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Novel method of hydrolysed collagen extraction from hide trimming waste

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Abstract

Hide trimming waste is a by-product of tannery. Collagen is the main structural protein in hide trimming waste (70%), and extensively utilised in numerous industries including food, non-food, cosmetics, and medical. Research related to the development of hydrolysed collagen (h-collagen) extraction methods from cowhide, especially from the hide trimming waste, is still limited. The present work thus aimed to develop a four-step method for extracting h-collagen from hide trimming waste, and examine the product's properties. The present work successfully developed a method for extracting h-collagen from trimming waste with a 20.35% yield. Analysis of molecular weight, FTIR, and amino acid composition confirmed that the product was h-collagen with a molecular weight of 16 - 23 kDa. This h-collagen had higher antioxidant activity than commercial h-collagen, with an IC_{50} value of 238.5 ppm.

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Introduction

Tannery generates between 50 and 60% of solid waste based on the total leather raw materials utilised (Matyasovsky *et al.*, 2012; Purba *et al.*, 2020). On average, cowhide and goatskin are utilised by the Indonesian leather industry around 264,000 tons per year. This generates around 145,000 tons of solid waste per year. Solid waste is generated from the fleshing, shaving, trimming, and buffing processes.

Hide trimming waste is a by-product of the tannery process involving smoothing salted hide before being tanned. Hide trimming waste is typically taken from the salted cowhide's head, tail, and edges. This procedure aims to produce a uniformly sized and shaped hide. Hide trimming waste accounts for over 75% of the solid waste generated by the tannery. The major components of hide trimming waste are the same as those of the original hide: 60% water, 25% protein, 2.5% fat, 2% carbohydrates, and 0.3% mineral salts (Sugihartono *et al.*, 2015).

Collagen is the main structural protein in skin, and also its main constituent (70%), including in hide trimming waste. Collagen is composed of various amino acids, usually a repeat of glycine, proline, and hydroxyproline ("-gly-pro-hyp-gly-"). Collagen has been widely used in various industries such as food, non-food, cosmetics, and medical. Extraction of collagen from raw materials containing collagen with hot water, acid, or alkaline produces a product known as gelatine. Further enzymatic hydrolysis of gelatine will produce hydrolysed collagen (h-collagen).

H-collagen has an extremely low molecular weight as compared to gelatine. It is water-soluble and biodegradable (Song and Li, 2017). H-collagen has long been used in medicines and foods in many countries and regions including the US, Europe, China, and Japan. In addition, the Food and Drug Administration Centre for Food Safety and Nutrition

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has classified h-collagen as a safe product (Generally Recognized as Safe) (Moskowitz, 2000). Based on *in vivo* studies, h-collagen exhibits significant antioxidant activity (Alemán *et al.*, 2011; Nakchum and Kim, 2016). In the non-food industry, h-collagen is utilised to produce biodegradable hydrogels as a natural polymer. The global collagen market is expected to expand at a compound annual growth rate (CAGR) of 9% from 2020 to 2028 (Grand View Research, 2021). This growth is believed to result from the increasing application in the food and beverage industry, cosmetic industry, healthcare industry, and the adaptation of collagen-based products by consumers.

Research on the development of h-collagen extraction methods from cowhide, especially from hide trimming waste, is still limited. Previous studies have concentrated mostly on collagen and gelatine extraction (Selvakumar *et al.*, 2012; Masilamani *et al.*, 2016). Therefore, the present work aimed to develop a method for extracting h-collagen from hide trimming waste in four stages, and evaluate the properties of the extracted h-collagen. The first stage was removing salt, hair, fat, and impurities. The second stage was acid or base pre-treatment to eliminate non-collagen proteins. The third stage was stepwise denaturation to produce gelatine, and the fourth stage was enzymatic hydrolysis to produce h-collagen. This method was expected to produce h-collagen with higher yield and better characteristics than existing methods.

Materials and methods

Materials and apparatus

The materials used were hide trimming waste, degreasing agent (DA), caustic soda (NaOH), ammonium sulphate ($(NH_4)_2SO_4$), bating agent, sodium chloride (NaCl), chloramine T ($C_7H_7ClNO_2Sna$), methyl cellosolve ($CH_3OCH_2CH_2OH$), citric acid monohydrate ($C_6H_{10}O_8$), glacial acetic acid (CH_3COOH), sodium acetate trihydrate ($C_2H_9NaO_5$), para-dimethylaminobenzaldehyde ($C_9H_{11}NO$), perchloric acid ($HClO_4$), hydrogen chloride (HCl), calcium chloride ($CaCl_2$), trypsin enzyme (HiMedia), DPPH, and ethanol (C_2H_5OH). The apparatus used were rotary drum, water bath shaker, centrifuge, freeze dryer, UV-VIS spectrophotometer, digital scale, volumetric pipette, and glassware.

