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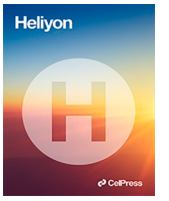

Research article

Crude proteolytic enzyme from *Bacillus halodurans* BCRC 910501 and its application in leather processing

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ABSTRACT

Enzymes are biological catalysts that exist in all living organisms. Proteases are one of the most important enzymes in the industry; microbial proteases are widely used in the food, textile, detergent, and leather industries. Traditionally, alkaline proteases are used in the leather industry for bating, however, due to environmental issues, there are many investigations for the application of proteolytic enzymes in other operations such as soaking, unhairing, and derma opening up. This study aimed to produce a proteolytic enzyme from *B. halodurans* BCRC 910501 and apply it to the soaking of salted hide. After cultivation, the crude enzyme was used for further analysis and leather processing. The results showed an enzyme with higher caseinolytic activity compared to keratinolytic activity. A soaking study found that enzyme improves rehydration, non-collagenous protein, and salt removal. Enzymatic soaking affected collagenous proteins more than the control, but did not significantly impact wet-blue leather properties. Using this enzyme in leather processing might be suitable to reduce the duration of soaking and the number of process steps.

1. Introduction

Enzymes are biological catalysts that exist in all living organisms. They are extremely important for different processes such as cell division, protein turnover, cell growth and differentiation, digestion, biomolecules synthesis, etc. [1]. Even though enzymes have been known and used for centuries, recent biotechnology improvements allow for a wider production and application of these proteins [2]. Biocatalysts can be used in biotechnology instead of inorganic catalysts due to their high specificity, activity and selectivity under mild conditions [3]. Now many different industries such as food, detergent, biofuels, leather, textile, chemical production, pharmaceuticals employ enzymes on a commercial scale [4]. Proteases are the most commercially used enzymes, as they take up around 60 % of the global enzyme market [5].

Proteases are involved in a broad spectrum of biological processes as digestion, synthesis of biomolecules, DNA replication, cell cycle, apoptosis, control size, shape, composition of fundamental proteins, and so on [6]. These biocatalysts hydrolyze peptide bonds between two amino acids of a polypeptide chain [5]. Proteolytic enzymes are difficult to classify because of their wide range of site of action and structure. Now, they are categorized based on their varied characteristics such as the reaction they catalyze, composition

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and structure, and chemical nature of the active sites. Principally there are two groups of proteases, depending on the substrate's catalytic site: endoproteases and exoproteases [7].

Proteolytic enzymes can be obtained from various sources such as plants, animals, and microbes. Today, proteases of microbial origin are used most widely due to their simple genetic manipulation, smaller cultivation space, lower production cost, higher productivity, and wide biochemical diversity; microbial proteases take almost two-thirds of commercially available proteolytic enzymes [8,9]. Catalytic activity, substrate specificity, pH, temperature, and other properties vary depending on the type of protease and the origin [10]. Currently, alkaline protease has the broadest application in different industries, such as the detergent industry, food, paper, and leather processing.

Alkaline proteases are enzymes that are most active in the neutral to alkaline pH range. These proteases can be obtained using different fermentation techniques as submerged-state or solid-state fermentation. Mostly, alkaline proteases are produced using alkalophilic microorganisms; media composition and used bacterial strains affect enzyme cultivation [11]. Traditionally in leather processing, alkaline proteases are used during bating; commercially available proteolytic enzyme preparations may consist of a combination of trypsin as trypsinogen, carboxypeptidase as procarboxypeptidases, and chymotrypsinogen [12]. The purpose of this process is to remove non-collagenous proteins and other substances that might affect the finished product. During bating fibre structure is opened up to improve the penetration of substances used in other processes as well as to obtain desired properties: elasticity, flexibility, softness, etc. [13].

To reduce pollution in the leather industry, applications of alkaline proteases are also studied in other processes; due to elastolytic and keratinolytic activities, biocatalysts can be used in soaking and unhairing [1,12,13]. Soaking is the first process in leather production. Its purpose is to rehydrate hide and remove salts, dirt, blood. Microbial protease can improve water absorption and reduce process duration [14], also, it may help to remove non-collagenous substances. Alkaline protease is used with other enzymes for better elimination of substances [13].

