

Amino Acids Isolation from α -keratin of Javanese Goat (*Capra hircus*) Hair and Garut Sheep (*Ovis aries*) Hair Waste Using Acid Hydrolysis Method as BCAA Supplement

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Abstract

Javanese goat and Garut sheep hair contain α -keratin, a protein that can be broken by hydrolysis to produce simpler amino acids. Feather waste generates millions of tons of α -keratin biomass originating from animal slaughterhouses, thereby raising health concerns. The utilization of acid hydrolysis is considered to be more cost-effective compared to enzymatic hydrolysis, and it provides a broader range of amino acid cleavage sites compared to enzymes, which exhibit specific cleavage. This study aimed to isolate amino acids from Javanese goat and Garut sheep hair through acid hydrolysis. The methods included hair sample preparation, acid hydrolysis used 6 M HCL at 110°C, reflux isolation, amino acid separation based on isoelectric pH 4.9 –5.4, functional groups analysis using FTIR, and analysis of amino acid content by HPLC methods. The results showed that the yield produced after isolation on Javanese goat hair samples was 0.92% and Garut sheep hair 0.32%, respectively. The FTIR spectrum showed amino acid functional groups in both samples, including carboxyl (COOH), amine (C-N primer), (C-S disulfide), and amide I (-CONH₂). Successful breakdown of α -keratin proteins into simpler amino acids was achieved for Javanese goat and Garut sheep hair. Amino acid analysis of Javanese goat hair isolates revealed the presence of aspartic acid, threonine, serine, glutamate, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine amino acids, respectively. The highest content was isoleucine at 0.60% w/w. In conclusion, the isolated amino acids from Javanese goat hair can be used as a halal supplement that serves as nutrition in the body.

Keywords: Javanese goat; Garut sheep; α -keratin; amino acid

1. INTRODUCTION

The growth of urbanization encourages and causes the food sector, especially the meat market, animal slaughter facilities, and the leather industry, to produce amounts of keratin biomass reaching millions of tons¹. Among countries, New Zealand, China, Australia, Iran, and Argentina stand out as leading wool producers^{2,3}. This global production translates to over 67.5 million tonnes of wool and feathers annually, with wool itself exceeding 2.5 million tonnes^{4,5}. Keratin biomass of sheep and goat hair waste from the slaughterhouse produces considerable hair waste that decomposers

cannot directly degrade, this waste can lead to pollution and other health problems, additionally, accumulation or landfill methods can disturb soil fertility and other functions and a large number of keratin biomass is a potential threat to the environment^{1,6}.

Hair is composed of abundant keratin protein and is known for its hardness and fibrous nature. It is a major component of mammals, reptilians, bird hair, nails, hair, horns, and beaks. It is the third most abundant polymer after cellulose and chitin⁷. Keratin is mainly found in two forms α and β -keratin. α -keratins are abundantly found in soft tissues such as sheep wool, skin,

and hair. These are rich in cysteine and contain fewer amounts of hydroxyproline and proline amino acids. However, β -keratins are present in the hard tissue protein of bird feathers, fish scales, nails, and others⁸.

Keratin proteins can be hydrolyzed into amino acids and simpler compounds through chemical and enzymatic hydrolysis. Enzymatic hydrolysis is more expensive and requires a longer time of operation⁹. Chemical hydrolysis is commonly employed for industrial purposes, although the results are less specific. Chemical hydrolysis is less influenced by environmental conditions, making it more cost-effective for industrial applications¹⁰. Keratin from sheep wool was successfully hydrolyzed using a combination of chemical (base) and enzymatic methods, resulting in a keratin hydrolysate of 24.05%-27.25% (w/w)⁶. Additionally, keratin from sheep hair was also successfully hydrolyzed using hydrogen peroxide through a conventional process, yielding hydrolysates of 64.99%, 64.30%, and 60.50%¹¹.

Essential amino acids are amino acids highly important and cannot be synthesized by the body. Branched-chain amino acids (BCAAs) comprising valine, leucine, and isoleucine, are essential amino acids. In addition to participating in de novo protein synthesis, BCAA functions as potent nutrient signal molecules for cellular metabolism and growth, such as glucose and lipid metabolism, gut health, and immunity¹². The unique properties of BCAAs make them of integral importance for skeletal muscle metabolism. Thus, BCAAs have long been viewed as potential candidates for supplementation. The BCAA facilitates the ability of muscle fibers to absorb blood sugar and modulate insulin signaling¹³. The global amino acids market is projected to hit USD 48.3 billion by 2030, driven by a steady CAGR of 8.5% from 2024 to 2030. This growth is attributed to the rising demand for Branched-Chain Amino Acids (BCAA) like leucine, valine, and isoleucine for their use in functional food & beverages and dietary supplements¹⁴. In addition, to its beneficial effects on muscles and the body, BCAA supplements are halal supplements that Muslim communities can consume because they are derived from the waste hair of Javanese goats and Garut sheep that have been slaughtered previously. According to The State of the Global Islamic Economy Report 2020/2021, the global halal product market reached USD 2.02 trillion¹⁵. This figure indicates that the halal industry has tremendous market potential, especially for BCAA supplements.

