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Purification and characterization of recombinant Streptokinase expressed in E.coli from Streptococcus equisimilis with N-terminal methionine

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HIGHLIGHTS

- Purification of recombinant streptokinase produced from S.equisimilis in E.coli with N- terminal methionine.
- Purification by three Chromatography steps: CM sepharose, DEAE sepharose, and Gel filtration.
- Ultrafiltration and diafiltration of the purified streptokinase.
- Streptokinase has two isomers in which one of the isomers is expressed with methionine that shows nil biological activity. Methionine is affecting the biological activity of Streptokinase.

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ABSTRACT

Purification of streptokinase produced from S.equisimilis in E.coli with N-terminal methionine was carried out in 3 Chromatography purification steps, 1) CM-Sepharose-FF at pH 4.2 followed by concentration and dialysis overnight with Tris-HCl pH 8.0. Partially purified dialyzed enzyme sample was loaded on to 2) DEAE-Sepharose-FF column. The Purified fractions of DEAE column were pooled and applied on to Sephadex G-100 column. SDS-PAGE and RP-HPLC confirmed enzyme purity.Its biological activity is determined by specific streptokinase assay and characterized the enzyme by Peptide mapping, MALDI-TOF, Isoelectric-focusing, and RP-HPLC. The isoelectric point (pI) of streptokinase is around 4.98. The results of characterization show that it contains two forms (Isomers) of streptokinase expressed in E. coli, which was analyzed by RP-HPLC and chromogenic assay. The variation is formed by isomer-1 in which 85% of Streptokinase expressed without methionine (85000IU/mg) and Isomer-2 in which 15% of streptokinase expressed with methionine (nil activity) in E. coli. This phenomenon shows that the presence and absence of methionine in isomers of streptokinase varying the catalytic activity of the enzyme.

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1. INTRODUCTION

In cardiovascular disease management, thrombolytic therapy plays an important role. A thrombus or a blood clot is a solid mass made up of the blood constituents from within the vascular system. These thrombi can break free into pieces and carry to a different location via the blood stream, which leads to the condition known as an embolism. Further, oxygen supply to the surrounding tissue is obstructed by these blockages causing degradation and death. Based upon the location of such thrombi and emboli, they may manifest themselves in the form of different thromboembolic disorders like stroke, pulmonary embolism, deep vein thrombosis, arterial thrombosis, acute myocardial infarction (AMI) and retinal artery occlusion. In 2012 around 17.5 million deaths occurred at an estimated rate of approximately 23.6 million by 2030, such cardiovascular diseases are progressively becoming the leading cause of morbidity and mortality worldwide. The low and middle-income countries are the most affected due to their socio-economic conditions and inaccessibility to efficient healthcare services. Alterations in the vascular wall, reduction in bloodstream velocity, and an increased tendency of blood coagulation are the three significant variables leading to thrombosis. The Major cause of cardiovascular complications are alcohol abuse, obesity, physical inactivity, unhealthy diet, lifestyle, tobacco use, hypertension, raised blood lipids, diabetes, air pollution, high salt intake combined with age-related risks.[1]

Streptococcus equisimilis GCS strain H46A (ATCC 12449) was the first streptococci to be introduced as a high-yield SK secreting bacteria for production purpose by Christensen et al. in 1945. Further, another Streptococcus. Equisimilis group C (ATCC 9542) was introduced by Estrada et al. as a Streptokinase production strain in 1992. These two strains were used as a principal source of SK gene for heterologous expression of the recombinant SK (rSK) in hosts like E. coli and yeast for SK production.[2]

Plasminogen activators cause thrombolysis via activation of plasminogen into active serine protease plasmin that can degrade the fibrin blood clot. The plasminogen activators mediate fibrin lysis via a direct or an indirect mechanism. The direct plasminogen activators are serine proteases that mediate a direct action on plasminogen to catalyze its activation, e.g., u-PA, pro-urokinase (pro-uPA), t-PA, reteplase (r-PA), tenecteplase (TNK-tPA), etc. The indirect plasminogen activators (SK, SAK), on the other hand, do not have any enzymatic activity of their own but form a 1:1 stoichiometric complex with plasmin or plasminogen. This activator complex then activates the plasminogen molecules present in the circulation.[3]

Plasminogen activators of human origin (t-PA and u-PA) are still in use for thrombolytic therapy, but their high cost and side effects have led researchers to look for alternate sources of fibrinolytic enzymes. Due to their low cost of production and fewer side effects, microbial fibrinolytic enzymes have gained proper attention. The enzyme is a single polypeptide that exerts its fibrinolytic action indirectly by activating the circulatory plasminogen. Streptokinase has a molar mass of around 47 kDa which is made up of 414 amino acid residues and exhibits its maximum activity at pH 7.5 without cystine, cysteine, phosphorous, conjugated carbohydrates and lipids.[4,5]

Streptokinase has internal sequence homology between the NH2-terminal 173 residues and a COOH-terminal 162-residue region between residues 254 and 415. These two regions have moderate homology in predicted secondary structures. The 80 residues of COOH-terminal region are deleted from the second half of the duplicated structures. The three-dimensional structure of streptokinase likely contains two independently folded domains, each homologous to serine proteases form these observations.[6]

Many thrombolytic agents have been identified and characterized from different sources, including fermented food products like Japanese natto,[7] Korean chunkook-

jang[8], and Chinese douchi[9], food grade microorganisms[10,11], insects[12], polychaetes[13], earthworms[9,14], and snake venom.[15] Microorganisms have various thrombolytic agents and gained particular medical interest because of their broad biochemical diversity, the feasibility of mass culture, and ease of genetic manipulation. Hence, a variety of microorganisms has been used for isolation of fibrinolytic enzymes, which include bacteria, fungi, and algae. Various methods of purifying streptokinase have been described which are based on quantitative differences in solubility, electrical charge, molecular size, and shape or non-specific physical interactions with surfaces.[16] Recently have produced untagged recombinant streptokinase and purified it with three chromatography purification steps.

2. MATERIALS AND METHOD

The materials used in the experiment include harvest culture of Streptococcus equisimilis group C, strain H46A (ATCC 12449, USA), High-speed Refrigerated centrifuge from Hitachi CR212GIII, Japan. A French press is from of Panda plus-Niro saovi, Italy; Refrigerated circulator is from Julabo, Germany. FPLC system (Akta prime plus) is from GE-Healthcare, 0.2 µm disc filters(PALL Life sciences), CM-sepharose-FF (GE-Healthcare), DEAE Sepharose-FF, Sephadex G100 (GE-Healthcare, USA) (GE-Healthcare, USA), Electrophoresis unit (AE-6500-Atto corporation, Japan) SDS PAGEHPLC (Agilent Technologies, USA), Chromogenic substrate S-2251 (Chromogenix laboratories, Italy), Buffer salts, acids and bases (Merck, Germany).

3. RESULTS AND DISCUSSION

3.1. Purification of streptokinase

Streptokinase was purified by 3 step chromatography. Following are the steps involved for purification. The cell harvest obtained from 2-liter fermentation batch was centrifuged at 4000 rpm, and the pellet was stored at -80ºC for cell lysis. Cell lysis was performed on French press (Niro saovi-Italy) at a pressure of 16 Kpsi (2 cycles.), lysis was carried out at 15°C using the refrigerated circulator (Julabo, Germany). The buffer used for cell lysis was 20 mM sodium acetate pH 4.2. After cell lysis, the lysate was clarified at 20000 g for 30 min at 4ºC and separated the supernatant from the cell debris. The separated supernatant was filtered with 0.2 µm disc filters and used for first purification step on CM-sepharose column (XK-26/20-40 ml). Loaded the filtered supernatant on to the column at pH 4.2 with a flow rate of 2.5 ml/min. Washed the column with wash buffer to remove other unbound proteins. The bound enzyme was eluted with a linear gradient of 0- 500 mM NaCl in 20 mM sodium acetate buffer. Partially purified streptokinase was eluted at 75 mM NaCl step which was further purified by anion exchange chromatography using DEAE Sepharose-FF at pH 8.0 with 20 mM Tris –HCl. The partially purified pooled fractions streptokinase were dialyzed with 20 mM Tris-HCl and loaded on to DEAE column and eluted with a linear gradient of 0-500 mM NaCl where the purified enzyme eluted at 150 mM NaCl step. Purified fraction of DEAE was passed on to Sephadex G100 column(length:30 cm, Diameter: 7.8 mm) to get a purity of >99 %.Buffer used for this column is 20 mM sodium phosphate,150 mM NaCl pH 8.0. All purifications were carried out on FPLC system (Akta prime plus, GE-Healthcare).

3.2. Quantification and electrophoresis of protein

The molecular mass and purity of the streptokinase were determined by 12% SDS polyacrylamide gel electrophoresis with Atto equipment (Japan). Proteins were visualized by staining with Coomassie Brilliant Blue R- 250 or with 0.1% (w/v) of silver nitrate.

Protein determination involves the binding of Coomassie Brilliant Blue G-250 to protein. The dye binding to protein causes a shift in the absorption maximum from 465

to 595 nm, and the increase in absorption at 595 nm is monitored. This assay is very reproducible and rapid with the dye binding process virtually complete in approximately 2 min with good color stability for 1 hr. Protein quantification was done by the Bradford dye binding assay with the bovine serum albumin as standard.[17,18]

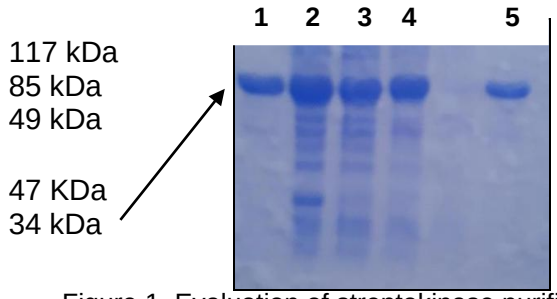

Figure 1. Evaluation of streptokinase purification by SDS-PAGE (Gel -1).
1)Standard; 2) Column 1-CM sepharose-FF; 3) Column 2-DEAE sepharose-FF; 4) Column 3- Sephadex G100; 5) Purified SK

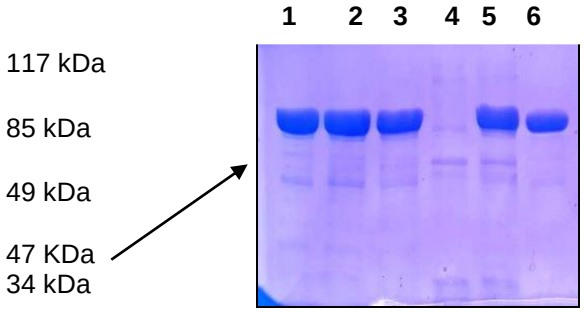

Figure 2. Evaluation of streptokinase purification by SDS-PAGE (Gel-2).
1)Column 3 sephadex G100; 2) Column 2 DEAE sepharose-FF; 3) Column 1-CM sepharose-FF; 4) Blank; 5) Purified SK (In-house); 6) Polished purified SK.

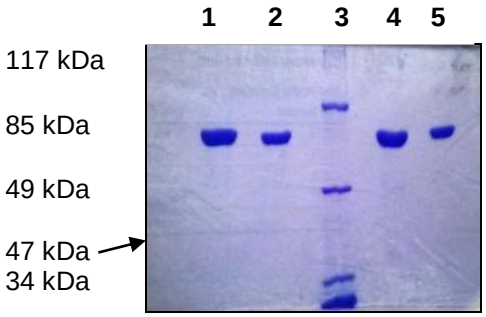

Figure 3. Evaluation of standard and purified streptokinase by SD-PAGE.
1)Standard-10 µg; 2) Standard-5 µg; 3) Molecular weight marker; 4) Purified Streptokinase-10 µg; 5) Purified Streptokinase-5 µg

Table 1. Purification table of Streptokinase

Sample	Total protein (mg)	Streptokinase (IU)	Streptokinase (IU/mg)	Fold purification	Protein Yield (%)	Activity Recovery (%)
Crude extract	400	6000000	15000	1	100	100
Purified, CM-Sepharose FF	252	5418000	21500	1.4	63	90
Purified, DEAE-Sepharose FF	105	4788000	45600	3.0	26	88
Purified, Sephadex-100	54	4584600	84900	5.7	14	96

The recombinant streptokinase mass analysis was done by using a Bruker Daltonics flex Analysis MALDI-TOF (matrix-assisted laser desorption ionization – a time of flight) mass spectrometer. Sinapinic acid matrix solution (10 mg) per ml in acetonitrile-water (1:1) was prepared, mixed with an equal amount (vol/vol) of dialyzed protein solution, and spotted on a sample plate. Then the mass spectra were recorded.[19]