Many studies have been conducted on the application of alkaline protease in fully enzymatic or coenzymatic unhairing. The use of enzymes in the unhairing helps to eliminate the use of chemicals during leather manufacturing and may improve the finished product. Enzymatic unhairing does not affect the hide surface and it is cheaper and easier to recycle wastewater and finished products [7,15]. Various bacterial species have been reported to produce proteases for effective dehairing. Most widely applied proteases are produced from different species of the *Bacillus* genus. Other species also showed applicability in unhairing for example *Pseudomonas, Streptomyces, Aspergillus*, etc. [16].

Bacillus spp. is widely used for enzymes production. This bacterium gains recognition because of a high variety of species that can produce different enzymes, rapid growth rates, and their ability to secret high amounts of proteins into the extracellular medium [17]. *Bacillus* spp. are Gram-positive bacteria, aerobes or facultative anaerobes, rod-shaped endospore-formers. Nowadays 2665 species of *Bacillus* genus are known (LPSN www.bacterio.net), that can be found in almost all natural habitats. Apart from *B. anthracis* and *B. cereus*, which are pathogenic, most species of this genus are considered nonpathogenic [18]. *Bacillus* species also have very high gene diversity with a genome size between 3.35 and more than 5.5 megabase and with % G + C content varying from 35 % to 46 % [19].

Even though *Bacillus* proteases are used in the leather industry, only a few studies have been done with *Bacillus halodurans*. In one of the studies, thermostable alkaline protease from *Bacillus halodurans* JB 99 was used for leather unhairing. The research showed high keratinolytic and unhairing activity. The protease effectively removed hair from goat and buffalo hide and did not damage the collagen layer and the color of the hide did not change significantly [20]. Another study with protease from *B. halodurans* PPKS-2 also showed the possibility of using enzyme in unhairing. Enzyme efficiently removed hair from goat hide. High pH allows the swelling of the hair roots; following a protease attack on the hair follicle protein which results in the removal of the hair. Despite alkaline conditions, the process with enzymes is safer than conventional in which a large amount of lime and sodium sulfide is used [21].

Due to growing concerns about the environment, it is important to find new enzymes for the leather industry and ways to use them in processing. Previous studies of *B. halodurans* protease showed high proteolytic and keratinolytic activity, however, there are no studies on the possibility of using such enzyme for salted hides and skins soaking. This study aims to produce a proteolytic enzyme from *B. halodurans* BCRC 910501 and study its application in soaking.

2. Materials and methods

2.1. Materials

The chemicals used for the enzyme production and analysis were of analytical grade, while analytical and technical grade materials were used for the technological processes. Bradford's protein assay reagent (Coomassie Brilliant Blue) and BSA standard were purchased from Bio-Rad. Peptone and yeast extract were obtained from Thermo Fisher Scientific. All other chemicals were purchased from either E.Merck or Riedel-de Haen. Bovine hide cured by NaCl salting (bull hide; 26 kg; purchased in Kėdainių Oda, Kėdainiai, Lithuania) was used for the investigation of the enzyme influence on a soaking process and subsequent assessment of the effect of the enzymatic soaking on chrome tanned leather properties.

2.2. Methods

2.2.1. Production of enzyme from B. halodurans BCRC 910501

The crude enzyme production (Fig. 1) was based on a previous study [22]. Horikoshii agar medium (glucose 10 g/L; peptone 5 g/L yeast extract 5 g/L; KH_2PO_4 1 g/l; $MgSO_4{\cdot}7H_2O$ 0.2 g/L; Na_2CO_3 10 g/L; Agar 15 g/L) was prepared and sterilized at 121 °C for 30 min.

After solidification, *B. halodurans* BCRC 910501 was subcultured from frozen stocks on the Horikoshii agar plate. The plate was incubated in an incubator (Labnet 311DS, Labnet International, USA) at 37 °C for 16 h; after 16 h a single colony on the plate was inoculated into 5 mL of medium for the crude enzyme production (lactose 15 g/L; soybean 6 g/L; $MnSO_4{\cdot}5H_2O$ 1.21 g/L; $MgSO_4{\cdot}7H_2O$ 1.23 g/L; $CaCl_2{\cdot}2H_2O$ 0.735 g/L). After mixing evenly, incubated in a 37 °C incubator at 175 rpm for 16 h. The 5 mL of the previously cultured bacterial solution was inoculated into a 500 mL Erlenmeyer flask containing 95 mL of the same medium and incubated for 48 h at 37 °C. After incubation cells and insoluble material were removed by centrifugation (benchtop centrifuge UNIVERSAL 32R, Hettich, USA) centrifuge at 10,000×*g*, 4 °C for 10 min. The cell-free supernatant was lyophilized and used as a crude source of proteolytic enzyme for leather processing.

