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Short communication

Proteolytic activity of cosmetic enzyme peel products*

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Our goal was to verify the proteolytic mode of action and activity levels among several commercial cosmetic facial peels advertised by manufacturers as "enzymatic". Eleven enzyme peels were analyzed for their proteolytic activity against casein as a generic substrate and compared to the activity found in pineapple and papaya fruits. The highest specific protease activity was observed in the flesh of a pineapple (5.88 U/g). Only two products demonstrated sufficient activity (0.924 and 0.238 U/g) to be called "enzyme peels". Three products showed trace activity (0.023–0.125 U/g), albeit too low to exert a significant exfoliating effect. Six preparations had no detectable enzyme activity.

Keywords: enzyme peels, cysteine proteases, proteolytic activity, spectroscopy, formulation/stability

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*This work is dedicated to the memory of the great musician, **Wojciech Karolak**.
Abbreviations: CDSN, corneodesmosin; CTSD, aspartate protease cathepsin D; CTSV, cysteine protease cathepsin L2; DSC1, desmocollin 1; DSG1, desmoglein 1; EDTA, ethylenediaminetetraacetic acid; KLK, kallikrein family serine peptidase; PEG, polyethylene glycol; TCA, trichloroacetic acid; ZO-1, Zonula Occludens Protein 1 (product of TJP1 gene)

INTRODUCTION

Enzymatic facial peels are increasingly popular because they reduce abrasion and redness, which often occur with mechanical or chemical peeling agents. Enzymatic skin exfoliation is promoted as safe for most skin types and recommended for individuals with sensitive skin who do not tolerate the α-hydroxy and β-hydroxy acids found in many chemical peels. Most enzyme peels exploit plant-based cysteine proteases, i.e., papain and bromelains. They cleave the extracellular domains of cell adhesion proteins constituting corneodesmosomes, the structures that bind the epidermal corneocytes together (Lopes *et al.*, 2008).

The extracellular parts of corneodesmosomal plaques are composed of at least three cell adhesion proteins, i.e., desmoglein 1 (DSG1), desmocollin 1 (DSC1), and corneodesmosin (CDSN) (Ishida-Yamamoto & Igawa, 2015). A few cell layers above the level at which the cells change from the granular to cornified phenotype, the cell surfaces become covered with corneodesmosomes (Naoe *et al.*, 2010). During corneocyte maturation, corneodesmosomes are progressively decomposed and are preserved only at the lateral rims of the cells, where they are protected from proteolysis by the cell-to-cell tight-junction structures (Igawa *et al.*, 2011).

The physiological degradation of corneodesmosomal proteins is controlled mainly by a cascade composed of the kallikrein family of serine peptidases. KLK5 cleaves CDSN, DSC1, and DSG1; KLK7 degrades CDSN and DSC1 (Caubet *et al.*, 2004); whereas the KLKs 1, 6, and 14 hydrolyze the DSG1 cadherin (Borgoño *et al.*, 2007). The aspartate protease cathepsin D (CTSD) (Igarashi *et al.*, 2004) and cysteine protease cathepsin L2 (CTSV) (Bernard *et al.*, 2003) are involved in desquamation processes by decomposing CDSN. The above proteases, along with their physiological polypeptide inhibitors, control the balance between the formation and desquamation of the outer layers of the stratum corneum (reviewed by Ishida-Yamamoto & Igawa, 2015).

Application of a peel enriched in external endopeptidases to the skin mimics natural enzymatic exfoliation and accelerates the process. For example, papain degrades the proteins of the tight junctions of human keratinocytes, i.e., ZO-1, claudin 4, and occludin (Stremnitzer *et al.*, 2015), as well as the proteinaceous components of corneodesmosomes (Lopes *et al.*, 2008). Likewise, the bromelain proteases, i.e., fruit bromelain, stem bromelain, and ananain, hydrolyze a vast array of skin cell-surface proteins (Hale *et al.*, 2005).