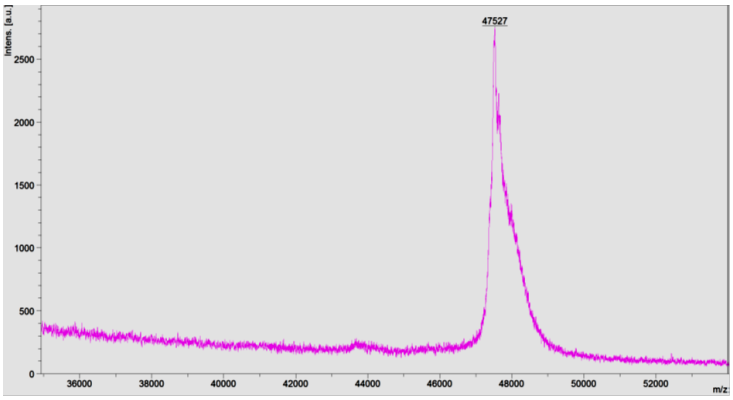

Figure 4. MALDI-TOF mass spectral analysis of recombinant streptokinase.
MALDI-TOF
Powder sample\0_E20\1\1Lin
BrukerDaltonicsflexAnalysisprinted: 11/16/2017 7:13:31 PM
Molecular weight for Streptokinase

3.4. Peptide mapping

Peptide mapping is a relative procedure because the information obtained, compared to a reference standard or reference material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability.[20]

Reagents and Chemicals using Acetronitrile and Orthophosphoric acid obtained from Sigma and Fisher scientific respectively, the water used for a reaction was Milli-Q Water (Millipore Corp.) with 18 mega ohms or less resistance. TPCK (L-l-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin was ordered from Worthington and N-ethyl morpholine and TLCK (N,-p-tosyl-L-lysine chloromethyl ketone)-treated chymotrypsin from the make Sigma. A Waters Associates liquid chromatograph equipped with gradient elution capability, a U6K sample injector, fixed dual wavelength detector, and a variable wavelength detector was employed. Performed all separations on reversed phase column (IO pm, 4 mm x 30 cm), from water associates.[15]

Elution of peptides was carried out by the use of a linear gradient, from 0.1% orthophosphoric acid (Solvent A) to acetonitrile (Solvent B) at a constant flow rate of 2 ml/min with 500 to 1000 p.s.i varying column pressure. Filtered the Phosphoric acid (0.l%, pH 2.2) through a 0.5~pm Millipore filter (Millipore Corp., Bedford, MA) and degassed under vacuum for 30 min, with stirring. Acetonitrile was used directly. Solvents were stirred continuously during runs to eliminate the formation of temperature gradients and performed all runs at room temperature.

The column precision can be maintained by pumping acetonitrile at 0.1 ml/min through the column overnight. The column was equilibrated with Solvent A for ten min.[15] Buffers such as 0.2 N N-ethyl morpholine acetate (pH 8.1) at 37°C were used for enzymatic digestion with an enzyme to protein ratio of 1 to 100 (w/w). Acetic acid was added to stop the digestion. The test samples were directly injected into the HPLC without any manipulation. The compositions of proteins and peptides were determined

on a Beckman model 119Cl analyzer equipped for single column methodology. The samples were lyophilized (1 to 5 nmol) in hydrolysis ampoules and vacuum hydrolyzed for 24 h at 1l0'C in 1.0 ml of constant-boiling HCl.[15]

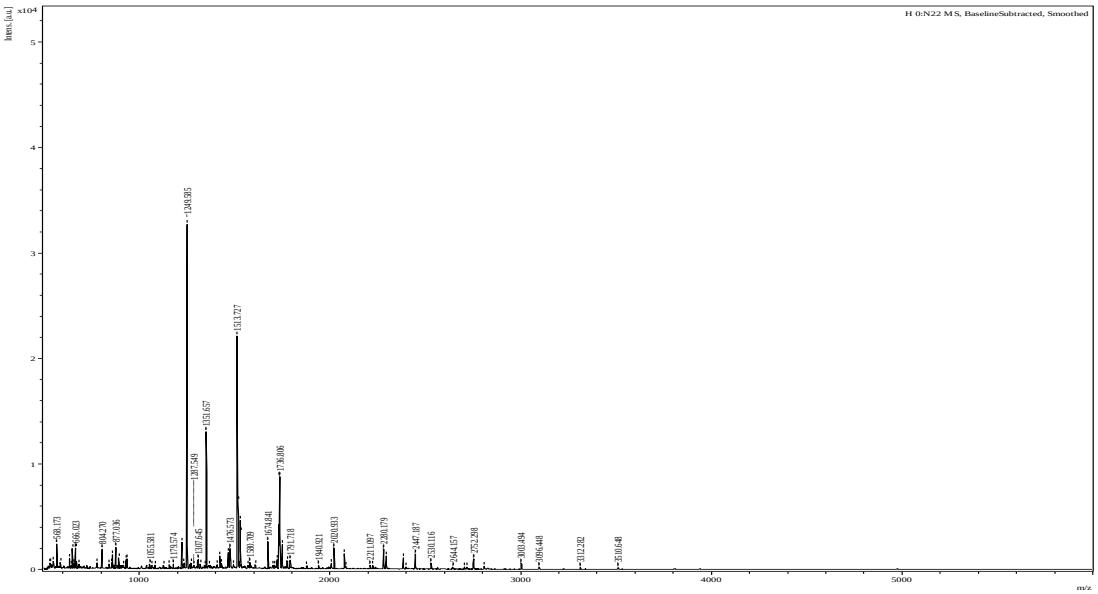

Figure 5. Peptide mapping of recombinant streptokinase.

3.5. Isoelectric focusing

Immobilized pH gradient (IPG) strip commercially available is used. The Acrylamide gel of IPG strip contains wide pores which prevent a sieving effect based on protein mass, with a pH gradient. Different gradients are available, with broader gradients, at pH 3-10 for whole proteome analysis, and narrower ranges, at pH 5-8 used for more specialist applications.[21] Equipments and Reagents using Gel casting apparatus for a horizontal electrophoresis cell, Fluorescent lamp, 30 % T, 3 % C Acrylamide stock solution, 50 % glycerol,0.1 % riboflavin-5'- phosphate(FMN)[c,]carrier ampholytes with a pH range spanning the p/s of the protein of interest,10 % APS and TEMED.[22] Equipments and reagents using Flat-bed electrophoresis cell-The GE multiphor,Power supply capable of delivering 2- 3000 V and 6 W at high voltage, Refrigerated water circulator, Electrode strips, sample application strips,1 N NaOH catholyte,1 N H3PO4 anolyte.[22]

Procedure using set up the IEF cell as recommended by the manufacturer. Connected water circulator and prepared the cooling platform and electrode strips. Placed the sample application strips on a glass plate and pipette 5 µl of a protein sample to each strip. Place the application strips 1 cm from the anode end of the gel. Positioned the gel in the IEF cell and make electrode contact as specified for the particular cell. Close the electrophoresis cell and connect the leads to the power supply. The red lead is the anode, and the black lead is the cathode. Set the running conditions as recommended by the manufacturer of the electrophoresis cell.[22]

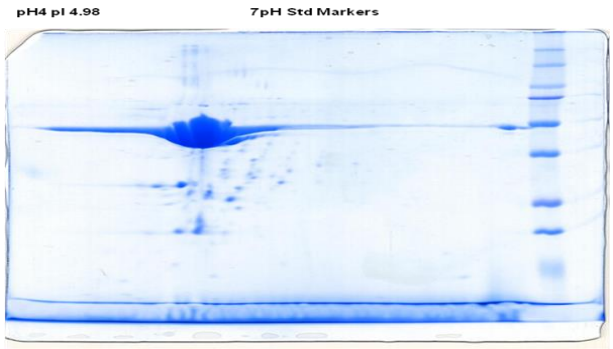

Figure 6. Isoelectric focusing of recombinant Streptokinase: 250 ug, 13cm, pI 4-7, CBB staining.

3.6. Reverse Phase - HPLC

The purified streptokinase sample is obtained 20mg/ml. Further, the sample was diluted in 1mg /ml with 20mM phosphate buffer pH 7.5. 100µl of sample is injected to Agilent HPLC system with C18 reverse phase column size 250mmx 0.46. The mobile phase solvent A is water with 0.001% TFA (Trifluro acetic acid), and mobile phase B is 40:60% of methanol and acetonitrile. The total run time process on HPLC is 70 minutes with gradient mode using the absorbance at 280 nm. The same procedure was followed for standard streptokinase.

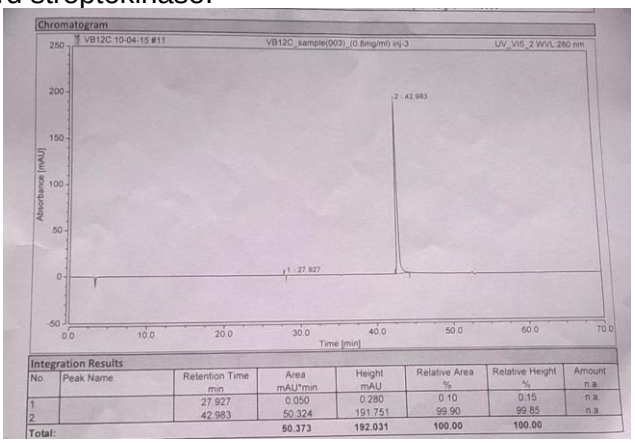

Figure 7. Reverse phase Chromatogram of Purified Streptokinase.
The retention time of purified streptokinase is 42.983 and the observed purified streptokinase purity is 99.9

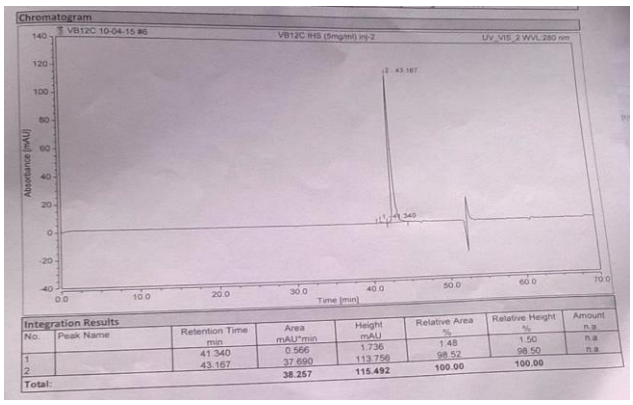

Figure 8. Reverse phase Chromatogram of standard purified Streptokinase.
The retention time of standard streptokinase is 42.167, and the observed standard streptokinase purity is 98.52.

3.7. Streptokinase chromogenic substrate assay (scsa)

Twenty-five microliter volumes of the STK and the sample solutions with concentrations between 2.5 and 40 IU/mL were added to the 96-well plate, respectively in triplicate, and equilibrated at 37°C in a water bath for 1 min. Incubated the plate for 2 min after adding 100 µL of the chromogenic substrate (diluted 1:1 in water) into each well, Then added 50 µL of the 1 mg/mL plasminogen. Stop the reaction by adding 90 µL of 20% acetic acid after 10 min and measured the absorbance at 405 nm in the microplate reader. Calculated the biological potencies against STK by the parallel line statistical method using the CombiStats software.[17, 23]

3.8. Clot lysis assay

The fibrin clot lysis method is based on the clotting of fibrinogen in the presence of plasminogen and streptokinase.

Two solutions prepared fibrin plate:

Solution 1: Dissolved 200 mg of agarose (Merck, USA) in 20 mL of phosphate buffer solution and pH adjusted to 7.2. Maintain the solution at 56°C.

Solution 2: Dissolved 50 mg of human fibrinogen (Fibrinogen, Fraction 1, Type 1 from Human Plasma, Sigma-Aldrich, USA) in 10 mL phosphate buffer solution (pH = 7.2).[24]

To Solution 1 2000 µL of 1 mg/mL human plasminogen (Human plasminogen, Hyphen Biomed, France) was added and to Solution 2, 500 µL of thrombin (10 NIH unit/vial, from human plasma, Sigma-Aldrich, USA) was added. By mixing solutions (1) and (2) in a Petri dish, clot formation was initiated for 30 min and maintained at 4ºC. Dilutions were prepared in the range of 100-1000 IU/mL, with Milli-Q water from the international standard and loaded, as duplicate, onto fibrin plate and incubated for eighth. The dose-response curve for streptokinase was obtained by measuring the zones of lysis produced on the fibrin plates. By reconstituting each vial with 1 mL of Milli-Q water, the test samples were prepared. Streptokinase activity of the samples using the dose-response curve was determined from zones of lysis that was produced by allocated dilutions of each sample loaded on fibrin plates.[23]

The results of the Streptokinase (SK) purification by CM-Sepharose FF, DEAE sepharose, and Sephadex G100 are shown in Table 1. In the first column purification by CM-sepharose the enzyme was purified 1.4 fold with a yield of 63 % and activity recovery of 90 %. In this step, 252 milligrams (mg) pure streptokinase was produced; the purified product had a specific activity of 21500 IU/mg. In the second column purification by DEAE, the enzyme was purified 3.0 fold with a yield of 26 % and activity recovery of 88 %. In this step, 105 milligrams (mg) pure streptokinase was produced; the purified product had a specific activity of 45600 IU/mg. In the third column purification by Sephadex G100, the enzyme was purified 5.7 fold with a yield of 14% and activity recovery of 96 %. In this step, 54 milligrams (mg) pure streptokinase was produced; the purified product had a specific activity of 84900 IU/mg. In an SDSPAGE analysis, the eluted product of three columns showed a single band of approximately 47 kDa (Figure 1 and Figure 2). In RPHPLC analysis purified Streptokinase showed a purity of ~ 99.9 % with a retention time of ~ 42.98 min where the standard streptokinase showed a purity of ~ 98.5 % with a retention time of ~ 42.16 min. The results of Mass spectral analysis of recombinant streptokinase by MALDI-TOF showed a molecular weight of ~47.5 KD. Based on the results of Isoelectric focusing the pI of recombinant streptokinase is ~4.98.The results of peptide mapping showed that the amino acid sequences of standard and recombinant streptokinase are identical. The results of RPLC showed that streptokinase was expressed in two isoforms (isomers). Isomer-1 has 85 % SK expressed without methionine, and Isomer-2 has 15 % SK expressed with methionine, which showed nil activity.