2.2.2. Protein quantification

The protein concentration in the supernatant was determined using the Bradford method [23]. The protein assay reagent was diluted five times and used for protein quantification. Protein samples and diluted protein assay reagent were mixed evenly at a ratio of 1:4. The measured OD595 nm (SP-880 spectrophotometer, Metertech In., Taiwan) value was within the calibration curve; for the calibration curve bovine serum albumin (BSA) was used as standard.

2.2.3. SDS-PAGE

For SDS-PAGE, 12.5 % acrylamide gel was made using H_2O (3.38 mL), 40 % acrylamide mix (2.5 mL), 1.5 mol Tris (pH 8.8, 2 ml), 10 % SDS (80 μL), 10 % APS (40 μL). The samples were mixed with 5X Sample Buffer (0.05 mol Tris·HCl, pH 6.8, 10 % SDS, 30 % (v/v) Glycerol, 10 mmol DTT, 0.25 % (w/v) Bromophenol Blue) at a 4:1 ratio and heated in a dry bath at 100 °C for 10 min. In the electrophoresis tank 1X Running Buffer (0.025 mmol Tris base, 0.192 mol glycine, 0.1 % SDS) was poured, and for the first hour the voltage was set to 60 V; after 1 h the voltage was set at 120 V and ran until the tracking dye was close to the bottom of the gel. The gel was stained with Coomassie Staining Solution (45.5 % methanol, 9 % acetic acid, 0.2 %(w/v) Coomassie Brilliant Blue) for 1 h using an orbital shaker at 100 rpm and it was destained with a Destaining Solution (10 % acetic acid, 30 % methanol, 60 % DI water) overnight.

2.2.4. Keratinolytic activity assay

To determine the keratinolytic activity, the soluble keratin substrate was prepared. First, the chicken feathers were removed from stems, washed with deionized water containing Triton ×100 (10 g/L), and dried. A gram of washed and dried feathers was weighed, immersed in a 25 mL solution of 6 mol Urea containing 0.2 mol β-mercaptoethanol, pH 10.5, and incubated in a water tank at 45 °C for 2 h. The cells were completely crushed by an ultrasonic cell crusher (LC-150Y, Biometer, China), and then placed in a 45 °C water bath for 30 min. The solution was centrifugated at 14,000×*g* for 10 min and the supernatant was collected. Finally, the supernatant was dialyzed three times against a 5 kD hollow fibre. Protein concentration was determined and a solution was diluted to 10 g/L and stored in a refrigerator at 4 °C.

The keratinolytic activity was assessed by a modification of the method of Kunitz [24]. 500 μL of 10 g/L soluble keratin was added to 250 μL 0.05 mol glycine-NaOH (pH 11), then 250 μL enzyme was added and mixed evenly. The mixed solution was placed in a 50 °C water bath, and after 30 min of reaction, 1 mL of 10 % TCA was added to the experimental group. After the reaction solution was centrifuged at 12,500×*g* for 10 min 400 μL of the supernatant was taken out and mixed with 400 μL of 10x diluted Folin's phenol reagent and 1200 μL of 0.7 mol sodium carbonate. The reaction solution was placed in a water bath at 50 °C for 30 min, and the OD660nm was measured using a spectrophotometer. The amount of tyrosine released by the reaction was calculated using the tyrosine calibration curve.

