) and defatted by supercritical carbon dioxide (SC-CO₂) extraction in a Nova Swiss extraction vessel (Nova Werke AG, Switzerland) with a Chematur Ecoplanning compressor (Chematur Engineering Ltd., Finland). The vessel was operated at 40°C, 300 bar for ca. 5 hours. After defatting the press cake was dry-milled twice in a 100 UPZ-II fine impact mill (Hosokawa Alpine Ag, Germany) with stainless steel pin discs at 17800 rpm. The milled material was air-classified in a Minisplit Air Classifier (British Rema Manufacturing Company Ltd., UK) at 15000 rpm, 220 m³ h inlet air flow and 25 rpm feed rate in order to separate the fine kernel fragments, for further use as PEF sample, from the coarse hull particles.

Enzymatic Hydrolysis of PEF to Obtain Rapeseed Protein Hydrolyzates (RPH)

Four commercial endopeptidase preparations of microbial origin were used for PEF hydrolysis - Protex 6L (alkaline serine endopeptidase from *B. licheniformis*, dosed at 1634 nKat g⁻¹ dry substrate), Protamex (alkaline serine and neutral metallo-endopeptidase from *B. licheniformis*/*B. amyloliquefaciens*, 2052 nKat g⁻¹), Corolase 7089 (neutral serine endopeptidase from *B. subtilis*, 2140 nKat g⁻¹) and Alcalase 2.4L FG (alkaline serine/glutamyl endopeptidase from *B. licheniformis*, 720.3 nKat g⁻¹). Their proteolytic activities in nKat g⁻¹ of dry substrate were determined at pH 7.5, 30 °C using casein as a substrate, and applying identical experimental conditions to allow for an activity comparison among the proteases. Commercial rapeseed protein isolate Isolexx was used as a reference material for protein hydrolysis and hydrolyzates characterization. The protein concentration of Isolexx and PEF was determined as the total nitrogen content according to the Kjeldahl procedure [24]. Briefly, the proteins were digested with sulfuric acid at 420°C using CuSO₄ and TiO₂ as catalysts. The obtained NH₃ was distilled and titrated using HCl. Protein concentration was calculated from the total nitrogen content using a conversion factor of 6.25. The PEF and Isolexx were treated with proteases in a single or

two-step process as follows: 1) Protex 6L (sample code RPH_Px), 2) Protamex (RPH_Pm), 3) Corolase 7089 (RPH_Co), 4) Alcalase 2.4L FG (RPH_Al), 5) Protex 6L followed by Protamex (RPH_Px-Pm), 6) Protex 6L followed by Corolase 7089 (RPH_Px-Co), and 7) Protex 6L followed by Alcalase 2.4L FG (RPH_Px-Al). Additionally, Isolexx and the PEF were subjected to aqueous treatment without enzyme to obtain non-hydrolyzed reference protein extracts (Ixx_NH and RP_NH respectively). The enzyme and reference treatments were performed at the natural pH of the substrate (ca. pH 6) without pH adjustment. Each step was carried out for 2 hours at 50 °C, 10% (w/v) consistency in water, with 10 mg total enzyme protein per g dry substrate. Before addition of the proteolytic enzymes, the substrate-water suspensions were boiled for 10 min in order to inactivate endogenous enzymes. The enzymatic hydrolysis was stopped by boiling the suspensions for 10 min. After the final treatments, the suspensions were centrifuged for 15 min at $15,281 \times g$ and the supernatants were freeze-dried to obtain RPH in the form of fine powders. The samples were kept at - 20 °C until required for further use.

Free Amino Nitrogen (FAN) Content and Molecular Weight (MW) Distribution in RPH

Liberation of peptides and amino acids during protease treatment of rapeseed materials was quantified by determining the free amino nitrogen (FAN) ends in the hydrolyzates, according to the method described in Analytica-EBC [3]. The degree of hydrolysis (DH) was calculated as the proportion of FAN out of total raw material nitrogen. MW range of proteins and peptides in Isolexx, PEF, and in the non-hydrolyzed extracts and hydrolyzates from these substrates was determined by reducing SDS-PAGE. The samples were dissolved in 1 M Tris-HCl buffer pH 6.8 containing 40% glycerol, 2% SDS and 1% mercaptoethanol by heating at 100 °C for 5 min, and run on a 16.5% Tris-Tricine Precast peptide gel (Bio-Rad) at 125 V for 95 min in Tris-Tricine buffer pH 8.3. The MW of the protein and peptide bands was determined based on the migration of 1400-26600 Da polypeptide standards (Bio-Rad).

