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Anti-elastase, anti-tyrosinase and antioxidant properties of a peptide fraction obtained from sorghum (*Sorghum bicolor* L. Moench) grain

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Abstract

Nowadays, the food industry are developing products that provide benefits to the skin health, thus representing a new potential market for the application of biopeptides. The aim of the present work was to investigate the elastase and tyrosinase inhibitory and antioxidant activities of the sorghum kafirins-derived peptide fraction (KAF-P) on human skin cultures exposed to ultraviolet B (UVB) irradiation *in vitro*. Sorghum kafirins were first hydrolysed with alcalase (degree of hydrolysis = 18%). For KAF-P separation, hydrolysates were fractionated by ultrafiltration with a molecular weight cut-off of 3 kDa. It was observed that exposure to UVB induced overactivity of elastase and tyrosinase, as well as decrease in the activity of the antioxidant enzyme system [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)]. Treatment with KAF-P inhibited elastase and tyrosinase. Moreover, KAF-P attenuated the reduction in the activities of SOD, CAT, and GPx; and maintained or enhanced the activity of these enzymes in normal human skin *in vitro*. These anti-elastase, anti-tyrosinase, and antioxidant properties of KAF-P were similar to those obtained with glutathione, which is a peptide that plays an important role in protecting skin cells against UVB. The results suggest that KAF-P is able to intervene in the biochemical reactions of the skin and could, therefore, be considered as a potential candidate for the design of new nutricosmetics and skin-care products.

Introduction

Skin aging in humans is an inevitable process that occurs as a consequence of the accumulated damage as humans age. It is caused by both intrinsic (physiological components and genetic predisposition) and extrinsic factors (exposure to chemical products, air pollution, tobacco oxidants, stress, and solar radiation) (Tobin, 2017). Excessive exposure of the skin to solar UV-radiation, especially UVA and UVB, is the main factor contributing to the development of skin diseases; because it accelerates the aging process by causing oxidative stress, inflammation, as well as damage to DNA and RNA (Tabor and Blair, 2009). Additionally, UV-radiation causes overexpression of proteases (elastase and collagenase), diminishing the elastic fibres and the collagen of the skin (Sultana and Lee, 2007); together with the aforementioned effect, it produces overactivity of tyrosinase (key enzyme implicated in melanin biosynthesis), causing pigmentation and spots that can be located in any part of the body (Ochiai *et al*., 2016). Hence, photoaging is a process characterised by the premature appearance of

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injuries on the skin such as wrinkles (fine and deep), desquamation, laxity, dryness, hyperpigmentation, pre-cancerous lesions and even skin cancer (Royer *et al*., 2013; Soeur *et al*., 2017). For this reason, the food industry has designed a new category of products that benefit the health and well-being of the skin, known as nutricosmetics (products ingested orally) (Taofiq *et al*., 2016). In past years, a great interest has arisen on the application of compounds of natural origin that could improve skin health, establishing as main strategy the incorporation of compounds with antioxidant activity (vitamins, polyphenols, carotenoids, probiotics, polyunsaturated fatty acids, among others) (Lorencini *et al*., 2014).

Sorghum (*Sorghum bicolor* L. Moench) grain is a cereal that contains, on average, 11% protein, of which 70 to 80% corresponds to kafirins (KAF; prolamins fraction), characterised by their high content of hydrophobic amino acids, being extensively crosslinked, and forming inter- and intra-molecular disulphide bonds (Wong *et al*., 2009). Kafirins are classified based on their structure, molecular weight (MW), and amino acid composition in four groups: α, β, γ, and δ–KAF (Belton *et al*., 2006). Several studies on the modification of KAF using chemical treatments (reducing agents), biotechnological processes (fermentation, enzymatic hydrolysis, and genetic improvement) and thermo-mechanical methods (irradiation and extrusion) have been performed to increase their nutritional quality and techno-functionality. However, there is still a great difference between KAF properties and their potential use (de Mesa-Stonestreet *et al*., 2010). Among the modification processes of proteins; enzymatic hydrolysis has shown to be an attractive technique for the production of peptides with different effects on health (antihypertensive, antioxidant, opioid, immunomodulatory, among others) (de Castro and Sato, 2015). Currently, sufficient scientific evidence is available on the bioactivity of peptides, which has raised the scientific and industrial interest in obtaining these biomolecules (Uhlig *et al*., 2014). Because peptides possess antioxidant activity, it is necessary to focus studies on proving their potential benefits on skin health. Likewise, it is also important to study new food sources for peptides. Hence, the aim of the present work was to investigate the elastase and tyrosinase inhibitory and antioxidant properties of a peptide fraction (< 3 kDa) obtained from sorghum grain on human skin cultures exposed to UVB.