Removal of salt, hair, and fat

The hide trimming waste was obtained from a tannery in Garut, West Java, Indonesia. The skin was obtained in the salted hide, and transported at room temperature. Upon sample arrival at the laboratory, it was stored in the freezer. The salt, hair, and fat removal process was based on the conventional pre-soaking, soaking, liming, fleshing, and deliming processes. The cleaned hide trimming waste was then stored in the freezer until they were used for further processing.

Size reduction

The hide trimming waste was cut into 5 × 0.5 cm using a knife. The hide was placed in a polyethylene bag, and frozen until needed. The frozen hide was thawed prior to use by allowing it to stand at 4°C for one night.

Alkaline or acid pre-treatment

Two different solutions were examined: alkaline (NaOH) and acid (CH_3COOH). At 25°C, the trimmed skin was immersed in 0.75 M NaOH with a skin/solution ratio of 1:10 (w/v) for 1, 2, 3, and 4 d. The mixture was manually stirred, twice daily. The skin was then washed with running water until neutral or slightly alkaline. Acid pre-treatment was performed by soaking the hide trimming pieces in 0.5 M CH_3COOH for 24 h, then neutralising with water to pH 5.0 - 6.0. Any other non-collagenous material discharged from the skin was eliminated during pre-treatment and washing.

Denaturation and enzymatic hydrolysis

The pre-treated hide trimming waste was put into a 250 mL Erlenmeyer flask, and heated gradually at different temperatures (60 - 90°C) for 9 h in a water bath. After that, it was cooled to 37°C. A certain amount of trypsin enzyme (0.6 - 1%) was added to the Erlenmeyer flask. The Erlenmeyer flask was then put into a water bath shaker incubator for 3 h at 37°C and 200 rpm. After that, it was removed, placed into a water bath, and heated at 80°C for 10 min to stop the enzymatic hydrolysis. After the temperature was lowered to room temperature, the h-collagen was separated from the solids using a centrifuge at 5°C, 12,000 rpm for 10 min. The liquid phase was collected and stored in the freezer, then dried with a lyophiliser (freeze-drying) to obtain h-collagen powder.

Yield

Collagen yield was calculated by comparing h-collagen's dry weight with hide trimming waste before extraction following the AOAC (1995) method.

Molecular weight

The molecular weight of the h-collagen protein was analysed using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The separating gel was prepared to harden in the glass for 60 min, then stacking gel was added. A total of 10 mL of sample and 5 mL of the marker were injected into the electrophoresis well. The marker used was a protein marker with a 10 - 250 kDa molecular weight. Electrophoresis was run constantly at a current of 13 mA and 100 volts for 2 h. The gel was removed from the glass slab, and staining and destaining processes were performed. Molecular weight detection was performed using Photocapt.

Amino acid composition

Ultra-performance liquid chromatography (UPLC) was used to analyse the amino acids. The UPLC device was cleaned for 2 - 3 h with eluent prior to use. The UPLC analysis of amino acids involved two stages: the preparation of a sample solution and a standard solution.

Functional group

A Fourier transform infrared spectrophotometer (FTIR) was used to analyse distinct functional groups in h-collagen. H-collagen was formed into pellets with a mixture of KBr. Infrared light was fired from the infrared spectrophotometer IR-408 nm onto the pellets. The frequency of the collagen wave number was read in the range of 500 - 4000 cm^{-1}.

Antioxidant activity

Antioxidant activity was determined using the DPPH method (1,1-diphenyl-2-picrylhydrazyl) as described by Shimada *et al.* (1992) with modification. A total of 0.5 mL of sample was added to 0.5 mL of 1 mM DPPH in ethanol. The mixture was shaken and left for 30 min at room temperature. The solution's absorbance was measured at 517 nm with a UV-VIS spectrophotometer. Ascorbic acid was used as a positive control. The DPPH blank was 10 mL of ethanol mixed with 0.5 mL of ethanol containing 1 mM of DPPH. The antioxidant activity of the sample was determined based on the absorption resistance of DPPH particles by calculating the percentage of DPPH absorption inhibition using the Molyneux formula (Molyneux, 2004). All experiments were carried out in triplicate.

