Recovery and Characterization of Protein Hydrolysate from Chrome Shavings by Microbial Degradation

by

C. Shanthi,[1*] Pradipta Banerjee,[1] N. K. Chandra Babu[2] and G. Suseela Rajakumar[3]

[1]*School of Bio Science and Technology, VIT University*
Vellore 632014, Tamil Nadu, India.

[2]*Tannery Division, CSIR-Central Leather Research Institute*
Adyar, Chennai-600 020, India

[3]*Emeritus Scientist, CSIR-Central Leather Research Institute*
Adyar, Chennai-600 020, India

Abstract

Chromium containing collagenous solid wastes from the leather tanning industry requires special attention because of the pressure exerted by environmental authorities for safe disposal. Such collagen-rich wastes can be recycled by adapting environmentally suitable and safer methods based on microorganism and/or enzymatic treatment, so as to obtain a collagen hydrolysate with potential applications. In the present study, chromium tolerant bacteria were isolated from tannery soil and screened for high proteolytic activity by zone-clearance assay and caseinolytic activity. The most potent bacterium, with a high proteolytic and chromium-tolerant ability was found to degrade about 90% of the chrome shavings in 120h and was identified as *Alcaligenes faecalis*. The hydrolysates at various time intervals of proteolysis were collected, chromium removed and characterized. The hydrolysate was found to have 12% ash and 80% protein or peptides contents after 120h of proteolysis with 3.14±2.0μg of chromium/g of protein. Molecular weight profiling done by gel filtration chromatography using sephadex G 25 and tricine-SDS-PAGE electrophoresis revealed that the major component of the hydrolysate comprised of small peptides in the molecular weight range of 3-30kDa.

Resumen

El cromo que contiene residuos sólidos de colágeno de la industria del curtido de pieles requiere una atención especial debido a la presión ejercida por las autoridades ambientales para su eliminación segura. Tales residuos ricos en colágeno se puede reciclar mediante la adaptación de métodos ambientalmente adecuados y más seguros basadas en microorganismos y/o tratamientos enzimáticos para obtener un hidrolizado de colágeno con aplicaciones potenciales. En el presente estudio, las bacterias tolerantes al cromo fueron aisladas del suelo de la curtiembre y seleccionados por alta actividad proteolítica mediante el ensayo de zona aislada y la actividad caseinolítica. La bacteria más potente, con una alta capacidad proteolítica y tolerancia al cromo degrada aproximadamente el 90% de las virutas de cromo en 120 horas y fue identificada como Alcaligenes faecalis. Los hidrolizados a intervalos de tiempo diferentes de la proteólisis fueron recogidos, el cromo eliminado y caracterizado. El hidrolizado fue encontrado conteniendo 12% de cenizas y un contenido de proteína o péptidos de 80% después de 120 horas de la proteólisis con 3.14±2.0 g de cromo/g de proteína. Perfiles de peso molecular realizados por cromatografía de filtración en gel utilizando Sephadex G 25 y la electroforesis SDS-PAGE en tricina reveló que el principal componente del hidrolizado está compuesto de pequeños péptidos en el intervalo de peso molecular de 3-30kDa

*Corresponding author e-mail: cshanthi@vit.ac.in, Fax: 91-416-2243092, Tel: 91-416-2202479
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INTRODUCTION

In India, there are more than 2000 tanneries and nearly 80% of them make use of chrome tanning as the main tanning system in leather production.[1] Processing leather from hides and skins leads to enormous amounts of chrome tanned wastes; one ton of wet hide yields only about 200kg of leather but close to 600kg of solid wastes as byproduct.[2] During leather production, chrome tanning is an essential process required for stabilization of the constituent skin matrix against microbial degradation. The resultant tanned leather is shaved to adjust the thickness of the finished product and the shavings obtained during this process are generally dumped indiscriminately.[3] These chrome shavings contain 75-85% proteins (on dry weight) along with chromium oxide, inorganic salts, fats and oils. A fraction of the shavings is utilized in the manufacture of leather boards but the major quantity remains unused and could pose a potential threat to the environment.[4] Recovery of the protein from chrome tanned wastes by either chemical or microbial methods is sought as an ideal solution to obtain value added products for reuse.

