Purification of Protein Hydrolyzate Recovered from Chrome Tanned Leather Shaving Waste

by

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Abstract

Environmental constraints have become key issue for sustenance of industries worldwide. Waste management approach insists tanners look for an innovative way of creating wealth from waste. Chrome tanned leather shavings (CTLS) are generated in leather making process and requires a major attention on disposal. There are developed processes for recovery of protein and chromium from CTLS through acid/alkaline/enzymatic hydrolysis process. However, the derived protein hydrolyzate contains impurities such as chromium and neutral salts. In this study, an attempt has been made to purify protein hydrolyzate through protein precipitation process. Protein purification studies have been carried out to study the influence of factors such as salt concentrations, pH and time. The purity of protein hydrolyzate before and after purification process is found to be 95 and 99.4% respectively. This is further evidenced from amino acid analysis of protein hydrolyzate before and after purification. The recovered protein hydrolyzate could be used in a wide range of products such as fertilizer, animal feed and bio-composites.

Introduction

Conversion of hides and skins into leather generates huge quantities of liquid and solid wastes.[1] Chrome tanned leather waste is consists of chromium and protein (collagen). Hide and skin contain type I collagen protein which is insoluble fibrillar form structure. Collagen is characterized with triple helical conformation which is formed by three interwoven α chains. Several researchers have developed various methods to recover chromium and protein fraction, which are generally based on the chemical,[2-10] and biochemical/enzymatic hydrolysis of waste such as CaO, MgO, NaOH, HCl, H_2SO_4 and enzymes.[11-21] Protein hydrolyzate derived from above hydrolysis process contains inorganics matters and trace amount of chromium. The possibility of transforming recovered crude protein hydrolyzate from chrome leather wastes into purified protein hydrolyzate seem to be an interesting challenge, being of economic value as well. High-purity and de-mineralized protein hydrolyzates have potential use in various field.[22-27] The pure form of protein hydrolyzate can be used as bio-fertilizer in agriculture industry.[16, 28-34] Cosmetic application demands protein hydrolyzate purity of about 99%.[35-36] Poultry feed requires protein hydrolyzate purity 98% with free of chromium.[37] These applications would enhance the value of derived protein hydrolyzate to multifold. Hence, purification of protein hydrolyzate is paramount.

The protein hydrolyzate obtained from conventional process contained chromium and inorganic salts; its purity is about 80 - 95%. The high content of salts of the collagen hydrolyzate obtained from alkaline hydrolysis, whose separation is expensive and difficult to recover pure protein. Protein purification can be done in a number of ways i.e precipitation (salting out), ion exchange, Semi permeable membrane (dialysis) or gel filtration, ultra filtration, electrophoresis and the best way is depends on application and requirement. Protein precipitation is an important operation for the laboratory and industrial scale recovery and purification of proteins. Protein precipitation is a technique that utilizes the differences of protein solubility to precipitate proteins into the solid phase from the liquid phase. It has been used extensively to separate and purify proteins. A number of methods exist for precipitating proteins the most common method are salting-out using ammonium salt[38] and it does not denature protein. The process of salting-out proteins is due to hydrophobic interactions.

In this study an attempt has been made to standardize the salting out based purification process for protein hydrolyzate derived from chrome shaving through biochemical based extraction method. Purification studies were carried out at different salt concentration, pH, time, temperature for process standardization. Pilot level study was also conduct with standardized parameters. All the standardization and salt recovery studies were done triplicate and reported average values.

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EXPERIMENTAL

Materials

Fresh chrome leather solid waste – chrome shavings were collected from Tata International Ltd, Dewas, Madhya Pradesh, India. Laboratory grade *bacterial protease* (15000 units/g) from *Bacillus licheniformis* and *α-amylase* (25000 units/g) from *Bacillus subtilis* were sourced from Sigma Aldrich. All the chemicals i.e. ammonium sulfate, ammonium chloride and ammonium oxalate used for experiments were of laboratory grade. Biuret reagent was prepared by adding with stirring, 300 mL of 10% (w/v) sodium hydroxide to 500 mL of a solution containing 0.3% copper sulfate pentahydrate and 1.2% sodium potassium tartrate, then dilute to one liter.