Cosmetologists frequently ask how to judge which of the commercial peel products act by proteolytic degradation of the cell adhesion proteins. We could not find any peer-reviewed publications addressing this issue directly. It is hard to answer this question, considering that revealing the specific activity of the enzyme peels is not required by EU regulations (Regulation (EC) No 1223, 2009; Commission Regulation (EU) No 655, 2013), nor is it voluntarily supplied by most manufacturers. Therefore, we decided to pursue our investigation by measuring the proteolytic activity of eleven representative facial peel products marketed as "enzymatic peels" that were available on the Polish market at the time.

MATERIALS AND METHODS

Chemicals and materials

Anhydrous sodium carbonate, L-tyrosine, potassium dihydrogen phosphate, proteinase K from *Tritirachium album* (lyophilized, >3 U/mg), sodium dihydrogen phosphate, sodium hydroxide, and Triton X-100, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine Hammarsten casein was from LOBA Feinchemie (Fischamend, Austria). Folin-Ciocalteu reagent and trichloroacetic acid were from POCH (Gliwice, Poland). High-purity water (18.3 MΩ/cm resistance) was produced with a Hydrolab HLP 10UV purification system (Straszyn, Poland).

Table 1. Forms and declared compositions of the enzyme peel products (emboldened are proteolytic enzymes and enzyme-containing extracts, chemical exfoliants are underlined)