Trivalent chromium is considered to be comparatively less toxic but hexavalent chromium (Cr^{6+}) is known to be a highly reactive carcinogen leading to chromium containing solid waste being categorized as hazardous.[5] Contemporary research is being focused on transformation of this hazardous waste into a resourceful material by eco-friendly means. Work has been carried out for effective removal of chromium and use of the recovered protein as feeds, fertilizers, biodegradable plastics, syntans in tanneries and microencapsulating material in many industries and for immobilizing enzymes.[6-10] The main protein present in leather is collagen, a major extracellular component of skin. A vital step in leather production is chrome tanning, or the formation of stable chromium - collagen cross-links, which impart protection to the leather against bacterial attack.[11] The chrome tanning reaction is favorable under mild conditions whereas the reverse process of removal of chromium requires use of strong and harsh chemicals, taking a heavy toll on environment. A comparatively better and eco-friendlier solution to this problem includes hydrolyzing the chrome shavings enzymatically to recover protein as hydrolysate and chromium as a cake-like deposit.[6,12-14] A wide spectra of enzymes including pepsin, trypsin, papain and chymotrypsin have been used to treat chrome shavings and by careful control of the enzyme concentration, gelatin with varying blooms has been isolated. The applicability of the obtained hydrolysate would largely depend on its molecular weight distribution which ultimately depends on the efficiency of the protease used. Most of the commercially available tryptic enzyme products have yet to be proven as cost effective.[14]

As the tanning is performed to impart stability to leather against bacterial attack, a bacterial source that can degrade such stabilized leather wastes becomes limited. Several authors have used chromium tolerant bacteria from tannery effluent for biodegradation with satisfactory results.[15-17] The present work deals with isolating a Cr^{3+} tolerant organism from tannery soil with high proteolytic activity, studying the usefulness of this organism on the hydrolysis of chrome shavings and characterization of the recovered protein hydrolysate to find its suitability for various end applications.

MATERIALS AND METHODS

Chrome shavings were obtained from the pilot tannery, Central Leather Research Institute, Chennai, India. Wet chrome shavings were air dried and stored at room temperature until use. The % contents of chromium and protein in chrome shavings were analyzed by standard procedures[18] and were found to be 4.46±0.06 and 86.49±1.02% (on dry weight basis) respectively. Peptone and nutrient broth were procured from Hi Media Ltd, India. Other chemicals used were of analytical grade.

Isolation of Organism

Soil samples around tanneries were collected. 1g of soil was suspended in 10ml of sterile saline water and serially diluted followed by plating on skimmed milk medium and incubated at 37°C overnight. The colonies showing largest zone of clearance were sub-cultured and maintained in glycerol stock at -20°C. The sub-cultured microorganisms were screened for protease production, as described in the following section.

Protease Activity

For screening, the selected microorganisms were sub-cultured in peptone medium and protease activity was measured after 24, 48, 72, 96 and 120h by the caseinolytic method.[19] The culture broths, after the required time periods, were centrifuged at 7000rpm for 15min and the supernatant used as enzyme source. Briefly, 0.5ml of this cell-free supernatant was mixed with 2ml of 2% casein dissolved in 0.15M phosphate buffer and the resultant solution incubated at 37°C for 30min. The reaction was arrested by addition of 2.5ml of 1.2M trichloroacetic acid (TCA). A control sample was prepared by adding TCA prior to enzyme addition and a blank was prepared with only buffer. The test, control and blank solutions were subsequently filtered through a Whatman No. I filter paper. 2.5ml of the filtrate was mixed with 2.5ml of 0.5M Na_2CO_3 and 0.5ml of 2:1 diluted Folin-Ciocalteu reagent was added and mixed thoroughly. Absorbance was measured at 660nm with a UV-Visible spectrophotometer (Shimadzu, Japan) and the activity was expressed as the amount of tyrosine liberated per ml of culture filtrate.