Chemical Composition of the RPH

The chemical composition of the RPH was analyzed in terms of protein, sugar and phenolic content. To analyze protein content, the Lowry method was performed using DC™ Protein Assay Kit. Briefly, the dried RPH were dissolved in water to 40 mg mL⁻¹, and alkaline copper tartrate solution and Folin reagent were added. Color development upon 20 min incubation was determined by measuring absorbance at 750 nm. BSA 0.2-1.5 mg mL⁻¹ (from Sigma) was used to plot a standard curve from which the protein concentration of the hydrolyzates was determined.

The total sinapic and ferulic acid content of the RPH was determined by liquid chromatography according to the method by Vuorela et al. [44]. The samples were hydrolyzed with 2 M NaOH to de-esterify bound phenolics, and phenolic acids were extracted with 0.6 M ethyl acetate. The extracts were analyzed by ultra-performance liquid chromatography with diode array detection (UPLC-DAD) using an Acquity™ Ultra Performance LC unit equipped with an Acquity UPLC BEH C18 column (Waters, USA). Sinapic and ferulic acid concentrations were calculated on the basis of their respective standard curves (0.1-1.0 mg mL⁻¹).

Neutral sugar (glucose, fructose, rhamnose, galactose, arabinose, mannose, xylose and sucrose) concentrations in the RPH were determined by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) using a ICS-3000 ion chromatography system equipped with a CarboPac PA1 column (Dionex, Sunnyvale, CA) based on their respective standard curves [20].

Anti-oxidant activity of RPH in vitro

DPPH radical scavenging activity assay.

DPPH radical scavenging activity of RPH were determined using a modified method described by Badakhshan et al. [29]. Briefly, 50 µL of RPH water solution (0.5 mg mL⁻¹) and 5 mL of 0.004% (w/v) DPPH solution in methanol were incubated in dark during 30 min at room temperature. The absorbance values were then measured at 517 nm using a microplate reader

TECAN infinite M200. Methanol was used as a blank and ascorbic acid (0.5 mg mL^{-1}) and sinapic acid were used as a positive control.

Oxygen radical absorbance capacity (ORAC) assay.

The ORAC assay was performed according to the method described by Alashi et al. [1] with modifications. Briefly, RPH were dissolved in 75 mM sodium phosphate buffer pH 7.4. The samples (final peptide concentration 0.5 mg mL^{-1}) were then mixed with 300 nM fluorescein in a 96-well tissue culture-treated polystyrene black plate (Nunc) followed by incubation of the mixture in the dark at $37 \text{ }^{\circ}\text{C}$ for 15 min. Thereafter, $50 \mu\text{L}$ (80 mM) 2, 2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was added to the mixture and the change in fluorescence due to AAPH-induced oxidation of fluorescein was measured at 1 min intervals during 90 min at 485-535 nm excitation and emission wavelengths respectively, using a fluorescence microplate reader.

Cell culture.

To determine the biocompatibility of RPH with skin, BJ-5ta cells were used. The cells were maintained in 4 parts Dulbecco's Modified Eagle's Media (DMEM) containing 4 mM L-glutamine, 4500 mg L^{-1} glucose, 1500 mg L^{-1} sodium bicarbonate, 1 mM sodium pyruvate and 1 part of Media 199, supplemented with 10% (v/v) of fetal bovine serum (FBS), and 10 g mL^{-1} Hygromycin B at $37 \text{ }^{\circ}\text{C}$, in a humidified atmosphere with 5% CO_2 , according to the recommendations of the manufacturer. The culture media was replaced every 2 days. At pre-confluence, cells were harvested using trypsin-EDTA (ATCC-30-2101, 0.25% (w/v) trypsin/ 0.53 mM EDTA solution in HBSS without calcium or magnesium).

Cellular oxidative stress.

Cellular antioxidant activity (CAA) was determined as described by Wolfe et al. [45]. Growth media per well, containing BJ-5ta cells, was seeded at a density of 1×10^5 per well on a 96-well

tissue culture-treated polystyrene black plate clear bottom (Corning Costar Corp.). The growth media was removed 24 h after seeding and the wells were washed with 100 μL of PBS. The cells were then treated for 1 h with 100 μL of 1% FBS media containing RPH (2 mg mL^{-1}) plus 25 μM DCFH-DA. After incubation, the wells were washed again with 100 μL of PBS followed by the application of 100 μL of HBSS containing 600 μM AAPH and fluorescence was immediately measured in a microplate reader at 37 $^{\circ}\text{C}$. Emission at 538 nm was measured with excitation at 485 nm every 5 min during 1 h. Negative control wells contained cells treated with DCFH-DA and AAPH. Ascorbic acid (1 mg mL^{-1}) was used as positive control.