Extraction of Protein Hydrolyzate from Chrome Shavings Through Biochemical Process[20]

About 20 kg (on dry basis) chrome shavings were feed into a stainless steel reactor equipped with an agitator 30 RPM and treated with 1% (w/w of chrome shaving) protease and 1% (w/w of chrome shaving) α-amylase and 2% (w/w of chrome shaving) lime at temperature 55°C for 18 h and followed by rise of temperature to 90°C for 2 h. The protein was separated out from reactor by passing through sieves and the protein content in the supernatant was determined using biuret reagent after proper dilution. [39-40] Protein hydrolyzate derived from above extraction was concentrated to 40% total solids at 100°C in atmospheric pressure. Prepared 40% protein hydrolyzate was used for purification study with proper dilutions.

Purification of Protein Hydrolyzate Derived from Biochemical Process: Optimization of Nature of Salt, Concentrations of Salt and Protein Hydrolyzate, Time and pH

15 g of each salt such as ammonium sulfate, ammonium chloride and ammonium oxalate were taken in three 250 ml beaker and mixed with 100 ml of 20% protein hydrolyzate. Mixed solutions were transferred to separating funnels and kept for 18h to form precipitation. 1 ml sample was taken from top of separating funnel and analyzed for protein by biuret method after proper dilution.[39-40] Different quantities of ammonium Sulfate (15, 20 and 25g) was mixed with 100 ml of 20% protein hydrolyzate and kept for 18h to form precipitation 1 ml of sample taken from the top and protein content measured. [39-40] Various concentrations of 100 ml protein hydrolyzate (10 - 35%) were mixed with 15 g of ammonium sulfate. Samples were kept for 18h to form precipitation. After precipitation, supernatant volume measured as partitioning volume, supernatant was taken and analyzed for protein and solid content (hot air oven dry at 105°C for 5 h). [39-41] Protein hydrolyzate (20%) of 100 ml each was mixed with 15 g of ammonium sulfate. 1 ml of supernatant was taken and analyzed for protein content at various time intervals (0.5 – 18h).[39,40] Protein hydrolyzate (20%) 100 ml taken and pH was adjusted to 3.0 – 9.0 employing dilute 0.1N hydrochloric acid and 0.1N sodium hydroxide. 15 g of ammonium sulfate was added to the pH adjusted solution and mixed well. Mixed solutions were kept for 4 hr for precipitation. 1 ml of supernatant was taken and analyzed for protein content. [39, 40] Protein precipitation efficiency is calculated based on below formula.

$$\%P = (A - B)/A$$

% P = percentage protein precipitation

A = Initial protein content

B = Protein content in supernatant

Pilot Trials

About 5kg of 20% protein hydrolyzate was taken for laboratory experiment. Protein hydrolyzate (20%) was mixed with 15% (w/w of protein hydrolyzate) ammonium sulfate. Mixed solution was kept for 4 h in 25 liter separating vessel. After precipitation, 1 ml of supernatant was taken and analyzed for protein content for measuring protein precipitation efficiency. [39, 40]

Characterization of Protein Hydrolyzate Before and After Purification

Precipitated protein from laboratory trial was dried in air oven at 100°C for 5 hrs and named as experiment. For comparison, un-precipitated protein was also dried in air oven at 100°C for 5 hrs and named as control. Experimental and control trial protein hydrolyzate were analyzed for total ash, protein and chromium content.[39,40,42,43] Amino acid content of control and experiment were determined using Hitachi amino acid analyzer model 835–50, Tokyo Japan, (1981).