Chromium Tolerance

The bacterium displaying the highest proteolytic activity was tested for chromium tolerance. Cr^{3+} salts have a tendency to

precipitate at pH above 5 [20] and to avoid such precipitation, the stock Cr solution (2g L^{-1}) was masked with citrate in 1:1 mole ratio.[21] The resultant solution was filtered through a 0.2 micron membrane and stored as 'stock'. The Cr stock was added to several 1% peptone-containing flasks to produce an array of final working concentrations; 100, 250, 500, 750 and 1000mg L^{-1}. The microorganism was pre-cultured in peptone broth for 16h and 20ml of this bacterial pre-culture containing 1x10^8 CFU was added to each flask. The flasks were incubated at 30°C in a shaker at 100rpm (Technico, India). After 24 and 48h, the culture supernatant was collected by filtration and protease activity measured according to the protocol given in section 2.2.

Degree of Hydrolysis (%) and Cr^{3+}Removal from the Chrome Shavings

The process of hydrolysis along with the microorganism used has been patented.[22] 100g of chrome shavings, 4g of calcium oxide along with 10g of peptone and 1L of water were added to 15 nos. of 3L Haffkines flasks. The pH of the chrome shaving medium was checked (7.2±0.25) and the flasks were autoclaved at 15psi and 120°C for 20min. As done before, a bacterial pre-culture containing 1x10^8 CFU was added to each of the flasks. The flasks were then incubated at 30°C in a shaker at 100rpm; at intervals of 24, 48, 72, 96 and 120h, the flasks (in triplicates) were taken off the shaker to collect the hydrolysate formed. The cells were separated by centrifugation at 7000rpm for 15min and the supernatant obtained was filtered with a sieve of 0.5mm diameter. The residue was dried and weighed. The percentage of hydrolysis was calculated on the basis of dry residue left on a moisture free basis. A similar process was followed for chromium removal. To the protein hydrolysate solutions collected in the above mentioned manner, 4% (w/v) of magnesium oxide was added and the solutions boiled at 100°C for 15min to precipitate the chromium. The resultant hydrolysate solutions were filtered through cheese cloth, labeled and stored in the refrigerator until use.

Analysis of the Protein Hydrolysate

Protein, total solid, ash and chromium content in the protein hydrolysates from different time intervals were determined. Nitrogen content was determined by Kjeldahl method and the protein content was determined by multiplying the nitrogen content by a factor of 5.4.[23,24] Solid content was determined by drying known volume of aliquots at 108°C and was expressed as% (w/v) solid in 100ml hydrolysate. The total ash was measured by ashing known aliquot of hydrolysate at 550°C for 4h in a muffle furnace.[25] The hydrolysate was digested for 6h in a sealed glass container with concentrated HCl and subsequently used for residual chromium determination by atomic absorption spectroscopy using an Analyst 200 atomic absorption spectrometer (Perkin Elmer, Massachusetts, USA).

pH Determination by Deionization

Deionization was done by batch method[26] using Amberlite MB-150, a mixed bed ion exchange resin. 1g of resin was added to 10ml of the hydrolysates obtained at 24 and 72h separately and stirred for 1h at room temperature. This procedure was repeated until there was no change in resin color. The mixture was filtered under suction to remove the resin and the pH noted.

Gel Filtration Chromatography

The 24 and 72h samples were subjected to a pretreatment as required for efficient resolution of collagen peptides.[27] 2ml of the sample was aspirated under aseptic conditions and mixed with 1.5ml of binding buffer (acetate buffer, 0.05M, pH 4.5). The resultant solution was heated in a water bath at 55°C for 3min and centrifuged at 2000rpm for 5min to remove the insoluble debris. The resulting clear supernatant was used for gel-permeation chromatography. The treated sample was applied to a 1.4 x 25cm Sephadex G25 column equilibrated in acetate buffer (0.05M, pH 4.5) and run using an ÄKTAprime plus unit (GE Healthcare, UK). The column effluent flow rate was maintained at 0.8ml min^{-1}, absorbance was monitored at 214nm and fractions of 3ml were collected.

Ion Exchange Chromatography

For ion-exchange chromatography, 0.25ml of the desalted 72h hydrolysate was mixed with 0.25ml of 0.1M acetate buffer, pH 4.5 and applied to a 1.8 x 13cm column of CM sepharose pre-equilibrated with the same buffer.[27] A linear gradient of NaCl from 0 to 0.3M was applied over a volume of 50ml. The flow rate was maintained at 1.5ml min^{-1}, absorbance monitored at 214 nm and fractions of 2ml were collected. The fractions were pooled, desalted, lyophilized in a Micro Modulyo-230 freeze-drier (Thermo Scientific, USA) and re-applied to a Sephadex G25 bed to determine their molecular weights.

