Keratin-Silica Matrix – A New Protein Filler from Chicken Feathers for Retanning

by

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Abstract

A Keratin-Silica matrix (KH-Si) has been prepared by treating chicken feathers with sodium silicate to overcome the known problems associated with conventional keratin hydrolyzate (KH) when used for retanning. Sodium silicate converts keratin into a water soluble product with desirable color and added properties like antimicrobial character but with retanning action similar to KH. A comparative study has been carried out between KH-Si and KH, which has been prepared with NaOH. Their physical and structural characteristics have been characterized using FT-IR, TGA, MALDI-TOF, XRPD, EDAX and Agar-well diffusion methods. The retanning performance of KH-Si and KH has been tested in wet blue goat skins. Performance of the resultant leather made with KH-Si was marginally better than KH. Computer aided color measurement on products and additional results are discussed.

Resumen

Una matriz de sílice-queratina (KH-Si) fue preparada por el tratamiento de las plumas de pollo con silicato de sodio para superar los problemas conocidos asociados con el hidrolizado de queratina convencional (KH) cuando es empleado en el recurtido. El silicato de sodio convierte a la queratina en un producto soluble en agua con el color deseable y agrega propiedades como carácter antimicrobiano, pero con acción recurtiente similar a la de la KH. Un estudio comparativo ha sido realizado entre KH y KH-Si, el cual ha sido preparado con NaOH. Sus características físicas y estructurales han sido caracterizadas mediante FT-IR, TGA, MALDI-TOF, XRPD, EDAX y métodos de difusión con Agar. La performance del recurtido de KH y KH-Si se ha probado en wet blue de pieles de cabra. La performance del cuero resultante realizado con KH-Si fue marginalmente mejor que con KH. Medición del color mediante computadora del producto resultante y resultados adicionales se discuten.

Introduction

Chicken feather is an abundantly available keratinous waste in many parts of the world and it has the potential for conversion to high value added products with a number of industrial applications.[1] Keratinous wastes are converted into keratin hydrolyzate by hydrolysis with sodium hydroxide to get a soluble hydrolyzate suitable for leather processing, particularly in retanning process.[2] Conventionally, keratin hydrolyzate (KH) prepared by alkali (e.g. NaOH) and acid (e.g. HCl) hydrolysis[3] requires neutralization to ensure that it is suitable for applications in animal feed, cosmetic and leather processing industries. Besides, the existing keratin hydrolyzate prepared by alkali hydrolysis and microbial degradation for retanning[4] of wet blue leathers imparts color to the leather, which is not desirable and moreover, the product does not have antimicrobial and tanning potency. This limitation of these products eventually affects the economy of application adversely.

The advantages of using silicates to convert keratin into water soluble keratin hydrolyzate for application in retanning are many fold. Conventionally, silicates have been used for the preparation of various industrial products exhibiting antimicrobial property. Silicate containing sheet with antimicrobial and antifouling properties[5] and a fibre structure comprising a zeolite layer and an alkyl silicate layer suitable for textiles[6] are some of the examples. Another advantage of using silicate is that it would enhance tanning action (shrinkage temperature) of the keratin hydrolyzate; as the use of alkali metal silicates for tanning of animal skins is well known.[7] Alkali silicates have also been used to improve the exhaustion of added auxiliaries, especially of chrome tannins in leather processing.[8] Silicates could also be used for the preservation of hides and skins.[9] Hence, there is a scope to develop keratin-silica based retanning agent that overcomes the existing problems in the conventional keratin hydrolyzate used as a retanning agent.

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Fiber structure of hide or skin is not uniform throughout the entire area and it is most common to fill the empty nature of chrome tanned leathers by retanning to improve the required properties of leathers,[10] which are intended for making footwear, garments, gloves, furniture and automotive upholstery etc. Today several developments are taking place in the field of retanning such as phenol formaldehyde and naphthalene formaldehyde condensates, melamine, dicyandiamide and carbodiimide based syntans, polymers of various types, such as acrylates, urethanes and melamine resins.[11-13] Most of these retanning agents are still suspected in their application due to release of high COD, TDS, free phenol and free formaldehyde. Proteins and protein hydrolyzates are finding increasing amounts of applications as fillers in retanning operations.[14] Protein based retanning agents offer better prospects as they fill loose areas such as belly, flanks and poor substance materials without contributing much load to tannery effluent. A synergetic effect of keratin with silica species has been considered advantageous. Thus the objective of the study is to develop a protein based retanning agent from chicken feather waste by hydrolyzing it with silica salt. Keratin protein hydrolysis with silica offers a light colored product with antimicrobial property and good tanning potency.

EXPERIMENTAL

Materials and Methods

Chicken feathers were collected from poultry processing units at Chennai. Sodium silicate lye (waterglass) (27% SiO_2, 10%NaOH), sodium hydroxide pellets (Extra pure AR, assay 98%) were purchased from SD fine chemicals, Chennai and other chemicals were purchased from standard dealers in Chennai. The design of experiments carried out on the chicken feather is illustrated in Figure 1.

