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Original Research

Keratinolytic Proteases in Biodegradation of Pretreated Feathers

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Abstract

The increasing generation of keratinic wastes by the poultry industry stimulated research on novel, cost-effective methods of keratin protein management respecting current environmental regulations. Enzymatic treatment of feather waste with microbial keratinases appears to be one of the most promising methods to obtain valuable products. Nevertheless, the action of keratinolytic proteases requires the support of disulfide bond-reducing agents to reveal their full potential. The presented study was aimed at evaluating effects of moderate thermo-chemical feather pretreatment, preceding hydrolysis with two crude microbial keratinases from *Bacillus cereus* B5esz and *B. subtilis* P22. Keratinases of both tested strains exhibited capability for degradation of native feathers, but substrate pretreatment resulted in significant improvement of the process. Application of 10 mM sulfite in the pretreatment remained to be the most appropriate option, leading to 160% or 95% activity enhancement of keratinases from *B. cereus* and *B. subtilis*, respectively. Pretreatment with 10 mM NaOH also gave a satisfactory effect. Pepsin digestibility was mostly influenced either by 1 mM sulfite pretreatment or by sole autoclaving. Crude keratinase from *B. cereus*, a potent keratinase producer, expressed only four times lower specific activity against raw feathers in comparison to purified commercial proteinase K, and the action of both enzyme preparations was influenced by sulfite pretreatment of the substrate. Additionally, the concentrated culture broth of *B. cereus* B5esz proved to be highly applicable in degradation of raw feathers, especially in the presence of sulfite or sulfite-pretreated feathers.

Keywords: keratinase, feather keratin, pretreatment, hydrolysis, *Bacillus* sp.

Introduction

Poultry feathers, along with other keratin-rich by-products are classified within category 3 according to European Parliament and Council Regulation (EC) 1774/2002. According to the document, keratinic waste must be subject to certain methods of disposal, such as incineration or landfill deployment after thermal treatment, but also can undergo conversion into valuable products. The main directions of bioconversion comprise: transformation into feed components for household and fur animals (with current restrictions referring to their eventual application in farm animal feed as introduced by regulation 142/2011), composting, biogas production, or application as soil fertilizers. The document, however, provides an open field for application of alternate methods of keratin waste treatments, but preceded by an established procedure [1, 2].

Presently, the increasing output of keratinic wastes from the poultry industry draws an attention to exploring novel, cost-effective ways of keratin protein management, respecting current environmental regulations. The main requirement for the bioconversion methods would be the employment of accessible and nontoxic reagents to obtain products of high animal nutritional values. Currently applied proce-

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dures include costly equipment, high temperatures, and prolonged processes, often incorporating high concentrations of hazardous chemicals [3]. Hence, numerous industrial applications of keratinolytic enzymes are being developed. From among them, a few directions constitute the dominating trend. The application of keratinolytic enzymes as well as keratin hydrolysates is of major interest in the leather processing industry. Benefits of keratinolytic proteases can serve as an alternative for high concentrations of lime and sulfide during the liming stage, responsible for 80-90% of pollution in the entire process of leather preparation, while keratin hydrolysates proved effective either in the improvement of chromium exhaustion from tannery bath or in enhancement of the retanning process. Keratinases can also amend hide dehairing in the non-lime process, which besides elimination of lime-sludge effluent – allows us to considerably reduce the concentration of sodium sulfide [4-6]. Equally important is the applicatory area for keratinolytic enzymes or their microbial host producers in bioconversion of keratinous by-products into protein hydrolysates intended as feed ingredients. Since conventional keratin meals, e.g. feather meal, exhibit limited digestibility to animals, the main objective would be to upgrade the nutritional value of these components. This could be achieved through liquid or solid state fermentation of keratinolytic microorganisms on keratins or ready meals. Likewise, application of cell-free enzyme extracts or purified keratinases also is feasible. Both methods not only improve protein digestibility but also modify the amino acid imbalance, concerning especially methionine, histidine, and tryptophan [7-9]. An alternate approach is based on the enrichment of keratin-supplemented feedstuff or animal diet with keratinolytic enzymes, which also boosts the digestibility of keratin [10]. A two-stage fermentation of feather waste also was successfully applied for the production of biogas, of which the first aerobic phase of feather degradation was carried out by a native *Bacillus licheniformis* or a recombinant *B. megaterium* [11].

