THE INFLUENCE OF ALKALINE EXTRACTION ON SOME KERATIN HYDROLYSATES PROPERTIES

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Keratin is a fibrous protein abundant in nature, being the component of wool, hair, hooves, horns, feathers, and claws. Keratin is one of the most valuable natural biopolymers due to its chemical versatility and biological performance. At the molecular level, keratin is distinguished from other biopolymers by its high concentration of cysteine-containing sulfur. Two keratin hydrolysate batches were obtained in alkaline medium, at a constant concentration of 8% NaOH and 75°C (KerNa8₇₅), 85°C (KerNa8₈₅), and 95°C (KerNa8₉₅), and at a fixed temperature of 99°C and different concentrations of NaOH, i.e. 3% (KerNa3₉₉), 5% (KerNa5₉₉), and 8% (KerNa8₉₉), respectively. Physical-chemical analyses showed that the protein content ranging between 83.60% for KerNa8₇₅ and 88.88% for KerNa3₉₉, while the total nitrogen was found 13.83% and 14.67% in the case of KerNa8₇₅ and KerNa3₉₉, respectively. Dynamic light scattering analysis showed that the particle sizes decreased with the increased concentration in the reaction medium. The average particle size was between 1352 nm and 1771 nm for the samples obtained at a temperature of 99°C and with lower values between 463.3 nm and 571.6 nm for the samples obtained with 8% NaOH. The Fourier transform infrared (FT-IR) spectra evidenced the specific bands of keratin-specific proteins and sulfur compounds. Experiments were also performed to evaluate the antioxidant activity and the growth of Tamino and Mirastar wheat plants by applying the treatments with 3% and 5% concentrations of KerNa8₉₉ on wheat seeds. These experiments showed an improvement in the wheat plant growth during 10 days of observation compared to control sample. The results recommend the potential use for keratin hydrolysates in the medical, pharmaceutical, cosmetics fields, and also as fertilizers in agriculture.

Keywords: keratin hydrolysates, alkaline hydrolysis, keratin properties

INTRODUCTION

Keratin is one of the most valuable natural biomaterials due to its chemical versatility and biological performance (Râpă *et al.*, 2020). Keratin is an abundant fibrous protein in nature being the component of wool, hair, horns hooves, feathers, claws and epithelial cells (Râpă *et al.*, 2020; Aluigi *et al.*, 2013). At the molecular level, the keratin is distinguished from other proteins (such as fibroin or collagen) by high concentration of cysteine - containing sulfur (7-20% of the total amino acid residues) (Hearle, 2000; Seghir *et al.*, 2020). Cysteine residues are present in oxidized form of cystine residues, characterized by covalent disulfide bonds, which gives the mechanical, thermal stability and chemistry properties (Brandelli, 2008; Feughelman, 2002). In particular, keratins extracted from wool can be classified into two groups: proteins with intermediate filaments and matrix proteins. Intermediate filament proteins have low sulfur keratins with molecular weights in the range of 40-60 kDa and α -helix structure. Matrix proteins are keratin with a lot of sulfur, and molecular weights in the range of 11–26 kDa. Matrix keratins surround keratins in proteins with intermediate filaments and interact with them through intermolecular disulfide bonds (Plowman, 2003). Keratins extracted from wool are biodegradable (Yamauchi *et al.*, 1996), support

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growth and adhesion of fibroblasts (Tachibana *et al.*, 2002) and osteoblasts (Tachibana *et al.*, 2005). Keratin is a structural protein with interesting properties in terms of biocompatibility and an absorbent ability for heavy metal ions and volatile organic compounds (Râpă *et al.*, 2020). Keratin is an ideal biopolymer for wound healing due to the amino acid sequence that induces cell adhesion, mitogenic and chemotactic activity and mediates changes in gene expression (Râpă *et al.*, 2020). The development of techniques for the efficient production of biomass from poultry farming or sheep breeding for keratin extraction should prove to be very useful for the sustainable management of huge waste. Denaturation techniques, such as methods based on reduction (Zeng and Lu, 2014), oxidation (Brown *et al.*, 2016) and enzymatic hydrolysis and ionic liquids (Rajabinejad *et al.*, 2018) are benchmarks for obtaining good yields and soluble keratin (Hearle, 2000).

In this paper we aimed to modify the properties of some keratin hydrolysates obtained by alkaline extraction under the influence of temperature and concentration variation of the working environment. Experiments were also performed to evaluate the antioxidant activity and bioactive properties by assessing the growth of Tamino and Mirastar wheat plants by applying the treatments with 3% and 5% concentrations of KerNa8₉₉.

MATERIALS AND METHODS

Materials

Pearl sodium hydroxide (NaOH) was acquired from Lachner, Neratovice, Czech Republic. Sheep wool was purchased from local sheep farmers. The other materials are analytical grade.