This study proposes a novel approach to harnessing α -keratin biomass from Javanese goats and Garut sheep hair waste as a potential source of Halal Branched-chain Amino Acids (BCAA). This approach entails isolating the waste material through an acid hydrolysis process. This research aims to isolate amino acids from Javanese goat hair (*Capra hircus*) and Garut sheep (*Ovis aries*) using acid hydrolysis. The isolated

amino acids from Javanese goat hair can be used as a halal BCAA supplement that serves as nutrition in the body and muscles.

2. RESEARCH METHODS

Instrumentations and Materials

The instruments used in this research were Fourier Transformation Infrared (FTIR) (BB MB3000), and High-Performance Liquid Chromatography (HPLC) (Shimadzu). The tools used in this study were oven (Mettler), aluminum foil (Klin), chemical glassware (Iwaki), Florence pumpkin 500 mL (Pyrex), digital balance (Kenko), Whatman filter paper no 40 (Cytiva), reflux equipment, titration equipment, thermometer (Pyrex), acid room, glass stirrer rod, heating mantle (Witeg), hot plate (B-ONE). The materials used in this study were Garut sheep (*Ovis aries*) hair taken from Sukabumi, West Java, 2 years old, and Javanese goat (*Capra hircus*) hair taken from Blitar, East Java, 2 years old. HCl (Supelco), activated carbon (Norit), NaOH (Merck), dan congo red, CuSO₄ 1%, Ortho phthalaldehyde (OPA), and (Potassium borate) BK₃O₃.

Preparation of raw materials

The main samples used are the hair of slaughtered Garut sheep taken from Sukabumi, West Java, aged 2 years, and the hair of slaughtered Javanese goats taken from Blitar, East Java, aged 2 years. The hair from sheep and Javanese goats intended for use is separated from impurities, such as leaf residues and soil. The sorted hair was washed with water, dried at room temperature for approximately 3 h, and stored in a refrigerator. Once dried, the hair was sorted again to select fibers free from impurities, and a 100-gram sample was then carried out to amino acid isolation¹⁶.

Amino Acid Isolation

The hair samples from sheep and Javanese goats were hydrolyzed in a 500 mL Florence flask with the addition of 400 mL of 6 M HCl solution. The flask was placed on a heating mantle and heated at 110°C. Samples were added in 10-gram increments until reaching the desired amount of 100 grams, heating continued until complete dissolution. After dissolution, reflux heating at 110°C was carried out for 5 hours. The entire process was conducted in an acid chamber. A small sample was subjected to the Biuret test. If positive, reflux heating at 110°C was repeated for 5 hours until the Biuret test result was negative. Activated carbon (6 g) was added to remove color, and the mixture was left to stand for 24 hours. After decantation to separate the activated carbon, the sample was filtered using a Buchner funnel, and any remaining solid residue was rinsed twice with distilled water¹⁷. The formed solid was dissolved in 100 mL of 40% NaOH with stirring. Congo Red indicator was added, and the mixture was titrated with 6 M HCl until turning blue. Samples were stored for 72 hours at room

temperature. Solids and liquids were separated using a Buchner funnel, dissolved in 150 mL of 1 M HCl, and treated with 4 g of activated carbon. After 24 hours, the solution was filtered, and the filtrate was collected. Congo Red test was performed, and if positive, 4 g of activated carbon was added again, repeating the process until the result was negative (blue). The solution then underwent separation with isoelectric pH until reaching the keratin pI. After titration, the precipitated amino acids were filtered, rinsed, and dried. The yield of isolated amino acids is calculated using the following formula:

$$\text{Amino acid yield (\%)} = \frac{\text{Amino acid weight} \times 100\%}{\text{Sample raw weight}}$$

Biuret Test

Sample solution was taken for 1.5 mL, and 1 mL of 1% CuSO₄ solution was added. Subsequently, 1 mL of 1% NaOH solution is added, and the solution is homogenized. Egg white is used as a positive control, and distilled water is used as a negative control. A positive result for the biuret test was indicated by a violet color¹⁸.