No.	Product's form	Product's composition specified by manufacturer
1	Light brown thick paste	Prunus Amygdalus Dulcis Oil, Elaeis Guineensis Oil, Theobroma Cacao Seed Oil, Butyrospermum Parkii Butter, Glyceryl Stearate, Lauryl Glucoside, **Papain**, **Bromelain**, Hydroxystearic Acid, Cymbopogon Schoenanthus Oil, Tocopheryl Acetate, Pelargonium Graveolens Oil, Citrus Limonum Peel Oil, Allantoin, Benzyl Alcohol, Dehydroacetic Acid, Geraniol.
2	Light brown thin cream	Aqua, Prunus Amygdalus Dulcis Oil, Glycerin, Simmondsia Chinensis Seed Oil, Sodium Acryloyldimethyl Taurate/acrylamide/vp Copolymer, Glycyrrhiza Glabra Root Extract, Humulus Lupulus Cone Extract, Rheum officinale Baill, Spiraea Ulmaria Extract, Salix Alba Bark Extract, Hibiscus Rosa-sinensis Flower/leaf Extract, Tropaeolum Majus Extract, Benzyl Alcohol, **Bromelain**, Lactobionic acid, Parfum, Ethylhexylglycerin, Lactic Acid.
3	Light yellow thin gel	Aqua, Glycolic Acid, Macadamia Ternfolia Seed Oil, Persea Gratissima (Avocado) Oil, Vitis Vinifera Seed Oil, Olea Europaea (Olive) Fruit Oil, Hydroxypropyl Starch Phosphate, Panthenol, Sodium Hydroxide, **Carica Papaya (Papaya) Fruit Extract**, **Papain**, Pyrus Malus (Apple) Fiber, Citric Acid, Lactic Acid, **Bromelain**, Eclipta Prostrata Extract, Daucus Carota Sativa (Carrot) Seed Oil, Guaiacum Officinale Wood Oil, Citrus Aurantifolia (Lime) Peel Oil, Melia Azadirachta Oil, Echinacea Purpurea Extract, Linoleic Acid (and) Oleic Acid (and) Linolenic Acid, Mandelic Acid, Saccharum Officinarum Extract, Glycyrrhiza Glabra (Licorice) Root Extract, Citrus Medica Limonum Fruit Extract, Salicylic Acid, Retinol, Lecithin, Rosmarinus Officinalis Leaf Extract, Iron Oxides, Benzyl Alcohol, Dehydroacetic Acid, Benzoic Acid, Sorbic Acid.
4	White powder (lyophilisate)	Talc, Oryza Saliva (Rice) Starch, Sodium Carrageenan, Algin, Cl 77891 (Titanium Dioxide), Sodium Carboxymethyl Starch, Lactose, Parfum (Fragrance), **Ananas Sativus Extract (Bromelain)**, **Papain**, Silica, Ascorbic Acid, Maris Aqua (Sea Water).
5	White thick cream	Sea Salt, Caprylic/Capric Triglyceride, Cetyl Alcohol, PEG-75 Lanolin, Zea Mays (Corn) Seed Flour, Aluminum Starch Octenylsuccinate, Laureth-4, Beeswax, Hydrogenated Castor Oil, Glyceryl Oleate, **Ananas Sativus (Pineapple) Fruit Juice**, PEG-45/Dodecyl Glycol Copolymer, Glycerin, Lecithin, Tocopherol, Hydrogenated Palm Glycerides Citrate, Ascorbyl Palmitate, Benzyl Alcohol, Quaternium-90 Bentonite, Salicylic Acid, Alcohol, Sorbic Acid, Parfum (Fragrance), Hexyl Cinnamal.
6	Light yellow thin gel	Water, Polyquaternium-6, Polyethylene, Propanediol, **Ananas Sativus (Pineapple) Friut Extract**, Glycosyl Trehalose, Hydrogenated Starch Hydrolysate, Carbomer, Phenoxyetanol, Sodium Polyacrylate, Propylene Glycol, Dipotassium Glycyrrhizate, Chlorphenesin, Polysorbate 20, Fragrance, Xantan Gum, Glycerin, **Papain**, Calcium Pantothenate, Caprylyl Glycol, Urea, Magnesium Lactate, Ethylhexylglycerin, Potassium Lactate, Serine, Alanine, Proline, Magnesium Chloride, Sodium Citrate, Cl 19140.
7	White thin cream	Aqua (Water), Ammonium Acryloyldimethyltaurate/VP Copolymer, PEG-7 Glyceryl Cocoate, Phenoxyethanol, Panthenol, Niacinamide, Octyldodecanol, Passiflora Edulis Fruit Extract, Citrus Limon Fruit Extract, Sodium Hyaluronate, Sorbitan Tristearate Propanediol, Alcohol Denat., Parfum (Fragrance), Ethylhexylglycerin, Glyceryl Stearate, Maltodextrin, Agar, Scierotium Gum, C20-24 Alkyl Dimethiocne, **Papain**, Allantoin, Sodium Bisulfite, Potassium Sorbate, Disodium EDTA, Methylparaben, Propylparaben, Ethylparaben, Xylitylglucosides, Anhydroxylitol, Xylitol, Glucose.
8	White thin cream	Aqua, Hydrogenated Polydecane, Isopropyl Myristate, Glyceryl Stearate, Hydrogenated Polydecene, Glycerin, Stearyl Alcohol, **Papain**, Carbomer, Algin, Aloe Barbadensis Leaf Juice Powder, Lecithin, Sorbitol, Ascorbyl Glucoside, Panthenol, Glucose, Trilaureth-4 Phosphate, Ascorbyl Tetraisopalmitate, Alcohol, Terminalia Ferdinandiana Fruit Extract, Xanthan Gum, Dimethicone, Ceteareth-20, Ceteareth-25, Sodium Hydroxide, Disodium EDTA, Phenoxyethanol, Hydroxyacetophenone, Octadecyl Di-T-Butyl-4-Hydroxyhydrocinnamate, Benzyl Salicylate, Citronellol, Hexyl Cinnamal, Hydroxycitronellal, Limonene, Linalool, Parfum, Cl 15985, Cl 19140.
9	White thin cream	Aqua (Water), Glycerin, Prunus Amygdalus Dulcis (Sweet Almond) Oil, Polyacrylamide, Parfum (Fragrance), C13-14 Isoparaffin, Panthenol, Laureth-7, Alcohol, **Papain**, Ethylhexylglycerin, Propylene Glycol, Lecithin, Guar Hydroxypropyltrimonium Chloride, Hydrogenated Starch Hydrolysate, Hydroxyethylcellulose, Malpighia Punicifolia (Acerola) Fruit Extract, Bambusa Vulgaris (Bamboo) Shoot Extract, Nelumbo Nucifera Flower Extract, Nymphaea Alba (Water Lily) Root Extract, Phenoxyethanol, Methylparaben, Mica, Cl 77891 (Titanium Dioxide), Cl 16035 (Fd&C Red No. 40), Cl 17200 (D&C Red No. 33).
10	White thin cream	Aqua, Cetearyl Alcohol, Glycolic Acid, Caprylic/Capric Triglyceride Glycerin, Octyldodecanol, Glyceryl Stearate, Cetearyl Glucoside, Sodium Hydroxide, Hydroxyethylcellulose, Angelica Archangelica Root Water, Prunus Armeniaca Kernel Oil, Mangifera Indica Seed Butter, Macadamia Ternifolia Seed Oil, Olea Europaea Fruit Oil, Sodium Stearoyl Glutamate, Benzyl Alcohol, Benzoic Acid, Sorbic Acid, Parfum, Hexyl Cinnamal, Cl 15985, Cl 16255.
11	Pink thin cream	Aqua (Water), Glycerin, Prunus Amygdalus Dulcis (Sweet Almond) Oil, Polyacrylamide, Parfum (Fragrance), C13-14 Isoparaffin, Panthenol, Laureth-7, Alcohol, **Papain**, Ethylhexylglycerin, Propylene Glycol, Lecithin, Guar Hydroxypropyltrimonium Chloride, Hydrogenated Starch Hydrolysate, Hydroxyethylcellulose, Malpighia Punicifolia (Acerola) Fruit Extract, Bambusa Vulgaris (Bamboo) Shoot Extract, Nelumbo Nucifera Flower Extract, Nymphaea Alba (Water Lily) Root Extract, Phenoxyethanol, Methylparaben, Mica, Cl 77891 (Titanium Dioxide), Cl 16035 (Fd&C Red No. 40), Cl 17200 (D&C Red No. 33).