Tricine SDS-PAGE of the Hydrolysate

Tricine SDS-PAGE was done for protein hydrolysate obtained after 72h based on the method of Schägger.[28] The deionized protein hydrolysate obtained was lyophilized in a Micro Modyulo-230 freeze dryer (Thermo Fisher, USA). The powdered sample at a concentration of 2mg ml^{-1} in sample buffer was loaded onto a 16%/6M urea polyacrylamide gel. SDS-PAGE electrophoresis was carried out using a Tris/tricine buffer system with a run time of 3.2h. Ultra low range molecular weight markers were also run under similar conditions.

Results and Discussion

The chrome tanning process generates chromium-containing wastes both liquid and solid, the disposal of which is now stringently regulated. Non-biodegradability of heavy metals such as chromium is responsible for their persistence in the environment. Though even low levels of chromium are toxic to majority of organisms, some organisms evolve tolerance. Sampling environments that contain elevated concentrations

of chromium are potential sources of toxic metal tolerant bacteria. By using such bacteria, potential toxic wastes like chrome shavings, could be recycled for further use, leading to a lowering of the pollution load.

Protease Activity, Identification and Chromium Tolerance of the Screened Bacterium

Growth of isolates in various protein media was measured as increase in absorbance of the broth at 600nm. Growth in nutrient broth and peptone were comparable and the protease activity was higher in peptone medium in comparison to other protein media, probably because of the higher protein content. The organism with highest proteolytic activity was identified at Indian Institute of Microbial Technology, Chandigarh, India as *Alcaligenes faecalis*. The bacterium has been pre-cultured in peptone broth for the rest of the study.

The effect of Cr on proteolytic activity was measured by culturing the isolate in varying concentrations of Cr (100 to 1000mg L^{-1}) in peptone medium and checking for protease activity at two different time periods. Citrate, a tridendate ligand has been chosen as the masking agent in the present study to prevent precipitation of Cr. From the results in Fig. 1, it was concluded that protease activity measured using casein as substrate in the presence of 100mg L^{-1} Cr was comparable to the activity in the absence of Cr ($p>0.05$, by t-test) and the organism could tolerate up to 500mg L^{-1} of Cr. A significant difference in activity with respect to time of reaction could only be noticed for control and100mg L^{-1} Cr and an increase in Cr concentration affected both the enzyme activity at 24 and 48h equally ($p>0.05$, by t-test). A drastic decrease was observed in protease activity at chromium concentrations higher than 500mg L^{-1}, particularly after 48h of incubation. The protease activity of the culture supernatant during chrome shaving hydrolysis (Fig. 2) was found to increase steeply after 48h, which corroborates with the%hydrolysis measured on the basis of unhydrolyzed mass.

Chrome Shaving Hydrolysis Process

For microbial degradation to occur, initial pretreatment of chrome shavings is important. Chromium stabilizes collagen by complexation with side chain carboxyl groups of aspartic and glutamic acid.[29] The degradation of chrome shavings could be brought about by breaking bonds between chromium and protein or by lysis of peptide bonds. The collagen triple helical structure is tightly coiled; this makes enzymatic degradation difficult as the enzyme cannot penetrate deep into such a cross-linked superstructure.[30] To solve this problem, the chrome shavings were autoclaved. Autoclaving served the dual purpose of sterilization of the shavings in addition to loosening of the fiber matrix, enabling the polypeptide chains to be susceptible to bacterial attack. The degree of hydrolysis was calculated from the unhydrolzed mass left after hydrolysis and as expected, the degree of hydrolysis increased with time (correlation=0.9581) as displayed in Table 1. A sharp increase in hydrolysis was observed around 48h. Initial delay in degradation could be due to the initial induction period required by the bacterium for protease synthesis. The supernatant recorded a greenish colour around 48h due to leaching of substantial amount of Cr^{3+}. After 96h, the degree of hydrolysis was 83.63±1.44% and the supernatant displayed a brownish tinge. The Cr released after hydrolysis probably precipitates to some extent as the pH of the medium is around 7.2-7.5. This eliminates toxic levels of Cr build-up that may inhibit the growth and activity of the organism. After 120h, 89.48±1.02% of the chrome shavings were degraded.