Experimental design

The present work used a completely randomised design with two factors. The first factor was the denaturation temperature consisting of three levels (S1: 60, 65, and 70°C; S2: 60, 70, and 80°C; S3: 60, 75, and 80°C). The second factor was the trypsin concentration consisting of three levels (T1: 0.6%; T2: 0.8%; and T3: 1%). Each treatment was repeated three times. If the treatment had a significant effect, the results were assessed through Duncan's multiple comparison test. The mathematical equation model is presented in Eq. 1:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk} \quad \text{(Eq. 1)}$$

where, i = 1, 2, 3 (denaturation temperature); j = 1, 2, 3 (enzyme concentration); k = 1, 2 (repetition); Y_{ijk} = observation of factor at level i and j, and repetition k; μ = average; α_i = effect of denaturation temperature factor at i level; β_j = effect of trypsin enzyme concentration factor at j level; $\alpha\beta_{ij}$ = interaction of factors at levels i and j; and ε_{ijk} = effect of error at i and j levels, and the k repetition.

Statistical analysis

A completely randomised experimental design was carried out with three replicates per treatment. Data were subjected to Bartlett's test for homogeneity of variances, analysis of variance, and Duncan's multiple range test using GenStat Release 11.1. Statistical significance was considered at $p < 0.05$.

Results and discussion

Alkaline and acid pre-treatment

Collagen found in animal connective tissue such as hide trimming waste has cross-links that make it very difficult to dissolve even in boiling water. Due to its triple-helix structure, collagen is resistant to most proteases including trypsin (Brodsky and Ramshaw, 1997). Therefore, pre-treatment with alkali or acid is required to disrupt the triple-helix structure, thus resulting in swollen insoluble collagen with an original disrupted structure (Stainsby, 1987). In addition, the quaternary structure (internal cross-

linking between tropocollagen molecules) is also disturbed. The present work tested two materials commonly used for collagen pre-treatment: NaOH and CH_3COOH. Results showed that after 24 h of soaking in 0.5 M CH_3COOH, the hide trimming waste was completely dissolved. Immersion in CH_3COOH causes the intramolecular hydrogen bonds that stabilise collagen's triple-helix structure to break (Ahmad and Benjakul, 2010). It is considered that the cause was the dissolution of the entire hide trimming waste and the high concentration of acetic acid. Hence, no material could be used for further processes. As a result, the CH_3COOH pre-treatment technique was not selected.

Pre-treatment with NaOH was carried out with several variations of immersion time namely 1, 2, 3, and 4 d. During the pre-treatment, the alkaline breaks down most of the telopeptide linkages (crosslinks at the N-terminal) in the collagen molecule (Yoshimura *et al.*, 2000). After 4 d of soaking, the hide trimming samples were almost totally dissolved, with only a small number of particles remaining after separation. The observations found that the weight of the hide trimming samples soaked for 1 and 2 d was not much different. As a result, immersion in 0.75 M NaOH for 1 d was chosen for the pre-treatment method.

Stepwise denaturation

Heat can interfere with the hydrophobic interactions and hydrogen bonds in collagen; heat increases kinetic energy, and causes the molecules to vibrate so fast and strong that the bonds that maintain the structure of collagen are disrupted. Heat breaks the hydrogen and covalent bonds during the stepwise denaturation process, transforming the collagen chain's triple-helix structure into a helix to coil transition structure that yields gelatine (Djabourov *et al.*, 1993). Covalent and non-covalent bonds are broken, releasing free oligomers and chains (Johnston-Banks, 1990). Furthermore, some amide bonds from the original collagen structure are broken during denaturation (Bailey, 1985). The resulting gelatine contains a mix of polypeptides with lower molecular weight.

Effect of enzymatic hydrolysis

Protein hydrolysis by proteolytic enzymes is prevalent in the food industry. Enzyme hydrolysis has several advantages including lower extraction temperatures, the production of relatively few salt by-products, and the absence of amino acid degradation (Marcet *et al.*, 2016). Protease enzymes hydrolyse gelatine by cleaving certain peptide bonds. Trypsin is a protease enzyme that has been widely employed in the production of h-collagen (Ketnawa *et al.*, 2017; Abdollahi *et al.*, 2018). Heat treatment given in the stepwise denaturation stage makes the peptide bond more susceptible to the enzyme's active site, thus allowing the enzyme to function more optimally (Zhang *et al.*, 2006). Zhang *et al.* (2013) found that heating bovine collagen can increase the hydrolysis process by most enzymes such as collagenase, thermolysin, trypsin, and proteinase.