Separation with Isoelectric pH

The solution, which has been added with 6 M HCl, is taken, and its initial pH is measured. It is then titrated with 0.05 M NaOH solution, and the amount of solution added is recorded until a precipitate form, and its pH reaches the keratin pI (4.9 –5.4)¹⁹. Further ANOVA analysis of precipitation yield between Javanese goat hair and Garut sheep hair samples²⁰.

Characterization of Isolated α -Keratin by FTIR

The spectrum of the isolated sample is analyzed using the potassium bromide (KBr) pellet as a matrix at a wavelength of 4000-400 cm⁻¹ on the ABB MB3000 FTIR spectrophotometer. Sample 1.5 mg is mixed with 300 mg KBr and ground with a special mortar. The mixture is pressed using the E-Z pressTM 12 Ton Hydraulic Presses from International Crystal Laboratories with a pressure of 4000 psi. The pellet was placed on a holder for subsequent readings^{21,22}.

Amino Acid Analysis with HPLC

The sample was dissolved in 5 mL of 0.01 N HCl and then added to a potassium borate solution at pH 10.4 in a 1:1 ratio. The solution was then placed in an empty vial at 50 μ L, and 250 μ L of OPA (Ortho phthalaldehyde) reagent was added and left to stand for 1 min in a dark room. Subsequently, the sample was injected into the HPLC column at 5 μ L, and then all

amino acids were separated for approximately 25 min^{23,24}.

3. RESULTS AND DISCUSSION

Isolation and Acid Hydrolysis of Amino Acids in Sheep and Javanese Goat Hair

Results showed that the sample solubility was not so high during the hydrolysis process (Figures 1a and 1b). To optimize the hydrolysis process, reflux was performed for 5 hours at 110 °C. The hair samples that underwent reflux completely dissolved in the acid, producing a dark brown solution, and leaving insoluble black powder at the bottom of the Florence flask (Figure 1a and 1b). Both samples released water vapor during the heating process, and the reflux process helped to retain the water vapor in the sample solution. The solubility process indicates that the protein compounds in sheep and goat hair have been successfully hydrolyzed. The chemical constituents in the hair are keratin, a structural protein commonly found in hair, fur, and nails²⁵. The keratin structure is stabilized by intermolecular and intramolecular interactions such as disulfide bridges, hydrogen bonds, hydrophobic interactions, and ionic bonds²⁶. The hair content is mainly dominated by the α -keratin protein type, while the β -keratin protein type dominates in structures like nails, and claws in reptiles, and beaks in birds¹.

α -Keratin is distinguished by its low sulfur content compared to β -keratin. Molecular analysis of α -keratin reveals helical chains that cause chain rotations and display a spiral shape due to the presence of disulfide bonds²⁷. α -keratin is mostly reported to consist of amino acids such as cysteine, methionine, phenylalanine, isoleucine, proline, and aspartic acid, while histidine, methionine, tryptophan, and tyrosine are rarely found^{28,29}. Adding 6 M HCl and high-temperature heating can break the disulfide bonds (S - S) and cleave some peptide bonds in keratin fibers. This process leads to the breakdown of the α -keratin protein structure into amino acids and simpler compounds rich in amino acids, making them soluble in acid (Figure 2). The hydrolysis process can break the peptide bonds in the protein structure³⁰. Combination of concentrated 6 M HCl at a temperature of 110°C can break down keratin into simpler amino acids¹⁷. After acid hydrolysis, a biuret test is conducted to demonstrate the presence of peptide bonds, indicating the presence of proteins in the hydrolyzed sample¹⁸. A positive biuret test is indicated by a color change to purple. Conversely, a negative or unchanged color in the biuret test indicates a negative result.