Anti-inflammatory activity of RPH

The effect of RPH on MPO activity was detected spectrophotometrically using guaiacol as a substrate. The samples were previously diluted in 50 mM PBS pH 6.6 at 0.2 mg mL^{-1} and were further incubated for 1 h at 37 $^{\circ}\text{C}$, with 0.24 units MPO and 10 mM guaiacol to a final volume of 180 μL . Thereafter, the reaction was initiated by adding 1 mM H_2O_2 (20 μL). The activity was determined by the increase of the absorbance rate per min at 470 nm, and expressed as a percentage of MPO inhibition.

Anti-ageing activity of RPH in vitro

The inhibitory effect of biopeptides on the HNE was determined according to the method described by Vasconcelos et al. [42] with modifications. Briefly, 15 μL of HNE (20 $\mu\text{g mL}^{-1}$) were incubated with 15 μL of RPH (20–80 μM) for 30 min at 25 $^{\circ}\text{C}$. The final volume of each sample was adjusted to 300 μL with 0.1 M HEPES, 0.5 M NaCl pH 7.5 assay buffer. 5 mM of N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide solution was prepared in DMSO. The reaction was initiated with the addition of the substrate previously diluted in the assay buffer (200 μM). Negative control was performed in the same conditions without inhibitor and 100 μM phenylmethylsulfonyl fluoride (PMSF) was used as a positive control. The residual activity was

determined by the increase of the optical density at 410 nm over 10 min using a microplate reader.

Biocompatibility of RPH

Cell viability was monitored using AlamarBlue® (Resazurin as an active compound) assay. Briefly, cells in growth media were seeded at a density of 1×10^5 cells per well into a 96-well tissue culture-treated polystyrene plate (Nunc). 24 h after seeding, cells were exposed to 150 μ L of growth media containing RPH (0.2 and 2 mg mL⁻¹) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. After 24 h of contact with cells, the RPH were removed and the cells washed twice with PBS. Subsequently, 100 μ L of 10% (v/v) AlamarBlue® in grow media was added to each well as a reagent for detecting cell viability. The absorbance at 570 nm was measured after 4 h of incubation at 37° C using 600 nm as a reference wavelength, in a microplate reader. Wells with media were used as the blank, wells with 500 μ M H₂O₂ was used as a positive control of cell death, and those seeded with BJ-5ta in media were the negative control. All tests were performed in duplicate. BJ-5ta cells relative viability percentage was determined for each concentration of RPH based on the proportionality of resorufin formation and the number of viable cells.

Statistical analysis

The experiments were performed in triplicate (MPO, DPPH, ORAC and CAA) or duplicate (HNE and biocompatibility) and results are presented as means with error bars representing the SD. The data were analyzed using the statistical software graph pad prism version 5.04 for windows (Graph Pad Software, San Diego, CA, USA). Differences between treatments were analyzed with one-way analysis of variance (ANOVA). Significant differences among means were determined by Dunnett post-hoc test with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

RPH generation by enzymatic hydrolysis of crude intermediate PEF

The commercial proteases Protex 6L, Protamex, Alcalase 2.4 FG and Corolase 7089 were used to hydrolyze rapeseed PEF and a commercial rapeseed protein isolate (Ixx) containing ~46% and ~90% protein respectively. The latter was used as a reference material to compare the efficacy of the enzymes for RPH production from a rapeseed protein isolate and a crude rapeseed PEF. Endopeptidases were selected due to their capability to cleave peptide bonds in the middle parts of the polypeptide chains, generating small and medium-size peptides, the expected size range for optimal bioactivity [12]. The hydrolyzates were analyzed for free amino nitrogen content to monitor the efficiency of the enzymatic hydrolysis, and using a SDS-PAGE gel electrophoresis to monitor the breakdown of the proteins.

The increase of the FAN levels in the hydrolyzates (Figure 1), in comparison with the non-hydrolyzed extracts, indicated the liberation of peptides or amino acids during the protease treatments. The hydrolysis was more pronounced on the commercial substrate Isolexx due to its higher protein content. Two-stage enzyme treatments (Px-Pm, Px-Co and Px-Al) hydrolyzed both substrates to a larger extent than single enzyme treatments, releasing more FAN in the form of peptides and most probably free amino acids.

Protex 6L and Protamex applied individually were also efficient on both substrates. This might be explained by their specificity towards hydrophobic amino acids which are the predominant residues in the major rapeseed proteins: cruciferins, napins and lipid transfer proteins [7,9].