H-collagen yield

The highest yield of h-collagen (20.35%) was obtained from the stepwise denaturation at 60, 70, and 80°C, and the trypsin enzyme concentration of 1% ($p < 0.05$). It was higher than that of h-collagen from other sources including collagen (12.59%) from chicken skin (Budiarti *et al.*, 2019), acid-soluble collagen (5.1%) and pepsin-soluble collagen (7.7%) from striped catfish skin (Singh *et al.*, 2011), pepsin soluble collagen (8.86%) from bamboo shark skin (Kittiphattanabawon *et al.*, 2010), acid-soluble collagen (6.4%) from large-eye snapper skin, acid-soluble collagen (9%) and pepsin-soluble collagen (4.7%) from red snapper skin (Jongjareonrak *et al.*, 2005), and pepsin-soluble collagen (6.2%) from fish yellowfin tuna (Fauzi, 2018).

It was suspected that increasing the extraction temperature to more than 70°C and extending the extraction period to 9 h would improve the yield of h-collagen. Increasing the extraction temperature up to 70°C could play an important role in breaking the bonds between the chains of the collagen molecule, thus resulting in more chains and chain dimers from the skin complex, which helps in extracting higher gelatine from the skin matrix (Sinthusamran *et al.*, 2014). The prolonged extraction time provides a greater opportunity to break down cross-links in collagen, thus facilitating gelatine extraction (Al-Kahtani *et al.*, 2017). Al-Hassan (2020) also reported that increasing the temperature and extending the extraction time could result in a higher gelatine yield. The higher gelatine yield from the stepwise denaturation process will increase the opportunity to obtain greater h-collagen during the trypsin enzymatic hydrolysis.

Analysis of variance (ANOVA) showed that the interaction between the denaturation temperature factor and the trypsin enzyme concentration had a

significant effect ($p < 0.05$) on the h-collagen yield (Table 1). Therefore, the most efficient denaturation temperature and trypsin enzyme concentration in producing h-collagen yield was stepwise denaturation at 60, 65, and 70°C, and trypsin enzyme concentration of 0.8%.

Table 1. Yield of h-collagen from hide trimming waste.

Treatment	Yield (% b/b)
S2 T1	14.15[a]
S3 T1	17.37[ab]
S3 T3	17.84[b]
S1 T3	18.78[b]
S2 T2	18.85[b]
S1 T1	19.39[b]
S3 T2	19.45[b]
S1 T2	19.69[b]
S2 T3	20.35[b]

Values are mean of triplicates ($n = 3$). Means followed by different lowercase superscripts are significantly different ($p < 0.05$).

Molecular weight of peptide chain in h-collagen

SDS-PAGE examination revealed bands with molecular weights of 23, 19, and 16 kDa in the samples (Figure 1).

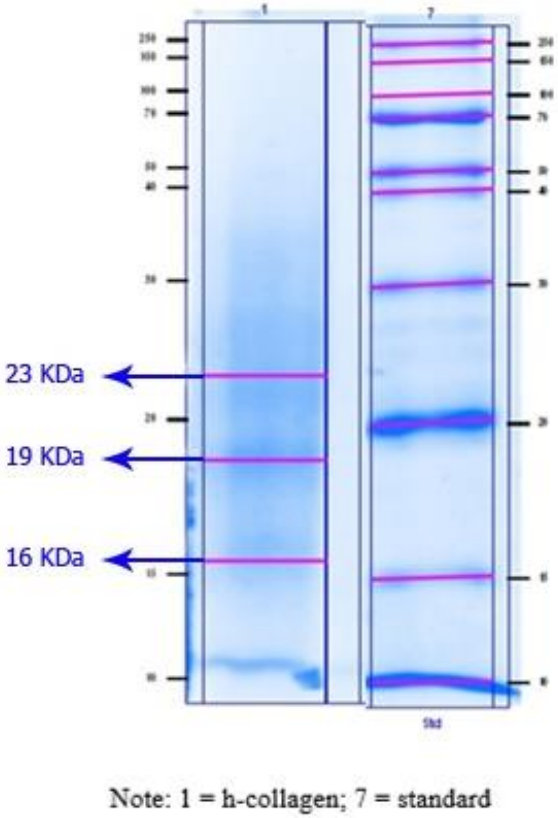

Figure 1. Protein bands of h-collagen from hide trimming waste.