To investigate the impact of an amino-terminal methionine on the catalytic activity of streptokinase, the mature native streptokinase sequence (rSK) was cloned. The N-terminal amino acid of native streptokinase started with Isoleucine (I), Alanine (A) and Glycine (G) and followed by Proline (P), etc. The first amino acid of Isoleucine is playing an essential role in the catalytic activity of streptokinase is binding towards inactive plasminogen to activate plasminogen. The specific activity of native streptokinase is 100000 IU/mg with the initial N- terminal isoleucine amino acid.

Despite the recombinant streptokinase begins with a proteinogenic amino-terminal methionine (rSK-Met) for expression and is not susceptible to processing during expression The presence of the amino-terminal methionine in rSKMet was confirmed by protein sequencing. Comparison of the specific activity of recombinant streptokinase shows only 85000 IU/mg than 100000 IU/mg native streptokinase. The reason behind this objective is that there are two forms (Isomers) of streptokinase are expressed in E. coli, which was analyzed by RP-HPLC and chromogenic assay. We have found that isomer-1 forms this variation have 85% of Streptokinase expressed without methionine (85000IU/mg) and Isomer-2 has 15% of streptokinase expressed with methionine (nil activity) in E. coli. This phenomenon is demonstrating that the presence and absence of methionine in isomers are varying the catalytic activity of streptokinase.

4. CONCLUSION

SK is one of the promising blood-clot dissolving agents for the treatment of patients suffering from a heart attack. It is crucial to produce this enzyme in large quantities for biochemical characterization and clinical trials. In this work, we found and observed that streptokinase has two isomers where one of the isomers has nil activity because of methionine. Hence methionine in isomers is affecting the activity of streptokinase. The production and purification of efficient and low-cost r-SK are possible only with one of the isomers for pharmaceutical applications. This technology or new finding plays an essential role in the successful production of bioactive r-SK and to scale up the production process using fermentation technology.

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Erythrocyte sedimentation rate in diabetic and non-diabetic patients of cardiovascular disease

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HIGHLIGHTS

The elevated level of ESR in CVD patients might indicate the prognosis of diabetes mellitus

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A B S T R A C T

The pervasiveness of global endemic of type II diabetes, driven by population development, increasing obesity, urbanization, and other additional unidentified factors may be crumpled in the next 20yrs. The purpose of this study was to determine the correlation of ESR in diabetic and non-diabetic patients of cardiovascular diseases. It is a co-relational study conducted at KRL hospital Islamabad from February 2018 to September 2018. The study population compromised a total of 180 cases aged 45 to 50 years were taken, in which 60 cases of CVD with DM, 60 cases of CVD without DM and 60 normal healthy population were taken. All patients and controls gave written informed consent. All subjects went through a general physical examination, and a questionnaire was used to collect the records about demographics and past medical history and existing use of medications. In the presented study, Pearson's Correlation between ESR of healthy and ESR of CVD without DM shows a weak correlation between these two variables i.e., 0.127 with a *p*-value of 0.503. We also establish that there is a direct intermediate relationship between ESR of healthy and ESR of CVD with DM. High level of ESR in CVD patients might indicate the prognosis of DM. To confirm this further researches and studies must be conducted in this area.

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1. INTRODUCTION

The pervasiveness of global endemic of type II diabetes, driven by population development, increasing obesity, urbanization, and other additional unidentified factors may be crumpled in the next 20yrs.[1] India, the world's second most crowded country, has made an astounding economic development in recent years but on the other hand however, currently it treasures itself lagging behind peers on healthcare consequences. Every fifth person in India is diabetic, and because of this, it made India the world's capital of Diabetics so this disease is pretentiousness a massive health issues in the country.[2, 3] Whereas, the pervasiveness of diabetes in the urban compared to the rural areas of Pakistan was 6.0% in men and 3.5% in women while 6.9% in men and 2.5% in women, correspondingly.[4]

On the whole glucose intolerance diabetes mellitus and impaired glucose tolerance (DM+IGT) was 22.04% in urban and 17.15% in rural areas of Pakistan.[4] At present, Pakistan defenses 6thbetween the countries with the whole load of diabetes mellitus.[1] In the previous 50 years, health care systems of the world have faced a new epidemic twofold disease: cardiovascular diseases and diabetes mellitus. At present, CVD is the foremost root of death in all western countries, and 60% of deaths for ischemic heart disease and stroke takes place in developing countries with setting up possessions, and the conspicuous relationship between peripheral artery disease, stroke, coronary artery disease, and diabetes have enforced physicians to explore the probable pathophysiological association amongst these dissimilar clinical circumstances. Latest and persuasive evidence has revealed the noteworthy and self-regulating role of inflammation, insulin resistance and subsequent endothelial dysfunction in the commencement and succession of atherothrombosis, superimposed on conventional risk factors.[5]

The importance of the erythrocyte sedimentation rate is also examined in the background of heart failure, even though previous studies recommended a poorer prognosis of chronic heart failure with low down ESR.[6] Current studies on patients with chronic heart failure treated with angiotensin-converting enzyme inhibitors showed that an elevated ESR might be an adverse prognostic indication, independent of patients' symptomatology and ventricular function. ESR is also positively associated with pro-inflammatory cytokines,[6,7] that have been revealed to forecast heart failure even in the asymptomatic populace, and that appears to be linked with worse prognosis in patients with heart failure.[8,9] In recent years, the relationship between inflammation and cardiovascular diseases has gained significant interest.[10] Numerous systemic markers of inflammation, together with an erythrocyte sedimentation rate (ESR), have been established to be predictors of coronary heart disease.[11] C-reactive protein, interleukin (IL)-6, and tumor necrosis factor (TNF)-alpha and all markers of cytokine-mediated inflammation, have been revealed to predict occurrence heart failure (HF).[12] Erythrocyte sedimentation rate, a wide-ranging indicator of inflammation an included measure of acute stage response is enlarged in several inflammatory circumstances.[13] Erythrocyte sedimentation rate is an extensively used, frequently executed, and low-priced laboratory test to find out the red blood cell accumulation. ESR in a diabetic patient may be raised in the nonexistence of unconcealed infection.[14] The purpose of the present study was to determine the correlation of ESR in diabetic and non-diabetic patients of cardiovascular diseases.

2. MATERIALS AND METHOD

This research performed a correlational study from February 2018 to September 2018, assessing the level of ESR in 60 stable patients of CVD without DM and 60 stable patients of CVD with DM from KRL Hospital Islamabad. ESR measurement was also done in 60 healthy subjects. All patients and controls gave written informed consent.

All subjects went through a general physical examination, and a questionnaire was used to gather the records concerning demographics and past medical history and existing use of medications. The diagnosis of CVD and DM was based on clinical presentation, standard investigations, and examination. Diabetes occurrence data for adults aged 45 to 50 years were taken. Patients were disqualified from the study if they were clinically unstable, were edematous, had dyspnea, or had severe renal failure. No subject had clinical signs of infection, rheumatoid arthritis, or cancer at the time of ESR measurement.

The ESR was determined by using the Westergren method. In brief, 4.5 mL of venous blood was drawn into a tube containing 3.8% solution of sodium citrate. The test tube was mixed gently right away following blood sampling and again just before measuring, which took place within 2 hours after the blood was drained. A standard 200-mm, Westergren method glass tube was filled to the zero marks at the top, placed in a vertical position, and left for 1 hour. The distance from the bottom of the surface of the tube to the top of the column was then calculated, and the result was articulated as millimeters in the first hour. The blood samples were then analyzed for ESR, total cholesterol, high and low-density lipoprotein cholesterol (HDL-C and LDL-C), plasma triglycerides, and homocysteine. Data were analyzed by using SPSS 21.

3. RESULTS AND DISCUSSION

The relationship between erythrocyte sedimentation rate (ESR) in diabetic (DM) and non-diabetic patients of cardiovascular diseases (CVD) were investigated in a total of 180 participants in which 60 patients of cardiovascular diseases with diabetes in group A, 60 patients of cardiovascular diseases without diabetes in group B and 60 healthy participants were included. ESR Strength of CVD with DM, CVD without DM and with normal healthy population was determined by using Pearson correlation ‘r' of the patients included in the study, 116 (64%) were male with the mean age was 61.8 ± 10.3 years and 64 (36%) were females with the mean age was 57.7±9.5 years. The mean values of ESR among these three groups were 35.23+5.20, 33.03+4.98 and 17.20+1.73, 33.03+4.98 mm/hr respectively.

Pearson's Correlation ‘r' between ESR of healthy and ESR of CVD without DM shows a weak correlation between these two variables i.e., 0.127 with a *p*-value of 0.503, as shown in Table 1. This result means that changing in one variable is weakly correlated with changing in the second variable. In our result, the Pearson Correlation ‘r' is 0.127, and no clear association establishes between ESR of healthy and ESR of CVD patients without DM. In the present study, we also determine that there is a direct intermediate relationship between ESR of healthy and ESR of Cardiovascular disease with diabetic patients. In this result, the Pearson ‘r' is 0.228 with a *p*-value of 0.225, as shown in table 01. So, there is no clear association or interaction between the ESR of healthy and ESR of CVD with DM patients. We also calculate Pearson correlation between ESR of CVD without DM and ESR of CVD with DM, and we determined that there is also a weak relationship between these two variables. Value of Pearson ‘r' between these two variables is 0.160 (see table 01). Probably, elevated ESR, CVD, and hyperglycemia are associated with worse prognosis by a different mechanism.

Results of comparing ESR of healthy samples with those of CVD patients without DM did not show anything to ponder on regarding ESR. Same was the case with healthy samples and diabetic patients with cardiovascular diseases.

Table 1. Correlations between ESR of healthy, ESR of CVD without DM and ESR of CVD with DM.

	ESR of healthy	ESR of CVD without DM	ESR of CVD with DM
Pearson correlation		.127	.228
P value		.503	.225
Pearson correlation	.228	.160	
P value	.225	.399	

ESR= Erythrocyte sedimentation rate, CVD= cardiovascular diseases, DM= Diabetes mellitus

The previous study based on inflammation measured by ESR and development of heart failure stated that ESR is an independent predictor of HF[15]; similarly, another research was done to find a relation between ESR and micro-inflammation, that research concluded that increased or decreased ESR is easy means to detect micro-inflammation.[16] Another research was done finding a relation between ESR and myocardial infarction, and according to the conclusions of that research, there is an association between raised ESR and long-term mortality in ST-elevated Myocardial infarction patients.[17] ESR and heart's connection was also tested by another study carried out based on a relation between ESR, atherosclerosis and cardiac mortality and result of that study also avers that ESR is an independent indicator of coronary atherosclerosis.[18]

Following previous studies, it was considered vital to determine the role of ESR in CVD with and without DM. So, this study was carried out to ascertain the role of ESR in cardiovascular disease and diabetes mellitus. After calculating ESR from samples of healthy people, those having CVD with DM and people having CVD without DM, results were compared and analyzed to find an association between them regarding ESR as a determinant of inflammation. When the results of the ESR of healthy and ESR of CVD patients without DM were compared, it was discerned that there is no lucid association between them. This means that no matter what ESR is, it has no significant effect on the onset of CVD without DM or its progress.

While comparing Pearson ‘r' with p values from samples of healthy and CVD patients with DM it was observed that there is no link or significant association between the ESR of them, means that there is also no noted association between ESR and CVD with DM. It was earlier thought that the same might be the case with ESR and CVD with DM, but results nullified this notion. Another study regarding ESR and coronary heart disease says that further studies and research is needed on ESR regarding its role in heart diseases.[19] Following those results, it was considered a probability that ESR might also play a role in CVD patients with and without DM. Now ESR of CVD patients with DM and CVD patients without DM were compared. Results after were somehow different. It showed a weak relationship between variables and elevated ESR, CVD, and hyperglycemia are associated. That meant that the increase in ESR in CVD patients might indicate the prognosis of DM.