Alcalase 2.4L FG and Corolase 7089 caused low DH (9.96% and 8.48%, respectively) towards PEF in comparison to other enzymes. Although majority of rapeseed protein bioactivities have been reported for small molecular weight peptides and hydrolyzates with relatively high DH [12,21,46], a low DH may be favorable for certain functional properties. Partial DH (3-10%) has been found to result in improved angiotensin converting enzyme-inhibitory activity and functional properties of the resulting hydrolyzates in comparison to non-hydrolyzed protein [48]. In addition to the choice of enzyme, the selected hydrolysis conditions are expected to have influenced the DH. In this study, all protease treatments were carried out in

water without pH adjustment to avoid introduction of ions into the hydrolyzates. Modification of the hydrolysis conditions towards the pH optimum of each enzyme preparation would most probably increase the hydrolysis efficiency, especially in case of the alkaline enzyme preparations Alcalase 2.4L FG, Protamex and Protex 6L.

The proteolysis generated small molecular weight peptides from both Isolexx and PEF as confirmed by SDS-PAGE (Figure 2). As a result of protease treatment, most of the bands with MW above 26.6 kDa and between 26.6-6.5 kDa disappeared and a large and clear band in the region below 1.4 kDa appeared, confirming that all enzymes and combinations thereof were able to hydrolyze the original fractions (Ixx and PEF) to low MW peptides.

The overall protein composition of Isolexx and PEF was determined by SDS-PAGE after dissolution of the proteins in SDS-PAGE sample buffer (2% SDS and 1% mercaptoethanol). Some of the bands with MW above 14.4 kDa present in the raw materials (“Ixx” and “PEF” columns in Figure 2) were not present in the non-hydrolyzed extracts (“No enzyme” columns in Figure 2), suggesting that the corresponding proteins (cruciferins) remained partially insoluble without enzymatic treatment and were thus not recovered in the non-hydrolyzed extracts.

The gel profile of the extract obtained from hydrolysis of PEF with Corolase 7089 showed attenuated bands with MW above 26.6 kDa, which were not found in the original fractions (PEF and Ixx column) or in the respective non-hydrolyzed extracts (“No enzyme” column). Similar results were observed after hydrolysis of Isolexx with Alcalase. This behavior suggests the breakdown of high MW proteins (e.g. cruciferins), not observed in the reference gel profile due to range of the used standard marker, into proteins around 26.6 kDa.

The bands around 6.5 kDa in PEF, Isolexx and the Alcalase-obtained hydrolyzates, corresponding to napin subunits [7], were attenuated or not present in the hydrolyzates obtained after other enzyme treatments. This suggests that albumins remained partly undigested after Alcalase treatment. In comparison to the other enzymes, Alcalase 2.4L FG may have possessed a weaker catalytic efficiency towards albumins which are stabilized by their rigid structure with

four disulfide bonds and have been reported to be more resistant to hydrolysis by Alcalase than rapeseed cruciferins [10].

Chemical composition of the RPH. The chemical properties of Isolexx and PEF-derived RPH samples were assessed in terms of protein, neutral sugars and sinapic acid content (Table 1). The non-hydrolyzed extract (NH) and hydrolyzates from Isolexx contained generally more protein per dry matter than the respective extracts obtained from PEF.

Due to the presence of carbohydrates, lignin, lipids, ash and phenolic compounds in rapeseed press cake [36], the PEF-derived extracts were expected to contain a larger share of co-solubilized non-protein components than Isolexx-derived extracts. This was accordingly shown as the higher content of neutral sugars in the PEF-derived extracts.

As a result of enzymatic hydrolysis, the obtained RPH were enriched in protein content while the content of neutral sugars and sinapic acid was significantly reduced (Table 1). The improved protein recovery is considered a common feature of the enzymatic hydrolysis, resulting from the higher solubility of peptides in comparison to intact rapeseed protein.

Effect of RPH on the anti-oxidant activity in vitro. As the largest organ in the body, skin provides a barrier against UV radiation, chemicals, microbes and physical pollutants [41]. Nevertheless, with advancing age, the cellular anti-oxidant potential as well as the absorption of nutrients, including scavengers of harmful free radicals, gradually diminishes [18]. The incorporation of anti-oxidants into skin conditioning products enhances the ability of other anti-oxidants within the skin to protect skin tissues, hinders the UV-induced immunosuppression, and could be used for the treatment and prevention of oxidative stress-mediated alterations [39]. The obtained RPH were studied for their antioxidant capacity using three different methods: i) DPPH radical scavenging activity assay using the non-biological radical DPPH was performed as a preliminary test, ii) ORAC assay, based on a hydrogen atom transfer (HAT) reaction mechanism, was carried out as it better mimics human biology than DPPH, and finally iii) a

cell-based anti-oxidant activity that better represents the complexity of biological systems was evaluated as it is shown in Figures 3, 4 y 5 respectively.

