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1 Executive summary

This deliverable, the first among 3 planned under this task, reports on results of WP3-Task 3.2, which aimed at exploiting different routes for the production of peptides from residues of poultry and fish industries at lab scale (TRL3). Specifically, microbial, enzymatic, and chemical protein hydrolyses have been applied for obtaining peptides from chicken feather (CF), fish scales (FS), and fish bones (FB). Then, the obtained hydrolysates have been characterized for their composition and bioactivities.

Regarding the CF microbial hydrolysis, marine bacterial isolates available at UNIBO collection, have been exploited. First, 79 isolates have been tested for their ability to hydrolyse milk proteins and produce extracellular proteases on solid and then in liquid growth media. Then, the 25 selected proteolytic isolates have been tested for growth with CF resulting in the selection of 10 able to efficiently degrade the substrate. The hydrolysates obtained after growth of the 10 most promising isolates have been used for evaluating keratinase production and different bioactivities. Based on the results of antimicrobial, antioxidant as well as keratinase activities, one marine isolate (MT) was selected for the production of CF bioactive hydrolysate. Such bioactive hydrolysate showed no cytotoxicity towards tested skin cells, exhibited high stability to thermal treatment and had high protein concentration. Through ultrafiltration (10 kDa cut-off) process, the recovery and separation of keratinase (in the retentate fraction) from antioxidant compounds (in the permeate fraction) was achieved. Moreover, whole genome sequencing of the isolate MT and LC-MS/MS analyses of the hydrolysate have been performed allowing, through bioinformatics analyses, the detection/identification of the different keratin peptides with antioxidant activity.

With regard to FS and FB, trials for peptides production have been performed by the marine isolate selected as most active on feather (MT) as well as by a desert one (DS). Specifically, six samples including 2 FS and 4 FB have been subjected to microbial hydrolysis. Bacterial growth and keratinase production (9-12 U/mL) were observed on all substrates. The hydrolysates exhibited antibacterial activities towards *S. aureus, S. epidermidis and E. coli* and a high antioxidant activity (ABTS scavenging activity) of up to 90% (abiotics, 20%). Through ultrafiltration, peptides (permeate fraction) having antioxidant activity have been separated from keratinases (retentate fraction) while antimicrobial compounds were distributed between the two fractions.

With regard to enzymatic hydrolysis, the first challenge to address was the intrinsic insolubility of keratin from CF in water. Since proteases require substrates in solution to effectively hydrolyze them, two solubilization treatments were developed. These treatments were designed to target the structural features that primarily prevent CF from solubilizing, thereby contributing to its mechanical durability: hydrogen bonding and disulfide crosslinks.(Ramya et al., 2020; Shavandi et al., 2017)

To cleave disulfide bonds, two reducing agents were tested: tris(2-carboxyethyl)phosphine (TCEP) and cysteine.

Using reaction conditions refined to improve keratin solubilization, CF were incubated in the two solubilization solutions for 24 h. Keratin was then precipitated using ammonium sulfate, purified by filtration, freeze-dried, and stored at −20 °C. Matrix-assisted desorption ionization mass spectrometry (MALDI–MS) analysis of the purified material, performed under optimized acquisition conditions, revealed a peak around 10,000 m/z, corresponding to the expected molecular weight of keratin.

Several proteases were evaluated for keratin digestion, including proteinase K, pronase E, bromelain, trypsin, pepsin, papain, and proteases from *Bacillus licheniformis* and *Streptomyces griseus*. Keratin

digestion was monitored by MALDI–MS at various reaction times. Peptide production was observed for all tested enzymes. Trypsin treatment predominantly yielded peptides with molecular weight values above 1000 Da, whereas papain generated peptides mainly below 1000 Da. The remaining proteases produced mixtures of low- and high molecular weight peptides.(Qiu et al., 2020)

The peptide-production process was subsequently optimized using purified keratin to enable upscaling. Ultimately, the concentrations of urea, TCEP, cysteine, and ascorbic acid were fine-tuned to permit direct addition of proteolytic enzymes to the solubilized CF without requiring intermediate purification steps.(Athwal et al., 2023)

Concerning chemical hydrolysis, different methods are known in the literature for the chemical treatment of chicken feathers, which allow obtaining hydrolysates containing a mix of peptides and amino acids after keratin hydrolysis (Alvaro Torices-Hernandez et al., 2025; Mengistu et al., 2024).

The two most well-known methods, particularly at industrial level, are:

- Acid hydrolysis using HCl (Alvaro Torices-Hernandez et al., 2025).

- Basic hydrolysis using NaOH (Shavandi et al., 2017).

As part of our work, both methods have been studied and tested. The best method was found to be basic hydrolysis, which is faster and allows for longer fragments.

This period covered the optimization of this method by testing first duck feathers and then chicken feathers.

The optimized conditions finally allowed obtaining hydrolysates in the form of a solution, which was subsequently neutralized.

According to MALDI-TOF analysis, the crude hydrolysates contain peptides ranging from 400-1980 Da, corresponding to fragments ranging from 4-18 amino acids.

To better understand the nature of the AA-fragments observed in MALDI-TOF upon chemical hydrolysis of poultry-feathers, we are developing a bioinformatic tool to correlate their origin with the original keratin AA-sequence. This tool proposes fragments accounting not only for amide-bond hydrolysis but also possible decomposition pathways for certain amino acids (Geiger et al., 1987; Rombouts et al., 2015; Kaiser et al., 2005; McKerchar et al., 2023).

The software is designed to suggest the AA-sequence that perfectly matches the studied fragment or the possible chemically modified sequence with the precise position of the modification, the type of modification and the amino acid concerned by this modification.

The quantities (0.12 kg) of chicken feather hydrolysate (solubilized peptides) requested by ANAVERIS for cosmetic products were produced as agreed. An optimized system was developed, using three different batches of 40 g of CF. Difficulty was encountered during lyophilization of hydrolysates due to the high concentration of salts contained in the final solution (< 30 % of the initial CF weight). Instead of delivering keratin-hydrolysate in the form of dry powder, it will be delivered in the form of solutions. In the meantime, the investigation of a method allowing removal/ reduction of salts is ongoing.

In a second objective we are seeking to develop and apply new approaches to allow for selective chemical cleavage of proteins. Towards this goal we are developing reactive groups that are capable of reacting selectively and covalently to lysine residues. Attached to such groups will be chelating agents which could induce hydrolytic cleavage of amide bonds in the neighborhood of modified lysine residues, providing a new, robust and selective tool for chemical protein cleavage. First results of selective modifications of lysine residues with new derivatives of ortho-phthalaldehyde (OPA) have shown that this approach is feasible and further studies are ongoing. Our chemical approach anticipates a plan for removing the bonded OPA by precipitation or similar methods, for a final application in cosmetics. Work is still ongoing, and further information is not available.

Overall, the results of Task 3.2 showed the feasibility of peptide production from CF, FS and FB by microbial routes. Enzymatic and chemical proteolytic treatment of CF have also been shown effective for the production of peptides, while hydrolysis of FS/FB through these two approaches is planned for the next months.

2 Introduction

Millions of tons of keratin-rich and protein-rich waste are produced globally every year. Improper disposal, as landfilling, of these recalcitrant by-products leads to environmental pollution and the loss of valuable nutrients. Therefore, there is a growing need for sustainable (bio)technological strategies to convert keratinous (e.g., feathers) and proteinaceous (e.g., fish scales and bones) residues into value-added products.

According to FAO (Food and Agriculture Organization of the United Nations), the global population of chickens was 29.2 billion birds alive at once in 2023, for both egg and meat production. Feathers, one of the main by-products of the poultry industry, accounts for 5-7% of the weight of the chicken. Indeed, around 9 million chicken feathers were generated in 2020 (de Menezes et al., 2023). The poultry industry is a growing sector, due to increasing awareness of customers towards the health benefits associated with their products and low cost, thus these numbers are expected to grow.

Feathers are rich in proteins and composed of more than 90% keratin (Mukesh et al., 2024; Qiu et al., 2020; Riaz et al., 2024), a very recalcitrant protein due to the high cross-linked structure, especially abundant in disulfide bonds. This robust structure makes keratin resistant to degradation by common proteases (Hassan et al., 2020; Li, 2019; Mazotto et al., 2022; Mukesh et al., 2024). Feathers used to be mainly landfilled, causing harm to the environment due to their recalcitrance and to human health since they carry pathogens (Li, 2019; Mukesh et al., 2024; Shestakova et al., 2021). Nowadays, feathers are managed through mechanical, physical, or chemical treatment, using high temperatures and chemicals (Gupta et al., 2013; Hassan et al., 2020; Mukesh et al., 2024). These treatments lead to the loss of thermolabile nutrients with the decrease in the nutritional value of the hydrolysate and require high energy and costs (Gupta et al., 2013; Hassan et al., 2020; Li, 2019; Mazotto et al., 2022; R. Sharma & Devi, 2018).

The fish industry is growing, with more than 196 million tons of fish predicted to be processed in 2025. This industry generates large amounts of by-products, such as heads, skin, scales (5%), trimmings, fins, viscera, bones (9-15%), and muscle, which are currently wasted (e.g., landfilling) or used to produce low-value-added products (e.g., fish meal and silage). The by-products account for more than 50% of the whole fish, but these values can vary significantly depending on the species and application (Gao et al., 2021; Rustad et al., 2011; Saleh et al., 2021). In 2016, around 52-80 million tons of this waste were produced (Saravanan et al., 2023).

Fish by-products are rich in proteins (8-35%) and represent a source of mainly collagen, but also gelatine, polyunsaturated fatty acids, amino acids, and enzymes. In particular, fish scales and bones are composed of approximately 70% protein, mainly type I collagen, which provides strength and structural resistance. The remaining 30% consists primarily of hydroxyapatite, along with smaller amounts of ash, fats, gum, vitamins, zinc, iron, calcium, phosphorus, polyunsaturated fatty acids, lecithin, and traces of other essential elements (Gill et al., 2025; Liu et al., 2024). Scales, more than bones, have an outer layer of hydroxyapatite and calcium carbonate, which makes them more stable (Suresh et al., 2025). Fish byproducts may be used to produce protein hydrolysate, rich in many bioactivities. Therefore, reducing the environmental impact of waste and obtaining an economic advantage by producing a value-added product (Gao et al., 2021).

A sustainable solution for feather and fish waste valorisation is the biotechnological approach, that is based on the action of keratinolytic microorganisms or their enzymes. These bacteria can convert low-cost by-products into value-added products, thereby reducing the environmental impact while preserving their nutritional values. The obtained hydrolysate, rich in peptides and amino acids, serves as a valuable nitrogen source for many industrial applications in the context of the circular economy (Aktayeva et al., 2022; Gill et al., 2025; C. Sharma et al., 2022). The microbial degradation of protein-rich waste represents an environmentally friendly process for producing high-value protein hydrolysates rich in bioactive peptides and enzymes (Das et al., 2024; Mukesh et al., 2024).

3 Peptides production by microbial routes-UNIBO

4.1 Production of chicken feather hydrolysates (CFH)

3.1.1 Selection of keratinolytic bacteria

The production of bioactive CFH was attempted by testing marine bacteria. For this, trials for the selection of bacterial isolates able to hydrolyze keratinous substrates and to produce keratinases have been performed. In specific, 25/79 isolates producing extracellular proteases have been selected after

a preliminary screening on agar plates and then in liquid growth media using skim milk/casein as substrate. Then, bacterial growth with chicken feathers as substrate was carried out and bioactivities (antibacterial and antioxidant) as well as keratinase production were evaluated on CFH resulting in the selection of five bacterial isolates as most promising. Indeed, after 72h incubation, the resulting feather hydrolysate exhibited significant keratinase activity (up to 9 U/mL, keratin azure assay (Cai et al., 2008)) and strong bioactivities, including antioxidant activity (75-85% ABTS radical scavenging (Xiao et al., 2020), increase of 60-70% compared to the abiotic, Figure 1) and antimicrobial effects against *Staphylococcus aureus* and *S. epidermidis* (well diffusion assay (Balouiri et al., 2016)).

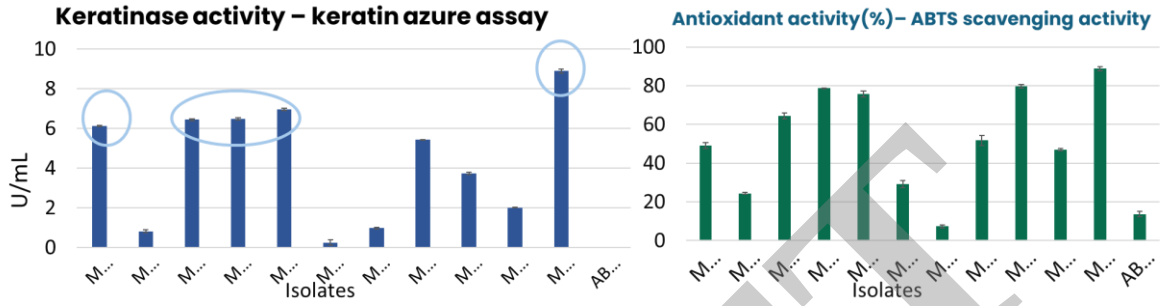


Figure 1 Feather hydrolysate bioactivities of the selected 10 proteolytic marine isolates, a) keratinase activity (U/ml), and b) antioxidant activity expressed as ABTS scavenging activity (%).