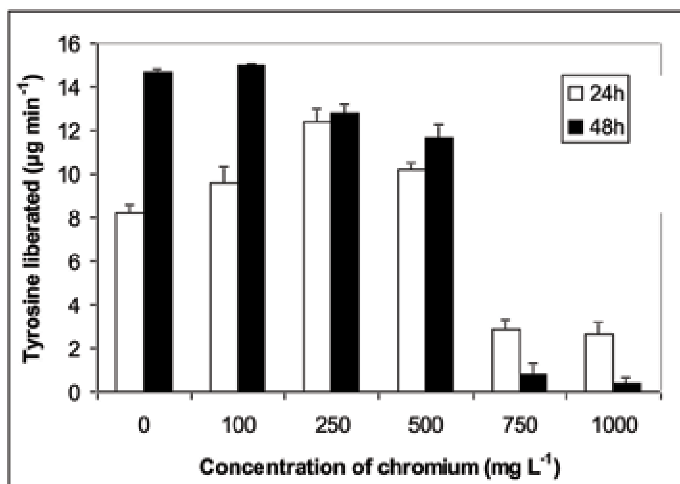

Figure 1. Effect of chromium concentration on protease activity. The Y-axis denotes protease activity in μg of tyrosine liberated min^{-1} units. The X-axis denotes increasing concentrations of chromium. Values are given as mean of triplicates± standard deviation.

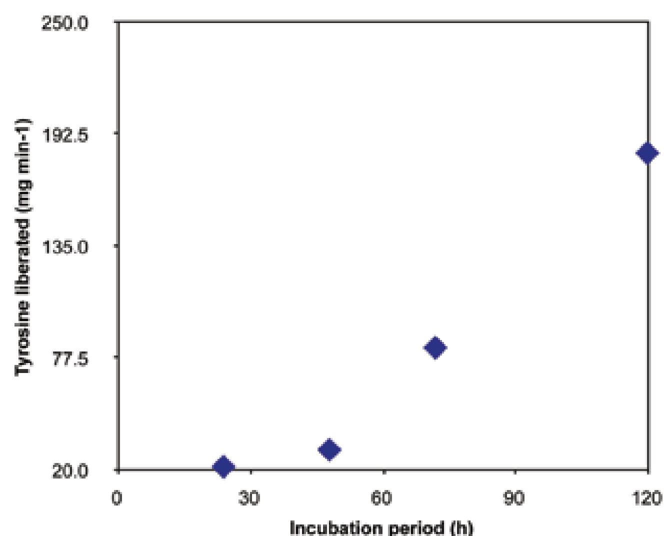

Figure 2. Effect of incubation period on the hydrolysis of chrome-shavings. The Y-axis denotes protease activity in μg of tyrosine liberated min^{-1} units and the X-axis denotes time in hours. The values given are in mean ± standard deviation.

After hydrolysis, the medium was made alkaline for chromium precipitation. The precipitants tried in earlier studies include sodium hydroxide, sodium bicarbonate, calcium oxide and magnesium oxide[31, 32] and the last two alkalis were observed to be good precipitating agents. MgO was eventually chosen as the most effective precipitant due to the shorter time period needed for precipitation in comparison to CaO. The precipitated Cr was removed by filtration and the protein hydrolysate was recovered as the filtrate. The recovered protein was characterized for different properties and the results are given in Table 1.

Characterization of Protein Hydrolysate

Proteins are biopolymers that have many end applications depending on the functional properties. Hence the recovered product was characterized, which would help in identifying possible uses. The amount of nitrogen present in the hydrolysate was measured by micro kjeldahl method and the nitrogen content followed a general trend of increasing with the time of hydrolysis (Table 1). A sudden increase in the nitrogen content after 48h hydrolysis was noted, probably indicating the onset of hydrolysis.