Naturally ripened pineapples (*Ananas comosus*) and papaya (*Carica papaya*) were bought in the local supermarket. The facial peel products were popular, widely distributed in Poland, clearly labeled as "enzymatic", and analyzed well within the specified periods of their minimum durability. The products were assigned consecutive numbers without revealing the identities of the peel preparations or their producers. Their forms and declared compositions are listed in Table 1. The author has had no relationships with the peel-producing companies or their employees.

Determination of L-tyrosine release

The L-tyrosine assay used the Folin and Ciocalteu method (Folin & Ciocalteu, 1927). A stock solution was prepared by dissolving 20 mg of L-tyrosine in 100 mL H_2O at 30°C. To determine a precise molar concentration of this solution, it was diluted three times with 20 mM phosphate buffer, pH 7.0. Its UV absorbance at 200–340 nm was measured against the same buffer. The absorbance at 274.5 nm was used to calculate the L-tyrosine concentration (1090.8 μM) based on its molar absorption coefficient (ε=1405 M^{-1} cm^{-1}) (Fig. 1a). Calibration solutions were prepared by diluting the L-tyrosine stock with water to the concentrations of 30, 60, 120, 240 and 300 μM.

The 0.5 mL aliquots of calibration solutions or enzymatic reaction samples were reacted at 37°C for 30 min with 1.25 mL of 0.5 M sodium carbonate and 0.25 mL of 4-times water-diluted Folin-Ciocalteu reagent. All samples were centrifuged for 10 min at 3850×*g* (room temperature), the resulting supernatants were analyzed spectrophotometrically. To generate a calibration curve, the absorbance values of the calibration samples were measured at 660 nm (Fig. 1b) against a blank containing a water equivalent in the place of the tyrosine solution (Fig. 1c). For enzymatic reaction mixtures, the measurements were performed against blank samples prepared as described below.