Methods

Extraction of Keratin Hydrolysate

Keratin hydrolysate was obtained by alkaline hydrolysis with NaOH. Two series of keratins were obtained by varying the temperature or concentration during the production process. Alkaline hydrolysis was made with mechanical stirring for 3h. The first batch of keratins was obtained at constant temperature of 99°C and different concentrations of NaOH: 3% (KerNa3₉₉), 5% (KerNa5₉₉) and 8% (KerNa8₉₉) (w/w). The second batch of keratins was obtained at a constant concentration of NaOH 8% (w/w) and modified temperatures, i.e. 75°C (KerNa8₇₅), 85°C (KerNa8₈₅), and 95°C (KerNa8₉₅).

Characterization of Keratin Hydrolysates

The physical–chemical characteristics for keratin hydrolysates obtained were analyzed according to the standardized and in-house methods: SR EN ISO 4684:2006 (dry matter), SR EN ISO 4047:2008 (ash content), SR ISO 5397:1996 (total nitrogen content and protein). The size particles and zeta potential of keratins were measured by Dynamic light scattering (DLS) technique with Zetasizer Nano-ZS device from Malvern (Malvern Hills, UK). The results of the analyses are expressed as the average values of three determinations. ATR-FT-IR analysis was performed with a FT-IR/ATR spectrometer - Jasco 4200 operating in the range of 4000 to 550 cm⁻¹, with spectral resolution of 0.5 cm⁻¹.

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The antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS^{•+}) free radicals for KerNa8₉₉ was also determined by measuring the maximum absorbance recorded at 517 nm and 750 nm, respectively. Experiments were also performed to evaluate the growth of Tamino and Mirastar wheat plants by applying the treatments with KerNa8₉₉ at two concentrations of 3% and 5% over a period of 10 days. Three samples were prepared for each wheat type consisted of 10 seeds: a control sample treated with water, a sample treated with 3% KerNa8₉₉ and a sample treated with 5% KerNa8₉₉.

RESULTS AND DISCUSSIONS

The main physical-chemical characteristics of keratins hydrolysate extracts obtained with 3%, 5% and 8% solution of NaOH and constant temperature at 99°C are shown in table 1.

Table 1. Physical-chemical characteristics of KerNa3₉₉, KerNa5₉₉ and KerNa8₉₉ hydrolysate extracts

Characteristics	KerNa3 ₉₉	KerNa5 ₉₉	KerNa8 ₉₉
Dry matter (%)	10.70	11.75	12.60
Ash (%)*	8.32	11.40	11.03
Total nitrogen (%)*	14.67	13.96	13.89
Protein content (%)*	88.88	84.60	84.21
pH (units of pH)	10.31	10.34	10.77

* Values reported at dry substance

The keratin hydrolysates obtained have values from 13.89% to 14.67% for total nitrogen content and between 84.21% and 88.88% for the protein content and the dry matter content increased from 10.70% to 12.60% with the increasing of concentration in the reaction medium (table 1).

Table 2. Particle sizes and zeta potential of KerNa3₉₉, KerNa5₉₉ and KerNa8₉₉ hydrolysate extracts

Keratin hydrolysate	Particle populations (%) and size (nm)						Average, d, nm	Pdl	Zeta potential, mV
	Majority population 1		Majority population 2		Majority population 3				
	Size	%	Size	%	Size	%			
KerNa3 ₉₉	446	27.1	2111	50	4723	22.9	1358	0.744	-11
KerNa5 ₉₉	117.3	11.5	798.6	88.5	-	-	1697	0.993	-12.1
KerNa8 ₉₉	142.9	6.7	614	73.4	5497	19.9	1771	1	-12.7

As the concentration of NaOH increased from 3% to 8%, the size of the constituent nanoparticles decreases in the keratin hydrolysate extraction medium (Table 2) due to the increase of the degree of hydrolysis of keratin chains. DLS analysis of keratin hydrolysates shows the presence of majority populations starting from 117.3 nm (11.5%) for KerNa5₉₉, 142.9 nm (6.7%) for KerNa8₉₉, and 446 nm (27.1%) for KerNa3₉₉ to 5497 nm (19.9%) for KerNa8₉₉. The zeta potential was between -11 mV for KerNa3₉₉ and -12.7 mV for KerNa8₉₉ (Table 2). The main physical-chemical characteristics of keratin hydrolysate extracts obtained at 8% NaOH and temperatures of 75°C, 85°C, and 95°C are shown in table 3.

The Influence of Alkaline Extraction on Some Keratin Hydrolysates Properties

Table 3. Physical-chemical characteristics of KerNa8₇₅, KerNa8₈₅ and KerNa8₉₅ hydrolysate extracts

Characteristics	KerNa8 ₇₅	KerNa8 ₈₅	KerNa8 ₉₅
Dry matter (%)	3.11	4.25	4.74
Ash (%)*	15.11	11.53	10.97
Total nitrogen (%)*	13.83	14.35	14.56
Protein content (%)*	83.60	87.06	88.19
pH (units of pH)	11.93	11.42	10.61

* Values reported at dry substance

The keratin hydrolysates obtained with 8% NaOH and different temperatures showed an increase of the total nitrogen values from 13.83% in the case of KerNa8₇₅ to 14.56% in the case of KerNa8₉₅ and of the protein substance from 83.60% in KerNa8₇₅ to 88.19% in KerNa8₉₅ (table 3).