Achieving advanced structural degradation of waste feathers could be feasible through enhanced sulfitolysis by chemical or microbial processes, or by the combination of the methods [12]. Evidence was assembled to show that only combined chemical and enzymatic treatment provides optimum results [13, 14]. Microbial treatment in feather waste hydrolysis appears to be the most promising method, allowing us to obtain valuable products. While feather degradation in bacterial cultures is usually a fast and efficient process, hydrolysis with separated keratinolytic proteases undergoes certain limitations. Enhancement of the enzyme activity and stability remain important issues, nevertheless cleavage of keratin disulfide bridges requires particular attention. According to numerous reports, complete keratin hydrolysis could be achieved not only by the sole action of keratinolytic proteases, but also requires a reducing environment. In microbial culture conditions it is accomplished through accumulation of reducing sulfur compounds, of which sulfite is known to play a crucial role [15]. Moreover, vast evidence is available that the process of sulfitolysis could be carried out by disulfide reductases [16, 17].

Bacillus cereus B5esz and *B. subtilis* P22 are keratinolytic strains with profound proteolytic activity [18, 19]. Their high capability of native keratin hydrolysis could be further enhanced by the substrate pretreatment. It could be performed most effectively due to sulfitolytic reaction or limited structural damage to keratin fibers. The goal of the inquiry was to estimate the suitability of cell-free crude keratinases of these bacterial strains for the *in vitro* hydrolysis of native chicken feathers and to assess the validity of moderate thermo-chemical pretreatment of the substrate involving the lowest effective pretreatment agent concentration.

Experimental Procedures

Microbial Strains and Enzymes

The microbial strains enquired in the study were *Bacillus cereus* B5esz and *B. subtilis* P22, isolated from keratin waste and characterized in previous studies [18, 19].

Commercial proteinase K (Sigma) was used as a reference enzyme exhibiting keratinolytic activity. Commercial porcine pepsin (International Enzymes Limited) was used to estimate approximate levels of protein digestibility.

Media and Culture Conditions

Microbial cultures were carried out in 500 ml Erlenmayer flasks, in 100 ml of medium at 30ºC with 180 rpm shaking. Nutrient broth culture (glucose 1%, nutrient broth 0.8%) of density 1.2×10^8 cfu/cm^3 was applied as inoculum, in 1 ml per flask. The culture medium consisted of (%): $MgSO_4$ 0.1, KH_2PO_4 0.01, $FeSO_4\cdot7H_2O$ 0.001, $CaCl_2$ 0.01, yeast extract 0.05, set to pH 7.1 prior to autoclaving (121ºC, 20 min). The main carbon and nitrogen source was whole, degreased white chicken feathers (1%).

The culture fluid, form the 3rd day of culture after removing feather debris by filtration through Whatman No. 2 filter paper and biomass separation by centrifugation (5,000 g, 20 min, 4ºC), served as the enzyme source for further experiments.

Concentration of culture fluid was carried out at the Labscale TFF System (Millipore) using a Pellicon XL 50 casette with Ultracel-10 PLCGC membrane (10 kDa cut-off).

Pretreatment of Feathers Prior to Enzymatic Digestion

Thermo-chemical pretreatment of chicken feathers (washed and degreased with methanol-chloroform 1:1) was performed by autoclaving whole feathers (121ºC, 1 atm. overpressure, 20 min), suspended in solutions of various agents (1 g feathers per 100 cm^3 of solution). The following substances were applied at concentrations of 1 mM, 10 mM and 100 mM: sodium sulfite (Na_2SO_3), sodium thiosulfate ($Na_2S_2O_3$), dimethyl sulfoxide (DMSO), sulfuric acid (H_2SO_4), and sodium hydroxide (NaOH). After the treat-

ment feathers were separated, extensively washed with tap water (followed by distilled water), and finally air-dried. Untreated feathers and feathers autoclaved in distilled water served as control experiments.

An additional method of biological pretreatment was accomplished by subjecting feathers to short-term culture of *B. cereus* (medium and conditions as described above). After 3 days of feather degradation in culture conditions, the solids were removed, washed with water and autoclaved to neutralize bacterial cells.

Analytical Methods

Keratinolytic activity against raw and pretreated feathers was determined in 4-hour reaction in a mixture containing: coarsly cut feathers 100 mg, buffer 8 cm^3 containing 2 mM $CaCl_2$ and 0.02% NaN_3, and enzyme solution 2 cm^3. The reaction was terminated with the addition of 10 cm^3 trichloroacetic acid (TCA) 8%, subsequently cooled for 30 minutes, filtered through a Marcherey-Nagel GF-1 glass-fiber filter, and centrifuged (12,000 g, 10 min). The absorbance of TCA-soluble hydrolysis products was measured at 280 nm wavelength against a control. One unit of keratinolytic activity was expressed as 1 µmol of released tyrosin calculated per 1 mg of protein within 1 hour. The following reaction conditions were used for each enzymatic preparation: *B. cereus* – Tris-HCl 0.05 M pH 7.5, 55ºC; *B. subtilis* – Tris-HCl 0.05 M pH 8.0, 60ºC; proteinase K – Tris-HCl 0.05 M pH 7.5, 37ºC; pepsin – glycine-HCl 0.05 M pH 2.2, 37ºC.