Preparation of Keratin–Silica Matrix (KH–Si)

Chicken feathers collected from local poultry units were washed thoroughly with water and dried in the sunlight. The dried chicken feathers weighing 1kg were treated with 350gms of sodium silicate lye with the addition of 3 liters of water in an industrial autoclave (Type LA 1000, MLW; 10lit capacity; Max. Temp 285°C; Germany) fitted with automatic temperature control. The temperature was maintained at 90-100°C for a period of 5h. The material obtained was filtered to remove the traces of polypeptides, centrifuged at 2500 rpm for 15 min to remove the larger fragments and dried in a drum dryer. The yield of the product is around 850grams.

Preparation of Keratin Hydrolyzate (KH) using NaOH

To make a comparative study with KH–Si, keratin hydrolyzate was prepared by using sodium hydroxide pellets. The preparation of KH was similar and used sodium hydroxide pellets (70 G.) instead of sodium silicate lye.

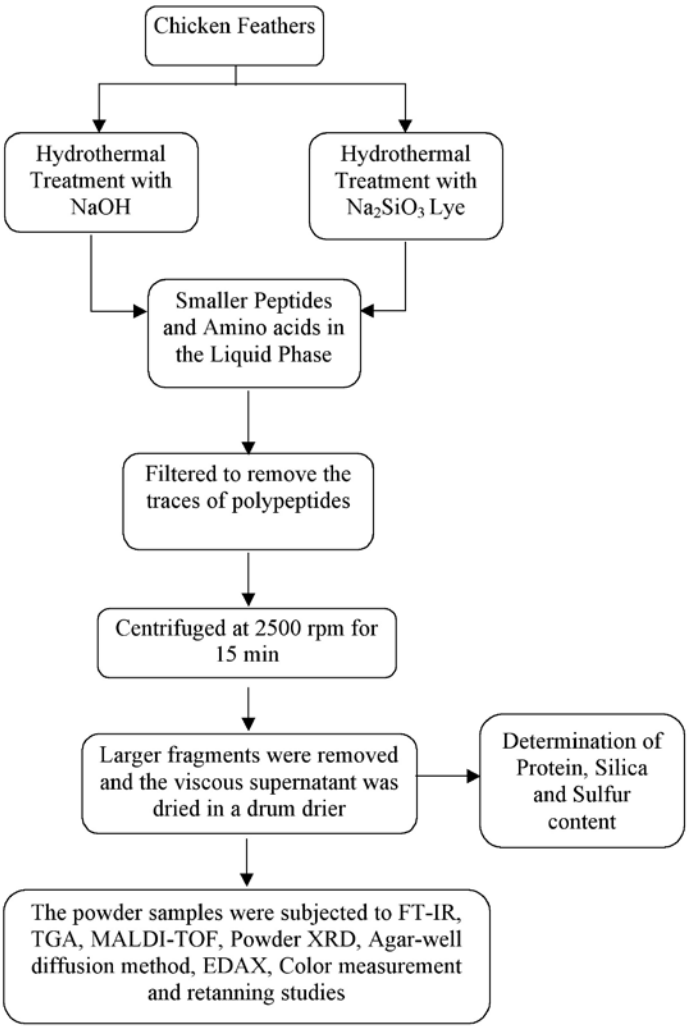


Figure 1. Flow chart for experiments on chicken feathers

Estimation of Silica Content

The silica content in the keratin-silica sample was estimated as per the official AOAC method.[15]

Determination of Protein Content

Both keratin preparations, KH and KH–Si were analyzed for their total protein content through Kjeldahl method.[16]

Estimation of Total Sulfur

The total sulfur present in KH–Si and KH samples was estimated by sodium peroxide method.[17]

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra of KH–Si and KH samples were recorded in a Thermo Nicolet avatar 320 FT-IR spectrometer (Nicolet Instrument Co. USA). The powder sample was mixed with KBr of spectroscopic grade and made in the form of pellets at pressure of about 1 MPa. The pellets were about 10 mm in diameter and 1 mm in thickness. The measurements were carried out in the mid-infrared range from 4000 to 400 cm^{-1}

after baseline correction and analyzed by OMNIC (Version 6.0) software supplied by Thermo Nicolet.

Thermogravimetric Analysis (TGA) of KH and KH–Si

TGA of KH–Si and KH samples were carried out using a TGA Q 50 thermogravimetric analyzer (TA instruments) under nitrogen atmosphere (80mL/min) in a platinum pan between 50 and 800ºC with a heating rate of 10ºC/min. Weight loss of these materials as a function of temperature was recorded.