Table 4. Particle sizes and zeta potential of KerNa8₇₅, KerNa8₈₅ and KerNa8₉₅ hydrolysate extracts

Keratin hydrolysate	Particle populations (%) and size (nm)						Average, d, nm	Pdl	Zeta potential, mV
	Majority population 1		Majority population 2		Majority population 3				
	Size	%	Size	%	Size	%			
KerNa875	93.51	9.2	510.3	79.3	5062	11.5	463.3	0.682	-18.9
KerNa885	96.17	10.3	590.8	76.7	5146	13	563.1	0.760	-18.3
KerNa895	73.66	8.3	541.8	81.6	5320	10	571.6	0.796	-17.1

Particle size measurement shows values from 73.66 nm (8.3%) in KerNa8₉₅ and 96.17 nm (10.3%) in KerNa8₈₅ to 510.3 nm (79.3) in KerNa8₇₅ and 590.8 nm (76.7%) in KerNa8₇₅ to 5062 nm (11.5%) in KerNa8₇₅ and 5320 nm (10%) in KerNa8₉₅. The potential zeta values were between -17.1mV at KerNa8₉₅ to -18.9 mV for KerNa8₇₅ (table 4).

Figure 1. ATR-FTIR spectra for: KerNa3₉₉, KerNa5₉₉, and KerNa8₉₉ (a) and KerNa8₇₅, KerNa8₈₅, KerNa8₉₅ (b) compared with the spectrum of sheep wool

The ATR-FTIR spectra showed in Figure 1 (a,b) the spectral bands specific to proteins and sulfur compounds resulting from the hydrolysis process by breaking the S-S bonds of cystine (Khosa and Ullah, 2014). Keratin is easier to break into smaller specific fragments after breaking these S-S bonds between macromolecular chains

(Pavia *et al.*, 2008). The spectral band from 3280-3274 cm^{-1} can be attributed to the stretching vibration of -O-H and -N-H (Amide A) (Mohanty *et al.*, 2005). The absorption peak around 2960 cm^{-1} is attributed to the asymmetric extent of -CH₂- (Amide B). The specific band from 1630-1637 cm^{-1} is assigned to the extension of -C=O (Amide I). The absorption peaks between 1517-1549 cm^{-1} and 1242-1237 cm^{-1} correspond to the N-H band coupled to the C-H (Amide II) range and the C-H (Amide III) range, respectively. The absorption bands at 670 cm^{-1} and 578-541 cm^{-1} can be attributed to the extension of the C-S bond and the S-S bond, as well as to the deformation of the C-C bond corresponding to keratin specific sulfur compounds.

KerNa8₉₉ hydrolysate extract showed antioxidant activity measured by DPPH method of $23.65 \pm 1.4\%$ and by ABTS method of $99.86 \pm 2.0\%$. The values are similar with feather origin keratin hydrolysates with 0.2 mg/mL concentration (Oluba *et al.*, 2019).

KerNa8₉₉ was selected to treat the wheat seeds because this type has a higher value for dry matter (12.60%) and important protein content (84.21%). Tamino wheat treated with both concentrations of KerNa8₉₉ increased more than the control sample and Mirastar wheat treated with 5% KerNa8₉₉ increased more compared to the control sample in the 10 days of the experiment. Tamino wheat increased in length by 44.3% in the case of treatment with 3% KerNa8₉₉ and by 19.9% when it was treated with 5% KerNa8₉₉ compared to the control sample (figure 2). Mirastar wheat seeds grew less, with 25.49% for treatment with 3% KerNa8₉₉ compared to the control sample, while in the case of 5% KerNa8₉₉ it increased by 50.3% compared to water treated seeds (figure 3).

Figure 2. The increase in Tamino wheat stem length (cm)

Figure 3. The increase in Mirastar wheat stem length (cm)

CONCLUSION

Two keratin hydrolysate batches were obtained from sheep wool by alkaline hydrolysis at different concentrations and temperatures of the reaction medium. The physical-chemical characteristics of the keratins showed values of the protein substance between 84.21% and 88.88% corresponding to total nitrogen content of 13.83% and 14.67%. Particle size measurements showed a decrease in particle size with increasing concentration of the reaction medium. The ATR-FTIR spectra showed spectral bands specific to proteins and sulfur compounds from keratin. KerNa8₉₉ hydrolysate extract had the highest antioxidant activity, due to the highest level of hydrolysis. Experiments performed on growth Tamino and Mirastar wheat plants by applying 5% KerNa8₉₉ treatments showed an improvement in the wheat plant growth in first 10 days of

observation compared with control sample. Keratin hydrolysates can be used in the medical, pharmaceutical, cosmetics fields, and also as fertilizers in agriculture.

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