Proteolytic activity was determined on casein (VWR), except for pepsin activity determined on hemoglobin (Merck), in a mixture of substrate solution 2 mg/cm^3 (1 cm^3), specific buffer, as described above (0.98 cm^3), enzyme preparation (0.02 cm^3) and incubated for 15 minutes at optimum temperature. The reaction was terminated with the addition of 2 cm^3 TCA 8%. The mixture was cooled for 30 minutes, centrifuged (12,000 g for 10 min) and the absorbance was measured at the wavelength 280 nm. One unit of proteolytic activity (PU) was defined as 1 µmol of released tyrosin calculated per 1 mg of protein in 1 minute.

The stability proteases in crude culture broths was determined for incubation time of 4 hours and 20 hours at optimum conditions.

Protein concentration was assayed using the method of Lowry [20].

Hydrolysis of native and pretreated feathers with the concentrated culture fluid was conducted for 24 hours in the reaction conditions described above, except for the final addition of TCA solution. Variants without enzyme served as control tests. Residual substrate was determined after separation and drying at 105ºC and concentration of soluble proteins was measured in reaction mixtures.

Thin layer chromatography (TLC) was performed in order to analyze amino acid liberation during the feather digestion experiment with the concentrated keratinase extract of *B. cereus* B5esz. The TLC separation was carried out on Silica Gel 60 plates and developed using a pre-staining method described by Qiu et al. [21]. The developing solvent was N-butanol:acetic acid:water (5:3:2) containing 0.4% ninhydrin and 3 µl of reaction supernatants without TCA solution were spotted onto a plate.

Statistical Analysis

All enzymatic reactions were performed in three replicates. Standard deviation values and significant differences determined with Duncan's test at p=0.05 (Statistica version 10) were given.

Results

Feather Pretreatment

The survey on the feather pretreatment method in relation to enzymatic hydrolysis of the keratinic substrate with bacterial keratinases and commercial enzymes revealed significant influence of the applied chemical agent and its concentration.

During the pretreatment process with the thermo-chemical method, limited solubilization of keratin fibres was an inevitable effect, leading to a certain degree of protein loss. Highest protein concentration in the working solution was observed in the presence of 100 mM NaOH and 100 mM Na_2SO_3, reaching 4.53 mg/cm^3 and 4.34 mg/cm^3, respectively (Fig. 1). In the first case complete substrate liquefying was achieved, which excluded this pretreatment variant from further analysis. In the latter, major structural change

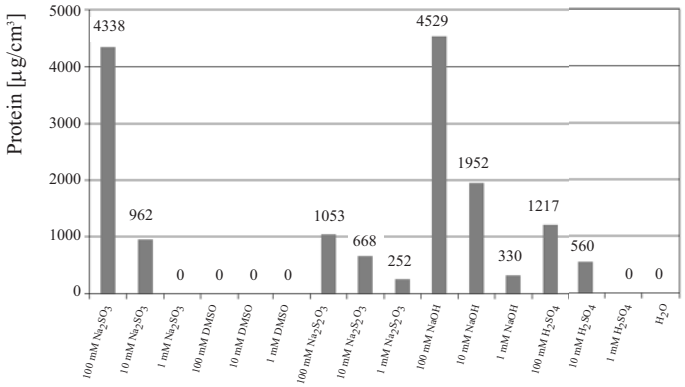

Fig. 1. Liberation of soluble proteins during the thermo-chemical treatment of feathers in the presence of various chemical agents.

Table 1. Stability of keratinolytic enzymes at optimum conditions. Percent residual activity given.