4. CONCLUSION

High level of ESR in CVD patients might indicate the prognosis of diabetes mellitus. To confirm this further researches and studies must be conducted in this area. If this is so, it can be beneficiary for CVD patients to detect their risk of developing DM by ESR, which is an inexpensive laboratory test.

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RETN rs3745368 polymorphism and resistin level in Javanese ethnic Indonesian obese: a case-control study

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HIGHLIGHTS

Resistin level has a negative correlation with Body Mass Index.

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A B S T R A C T

Obesity has become a global public health problem. It occurs due to a positive energy balance leading to adipose tissue expansion. White adipose tissue was an endocrine organ which secreted resistin. Resistin also produced by immune cells due to low chronic level inflammation might cause higher resistin level in obese people. Polymorphism +62G>A RETN gene was reported has a relationship with low resistin level and A allele as a protective allele. This study aimed to determine genotype and allele frequency distribution concerning resistin level. Another objective aimed to know the correlation between resistin level with body mass index. The design of the research was a case-control study with 122 people (18-40 y.o.), divided equally in the case group (BMI ≥ 27 kg/m2) and control group (BMI 18.5-24.9 kg/m2) without diabetes mellitus. Blood was taken after fasting a minimal 8 hours. Plasma was used to measure the resistin level. DNA genotyping was analyzed using PCR-RFLP. Genotyping result showed three genotypes of RETN gene +62G>A polymorphism (GG, GA, AA). There was no significant difference in genotype and allele frequency distribution related to obesity status (p=0.680; p=1) and resistin level (p=0.537) between case and control group. There was no significant difference in resistin level between case and control group (p=0.770). Resistin level was correlated with BMI in obese group (p= 0.05; r= -0.25). The present study concludes that there is no significant difference in genotype and allele frequency distribution related to obesity status and resistin level. Resistin level has a negative correlation with BMI.

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1. INTRODUCTION

Obesity has become a global public health problem. The prevalence of obesity is roughly around 1.2 million and increased three-fold or more since 1980 in the Middle East, the Pacific Islands, Australasia, and China based on data from the World Health Organization (WHO).[1] The prevalence of obesity and overweight in the Yogyakarta region is higher than the national average.[2] The Javanese ethnic is the dominant ethnic in Yogyakarta.

Obesity occurs due to positive energy balance caused by weight gain.[3] Adipose tissue expansion was significantly affected physiological response and can interfere in its function. Adipose tissue hypertrophy, ectopic fat deposition, hypoxia, and chronic stress occur in the state of obesity. White adipose tissue is known as an endocrine organ secreting signalling molecules called adipokines. Adipose tissue hypertrophy secreted pro-inflammatory adipokines.[4] One of the pro-inflammatory adipokines elevated in an obese state is resistin. Changes in the chronic secretion of adipokines show adipose tissue dysfunction and developed into metabolic diseases, cardiovascular, inflammatory, and malignant disease.[5] Hypertrophy of adipose tissue in obese individuals increases the infiltration of immune cells that secrete pro-inflammatory mediators, causing chronic low-level systemic inflammation.

Resistin is the link between obesity, insulin resistance to diabetes based on animal studies. On mice injected recombinant resistin, its expression was increased and interfere with glucose tolerance and insulin action.[6] Some research shows that serum resistin levels in the obese subjects were higher than the normal subject, which positively correlated with changes in Body Mass Index (BMI) and visceral fat.[7] However, other studies reported no association between serum resistin level with body fat percentage, visceral adipose tissue, and BMI.[8] The role of resistin in obesity, insulin resistance and diabetes mellitus as a risk factor still controversial and the mechanisms underlying the expression, regulation, secretion, and resistin still unclear.[9]

Genes encoding resistin is RETN gene.[6] Several single nucleotide polymorphisms (SNPs) RETN gene related to levels of circulating resistin.[10] Polymorphism +62G>A RETN gene (rs3745368) associated with low resistin levels.[11,12] This study aimed to determine genotype and allele frequency distribution concerning resistin level in obese and control group. Another objective aimed to determine the difference of resistin level between obese and control group and to knowing the correlation between resistin level with body mass index. Research on polymorphism + 62G> A RETN in the Javanese ethnic had never been done before.

2. MATERIALS AND METHOD

The design of the research was a case-control study with 122 people (18-40 y.o.), divided equally in the case group (BMI≥ 27 kg/m$^{2)}$ and the control group (BMI 18.5-24.9 kg/m^2) according to obesity classification WHO the Asia Pacific. Subjects were Javanese ethnic and without diabetes mellitus. Blood was taken after minimal 8 hours fasting. Informed consents were obtained from all the subjects before their inclusion in the study. Studies were conducted under the guidelines set by The Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Gadjah Mada University, Dr Sardjito General Hospital, Yogyakarta, Indonesia.

2.1. Anthropometric measurement

Obesity status was determined by body mass index (BMI). Height and body weight were measured without shoes, and the subjects wore light clothes. BMI of the obese subject was ≥ 27 kg/m^2, and the control subject was 18.50-24.99 kg/m^2.

2.2. Biochemical parameters

Fasting blood from subjects was collected in EDTA tube then is centrifuged. Blood plasma was used to measure fasting glucose using spectrophotometer with a commercially available kit (Dyasis), and resistin level was measured using ELISA method (RayBiotech).

2.3. Genotyping

DNA genotyping was analyzed using PCR-RFLP. Genotyping was carried out by PCR amplification of peripheral blood genomic DNA extracted using blood isolation kit (Promega) followed by restriction enzyme used in the previous study [13]. DNA was PCR amplified using the forward primer, 5'-AGAGTCCACGCTCCTGTGTT-3' and the reverse primer, 5'-TCATCATCATCATCTCCAGGTT-3'. The amplified products were digested with a restriction enzyme, BseRI (New England BioLabs). PCR product size for AA was 238 bp and 21 bp (homozygous mutant allele), 259 bp for wild-type allele; 259, 238 and 21 bp for heterozygote allele.

2.4. Statistical analysis

Normality and variance of data were analyzed using Kolmogorov-Smirnov. Differences between variables were computed using Mann-Whitney and Kruskal-Wallis test. Genotype and allele frequency was determined using the chi-square test or Fisher exact test for an expected value less than 5. The Odds ratio was determined by logistic regression analysis. P-value was considered to be statistically significant $p<0.05$. Correlation resistin level with BMI using Spearman Correlation.

3. RESULTS AND DISCUSSION

Subject's baseline characteristics are presented in Table 1. The number of men and women was the same in both groups, with no significant difference in age. Anthropometric measurement, including BMI, was significantly higher in the obesity group than in control. Fasting plasma glucose and resistin level both groups showed were not significantly different.

Table 1. Subjects baseline characteristics

Variables	Obesity (n= 61)	Control (n= 61)	*p-value*
Sex			
Men	26 (42.62%)	26 (42.62%)	
Women	35 (57.38%)	35 (57.38%)	
Age (years)	22 (18-40)	23 (19-38)	0.934
18-20	12 (19.7%)	13 (21.3%)	
21-30	30 (49.2%)	30 (49.2%)	
31-40	19 (31.1%)	18 (29.5%)	
Body Mass Index (kg/m^2)	30.50 (27-41.8)	21 (18.5-24.2)	<0.0001*
Fasting blood glucose (mg/dL)	86(47.06-125)	87(50.48-114)	0.654
HOMA-IR	3.71(1.04-15.98)	1.96(0.44-12.60)	<0.0001*
Resistin (pg/mL)	374(19.20-1645)	363(3.55-1955)	0.770

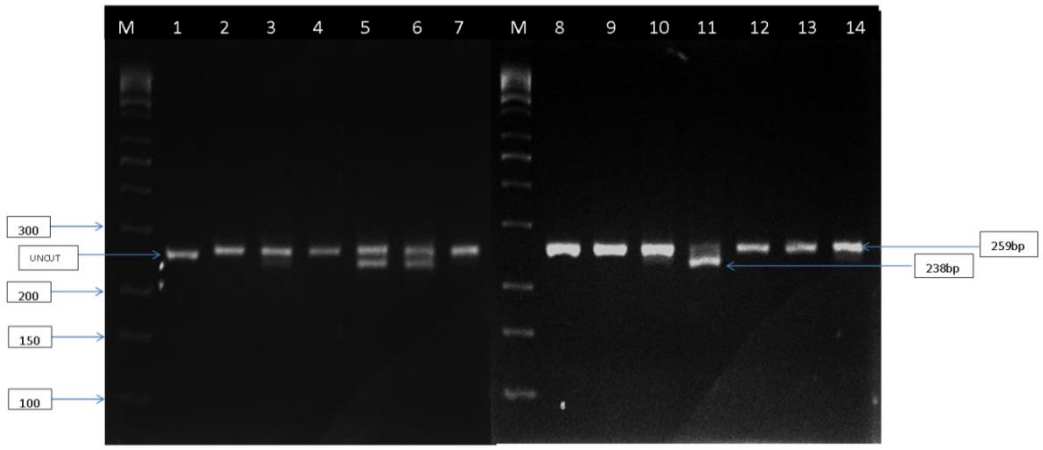

Figure 1.Result of PCR-RFLP *RETN* gene polymorphism +62G>A. Lane M is a marker. PCR product was 259 bp (lane 1 and 8). Restricted fragment by BseRI enzymes was wild type or GG (lane 2,4,7,9,10,12,13,14), heterozygote mutant or GA (lane 3,5 and 6), and homozygote mutant or AA (lane 11). No 21 bp in the figure.

Allele and genotype frequency distribution was not statistically different (p=0.187 and p= 1) between groups as well as wild-type genotype and T allele carrier frequency (p= 0.680) (Table 2). Odds ratio test showed that SNP +62G>A has neither risk factor nor a protective role in the studied population.

Table 2. Genotype and Allele Distribution of *RETN* Gene Polymorphism +62G>A

Variables		Obesity (n=61)	Control (n=61)	*p*	OR (CI 95%)
Genotype	GG GA+ AA	59(96.72%) 2(3.28%)	57(93.44%) 4(6.56%)	0.680	2.070(0.365-11.747)
	GG GA AA	59(96.72%) 1(1.64%) 1(1.64%)	57(93.44%) 4(6.56%) 0	0.187	Reference 4.14 (0.449-38.172) 0 (0.00-18.47)
Allele	G A	119(97.54%) 3(2.46%)	118(96.72%) 4(3.39%)	1	1.345(0.295-6.138)

Resistin levels were not significantly different between obese group than the control group (Table 1). Table 3 shows that on the whole subject of the study, it has no significant differences in levels of resistin between genotype GG, GA, and AA (p = 0.537).

Table 3. Resistin Level among Genotypes

	GG (n=116)	GA (n=5)	AA (n=1)	p
Resistin level (pg/mL)	366(3.55-1955)	142(15.42-1536)	776	0.537

Resistin level (Table 4) has no significant difference with the gene polymorphism RETN + 62G>A in this study. Resistin level in GG genotype had no significant difference between obese with control groups (p= 0.816) as well as individuals with GA genotype (p=1). Resistin level of genotype A allele carrier among the obese group and control group was also not significantly different (p=0.643).

Table 4. Resistin Level In Obese and Control Group

	Resistin Level (pg/mL)			p		P
	GG	GA	AA		GA+A	(GG/GA+AA)
Obese (n= 61)	374 (19.20-1645)	142	776		459 (142-776)	0.815
Control (n= 61)	363 (3.55-1955)	0.364	0		378 (15.42-1536)	0.830
p	0.816		-		0.643	
		0.830				

Plasma resistin levels in the obese group were associated with body mass index. The relationship between plasma resistin levels with a body mass index in the obese group and the control group can be seen in the scatter diagram below.