MALDI–TOF Analysis of KH and KH–Si

To determine the molar mass range of the species present in the KH and KH–Si samples, matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOF-MS) analysis was carried out. The matrix used was cyano-4-hydroxycinnamic acid (CHCA) at a concentration of 10mg/mL in demineralized water. The sample was dispersed in acetonitrile mixed with matrix in 1:5 volume ratio. A volume of 1µl of prepared sample was spotted on sample plate (stainless steel) and allowed to air-dry[18] and the MALDI-TOF analysis was conducted using Voyager DE PRO Biospectrometry work solution (Applied Biosystems).

X-Ray Powder Diffraction (XRPD) Analysis

The KH and KH–Si samples were analyzed on Philips X' Pert Pro X-ray diffractometer, equipped with a Ni filtered Cu-Kα (λ=0.154 nm, 40kV, 30mA) radiation and X' celerator detector. The fine powder sample was placed on a zero background quartz sample holder. The pattern was recorded in the 2θ range of 10-70º.

EDAX Analysis of KH–Si

In order to demonstrate the elemental composition of keratin-silica matrix, EDX analysis has been carried out using a Hitachi S-3400 N instrument.

Color Measurement Analysis

The color characteristics of KH and KH–Si samples in terms of CIE color coordinates L, a, b, C, and h were studied using a computer controlled Gretagmacbeth spectrolino instrument.

Variables ΔL, Δa and Δb were calculated as follows

- $\Delta L = L_{KH-Si} - L_{KH}$ (if $+\Delta L$, KH–Si is lighter than KH)
- $\Delta a = a_{KH-Si} - a_{KH}$ (if $+\Delta a$, KH–Si is redder than KH)
- $\Delta b = b_{KH-Si} - b_{KH}$ (if $+\Delta b$, KH–Si sample is yellower than KH)

The color difference between KH–Si and KH was calculated in terms of ΔE, the overall color difference using standard equation.[19, 20]

$$\Delta E = \sqrt{L^2 + a^2 + b^2}$$

Antimicrobial Activity of KH and KH–Si by Agar-Well Diffusion Method

Ageing the protein filler treated leathers (pile up in wet condition for 2 or more days before setting, drying etc.) frequently cause problems to the tanners, because there is a chance for bacterial attack, also in the time of monsoon seasons where the crust leathers take too much time to dry. To avoid bacterial damage, protein filler treated leathers are commonly treated with a suitable biocide. Hence, there is a necessity to test the antimicrobial activity of newly prepared protein based materials used for retanning purpose. Antimicrobial activity of KH and KH-Si was tested by the agar-well diffusion method[21] using *Bacillus substilis* inoculum.

Retanning Studies

The KH and KH–Si samples rich in protein content were used as protein filler in the retanning of chrome tanned leathers. Shaved wet blue goat leathers having 1mm thickness were used as raw material for retanning trials. The wet blues were cut into the sides on the backbone and marked as 1L, 1R, 2L, 2R, 3L, 3R. The wet blues marked as 4 and 5 were used as such (without cut into sides). The leathers were washed and neutralized to pH 5.2 and washed twice. The wet blues 1L, 2R, 3L and 4 were retanned using 7% KH and the wet blues 1R, 2L, 3R and 5 were retanned using 7% KH–Si (% based on shaved weight of wet blues) for 30 min. The remaining procedure (dyeing and fatliquoring) was common for both control (KH treated) and experimental (KH–Si treated) leathers. 2% acid dye and 9% commercial fatliqouring agent was used and finally fixed with formic acid. The whole process was repeated for 2 more times using the same number of goat skins and following the same process as described above.

Scanning Electron Microscope Analysis

The scanning electron microscopic analysis was carried out on the KH and KH–Si retanned crust leathers using a FEI-Quanta 200 scanning electron microscope.

Physical Analysis and Visual Assessment

The samples for physical testing were cut from both KH and KH–Si retanned crust leathers according to the official sampling position.[22] The samples were conditioned at 80±4°F and 65±4% R.H. for 48 h. The tensile and tear strengths were measured as per the IULTCS method.[23,24] Experienced technologists assessed the organoleptic properties such as fullness, feel, grain tightness and general appearance. The leathers were rated on a scale of 0-10 points for each functional property, where higher points indicate better property.

Determination of Tanning Potency of KH and KH–Si

In order to determine the tanning potency of the newly prepared protein filler, the delimed goat skins (pH 7.5-8.0)

were treated with 12% of KH and KH–Si (% on pelt weight) for 2.5 hours and the final pH of the pelts was adjusted to 4.0. One of the benchmarks for ensuring and monitoring of tanning is the increase in hydrothermal stability.[25] The denaturation temperature of KH–Si and KH treated goat skins was recorded using a DSC Q 200 differential scanning calorimeter (TA Instruments).

RESULTS AND DISCUSSION

Chemical Characteristics of KH and KH–Si

Sodium silicate solutions (pH 11.0) contain a large number of polysilicate anions[26] such as $[Si_4O_8(OH)_4]$ and are considered crosslinking agents since they interact through both electrostatic interactions and hydrogen bonding with proteins[27]. Hydrothermal treatment of chicken feathers in the presence of silicate species leads to the formation of keratin–SiO_2 matrix. Electrostatic interactions between positively charged peptides or proteins and negatively charged silicate species favor the formation of keratin-silica matrix at pH 8.0-8.5 without further neutralization.