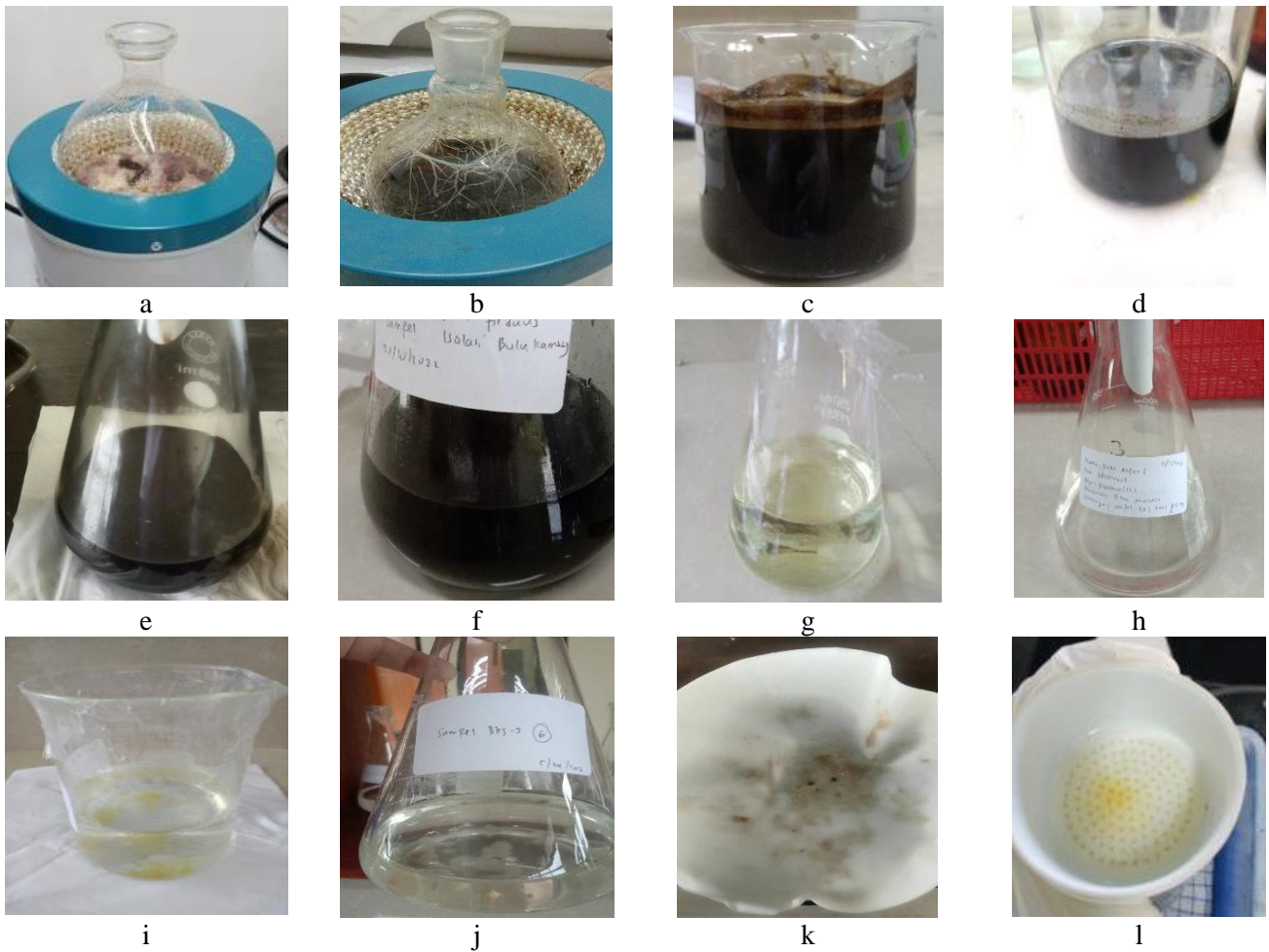


Figure 1. Amino acid isolation process from Garut sheep hair and Javanese goat hair. (a) Acid hydrolysis process of Garut sheep hair sample, (b) Acid hydrolysis process of Javanese goat hair sample, (c) Javanese goat hair sample dissolved in acid, (d) Garut sheep hair sample in acid, (e) Garut sheep hair sample after adding 6 grams of activated carbon, (f) Javanese goat hair sample after adding 6 grams of activated carbon, (g) Garut sheep hair sample after adding 4 grams of activated carbon, (h) Javanese goat hair sample after adding 4 grams of activated carbon, (i) Precipitate of Garut sheep hair sample, (j) Precipitate of Javanese goat hair sample, (k) Yield of Javanese goat hair sample, (l) Yield of Garut sheep hair sample.

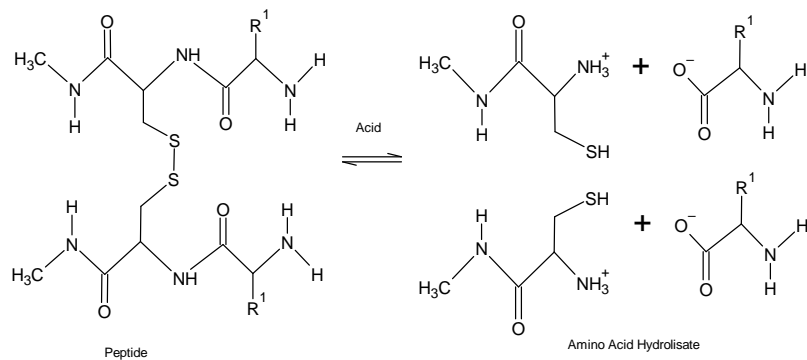


Figure 2. Peptide chain cleavage mechanism in keratin through acid hydrolysis³¹.