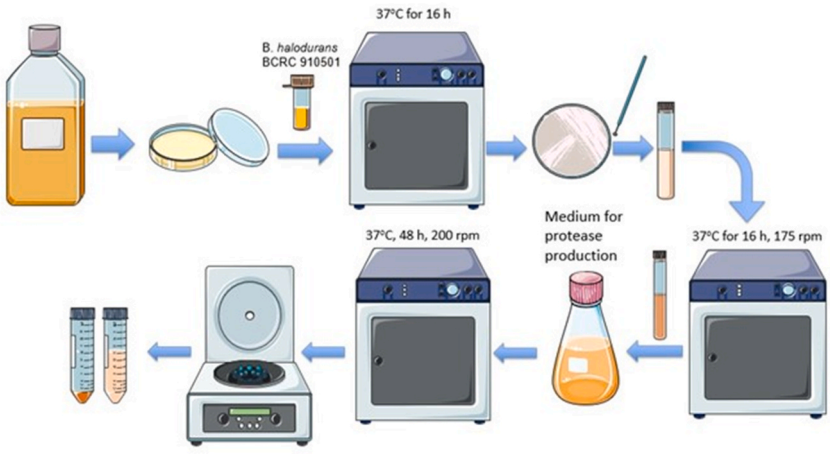

Fig. 1. Production of crude protease from *B. halodurans* BCRC 910501. The figure was designed using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

2.2.5. Caseinolytic activity assay

Caseinolytic activity was determined in the same way as keratinolytic. Casein was used as a substrate [24]. 0.1 g of casein was mixed with 10 mL 50 mmol glycine-NaOH (pH 11) buffer solution and stirred in 80 °C water bath until casein was completely dissolved.

2.2.6. Leather soaking

For soaking exploration, bovine hide was cut into pieces (8 × 8) cm and a series of samples were prepared from these pieces. The series were formed in such a way that samples from all the hide parts would be presented in each series. The process was carried out at room temperature (20 ± 1 °C). Other parameters are presented in Table 1.

After soaking enzymatic effect was evaluated by moisture content in the samples, removed collagenous proteins, non-collagenous proteins, and NaCl. After soaking other conventional leather processing operations were performed and the properties of the chromed leather (wet-blue) were evaluated.

2.2.7. Leather processing

To determine the influence of enzymatic treatment on the following processes, after soaking conventional leather processing was carried out as described in Supplementary Table S1 and the wet blue characteristics were defined.

2.2.8. Analysis methods

2.2.8.1. Determination of removed collagenous protein. The amount of removed collagenous proteins was estimated from the amount of hydroxyproline in the soaking solution using a photocolorimetric method [25]. Samples of the soaking solution after the process were hydrolyzed with 6 N of HCl at 120 °C for 10–12 h. The formation of a colored-soluble product was based on the reaction of hydroxyproline with *p*-dimethylaminobenzaldehyde. Absorption was measured with a GENESYS-8 spectrophotometer (Spectronic Instruments, Cheshire, UK) at a wavelength of 558 nm.

2.2.8.2. Determination of removed non-collagenous proteins. The amount of non-collagen proteins removed was determined using the Lowry method to analyze the soaking solution as well [26]. It is a colorimetric assay based on the interaction of the protein with an alkaline copper tartrate solution and a Follin reagent. The absorption of the blue-colored reaction product was measured with a spectrophotometer at a wavelength of 660 nm.

2.2.8.3. Determination of the amount of NaCl. The determination of the amount of NaCl in the solution was evaluated using titration with a $HgNO_3$ solution 0.1 mol/L [27]. Mercuric and chloride ions form a highly stable soluble complex; excess mercuric ions react with diphenylcarbazone (indicator) to form a blue-violet complex.

2.2.8.4. Evaluation of chromed leather characteristics. After chroming wet-blue was washed and used for analysis. Dehydration with acetone was performed before determination of physical properties to fix the structure of the leather [28]. Chromium compound exhaustion was assessed by determining the concentration of chromium in the initial chroming solution and a mixture of the used chroming solution and washing (after chroming) solution. The assessment was based on the method outlined in the literature, which involves oxidizing the chromium in the solution to its hexavalent state with hydrogen peroxide and analyzing the solution by iodometric titration [29].

The shrinkage temperature of the hide was determined according to the standard [30], and for chromed leather samples the shrinkage temperature was determined by the same method replacing the distilled water with glycerol [29].

The moisture content in the leather, the strength properties, and the amount of chrome compounds in the leather were determined according to the standards [31–33].

2.2.9. Statistical analysis

All data were expressed as the average value of measurements performed in triplicate. One sample was used for one measurement. Standard deviations did not exceed 5 % for the values obtained.