Materials and methods

Raw material

White sorghum grain (variety Perla 101) obtained from the National Research Institute for Forestry, Agriculture and Livestock (INIFAP), Campus Culiacan, Mexico, was used in the present work. The whole grains (3 - 4 kg) were milled (IKA, Germany) and sieved through a 300-µm mesh. The proximate analysis of the resulting flour was carried out following the AOAC (2000) methods. The proximate contents were (%, dry basis): crude protein (%N × 6.25 = 8.4 ± 0.2), ash (3.4 ± 0.1), ether extract (1.4 ± 0.1), crude fibre (1.8 ± 0.4) and carbohydrates (85.0 ± 1.4).

Extraction of KAF

KAF extraction was performed according to Kamath *et al*. (2007) with some modifications. Sorghum flour (100 g) was mixed with 500 mL tert-butanol containing 5% (w/v) of sodium metabisulphite and stirred for 6 h at 37°C. The slurry was centrifuged at 10,000 rpm for 15 min at 4°C. The residue was re-extracted with the same volume of solvent, and the two supernatants were pooled and placed on a rotary evaporator model RE100-Pro (DLAB Scientific, Riverside, California, USA) at 60 rpm for 2 h at 40°C. All KAF extracts were stored at -20°C until further use. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard (Sigma-Aldrich, St. Louis, Missouri, USA) at a concentration range from 0 to 1 mg/mL.

Enzymatic hydrolysis of KAF

KAF extract (3 mg/mL, final concentration) was hydrolysed with alcalase (EC 3.4.21.62, protease from *Bacillus licheniformis*, specific activity 2.4 U/g, Sigma-Aldrich) at pH 7.5 and 50°C. The effect of two factors, hydrolysis time (*t*) and enzyme-substrate ratio (ES) on the degree of hydrolysis (DH) was studied using a full factorial design: 3^2. By using this design, the levels for the *t* factor (X_1) were: 3, 6 and 9 h, and for the ES factor (X_2) were: 0.8, 1.2 and 1.6 U/g protein. The response variable DH was determined by the reaction of free amino groups with the 2,4,6-trinitrobenzenesulfonic acid (TNBS) reagent (Adler-Nissen, 1979), using L-leucine as standard (Sigma-Aldrich, USA) at a concentration range from 0 to 2.5 mM. DH values were calculated using Eq. 1:

$$DH\ (\%) = \left[\frac{(L_t - L_0)}{(L_{max} - L_0)} \right] \times 100 \qquad \text{(Eq. 1}$$

where, L_t = amount of free amino groups released after enzymatic hydrolysis, L_0 = amount of free

amino groups in the KAF extract, and L_{max} = total amount of free amino groups in KAF obtained after total hydrolysis.

Table 1 shows the experimental matrix design, with the results obtained. The model proposed for response variable (Y) was:

$$Y_{\%DH} = \beta_0 + \beta_1 t + \beta_2 ES + \beta_{1,2}\, tES + \varepsilon \qquad \text{(Eq. 2)}$$

where, β_0 = intercept, β_1 and β_2 = linear coefficients, $\beta_{1,2}$ = interaction coefficient, and ε = experimental error. The parameters of the model were estimated by multiple linear regression using the Statistica v. 10 software (StatSoft, Palo Alto, California, USA).

Table 1. Matrix of experimental design to evaluate the effect of hydrolysis conditions on the degree of hydrolysis.

Run	Experimental factors		Response variable
	t (h)	ES (U/g protein)	DH (%)
1	3	0.8	5.1 ± 0.0[a]
2	6	0.8	10.2 ± 0.1[b]
3	9	0.8	13.3 ± 0.0[d]
4	3	1.2	10.0 ± 0.1[b]
5	6	1.2	14.6 ± 0.0[e]
6	9	1.2	16.6 ± 0.0[g]
7	3	1.6	11.2 ± 0.1[c]
8	6	1.6	15.3 ± 0.1[f]
9	9	1.6	18.1 ± 0.0[h]

Data are means ± standard deviations of three replicates (n = 3). Means with different superscripts are significantly different ($p < 0.05$). t = hydrolysis time, ES = enzyme-substrate ratio, DH = degree of hydrolysis.

Ultrafiltration

For peptide fraction separation with a M_W lower than 3 kDa, KAF hydrolysate (KAF-H) obtained at the optimal hydrolysis conditions was filtered through a stirred cell filtration module (Model 8400, volume of 400 mL for 76 mm filters, EMD Millipore, Bedford, Massachusetts, USA) with a regenerated cellulose membrane (3 kDa M_WCO). Ultrafiltration (UF) was performed at a pressure of 200 kPa and stirring speed of 250 rpm (at room temperature). The permeate flux (J) was maintained constant (6.9 ± 0.1 kg/m^{2}h) and peptide concentration was determined by the TNBS method. Ultrafiltered peptide fraction (M_W < 3 kDa) was denominated KAF-P, lyophilised and stored at -20°C until further analysis.