The anti-oxidant efficacy of RPH obtained from the CAA and ORAC assays was in good agreement with that measured in the DPPH radical scavenging activity assay. In general, all RPH showed remarkably higher radical scavenging ability when compared with the non-hydrolyzed reference extract from Isolexx (Ixx_NH).

Interestingly, the non-hydrolyzed extract from PEF showed a similar level of DPPH activity as the hydrolyzates. Knowing that this sample contained negligible amounts of low-Mw peptides (Figure 2) and had a high content (3.5% d.m.) of sinapic acid (Table 1) in comparison to the hydrolyzates, the presence of phenolic compounds, is suggested to play an important role in its antioxidant activity. Phenolics have been extensively reported to have good anti-oxidant properties neutralizing free radicals by acting as donors of a hydrogen atom to radicals [2], and sinapic acid, the main phenolic compound in rapeseed press cake, is a well-known antioxidant [23]. Hydrolyzates from RPH_Px and RPH_Al treatments showed the highest anti-oxidant activities in CAA assay (~80%) at a concentration of 2 mg mL⁻¹, while the same samples reached 50-60% antioxidant activity in the ORAC assay at 0.5 mg mL⁻¹ (Figure 5). These results correlate well with a number of studies reporting potent anti-oxidant activities of Alcalase-derived hydrolyzates when compared to other enzyme samples. The hydrolyzates obtained using a combination of these two alkaline serine endopeptidases (Protex and Alcalase) also contain a higher sinapic acid content (Table 1) which might have enhanced the overall anti-oxidant activity of the hydrolyzates [34].

On the other hand, the two-stage protease treatments showed lower radical scavenging activity. This was probably due to the extensive proteolysis, as observed in Figure 1, which might have resulted in a decrease of the amount of active peptides and an increase of free amino acids which are considered ineffective as anti-oxidants [17].

By analyzing the antioxidant properties of the obtained hydrolyzates, it could be concluded that the presence of phenolic acids, found in both hydrolyzed and non-hydrolyzed PEF samples, boosts the antioxidant properties of the proteins/peptides. Especially in the non-hydrolyzed extracts, antioxidant activity can be assigned to the higher content of phenolic compounds. After proteolysis, the antioxidant activity is believed to be mainly due to the presence of low MW peptides that act synergistically with sinapic acid for antioxidant activity as observed for the RPH_Px and RPH_AI hydrolyzates. The differences in the antioxidant properties of the other hydrolyzates are suggested to be affected mainly by the specificity of the enzyme preparation used and the extent of hydrolysis, influencing the nature and the composition of the obtained peptides and consequently their functional properties.

Anti-inflammatory activity - MPO activity inhibition. Skin damage is a cosmetic issue but also a medical problem. Severely damaged skin is prone to bruising and chronic inflammation, which together with UV exposure and other environmental factors may trigger an imbalance between ROS and endogenous anti-oxidant systems, stimulation the production of neutrophils and thus increasing the activity of myeloperoxidase (MPO). This enzyme is involved in a wide range of body-regulating activities, including infection protection, but when overexpressed increases the susceptibility of skin to inflammation [13]. Previous studies have suggested that the presence of MPO in inflamed skin was much higher compared with normal skin, providing evidence for its role in the inflammatory process [26]. Moreover, the MPO activity is commonly used as a measure of total infiltrating neutrophil content found in inflamed UV-irradiated skin [8,25]. Therefore, a moderated MPO inhibition is desired to provide inflammation control, without inducing unbalanced skin functions.

In general, all RPH inhibited moderately the MPO activity (Figure 6), showing potential in counteracting skin inflammation. The RPH_Px and RPH_AI hydrolyzates inhibited MPO by 36%, which indicates that apart from being efficient antioxidants they could act as anti-inflammatory agents as well. Surprisingly, the RP_NH inhibited around 80% of the MPO

activity, suggesting that the phenolic compounds present in high concentration in this non-hydrolyzed sample may act as competitive substrates for MPO.

The propensity of plant polyphenolic extracts to bind proteins presumably accounts for the fact that polyphenols inhibit virtually every enzyme tested *in vitro* [19]. However, such strong inhibition is not desired due to the biological functions of MPO, and thus moderate inhibition by peptides is considered more favorable. Out of the hydrolyzates, the RPH_Px-AI treated sample showed strongest activity inhibiting the MPO enzyme up to 65%, a level which could be considered a balanced MPO inhibition for treatment of skin irritation.