Table 1
Hide soaking parameters.

Process	Material, %[a]	Process duration
Washing	H_2O – 200	1 h
Soaking a)Control	a) H_2O – 160;	a) 6 h
b)Experimental	b) H_2O – 160; crude protease from *B. halodurans* – 0.6	b) 6 h

[a] Amounts of the materials for the processes are based on fresh hide mass.

3. Results and discussion

3.1. The crude enzyme characteristics

For the crude enzyme production, *B. halodurans* BCRC 910501 was used. After bacterial culture cultivation, the protein supernatant was collected and used for further analysis as a crude enzyme. First, the protein concentration in the supernatant (Table 2) was determined. It was found that protein concentration in bacterial fluid was 0.54 mg/mL. The results of proteolytic activities show more than six times higher caseinolytic activity compared to those of keratinolytic.

For the determination of molecular weight, SDS-PAGE was used (Fig. 2). The crude enzyme had a wide range of proteins of different sizes, with more at 63 kDa, 25 kDa, 23 kDa, and 13 kDa masses. However, the greatest number of proteins was obtained at 48 kDa. It is known that *Bacillus* protease has a broad molecular weight; typically ranging from 27 to 71 kDa [17]. There are studies with different size proteases that can be effectively used in leather processing. For example, during one of the studies, a 39.5 kDa size protease was produced, that can be used in dehairing; in different research, B. *halodurans* protease was purified with 29 kDa [20,34]. The molecular size distribution in the protease mixture may affect protein hydrolysis. Bigger size molecules can hydrolyze the surface proteins while letting smaller molecular size proteins diffuse deeper inside the structure and hydrolyze inner proteins. The presence of various proteases with varying molecular weights might enhance the synergistic effect [35].

3.2. Soaking studies

Soaking studies were performed with lyophilized cell-free supernatant; it was used as the crude proteolytic enzyme. The soaking process aims to rehydrate hides/skins to fresh hide moisture, which is 60–70 % depending on animal age, breed, etc. [36]. For the experiment, the duration time of the process was chosen to be 6 h because when the soaking time is extended for a long period, the distinctions between the rehydration effect become insignificant [37]. Furthermore, a shorter process results in reduced energy consumption. After the experiment, firstly, the moisture content in the hide during soaking was investigated (Fig. 3). Soaking of hide is important not only for rehydration of hide but also for following processes; uneven or poor operation can cause unequal chemical penetration into the derma during subsequent processing and defects in the finished product [38].

It was observed that the enzyme influences rehydration during soaking, after the first hour moisture reaches 57.8 % while control moisture is 50.7 %. During the 6-h process, the moisture in control sample did not reach experimental values; at the end moisture in a control hide reached 56 % while an experimental – 64.8 %. Using enzymes in rehydration had an advantage of a higher moisture content in a shorter time.

The next important index was to evaluate removed non-collagenous proteins. As mentioned before, during soaking not only rehydration is performed, but also other non-collagen substances are removed. Fig. 4 shows the kinetics of removal of non-collagenous proteins through soaking. Interestingly, after the first hour using only water more proteins were eliminated, however, after 2 h protease had a greater influence on the process, and in the end, the enzyme showed a better effect on non-collagenous proteins compared to control. These results might explain the higher percentage of moisture in the experimental sample; it occurred due to better breakdown of proteins, particularly globular proteins that are found in the hide [39].

After 4 h enzyme activity towards interfibrous proteins declines, amount of removed non-collagenous proteins after 6 h is very similar to after 4. Meanwhile, in the control removal of non-collagenous proteins increases with every hour; the process is slower but continuous.

Proteolytic enzymes can also attack collagen, damaging fine fibres in grain enamel [38]. Therefore, it is important to investigate whether the applied crude enzyme can perform soaking without too much hide damage. Results (Fig. 5) show protease higher effect on collagenous proteins compared to a control sample. After the first hour the amount of removed protein is more than 2.6 times higher than the control, however, after 6 h difference between the experimental and control is around 1.9 times. Even though enzymatic process eliminated more collagen the amount is not greater than in conventional unhairing; during which amount of removed collagenous proteins ranges from 0.2 to 0.5 % [40,41]. Nevertheless, after these findings, it is essential to evaluate further processes, if a greater removal of collagenous proteins does not affect the quality of the leather.