	4 hours		20 hours	
	keratinases	proteases	keratinases	proteases
B. cereus B5esz[a]	77.4±14.4	81.5±17.5	28.2±12.0	29.6±5.1
B. subtilis P22[b]	55.1±13.2	44.5±7.0	33.1±15.4	35.8±6.7

[a]initial activities in crude culture fluid: keratinolytic 47.2±4.2 μmol/cm^3·min, proteolytic 429±30
[b]initial activities in crude culture fluid: keratinolytic 18.1±3.0 μmol/cm^3·min, proteolytic 45.7±8.7

of feather substrate was observed. Nevertheless, it remained a solid material. One-fold decrease in concentration of NaOH allowed reduction of protein loss to a level below 2 mg/cm^3. Other chemical factors like 10 mM Na_2SO_3 or 100 mM $Na_2S_2O_3$ and 100 mM H_2SO_4, caused a protein release in the range of 0.96-1.22 mg/cm^3. Factors like DMSO or 1 mM concentration of Na_2SO_3, $Na_2S_2O_3$, and H_2SO_4, as well as control pretreatment in water, did not cause protein solubilization.

Stability of Keratinolytic Enzymes

In order to estimate appropriate feather hydrolysis reaction time, in optimum conditions elucidated in previous studies, the stability of keratinolytic proteases was measured. Enzymes of *B. cereus*, tested at 55ºC, exhibited considerable stability, retaining 77% keratinolytic activity and nearly 82% proteolytic activity after 4 hours (Table 1). In 20 hours residual activity in each case remained at nearly 30%. The activity of *B. subtilis* keratinases and proteases at 60ºC descended more rapidly within 4 hours to a level of 55% and 45%, respectively. Nevertheless, after 20 hours, incubation persisted above the level of 30%.

Keratinases of *Bacillus cereus* B5esz in Hydrolysis of Native and Pretreated Feathers

Determination of keratinolytic activity of proteases present in crude culture fluid conducted on native and pretreated feathers was aimed at identification of the optimal pretreatment method to support enzymatic keratin breakdown. Keratinases biosynthesized by *B. cereus* exhibited excessive ability of native feather keratin decomposition, in the absence of active bacterial cells. The assayed keratinolytic activity was 0.10 KU (Fig. 2). The pretreatment limited to autoclaving in water had a minor but significant

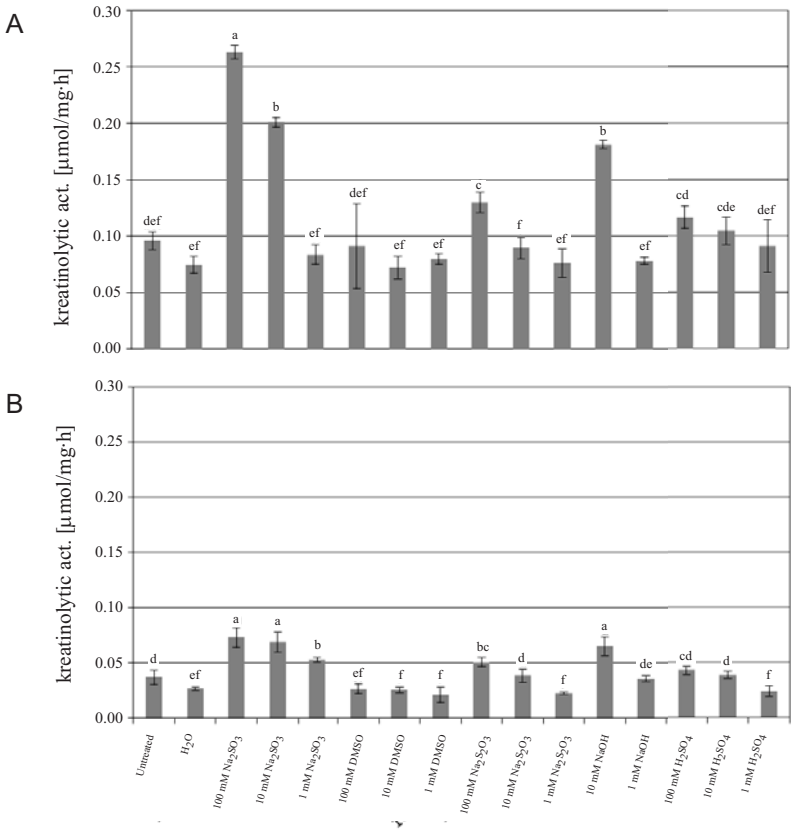

Fig. 2. Activity of crude keratinases of *B. cereus* (A) and *B. subtilis* (B) in hydrolysis of feathers subject to a thermo-chemical pretreatment with different chemical agents.

negative influence on keratinolytic activity. The highest effect from among tested chemicals was posed by Na_2SO_3 at 100 mM concentration, resulting in 160% increase of activity, followed by Na_2SO_3 10 mM and NaOH 10 mM with an effect below 100%. Moderate pretreatment effect was observed for a group comprising $Na_2S_2O_3$ 100 mM, and H_2SO_4 at 10 mM and 100 mM. The performance of remaining variants was below the significant difference in comparison to the control.