Among these, one marine isolate MT showed the highest performance and was therefore selected for further characterization. The keratinase displayed optimal activity at pH 8.5 and 50-55 °C, remaining also stable under a wide range of conditions, between 4-50°C and pH 4-10 (Figure 2). On the other hand, the keratinase was inhibited by serine and metalloprotease inhibitors, thus it probably belongs to these enzyme families.

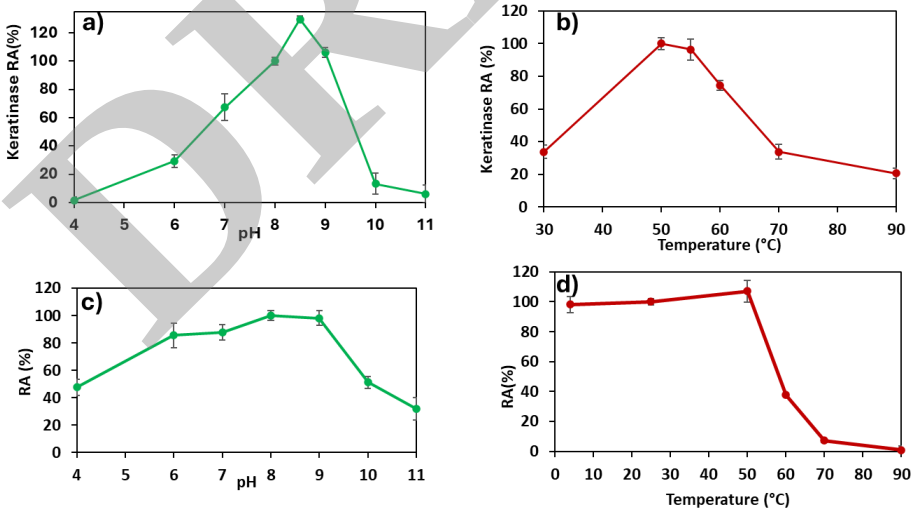


Figure 2 Characterization of isolate MT keratinase in terms of pH a) optimal activity c) stability; and temperature (°C) b) optimal activity and d) stability expressed as Relative activity (%). The keratinase activity detected under standard conditions (pH 8, 50°C) has been considered as 100% activity.

The incubation conditions were then optimized, and a scale-up of the process was performed (1 L flask), and all the results obtained in small scale (250 ml flask) were reconfirmed, in terms of

bioactivities and enzymatic activity. Through this hydrolysis process, a protein concentration (Lowry assay) of 2.01±0.14 mg/mL was achieved.

Moreover, the feather hydrolysate was proven to be safe, showing no cytotoxicity toward skin cells after dilution or autoclaving, which is very important for cosmetic application. Specifically, on dermal fibroblasts (HDF), no cytotoxicity was observed when the hydrolysate was autoclaved. While keratinocytes (HaCaT), were less sensitive, in fact no cytotoxic effect was observed when the hydrolysate was diluted to 0.37-fold.

Afterwards, ultrafiltration of the hydrolysate enabled the separation of enzymes (retained in the high-molecular-weight fraction) from low-molecular-weight bioactive peptides, with Amicon Ultracentrifugal Filters. Indeed, the antioxidant activity was isolated in the permeate while the antimicrobial activity redistributed between both permeate and retentate. The keratinase activity was concentrated in the retentate (Figure 3).

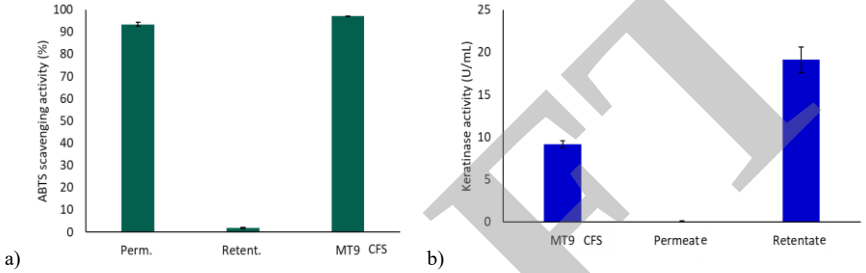


Figure 3 Feather hydrolysate fractions after ultrafiltration with a 10 kDa cut-off membrane, in terms of a) antioxidant activity (%) and b) keratinase activity (U/mL).

The enzyme-rich retentate was freeze-dried and reused for feather hydrolysis (5 g/L) at varying enzyme concentrations, under optimal keratinase activity. As reported in Figure 4, around a 50% increase in antioxidant activity (compared to T0h) was measured within 3 h when 1 U of crude enzyme/keratinase was used (1X), while no enhancement in antimicrobial activity was detected. This suggests that antioxidant compounds are released from the substrate, whereas antimicrobial molecules are produced directly by the microorganism during growth.

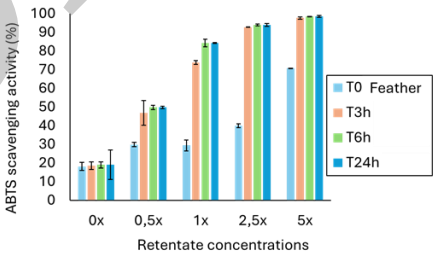


Figure 4 Antioxidant activity of CFH obtained by enzymatic hydrolysis with recovered retentate (crude enzyme/keratinase) at different enzyme concentrations, time course, expressed as ABTS scavenging activity (%).

The second phase of the work focused on the characterization of the keratinolytic strain and its feather hydrolysate and was performed in collaboration with FHNW. Whole-genome sequencing (WGS) was performed to identify the enzymes involved in keratin degradation. Bioinformatic analysis revealed the presence of numerous serine and metalloproteases, and a promising keratinase candidate was identified through multiple sequence alignment using the MUSCLE tool, based on the matching of conserved regions with an S8 family peptidase of 39 kDa from the WGS, consistent to what was

observed during the enzyme characterization. Genome mining with antiSMASH and BAGEL further indicated the potential synthesis of antimicrobial compounds.

In parallel, SDS-page, MALDI-TOF, and LC-MS/MS analyses were used to characterize the CFH, rich in peptidases, including the predicted keratinase, and to identify bioactive peptides. Indeed, the SDS page (Figure 5a) confirmed the presence of peptide at low MW in the permeate, below 9 kDa, in lane 7 and different bands at higher MW in the retentate, one around 30 kDa, coherently to the predicted keratinse. On the other hand, the MALDI-TOF (Figure 5b) and LC-MS/MS analysis allowed the detection of bioactive peptides having MW between 400-1400 Da (coherently with the previous results) and high hydrophobic amino acid content; their potential for bioactivities were predicted via PeptRanker.

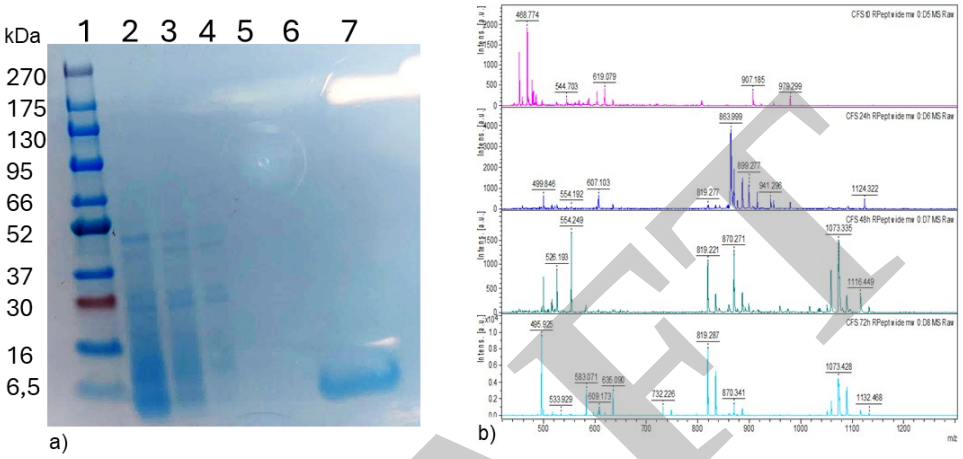


Figure 5 Characterization of feather hydrolysate peptides and proteins MW, through a) SDS-page (4-20% polyacrylamide) and b) MALDI-TOF spectrum.

4.2 Production of fish scales (FS) and fish bone (FB) hydrolysates

For FS and FB hydrolysis, two bacterial isolates including 1 marine (MT) selected as most active on feathers and one desert (DS isolate) were tested on fish-processing residues as substrates, scales and bones of sea and river fish. In a preliminary small-scale screening (250 ml flask), a higher (compared to FB) antioxidant activity (80-90% ABTS scavenging activity, Figure 6) and antimicrobial effect against *S. aureus* were detected for hydrolysates obtained by both isolates on FS as substrates. Concerning the keratinase activity, all substrates induced a keratinase production between 6-12 U/mL.

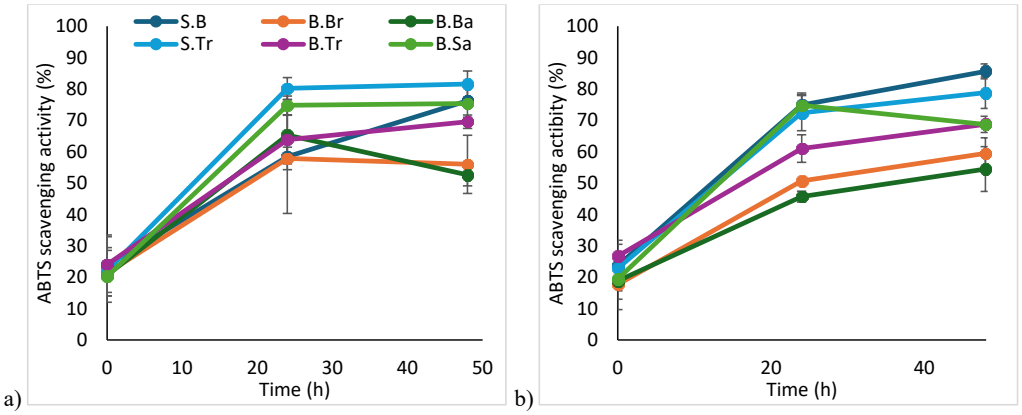


Figure 6 Antioxidant activity time course of different fish waste substrates (S. scales, and B. bones), produced via microbial routes by a) a marine isolate MT and b) a desert isolate DS, expressed as ABTS scavenging activity (%).

Due to their higher bioactivities and keratinase activity, the experiments were further scaled up (1000 ml flask) using FS (trout scales-S.Tr and a mix of seabream+seabass scales-S.B) as substrates. The hydrolysates maintained high antioxidant activity showing an increase of around 55% (S.T) and 40% (S.B) compared with the abiotic control after 48 h incubation. The marine hydrolysates exhibited antimicrobial activity against *Escherichia* coli *and S. aureus*, while the desert isolate showed activity only against *S. aureus*, on both scale substrates. Furthermore, the antioxidants remained stable while the antimicrobial activity was lost after autoclaving. All hydrolysates displayed notable keratinase activity, from 7.2 ± 0.6 to 11.36 ± 0.5 U/mL on both scales. Through this hydrolysis process, a protein concentration (Lowry assay) of 2.80±0.02 mg/mL was achieved.

Bioactive peptides and enzymes were then successfully separated by ultrafiltration, using a 10 kDa cut-off membrane. At low molecular weight (MW), below 10 kDa, the permeate fraction isolated the antioxidant activities (Figure 7), with a permeate activity comparable to the original hydrolysate before ultrafiltration (CFS), for both isolates, but the activity was higher on the S.Tr also in the retentate. While only the retentate, at higher MW above 10 kDa, showed a concentrated keratinase activity, compared to the original (CFS), higher on SB for the desert isolate and on STr for the marine isolate.

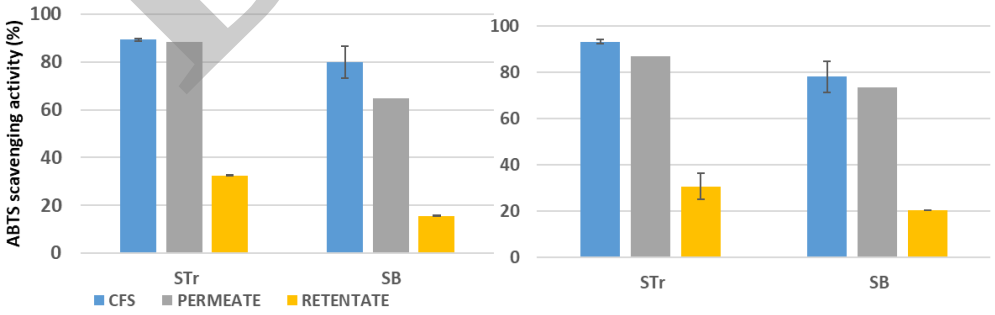


Figure 7 Antioxidant activity of fish scales hydrolysates fractions after ultrafiltration with a 10 kDa cut-off, of a) desert DS isolate and b) marine MT isolate.