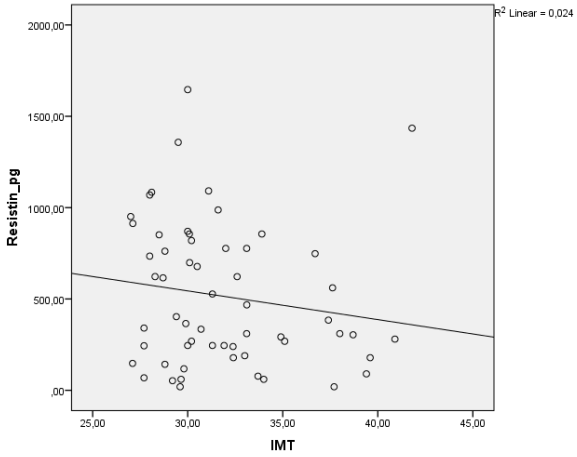

Figure 2. Scatter diagram resistin level vs BMI in obese group

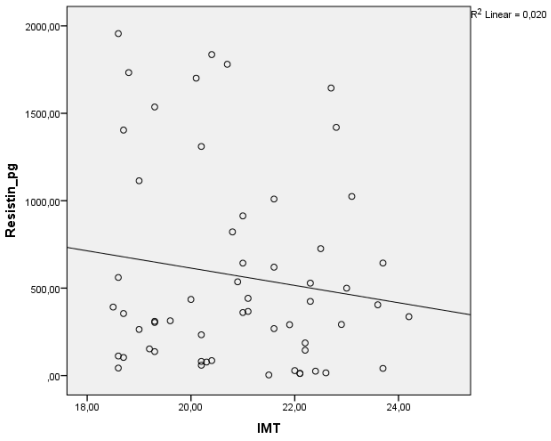

Figure 3. Scatter diagram resistin level vs BMI in obese group

Genotyping results showed three genotypes (GG, GA, AA) in the studied population (Figure 1). All three genotypes were also discovered in other Asian population [12,14,15], Caucasian,[16] and the German population.[17]

Allele and genotype frequency distribution was not statistically different between groups as well as wild-type genotype and T allele carrier frequency (Table 2). The

present result showed no significant relationship between genotype and allele with obesity status. Results of other studies also showed no association between gene polymorphisms RETN +62G>A with the parameters of the metabolic syndrome[16] and the An allele carriers had a body mass index higher than the G allele carrier.[15]

The results showed there was no significant relationship between the genotype and allele with obesity status. Results from other studies also showed no association between gene polymorphisms RETN +62G> A with the parameters of the metabolic syndrome.[16] An allele carrier had a body mass index higher than G allele carrier.[15] Obesity is caused by genetic factors, environmental, and other factors, such as decreased physical activity and increased food intake.[18] Polygenic obesity results from the combined effects of multiple genes influenced by environmental factors.[19] RETN gene variations differ in various ethnic groups and different samples. AA genotype is less common in Europe than in Asia. Environmental exposure differences (e.g., differences in dietary habits) and genetic background may also have a role in this polymorphism.[20] In this study, genotype and allele frequency differences were not significant in the obesity group compared with normal BMI because gene polymorphism RETN + 62G>A might not directly be related to the status of obesity. Other research showed polymorphism +62G>A RETN gene associated with increased levels of adiponectin, resistin levels, markers of metabolic and body fat stores.[13] This polymorphism was reported associated with hypertension and diabetes mellitus.[14]

Polymorphism RETN gene +62G> A in this study was not related to resistin level. Asano (2010) compared plasma resistin level with other RETN gene polymorphism obtained that this polymorphism had p-value bigger among RETN gene polymorphism than other areas. So it can be said that polymorphism in the promoter region -537A> C (rs34124816) and -358G> A (rs3219175) has a more significant effect on resistin levels than in the 3' UTR (rs3745368). Promoter area polymorphisms affect resistin level. SNPs -358G>A and -638G>A has a strong linkage disequilibrium with -420C> G affected circulating resistin level among other SNPs at the same locus RETN. Polymorphism RETN gene -420C>G activity increased by inducing promoter activity bind to Sp1/3.

Transcription factor Sp1 and Sp3 bind specifically to DNA elements associated with -420G. Over-expression Sp1 and Sp3 increased the activity of gene promoter -420G RETN. Polymorphism RETN gene -638G>A (rs34861192) bind to SREB1c RETN as regulators of gene expression.[12] This study was not finding significant differences may be due to gene SNP RETN +62G>A in this population has no linkage disequilibrium (LD) with functional genes that it directly influences resistin level. This study only screened +62G>A RETN gene, and therefore it cannot exclude the role of resistin other variant or variants of the nearby genes that linkage disequilibrium with SNP +62G>A RETN gene. LD structure differences may cause research of resistin level vary between populations.[14]

Correlation resistin level with body mass index in this study showed resistin levels inversely related to BMI.[21] A weak correlation between resistin levels with BMI was also found in the other study.[22] Resistin levels in this study did not differ significantly between the obese with the control group. Others studies reported no association between serum resistin levels with the percentage of body fat, visceral fat tissue, and body mass index.[8] Serum resistin reported unchanged after bariatric surgery despite significant weight loss.[23] Increased serum resistin during weight loss in overweight subjects was also reported from other studies.[24]

Resistin did not have a role in obesity and insulin resistance in human studies. Resistin mRNA in adipose tissue throughout the obese subjects increases, but lower resistin mRNA levels in isolated adipose tissue. No relationship of resistin gene expression in human adipose tissue with insulin resistance and BMI.[25] Structure and physiological human resistin and mice were differences. Human resistin gene is located on chromosome 19p13.3 in an area that was not related to obesity and insulin resistance. Differences between rodents and humans resistin because there were significant

differences between the two species in the gene and protein structure, gene regulation, distribution in a specific tissue, and the induction of insulin resistance which the lack of evidence relating to the receptor resistin and mechanisms of action of downstream signalling.[27]

Resistin level results differences may because from various confounders such as age and sex, drug use, and measurement methods.[28] Reference and sensitivity of the ELISA kit. In several studies, the cross-react with the ELISA kit RELM also affect the diversity of serum resistin concentrations. Other studies showed resistin, a hormone produced by adipose tissue that works not throughout the body. The expression of mRNA may cause this was not directly associated with the expression of proteins, which were modified post-transcriptional and post-translational affect resistin level. Serum resistin increased the average degradation of transcription through a negative feedback mechanism and the recruitment of translational inhibitors. Resistin secreted paracrine and act at the protein level. Effect of obesity on the expression of resistin in humans remains unclear.[9]

Resistin has a role in the pro-inflammatory process associated with obesity and insulin resistance. Resistin expression in peripheral blood mononuclear cells (PBMC) from several studies were affected by IL-1, TNF-α, and IL-6. The relationship between the expression, secretion of resistin, and other inflammatory markers include IL-6, leptin, and CRP were also reported in patients with severe inflammatory diseases.[27] Increased production of pro-inflammatory cytokines TNF-α and IL-12 were obtained from macrophages incubation with recombinant human resistin.[28] The study showed that this induction is mediated by transcription factor nuclear factor-kappa B (NF-κB). Low levels of tissue inflammation is a significant cause of insulin resistance induced by obesity and the molecular crosstalk in inflammation pathways and insulin signal delivery. Circulating resistin may be a central messenger between inflammation and insulin homeostasis.[29] In this study, subjects were not in inflammation condition from clinical symptoms but not measured inflammatory markers so it cannot be measured the influence of inflammatory markers such as IL-1, TNF-α, and IL-6 which could also affect plasma resistin level.

4. CONCLUSION

There was no significant difference in genotype and allele frequency distribution related to obesity status and resistin level between the case and the control group. Resistin level has a negative correlation with BMI.

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Physiochemical properties, sensory evaluation and shelf life of corn flour supplemented with Acheta gossypii (cricket) flour

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HIGHLIGHTS

Cricket supplemented flour was more acceptable than sensory evaluation.

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Most consumed diets are made from corn and are deficient in proteins. Corn flour was made from fermented and dried yellow corn and supplemented with 5 and 10 % proteins of crickets. The diets were analyzed for the following physiochemical properties: swelling index, wettability, bulk density, solubility, and water holding capacity as well as sensory characteristics in a 7 point hedonic scale and the maintaining quality of each diet. Standard methods for physiochemical properties were used, and the microbial assay was also to analyze the diets at refrigerated and non-refrigerated conditions. The swelling index(1.83-2.00 g), gelatinization temperature (66.3 -72.3°C), wettability (22.0-120 seconds), and bulk density (0.59 - 0.67 mg/mL), significantly increased but solubility (14.1 - 3.00%), and oil-water holding capacity (90-65 %) reduced significantly in the cricket supplemented diets over the corn flour ($p< 0.05$). The sensory characteristics from both the adult and children scored above average on the 7 points hedonic scale, with a major preference for the 5 % cricket supplemented the diet. It is noteworthy that in the 4 weeks of shelf life experiment, all the diets were found to be devoid of the growth of coliforms, yeast and the only occurrence of the mold is below the harmful threshold except with the 10 % cricket supplemented non-refrigerated diet with the growth of *B. subtilis* on the 3rd week of the experiment. However, the growth of this bacterium is below a harmful threshold. Proper handling and processing are safest before consumption. Supplementations of corn flour with cricket have been found to improve the nutritional quality of corn and may, therefore, be able to solve the problem of malnutrition.

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1. INTRODUCTION

Availability of food for consumption remains a significant concern of the world.[1] This is because global malnutrition level is still high[2] most consumed diets are made of staples which are deficient in nutrients; especially the cereals may lead to malnutrition.[3] Cereals such as maize, rice, millet, sorghum, oats, barley and wheat; in gravy, paps, milk or tea and rice are widely consumed around the world.[4] Maize is one of the essential staple diets of the Nigerian population[5] and third most used after rice and sorghum.[4] The proximate composition of the grains showed that maize contained low levels of crude protein and fat but high levels of carbohydrate and therefore, require supplementation for increased protein composition.[6] Crickets and other insects are a good source of proteins.[7]

However, insects, and specifically crickets, have long been overlooked and underutilized as a source of an essential nutrient in the modern world. Many species have been researched on, and the nutritional values determined. These include species such as the field cricket (*Gryllus* genus), and the house cricket (*Acheta* genus). Thailand's field cricket *Gryllus bimaculatus* (raw), for example, contains an energy value of 120 kcal/100g, which is comparable to the 150 kilocalories per100grams of skinless chicken breast. The proximate protein and fat composition of a single field cricket (*Gryllus* genus) is also 58 % protein and 10 % fat. The composition of single house cricket (*Acheta domestica*) is higher 65 % protein and 20 % fat.[8] Field crickets provide more than the minimum amino acid profile reported by the World Health Organization in order to be an adequate source of essential amino acids. In one study, it was observed that the protein of the house cricket was superior to soy protein for amino acid intake when fed to rats.[9] Vitamin B_{12}, which is only naturally present in food of animal origin, andis vital for human health, is found in sufficient amounts in house crickets (5.4µg per 100 g in adults and 8.7 µg per 100 g in nymphs); the recommended dietary amount is 2.4 µg daily.[10] The mineral composition of insects is also comparably high. The majority of insects show high amounts of potassium, calcium, iron, magnesium, and selenium, as well as zinc. *Acheta domesticus* contains about six to eleven milligram of iron per 100 gram while ground beef contains about 2.2 milligrams of iron per 100 gram.[11] This makes cricket a suitable alternative to conventional meat such as beef products[12] for supplementation of protein deficient staples.

As with all other edible products, the safety of consuming cricket products is an important question to reflect on.[10] One presumption on why there is such a prevalent caution against edible insects is that the swing from hunting and gathering to a more sedentary farming routine resulted in the view of insects as pests besides a risk to health.[13] If the safety of insect-related foods as related to processing, preservation, packaging is considered and found healthy, the consumption can be enhanced.[14,15,16] Neo-phobia for foods is another determinant of subjects' interest to eat insect-based food.[17] The essential factor in promoting entomophagy (compared to pricing, value, profit, risks, naturalness, confidence, approach, and traditions) is the opportunity for sensory evaluation.[13] Hence crickets could add its nutritious value to the vast array of foods targeted for supplementation.

2. MATERIALS AND METHOD

All the chemicals used were of general purpose grade manufactured by the British Drug House (BDH) Limited, Poole, England, and Sigma Aldrich Chemical Company Incorporation, Wisconsin, USA.

2.1. Samples Treatment

The (Yellow) corn (*Zea mays)* samples were bought from Maikunkele Market, Bosso Local Government, Minna, Niger State. The roasted cricket (*Acheta gossypii*) flour sample was commercially obtained from Ankpa roadside market, Ankpa local

Government, Kogi State, Nigeria. Both the (Yellow) corn (*Zea mays)* and the cricket (*Achetagossypii*) samples were also identified in the Department of Animal Biology, Federal University of Technology, Minna, Nigeria. The cricket roasted cricket flour after the collection was stored in plastic containers for analyses. The corn kernels were cleaned, steeped in a plastic container to ferment for 68 hours to allow for fermentation without sprouting. The fermented product was rinsed with clean tap water and sundried after which they were wholly ground severally to powder using an electric grinder (Henry west G160, 5.5 HP). This was kept in a plastic container for further analyses.

The diets were formulated into 5 and 10 % cricket supplements based on the percentage inclusion of 5 and 10 % proteins from cricket. The formulation was done based on the % protein content of the original fermented corn and cricket flour respectively to yield an increment of 5 and 10 % cricket content in the experimental diets. The diets were kept in plastic containers for further analyses. Paps were made from the corn flour, 5 % and 10 % cricket supplemented diets. Each pap sample was made with the powdered diets (4g) containing 1g of sugar and 6 mL of boiling water. This was used for sensory evaluation.

2.2. Physiochemical Properties

Food physiochemical properties are those food characteristics that describe how food responds to physical, thermal, and chemical treatments. These properties use various methods to define, quantify, and predict the behavior of new food material that is useful in determining food quality and safety. The properties carried out in the experiment (includes: bulk density, wettability, oil, and water absorption capacity, solubility temperature, and swelling index) of the corn and cricket supplemented diets.