Keratinases of *Bacillus subtilis* P22 in Hydrolysis of Native and Pretreated Feathers

The activity of keratinases in culture fluid of *B. subtilis* was nearly twice lower compared to *B. cereus*, but still sufficient to sustain the keratinolytic potential of the tested strain (Fig. 2). Likewise, in the case of *B. cereus* enzymes, thermal treatment in water resulted in the slight deterioration of feather keratin decomposition. However, application of Na_2SO_3 at a concentration of 10 mM and 100 mM, as well as NaOH 10 mM, proved to be the most effective pretreatment method, triggering the keratinolytic activity for 75÷97%. The effective pretreatment also was observed in the case of Na_2SO_3 1 mM and $Na_2S_2O_3$ 100 mM.

Keratinolytic Activity of Commercial Proteases

Keratinolytic activity of two commercial enzymes, pepsin and proteinase K, was ascertained on native and pretreated feathers. The measurement of pepsin activity could serve as an approximation of *in vitro* digestibility of feather keratin and its changes in relation to feather keratin pretreatment. Low initial activity of pepsin against native feathers (0.006 KU) was increased twice with thermal treatment in water (to 0.013 KU) or treatment with Na_2SO_3 1 mM (to 0.016 KU). Other pretreatment methods produced less important effects on pepsin digestibility (Fig. 3). An attempt to apply a microbiological treatment in the form of a short-term culture of *B. cereus* B5esz did not provide the expected outcome.

Proteinase K, the enzyme of confirmed keratinolytic activity, was included in the presented study for comparative purpose, only for selected pretreatment variants (Fig. 4). The tested enzyme revealed high keratinolytic activity, 0.45 KU, on native feather keratin. This result was further enhanced by substrate pretreatment with Na_2SO_3 10 mM to the level of 0.86 KU. The disadvantageous nature of microbiological treatment was confirmed for reaction, including proteinase K as well.

The analysis of inquired proteases in terms of activity on casein revealed that enzymes in culture fluid of *B. cereus*, despite lower activity on raw feather keratin in comparison to proteinase K, dominated in the field of caseinolytic activity (Fig. 5).

Table 2. Proteolytic activity after concentration of culture fluid of *B. cereus*.

	Proteolytic activity	
	μmol/cm³·min	μmol/mg·min
Culture fluid	429±30	1,191±84
Concentrated on 10 kDa membrane	15,580±713	9,213±421
Permeate	3±1	11±4

Enzymatic Digestion of Native and Pretreated Feathers

Concentrated culture fluid of the potent protease producer *B. cereus* (Table 2) was applied in hydrolysis of raw feathers and feathers pretreated with Na_2SO_3 10 mM. In addition, suitability of using Na_2SO_3 10 mM as a supplement of enzymatic reaction on raw feathers was evaluated. During 20-hours reaction as much as 86% substrate loss of sulfite-pretreated feathers was observed, which remains a significant improvement, as compared with 17% loss of untreated feathers. The level of liberated proteins in reaction fluids reflected the degree of substrate decomposition (Table 3). The TLC analysis of keratin-derived amino acid also confirmed the superiority of the sulfite pretreatment method (Fig. 6). The result not only verifies the convenience of the pretreatment method, but also confirms high

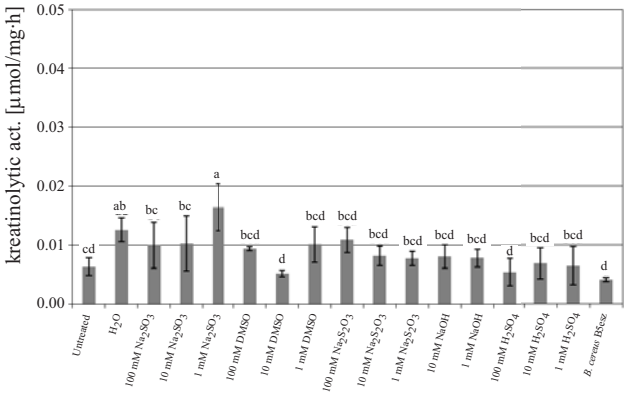

Fig. 3. Activity of pepsin in hydrolysis of feathers subject to a thermo-chemical pretreatment with different chemical agents and to microbial treatment in *B. cereus* culture.