Preparation of samples for the proteolytic activity assay

Four-gram portions of each enzyme peel, or the fruit flesh samples, were homogenized on ice with 12 mL of cold 50 mM KH_2PO_4-NaOH buffer, pH 7.5, containing 1% Triton X-100, in a motor-driven 50-mL Potter-Elvehjem homogenizer. The lyophilized product No. 4 was suspended in the manufacturer-specified amount of water. The homogenates were centrifuged for 15 min at 3850×*g* (10°C) in a 50-mL screw-capped polypropylene tubes. The water phases of the supernatants (referred to hereafter as the "extracts") were gently aspirated and immediately analyzed for proteolytic activity.

Determination of the proteolytic activity

The proteolytic activity assay was based on the method developed by Maeno *et al.* (Maeno *et al.*, 1959). Casein, 0.65% (m/v), in 50 mM KH_2PO_4-NaOH buffer, pH 7.5, was used as a substrate. To prepare the substrate solution, 650 mg of casein was gently stirred into 100 mL of the above buffer while gradually increasing the temperature to 80°C. The substrate solution, 14 mL in 50-mL screw-capped polypropylene tube, was pre-incubated to 37°C in a circulating water bath. The enzymatic reaction was initiated by adding 0.7 mL of peel extract. The progress of the reaction was monitored after 10, 20, 30, 60, 90, and 120 min by transferring a 2.1 mL aliquot of the reaction mixture to a 15-mL screw-capped polypropylene tube containing 2 mL of 110 mM trichloroacetic acid (TCA). The samples were vigorously vortexed and then incubated at 37°C for at least 30 min to precipitate non-hydrolyzed casein. A blank sample was prepared by the sequential mixing of 2 mL of 110 mM TCA, 2 mL of the substrate solution, and 0.1 mL of the analyzed extract (TCA inhibits the activity of the proteases) followed by incubation at 37°C for 120 min. All TCA-treated samples were centrifuged for 30 min (10°C) at 3850×*g* and the released L-tyrosine in the collected supernatants was determined as described above. The absorbance values at 660 nm were measured in the enzymatic reaction samples against the corresponding blank samples containing the same peel or fruit extract.

Determination of the specific activity

Enzymatic activity was calculated from the initial rate of the reaction (measured 10 min after initiation) and expressed as micromoles of L-tyrosine released per min in one mL of the reaction mixture. The enzymatic activity unit (U) was defined as the amount of activity liberating 1 μmol of L-tyrosine per min in a 14.7-mL reaction mixture under the reaction conditions defined above. The specific activity was expressed as the activity units contained in one gram of peel product or fruit flesh.

RESULTS

To confirm the accuracy of the well-established assay that we used for general proteolytic activity (Folin & Ciocalteu, 1927; Maeno *et al.*, 1959), we applied it to follow the progress of casein hydrolysis by a commercial preparation of proteinase K from *Tritirachium album* (1 mg/mL) under our standard reaction conditions.