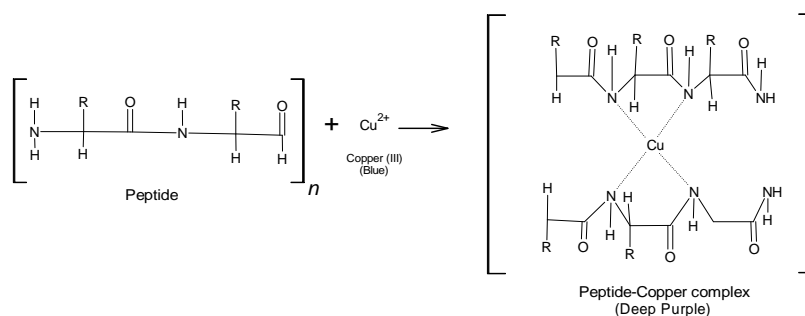


Figure 3. Complex Formation Reaction in the Biuret Test ³².

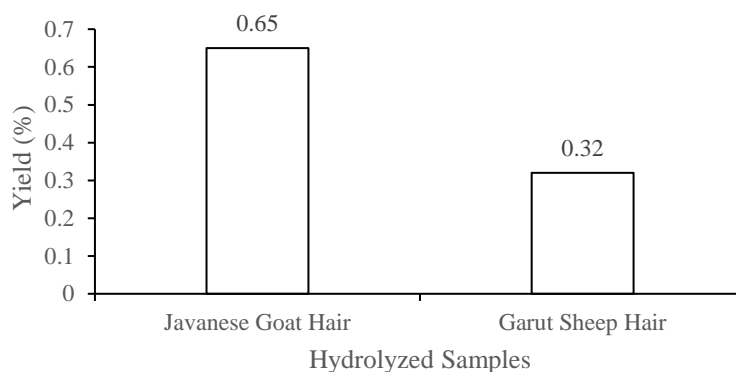


Figure 4. Comparison of the quantity of yields from Garut sheep hair and Javanese goat hair.

The results of the biuret test for both samples, sheep hair and goat hair, showed negative results, marked by no color change to purple. This indicates hydrolysis has progressed well, and the proteins have transformed into simpler compounds. Color change to purple occurs in the egg white control tube (Figure 3). This change is formed from the bond between Cu^{2+} and peptide bonds ³³. Meanwhile, distilled water as a negative control produces a negative reaction with a blue color. The hydrolyzed samples of goat and sheep hair did not show a purple color change, indicating that the peptide bonds have been broken and that the α -keratin protein has undergone hydrolysis.

The samples produced after acid hydrolysis appeared dark brown. To remove this color, activated carbon was added in two stages. In the first stage, 6 grams of activated carbon were added. The color removal process took place for 24 hours. The addition of activated carbon to the sheep hair sample resulted in two phases. The lower phase was dark brown, while the upper phase was clear. Similarly, the Javanese goat hair sample's lower phase was dark brown, and the upper phase was less visibly clear. The mixture was then filtered using a Buchner funnel, resulting in a lighter brown filtrate and a black residue (Figure 1c and 1d). The residue was dissolved by adding 100 mL of 40% NaOH and stirring with a stirring rod. The result was a residue that could dissolve in the base. Color removal from the sample is necessary for easier observation of the amino

acid precipitate produced during separation using isoelectric pH, so the liquid sample is treated with decolorization by adding activated carbon ³⁴.

The sample was then filtered using a Buchner funnel, and the black-colored residue was dissolved by adding 150 mL of 1 M HCl. The sample still appeared turbid brown, so a second addition of 4 grams of activated carbon was made and left to stand for 24 hours. This was done to remove the color from the sample so that the precipitation with the isoelectric pH of amino acids could be easily observed. Both samples formed two phases, with the lower phase being a black precipitate and the upper phase being a clear solution. The sample was filtered using a vacuum pump and a Buchner funnel (Figures 1e and 1f). A clear 150 mL filtrate was taken for precipitation using the isoelectric pH.