Effect of RPH on human neutrophil elastase (HNE) activity *in vitro*. The process of skin ageing induces changes in the elasticity and thickness of the skin over time due to the degradation of collagen and elastin components of the extracellular matrix (ECM). These changes result in visible alterations such as wrinkles, pigmentation and skin thickness [2]. In normal conditions, the presence of proteolytic enzymes such as elastase and collagenase on skin are necessary to maintain skin balance [18]. Nevertheless, UV exposure and other environmental conditions can over-induce their activity causing irreversible skin damage and premature ageing [26]. In terms of anti-ageing, finding inhibitors of these proteolytic enzymes could prevent the loss of skin elasticity and thus skin ageing. Therefore, the ability of natural sources such as RPH to act as anti-ageing and skin repairing agents by blocking their activity and altering or inhibiting their metabolic pathways was evaluated. RPH_AI, RPH_Pm and RPH_Px hydrolyzates inhibited the elastase activity by 88%, 87% and 83%, respectively (Figure 7). Nevertheless, good inhibitory activities were also found for the other enzyme-obtained hydrolyzates, including the non-hydrolyzed sample (~65%). The low MW peptides generated by the hydrolysis may act as competitive substrates for elastase, deviating its activity from the elastin substrate.

During the last decades, an intense effort has been directed towards the development of inhibitors to supplement the body's elastase inhibitory capacity. The most pursued approach was the development of low MW recombinant endogenous or synthetic elastase inhibitors that act as competitive substrates [6,32]. Recently, a large number of natural compounds, comprising phenolics, terpenoids, fatty acids and carbohydrates have also been reported as elastase inhibitors [40]. Therefore, the higher inhibitory activity found for the reference treatment might be related to the higher amount of phenolic acids compared to the hydrolyzates. On the other hand, RPH_Px-Pm hydrolyzate induced only ~15% elastase inhibition, possibly due to its high DH (Figure 1) which may have resulted in generation of free amino acids unable to act as competitive substrates.

Biocompatibility of RPH with human foreskin fibroblasts. Aiming at cosmetic applications, the biocompatibility of RPH is an essential parameter to be assessed. It is important to ensure that they do not cause adverse effects upon interaction with human cells. The biocompatibility of the obtained peptides was assessed in human skin fibroblasts. Two different RPH concentrations (0.2 mg mL^{-1} and 2 mg mL^{-1}) were used in the biological activity assays performed after 24 h contact with the cells (Figure 8).

The results confirmed that both the lowest and highest sample concentrations used in the assay did not damage the cells except for the non-hydrolyzed sample RP_NH. This one induced toxicity to the cells at 2 mg mL^{-1} after 24 h contact (53%), probably due to the relatively high concentration of sinapic acid (3.5% d.m.) or the expected presence of other phenolic compounds deriving from PEF. In all other cases, the cell viability was above 80% indicating that at these concentrations the RPH are safe for skin conditioning purposes.

In the present study, microbial endoproteases with different substrate specificity were applied individually or in combination to hydrolyze proteins in the rapeseed press cake, generating peptides which could probably act synergistically with the antioxidant phenolic acids and thus boosting the anti-oxidant, anti-wrinkle and anti-inflammatory activity of the extracts. In

addition, the dry fractionation (i.e. SC-CO₂ extraction, milling and air classification) eliminated any potential toxic compounds encountered in rapeseed press cake, as confirmed by the high biocompatibility of the hydrolyzates with skin fibroblasts. This study presents the rare example of an underutilized natural product transformed into suitable bioactive ingredients that have shown interesting activities and can be used in topical skin care applications for improving its properties.

Conclusions

In the present work, bioactive peptide-rich extracts suitable for skin care applications were obtained from a co-product of rapeseed oil processing industry using enzyme technology. Microbial endoproteases with different substrate specificity, i.e. Alcalase 2.4L FG, Protex 6L, Protamex and Corolase 7089 were applied individually or in combination to hydrolyze proteins in the rapeseed press cake. All enzymes liberated peptides from the rapeseed press cake. The DH was generally higher when a combination of two enzymes, namely Protex 6L followed by another enzyme, was used. Such extensive hydrolysis, however, resulted in less bioactive extracts when compared with the hydrolyzates obtained using a single enzyme, most probably due to the generation of non-active free amino acids rather than peptides. One-step single-enzyme hydrolysis was thus found to generate peptides with better anti-oxidant, anti-wrinkle and anti-inflammatory properties suitable for skin anti-ageing. The dry fractionation (i.e. SC-CO₂ extraction, milling and air classification) and hydrolysis process eliminated any potential toxic compounds encountered in rapeseed press cake, as confirmed by the high biocompatibility of the hydrolyzates with skin fibroblasts. Enrichment of protein content and reduction of the amount of phenolic acids and neutral sugars were observed after the proteolytic treatment.