Electrophoresis

Polyacrylamide gel electrophoresis (SDS-PAGE)

KAF profile under reducing conditions was carried out according to Mazhar *et al*. (1993), using a 12% separating gel (w/v polyacrylamide) and a stacking gel (4% w/v polyacrylamide). Gels were run in mini-slabs (Bio-Rad, Mini Protean III® Model, Hercules, California, USA) at 200 V for 45 min. The M_W of the bands was determined using a protein standard (SDS-PAGE Standards, Broad Range, Bio-Rad, Catalog Number 161-0317).

Tricine-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

KAF-H and KAF-P profiles under reducing conditions were carried out according to Schägger (2006), employing 16%, 10%, and 4% (w/v polyacrylamide) separating, spacing, and stacking gels, respectively. Gels were run at 90 V for 3 h. The M_W of the bands was determined using a standard (Polypeptide SDS-PAGE Standards, Bio-Rad, Catalog Number 161-0326).

Preparation of human skin cultures (in vitro model)

In the present work, human skin cultures were used as an *in vitro* model. Human skin samples (n = 6) were obtained from healthy women (25 to 35 years of age) undergoing abdominal plastic surgery. Skin samples were prepared following the procedure reported by Alcántara Quintana *et al*. (2015) with slight modifications. Skin explants were cleaned in 70% ethanol for 30 s and immediately placed in cold Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich). Subsequently, the hypodermis (subcutaneous layer) was removed and fragments of 2 mm^3 were cut. Skin explants were placed with the dermal side down in 2 cm^2-plates with 1.5 mL of medium DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin, and 2% foetal bovine serum; and stored in a humidified incubator containing 5% CO_2 at 37°C for 36 h. The medium was changed after 24 h.

Application of UVB irradiation

The human skin cultures were exposed to UVB (30 mJ/cm^2) for 30 min using an UVB lamp (VL-6.LM, Vilber Lourmat, France). Following UVB irradiation, the skin samples were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 36 h. Subsequently, the skin samples were homogenised in Tris-HCl buffer containing proteinase K, and centrifuged at 10,000 rpm for 15 min at 25°C. Supernatants obtained were used for the bioassays.

The skin samples were divided into the following groups: non-UVB-exposed skin as negative control (C-), UVB-exposed skin as positive control (C+), non-UVB-exposed skin plus KAF-P (treatment 1,

T_1), and UVB-exposed skin plus KAF-P (treatment 2, T_2). KAF-P dissolved in 1 mM dimethyl sulfoxide (DMSO) was applied at a concentration of 100, 200, and 300 µg/mL.

Determination of elastase and tyrosinase inhibitory activity

Elastase

The inhibitory activity of elastase (EC 3.4.21.36) was assessed spectrophotometrically following the method reported by Royer *et al*. (2013) with some modifications. N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Suc-Ala-Ala-Ala-pNA, Sigma-Aldrich) was used as the substrate, and the released *p*-nitroaniline was quantified using an absorbance of 415 nm ($\varepsilon = 8800\ M^{-1}\cdot cm^{-1}$). The reaction was carried out by adding 150 µL of 0.2 M Tris-HCl buffer (pH 7.5), 20 µL of sample (C-, C+, T_1, or T_2), and 30 µL of 1.4 mM Suc-Ala-Ala-Ala-pNA (dissolved in 0.2 M Tris-HCl buffer). The mixture was incubated at 37°C for 20 min, and the absorbance was measured at 415 nm. The percentage of elastase inhibition was calculated using Eq. 3:

$$Inhibition\ (\%) = \left[1 - \left(\frac{A}{B} \right) \right] x\ 100 \qquad \text{(Eq. 3)}$$

where, A = enzyme activity with KAF-P, and B = enzyme activity without KAF-P.

Tyrosinase

The inhibitory activity of tyrosinase (EC 1.14.18.1) was assessed spectrophotometrically following the method reported by Ochiai *et al*. (2016) with some modifications. L-tyrosine was used as the substrate, and the released dopachrome was quantified using an absorbance of 475 nm ($\varepsilon = 3700\ M^{-1}\cdot cm^{-1}$). The reaction was carried out by adding 145 µL of 0.1 M phosphate buffer (pH 6.5), 20 µL of sample (C-, C+, T_1, or T_2), and 35 µL of 2 mM L-tyrosine (dissolved in 0.1 M phosphate buffer). The mixture was incubated at 30°C for 10 min, and the absorbance was measured at 475 nm. The percentage of tyrosinase inhibition was calculated by the same equation used for elastase.