Separation and Precipitation of Hydrolysate Samples from Sheep Hair and Javanese Goat Hair with Isoelectric pH

The yield results obtained from the isoelectric point (pI) titration on the sheep's hair sample are 0.32%, and the results obtained from the goat's hair sample are 0.65% (Figure 4). The goat hair isolate as the highest yield sample is used for analysis with HPLC to determine the concentration and types of amino acids.

The ANOVA results indicate that there is no statistically significant difference between the groups in terms of the amount of variation between groups ($F =$

0.6433, $p = 0.4293$). This suggests that the mean amount of variation between groups is not significantly different from the mean amount of variation within groups.

The obtained results are still lower compared to sheep hair hydrolyzed with NaOH and H₂O₂ which were 60.50–64.99%¹¹, and was also 2.88% lower than that of chicken hair keratin⁴⁰. However, it is **still higher** than the keratin yield from sheep hair hydrolyzed with Br ions at 100°C and sheep hair hydrolyzed with choline thioglycolate at 130 °C³⁸ (Table 1). This is attributed to the influence of the temperature used during hydrolysis^{36,39}. Keratin precipitation occurs due to the addition of HCL. Proteins in an HCl solution have a positive charge, and the number of positive and negative charges on proteins at a specific pH range can be the same⁴¹. Adding NaOH in a certain amount and concentration will neutralize it, causing proteins and amino acids to coagulate and precipitate⁴². Precipitation results using the base produce a white precipitate (see Figure 1g and 1h).

Functional Groups of Compounds Isolated from Sheep and Javanese Goat Hair

The FTIR results on sheep hair samples (Figure 5) show the presence of amine groups C-N stretch (primary amine) at wavelengths 1056.91 cm⁻¹, 1110.92 cm⁻¹, and 1164.92 cm⁻¹ in amide I. Wavelengths 3363.61 cm⁻¹ and 3417.61 cm⁻¹ indicate stretching of carbon groups C-H alkane. Carboxylate groups (COOH) are observed at a wavelength of 1373.22 cm⁻¹. The R-group of amino acids is observed at wavelengths 609.46 cm⁻¹ and 702.04 cm⁻¹, representing the stretching bonds of C-S (disulfide) and C-S (thiol). The presence of carboxylate groups (COOH), amine groups C-N stretch (primary amine), and side chains C-S (disulfide) and C-S (thiol) of methionine indicates the presence of amino acids that have been degraded from keratin protein after hydrolysis^{6,43,44}.

Table 1. Comparative Analysis of Keratin Yield from Chicken Feathers and Sheep Wool under Different Hydrolysis Conditions

Source	Hydrolysis	Temperature	Yield
Chicken Feathers	NaOH and NaHSO ₃	90°C	39.60% ³⁵
Chicken Feathers	Alkaline	80°C	86.57% ³⁶
Sheep Wool	NaOH and H ₂ O ₂	45°C	60.5-64.99% ³⁷
Sheep Wool	Choline thioglycolate	130°C	- ³⁸
Sheep Wool	Cl	180°C	18% ³⁸
Sheep Wool	Br	100°C	- ³⁹
Chicken Feathers	-	60°C	2.88% ⁴⁰

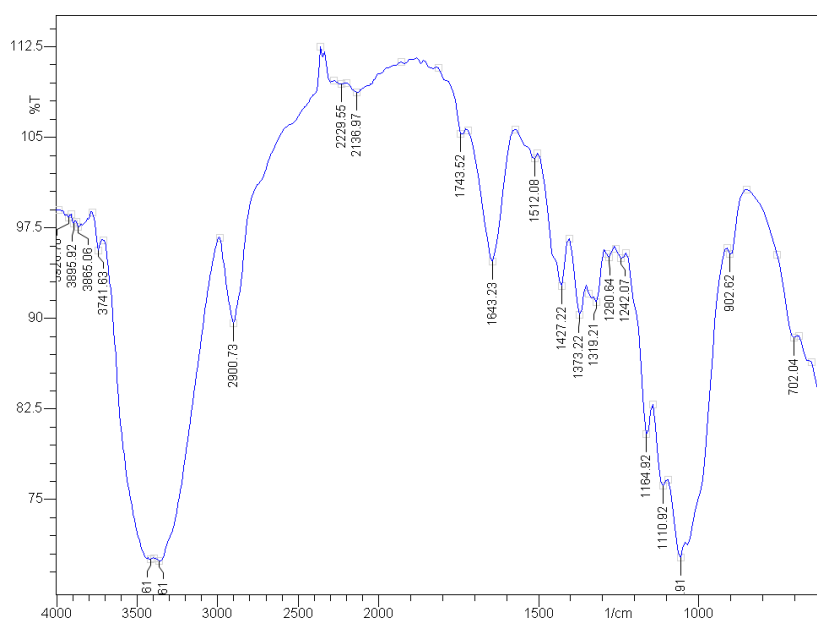


Figure 5. FTIR spectrum of sheep hair sample.