Table 3. Application of concentrated culture fluid of *B. cereus* in 20-hour digestion of native and pretreated feathers.

		Feather keratin loss	Protein concentration
		%	mg/cm^3
Native feathers	Reaction	16.7	1.81±0.29
	Control	1.5	0.25±0.02
Feathers pretreated with 10 mM Na_2SO_3	Reaction	86.3	4.33±0.08
	Control	6.0	0.30±0.13
Native feathers, addition of 10 mM Na_2SO_3 in reaction	Reaction	19.7	0.83±0.18
	Control	1.5	0.38±0.03

keratinolytic potential of enzymes produced by *B. cereus* B5esz. On the contrary, the presence of additional sulfite caused moderate effect, providing limited extent of the substrate sulfitolysis.

Similar reaction conditions were maintained in order to visualize enzymatic digestion of single raw contour feathers. An elongated hydrolysis period of 24 hours allowed us to achieve complete feather hydrolysis, including feather rachis, in a reaction mixture enriched with Na_2SO_3 10 mM. Partial digestion was observed in the presence of Na_2SO_3 1 mM. However, the effect was comparable to the action of sole enzyme preparation (Fig. 7).

Discussion

The waste-processing practice constantly employs feather keratin conversion methods based on either thermal or thermo-chemical treatment. Thermal techniques usually involve prolonged steam cooking at temperatures exceeding 130ºC, at elevated pressure. Fragmentation of feather material often is required prior to introducing it into processing installation [22]. If additional chemicals like lime or sodium hydroxide are introduced into a thermo-chemical pretreatment process, their concentration commonly remains above 50 mg per 1 g of solid waste [23].

In the presented study several reducing or oxidative substances were introduced into a thermo-chemical pretreatment at three relatively low concentrations at fold-differing levels. The oxidative compounds mode of action on keratin fibers should be relaxation and destabilizing of its structure, whereas reducing agents should take part in the process of sulfitolytic cleavage of disulfide bonds. Since the main purpose of the pretreatment was not the complete liquefaction of the substrate but its preparation for the subsequent hydrolysis with microbial keratinases, the lowest effective concentration was approved as satisfactory. According to this assumption, the application of factors like NaOH 100 mM and Na_2SO_3 100 mM causing entire or nearly entire liquefying of feather keratin were rejected. The major extent of protein extraction during pretreatment occurred in the presence of NaOH at each concentration, the factor most commonly used for effortless keratin disintegration [3]. The remaining chemicals at 10-100 mM also caused structural changes to the keratinous substrate, but the protein extraction was considered acceptable. It is notable that, despite various levels of soluble protein released into the pretreatment fluid, it does not necessarily reflect changes in the susceptibility of keratin toward proteolytic cleavage.

The stability of commercial enzymes is a crucial factor determining their functionality. The stability of proteolytic enzymes of both tested microbial strains (tested at elevated, optimum temperature) was considerable, especially during the 4-hour test reaction. It proves to be adequate for application in single-day hydrolysis periods, but 30% of residual activity allows for prolonging the reaction. Stability of purified keratinases from mesophilic bacteria varies from 100% residual activity at 60ºC, 120 min in the case of alkaliphilic *B. halodurans* PPKS-2 [17], 95% activity at 60ºC, 45 min

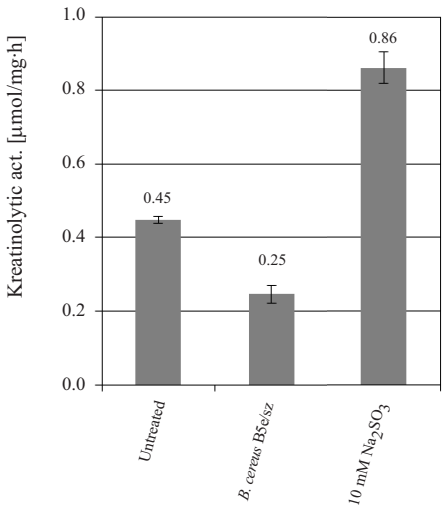

Fig. 4. Activity of proteinase K in hydrolysis of sulfite-pretreated feathers and feathers pretreated in *B. cereus* culture.