Assessment of the antioxidant enzyme system

The activities of the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined with test kits following manufacturer´s instructions: CAT (EC 1.11.1.6) Assay Kit (Cayman Chemical Item No. 707002, Ann Arbor, Michigan, USA), SOD (EC 1.15.1.1) Assay Kit (Item No. 19160), and GPx (EC 1.11.1.9) Assay Kit (Item No. CGP1) (Sigma-Aldrich, USA). Data was expressed as units per milligram of protein (U/mg protein).

Bioactivity of KAF-P was compared to glutathione (GSH), which is a peptide that plays an important role in protecting skin cells against damage by free radicals and UVB radiation (Tabor and Blair, 2009; de Castro and Sato, 2015).

Statistical analysis

Results were reported as means ± standard deviations. Significant differences among treatments were determined by analysis of variance and Fisher´s least significant difference (LSD) tests ($p < 0.05$) using the Statistica v. 10 software (StatSoft, Inc., USA).

Results and discussion

KAF extraction

The crude protein content of the Perla 101 sorghum grain was 8.4% (dry basis), which was within the range previously reported (8.2 - 8.6%), as well as for other white varieties (Costeño 201, Fortuna, Istmeño, Mazatlan 16, and RB Paloma) of 8.3 - 11.8% (Montes-García *et al*., 2010). The KAF extraction yield was 2.5 ± 0.1 g/100 g flour; to our knowledge, there are no reports about the content of KAF in the Perla 101 grain. According to our results, the extraction yield was within the range reported for white varieties, where values between 0.5 and 6.0 g/100 g of flour have been obtained (Kamath *et al*., 2007; Espinosa-Ramírez and Serna-Saldívar, 2016). The yield of extracted protein depends on several factors such as the variety and the decortication degree of the grain, the extraction method, type and concentration of the solvent, reducing agent, and batch size.

Figure 1(A) shows the electrophoretic SDS-PAGE profile of the KAF extract, which shows two main bands with a relative molecular weight (M_{Wr}) of 25 - 27 kDa and 23 kDa. Based on previous reports, α-KAF presents two subunits: α_1 and α_2 with M_{Wr} of 24 - 28 kDa and 22 - 23 kDa, respectively (de Mesa-Stonestreet *et al*., 2010; Espinosa-Ramírez and Serna-Saldívar, 2016). The KAF profile also showed a band (of lower intensity) of 19 kDa, which has been identified as β-KAF (Mazhar *et al*., 1993; Belton *et al*., 2006). The band corresponding to γ-KAF (M_{Wr} of 27 - 28 kDa) could not be distinguished clearly; based on previous studies, this would indicate that the γ-KAF fraction is bound to the α_1-KAF fraction by means of disulphide bonds (Belton *et al*., 2006),

and probably, these were not hydrolysed during the extraction, as there are covalent bonds between the thiol groups (—SH) of cysteines. Therefore, the electrophoretic analysis showed the presence of three subunits (α, β, and γ) constituting the KAF of the Perla 101 sorghum grain.