4 Peptides production by enzymatic routes-FHNW

Proteolytic enzymes hydrolyze proteins in solution; however, the insolubility of keratin makes feathers a poor substrate for enzymatic action. Therefore, solubilizing feathers is a necessary step in developing an effective enzymatic hydrolysis process. Keratin's insolubility is primarily due to the presence of multiple disulfide bonds in its structure. To address this, a 100 mM solution of tris(2-carboxyethyl)phosphine (TCEP) was used to reduce the disulfide bonds to thiol groups. TCEP is a water-soluble, thiol-free reducing agent commonly used in biochemistry for the selective reduction of disulfide bonds. Unlike traditional reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol, TCEP is odorless, highly stable in aqueous media, and resistant to oxidation. It reduces disulfide bonds by donating electrons from its phosphine group, generating two free thiols while undergoing oxidation to the corresponding phosphine oxide.(Burns et al., 1991)

Subsequently, 18 mM iodoacetamide was added to alkylate the thiol groups, preventing the reformation of disulfide bonds (*cf.* Figure 8).

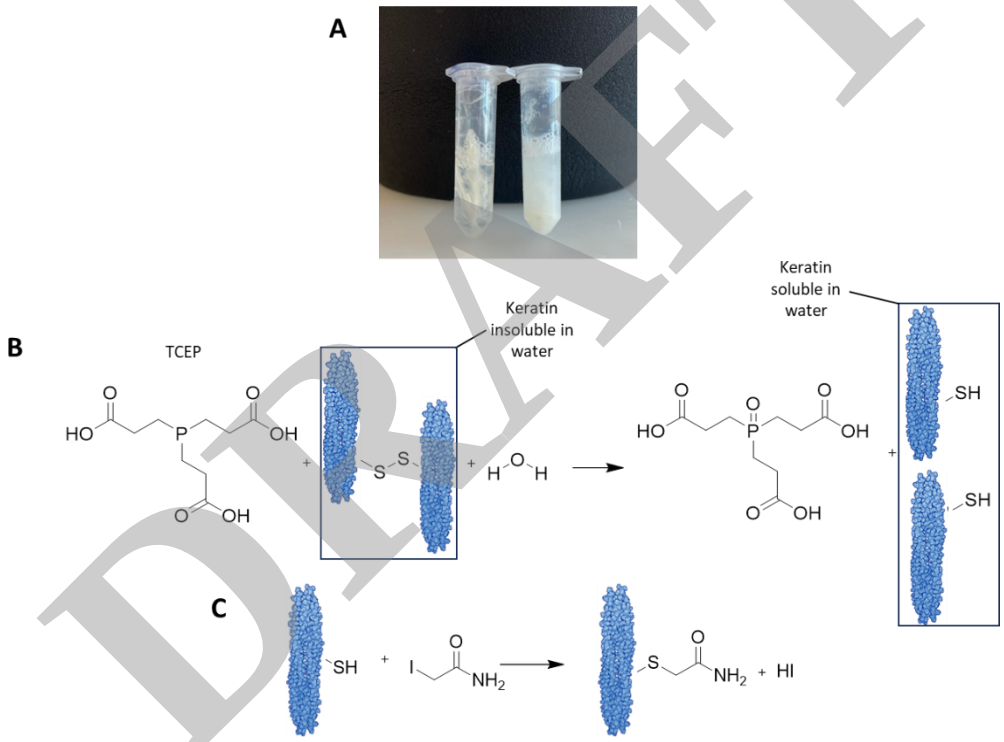


Figure 8 Feather solubilization and reduction/alkylation mechanisms. (A) Feathers with (right) and without TCEP (left) after 24h in buffer; no intact feathers are left in samples treated with TCEP. Moreover, TCEP-treated samples are turbid, in contrast with the control sample. (B) Reaction mechanism of disulfide bond reduction by TCEP. (C) Reaction mechanism of keratin alkylation by iodoacetamide.

After 24 hours incubation, visual inspection indicated that the CFs had undergone partial solubilization. Trypsin (100 U/mL) was then added to the solution and allowed to react for an additional 24 hours. Following this enzymatic treatment, the feather solution was analyzed using MALDI-MS and compared to control samples (*cf.* Figure 9)

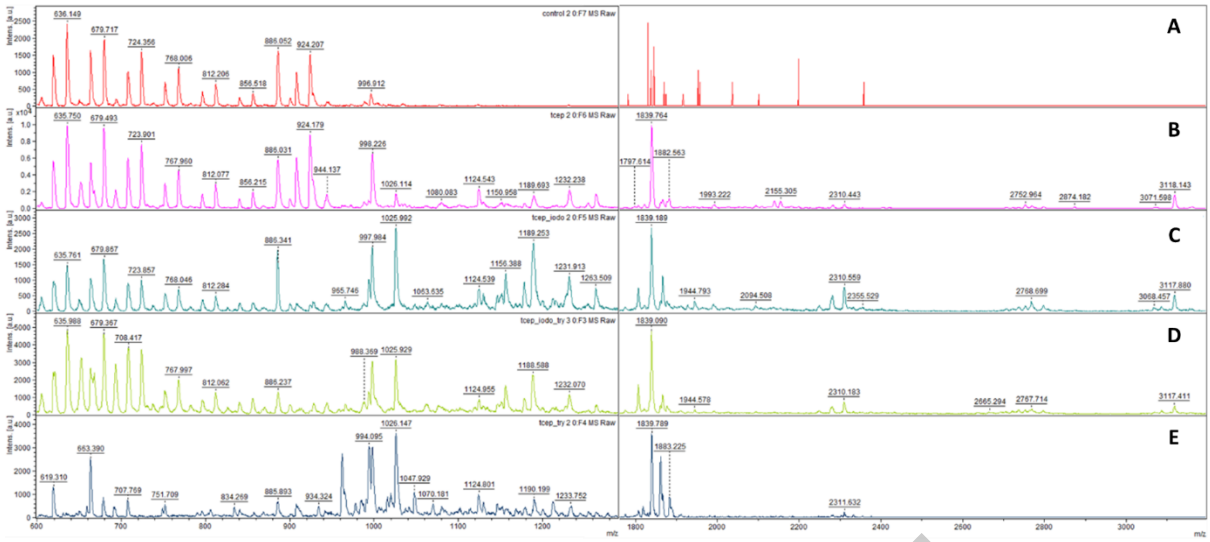


Figure 9 MALDI-MS spectra of CFs incubated in TRIS buffer (A), treated with TCEP alone (B), treated with TCEP and iodoacetamide (C), treated with TCEP, iodoacetamide and trypsin (D) and treated with TCEP and trypsin (E).

All samples treated with TCEP exhibited distinct peaks compared to the untreated feather sample indicating the reduction of SS bonds and release of soluble keratin proteins/peptides. However, the spectra of samples treated with various combinations of TCEP, trypsin, and iodoacetamide showed no significant differences, indicating the lack of proteolytic activity of trypsin in the tested conditions. This outcome may be explained by trypsin deactivation in the presence of high concentrations of TCEP and iodoacetamide, or by the fact that keratin is not a good substrate for trypsin. Additionally, no disulfide bond reformation is observed. No new peaks were observed in the spectra of samples treated with both TCEP and iodoacetamide compared to those treated with TCEP alone. To improve solubilization of the feather substrate and allow proteolytic activity, the process was optimized by reducing the concentration of the reducing agent and omitting iodoacetamide. Chicken feathers were then incubated in solutions with varying TCEP concentrations, ranging from 2 to 100 mM. CFs were successfully solubilized within 24 hours when samples were treated with TCEP concentration from 10 mM to 100 mM. Following solubilization optimization, various proteases were evaluated for their ability to hydrolyze keratin. To perform this evaluation, CFs were first incubated in a 10 mM TCEP solution to solubilize keratin. The resulting keratin solution was then diluted (10 X) to further decrease the TCEP concentration and incubated with seven different proteases (papain, bromelain, trypsin, pronase, proteinase K, protease form *Bacillus licheniformis*, protease form *Streptomyces griseus*) at 100 U ml^{-1}. Samples were then reacted for 24 hours and analyzed at MALDI-MS (*cf.* Figure 9).

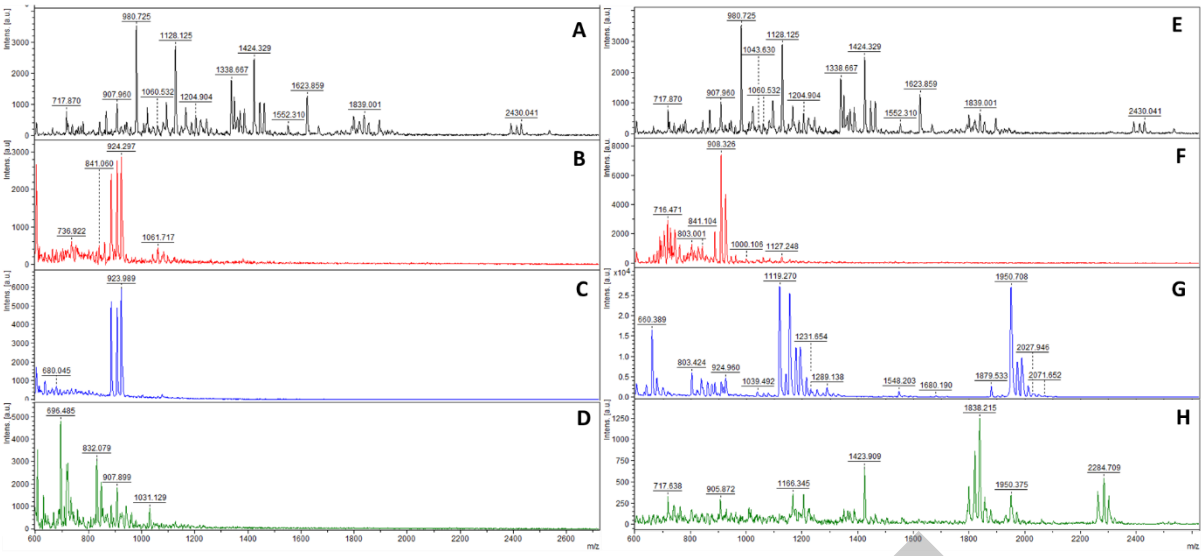


Figure 10 MALDI-MS spectra of solubilized CFs treated with papain (from A to D) and trypsin (from E to H). A and E spectra represent CFs treated with TCEP alone. B and F represent spectra of CFs treated with proteases alone. C and G represent spectra of proteases' self-digestion. D and H represent spectra of feathers treated with TCEP and proteases.

Samples treated with trypsin and papain displayed significantly different spectra compared to the controls, indicating effective keratin hydrolysis. In contrast, the other proteases did not yield conclusive results. This may be due to a high contribution to the spectra of the proteases' self-hydrolysis, which can overshadow signals from feather-derived peptides.