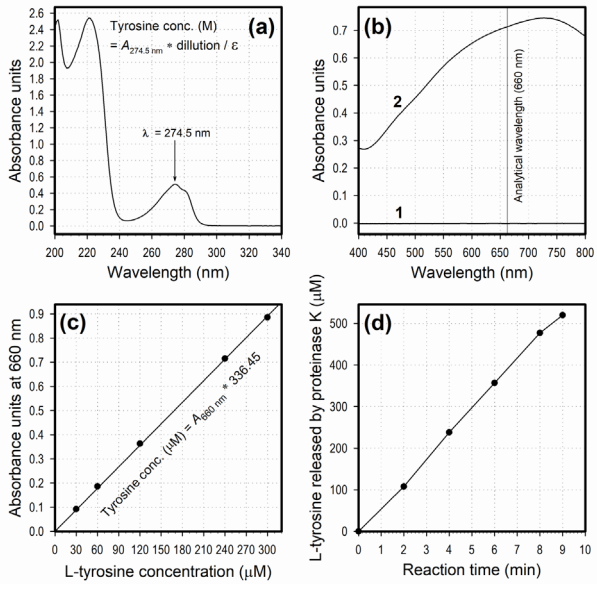

Figure 1. An assay of general proteolytic activity based on the liberation of tyrosine from a casein substrate:
(**a**) The UV absorbance spectrum of an L-tyrosine stock solution and the determination of its molar concentration based on its absorbance at 274.5 nm. (**b**) The visible light absorbance spectra of the Folin-Ciocalteu reagent before (1) and after the reaction with tyrosine (2), both measured against water. (**c**) The calibration curve for L-tyrosine determination with the Folin-Ciocalteu method. (**d**) The time course of the casein hydrolysis by proteinase K, expressed as liberated tyrosine.

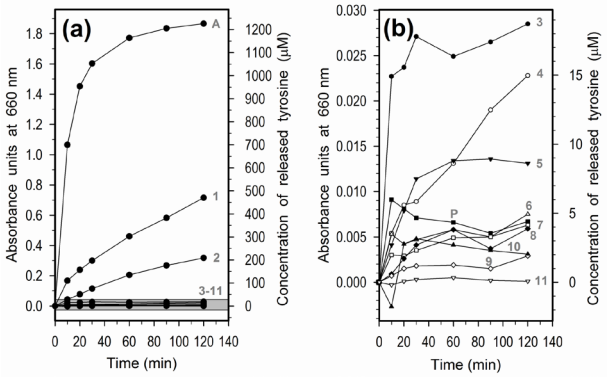

Figure 2. A degradation of casein by the extracts of enzyme peels and fruits:
(**a**) Time-course curves of casein hydrolysis catalyzed by the extracts of eleven commercial enzyme peels (Table 1) and two fruit extracts (A – pineapple, P – papaya). (**b**) A close-up view of the grey portion of the graph (a).

Fig. 1d shows the linear time course of casein degradation up to a 500 µM concentration of liberated tyrosine.

Since papain and fruit bromelain effectively hydrolyze casein at near neutral pH (Barbosa *et al.*, 2004; Corzo *et al.*, 2012), we confirmed the suitability of our method by checking the proteolytic activity of the flesh of the ripe pineapple collective fruit, as well as of the pulp of the ripe (yellow) papaya fruit. In agreement with a previous report (Martin, 2017), our pineapple's extract was found to rapidly hydrolyze casein (Fig. 2a), with a specific activity of 5.88 U/g of tissue (Fig. 3), unlike the papaya extract (Fig 2b), which demonstrated about 120-fold lower activity of 0.05 U/g of tissue (Fig. 3).

All the commercial peels degraded casein far less efficiently than the pineapple extract did. Of the eleven peels we examined, only No. 1 and 2 markedly hydrolyzed casein over 120 min period (Fig. 2a). Their specific activities were 0.924 and 0.238 U/g, respectively (Fig. 3). Considering the reaction curves (Fig. 2b), products 3, 4, and 5 demonstrated very low, but detectable, proteolytic activities, corresponding to specific activities of 0.125, 0.030, and 0.023 U/g, respectively (Fig. 3). Peels 6 through 11 appeared to be virtually devoid of protease activity (Fig. 2b and Fig. 3).

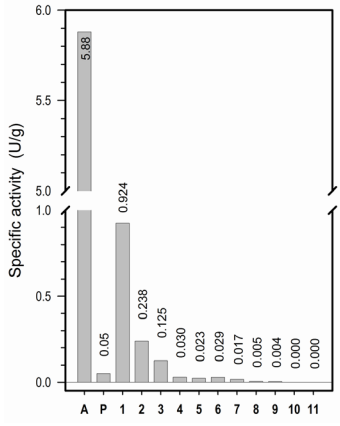

Figure 3. Specific proteolytic activities of commercial enzyme peels and fruit extracts are expressed as numbers of activity units contained in a gram of specimen.
Numbers 1 through 11 correspond to the peel products listed in Table 1; A – pineapple, P – papaya.