Analytical values of total protein, total sulfur and silica content of KH–Si and KH are listed in Table 1. The silica content as SiO_2 in KH–Si sample was found to be 5.21 ± 0.26% w/w. The sulfur content in KH sample is 2.24 ± 0.16% w/w, whereas in KH–Si it is 1.63 ± 0.13% w/w. Cystine, cysteine and small amounts of methionine are the sulfur containing amino acids present in chicken feather. The amount of sulfur in keratin preparations is mainly decided by the sulfur of the disulphide cystine. Both KH and KH–Si have been prepared by breaking the disulphide bonds in cystine, but KH–Si employs a higher percentage of silicate salt (35% on the weight of chicken feather) in the hydrothermal process to break the disulphide bonds, results in smaller percentage of sulfur in the final product. The EDX spectrum obtained from focusing keratin–silica particles is shown in Figure 2, and indicates that the particles contain significant amount of silica. The elements sulphur, sodium, nitrogen and oxygen are concentrated more in the KH-Si particle, where the electron beam is bombarded.

FT-IR Spectra of KH and KH–Si

Infrared absorption spectra of KH and KH–Si presented in Figure 3 show characteristic absorption bands assigned mainly to the peptide bonds (–CONH–). The amide I of KH and KH–Si peaks at 1652.66cm^{-1}. The amide I peak of chicken feather is reported to be 1641cm^{-1}. [28] The amide I peak of KH and KH–Si shifted to higher wave number was an indicative of more disordering structure. The amide II of KH peaks at 1542cm^{-1}, and KH–Si is at 1548cm^{-1}. The absorption near at 1402.13 cm^{-1} for KH and 1415.67 cm^{-1} for KH–Si is the characteristic absorption band of cis-peptide bond. The amide III of KH peaks at 1239cm^{-1}. The band at 1085.19cm^{-1} in KH–