Kjeldahl method was also used to determine the protein content of the chromium cake recovered at various intervals, and it was found to be between 2 to 3% of the amount of protein obtained in the hydrolysate. This indicated that some amount of protein still remained bound to the chromium. The chromium content in the hydrolysate after precipitation by magnesium oxide was found to be in the range of 2.5 to 6.0μg per gram of protein using atomic absorption spectroscopy. So the hydrolysate could possibly be used in the feed and for other industrial uses. The pH of the deionized protein hydrolysate was found to be 4.4 in case of samples hydrolyzed for varying intervals of time. Earlier studies on gelatin recovered by alkaline hydrolysis from skins and bones have reported the hydrolysate pH to be 4 - 5.[33]

Determination of Molecular Weight of Protein Hydrolysate by Gel Filtration and Gel Electrophoresis

To study the quality of the protein that was recovered during hydrolysis, the 24h and 72h hydrolysate were subjected to gel filtration using sephadex G 25. The column was previously calibrated by running standard chromatography markers in 50mM phosphate buffer containing 25mM NaCl. Acetate buffer of pH 4.5 was used as the eluant as the hydrolysate was comprised mainly of collagen, which is known to be soluble in acetic acid. As displayed in Fig. 3a, two small peaks could be observed in void volume, probably arising due to a small amount of unhydrolyzed, intact collagen. A major peak was observed at 85kDa (fraction number 6 and 7), which probably corresponds to initial hydrolyzed products of collagen. The 72h hydrolysate shows the peak at 85kDa to be reduced as a shoulder (fraction number 6 and 7) to a major peak spread in the region of 50-10kDa (fraction number 12-14) as displayed in Fig. 3b. The chromatograms confirm the fact that the degradation of collagen resulted in low molecular weight peptides but they were not separable from each other, probably because of their recoiling tendency. This interaction among gelatin network is characteristic of the well-conserved glycine-proline-hydroxyproline sequence prevalent throughout the polymer. The pyrrolidine rich sequences serve as inter-chain hydrogen bond formation sites and thereby partially reform the collagen triple helix.[34] Another and even more likely possibility is that non-specific cleavage resulted in non-identical but similarly sized peptides. But overall, the gel permeation studies were indicative of the fact that the 72h sample constituted numerous small peptides and was further substantiated by ion-exchange chromatography and gel electrophoresis.

TABLE I
Characterization of the recovered protein hydrolysate.

Hydrolysis time (h)	24	48	72	96	120
Hydrolysis(%)	22.94±8.13	53.50±9.32	72.48±6.72	83.63±1.44	89.48±1.02
Total solids (%)	3.71±0.58	4.388±0.41	4.860±0.40	4.53±0.37	6.20±0.86
Total ash (%)	9.17 ± 0.40	8.73 ± 0.25	10.08± 1.77	14.37± 0.60	12.41± 1.92
Nitrogen Content (%)	3.07±0.11	8.60±0.28	11.43±1.62	13.90±0.90	17.45±1.88
Chromium Content (as Cr (μg/g of protein)	3.9±0.92	2.5±1.46	6.0±2.7	3.74±2.7	3.14±2.0
Protein from N Content (%)	18.64±1.10	46.55±1.49	61.72±8.74	75.04±4.87	82.43±8.88

