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Influence of Germination and Morphological properties on Microscopic Characteristics of protein isolates from Two (2) Solojo Cowpea (Vigna Unguiculata L. Walp) Varieties in Nigeria

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Abstract
Structural morphologies of total protein isolate of the two varieties of solojo cowpea were studied by scanning electron microscope (SEM). This has been established to be a valuable tool for examining the microstructures of grains and products produced from them as well. The morphological properties of raw and germinated protein isolates of DAS and BS are presented in Figures. 4.42 – 4.53. Results showed the apparent structural differences between them. The Raw DAS showed plate like structure with relatively large smooth gutter-like cavities having bulky particle sizes and irregular geometry shapes and Raw BS showed flaky structure like that of Albumins. This pattern is similar to that of albumin shapes of the Great Northern Bean, field pea protein and that of Crotalaria pallida. After six hours of modification, a change in morphology of the original isolate was observed with gradual disappearance of the plate-like and flake-like structure and the vacuum space; DAS 24 h protein isolates then exhibited a spongy plate like structure. Rise in the germination period to 36 h led to disappearance of the cloudy mass and formation of smooth non homogeneous mass. Further increase in the germination time to 48 and 72 h led to smoother mass with a few dispersed particles with cracked surface like that observed for bambarra. The observation of the microstructure presentation from 36 h is also comparable to that obtained for bitter vetch (Vicia ervilia) protein films strengthened by microbial trans-glutaminase. In this case, the images of BVPC films containing mTGase clearly indicate a more compact microstructure, with evident continuous zones. The BS isolates morphology which presented a thin wafer-like structure like that of albumin, possessing wide surface area could in part justify the great solubility of albumins in neutral environment, enabling better access to water molecules. The structure was observed to become more homogeneous and compact, with no cavities as germination proceeded. This is like the image found for the outer and inner depths of chorizo stuffed in natural and synthetic casings before and after 5 days of smoking both protein isolate presented irregular, rectangular-shaped particles, which were agglomerated and had a thick mass with limited pores. The likeness between the particles in these micrographs is in agreement with that of loss of albumin proteins from the isolate extracted at pH 9. This isolate is envisioned to have most of the features of globulins. The patterns with increase in germination time clearly indicate a more compact microstructure, with evident continuous zones. The DAS protein isolates presents micrograph similar to those of white bambarra (WB) and black bambarra (black bambarra) surfaces which presented cracking. The micrograph of BS 6 h germinated protein isolate, which presents a flaky plate like structure is comparable to that of commercial textured Glycine max protein. It was also observed that the micrograph of freeze-dried pea protein isolate presented an irregular shaped structure, with denser mass and some pores which was observed to form a more compact structure with increase in germination time.

Keywords: *Solojo Cowpea, Underutilised legumes, Protein isolate, Antinutrients, DAS, BS, microstructure*

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I. Introduction

Cowpea (Vigna unguiculata (L.) Walp.) is an important legume crop growing across the world mainly in tropical and subtropical regions of Africa including Ethiopia and Nigeria. Besides its noticeable production, little is known about its yield, productivity, importance, and distribution in Nigeria. Cowpea is one of the most ancient crops known to man. The main subspecies is Vigna unguiculata (L.) Walp. subsp. unguiculata (L.) Walp. Cowpea is often called "black-eyed pea" due to its black- or brown-ringed hylum. Cowpea is called the "hungry-season crop" because it is the first crop to be harvested before the cereal crops **(Gomez, 2004).**

Among the grain legumes, cowpea (Vigna unguiculata (L.) Walp.) is an important food legume growing in tropical and subtropical regions of the world. Its fresh or dried seeds, pods and leaves are commonly used as human food. Since they are highly valuable as food, cowpeas are only occasionally used to feed livestock but the hay and silage can be an important fodder **(Alemu et al., 2016).**

Cowpea has great flexibility in use: farmers can choose to harvest them for grains or to harvest forage for their livestock, depending on economical or climatological constraints. Dual-purpose varieties have been developed in order to provide both grain and fodder while suiting the different cropping systems encountered in Africa **(Tarawali et al., 1997).** Cowpea by-products such as cowpea seed waste and cowpea hulls (which result from the dehulling of the seeds for food) have been used to replace conventional feedstuffs in some developing countries **(Ikechukwu, 2000).**