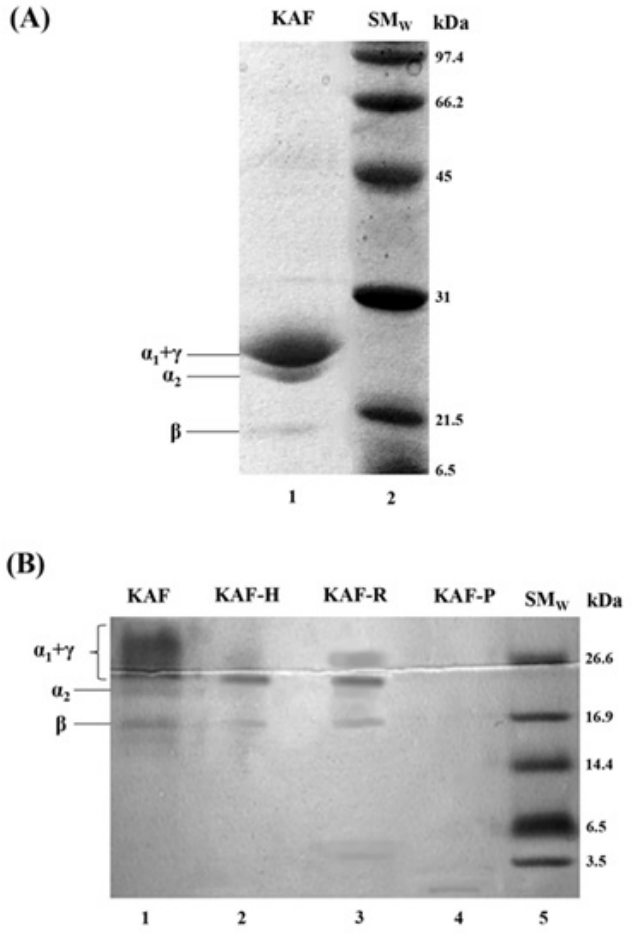

Figure 1. (A) SDS-PAGE profile of KAF from sorghum grain (variety Perla 101). Lane 1 = kafirins extract (KAF), lane 2 = protein standard (SM_W). (B) SDS-PAGE profile of KAF-H and ultrafiltration fractions. Lane 1 = kafirins extract (KAF), lane 2 = kafirins hydrolysate obtained at optimal hydrolysis conditions (KAF-H), lane 3 = retentate fraction (KAF-R), lane 4 = permeate fraction (KAF-P), lane 5 = polypeptide standard (SM_W).

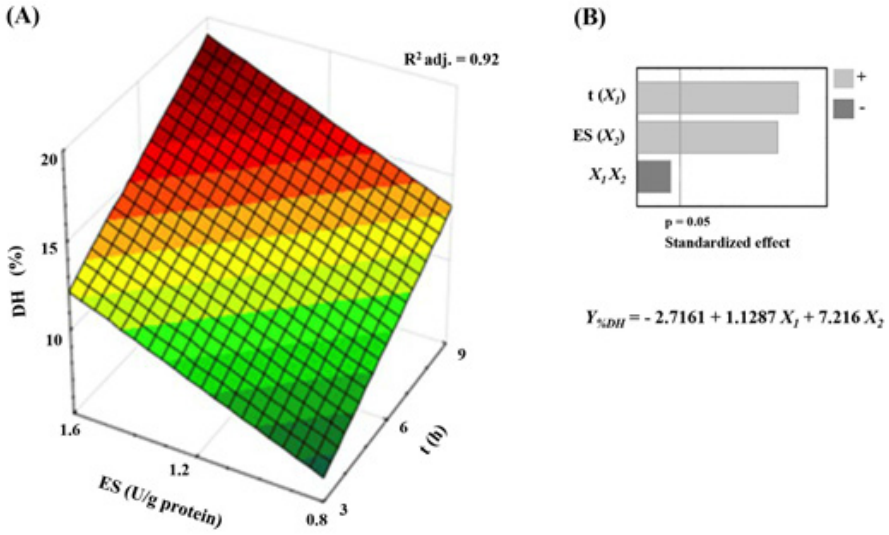

Figure 2. (A) Response surface plot indicating the effect of hydrolysis time (*t*) and enzyme-substrate ratio (ES) on the degree of hydrolysis (DH). (B) Pareto chart and polynomial equation that fitted to the response surface model.

Optimal hydrolysis conditions of KAF

In the present work, KAF hydrolysis was accomplished with alcalase, which is an endopeptidase of microbial origin that presents broad specificity, liberating peptides with hydrophobic amino acids at their C-terminal. It has been frequently used to obtain protein hydrolysates and biopeptides (de Castro and Sato, 2015). Table 1 depicts the DH values obtained for the combinations of *t* (h) and ES (U/g protein) used in the hydrolysis of KAF with alcalase. DH depends significantly ($p < 0.05$) on *t* and ES, in which as variables increased, DH also increased. DH values were 5.1 - 18.1%, pointing out that in 8 of 9 combinations of the hydrolysis conditions, a DH ≥ 10% was obtained, which, according to Agyei *et al*. (2016), leads to a release of biopeptides. It should be noted, that this is the first time that KAF are hydrolysed with alcalase and their DH is determined.

Figure 2(A) shows the surface plot for the response variable (DH) as a function of the factors *t* (X_1) and ES (X_2) of KAF hydrolysis with alcalase. The estimated model presented an adjusted determination coefficient (R^2_{adj}) of 0.92, indicating that more than 90% of the variation of the response variable can be explained by this model. The standardised Pareto chart (Figure 2B) shows the effect of each factor (X_1 and X_2) on the DH (%); in this case, their interaction (X_1X_2) was not significant ($p < 0.05$), thus, it was not considered in the final polynomial equation (Figure 2B).

t (X_1) = 9 h and ES (X_2) = 1.6 U/g protein were the conditions providing the greatest DH with a prediction value of 18.6% and an experimental value of 18.1%. Therefore, these conditions were used for the production of KAF-H.