2.3. Determination of bulk density

The method described by Onwuka[18] was adopted. The determination was carried out by placing each sample (50 g) into 100 mL measuring cylinder, making sure that the sample was well compacted (by knocking the bottom of the cylinder). The absolute value obtained was recorded in mg/mL. This is given as:

Bulk density = Bulk mass (mg) Bulk Mass (mg) Bulk volume (mL) …equation 1

2.4. Determination of wettability

The method described by Onwuka[18] was used for the determination. Each sample (1 g) was measured into a measuring cylinder (25mL) whose diameter is 1cm. The cylinder was inverted and clamped at 10 cm directly above the surface of 600 mL beaker containing distilled water (500 mL) as a finger was placed at the open end. Then the finger was removed, and the sample was allowed to be let into the water. The time taken for each sample to be thoroughly wet was noted.

2.5. Determination of oil and water absorption capacity

The method described by Sandra[19] was used to determine the oil and water absorption capacity of the samples. Using Oil (whose density is 0.9 g per mL) and or water (1 g per mL), the absorption capacity is given by the volume of a supernatant left after the sample and water are well mixed. Distilled water (10 mL) was added to each sample (1g). The combination was carefully mixed for 60 seconds and left to stand for 30minutes, and time the volume of the supernatant was recorded. The volume of the supernatant obtained is equal to the oil and water absorption capacity.

2.6. Determination of solubility

The method of Udensi and Onuora[20] was adopted. Dispersion of powder (10 % w/v) of each of the samples in distilled water was made by spreading 1g of dry sample

powder in 5mL of distilled water and made up to 10mLs then left to stand for 1hour being stirred every 10 minutes. After that, it was left to stand for another 15 minutes then filtered. The filtrate (2 mL each) was weighed in a Petri-dish (clean and dry) heated to evaporation and reweighed. The difference in mass is the total soluble solids.

Solubility = $TSS\ (\%)\big((VsMe - Md)x\ 100\big) / 2Ms\ X\ 1$ …… equation 2

Where
TSS= Total soluble solids
Vs = Total supernatant/ filtrate
Md = Mass of empty, dry Petri dish
Me = Mass of Petri dish plus residual solid after evaporative drying
Ms = mass of powder sample used in the preparation of the dispersion.

2.7. Determination of pasting temperature

The method of Nwosu[21] was modified and used. On heating, the starch granules (and protein molecules) absorb water molecules and form paste gels at a given range of temperature (gelatinization temperature). The temperature at which gelatinization onsets is the pasting temperature. Each powder (1 g) sample was dispersed in distilled water (5 mL) contained in a test-tube and heated in a water bath. The temperature of the onset of gelatinization was noted and recorded.

2.8. Determination of swelling index

The method described by Ukpabi and Ndimele was used for the analysis.[22] Dry powder portions (3 g) each was put into dry clean 50 mL graduated cylinders, and the initial volume noted after being carefully leveled. Each sample was then made up to 30mL with distilled water. The content was shaken then let to rest for 1hour while the change in volume (swelling) was noted for every 15 minutes.

2.9. Determination of organoleptic Score

The nutritional quality of a new diet is also a function of the sensory evaluation and opinion of panelists familiar with a similar diet by the aid of their senses of sight, feel, smell, and taste. In this work, the 7-grade organoleptic score was used according to Adegunwa.[22] A panel of adults (10) familiar with pap was constituted. Each person in the panel was served a pap sample and given a score sheet to mark their like or dislike. Another panel of 10 was set up for children familiar with pap. For the children, it was slightly modified with smileys where they marked their like or dislike. A seven-point scale was determined in a scale where 7 scored highest. The seven-point sensory score was based on general appearance, color, smell, taste, texture, and general acceptance. Informed consent of the parent, guardian of the children, and the ethical committee of the Federal University of Technology, Minna was obtained.

2.10. Determination of Shelf Life

The corn flour and the cricket supplemented diets were analyzed for shelf life. The shelf life for each sample was monitored at both refrigerated and room temperatures using microbial analysis. The spread plate method, as described by Ambreen, was used.[23] The spread plate method allows the secure transfer and quantifying of microorganisms on reliable mediums.

Both refrigerated and non-refrigerated powder samples were stored for 28 days and analyzed every seven days. Sample serial dilution was set up by the weighing of 1 g of each sample to 9 mL of sterilized phosphate buffer in the test tube. Following every dilution, the contents were blended for 10 seconds in a vortex mixer. 1 mL from each

dilution was added into a plate count agar in a petri dish and mixed thoroughly. Once solidification was achieved, the Petri dishes were incubated at 35° C for 48 hours for total plate count and colonies formed on the surface and in the media were calculated as colony forming unit (CFU per mL). For coliform, fungi (yeast), and mold counts the spread plate technique was also used. However, In the determination of counts for coliform, MCA (Macconkey agar) was used while that of yeast and mold was done using SDA (sabouraud dextrose agar) respectively. The Yeast growth was corrected by 40 ppm Chloramphenicol (put in as antibacterial agent) whereas only sabouraud dextrose agar was utilized for molds recognition. The media was incubated at 25 °C for 96 hours.

2.11. Statistical Analysis

Data obtained were subjected to statistical analysis for the determination of mean and standard deviation values using Microsoft Excel. Separate determinations for the significant differences ($p < 0.05$) between the mean values were analyzed using the Duncan's Multiple Range Test.

3. RESULTS AND DISCUSSION

The physiochemical properties of the diets are shown in Table 1. The swelling index ranged between 1.83 to 2.00(g/g) with the 10 % cricket diet having the highest value while the 5 % cricket supplemented diet had the lowest. There was no significant difference between the swelling index of corn flour and the 5 % cricket supplemented diet ($p > 0.05$). The corn flour diet had the lowest solubility (%) while the 10 % cricket supplemented diet had the highest solubility, which was significant from the other diets ($p < 0.05$).

There was also a significant difference in the gelatinization temperature of the diets ($p < 0.05$). It was lowest in the, and highest in the 10 % cricket supplemented the diet. The wettability and bulk density of each diet sample follows the same trend as the solubility. However, the difference in bulk density between the cornflour and the 5 % cricket supplemented diet was not significant ($p>0.05$). The oil or water absorption capacity varied significantly ($p < 0.05$) in all the diets. The corn flour had the highest oil, while the 10 % percent cricket diet has the least value (Table 1).

Table 1. Physiochemical Properties of the Experimental Diets

Diet	Swelling index(g/g)	Solubility (%)	Gelatinization Temperature (°C)	Wettability (seconds)	Oil/Water absorption capacity (%)	Bulk density(mg/mL)
Corn flour	1.92±0.40^{a}	14.0±1.50^{c}	66.3±0.70^{a}	22.0±12.0^{a}	90±10.0^{c}	0.59±0.09^{a}
5 % cricket	1.83±0.50^{a}	9.00±1.50^{b}	68.0±0.01^{b}	60.0±12.0^{b}	70±12.0^{b}	0.62±0.08^{a}
10 % cricket	2.00±0.80^{a}	3.00±0.80^{a}	72.3±0.70^{c}	120±15.0^{c}	65±10.0^{a}	0.67±0.08^{b}

Values are means (± Standard deviation) of triplicate determinations. Values with different superscript along each column are significantly different from each other ($p < 0.05$).

Tables 2a to 2 e show the microbial content of the experimental diet during storage. At the onset of the experiment (Table 2a), there was no identified bacterial, coliforms, and fungal count on the cornflour diet. However, both the 5 % and 10 % cricket diet showed the growth of *Lactobacillus bulgaricus* a fermentation microbe. The 10 % cricket supplemented diet additionally grew *Micrococcus luteus.* The organism identified were not in the list of those mentioned the Food and Drug Agency (FDA) to affect food safety.

Storage for one week at refrigerated temperature and room temperature (average of 24.9 °C) is shown in Table 2b. There was no microbial growth in the cornflour diet both at room temperatures refrigerated and at conditions. The total viable count for bacteria in the cricket diet was found to be lower than in week 1. The values in the shelved diets were much higher than the refrigerated diet. There was no significant difference between the 5 % cricket (shelved) and 5 % cricket(refrigerated) diets, but the shelved 10 % cricket supplemented diet was significantly higher than the 10 % cricket(refrigerated) diets ($p< 0.05$).

Table 2c shows that the cornflour diet still had no microbial growth after two weeks of storage. The total microbial count in the 5 % cricket diets was decreased, especially in the refrigerated diet, which had the lowest count. There was no significant difference between the total fungal count in the 5 % cricket diets (refrigerated) and 5 % cricket diets (shelved). However, the fungal count increased in the 10 % cricket with the 10 % cricket proteins (shelved) diets having the highest load ($p< 0.05$). The result for microbial assay for the third week is as shown in Table 2d. The corn flour diet remained free of microbes while the bacterial counts of the 5 % cricket diets (shelved) 5 % cricket diets (refrigerated) and 10 % cricket diets (refrigerated) diets are not significantly different($p> 0.05$). However, the bacterial count in the 10 % cricket supplemented diet (shelved) was higher than the other diets as it indicated the growth of *Micrococcus luteus, Lactobacillus bulgaricus, Bacillus subtilis*, and *Aspergillusniger.*

The result for microbial assay for the fourth week is as shown in Table 2e. In the corn flour diet (shelved), there was an appearance of *Bacillus subtilis* although within the safe limit for *Bacilus subtili*s. In the 5 % cricket supplemented diet (shelved), the growth of *Lactobacillus bulgaricus* was reduced but increased in the 10 % cricket diets (refrigerated). The 5 % cricket supplemented diet (refrigerated) had a lower growth of bacteria than in the 10 % cricket diets (refrigerated), which was significantly different ($p < 0.05$). The growth of the fungus, Mucor species was also noticed in the 5 % cricket supplemented diet (refrigerated) for the first time in the experiment.

Table 2a. Microbial Content of the Diet at Onset of Storage (week 0)

Sample	TVC $x10^3$ (cfu/g),NA	TCC $x10^3$ (cfu/g),MCA	TFC $x10^3$ (cfu/g), SDA	Microorganism Identified
Corn flour	Undetected	Undetected	Undetected	None
5 % cricket	4.09 ± 0.2^a	Undetected	Undetected	*Lactobacillus bulgaricus*
10 % cricket	3.00 ± 0.1^b	Undetected	Undetected	*Micrococcus luteus, Lactobacillus bulgaricus*
*Standard safe limit	0.10(a)	0.01(b)	10(c),0.1(d)	(a) *Bacillus subtilis* (b) *Coliforms* (c) Mould (d) *Saccharomyces spp*

Values are means (± Standard deviation) of triplicate determination. Values with different superscript along the column are significantly different from each other ($p< 0.05$). Alphabet in brackets represents organisms identified.

Key:
TVC -total viable count using nutrient agar (NA) for general bacterial
TCC - total coliforms count using MCA (Macconkey agar)
TFC -total fungal count using SDA (sabouraud dextrose agar)
cfu/g - colony forming units (S.I unit of microbial count) per gram of sample

Table 2b. Effect of Storage on the Microbial Content of the Experimental Diets (Week 1)

Sample	TVC x10^3 (cfu/g),NA	TCC x10^3 (cfu/g),MCA	TFC x10^3 (cfu/g), SDA	Microorganism Identified
Corn flour, shelved	Undetected	Undetected	Undetected	None
5 % cricket, shelved	2.70(±0.08)^c	Undetected	Undetected	*Lactobacillus bulgaricus*
10 % cricket, shelved	1.70(±0.08)^b			*Micrococcus luteus, Lactobacillus bulgaricus*
Corn flour, refrigerated	Undetected	Undetected	Undetected	None
5 % cricket, refrigerated	2.60(±0.1)^c	Undetected	Undetected	*Lactobacillus bugaricus*
10 % cricket, Refrigerated	1.30(±0.02)^a	Undetected	Undetected	*Lactobacillus bulgaricus*
*Standard safe limit	0.10(a)	0.01(b)	10(c),0.1(d)	(a) *Bacillus subtilis* (b) *Coliforms* (c) Mould (d) *Saccharomyces spp*

Values are means (± Standard deviation) of triplicate determinations. Values with different superscript along the columns are significantly different from each other (p< 0.05). Alphabet in brackets represents organisms identified.