Since skins of animals are made of collagen fibres, shrinkage temperature is one of the leather's quality parameters. This parameter is connected to collagen stability; with decreased stability less energy is needed to effect the molecule chains, leading to a lower shrinkage temperature [42]. Table 3 shows no considerable differences in shrinkage temperature between the control and experimental sample. Even though the addition of enzymes leads to higher removal of collagen proteins, this does not mean that the damage to collagen fibres is at such a level that it could be reflected as a significant decrease in shrinkage temperature. Presumably, the crude enzyme affected telopeptide regions in collagen structure that are responsible for bonding and flexibility, however, they do not play a significant role in preparative processes or stabilizing reactions [43].

Finally, NaCl in the solution was evaluated before further processing of the leather. For good quality products it is important to

Table 2
B. halodurans BCRC 910501 supernatant protein concentration and proteolytic activities.

Protein concentration, mg/mL	Keratinolytic activity, U/mL	Caseinolytic activity, U/mL
0.54 ± 0.02	9.91 ± 0.05	59.57 ± 1.16

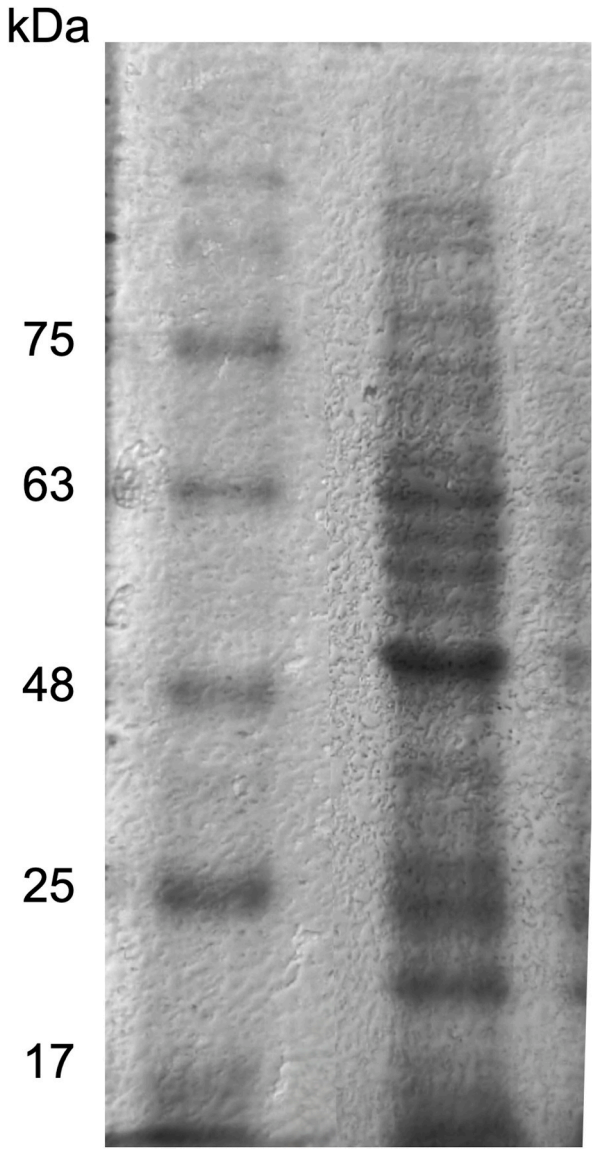

Fig. 2. Molecular weight by SDS-PAGE; on the left – marker, on the right – *B. halodurans* BCRC 910501 protease.

remove inorganic salts; usually for this aim two or three stages of soaking are performed [44]. During enzymatic soaking, salt is removed more efficiently (Fig. 6). After half an hour, the NaCl concentration in the experimental solution reaches 2.32 % while in control it reaches 1.74 %. At the end of the process, the difference in NaCl concentration between samples is 1.7 times. During enzymatic process more non-collagenous substances are eliminated [39]; due to this, possibly, more space appears in the hide structure for water to enter and dissolve sodium chloride. A better removal of salt with an enzyme might help reduce the steps in the soaking, and with that process time and water consumption can be decreased.