Figure 1(B) shows that KAF-H presents a different electrophoretic tricine-SDS-PAGE than KAF; additionally, a decrease in the intensity of the bands corresponding to α_1, β, and γ-KAF, as well as the absence of the band corresponding to α_2-KAF was observed. Based on this profile, the different protein groups that conform the KAF were hydrolysed by alcalase.

Separation of KAF-P

The hydrolysis of proteins is a process of simultaneous reactions, during which a release of peptide species of different M_W occurs. In this regard, the membrane filtration technology is an available process for the separation and enrichment of peptides with a specific range of M_W. Since it has been demonstrated that peptides with greater biological activity would have low M_W, containing from 2 to 20 amino acids (de Castro and Sato, 2015), the separation of < 3 kDa peptides present in KAF-H was accomplished by an UF module. The content of peptide in the ultrafiltered peptide fraction (KAF-P; < 3 kDa) was 901.8 ± 4.1 µg/mL. Zhuang *et al*. (2013) obtained peptides under 3 kDa, through sequential UF (membranes with M_WCO from 30 to 10 kDa) from corn gluten meal hydrolysates obtained with flavourzyme, resulting in a peptide concentration of 500 µg/mL, which is significantly lower than that obtained in the present work. Likewise, Meza-Espinoza *et al*. (2018) used a sequential separation with M_WCO from 30 to 1 kDa to obtain antioxidant peptide fractions from soybean, egg, and milk hydrolysates at concentrations between 140 - 981 µg/mL. Hence, under the conditions used in the present work, the separation of a peptide fraction (< 3 kDa) at a suitable concentration for the bioassays was achieved through the use of a single membrane.

Figure 1(B) shows the electrophoretic profiles (Tricine-SDS-PAGE) of KAF-H fed into the UF module, as well as the retained (KAF-R) and permeated (KAF-P) fractions obtained. It was observed that the KAF-R profile was similar to that of KAF-H, although a band between 3.5 and 6.5 kDa was found, which corresponded to the release of polypeptides with MW greater than 3 kDa that became concentrated because of the UF. It must be mentioned that in the KAF-R profile, no bands below 3.5 kDa were observed. On the other hand, the KAF-P profile presented only one band below 3.5 kDa, confirming the presence of < 3 kDa peptides. The electrophoretic analysis yielded similar results to those reported for soy hydrolysates fractionated with sequential UF through membranes of different M_WCO: 50, 10, 5, and 3 kDa (Jiménez-Ruiz *et al*., 2013). It must be pointed out that we did not apply sequential fractioning, thus indicating that using just one membrane was sufficient to obtain peptide fractions at the concentrations required for the bioassays.

Capacity of KAF-P to inhibit elastase and tyrosinase

Elastase inhibition

Initially, the skin samples exposed to UVB (C+) presented a 57% increase in the elastase activity as compared to samples not exposed to UVB (C-) (data not shown), demonstrating that UVB induces elastase expression and activation in human skin, as described by Cavinato and Jansen-Dürr (2017) and Fehér *et al*. (2016). Figure 3(A) shows that KAF-P inhibited elastase activity in a concentration-dependent effect, revealing that the highest inhibition percentage of this enzyme occurred when exposed to UVB (T_2), reaching 27.2% of inhibition at a peptide concentration of 300 µg/mL, which was similar to that obtained with GSH (27.6%) at the same concentration. Although KAF-P

inhibited the elastase activity at a lower percentage when not exposed to UVB (T_1), this was able to inhibit the enzyme as compared to GSH, which did not show inhibitory activity. The inhibitory values of KAF-P on elastase are within the reported range for polyphenolic extracts of different vegetal sources (Royer *et al*., 2013; Hong *et al*., 2014; Jamaluddin *et al*., 2014), synthetic peptides (Vasconcelos *et al*., 2011; Barros *et al*., 2012) and peptides derived from donkey hide gelatine (Kim *et al*., 2018) evaluated through *in vitro* assays and human skin fibroblasts. Likewise, the results indicate that KAF-P produces a higher or equal inhibitory effect on elastase to that showed by GSH. Since the inhibition of elastase is used as a helpful mechanism to protect the skin against aging induced by UVB (Tsukahara *et al*., 2001), KAF-P could be considered a potential protective agent, avoiding the accelerated degradation of elastin (main component of the elastic fibres of the dermis), thereby preventing the appearance of wrinkles and the loss of skin elasticity.