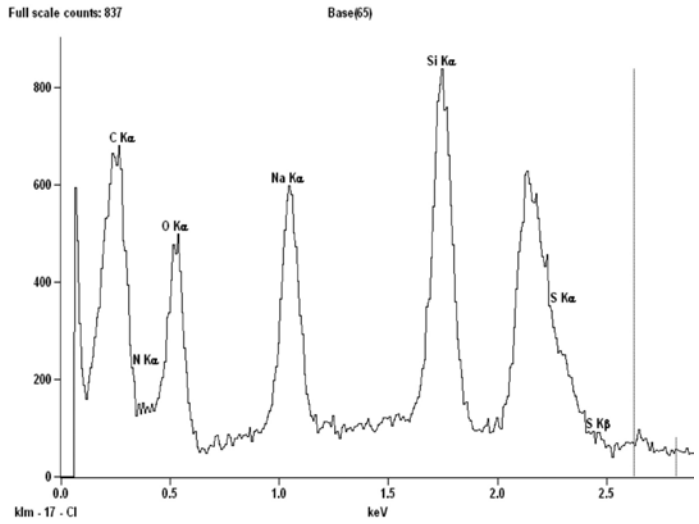


Figure 2. EDAX spectrum of KH–Si

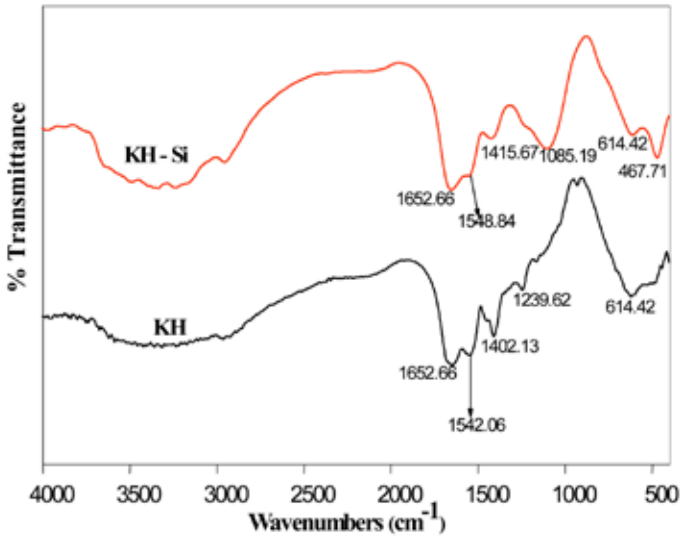


Figure 3. FT-IR Spectra of KH and KH–Si

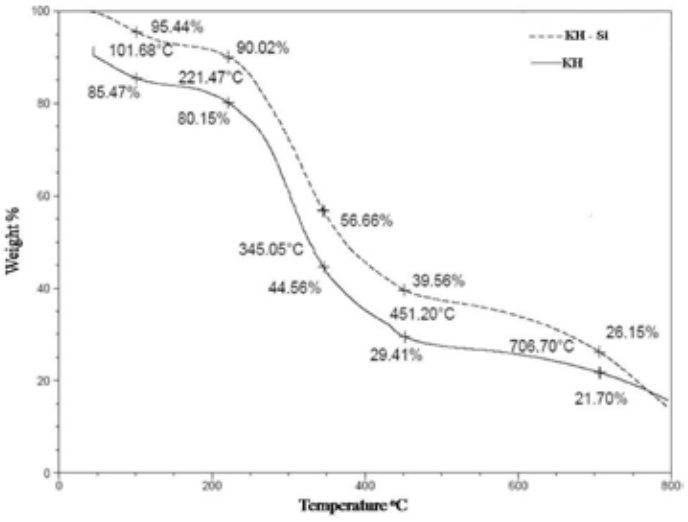


Figure 4. Thermogravimetric analysis results of KH and KH–Si

TABLE 1

Chemical characteristics of KH and KH-Si

Sample	Protein Content % w/w	Sulfur Content % w/w	Silica Content as SiO_2 %w/w
KH-Si	61.52 ± 2.46	1.63 ± 0.13	5.21 ± 0.26
KH	76.33 ± 2.82	2.24 ± 0.16	—

TABLE 2

CIE color values for KH and KH–Si

Sample	L	a	b	C	h
KH–Si	75.792	1.026	23.635	23.657	87.515
KH	34.338	9.158	28.634	30.063	72.263

Si sample corresponds to Si–O stretching and at 467.71cm^{-1} corresponds to Si–O bending[29] which are not present in KH. The interaction expected between peptides and silica species could be possibly of electrostatic attraction between Si–O^- and NH_3^+ of amino acids or peptides and hydrogen bonds. A similar kind of band for Si–O stretching at 1080cm^{-1} and for Si–O bending at 470cm^{-1} has been studied previously for silica network.[27]

Thermal Properties of KH and KH–Si

The thermogravimetric profiles of KH and KH–Si obtained under inert atmosphere are shown in Figure 4. The curves indicate the weight percent of residual composite at different temperature. Generally, the TGA curves of the KH and KH–Si are showing a gradual weight loss due to absorbed moisture upon initial heating up to around 100ºC, followed by a slow weight loss until around 220ºC and the final degradation of the peptides occurs from around 220 - 800ºC. KH–Si has a relatively low decomposition temperature compared to KH. At 100ºC the initial weight loss for KH accounted for about 15% of the total weight where as for KH–Si it is only about 5% weight loss. KH lost about 56% of its total weight up to 345ºC whereas KH–Si lost 44% only. From the TGA results it has been confirmed that the presence of silica species in KH–Si sample keeps the protein filler in dry condition for a prolonged period of time and is thermally stable compared to KH. Many retanning agents used in tanneries have ability to absorb moisture and become solid or a thick paste after some period of time when it is kept under open conditions. The presence of moisture also encourages the growth of mold and spoilage of protein fillers. Thus from the TGA results it is confirmed that the presence of silica species in KH–Si enhances the storage stability, an important property of protein filler used by the leather industry.

Color Measurement Data

The CIE color coordinates L, a, b, C and h for KH and KH–Si samples are given in Table 2. From the table, it is observed that, the lightness value 'L' is less for KH compared to KH–Si indicating that KH is darker than KH–Si. The red value 'a' and yellow value 'b' are more for KH sample compared to KH–Si. Variables of L, a, and b represented as ΔL, Δa and Δb in addition with overall color difference ΔE, chromaticity difference ΔC and hue difference Δh are presented in Table 3. From the table it is clear that the overall color difference between KH and KH–Si is more (42.539) indicating that KH–Si is lighter compared to KH. Thus it is clear that the use of silicate for the preparation protein filler from chicken feathers results in a light colored product preferred by the commercial tanners.

XRPD Analysis of KH and KH–Si Samples

In general, XRD pattern of KH and KH–Si reveals that all are amorphous body, and their crystallinity is very poor. In the XRD pattern of KH–Si sample, a broad hump peak centered at 2θ angle of 21.94◦ whose interplanar spacing d at 4.04874 is assigned to cristobalite phase of silica[30] which is not present in KH. The XRD pattern of KH sample shows the presence of very poor absorption peaks at 11.9024, 18.6776 and 28.6065, whose interplanar spacing d are 7.42947, 4.74697 and 3.11794 respectively, indicating the disordered structure of α helix and β sheet and it is difficult to be reserved due to hydrothermal treatment of chicken feathers. The crystal structures of the raw chicken feather analyzed previously by XRD show a diffraction peak at 2θ of about 9.8◦ corresponding to the α helix structure, and the peaks at 2θ of 19.5 and 21.2◦, corresponding to the β-sheet. A similar kind of result was obtained for previously reported feather hydrolyzate.[28]