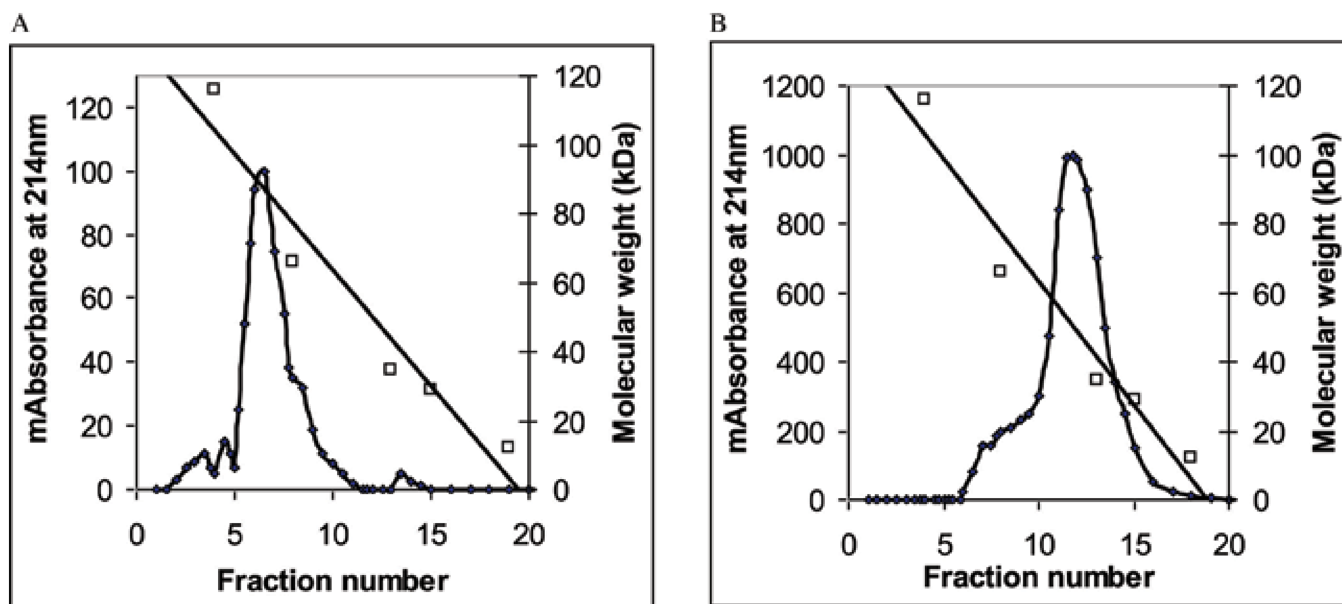

Figure 3. a. Gel filtration chromatogram of the 24h hydrolysate. The elution patterns displays two small peaks in void volume, probably due to intact collagen and a major peak at 85kDa possibly for partially hydrolyzed collagen.

b. Gel filtration chromatogram of the 72h hydrolysate. The elution pattern displays a small shoulder peak near 85kDa and a major peak at 55-10kDa. The Y-axis represents milli-absorbance and the X-axis, the fraction number. The molecular weights are represented in the secondary Y axis. Markers used were β−galactosidase (116kDa), BSA (66kDa), pepsin (35kda) carbonic anhydrase(29kDa) and cytochrome c(12.4kDa).

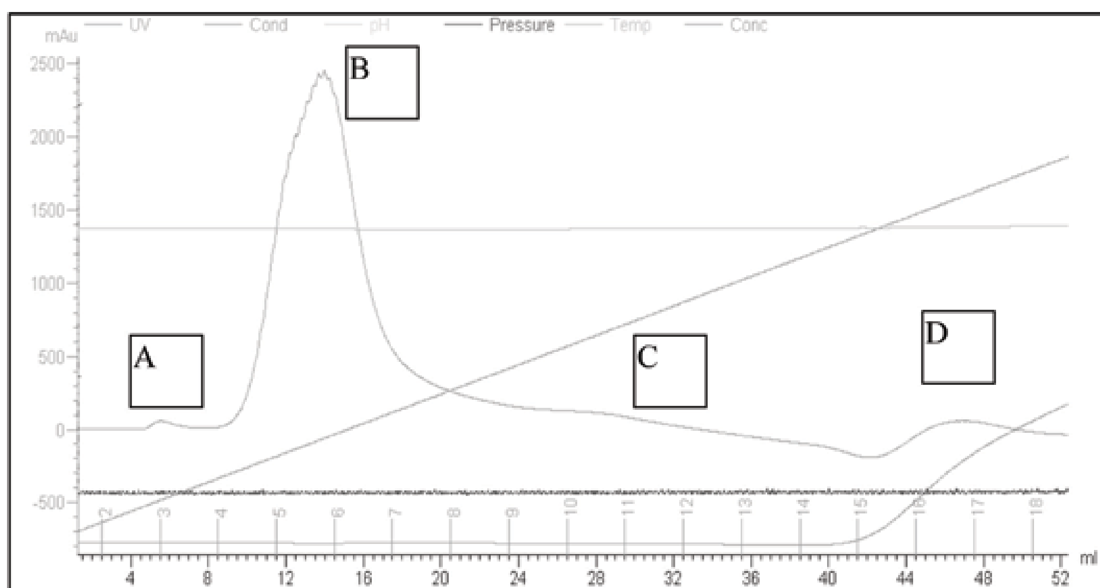

Figure 4. Ion-exchange chromatogram of the 72h protein hydrolysate. Four peaks were observed and labeled as A, B, C and D. The diagonal line represents the increasing NaCl concentration and the horizontal line indicates temperature (the last horizontal line from x-axis). The four peaks obtained were collected and run in sephadex G25 to determine their molecular weights.