To obtain peptide mixtures generated by distinct proteases, the extraction procedure was optimized. Instead of adding the enzymes directly to the solubilization solution, keratin was first purified following solubilization. CF were incubated for 24 h in 8 M urea and 100 mM TCEP. Urea was employed to disrupt hydrogen bonding. As a chaotropic agent widely used in molecular biology, urea denatures proteins by destabilizing their native conformation. It preferentially interacts with the peptide backbone and side chains, thereby shifting the equilibrium toward the unfolded state.(Zhang et al., 2022)

Solubilized keratin was subsequently precipitated by adjusting the pH to 4 and saturating the solution with ammonium sulfate. The resulting suspension was vacuum-filtered, and the solid fraction was washed three times with water to remove residual urea, TCEP and ammonium sulfate. Electrophoretic analysis and MALDI–MS confirmed that the recovered solid corresponded to keratin (*cf.* Figure 10)

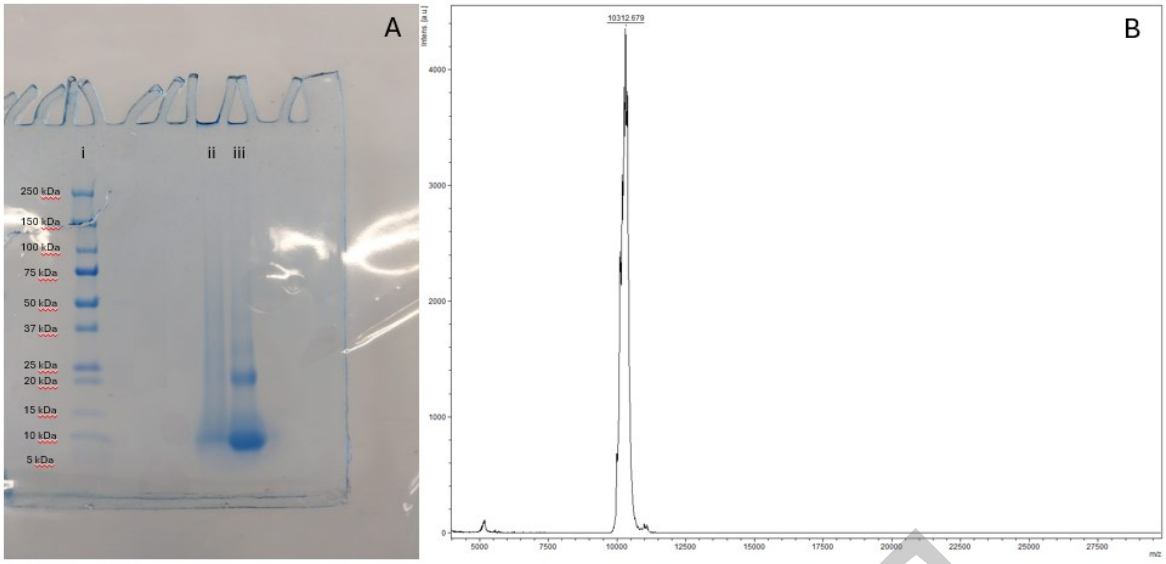


Figure 11 (A) Polyacrylamide gel after electrophoresis and staining. Lanes: (i) molecular weight ladder, (ii) crude solubilized fraction, and (iii) purified keratin after dissolution in Tris buffer. (B) MALDI–MS spectrum of purified keratin dissolved in Tris buffer, showing a peak at 10,312 m/z corresponding to chicken keratin.

SDS–PAGE analysis revealed a band at approximately 10 kDa, consistent with the expected molecular weight of chicken keratin. This result was corroborated by MALDI–MS, which displayed a peak at 10 kDa. Purified keratin was dissolved in 100 mM Tris buffer (pH 8) and incubated with papain or trypsin (100 U mL^{-1}) at 37 °C for 24 h. Samples collected at the end of the reaction were analyzed by MALDI–MS (*cf.* Figure 11).

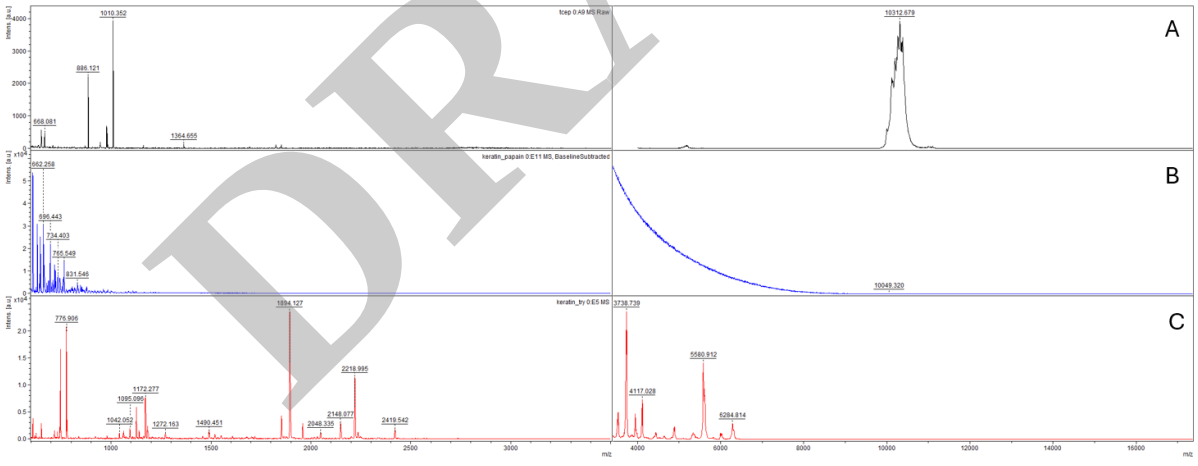


Figure 12 MALDI-MS spectra of purified keratin (A), keratin treated with papain (B), keratin treated with trypsin (C).

MALDI–MS spectra indicated complete keratin digestion for both enzymes, with new peaks corresponding to keratin-derived peptides. Moreover, the molecular weight distribution of the resulting peptides varied markedly depending on the protease employed. Papain generated exclusively low-molecular-weight peptides (<1000 Da), a class often associated with bioactive properties in cosmetic applications.(Pintea et al., 2025) In contrast, trypsin digestion produced a broader mixture of peptide sizes after 24 h, yielding both low- and higher-molecular-weight fragments.

A second solubilization method was developed by incubating CF for 24 h in a solution comprising 500 mM L-cysteine, 200 mM ascorbic acid, and 8 M urea. Cysteine, although a milder and less stable reducing agent, can also participate in thiol–disulfide exchange reactions. Under slightly basic conditions, its thiolate form acts as a nucleophile that attacks disulfide bonds, forming a mixed disulfide intermediate and ultimately releasing two free thiol groups.(Mthembu et al., 2020) However, cysteine is readily oxidized to cystine, which is sparingly soluble and can precipitate from solution. To mitigate this issue, ascorbic acid was included in the solubilization mixture. Ascorbic acid prevents cysteine oxidation due to its strong electron-donating capacity, conferred by its enediol structure. It is easily oxidized to dehydroascorbic acid while reducing reactive oxygen species. Additionally, as a mild reducing agent, ascorbic acid can convert cystine back to cysteine, thereby limiting precipitation.

Keratin was precipitated, filtered, and analyzed by MALDI–MS, which confirmed the presence of intact keratin and validated the efficiency of the new procedure.

Both solubilization and proteolytic digestion steps were subsequently optimized to enable a one-pot workflow. CF were treated with either solubilization mixture described above, then diluted 1:8 prior to protease addition. Trypsin, papain, proteinase K, and pepsin were evaluated under identical digestion conditions. Samples were collected after 1, 4, and 24 h and analyzed by MALDI–MS.

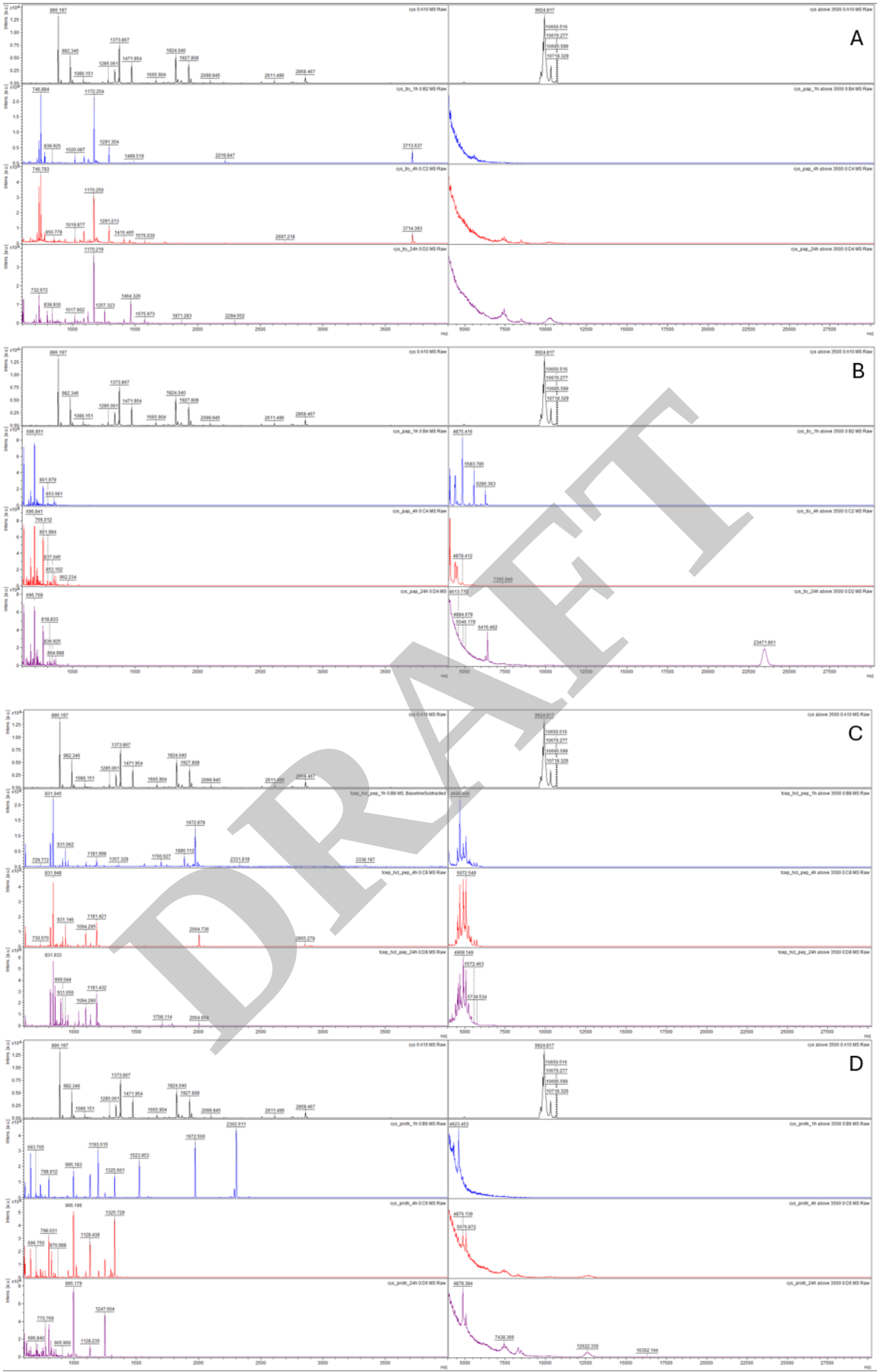


Figure 13 MALDI–MS spectra of purified keratin digested with papain (A), trypsin (B), pepsin (C), and proteinase K (D). The spectrum of untreated keratin is shown in black. Digestion profiles collected at different time points are colour-coded as follows: 1 h (blue), 4 h (red), and 24 h (purple).

Across all proteases and solubilization approaches, MALDI–MS spectra showed complete keratin digestion within 1 h. Notably, trypsin generated peptides predominantly above 1000 Da during short incubations, which are often included in cosmetic formulation for their hydration or film-forming properties.(Veiga et al., 2023) in contrast, papain produced peptides mainly below 1000 Da after prolonged reactions, confirming previous results. Other proteases yielded a broader range of peptide sizes.

Finally, the extraction and digestion procedures were scaled to process 40 g of CF per batch. The workflow remained largely unchanged, with optimization applied only to the dissolution of purified keratin prior to protease treatment. To enhance solubility and inhibit disulfide bond reformation, keratin was dissolved in 63 mM cysteine and 25 mM ascorbic acid at 37 °C. Using this optimized protocol, 20 g of purified keratin, 20 g of peptides <1000 Da, and 20 g of peptides >1000 Da were obtained and will be supplied to ANAVERIS for cosmetic applications.

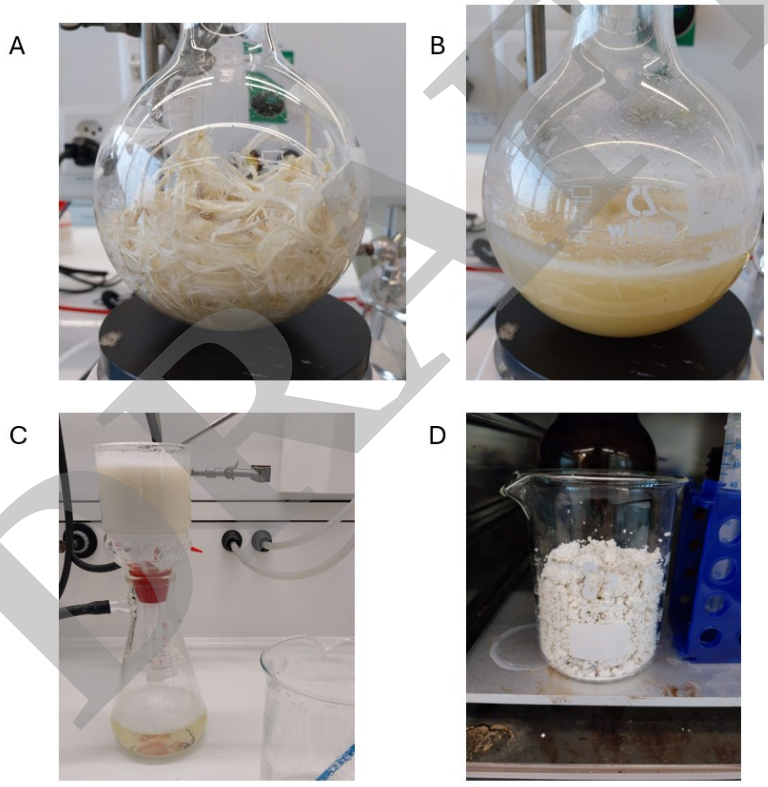


Figure 14 Photographs of the different stages of the scale-up process: (A) CF prior to solubilization, (B) CF after solubilization, (C) vacuum filtration of precipitated keratin, and (D) purified keratin before freeze-drying.