Determination of Molar Mass of Peptides in KH–Si and KH by MALDI–TOF

To elucidate the molar mass range of oligopeptides present in KH and KH–Si, MALDI–TOF analysis has been carried out. The MALDI–TOF mass spectra of KH and KH–Si samples are presented in Figures 5 and 6 respectively. The molar masses of the peptides present in KH is in the range of 211–655 Da, whereas for KH–Si it is in the range of 211–684 Da. From the MALDI-TOF results it is revealed that both KH and KH–Si samples are having low molecular weight peptides and a considerable portion of amino acid sequence is lost in both keratin preparations. These small molecules can achieve a uniform dispersion across the skin thickness much faster than the larger molecules. Since the interaction between silica species and peptides is due to electrovalent attraction, when the sample is dissolved in acetonitrile and CHCA mixture for MALDI–TOF analysis, the electrovalent linkage is expected to be broken and it is positively concluded that the additional peak (684 Da) present in the MALDI–TOF mass spectra of KH–Si sample is for peptide.

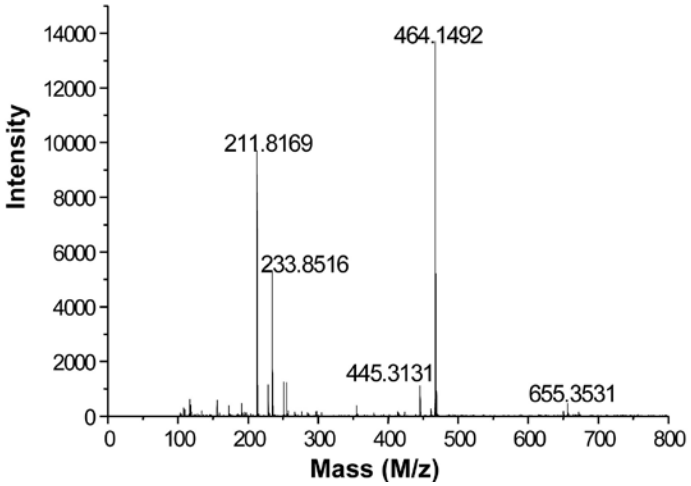


Figure 5. MALDI–TOF data of KH

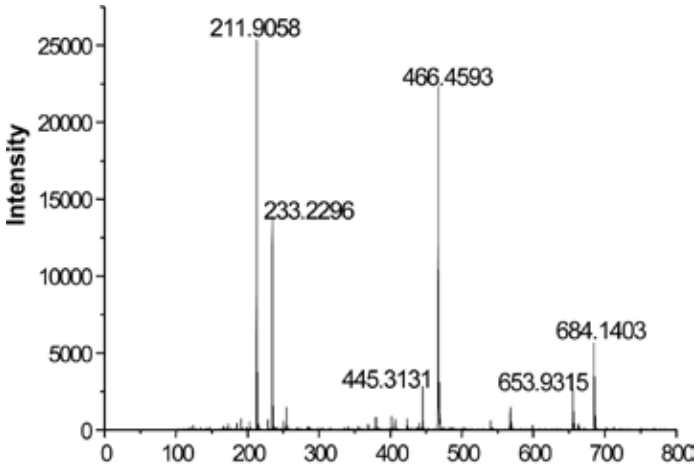


Figure 6. MALDI–TOF data of KH–Si

Effect of Keratin-Silica Matrix on Antibacterial Activity

The antibacterial activity of KH and KH–Si samples against *Bacillus substilis* are presented in Figure 7. KH–Si shows a spectrum of significant antibacterial activity by producing a clear zone of inhibition (14.15mm, including well diameter) whereas KH has found to be sensitive to *Bacillus substilis* due to the growth of bacteria near the surroundings of well area. The bacteria *Bacillus substilis* was selected to test the antimicrobial activity because it has been effectively used for the degradation of keratinous material such as feather, horn meal etc.[4,31] Thus from the results it is confirmed that the presence of silica component in KH–Si sample inhibits the bacterial growth which gives clear indication about its antibacterial property; where as KH has no antimicrobial activity against *Bacillus substilis*. Hence the storage stability of KH–Si is comparatively better and it is expected that it may not promote any bacterial growth after treatment with leathers during retanning.