Tyrosinase inhibition

Tyrosinase is the key enzyme implicated in melanin biosynthesis; therefore, its inhibition is of utter relevance for the protection of the skin against the alterations caused by the exposure to UVB. Under the conditions evaluated in the present work, the skin samples exposed to UVB (C+) presented an 11% increase of tyrosinase activity as compared to samples not exposed to UVB (C-), indicating that UVB produces an increase in the enzyme's activity. Figure 3(B) shows that in the treatment exposed to UVB (T_2), KAF-P was able to inhibit tyrosinase in a similar behaviour to that shown for elastase, that is, a concentration-dependent effect, with the highest inhibition (6.9%) at a peptide concentration of 300 µg/mL. However, in this case, GSH presented a greater inhibitory capacity (22.9%). On the other hand, in the treatment not exposed to UVB (T_1), it was observed that the inhibitory effect of KAF-P did not depend of the concentration, obtaining inhibitory values of 0.5 to 2.4%, significantly higher ($p < 0.05$)

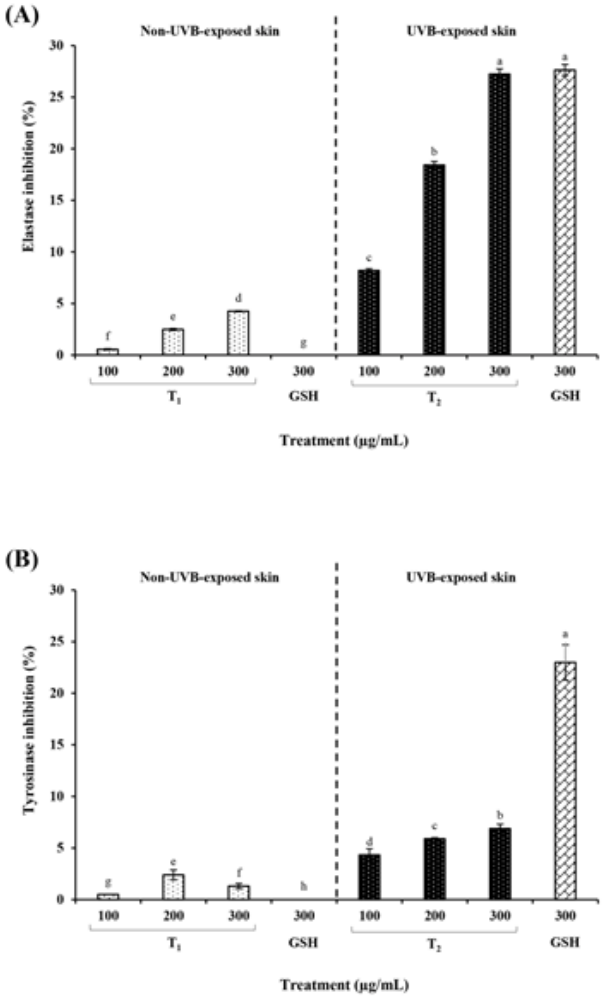

Figure 3. Elastase and tyrosinase inhibitory activity of KAF-P. (A) = elastase inhibition, (B) = tyrosinase inhibition. Data are means ± standard deviation of six replicates ($n = 6$). Means with different superscripts are significantly different ($p < 0.05$). T_1 = non-UVB-exposed skin plus KAF-P, T_2 = UVB-exposed skin plus KAF-P, GSH = glutathione.

than GSH, which did not present inhibitory activity. GSH is not only an antioxidant peptide but also an inhibitor of tyrosinase activity, which makes it an effective protective agent against UVB (Tabor and Blair, 2009). Additionally, *in vitro* studies have showed a tyrosinase inhibition between 1.7 and 80% when evaluating polyphenolic extracts (Royer *et al*., 2013; Jamaluddin *et al*., 2014) and synthetic peptides (Ubeid *et al*., 2012; Hsiao *et al*., 2014; Ochiai *et al*., 2016) at concentrations similar to those used in the present work. Likewise, results indicate that KAF-P produces an inhibition of tyrosinase and may exert a protective effect against UVB, which could avoid the appearance of age spots and prevent the hyperpigmentation and formation of melanomas.

KAF-P showed the capacity to inhibit the activities of elastase and tyrosinase in both treatments (T_1 and T_2). Since the highest inhibition was obtained at a concentration of 300 µg/mL, it was decided to assess its effects on the activity of antioxidant enzymes at this concentration.