5 Peptides production by chemical routes-FHNW

The main objective is the chemical hydrolysis of keratin. To do this, different conditions were tested initially, in order to determine the best conditions before moving on to the optimization step. The conditions were chosen based on the procedures reported in literature for poultry feathers.

Hydrolysis conditions applied to DF

A table summarizing all the tested conditions is given below.

Table 1 Table summarizing the hydrolysis conditions tested

Conditions	T (°C)	Time
1% NaOH + 1 % H_2O_2	r.t, 40 °C and 60 °C	2h, 4h, 6h, 24h, 48h and 72 h
10 % NaOH	r.t, 40 °C and 60 °C	2h, 4h, 6h, 24h, 48h and 72 h
12% HCl	r.t, 40 °C and 60 °C	2h, 4h, 6h, 24h, 48h and 72 h
1M thioglycolic acid + 0,3 M urea (v/v 1:3)	r.t, 40 °C and 60 °C	2h, 4h, 6h, 24h, 48h and 72 h

MALDI-TOF analysis of hydrolyzed DF

The MALDI-TOF results show that the best tested conditions are aqueous NaOH compared to acidic and thioglycolic acid conditions. These conditions allow faster and more efficient hydrolysis as well as the obtaining of longer fragments under same temperature and time conditions, as shown in the results in Figure 15.

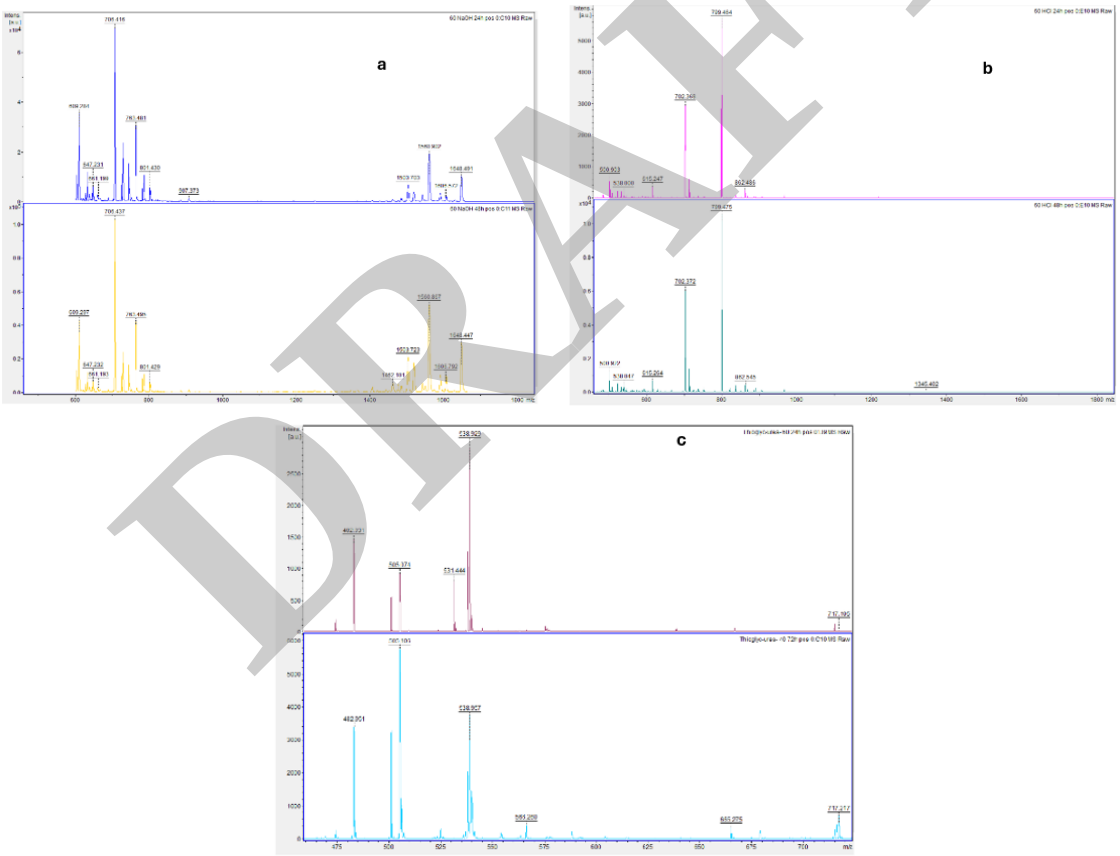


Figure 15 Basic hydrolysis 10% NaOH of duck feathers after 24 and 48h (a), 12% HCl after 24 and 48 h (b) and 1M thioglycolic acid + 0.3 M urea (v/v 1:3) after 24 and 72h (c).

All these results are given for the same temperature (60 °C) and reaction time: 24h, 48h for a and b and 24 h, 72 h for c. It is possible to clearly see that 10% NaOH gives longer fragments and has shorter reaction times of 4-6h (Figure 16).

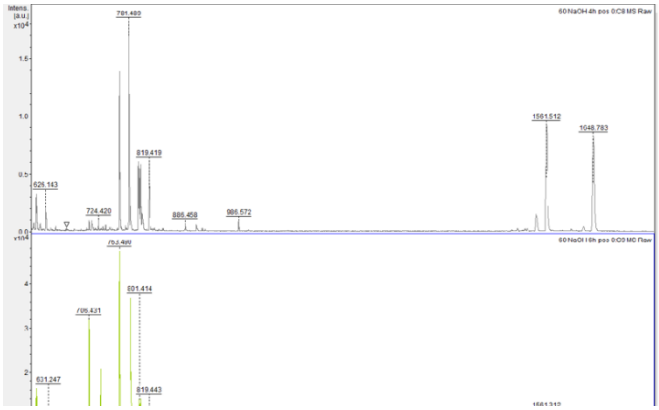


Figure 16: Basic hydrolysis 10% NaOH of duck feathers at 4h and 6h

This spectrum shows th[illegible] longer fragments (compared to H[illegible] acid conditions) of 1562 and 1649 Da are already obtained after only 4 hours of reaction, which favors this basic hydrolysis for the next optimization steps.

Once the best conditions have been determined, the objective is to optimize this process as much as possible in order to make it reproducible and with good yields.

To do this, different concentrations as well as different equivalents of NaOH were tested.

The objective here is to possibly obtain longer fragments, using fewer NaOH equivalents for hydrolysis.

We use different of equivalents of NaOH with respect to all 94 amide bonds present in Keratin-B4 (95 AAs, 9,719 Da).

Hydrolysis conditions' optimization

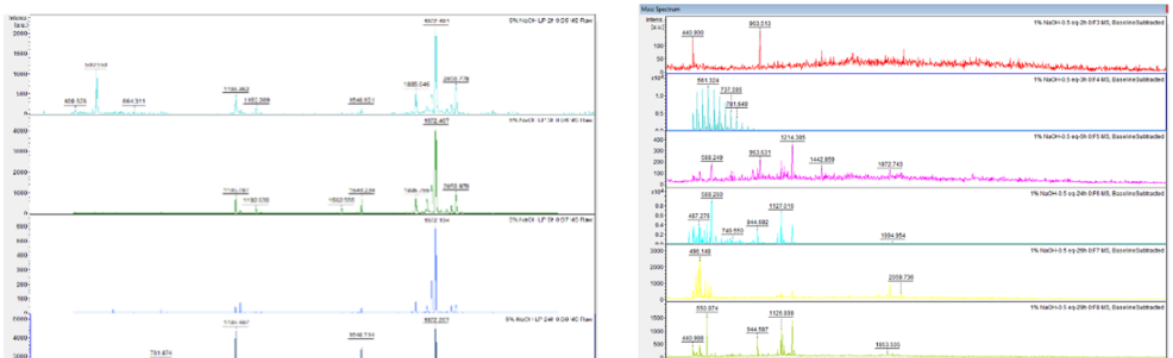
Moreover, by using more diluted solutions, we play both on the amount of salt remaining in the final product, as well as on the volume available to cover all the feathers for more efficient and optimal hydrolysis.

Table 2 Table summarizing optimization conditions tested for initial 1g CF

Solution	n equiv.	V (mL)
5% NaOH	0.5	3.6
5% NaOH	0.1	0.72
1% NaOH	0.5	18
1% NaOH	0.1	3.6

The MALDI-TOF results confirmed our expectations. Using 0.5 equivalents of NaOH, longer fragments are obtained (1900-2000 Da) (Figure 17 b and c) compared to 5% NaOH spectra (1.0 equiv.) where the obtention of shorter fragments (maximum 1648 Da) was observed (Figure 17 a).

a b



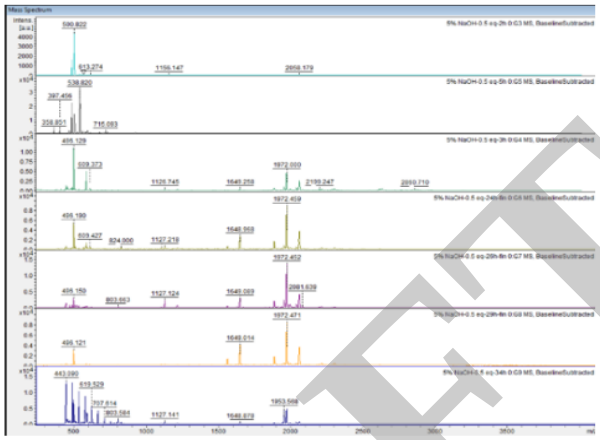


Figure 17: MALDI-TOF analysis of duck-feather hydrolysates (2h, 3h, 24h, 26 h, 29 h, 34h at 50°C) at different concentrations and equivalents of NaOH, as summarized in Table 2: 5% NaOH (1.0 eq) (a), 1% NaOH (0.5 eq) (b) and 5% NaOH (0.5 eq) (c).

For 1% NaOH, it is possible to see that the intensity of the peaks is lower for the longer fragments, hence the choice of intermediate NaOH concentration for the final application to chicken feathers.

For this reason, the optimized conditions chosen for application to chicken feathers are: **2% NaOH (0.5 equiv.), 50 °C, 37 h**.

Optimized conditions applied to CF

The final procedure followed for this work is represented by the scheme in Figure 18 below.

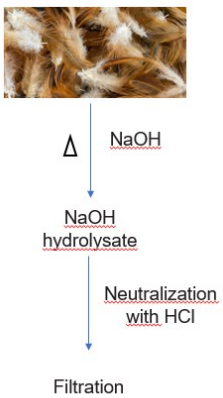


Figure 18: Procedure of chicken feathers chemical hydrolysis

The optimized procedure to hydrolyze chicken feathers is shown in Figure 19 below. CF were washed several times with water and methanol, dried overnight to reduce the odor and residual impurities (skin, blood etc.). The amount of washed and dried CF was divided into three portions to avoid the risk of breaking and losing material. A water bath was used instead of heating up directly the flask, to

reduce breaking risk. It is possible to see that after 34h we obtain complete solubilization of the starting CF.

After hydrolysis, samples were taken from the solution for MALDI-TOF analysis and frozen at -20 °C. The final hydrolysates (divided into 3 portions) were neutralized and filtered to remove the residual non-hydrolyzed feathers.

The final step was the freeze-drying of the neutralized and filtered hydrolysate. As shown in Figure 19 d only 3.5 g (Figure 19 d, orange arrow) were freeze-dried successfully and the rest stayed in the form of a solution because of the high amount of salts. The obtained 0.12 kg of CF hydrolysates will be shipped to ANAVERIS for cosmetics applications. A method for removing or reducing salt amount is investigated.