Table 2c. Effect of Storage on the Microbial Content of the Experimental Diets (week 2)

Sample	TVC x10^3 (cfu/g),NA	TCC x10^3 (cfu/g),MCA	TFC x10^3 (cfu/g), SDA	Microorganism Identified
Corn flour, shelved	Undetected	Undetected	Undetected	None
5 % cricket, shelved	1.20±0.09^b (a)	Undetected	0.20±0.03^a(b)	(a) *Lactobacillus bugaricus,* (b) *Muccorpussillus*
10 % cricket, shelved	18.0±1.0^c (a, b)	Undetected	0.70±0.01^b(c)	(a) *Lactobacillus bulgaricus,* *(b) Bacillus subtilis and* (c) *Aspergillusniger*
Corn flour, refrigerated	Undetected	Undetected	Undetected	None
5 % cricket, refrigerated	0.11±0.03^b (a)	Undetected	0.10±0.03^a(b)	(a) *Lactobacillus bulgaricus,* (b*) Miccorpussillus*
10 % cricket, refrigerated	1.70±0.12^a	Undetected	Undetected	*Lactobacillus bulgaricus*
*Standard safe limit	0.10(a)	0.01(b)	10(c),0.1(d)	(a) *Bacillus subtilis* (b) *Coliforms* (c) Mould, (d) *Saccharomyces spp*

Values are means (± Standard deviation) of triplicate determinations. Values with different superscript along the columns are significantly different from each other (p< 0.05). Alphabet in brackets represents organisms identified.

Table 2d. Effect of Storage on the Microbial Content of the Experimental Diets (week 3)

Sample	TVC x10^3 (Cfu/g),NA	TCC x10^3 (Cfu/g),MCA	TFC x10^3 (Cfu/g), SDA	Microorganism Identified
Corn flour shelved	Undetected	Undetected	Undetected	None
5 % cricket, shelved	1.21±0.11[a] (a)	Undetected	0.18±0.04[b] (b)	(a) *Lactobacillus bulgaricus,* (b) *Muccorpussillus*
10 % cricket, shelved	17.9±1.20[b] (a, b, c)	Undetected	0.70±0.05[c]	(a) *Micrococcus luteus* (b) *Lactobacillus bulgaricus* (c) *Bacillus subtilis* (d) *Aspergillusniger*
Corn flour, refrigerated	Undetected	Undetected	Undetected	None
5 % cricket, refrigerated	1.11±0.11[a] (a)	Undetected	0.10±0.08[b](a)	(a) *Lactobacillus bulgaricus,* (b)*Muccorpussillus*
10 % cricket, refrigerated	1.67±0.30[a]	Undetected	Undetected	*Lactobacillus bulgaricus*
*Standard safe limit	0.10(a)	0.01(b)	10(c),0.1(d)	(a) *Bacillus subtilis* (b) *Coliforms* (c) Mould, (d) *Saccharomyces spp*

Values are means (± Standard deviation) of triplicate determinations. Values with different superscript along the columns are significantly different from each other (p< 0.05). Alphabet in brackets represents organisms identified.

Table 2e. Effect of Storage on the Microbial Content of the Experimental diets (week 4)

Sample	TVC x10^3 (cfu/g),NA	TCC x10^3 (cfu/g),MCA	TFC x10^3 (cfu/g), SDA	Microorganism Identified
Corn flour, shelved	0.01±0.00[a]	Undetected	Undetected	*Bacillus subtilis*
5 % cricket, shelved	1.19±0.07[a](a)	Undetected	0.19±0.02[a](b)	(a) *Lactobacillus bulgaricus* (b) *Muccorpussillus*
10 % cricket, shelved	18.3±2.00[b] (a, b, c, d)	Undetected	Undetected	(a) *Micrococcus luteus* (b) *Lactobacillus bulgaricus* (c) *Bacillus subtilis* (d) *Aspergillusniger*
Corn flour, refrigerated	Undetected	Undetected	Undetected	None
5 % cricket, refrigerated	[a]1.19±0.05[a]	Undetected	[b]0.10(±0.02)[a]	(a) *Lactobacillus bulgaricus* (b) *Muccorpussillus*
10 % cricket, refrigerated	1.73±0.20[a]	Undetected	Undetected	*Lactobacillus bulgaricus*
*Standard safe limit	0.10(a)	0.01(b)	10(c),0.1(d)	(a) *Bacillus subtilis* (b) *Coliforms* (c) Mould, (d) *Saccharomyces spp*

Values are means (± Standard deviation) of triplicate determinations. Values with different superscript along the columns are significantly different from each other (p< 0.05). Alphabet in brackets represents organisms identified.

The values for the organoleptic score taken for both children and adults are shown in Figures 1 and 2. Figure 1 shows the mean sensory characterization of the experimental diets by children. The mean scores were above average in the 7-point hedonic scale. There was no significant difference in the acceptance of the pap made from all the experimental diets, but the taste score for the corn flour was significantly lower (p< 0.05) when compared to the other diets. The 5 % cricket diet scored highest in smell, color, taste, and texture; its high score for general appearance was not significantly different with the 10 % cricket supplemented diet. The corn flour diet pap scored the least in all the parameters except for the value in the general appearance.

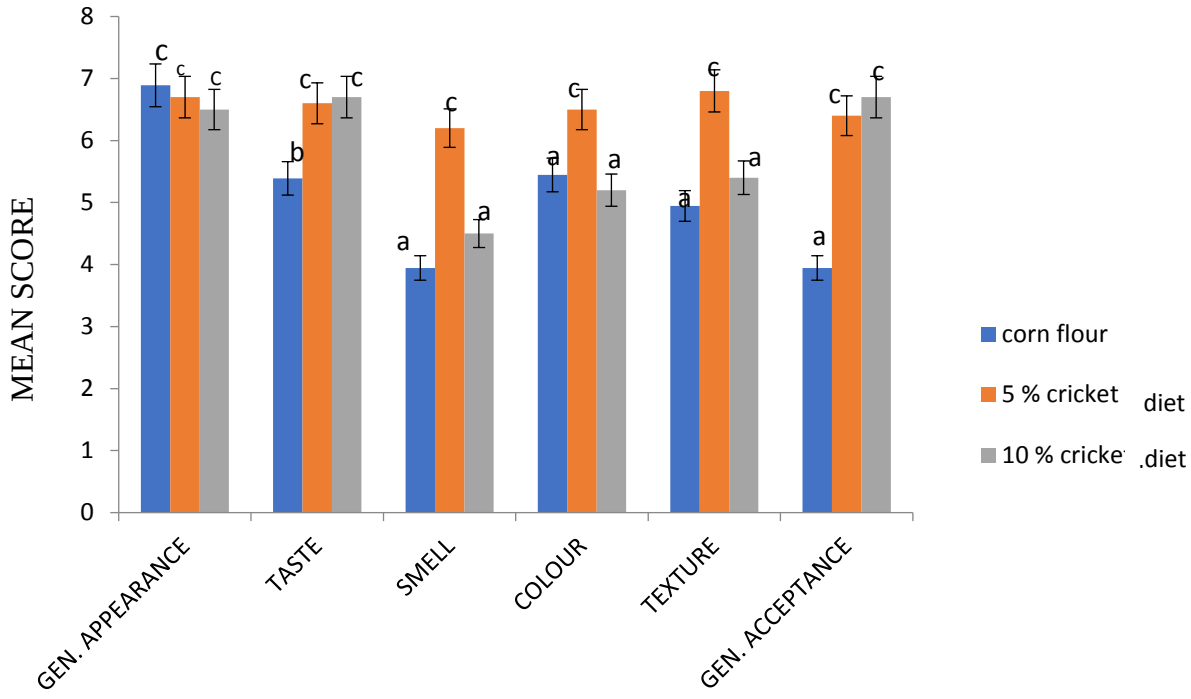

Figure 1. Sensory Characterization of Pap by Children, made from Experimental Diets.

Figure 2 which is the sensory evaluation scores of adults on pap made from the experimental diets shows that the 10 % cricket diet had the highest score for general appearance while the corn flour diet was the lowest, although these differences are not significant (p> 0.05). The organoleptic score for taste was not significantly different in the 10 % and 5 % cricket diets both were significantly higher than the corn flour for taste. The 10 % cricket supplemented diet scored highest in smell (p< 0.05). The 5 % cricket supplemented diet was rated highest for color and texture; the two parameters were not significantly different when compared in other diets. There was no significant difference between the 5 % cricket supplemented the diet and the corn flour for color texture (p> 0.05).In general, the scores for the parameters in each sample ranged between 4.8 and 6.3 on the 7-point scale. The sensory evaluation from both children and adults were similar both in the trend of values, general appearance, and smell of the pap sample.

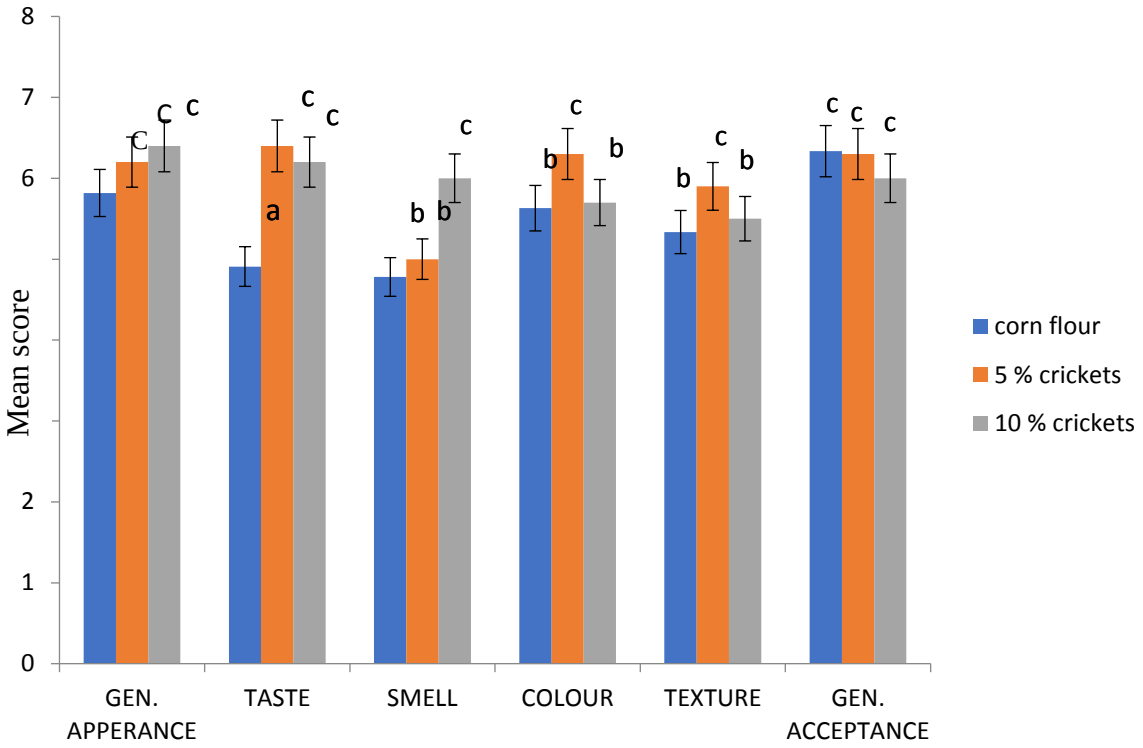

Figure 2. Sensory Characterization of Pap, by Adults made from Experimental Diets.

The physiochemical properties describe the functional properties of the diets for potential use in the food industry. The higher swelling value with 10 % cricket over the other diets might indicate that the interaction of the molecules such as proteins and fats within the amylase portion of the starch granules is non covalent as similarly reported by Adegunwa.[22] The reduction in solubility from the cornflour to the 10 % cricket diet indicates that more soluble matter such as sugars (amylose, sucrose, and other soluble carbohydrates), soluble proteins and salts may have been leached into the solution. The higher lipid content in the 5 % and 10 % cricket diet compared to the cornflour diet might be responsible for the decreased solubility in the supplemented diets which is similar to the findings of Chandra.[24]

The higher gelatinization temperature of the supplemented diets over the cornflour diets can also be related to the report of Chandra[24] that higher cooking temperature of carbohydrates increases with supplementation. This might be due to incorporation through the supplements other less polar long-chain molecules like the lipids.

The trend in wettability, which can be linked with the higher soluble matter found in the diets, might affect their storage properties as reported by Nwosu.[21] In humid conditions, easily wet samples might get contaminated with microbes. The lower value of absorption of water or oil with reducing carbohydrate content is expected. The water absorption capacity of the flour sample is influenced by increasing carbohydrates content but reduced lipid content. This is similar to Adegunwa[22] who reported that free sugars and some proteins are capable of binding a large amount of water through hydrogen bond formation as a result of the partial forces of attraction between OH^- and H^+.[21]

The increase in the bulk density from the corn flour through the 5 % cricket diet to the 10 % cricket diet may be as a result of a likely increase in relatively non-compressible components like the lipid. This implies a decrease in the compressibility of the flour sample for packaging and other related processes that require compression of food samples, and higher bulk density might also reflect higher energy value on the diet as similarly reported by Nwosu.[21]

The microbial load observed in this work (Tables 2a-2e) is similar to the findings of Ambreen[23]; Amadi and Kiin-Kabari[25]; Ajibola[26]. The microbial assay for week 0, in the supplemented diets, indicated organisms that are gut-friendly and therefore not harmful. The growth of the microorganisms noticed in the diets could be as a result of growth encouraging conditions arising from supplementation (presence of higher amounts of proteins). The general reduction of the microbial load at week 1 reflects the presence of inhibitory substances like organic acids and alcohols in the food. The presence of *B subtilis* in the 10 % cricket diet is an indication of a higher tendency of the diets to spoilage. This is not unexpected as the diet is rich in proteins, amino acids, and other nutrients which make it better substrates for microbe growth. Thao observed that a higher nutrient in diets increases the microbial composition of the diets.[27] The continued presence of *BACILLUS subtilis* and other organisms may not be of great concern since the food is to be processed by heating which destroys the microbes as reported in the findings of Ajibola.[26]

At the end of the 4th week of storage, the microbial growth noticed in a most shelved sample including the corn flour was an indication of increase conditions for growth such as mesophilic temperature, and nutrients allowed for the maturity and growth of spores into adult organisms which were previously not detected. It is noteworthy that in the 4th week's assay, apart from 10 % cricket diet (shelved) diet, all the diets are safe because they were void of coliform, yeast and the only occurrence of the mold was below the harmful threshold.[28] However, refrigeration condition is best for the samples.