Effect of KH and KH–Si on the Retanning of Wet Blues

From the various parameters studied, both KH and KH–Si have been successfully employed as protein filler in the retanning of wet blue goat leathers. It is found that both keratin preparations (KH and KH–Si) have low molecular weight peptides (< 690 Da) which penetrate through the pores, deep into the layers and fills the available gap present in the looser portions of the wet blue goat leathers. The general assessment and physical properties of the crust leathers retanned with KH–Si and KH show encouraging results. But comparing with KH (control), the physical properties such as tensile, tear strength and organoleptic properties such as fullness, grain tightness and general appearance of the crust leathers retanned

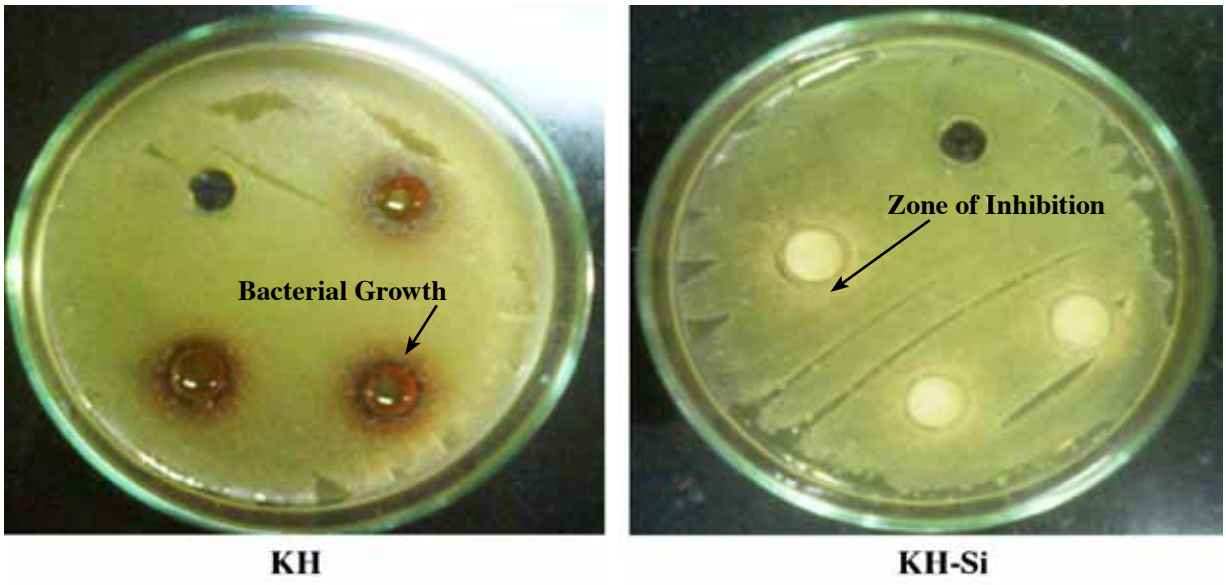


Figure 7. Effect of KH and KH–Si on the growth of *Bacillus substilis* in agar-well diffusion assay

TABLE 3

Variables of CIE color values for KH and KH–Si

ΔL	Δa	Δb	ΔC	Δh	ΔE
Lighter 41.454	Less red -8.133	Less yellow -4.999	Weaker -6.406	Increase 7.078	42.539

with KH–Si show marginally better values as compared to KH. The intensity of color is comparatively more in KH-Si retanned leathers due to presence Si complex which helps to improve the uptake of dye. The physical characteristics and visual assessment data for both KH–Si and KH retanned leathers are given in Tables 4 and 5 respectively. The use of KH and KH–Si in retanning process also influences lubricating effect that enhances the grain smoothness and softness characteristics of the leathers.

Effect of KH and KH–Si on Collagen Fibers

The scanning electron microphotographs of KH and KH–Si retanned leathers showing their cross section at a magnification of 1000x are given in Figure 8. From the figure it is evident that the fibre structure of KH and KH–Si retanned crust leathers do not show any adverse physical change. From the micrograph pictures it is observed that most of the interspaces are filled up with both keratin preparations, KH and KH–Si but the filling effect is better for leathers retanned with KH–Si. Fibre compactness is an indirect measure of fullness which is clearly evident from the visual assessment data of KH–Si retanned leather.

TABLE 4

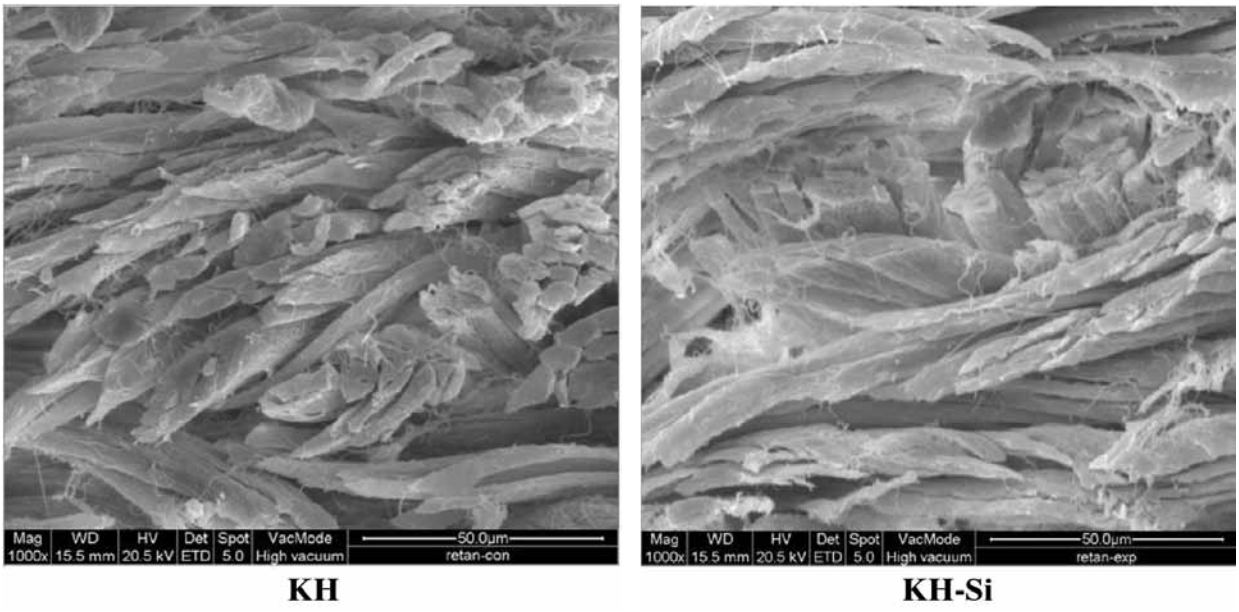
Physical testing data of KH and KH–Si retanned leathers