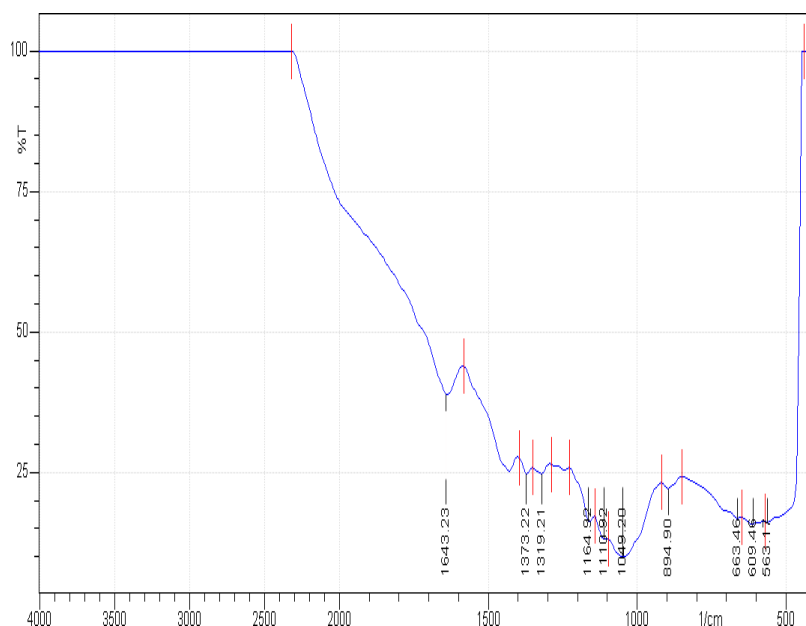


Figure 6. FTIR spectrum of javanese goat hair sample.

The FTIR spectrum on Javanese goat hair (Figure 6) shows the presence of C-S groups (disulfide) at wavelengths 563.17 cm^{-1} , 609.46 cm^{-1} , and 663.46 cm^{-1} . Carboxylate groups (COOH) are observed at a wavelength of 1643.23 cm^{-1} , and C-N groups (alkyl amine) are seen at wavelengths 1319.21 cm^{-1} , 1373.22 cm^{-1} , 1110.92 cm^{-1} , 1049.2 cm^{-1} and 1164.92 cm^{-1} is amide I, representing C-N stretch groups (primary amine). The presence of carboxylate groups (COOH), amine groups C-N stretch (primary amine), and side chains C-S (disulfide) of methionine indicates the presence of amino acids that have been degraded from keratin protein after hydrolysis^{6,43,44}.

Amino Acid Composition of Javanese Goat Hair

The HPLC data indicates the amino acids content that can be isolated from the most abundant include cysteine at 0% w/w, methionine at 0.02% w/w, proline at 0.05% w/w, threonine at 0.075% w/w, alanine at 0.075% w/w, valine at 0.075% w/w, serine at 0.08% w/w, tyrosine 0.085% w/w, aspartic acid 0.11% w/w, lysine 0.11% w/w, glycine 0.065% w/w, phenylalanine 0.135% w/w, arginine 0.135% w/w, glutamic acid was 0.155% w/w, leucine was 0.385% w/w, histidine was 0.4% w/w, and isoleucine was 0.60% w/w. The highest amino acid content was obtained for isoleucine at 0.60% w/w, while the lowest was obtained for cysteine at 0.00% w/w (Figure 7). Analysis reveals high isoleucine content in Javanese goat hair. This finding has significant implications for understanding the amino acid composition of animal hair and its potential in various industrial applications, particularly in the context of developing nutritional supplements. Isoleucine, as a major component of Branched-Chain Amino Acids (BCAAs), plays a crucial role in supporting athletic performance, muscle recovery, and overall health¹².

The sample with the highest yield was used in HPLC analysis to determine the concentration and types of its amino acids (Figure 7). The pH range during precipitation significantly influences the amount of precipitate obtained and the types of amino acids. α -Keratin is classified into two types based on its isoelectric point (pI) range: type I (pI = 4.9 - 5.4) and type II (pI = 6.5 - 8.5). α -Keratin containing more acidic amino acids falls into type I, while those containing more basic amino acids belong to type II. Proteins in an HCl solution carry a positive charge; the number of positive and negative charges on proteins at a specific pH range can be equal²⁸. The addition of NaOH in a specific amount and concentration neutralizes it, causing proteins and amino acids to coagulate and precipitate⁴². The precipitation results using a base produce whitish-yellow and whitish-gray precipitates in sheep and Javanese goat hair samples (Figure 2).