Effect of KAF-P on the antioxidant enzyme system

Antioxidant enzymes, SOD, CAT, and GPx, form a ROS-reducing coupled system that allows the avoidance of the damage caused by oxidative stress (de Castro and Sato, 2015). Figure 4 shows that in the skin samples exposed to UVB (C+), SOD, CAT, and GPx activities decreased by 30%, 18%, and 53%, respectively, as compared to the samples not exposed to UVB (C-), indicating that the cells underwent oxidative stress. However, in the treatment exposed to UVB with KAF-P (T_2), the SOD, CAT, and GPx activities increased by 22%, 14% and 34%, respectively, with respect to C+, which indicates that KAF-P avoided the diminution of CAT activity (Figure 4B) and restored the SOD and GPx activities (Figure 4A and C). Regarding GSH, a similar behaviour to that of KAF-P was observed in the SOD and CAT activities; however, the restoration of GPx activity was significantly higher ($p < 0.05$) in the presence of KAF-P, as compared to GSH. On the other hand, in the treatment not exposed to UVB with KAF-P (T_1), the SOD and GPx activities were kept constant, without significant differences ($p > 0.05$) with respect to the control (C-) and GSH. It is worth mentioning that for CAT, a 23% increase in the enzymatic activity was obtained, which was not observed with GSH.

Decreases in the activities of antioxidant enzymes

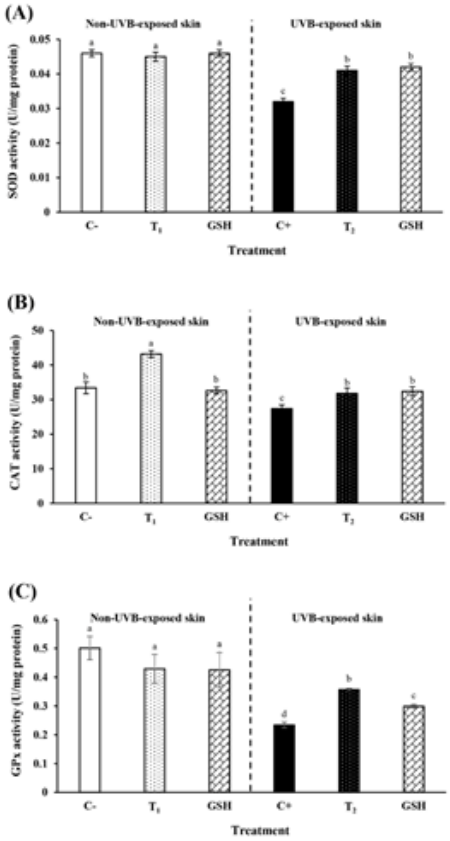

Figure 4. Effect of KAF-P on the antioxidant enzyme system. (A) = superoxide dismutase (SOD), (B) = catalase (CAT), (C) = glutathione peroxidase (GPx). Data are means ± standard deviation of six replicates ($n = 6$). Means with different superscripts are significantly different ($p < 0.05$). (C-) = negative control (non-UVB-exposed skin), (C+) = positive control (UVB-exposed skin), T_1 = non-UVB-exposed skin plus KAF-P, T_2 = UVB-exposed skin plus KAF-P, GSH = glutathione. Final concentration of KAF-P and GSH in the bioassays was 300 µg/mL.

(SOD, CAT, and GPx) significantly affected the susceptibility of the skin to oxidative damage, which is associated with different alterations and lesions, such as premature wrinkles, desquamation, irregular dryness, and hypo- and hyper-pigmentation (Tabor and Blair, 2009; Royer *et al*., 2013; Lorencini *et al*., 2014). Therefore, KAF-P, by restoring the activities of the antioxidant enzymes in human skin cultures subjected to oxidative stress induced by UVB and by maintenance or enhancement of the levels of enzymatic activity in the skin not exposed to UVB, showed a protective effect against oxidative damage, which was similar to that observed with GSH, a peptide considered as one of the main antioxidant agents in humans and contributes to the regulation and maintenance of the levels of antioxidant enzymes.

Our results are similar to those found previously by Alcántara Quintana *et al*. (2015) while evaluating albumin hydrolysates from amaranth grain in human skin cultures, as well as to other studies that have reported a protective effect against H_2O_2 oxidative damage by peptides obtained from dry-cured ham (Hu *et al*., 2016) and whey (Kong *et al*., 2012) in PC12 cells and MRC-5 fibroblast cells, respectively. Thus, KAF-P could be an antioxidant agent to counteract the damage caused by UVB, preventing photo-oxidative stress and, consequently, the respective skin alterations.

Conclusion

KAF-P showed a protective effect by maintaining and restoring the antioxidant enzyme system and inhibiting elastase and tyrosinase enzymes in human skin cultures exposed to UVB. Therefore, KAF-P has the potential to be considered as a new UVB-protection natural agent. Further studies are nevertheless needed on the molecular characterisation of KAF-P, as well as the elucidation of their mechanisms of action. Additionally, *in vivo* studies must also be made to determine the functional stability of this peptide fraction as an active ingredient in formulations of new nutricosmetics and skin-care products.

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