Parameter	KH retanned	KH-Si retanned
Tensile Strength kg/cm^2	256 ± 6	262 ± 4
Elongation at break %	66 ± 3	68 ± 4
Tear Strength kg/cm	57 ± 5	62 ± 3
Grain crack strength, Load (kg)	22 ± 2	24 ± 1
Distension at grain crack in mm	10.5 ± 0.2	11.2 ± 0.1

TABLE 5

Visual Assessment Data (scale 1-10)

Organoleptic Properties	KH	KH-Si
Fullness	7± 1	8±0.5
Grain Tightness	7±1	8±1
Softness	8±0.5	8±0.5
Grain smoothness	8±1	8±1
General Appearance	7± 1	8±0.5
Color intensity	7± 1	8±0.5



KH KH-Si

Figure 8. Scanning electron micrographs of KH and KH–Si retanned leather at a magnification of 1000x

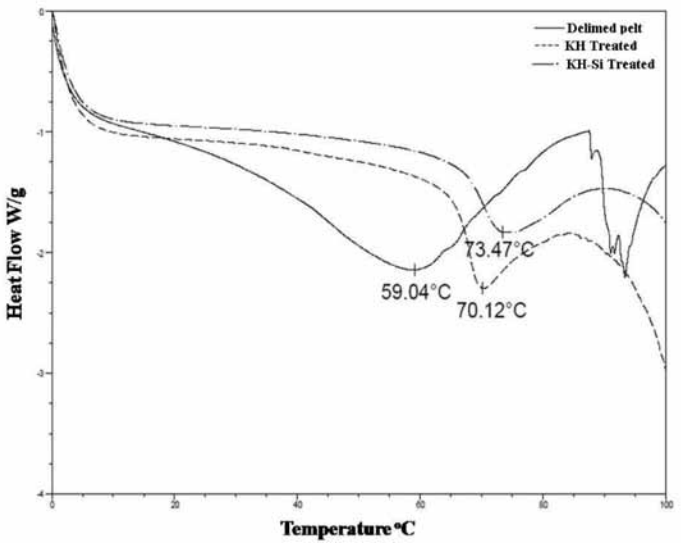


Figure 9. DSC profile for KH, KH-Si treated and delimed skin

Effect of KH and KH–Si on the Thermal Stability of Goat skin by DSC

In order to evaluate the tanning potency of KH and KH-Si, a delimed goat skin was cut into two sides along the backbone. One side was retanned with 12% of KH–Si and other side was with KH. The DSC profiles showing the denaturation temperature of KH–Si, KH treated and delimed skin recorded by DSC is presented in Figure 9. Denaturation temperature of KH–Si treated skin shows higher value (73.5ºC) compared to KH treated, and it is due to the fixation of silica species. Therefore from the DSC results it is confirmed that the retanning efficiency of KH–Si is comparatively better than KH. The use of silicates in pretanning processes would improve the uptake of auxiliaries especially of tannins, fatliqours and dyestuffs.[8,32] Thus from the DSC results it is revealed that KH–Si would also be used as a tanning agent for the stabilization of delimed pelts.

CONCLUSION

This investigation offers a method to use chicken feathers for the preparation of protein filler for use in retanning. The advantages of using silicate to convert keratin into water soluble keratin hydrolyzate are many fold. The use of silicate in the hydrothermal treatment of chicken feathers results in a light colored product. The low molecular weight peptides and amino acids present in KH–Si are preserved by silica species. These species impart antimicrobial character to the protein filler and also enhance its storage stability. The retanning action of the product KH-Si was marginally better than KH. In conclusion this study allows us (the authors/CLRI/ inventors) to offer tanners a commercially viable leather protein filler from waste chicken feathers.

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