Hydrolyzates obtained with either Alcalase 2.4L FG or Protex 6L showed potential as anti-oxidants scavenging the cellular free radicals. This was most probably related to the substrate specificity of these alkaline serine endopeptidases, promoting the liberation of antioxidant peptides. The peptides may in addition act synergistically with the antioxidant phenolic acids present at higher concentration in these extracts than in the other hydrolyzates. Alcalase 2.4L FG and Protex 6L hydrolyzates also showed anti-inflammatory and anti-wrinkle properties by inhibiting myeloperoxidase and elastase activity, most probably acting as competitive substrates. Ruled by the same mechanism, the non-hydrolyzed extracts showed good anti-inflammatory and anti-wrinkle properties as well. However, toxicity of the non-hydrolyzed extract from the protein enriched fraction of rapeseed press cake (PEF) towards human cells makes it unsuitable for skin care applications. This study presents the rare example of an underutilized natural product, obtained from a rapeseed oil pressing co-product, and subsequently enzymatically transformed into suitable bioactive ingredients that have shown interesting activities and can be used in topical skin care applications for improving its properties.

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Figure captions

Figure 1. Degree of enzymatic hydrolysis in the hydrolyzates of commercial rapeseed protein isolate Isolexx (Ixx) and crude rapeseed protein-enriched fraction (PEF) determined as the proportion of FAN out of total raw material nitrogen (N) in the hydrolyzates.

Figure 2. SDS-PAGE in reducing conditions of Isolexx (Ixx) (left side) and PEF (right side) and of extracts obtained from these materials under different enzymatic treatments: No enzyme: non-hydrolyzed extract, Px: Protex 6L, Pm: Protamex, Co: Corolase 7089, Al: Alcalase 2.4L FG, Px-Pm: Protex 6L followed by Protamex Px-Co: Protex 6L followed by Corolase 7089 and Px-Al: Protex 6L followed by Alcalase 2.4L FG. Proteins were dissolved in SDS buffer and disulfide bonds in proteins were reduced with 1% mercaptoethanol.

Figure 3. DPPH radical scavenging effect of RPH and non-hydrolyzed extracts from Isolexx (Ixx_NH) and PEF (RP_NH).

Figure 4. Oxygen radical absorbance capacity (ORAC) of RPH and non-hydrolyzed extracts from Isolexx (Ixx_NH) and PEF (RP_NH) at 0.5 mg mL⁻¹.

Figure 5. Cellular anti-oxidant activity of RPH at a concentration of 2 mg mL⁻¹ assessed in skin fibroblasts (BJ-5ta) cells. Peroxyl radical-induced oxidation of DCFH to DCF (% of dichlorofluorescein released) in skin fibroblasts cells, without anti-oxidant (C-), upon contact with RPH, 1 mg mL⁻¹ ascorbic acid and non-hydrolyzed sample.

Figure 6. Inhibition of MPO activity by RPH at 0.2 mg mL⁻¹.

Figure 7. Inhibition of human neutrophil elastase by the RPH (0.5 mg mL⁻¹).

Figure 8. Relative cell viability in presence of 0.2 mg mL⁻¹ and 2 mg mL⁻¹ of RPH after 24 h incubation.

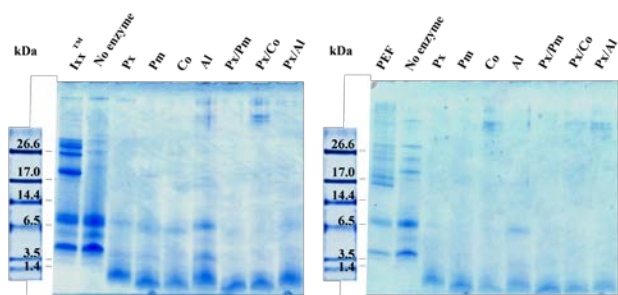
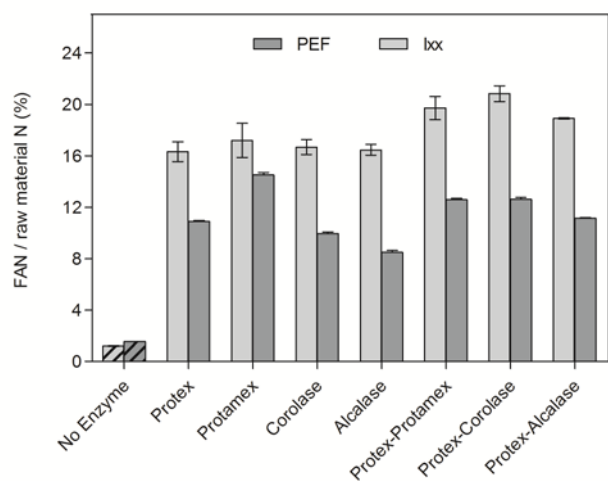
Tables