Cysteine residues, cysteine, and serine can be immediately hydrolyzed at high pH and undergo β -elimination reactions to produce AMD (Dehydroalanine) residues. Furthermore, AMD residues react with cysteine and lysine residues to form LAN (Lanthionine) and LAL (Lysinoalanine), which are highly stable⁴⁵ (Figure 8). This explains the absence of cysteine after the hydrolysis process. The isolated essential and non-essential amino acids can be utilized as supplements for animal feed as they serve beneficial functions for the bodies of animals. The composition of amino acids in animal hair offers exciting possibilities for industrial applications, especially in the development of halal nutritional supplements rich in BCAAs like isoleucine, which benefit athletic performance, muscle recovery, and overall health¹².

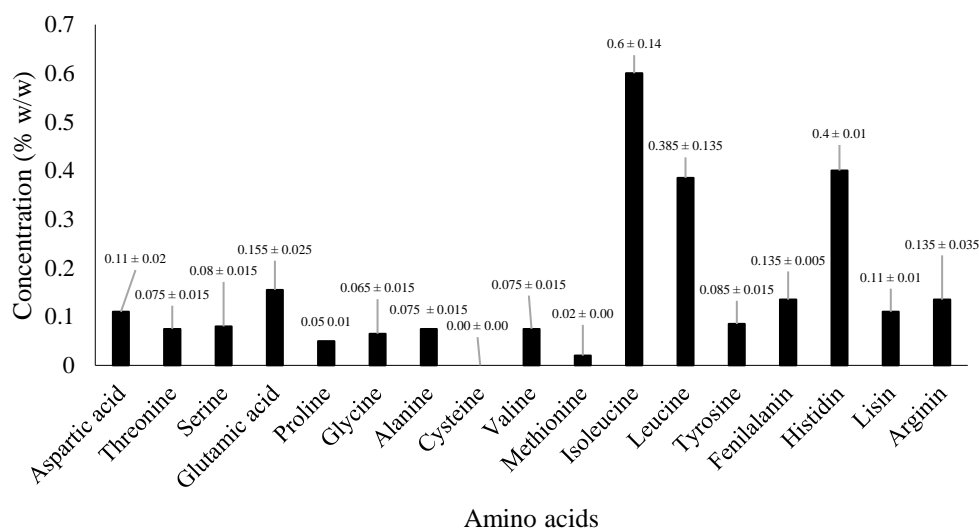


Figure 7. Concentration of amino acids from HPLC analysis.

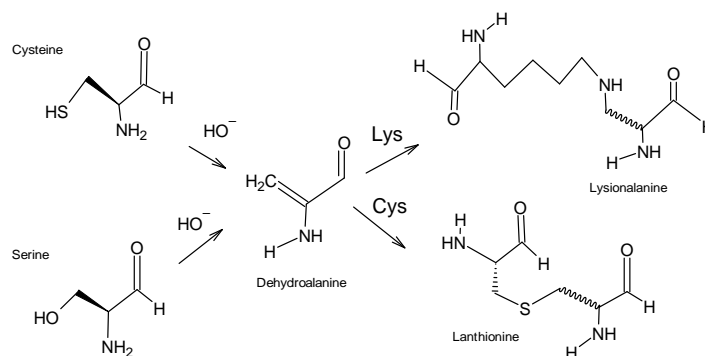


Figure 8. Formation of Cysteine into AMD (Dehydroalanine) and Subsequently into LAN (Lanthionine) and LAL (Lysinoalanine) ⁴⁵.

4. CONCLUSIONS

The peptide bonds of keratin protein in Javanese goat hair and Javanese sheep hair have been successfully broken down into simpler amino acids. The yield produced after isolation on Javanese goat hair samples was 0.92% and Garut sheep hair 0.32%. FTIR spectrum shows amino acid functional groups in both samples, including carboxyl (COOH), amine (C-N primer), (C-S disulfide), and amide I (-CONH₂). The isolation of amino acids from Javanese goat hair has revealed the presence of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine. Amino acid isolation has been successfully performed, showing the highest isoleucine content at 0.60% w/w. Isoleucine, leucine, and valine content can potentially be used as a halal BCAA supplement that serves as nutrition in the body and muscles.

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