RESULTS AND DISCUSSION

Underlying Principles of Protein Precipitation Through Salting Out

Salting out is a method of separating proteins based on the principle that proteins are less soluble at high salt concentrations. This process is also used to concentrate dilute solutions of proteins. When the salt concentration is increased, some of the water molecules are attracted by the salt ions, which decreases the number of water molecules available to interact with the charged part of the protein. As a result of the increased demand for solvent molecules, the protein-protein interactions are stronger than the solvent-solute interactions; the protein molecules coagulate by forming hydrophobic interactions with each other. This process is known as salting out.[44, 45]

Effect of Nature of Salts

Nature of salts plays a vital role on protein precipitation process as discussed above. Hence, different natures of salts

such as ammonium chloride, ammonium oxalate and ammonium sulfate have been tab Iken for conducting this study. Concentration of salts and protein hydrolyzate were kept constant. It is observed from Table I that ammonium sulfate have ability to precipitate protein and other selected salts were found to be nil. Sulfate ion has more tendencies to precipitate the protein molecules than chloride, oxalate and nitrate.[46] Hence, further experiments were carried out employing ammonium sulfate as salting out agent.

Optimization of Salt Concentration for Protein Precipitation

Various quantities of ammonium sulfate were mixed with constant protein concentration. The percentage protein precipitation is shown in Figure 1. The protein precipitation level is increasing with increasing the concentration of salt. This is due to nature of protein folding with hydrophobic ends. Also salt content in supernatant is increased with respect to higher salt usage. There is no significant increase in protein precipitation in 20 and 25 g of salt used. Hence, 15 g salt with 20% of 100 ml protein solution would provide optimum protein precipitation.

TABLE I
Effect of Nature of Ammonium Salts on Protein Precipitation.

Nature of Ammonium Salts	Protein precipitation occurs (Y/N)
Sulfate	Y
Chloride	N
Oxalate	N
Nitrate	N

Note: Y – Yes; N – NO

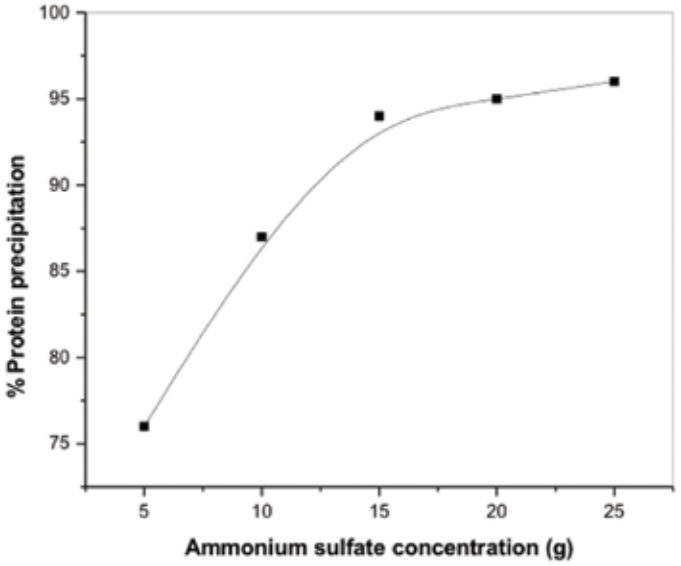

Figure 1. Effect of Ammonium Sulfate Concentration Protein Precipitation.

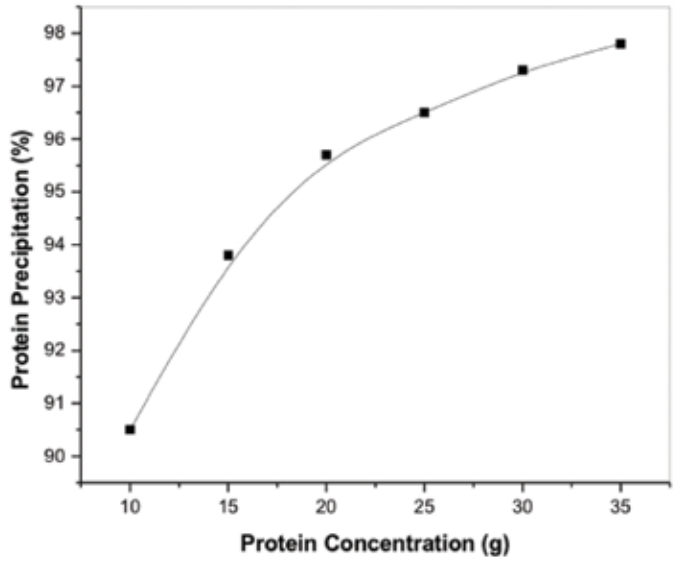

Figure 2. Effect of Protein Concentration on Protein Precipitation.