3.3. Characteristics of chromed leather

As mentioned before, it is important to evaluate if enzymatic soaking influences the following processes. To do so, after enzymatic treatment, further conventional operations such as lime-unhairing, bating, pickling, and chroming were performed. Table 4 findings indicate that wet-blue properties are very similar despite different soaking (with crude enzyme and without). It shows that although the enzymatic treatment led to a stronger effect on collagen, it did not have a worsening impact on wet blue properties.

4. Conclusions

A crude proteolytic enzyme from *B. halodurans* BCRC 910501 was cultivated and used in the soaking process. SDS-PAGE showed a

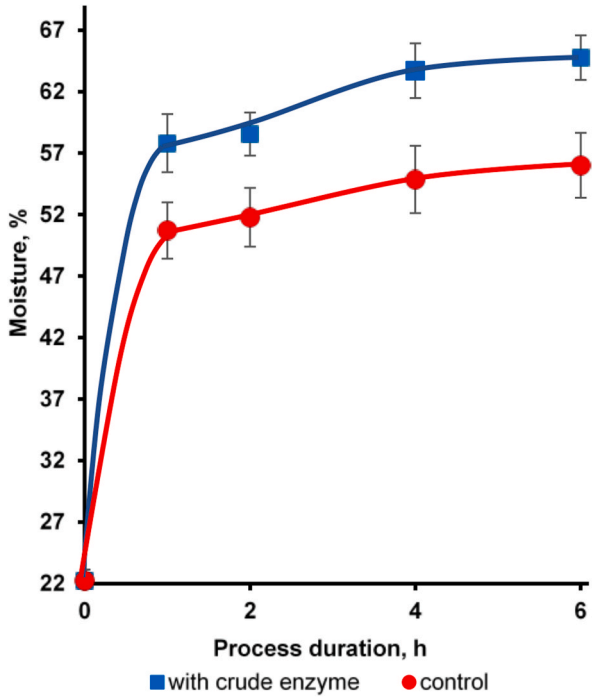

Fig. 3. Moisture changes in the hide during soaking with crude enzyme or without (control).

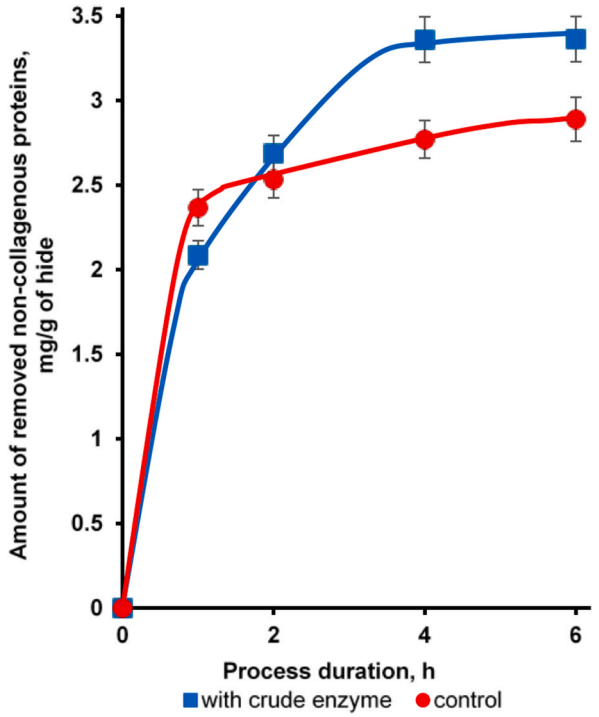

Fig. 4. Dependence of removal of non-collagenous proteins from hide during soaking with crude enzyme or without.

wide range of protein molecular masses. The enzyme activity results indicated a higher caseinolytic activity compared to keratinolytic activity. The crude protease source produced from *B. halodurans* showed promising results in hide soaking. Faster and better rehydration was achieved by using the enzyme in the process. Furthermore, enzymatic treatment led to better removal of NaCl and non-