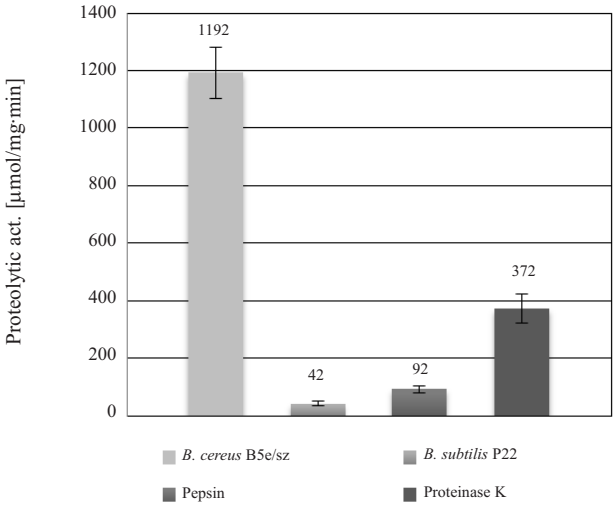

Fig. 5. Comparison of proteolytic activity of tested enzyme preparations, determined on casein.

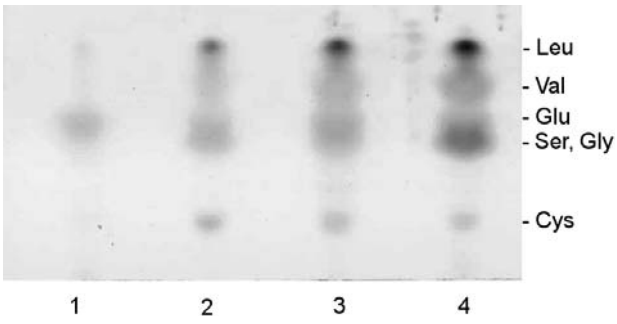

Fig. 6. The TLC analysis of amino acid liberation in 20-hour native and pretreated feather digestion with the concentrated keratinase extract of *B. cereus*: line 1 – sole concentrated keratinase extract, 2 – digestion of native feathers, 3 – digestion of native feathers in the presence of 10 mM sulfite, 4 – digestion of 10 mM sulfite-pretreated feathers.

for mukartinase of a mutant *Brevibacillus* sp. AS-S10-II [24] to 53% at 55ºC, 30 min for keratinase of *B. cereus* MCM B326 [25]. Enhanced performance of keratinolytic proteases may be obtained through various techniques, including immobilization on solid carriers [26, 27] or modifying enzymes autolytic sites [28].

Thermal treatment of feathers in the atmosphere of saturated steam and elevated pressure is one of the commercially available pretreatment methods. Nevertheless, autoclaving of keratinic substrates is routinely used by researchers in microbiological studies involving pure cultures of keratinolytic microorganisms. This kind of treatment should have a certain positive effect on the substrate accessibility to lytic enzymes. Still, it appears to depend on the enzyme properties. In the presented study standard autoclaving had a minimal negative influence on the hydrolysis with keratinases of both tested bacterial strains, but slightly increased pepsin digestibility.

Several reports highlight the fact that a sole keratinolytic protease is incapable of complete keratin hydrolysis [29-32]. The clear support for the validity of the theory included in the early research of Kunert [33] is the stimulation of the activity of keratinolytic proteases through sulfitolytic cleavage of disulfide bonds by non-enzymatic factors. In most cases either sulfite or 2-mercaptoethanol appear to be the optimal reducing agents to incorporate into reaction mixture during hydrolysis of raw feathers. Sulfite 0.1% stimulated feather degradation with crude keratinase of *B. halodurans* PPKS-2, pH 11, 45°C, from 50% to 75%, but the application of other reducers like 2-mercaptoethanol, dithiothreitol, or cysteine gave comparable effect [34]. Keratinolytic activity of *B. subtilis* KD-N2 protease on feather meal was largely enhanced by 5 mM 2-mercaptoethanol (approximately 20 times) and dithioerythritol (nearly 16 times), while the effect of sulfite was less distinct [35]. The same reducing agents at 5 mM proved to be effective during application with *B. subtilis* KS-1 keratinase, resulting in nearly 140% and 170% residual activity, respectively [31]. The reducing environment provided by 2-mercaptoethanol 1% was a necessary condition for effective feather hydrolysis at 80ºC by a thermophilic subtilase WF146 [28]. On the contrary, Okoroma et al. [36] revealed the case where concentrated keratinase extract of *B. licheniformis* N22 caused 80% weight reduction of melanized feathers within 48 hours, in the absence of reducing supplements. The application of sulfite 10 mM in whole native feather digestion with concentrated keratinase extract of *B. cereus* B5esz allowed for a significant improvement of the process, resembling the results of authors mentioned above. Nevertheless, the extent of sulfitolysis in the conditions of thermal treatment with sulfite seems to far exceed the one obtained in reaction mixture, resulting in nearly complete substrate hydrolysis with *B. cereus* keratinase. Despite high convenience of sulfite application in reaction conditions, authenticated by visible effects (Fig. 8), thermo-chemical pretreatment with sulfite gives an opportunity for an extensive improvement of keratin susceptibility towards proteolytic cleavage. This kind of substrate preparation is even more valid with the application of crude keratinase of *B. subtilis* P22. Mazotto et al. [37] managed to visualize liberation of amino acids from feathers in cultures of three *Bacillus* sp. strains using TLC separation, as a measure of keratin biodegradation. In the presented study we acquired comparable evidence of feather keratin hydrolysis using a cell-free keratinase extract.