The sensory evaluations of both children and adults that are significantly above the 3.5 average scores of the 7 points sensory scale were similar to the findings of Andrew[29] and Ambreen[23]. From the adult scores, the organoleptic evaluation for the cornflour pap was the least, and the most were the 5 % cricket diet. Igyor[30] stated that higher mean sensory values in the supplemented diets might be traceable to the effects of supplementation. It is possible that the blend of the roasted cricket and fermented corn flour can enhance the sensory receptors because they might have emitted volatile substances. This might have reached an optimum at the 5 % protein supplementation of the corn diet with the cricket flour. The organoleptic values for both children and adults were similar, suggesting the diets were acceptable and beneficial to all age groups.

4. CONCLUSION

The pap made from the 5 % cricket supplemented flour was more acceptable from the sensory evaluation compared to the pap made from corn flour, and 10 % cricket supplemented diets. The biological parameters showed that higher growth performance, total protein intake was found in the 10 % cricket supplemented diet. Moreover, the physiochemical parameters of the diets were all significantly different ($p<0.05$) except for the swelling index. Finally, studies on the shelf life showed that refrigeration was a better efficient method of storage when compared to the shelf storage at room temperature (27-29°C).

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The concentration of NaCl soaking to decreasing cyanide levels in Gadung (Dioscorea hispida Dennst)

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HIGHLIGHTS

Soaking 5% NaCl in G*adung* tubers containing cyanide can reduce cyanide levels by 99.70%

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Cyanide is one component contained in *gadung* tubers, and therefore, consuming gadung that contains more than 1 ppm of cyanide can cause nausea and intoxication. This research aims to study the effect of concentration variation of NaCl soaking to the decrease of cyanide levels in *gadung*. This research is an experimental study with three treatment. Testing is only done once. Samples of *gadung* were soaked by NaCl (0% 1% 3% 5%) for 3 days. Determination of cyanide content in *gadung* used the UV-Vis spectrophotometry method by adding Ninhydrin 0.1% in Na_2CO_3 2% and NaOH 0.1 % in the sample filtrate. Result of this research had shown that cyanide content in *gadung* before NaCl soaking was 4.42 ppm and after NaCl soaking (0% 1% 3% 5%) were 3.52; 2.53; 0.43 and 0.01 ppm, respectively.

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1. INTRODUCTION

Gadung plants are easy to grow in critical land without special care.[1] *Gadung* plants grow wild in dry forestlands and can be cultivated in a home garden. This plant grows at an altitude of 850 - 1,200 meters above sea level.[2] Yogyakarta people process *gadung* tubers into chips.[3] In Nusa Tenggara and Maluku, *gadung* tubers are consumed to substitute sago and corn during the dry season.[2] Gadung is also found in other countries, and the people commonly use it for traditional medicine due to its efficacy in medication for illnesses, such as leprosy, dermatitis, abdominal spasms, as well as in the making process of oral contraception.[4]

Gadung tuber contains cyanide (CN^-), which has a negative effect on health.[5] Results of the study have uncovered that fresh *gadung* tubers contain cyanide levels of 469.5 ppm.[6] Consuming *gadung* tuber with a very high level of cyanide potentially causes

nausea, motion sickness, even death.[5] Based on SNI (Indonesian National Standard), the maximum limit of cyanide levels in food products is 1 ppm.[7] The cyanide content (CN^-) in *gadung* tubers needs to be minimised and eliminated by several processes so that the *gadung* tubers are safe and suitable for consumption.

Some alternative processing methods commonly carried out by the community are soaking tubers in water and smearing them with ash. However, the immersion method takes about 3-5 days and needs to be done so that it is considered less efficient.[6] The process smearing with ash is inefficient because it requires many stages of treatments and takes up to 10 days. Another method is soaking *gadung* tubers into a salt solution.[8] The use of salt solutions in soaking *gadung* tubers can cause differences in osmosis pressure inside and outside the material so that the osmosis diffusion process occurs from the inside part into the outside part of the dissolved material.[8] When the concentration of the salt solution is too high, the osmosis process between water and solute (cyanide) gets faster.

Previous studies on *gadung* immersion used table salt and calcium solutions. The use of NaCl solution with various levels of concentration in the immersion in this research has never been applied in the previous research. The purposes of this study were to determine cyanide levels in *gadung* tuber samples before and after NaCl immersion and to determine the optimum concentration of NaCl which can reduce cyanide levels (CN^-) in *gadung* tuber samples.

2. MATERIALS AND METHOD

This research is an experimental study with three treatment. Testing is only done once.

2.1. Tools and materials

The tools used for determining cyanide levels in the sample: UV-vis spectrophotometric. Reagent materials needed: distilled water, NaCl (1%, 3%, 5%), CN p 10 ppm, 0.1 M NaOH, 0.1% Ninhydrin, and 2% Na_2CO_3. The research material was *gadung* tuber obtained from Magetan Regency.

2.2. Procedure

2.2.1. Sample Preparation

Gadung tubers were cut 3 mm thick, pounded using mortar. The sample was then weighed five grams and added 10 ml of distilled water. Next, it was filtered using Whatman filter paper no.42 until a clear filtrate was obtained. The filtrate was later tested qualitatively and quantitatively.

2.2.2. Qualitative Analysis

Qualitative analysis of cyanide was done by pipetting 2 ml of *gadung* tuber filtrate to the test tube and adding 1 ml of 0.1% ninhydrin solution in 2% Na_2CO_3. Then, 15 drops of 0.1 M NaOH were added and homogenized. Finally, the colour changes occurred.[9]

2.2.3. Determination of Maximum Wavelength

Solution (CN^-) of 1 ppm was pipetted as much as 2 ml and put in the test tube. 1 ml of 0.1% ninhydrin solution was added in 2% Na_2CO_3. Then, 15 drops of 0.1 M NaOH were added and homogenized. After the materials reacted, the colour changes occurred. The maximum wavelength was read using a UV-Vis spectrophotometer in the range 400 - 620 nm.[9]

2.2.4. Determination of Sample Level

Gadung tuber samples were cut with 3 mm thickness. The cuts were later soaked in NaCl solution (with concentration of 0%, 1%, 3%, and 5%) for three days. A total of 5 grams of refined *gadung* tuber samples were soaked in 10 mL of distilled water. Then, it

was filtered using Whatman filter paper no.42 until a clear filtrate was obtained. Next, 2 ml of the filtrate is pipetted and put into the test tube. 1 ml of 0.1% ninhydrin solution was added into 2% Na_2CO_3. Finally, 15 drops of 0.1 M NaOH were added and homogenized. Finally, the colour changes occurred.[9]

3. RESULTS AND DISCUSSION

The results of the qualitative test on *gadung* tuber samples before immersion can be seen in Figure 1. The findings show that an identical purple/blue colour was formed both in the sample and in positive cyanide control.

A B C

Figure 1. Qualitative Cyanide Test (CN^-)

Description:
A = *gadung* tuber sample before immersion
B = positive control (containing cyanide)
C = negative control (not containing cyanide)

Qualitative tests carried out by reacting the *gadung* tuber extract with 0.1% ninhydrin reagent in 2% Na_2CO_3, and 0.1 M NaOH demonstrate that the sample of *gadung* tuber was identified containing cyanide (CN^-). The reaction between cyanide in *gadung* tuber with ninhydrin and NaOH resulted in purple/blue discolouration. The formation of purple/blue colour is obtained, exposing that ninhydrin forms red colour if reacted with cyanide. When NaOH is added, it forms a purple/blue colour.

Determination of the absorption value of a sample must be at the maximum wavelength so that the maximum value is obtained. The results of determination of the maximum wavelength using a UV-Vis spectrophotometer in the wavelength of 498 nm. The determination of the calibration curve was obtained from the concentration of 10 ppm of cyanide stock solution (CN^-). The solution was then diluted to five concentrations, namely 1 ppm, 2 ppm, 3 ppm, 4 ppm, and 5 ppm. The absorbance levels of the five concentrations of standard solutions were measured at a wavelength of 498 nm. From the calibration curve, a regression equation $y = 0.1273X + 0.022$ was obtained.

The determination of initial levels of cyanide in *gadung* tuber samples before treatment was carried out three times. The absorbance obtained were: 0.572; 0.596, and 0.586. The average absorbance value was: 0.5847. The value of cyanide concentration in *gadung* tubers before treatment was: 4.42 ppm. Cyanide content in *gadung* tuber samples was still above the SNI standard value with a maximum cyanide content of 1 ppm.[7]

The results of immersion of *gadung* tubers with 0%, 1%, 3% and 5% NaCl are demonstrated in Table 1. *Gadung* tuber samples were soaked for three days in NaCl solution (0%, 1%, 3%, and 5%), and purple/blue colour that was less bright than the colour before immersion treatment was obtained. This research shows that the cyanide level in the sample after treatment decreased. The absorbance value of the sample proves it before treatment, which was higher than that after immersion.

Table 1. Determination of Cyanide Levels after Immersion with NaCl Concentration

Variation of Concentration (%)	Average Cyanide Level (ppm)	Decrease Percentage (%)
NaCl 0	3.52	20.26
NaCl 1	2.53	42.77
NaCl 3	0.43	90.28
NaCl 5	0.01	99.70

Based on research carried out, *gadung* tubers immersion using 3% and 5% NaCl can reduce cyanide levels up to 90.28% and 99.70%, with cyanide levels of 0.43 ppm and 0.01 ppm. Therefore, it can be implied that the immersion of 3% and 5% NaCl can reduce cyanide levels in *gadung* tubers to the safe limit of cyanide content in food products SNI (Indonesian National Standard) year 2015 which stipulates that the maximum limit of HCN level is 1 ppm.[7] Some previous studies have revealed that the immersion result of *gadung* tuber using table salt 5% for three days is 66.99%.[9] To compare two variables of the immersion with the same concentration (5%), NaCl is considered more effective because it can reduce the cyanide level-up to 99.70%.

The decrease of cyanide level is due to the osmosis diffusion process when the immersion of *gadung* tuber using salt solution is carried out.[10,11] The cyanide (HCN) compounds formed in *gadung* tuber undergo the osmosis diffusion and then bind to Na^+ on NaCl to form NaCN. According to the research in which *gadung* is immersed using $Ca(OH)_2$, it is found that Ca^{2+} can bind to HCN to produce $Ca(CN)_2$, which is easily dissolved in water.[12] The difference in the immersion between NaCl solution and table salt solution is because table salt solution is composed of several types of salt. The most prominent component of the table salt is NaCl compound, but there are other compositions such as $CaSO_4$, $MgSO_4$, $MgCl_2$, KCl, NaBr, and iodine.[13] These new salt compounds cause the osmosis diffusion process in table salt immersion less efficient compared to that using NaCl, which does not contain any other disturbing compounds. One of the factors that affect the osmosis reaction is that the molecules which are smaller than the centre line of the membrane hole will be more easily absorbed. Some salt compounds can be hygroscopic, that they absorb water from the tissue and damage cell walls and cause plasmolysis (cell membrane rupture due to lack of water).[14]

The practical application developed in this research is the use of table salt, especially NaCl, which can serve as the alternative method to reduce the cyanide level in *gadung* tuber before the technological processing is carried out. Further studies regarding the importance of organoleptic of *gadung* tuber can be improved. The parameter of organoleptic aims to determine the colour, scent, and taste of *gadung* tuber after the immersion using NaCl, for instance, by steaming, boiling, and frying. The processing of *gadung* tuber by frying can also reduce the cyanide level because the cyanide that is dissolved in water will evaporate with water particles during the frying process.[15] The processing method by drying the tuber starch can also reduce some toxics.[15,16] Several parameters that can be applied in further research are starch, water, ash, fibre, and nutrient in *gadung* tuber after treatment.

4. CONCLUSION

The results of the qualitative test show that *gadung* tubers contain cyanide. Quantitatively, the cyanide levels in *gadung* tubers before immersion treatment were 4.42 ppm. The optimum decrease in cyanide levels correlates with the immersion of 5% NaCl by the percentage of 99.70%.

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