Gelatine derived from cowhide has a chain of approximately 100 kDa, a component of approximately 200 kDa, and is classified as type I collagen (Gómez-Estaca *et al.*, 2009). These findings indicated that the method proposed in the present work was capable of hydrolysing all main collagen components including the α and β chains into peptides with molecular weights ranging from 23 to 16 kDa. This was in accordance with several previous studies using trypsin in producing h-collagen. Li *et al.* (2013) reported that h-collagen isolated from the skin and bones of Spanish mackerel with trypsin at 37°C for 3 h produced h-collagen with a molecular weight of less than 20 kDa. Abdollahi *et al.* (2018) found that hydrolysis with the combination of pepsin and trypsin on the skin and bones of silver carp produced h-collagen with a molecular weight between 15 and 10 kDa.

Amino acid analysis

The amino acid content of h-collagen was analysed using UPLC with 17 amino acid standards. Amino acids are a group of hydrogen-peptides that form a bond as the main component of protein. Based on their structure, amino acids consist of an amino group (NH_2), a carboxyl group (COOH), a hydrogen atom (H), and a radical I group bonded to a C atom (Voet *et al.*, 2013). Results indicated that h-collagen consisted of various types of amino acids with the largest concentrations of glycine (30.01%), proline (15.26%), arginine (10.19%), and glutamic acid (8.96%). This was nearly identical to the findings of earlier investigations. Balti *et al.* (2011) and Lassoued *et al.* (2014) discovered that halal beef gelatine contained 34.1 and 34.48% glycine, respectively, and 12.3 and 13.39% proline, respectively. The molecular band of h-collagen from hide trimming waste is presented in Figure 2.

Glycine is the main amino acid that affects the formation of the triple-helix structure of proteins (Piez, 1984). The amino acid glycine is also the largest constituent of collagen, which corresponds to the (Gli-X-Y)n chain pattern in collagen, with X being proline and/or Y being hydroxyproline. Moreover, glycine in collagen represents one-third of the total residues. It occurs in every one-third of the residues in collagen, except the first 14 amino acid residues of the N-terminus, and the first ten residues of the C-terminus (Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2010). The presence of polar amino acids, notably asparagine, glutamic acid,

arginine, and lysine is a further hallmark of collagen's amino acid composition (Gauza-Włodarczyk *et al.*, 2017). Therefore, it can be stated that h-collagen derived from trimming skin has a suitable amino acid profile.

Functional group

The h-collagen spectrum from the hide trimming waste showed the position of amide A band (associated with the N-H stretch) at 3360 cm^{-1} (Figure 2). The presence of amide A band confirmed the presence of hydrogen bonds (Zhang *et al.*, 2016). Amide B band of h-collagen was observed between 3070 - 2937 cm^{-1}. Amide I band of h-collagen was observed between 1650 - 1700 cm^{-1}. Amide I band indicates the secondary structure and the bond between the N-H (position X) and CDO (Gly) strains (Veeruraj *et al.*, 2015). The FTIR spectrum showed the secondary structure of collagen with the appearance of amide II bands (N-H bending vibration coupled with C-N stretching vibration) typical of collagen at 1500 - 1550 cm^{-1}. Santos *et al*. (2013) found that collagen from the bovine pericardium had amide I band between 1680 - 1800 cm^{-1}, and amide II band between 1470 - 1570 cm^{-1}. Amide III (associated with C-N deformation and N-H stretching) was found at 1242 cm^{-1}. FTIR investigations showed that the spectrum obtained for h-collagen from skin trimming was similar to collagen from other sources. Overall, FTIR represented a typical amino acid and collagen molecule.