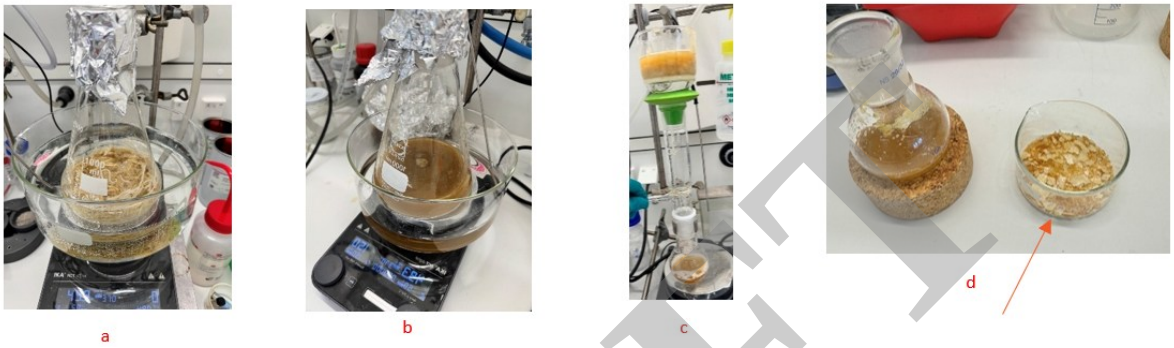


Figure 19: Strating washed CF + 2% NaOH after 5h (a), CF + 2% NaOH after 34h (b), filtration of neutralized hydrolysate (c), freeze-dried and residual hydrolysate (d)

For the keratin contained in chicken feathers, complete hydrolysis was slower; 48 hours instead of 34 hours for duck feathers. Moreover, the fragments obtained following hydrolysis are longer. A summary table of the main differences between the treatment of chicken feathers (first values) and ducks (second values) is given below.

Table 3: Summary table of optimized conditions applied to chicken and duck feathers respectively.

[NaOH]	n ° equiv.	T (°C)	Time (hours)	Peptide fragments range (Da)
2% vs 5%	0.5 for both	50 vs 60	48 vs 34	400-3600 vs 400-1980 Main fragments have similar lenghts in both CF and DF

In chicken feathers we have three different keratin types (P1, P2 and P3) with different compositions in amino acids as well as different lengths.

For a better understanding of proteins' differences, the sequences of CF-P1, CF-P2 and CF-P3 are defined as:

CF-P1:
EELSFVMQRWQCDLENVKKRVSSLRTSVSDTEQRGDRALKDAREKHTELQNALQKAKDELACMLRDYQELLNVKL A (76 AAs, **8908.11 Da)**

CF-P2:
MSCYDLCLPASCSPRPLATSCNEPCYRRCWDSTTVIQPPTVVVTLPGPILSSYPQSTTVGSTASAAVGSYLRCCGVPV ASGGTRGLGKGALLCLGGG (98 AAs, **9814.34 Da)**

CF-P3:
DYQELLNVKLALDIEIATYRTLLEGEESRICTGNPVSVAVVSGGGTVGECRSLSGIGGKCSVKTGASSGLGGVVSSFG VSGAGFSARSVDCVPRVGGGFGARSAASCVGREVLSGADGLQCAAGVGNLVCAGVEQCSPGTVIIPGPGVCGGG NRYSTAVRVVVRTSR (166 AAs, **16287.43 Da).**

A graph comparing the composition of duck and chicken feathers is given in Figure 20.

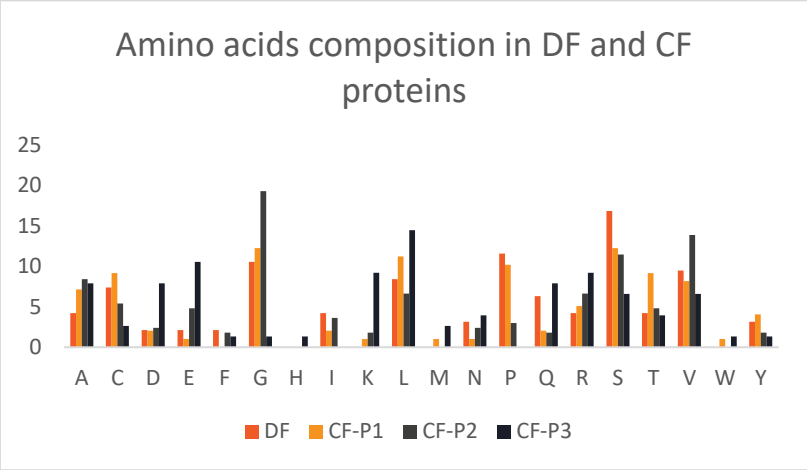


Figure 20: Composition in amino acids of CF chicken feathers and DF duck.

The MALDI-TOF results (Figure 21) comparing these two proteins, showed the obtention of certain fragments in common, but the majority of the fragments are different. The most remarkable result is the obtaining of shorter fragments for duck feathers and, on the contrary, longer for chicken feathers.

Moreover, after complete hydrolysis, no keratin peak is detectable, which means that all the keratin has been hydrolyzed.

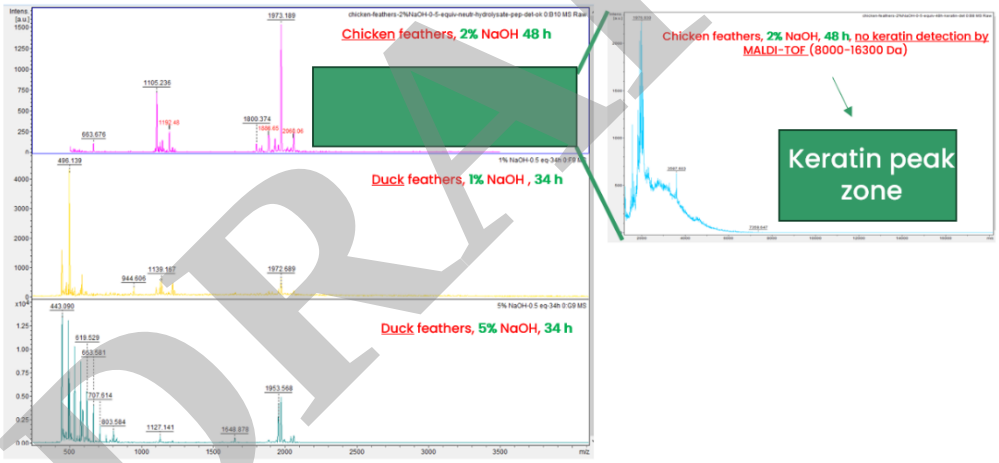


Figure 21 MALDI-TOF results comparing the final peptide composition of chicken and duck feathers after complete hydrolysis.

These results have therefore shown that the optimized conditions for duck feathers are effectively applicable to chicken feathers as well.

MALDI-TOF results allow us to have an idea on the length of the fragments obtained. It is now important to determine the sequence of these fragments.

Bioinformatics tool development

There is no known software/tool for the extraction of fragment sequences by chemical cleavage.

As said previously, we are in the process of developing a bioinformatic tool capable of doing this as well as providing a diagram refactoring the cleavage sites as well as statistics regarding fragile amino acids where chemical cleavage mainly takes place. Depending on the mass of the peptide fragments obtained by MALDI-TOF, the tool is capable of giving the length of the fragments as well as possible sequences, those that perfectly match with the given mass or chemically modified peptide sequences,

by specifying the type of modification, its position on the sequence of the protein, as well as the amino acids involved through chemical modification.

The software is still being optimized and the results obtained are not fully conclusive with the current version of the software.

OPA approach development

Above, we have discribed the optimization of non-selective chemical CF- and DF-keratin hydrolysis , based on literature reports (Tursunova et al., 2024; Gupta et al.2020; Fitriyanto et al., 2022). To develop more selective cleavage protocols, another of our main objectives is to develop a method for selectively cleaving proteins in order to obtain well-defined fragments through more controlled cleavage reactions. Different methods for hydrolytic cleavage of proteins are reported in the literature (Els Cartuyvels et al., 2007; Ly et al.,2018), but their cleavage efficiency is typically rather low as long reaction times are required (Loosen et al., 2020) (> 36-48 h).

Figure 22 explains the concept of our approach:

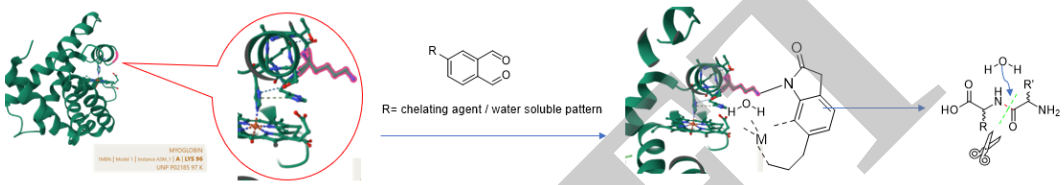


Figure 22: Diagram explaining the principle of hydrolytic cleavage induced by the OPA system-chelating agents

Ortho-phthalaldehyde (OPA) allows the formation of a covalent bond with proteins through lysine residues present on the surface and easily accessible (Figure 22). These OPAs were chosen for their high and fast reactivity towards lysine residues. Indeed, OPAs carry two aldehyde groups capable of reacting with the amino -NH_2 groups of lysines to form indoles.

We link these OPAs to chelating agents capable of coordinating specific metals that can catalyze amide bond hydrolysis, thus allowing selective cleavage of peptide bonds neighboring covalent reaction sites. This will therefore promote cleavage in proximity to lysine residues that react with such OPA-analogs.

An explanatory scheme representing our method and comparing it with the methods known so far in the literature is given in Figure 23.

An explanatory scheme representing our method and comparing it with the methods known so far in the literature is given in Figure 23.

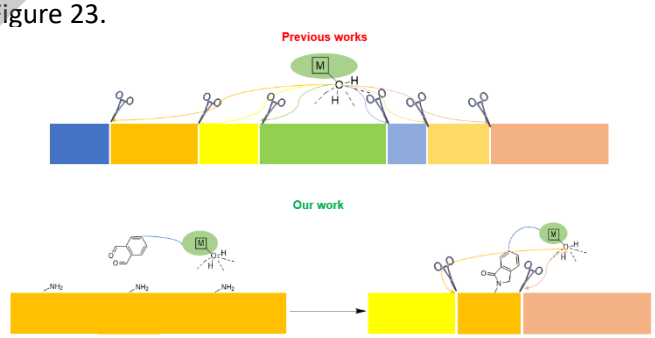


Figure 23: Scheme comparing the principle of our work with those already known in literature

This approach allows a selective reaction with the lysine residues on the surface of proteins. Depending on the OPA-analog used, we aim to covalently modify selected lysins in a given protein,

thus allowing for more targeted protein cleavage reactions compared to previously reported approaches, which rely on diffusion control and non-covalent affinity of the metal complexes to the protein. Consequently, we expect faster and more selective protein hydrolysis (Figure 23).

Test Reactions

In order to study the reactivity of these molecules with amino groups and have a broader overview, we set up different model-reactions (Figure 24).

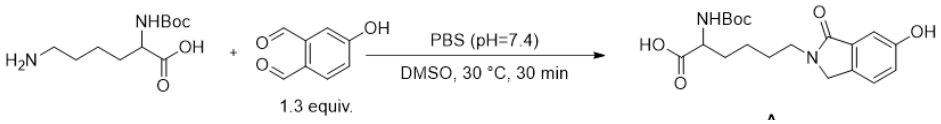


Figure 24: Reaction test of OPA-phenol with Boc-Lys-OH

Initially, our OPA-phenol reacted with Boc-protected lysin. The reaction gave a complete conversion to A, 2-((tert-butoxycarbonyl)amino)-6-(6-hydroxy-1-oxoisoindolin-2-yl)hexanoic acid, under optimized conditions: 1.3 equivalents of OPA-phenol after 30 minutes at 30 °C.

These optimized conditions were then applied to different model peptides at different concentrations.

Some of the results obtained are given in the figure below:

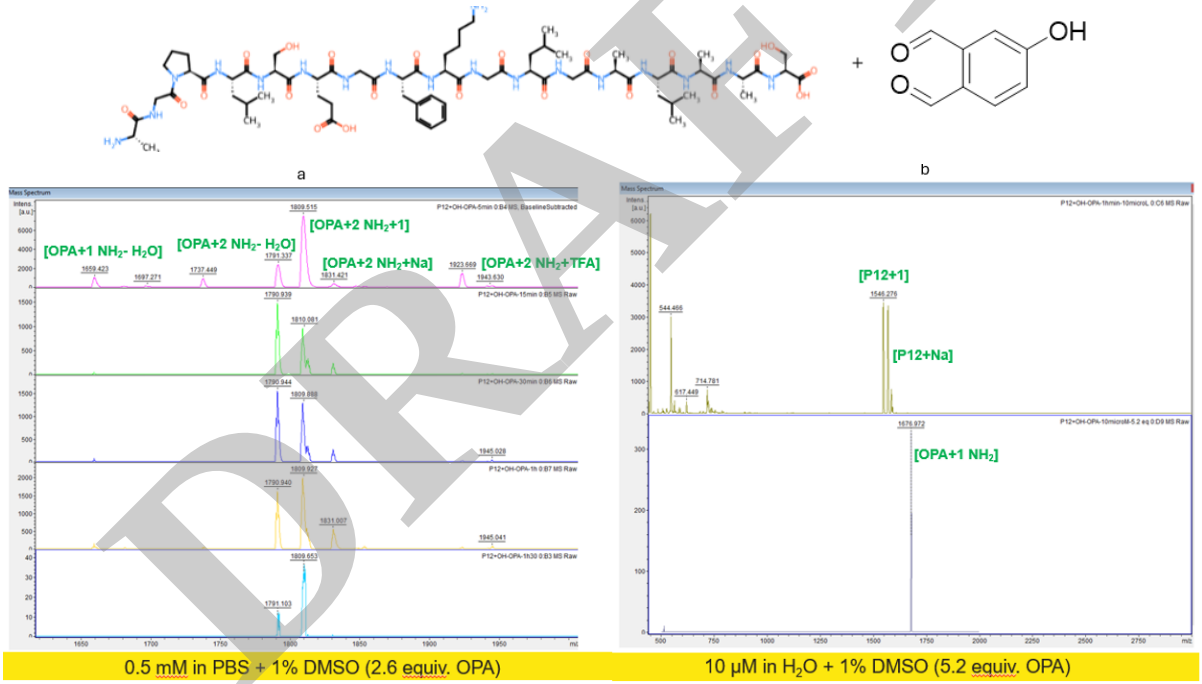


Figure 25: OPA-phenol test reaction with a P12 model peptide bearing two amino groups lysine and an NH_2-termius in PBS (a) and H_2O (b)

The reaction was carried out at two different concentrations: 0.5 mM (a) and 10 µM (b) in two different solvents PBS (a) and H_2O (b). The two MALDI-TOF spectra in Figure 25, show that the total conversion of our peptide takes place after only 5 minutes in both cases. For the concentration of 10 µM we managed to obtain a total conversion only by doubling the equivalent number of our OPA-phenol (5.2 instead of 2.6 equiv.). However, an interesting result was observed; under these conditions we have better selectivity. Indeed, according to the spectrum (b), only one lysine residue reacted with OPA-phenol, whereas in the spectrum (a), we observe a mixture of products derived from reaction of a single NH_2 and both.