Table I. Chemical composition of Isolexx and PEF-derived RPH in terms of protein, neutral sugars and sinapic acid content after enzymatic treatment

Hydrolyzate	Protein (% d.m.)	Neutral Sugars (% d.m.)	Sinapic Acid (% d.m.)
Ixx_NH	68.8	2.1	Na
RP_NH	38.1	31.2	3.5
RPH_Px	68.5	23.1	2.0
RPH_Pm	59.1	21.7	1.6
RPH_Co	57.3	24.1	1.6
RPH_Al	63.2	25.5	2.2
RPH_Px- Pm	54.8	17.8	1.4
RPH_Px- Co	59.2	21.9	1.5
RPH_Px- Al	65.8	16.3	1.7

Ixx_NH: Non-hydrolyzed extract from Isolexx. RP_NH: Non-hydrolyzed extract from protein-enriched fraction. RPH: Rapeseed protein hydrolyzates from protein-enriched fraction. Px: Protex 6L. Pm: Protamex. Co: Corolase 7089. Al: Alcalase 2.4L FG. Results are expressed as dry matter percentage (% d.m.). Na: not analyzed

Figures



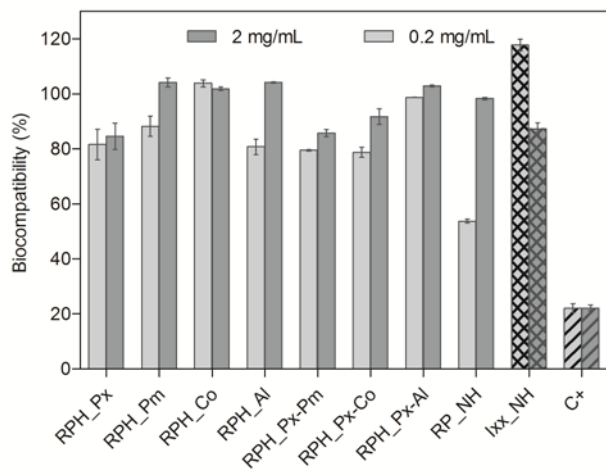
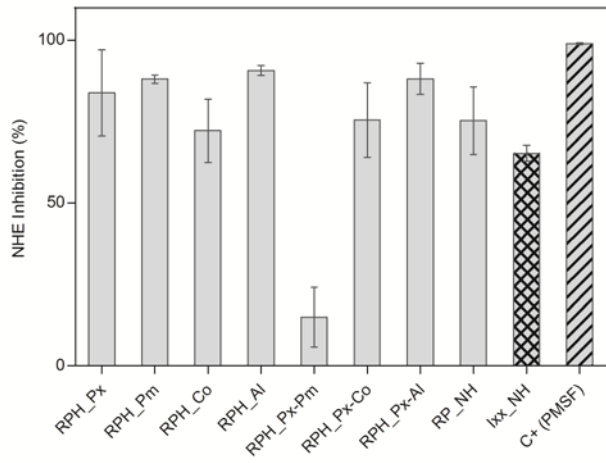
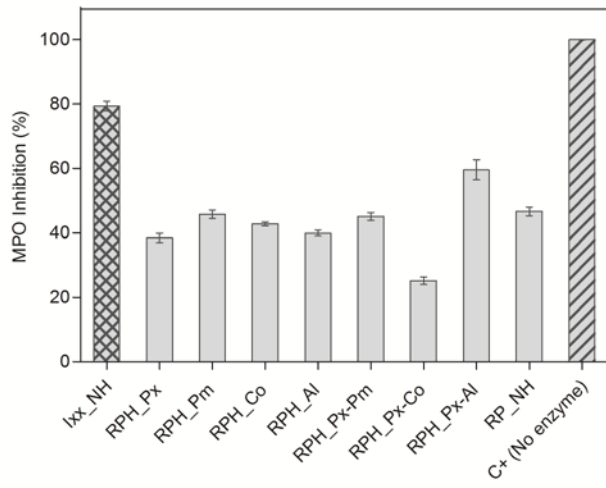


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