TABLE II
Effect of Protein Hydrolyzate Concentration on Precipitation.

Protein concentration (%)	10	15	20	25	30	35
Salt Input (g)	15.0	15.0	15.0	15.0	15.0	15.0
Salt content in supernatant[a] (%)	27.3±0.1	28±0.4	32.5±0.3	34.6±0.1	37.9±0.2	36.4±0.3
Partitioning volume[a] (ml)	96.0±0.2	97.0±0.3	98.0±0.2	95±0.3	96±0.2	98±0.3

[a]Average values of three values

Optimization of Concentration of Proteinous Substance

Various concentrations of protein hydrolyzate were mixed with 15 g ammonium sulfate. The salt content in supernatant and volume of supernatant partitioning is given in Table II and it shows that the partitioning volume for all the experiments were similar. It reveals the optimum salt concentration of salt precipitates low (10%) to high (35%) protein concentration. The salt content in supernatant is increasing due to salt present in protein hydrolyzate. Effect of protein precipitation efficiency on various concentration of protein hydrolyzate is shown in Figure 2. It shows that protein precipitation efficiency is increasing with respect to higher concentration of protein hydrolyzate. This is due to higher protein concentration, which enables protein folding rapidly and allows salting out process effectively. This has been evident from partitioning volume is 98 ml (Table II) and precipitation volume of higher protein concentration (1 ml). Overall the protein concentration significantly influences precipitation process.

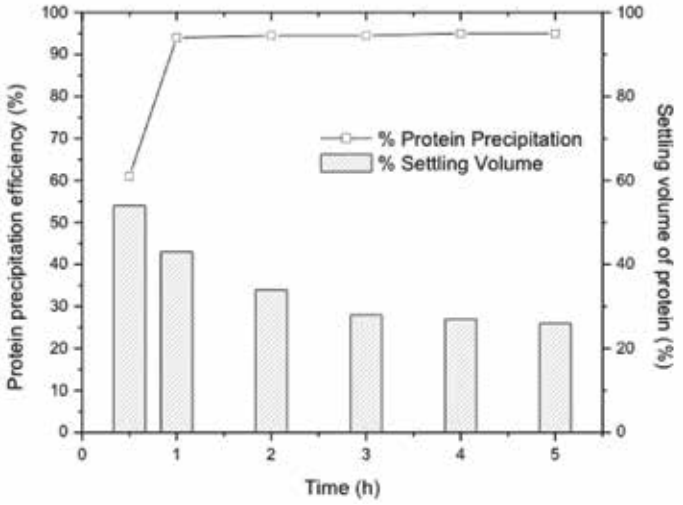

Figure 3. Effect of Time on Protein Precipitation and Settling Volume.

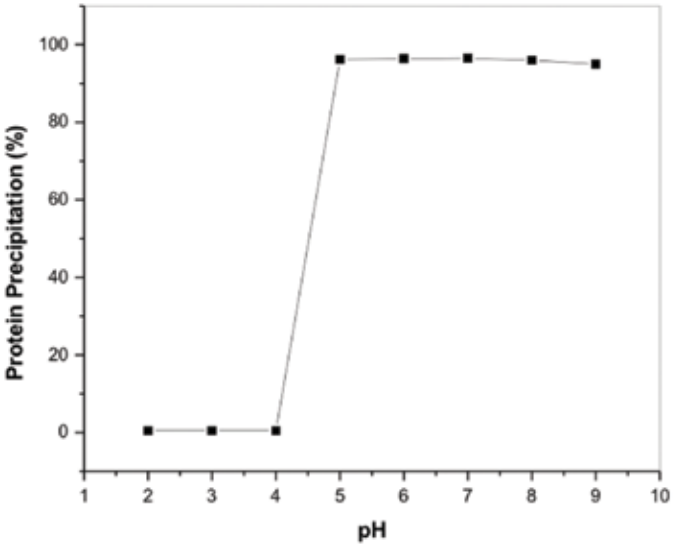

Figure 4. Effect of pH on Protein Precipitation.