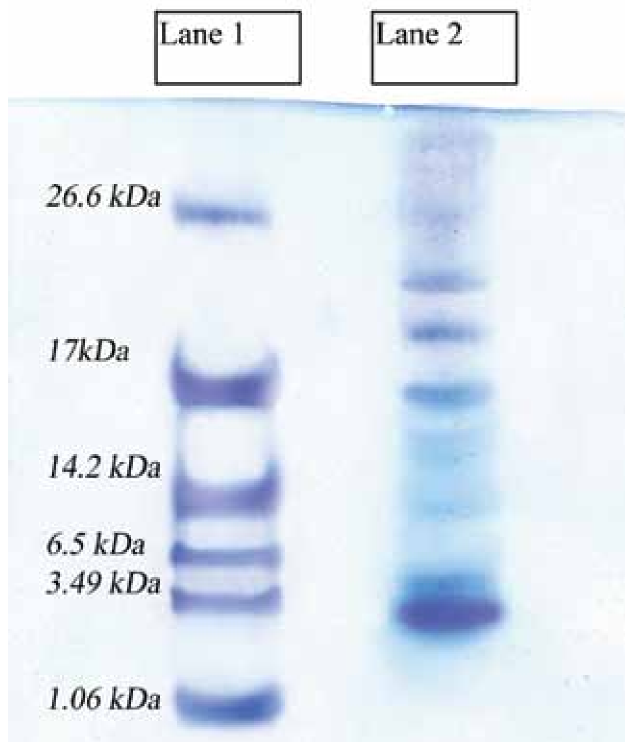

Figure 5. Tricine-SDS PAGE electrophoresis of 72h protein hydrolysate:
Lane 1 – Ultra low molecular weight marker (from 26.6kDa to 1.06kDa).
Lane 2 – Hydrolysate.

Collagen peptides are known to have a mild positive charge under acidic conditions and could be resolved by running through CM-sepharose, a weakly charged cation exchanger. Running the 72h hydrolysate in CM-sepharose resulted in the sample resolving into four peaks, as seen in Fig. 4, marked A, B, C and D. All four fractions on Sephadex G25 bed were found to comprise of peptides having molecular weight in the range of 5-50kDa. A tricine based SDS-PAGE gel was used for separation of the peptides, as this is generally preferred when trying to resolve small peptides.

As seen in Fig. 5, the 16% gels with 6M urea were found to be suitable for the separation of the recovered protein. In the presence of both SDS and urea, the protein bands were found to be spread in the region of molecular weights of 3 to 25kDa, although a small amount of high molecular weight bands, low in concentration was also observed (Fig. 5, Lane 2). Studies carried out by Cabeza et al.[13, 36] on degradation of chrome shavings with trypsin and pepsin for relatively much shorter duration had shown that the recovered protein hydrolysate had 3 molecular weight ranges namely 85- >208kDa, 55-85kDa and <7.2-5.5kDa; the results of this study confirm the presence of the last band.

Conclusion

The degradation of chrome shavings carried out in this investigation is less cumbersome and environmentally safer and cost effective method for chrome shaving hydrolysis as it involves the use of microorganisms instead of enzymes. It has been reported that peptides liberated by partial proteolysis of collagen macromolecules are able to regulate cell movement, differentiation and apoptosis.[39, 40] Collagen peptides have been shown to have biological properties including anti-oxidative activity[27] along with blood pressure lowering properties[41]. However, the chrome shaving hydrolysate peptides due to the possible contamination with residual biocides and other chemicals including chromium and contamination from bacterial culture may not be an ideal starting material for pharmaceutical applications. But the peptides may find possible applications in numerous other industries. Further work is being carried out to study their properties to determine their possible uses.

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