The reference experiment based on feather digestion with proteinase K, the commercial enzyme of documented keratinolytic activity, confirmed observations made in tests with crude keratinase extracts. According to Nam et al. [38], proteinase K is readily active on feather meal, while Liang et al. [28] supplement the reaction mixture with 2-mercaptoethanol 1% in order to achieve complete feather degradation. Proteinase K applied in the presented study,

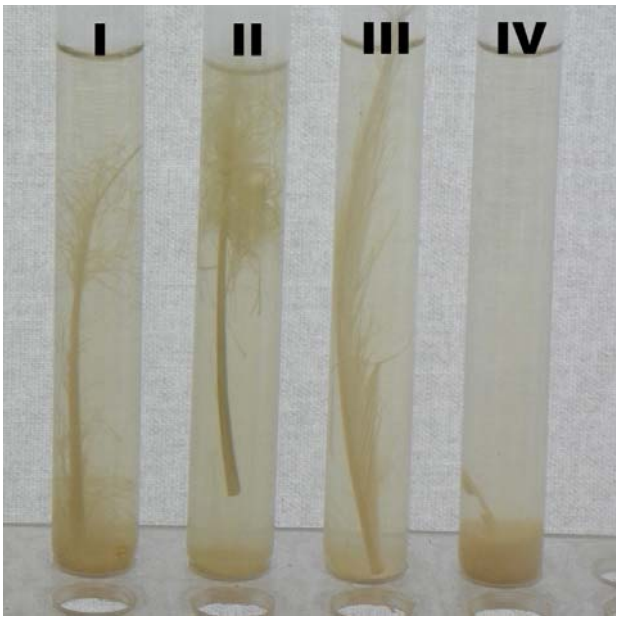

Fig. 7. Visualization of raw feather degradation with concentrated *B. cereus* culture fluid after 24 hours, at 55ºC, pH 7.5: I – unsupplemented mixture, II – addition of sulfite 1 mM, III – addition of sulfite 10 mM in the absence of the enzyme, IV – addition of sulfite 10 mM.

exhibiting high proteolytic activity on casein (371.8 PU), was active on native whole feathers (0.45 KU) in the absence of a reducer. Thermo-chemical substrate treatment with sulfite further enhanced keratinolytic activity nearly twice. In contrast, microbiological treatment in short-term culture of *B. cereus* appears to be an ineffective approach.

Application of thermal, chemical or enzymatic methods in keratin pretreatment is of great interest in terms of improving its limited digestibility and thus suitability as a feed ingredient. Kim et al. [14] improved pepsin digestibility and protein solubility of feathers by treatment with *B. subtilis* crude protease extract or sodium hydroxide. Nevertheless, the best result was obtained after combining these two methods. Despite the microbiological treatment in *B. cereus* B5esz culture did not improve pepsin digestibility of feathers in the presented study a few variants of the pretreatment proved to be effective. Typical autoclaving in the absence of a reducing agent gave the best results, along with the lowest sulfite concentration.

Further enhancement of enzymatic feather digestion could also be obtained for at least 10% by mechanical disintegration of keratin fibers [17], however the presented study aimed at degradation of whole feathers omitting the additional operation of grinding.

Presented pretreatment methods involving autoclaving of feathers in the presence of reducing agents, followed by a digestion stage with keratinolytic proteases, poses an alternative to standard preparation methods, allowing us to obtain valuable products. The investigated treatments offer application of moderate temperature and pressure, as well as low concentration of relatively safe sulfite, in comparison to current industrially used parameters, combined with hydrolysis by crude keratinase extracts of *B. cereus* B5esz or *B. subtilis* P22, two bacterial strains of profound keratinolytic potential, leading to the acquisition of feather keratin hydrolysates. Nevertheless, the influence of pretreatment on keratin digestibility or nutritional value, as well as other properties of obtained hydrolysates require further evaluation.

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