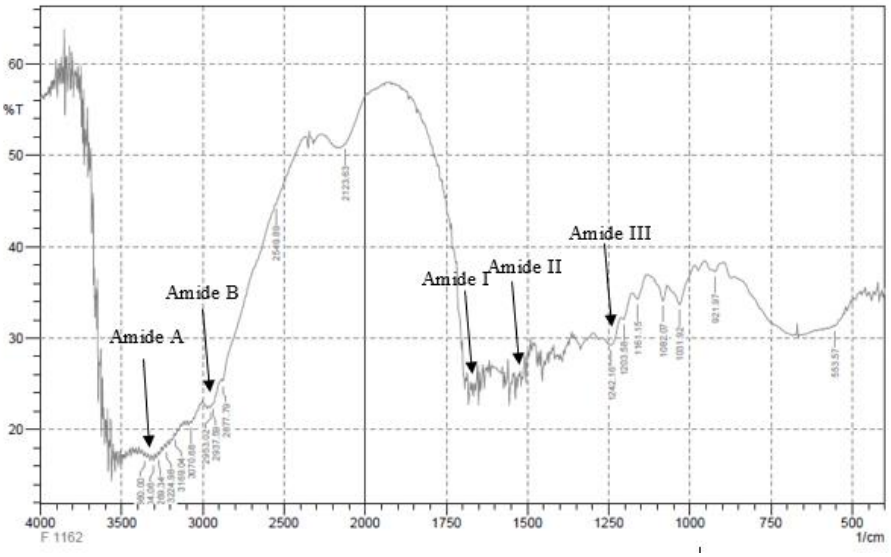

Figure 2. Infrared spectra of h-collagen from hide trimming waste.

Antioxidant activity

In the present work, the antioxidant activity of h-collagen was measured by using the DPPH method. The DPPH radical scavenging assay method was chosen because it is fast, easy, and efficient in predicting the antioxidant activity of protein hydrolysates (Li *et al.*, 2008). Free radical scavenging is the main mechanism by which antioxidants inhibit oxidative processes. Single-electron DPPH showed a strong modena and absorbance at a wavelength of 517 nm in ethanol solution. This absorbance value gradually decreases when DPPH meets proton donor substrates such as antioxidants (Li *et al.*, 2013; Jridi *et al.*, 2014).

The IC_{50} value was obtained from the regression curve between the absorbance measured at 517 nm and the sample concentration. The IC_{50} value of h-collagen extracted in the present work was 238.5 ppm. It was higher than the IC_{50} value of h-collagen from yellowfin tuna skin, collagen peptides from croceine croaker fish scales, and commercial h-collagen (Table 2). Therefore, h-collagen from hide trimming waste could have good antioxidant activity. The ability of h-collagen as an antioxidant is influenced by its hydrophobic amino acids, which were reported to enhance the reduction power of peptides (He *et al.*, 2013). The h-collagen from the present work mainly contained glycine and proline, which have reduction power (Table 3). This was in agreement with Mendis *et al*. (2005) who reported that hoki skin gelatine peptides (His-Gly-Pro-Leu-Gly-Pro-Leu) were able to act as a strong radical scavenger.

Table 2. IC_{50} value of h-collagen from hide trimming waste.

Material	IC_{50} to DPPH (ppm)	Source
H-collagen	238.5	Hide trimming waste
H-collagen[a]	251.23	Yellowfin tuna skin
Collagen peptide[b]	283	Croceine croaker fish scales
Commercial h-collagen[a]	> 400	-
Commercial collagen[a]	> 1000	-
Ascorbic acid[a]	2.88	-

[a]Fauzi (2018); [b]Li *et al.* (2008).

Table 3. Amino acid content of h-collagen from hide trimming waste.

Amino acid	Concentration (mg/kg)	Percentage (%)
Glycine	245,200.80	30.01
L-proline	124,695.54	15.26
L-arginine	83,279.59	10.19
L-glutamic acid	73,196.06	8.96
L-alanine	72,432.74	8.87
L-aspartic acid	37,193.39	4.55
L-serine	33,137.46	4.06
L-leucine	28,434.77	3.48
L-lysine	26,073.43	3.19
L-valine	21,713.16	2.66
L-phenylalanine	20,789.60	2.54
L-threonine	15,715.13	1.92
L-isoleucine	14,518.76	1.78
L-histidine	7,600.43	0.93
L-methionine	7,404.43	0.91
L-tyrosine	5,074.26	0.62
L-cysteine	506.28	0.06

Conclusion

The present work succeeded in developing a method of extracting h-collagen from hide trimming waste, solid waste of the leather tanning industry. This method consisted of four stages. The first stage involved removing salt, hair, and fat, followed by pre-treatment with NaOH (second stage). The third stage involved stepwise denaturation at 60, 65, and 70°C for 3 h each. The fourth stage involved enzymatic hydrolysis with 0.8% trypsin. This extraction method could produce h-collagen with a yield of 20.35%. Analysis of molecular weight, FTIR, and amino acid composition confirmed that the resulting product was h-collagen with a molecular weight of 16 - 23 kDa. The extracted h-collagen had higher antioxidant activity than commercial h-collagen with an IC_{50} value of 238.5 ppm. H-collagen can be used to manufacture superabsorbent hydrogels (SAP) for applications in various sectors such as hygienic products, agricultural applications, and controlled drug delivery systems. In addition, the h-collagen produced can also be used for health and beauty products because of its good antioxidant value.

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