DISCUSSION

We tested the proteolytic activity of eleven common commercial cosmetic facial products clearly labeled as "enzymatic peels". Table 1 shows nine of the products listed protease(s), i.e., papain, bromelain, or both, in their preparation. Product no. 5 contained pineapple juice as the sole source of proteases, whereas the list of ingredients for product no. 10 surprisingly had no potential protease source. In our opinion, only two of the eleven commercial peels demonstrated a proteolytic activity sufficient to qualify as an "enzyme peel". Our findings do not mean that the remaining nine products were unable to have positive peeling effects, just that these effects could not be attributed to proteolytic causes. Five peels (No. 2, 3, 5, 6, 10) contained alpha and/or beta hydroxy acids that induce chemical exfoliation. Additional product ingredients may improve the general condition of the skin - but not by the measurable action of proteases.

Since the manufacturers do not specify the originally intended units of the proteolytic activity of their peel products, it is impossible to determine the cause of the poor or absent activity, i.e., whether the proteolytic activity was negligible to begin with or was lost post-production. Cysteine proteases are known to have limited stability in water solutions. For instance, the activity of an unconjugated papain solution was shown to drop by more than 90% after one month at 25°C (Sim *et al.*, 2000). Bromelain in oil/water emulsions and gels was significantly more stable, but it still lost up to 70% of its initial activity after six months at 25°C. Moreover, certain gel formulations have been demonstrated to immediately inhibit up to 50% of bromelain's activity (Lourenço *et al.*, 2016).

Papain has better depilatory efficacy in creams than in gel formulations (Traversa *et al.*, 2007). Two of the peel products we investigated (No. 3 and 6) were in gel form (Table 1). As shown in Fig. 2 and 3, product No. 3 had low but detectable proteolytic activity, whereas No. 6 was inactive. Since the other five inactive peels (No. 7–11) were in the form of an emulsion cream, it would suggest that the amount of active enzyme added to the products during manufacture was what most likely determined their measured proteolytic performance. The exceptionally low activity of peel No. 4, sold in a lyophilized form, normally highly stable, further emphasizes the need for strict regulation of the enzymatic activity introduced during the production process. Product No. 1, the most enzymatically active of the peels tested, was the only water-free product. It was based on a mixture of oils and meant to be applied to wet skin. Our study found this formulation advantageous for this specific enzyme peel product, most likely by producing a longer shelf-life, although we have not conducted a shelf-life analysis.

Within the limited number of peel products investigated, most were labeled incorrectly as enzymatic. Since quality control of enzyme-based cosmetics is not mandatory under current commercial regulations, a consensus recommendation for verifying the effectiveness of commercial enzyme peels is needed from cosmetology and dermatology professionals. In our opinion, manufacturers should verify whether the right amount of active enzyme is added to their preparations and how other ingredients, further processing, and storage conditions affect the stability of the cysteine protease activities of their products.

Cysteine proteases extracted from papaya (papain, chymopapain, glycyl endopeptidase, caricain) and pineapple (stem bromelain, fruit bromelain, ananain, comosain)

have a broad substrate specificity (Choe *et al.*, 2006) and are able to cleave multiple peptide bonds in most proteins. Therefore, a proteolytic activity of the peel product toward casein is a good measure of its potential to cleave cell adhesion proteins of the skin. The enzymatic activity assay used in this study is simple, cheap, and capable of being performed in any biochemical laboratory with basic equipment. The two most commonly used enzyme exfoliants are papain and bromelain, and bovine casein is degraded at the neutral pH by both, as well as by fresh extracts of papaya and pineapple fruits (Barbosa *et al.*, 2004; Corzo *et al.*, 2012; Martin 2017). Tyrosine release determination with Folin-Ciocalteu reagent is sensitive and suitable for the detection of proteolysis. This analytical procedure can be easily reproduced by the manufacturers to verify the proteolytic potential and relative shelf-life of their cosmetic peel products.

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