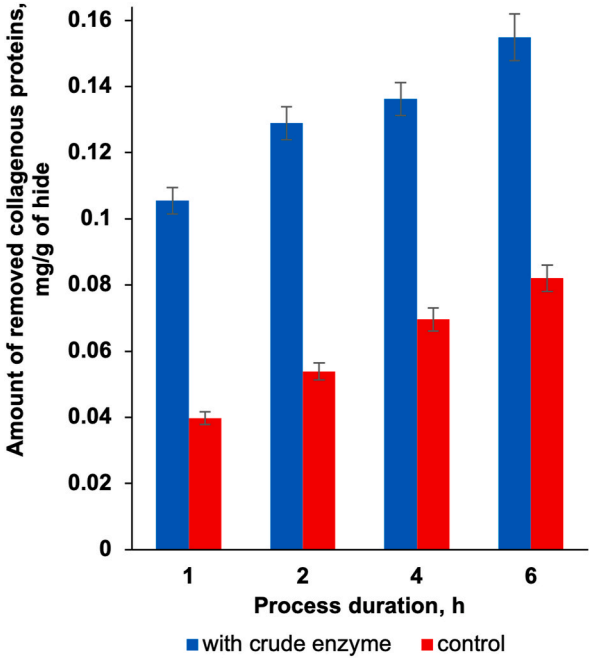

Fig. 5. Amount of the removed collagenous proteins during soaking with crude enzyme or without.

Table 3
Influence of soaking duration on shrinkage temperature of hide.

Process	Shrinkage temperature, °C			
	after 1 h	after 2 h	after 4 h	after 6 h
Control soaking (without enzyme)	61.5 ± 0.50	62.2 ± 0.29	62.0 ± 0.42	62.0 ± 0.44
Enzymatic soaking	62.3 ± 0.57	63.0 ± 0.40	62.3 ± 0.57	61.0 ± 0.35

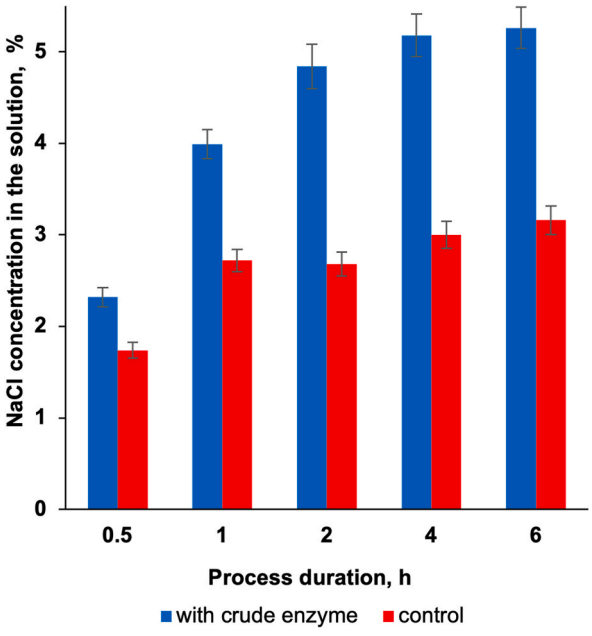

Fig. 6. Change of NaCl concentration in the soaking solution using crude enzyme or without it dependently on the duration of the process.

Table 4
Characteristics of chromed leather depending on the soaking method.

Indexes	Soaking	
	With crude enzyme	Control[a]
Moisture content, %	12.2 ± 0.58	12.4 ± 0.55
Shrinkage temperature, °C	108.6 ± 0.41	108.0 ± 0.40
Cr_2O_3 content, %	4.28 ± 0.18	4.24 ± 0.19
Cr_2O_3 exhaustion, %	86.2 ± 3.44	85.1 ± 3.27
Tensile strength of leather, N/mm^2	22.4 ± 1.28	22.7 ± 1.22
Grain strength, N/mm^2	22.4 ± 1.28	22.7 ± 1.22
Relative elongation of leather at the strain 10 N/mm^2, %	32.8 ± 2.04	31.6 ± 1.97

[a] For control all operations were performed as in conventional processes.

collagenous proteins. Although more collagen was affected in the enzymatic soaking compared to the control, the wet-blue properties after chroming were similar. Any worsening of the important exploitation properties of leather was not observed. These results show a high potential of *B. halodurans* protease in leather processing. The duration of the soaking process and operation steps can be reduced by using the enzyme.

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Data availability statement

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Renata Biškauskaitė: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Wen-Chien Lee:** Writing – review & editing, Validation, Supervision, Methodology. **Virgilijus Valeika:** Writing – review & editing, Validation, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35842.

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