So far, two previously unknow OPAs have been synthesized by our group (Figure 26); in order to study the effect of OPA's carrying negative or positive charges on the selectivity for lysine residues present in model peptides and proteins: Myoglobin and BSA.

Firstly, we are interested in the study of selectivity according to the load and charge of our OPAs, without incorporation of chelating agents. For this reason, OPA-1, which carries a negative charge and OPA-2 which carries a positive charge under physiological conditions, were studied for the reaction with the model peptides and proteins.

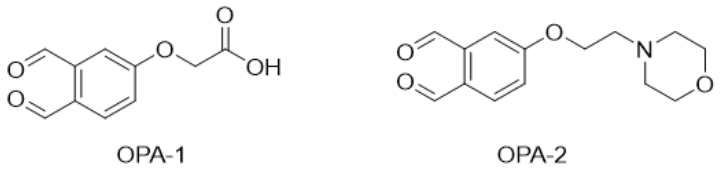


Figure 26: Two synthesized OPAs for reaction test with lysine residues in model peptides and proteins

Taking the optimized conditions used for reactions with OPA-phenol, the same reactions were applied to OPA-1 and OPA-2.

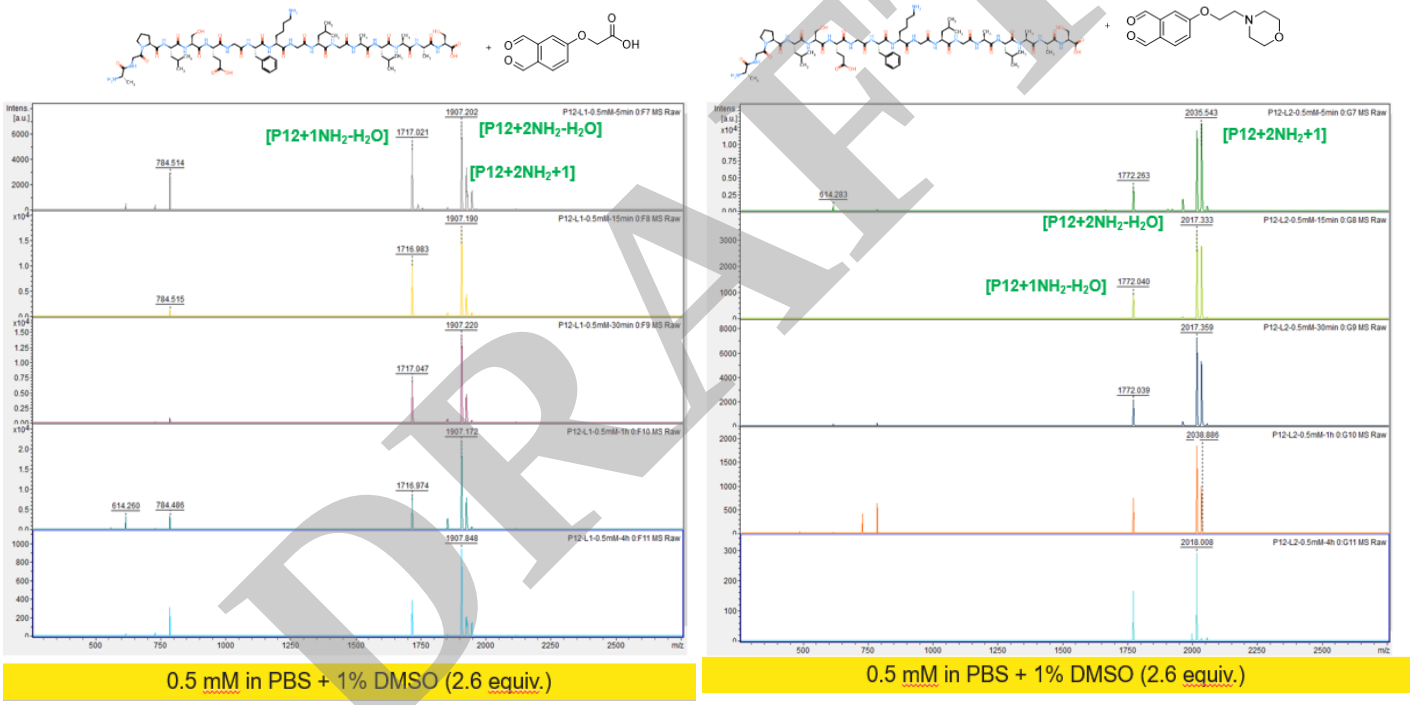


Figure 27: MALDI-TOF spectra reaction P12 with OPA-1 (left) and OPA-2 (right)

As shown in Figure 27, the same conditions described previously were applied to OPA-1 and OPA-2 and the reactivity of these compounds was confirmed by the final conversion of the starting peptide after only 5 minutes.

This shows and confirms the high reactivity of these compounds towards lysine residues at high and low concentrations, which are compatible with conditions applied to proteins.

For the lower concentrations (10 µM), it was necessary to use 10.0 equivalents of OPA, and the reaction was slower in H_2O compared to PBS. This result was observed for the two OPAs tested.

To confirm the results obtained for the first model peptide, another peptide was tested only with OPA-2:

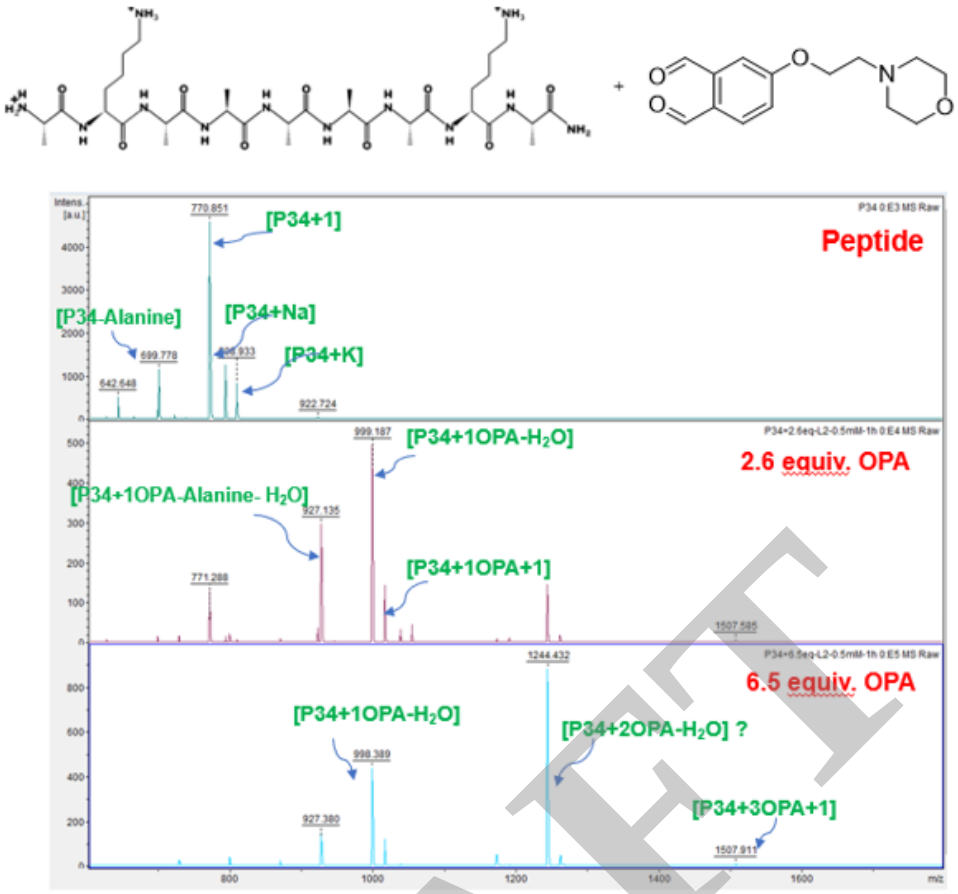


Figure 28: MALDI-TOF reaction spectra P34 with OPA-2

The MALDI-TOF spectrum of Figure 28 shows a complete conversion of P34 after only 5 minutes, in line with the results described previously.

The next step will then be the synthesis of OPAs incorporating 4 different chelating agents in order to study peptide cleavage in our model systems (peptides, BSA and Myoglobin). After optimization and finalization the approach will be used on CF keratin.

6 Conclusion and next steps

In conclusion, through microbial routes, we were able to obtain protein hydrolysates both from feather and fish byproducts, rich in enzymes and bioactive compounds. Hydrolysis of CF, FB and FS was performed by testing bacteria isolated from unconventional environments (i.e., desert and sea) since there have been very few studies on their use for the purposes of ONE EARTH project compared to most available studies that used bacteria deriving from conventional environment such as soil. Specifically, antimicrobial activity was detected against different *Staphylococcus* species and *E. coli*, antioxidant activity, and keratinase activity. It was also possible to separate the antioxidant compounds from the enzymes by ultrafiltration, a cheap and easy method of recovery; therefore, the different compounds can be exploited differently. The next steps will be to further characterize the bioactivities of FSH and to scale up the process in a 3-liter bioreactor using the different substrates (CF and FS) and the two most active isolates MT and DS.

With regards for enzymatic hydrolysis, two methods for keratin purification were developed, with efficiency around 60%. Moreover, multiple enzymes were validated to be used

For chemical treatment, a reliable, optimized, and reproducible method has been developed: alkaline hydrolysis.

Different tests were carried out first on duck feathers, the closest to chicken feathers in terms of composition. These were used for faster availability and especially for more comfortable working conditions.

Three different analytical methods were used to characterize the final composition of our hydrolysates: SDS-PEG, MALDI-TOF and LC-MS.

The SDS-PEG was found to be unsuitable because short fragments were not detectable.

MALDI-TOF was the main analysis technique and LC-MS is under study and exploitation for the quantitative detection of possible free amino acids.

A bioinformatics tool is also under development for the determination of the sequences of the different peptides obtained in the final hydrolysates. It was designed to provide as much reliable and complete information as possible. This is a big step in this field, because there are no similar tools known for processing data from chemical peptide cleavage, which is already the case for enzymatic peptide cleavage, for example. Further studies and optimization process are ongoing.

Regarding the products requested by our partners, the quantity of 120 g for cosmetic application by ANAVERIS has been prepared and will be delivered.

This approach was favored compared to the one proposed initially, considering all the difficulties described in the literature and the lack of reproducibility at a multi-gram scale, which is our case of interest.

The results obtained show that it is a more advantageous method, effective, inexpensive, and easily reproducible, even on a large scale.

Finally, for a more sophisticated study, a novel approach is being studied in order to obtain a more controlled and selective hydrolytic peptide cleavage, and further studies are ongoing.

7 References

Aktayeva, S., Baltin, K., Kiribayeva, A., Akishev, Z., Silayev, D., Ramankulov, Y., & Khassenov, B. (2022). Isolation of Bacillus sp. A5.3 Strain with Keratinolytic Activity. Biology, 11(2). https://doi.org/10.3390/biology11020244

Álvaro Torices-Hernández; Gallego, M.; Mora, L.; Toldrá, F. Microwave Irradiation Pre-Treatment as a Sustainable Method to Obtain Bioactive Hydrolysates from Chicken Feathers. *International Journal of Molecular Sciences* **2025**, *26* (13), 6344–6344. https://doi.org/10.3390/ijms26136344.