Optimization of Time for Protein Precipitation

Effect of time on protein precipitation was carried by conducting different time interval based experiments. The influence of time on precipitation of protein is shown in Figure 3. It is interesting to note that there is no significant effect on precipitation of protein with respect to time interval (1– 5h). However, it requires minimum 1 h time to precipitate 94% protein. Further, the protein precipitate takes time to settle and % settling volume was measured and shown in Figure 4. Compact settling of protein precipitate happens about 4 h. Hence, the duration for precipitation of protein could be optimized as 4 h.

Effect of pH on Protein Precipitation

Iso-electric point of protein is primarily driven by its amino acid composition. The ionic behavior of protein varies with pH and also relates with protein folding based on ionic interaction between amino and carboxylic acids. It is imperative to assess the effect of pH on the protein precipitation. The percentage precipitation of protein with different pHs is shown in Figure 4. It is evident from the graph that there is no precipitation of protein occurred in between pH 2 to 4. This is primarily due to iso-electric point of protein hydrolyzate; where carboxyl and amino groups are equal. This does not allow protein to fold make it to precipitate. Whereas pH 5 to 9, the iso-electric point of protein molecules is shifted and allows folding and precipitating faster. This is evident from Figure 4 that percentage precipitation is about 96% and almost constant up to pH 9. Hence, pH 5 to 9 favors precipitation of protein in salting out process.

TABLE III
Comparison of Ash Content of Control and Experiment.

Parameters	Control	Experiment	Methods
Ash content (%)	7.3±0.8	1.7±0.2	[42]
Chromium (ppm)	240±12	ND	[43]
Protein (%)	90±0.7	98±0.2	Biuret Method [39,40]

Note: ND – Not Detectable

Pilot Trials

Laboratory trial was conducted with 5kg of Protein hydrolyzate (20%) with optimized protein purification process. Protein precipitation efficiency is found to be 96%. It could be seen from Table III that control sample exhibits higher ash content and chromium as compared to experimental sample. This is primarily due to purity of protein from experimental is high. Further evidenced from amino acid analysis is given in Table IV. The purity of experiment protein is 99.4% whereas control is 95%.

TABLE IV
Amino acid Composition of Protein Hydrolyzate Before and After Purification.

Amino acid	Collagen Type 1	Control	Experiment
Thr	1.6	0.503	0.543
Val	2.3	3.411	3.411
Leu	2.5	2.721	2.721
Ile	1.2	1.101	1.101
Lys	2.8	2.112	2.112
Hyl	0.7	0.923	0.963
Tyr	0.4	0.312	0.312
Phe	1.3	1.812	1.812
Met	0.6	0.742	0.742
Gly	32.7	31.014	33.124
Hyp	8.6	6.012	6.012
Pro	13	12.356	15.016
Ala	11.4	12.724	13.124
Arg	5.2	3.867	3.867
Asp	4.6	4.012	4.512
Cys	0	0.452	0.452
Glu	7.5	7.121	7.121
His	0.5	0.473	0.483
Ser	3.1	1.725	2.025
Ammonia	-	1.213	-
Unknown	-	0.416	-
Total	100	95.022	99.453

CONCLUSIONS

The solid waste generated from leather making process particularly chromium based requires a key attention on disposal. There are established processes for recovery of protein hydrolyzate from chrome shavings; however the purity of proteinous matter limits its application. In this study, protein purification process has been standardized to achieve higher purity of protein. Standardization study showed that salt concentrations 15%, pH 5-9 and time 4h to achieve maximum protein purification efficiency. The purity of protein hydrolyzate before and after purification process is found to be 95% and 99.4% respectively. This has been evidenced from amino acid analysis of protein. Purified protein hydrolyzate could be used in a wide range of products such as fertilizer, animal feed and bio-composites.

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