Athwal, S., Sharma, S., Gupta, S., Nadda, A. K., Gupta, A., & Husain, M. S. B. (2023). Sustainable Biodegradation and Extraction of Keratin with Its Applications. In S. Thomas, A. Ar, C. Jose Chirayil, & B. Thomas (Eds.), Handbook of Biopolymers (pp. 713-747). Springer Nature Singapore. https://doi.org/10.1007/978-981-19-0710-4_27

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. In Journal of Pharmaceutical Analysis (Vol. 6, Issue 2, pp. 71–79). Xi'an Jiaotong University. https://doi.org/10.1016/j.jpha.2015.11.005

Burns, J. A., Butler, J. C., Moran, J., & Whitesides, G. M. (1991). Selective reduction of disulfides by tris(2-carboxyethyl)phosphine. The Journal of Organic Chemistry, 56(8), 2648-2650. https://doi.org/10.1021/jo00008a014

Cai, C. G., Chen, J. S., Qi, J. J., Yin, Y., & Zheng, X. D. (2008). Purification and characterization of keratinase from a new Bacillus subtilis strain. Journal of Zhejiang University: Science B, 9(9), 713–720. https://doi.org/10.1631/jzus.B0820128

Das, S., Das, A., Das, N., Nath, T., Langthasa, M., Pandey, P., Kumar, V., Choure, K., Kumar, S., & Pandey, P. (2024). Harnessing the potential of microbial keratinases for bioconversion of keratin waste. Environmental Science and Pollution Research, 31(46), 57478–57507. https://doi.org/10.1007/s11356-024-34233-6

de Menezes, C. L. A., Boscolo, M., da Silva, R., Gomes, E., & da Silva, R. R. (2023). The degradation of chicken feathers by Ochrobactrum intermedium results in antioxidant and metal chelating hydrolysates and proteolytic enzymes for staphylococcal biofilm dispersion. 3 Biotech, 13(6). https://doi.org/10.1007/s13205-023-03619-7

Els Cartuyvels; Absillis, G.; Parac-Vogt, T. N. Questioning the Paradigm of Metal Complex Promoted Phosphodiester Hydrolysis: $[Mo_7O_{24}]^{6-}$Polyoxometalate Cluster as an Unlikely Catalyst for the Hydrolysis of a DNA Model Substrate. *Chemical Communications* **2007**, No. 1, 85–87. https://doi.org/10.1039/b714860g.

Fitriyanto, N. A.; Y Ramadhanti; None Rismiyati; I Rusyadi; A Pertiwiningrum; Prasetyo, R. A.; Y Erwanto. Production of Poultry Feather Hydrolysate Using HCl and NaOH as a Growth Medium Substrate for Indigenous Strains. *IOP conference series. Earth and environmental science* **2022**, *951* (1), 012064–012064. https://doi.org/10.1088/1755-1315/951/1/012064.

Gao, R., Yu, Q., Shen, Y., Chu, Q., Chen, G., Fen, S., Yang, M., Yuan, L., McClements, D. J., & Sun, Q. (2021). Production, bioactive properties, and potential applications of fish protein hydrolysates: Developments and challenges. In Trends in Food Science and Technology (Vol. 110, pp. 687–699). Elsevier Ltd. https://doi.org/10.1016/j.tifs.2021.02.031

Geiger, T.; Clarke, S. Deamidation, Isomerization, and Racemization at Asparaginyl and Aspartyl Residues in Peptides. Succinimide-Linked Reactions That Contribute to Protein Degradation. *The Journal of biological chemistry* **1987**, *262* (2), 785–794. https://doi.org/10.1016/s0021-9258(19)75855-4.

Gill, J. M., Hussain, S. M., Ali, S., Ghafoor, A., Adrees, M., Nazish, N., Naeem, A., Naeem, E., Alshehri, M. A., & Rashid, E. (2025). Fish waste biorefinery: A novel approach to promote industrial sustainability. In Bioresource Technology (Vol. 419). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2025.132050

Gupta, A.; Kumar, P.; Yunus, R.; N. Kamarudin. *Extraction of Keratin Protein from Chicken Feather,* **2020**. https://www.semanticscholar.org/paper/Extraction-of-Keratin-Protein-from-Chicken-Feather-Gupta-Kumar.

Gupta, R., Rajput, R., Sharma, R., & Gupta, N. (2013). Biotechnological applications and prospective market of microbial keratinases. In Applied Microbiology and Biotechnology (Vol. 97, Issue 23, pp. 9931–9940). https://doi.org/10.1007/s00253-013-5292-0

Hassan, M. A., Abol-Fotouh, D., Omer, A. M., Tamer, T. M., & Abbas, E. (2020). Comprehensive insights into microbial keratinases and their implication in various biotechnological and industrial sectors: A review. In International Journal of Biological Macromolecules (Vol. 154, pp. 567–583). Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2020.03.116

Kaiser, K.; Benner, R. Hydrolysis-Induced Racemization of Amino Acids. *Limnology and Oceanography: Methods* **2005**, *3* (8), 318–325. https://doi.org/10.4319/lom.2005.3.318.

Li, Q. (2019). Progress in Microbial Degradation of Feather Waste. In Frontiers in Microbiology (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2019.02717

Liu, X., Hu, Q., Shen, Y., Wu, Y., Gao, L., Xu, X., & Hao, G. (2024). Research Progress on Antioxidant Peptides from Fish By-Products: Purification, Identification, and Structure–Activity Relationship. In Metabolites (Vol. 14, Issue 10). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/metabo14100561

Loosen, A.; Francisco de Azambuja; Smolders, S.; Moons, J.; Simms, C.; Dirk De Vos; Parac-Vogt, T. N. Interplay between Structural Parameters and Reactivity of Zr_6-Based MOFs as Artificial Proteases. *Chemical science* **2020**, *11* (26), 6662–6669. https://doi.org/10.1039/d0sc02136a.

Ly, H. G. T.; Fu, G.; Kondinski, A.; Bueken, B.; De Vos, D.; Parac-Vogt, T. N. Superactivity of MOF-808 toward Peptide Bond Hydrolysis. *Journal of the American Chemical Society* **2018**, *140* (20), 6325–6335. https://doi.org/10.1021/jacs.8b01902.

Mazotto, A. M., Cedrola, S. M. L., de Souza, E. P., Couri, S., & Vermelho, A. B. (2022). Enhanced keratinase production by Bacillus subtilis amr using experimental optimization tools to obtain feather protein lysate for industrial applications. 3 Biotech, 12(4). https://doi.org/10.1007/s13205-022-03153-y

McKerchar, H.; Dyer, J. M.; Gerrard, J. A.; Maes, E.; Clerens, S.; Dobson, R. C. J. Characterizing Lysinoalanine Crosslinks in Food Systems: Discovery of a Diagnostic Ion in Model Peptides Using MALDI Mass Spectrometry. *Food Chemistry X* **2023**, *19*, 100800–100800. https://doi.org/10.1016/j.fochx.2023.100800.

Mengistu, A.; Getaneh Andualem; Mikiyas Abewaa; Birhane, D. Keratin Extraction Optimization from Poultry Feather Using Response Surface- Box-Behnken Experimental Design Method. *Results in Engineering* **2024**, *22*, 102360–102360. https://doi.org/10.1016/j.rineng.2024.102360.

Mthembu, S. N., Sharma, A., Albericio, F., & de la Torre, B. G. (2020). Breaking a Couple: Disulfide Reducing Agents. ChemBioChem, 21(14), 1947-1954. https://doi.org/https://doi.org/10.1002/cbic.202000092

Mukesh, K., Kannan, P., & Saleena, L. M. (2024). Keratin hydrolysates: a sustainable product in biotechnology sectors by microbial conversion. In Biologia. Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s11756-024-01725-2

Pintea, A., Manea, A., Pintea, C., Vlad, R.-A., Bîrsan, M., Antonoaea, P.,…Ciurba, A. (2025). Peptides: Emerging Candidates for the Prevention and Treatment of Skin Senescence: A Review. Biomolecules, 15(1).

Qiu, J., Wilkens, C., Barrett, K., & Meyer, A. S. (2020). Microbial enzymes catalyzing keratin degradation: Classification, structure, function. In Biotechnology Advances (Vol. 44). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2020.107607

Ramya, K. R., Thangam, R., & Madhan, B. (2020). Comparative analysis of the chemical treatments used in keratin extraction from red sheep's hair and the cell viability evaluations of this keratin for tissue engineering applications. Process Biochemistry, 90, 223-232. https://doi.org/https://doi.org/10.1016/j.procbio.2019.11.015

Riaz, A., Muzzamal, H., Maqsood, B., Naz, S., Latif, F., & Saleem, M. (2024). Characterization of a Bacterial Keratinolytic Protease for Effective Degradation of Chicken Feather Waste into Feather Protein Hydrolysates. Frontiers in Bioscience-Elite, 16(3), 22. https://doi.org/10.31083/j.fbe1603022

Rombouts, I.; Lambrecht, M. A.; Carpentier, S. C.; Delcour, J. A. Identification of Lanthionine and Lysinoalanine in Heat-Treated Wheat Gliadin and Bovine Serum Albumin Using Tandem Mass Spectrometry with Higher-Energy Collisional Dissociation. *Amino Acids* **2015**, *48* (4), 959–971. https://doi.org/10.1007/s00726-015-2139-2.

Rustad, T., Storrø, I., & Slizyte, R. (2011). Possibilities for the utilisation of marine by-products. In International Journal of Food Science and Technology (Vol. 46, Issue 10, pp. 2001–2014). https://doi.org/10.1111/j.1365-2621.2011.02736.x

Saleh, N. E., Wassef, E. A., & Abdel-Mohsen, H. H. (2021). Sustainable Fish and Seafood Production and Processing. In Sustainable Fish Production and Processing (pp. 259–291). Elsevier. https://doi.org/10.1016/B978-0-12-824296-4.00002-5

Saravanan, A., Yuvaraj, D., Senthil Kumar, P., Karishma, S., & Rangasamy, G. (2023). Fish processing discards: A plausible resource for valorization to renewable fuels production, optimization, byproducts and challenges. Fuel, 335. https://doi.org/10.1016/j.fuel.2022.127081

Sharma, C., Timorshina, S., Osmolovskiy, A., Misri, J., & Singh, R. (2022). Chicken Feather Waste Valorization Into Nutritive Protein Hydrolysate: Role of Novel Thermostable Keratinase From Bacillus pacificus RSA27. Frontiers in Microbiology, 13. https://doi.org/10.3389/fmicb.2022.882902

Sharma, R., & Devi, S. (2018). Versatility and commercial status of microbial keratinases: a review. In Reviews in Environmental Science and Biotechnology (Vol. 17, Issue 1, pp. 19–45). Springer Netherlands. https://doi.org/10.1007/s11157-017-9454-x

Shavandi, A.; Silva, T. H.; Bekhit, A. A.; Bekhit, A. E.-D. A. Keratin: Dissolution, Extraction and Biomedical Application. *Biomaterials kmkScience* **2017**, *5* (9), 1699–1735. https://doi.org/10.1039/c7bm00411g.

Suresh, S., Umesh, M., Nag, A., James, N., Santhosh, A. S., & Stanly, L. M. (2025). Bioprospecting of fish scale waste as a cost-effective substrate for crude protease production using *Stenotrophomonas koreensis* SH32 and its multifunctional applications: A sustainable strategy for circular bioeconomy. Biomass and Bioenergy, 197. https://doi.org/10.1016/j.biombioe.2025.107811

Tursunova, D.; Maksumova, O. Analysis of the Amino Acid and Protein Composition of Feather Keratin Hydrolyzed Using Alkali and Sodium Hydrosulfite. *Results in Chemistry* **2024**, *9*, 101621. https://doi.org/10.1016/j.rechem.2024.101621.

Xiao, F., Xu, T., Lu, B., & Liu, R. (2020). Guidelines for antioxidant assays for food components. In Food Frontiers (Vol. 1, Issue 1, pp. 60–69). John Wiley and Sons Inc. https://doi.org/10.1002/fft2.10

Veiga, E., Ferreira, L., Correia, M., Pires, P. C., Hameed, H., Araújo, A. R. T. S.,…Paiva-Santos, A. C. (2023). Anti-aging peptides for advanced skincare: Focus on nanodelivery systems. Journal of Drug Delivery Science and Technology, 89, 105087. https://doi.org/https://doi.org/10.1016/j.jddst.2023.105087

Zhang, N., Wu, Z., Jiang, Z., Zhou, M., Yu, Y., Wang, P., & Wang, Q. (2022). pH Mediated L-cysteine Aqueous Solution for Wool Reduction and Urea-Free Keratin Extraction. Journal of Polymers and the Environment, 30(7), 2714-2726. https://doi.org/10.1007/s10924-022-02383-8