According to modern nutrition recommendations, human beings ought to depend majorly on proteins of vegetable and legume origin for their dietary protein needs **(Oreopoulou and Tzia, 2007; Sibt-e-Abbas et al., 2015).** Pulses have been found to play very essential role in achieving the required nutritional recommendations, particularly in emerging and third world countries where the consumption of mammalian protein is low because of the high cost **(Singhal et al., 2016).** Apart from the high cost, large amounts of saturated fat and cholesterol are other problems associated with animal protein sources **(Klupšaitė, and Juodeikienė, 2015).**

Concerns about high-cholesterol, has necessitated the recommendation of regular consumption of vegetable protein as opposed to animal protein by nutritionists. This has led to a renewed interest in legume protein because of their high level of protein which ranged between 20 and 60% **(Aletor and Ojelabi, 2007).** They also have good protein quality in respect to their digestible and nutritional characters **(Cheng-mei et al., 2018).** Apart from this, the level of fibre in the body also increases with increased consumption of more plant food that helps in reducing the danger of bowel diseases, as well as cancer of the colon and prevalence of osteoporosis Compared to cereal grains, legume grains are also very excellent source of weight reduction fibers **(Ahmed et al., 2011; Mune et al., 2013; Mudryj et al 2014; Aghajanpour et al., 2017).**.

Several efforts by researchers have gone into methods of improving the functionality of protein by different modification methods. Physical modification offood proteins to enhance their capabilities, example, gel formation, adhesiveness, emulsification and foaming has been studied **(Hughes et al., 2011; Sharif et al., 2017)**. Instances of the physical modification of proteins include altering the preparation parameters such as temperature and pH. This is done by bringing about partial denaturation of proteins using heat (dry or moist). Denaturation is believed to result in limited unraveling of the tightly crammed structure of the storage globulin proteins or in the regulated unravelling of the poly-peptides which brings about increased availability of sensitive areas of the molecules previously buried, thereby improving the protein functionality **(Shimelis and Rakshit, 2007).** Physical modification using heating, freezing, or extrusion has also been carried out, and this has been found to denature protein structure causing reduced solubility and functionality **(Mirmoghtadaie et al., 2016).** Protein modification by high-pressure homogenization, causes insolubilization of proteins (Marco-moles et al., 2012). Other modifications that have been investigated include genetic modification **(D'Astolfo et al., 2015; Krall et al., 2015)** enzymatic modification **(Alashi et al., 2011)** and chemical modification **(Lawal and Adebowale, 2006; MacDonald et al., 2015).**

The SEM is a powerful and versatile analytical instrument for material characterisation. It is a microscope that generate images pertaining to a specimen by browsing the surface using a concentrated ray of electrons. SEM require running a strong energy ray of primary electrons over the exterior of an aggregate sample that inspires discharge of subsidiary electrons. The released trapped electrons are electronically transformed to a representation of facial topography, exhibited through a cathode beam tube in raster form. The electrons react with atom in the material, generating varied signs containing facts concerning the surface area and structure of the material **(Stokes, 2008)**. The image produced by SEM is due to the signal obtained by the interaction at varied depth within the sample of the electron ray with atoms at these varied levels. The signals produced comprise of; Secondary electrons (SE), characteristic X-rays and light (cathodoluminescence) (CL), reflected or back- scattered electron (BSE), transmitted electrons and absorbed current (specimen current). SE detectors are part of the standard equipment in all Scanning Electron Microscopic instruments **(Keshri, 2014).**

It is very useful for small material analysis. It uses electrons for imaging. It is used to determine the morphological property of flour, concentrates and isolates of protein. It has been established as a valuable

instrument for analysing the micro-structures of cereal grains, imitation cereal seeds as well as allied produce. SEM has also been utilised in the determination of the internal microstructure of beef products, soybean foods, dairy products by **(Horita et al., 2014; Chen et al., 2014; Zhou et al., 2014; Ledesma et al., 2016)** respectively. It has also been utilised in the investigation of the characteristic framework of starch, flours and proteins of legumes such as vigna subterranea **(Kaptso et al., 2014; Yeboah-Awudzi et al., 2018)**, glysine max isolate **(Zhang et al., 2016)**; onion sprout **(Majid et al., 2018).** The internal microstructure, shapes, dimensions and distinguishing facial characteristics have also been investigated for foods in powdery form.

Freeze dried raw and sprouted Solojo isolates and their morphological properties were analysed using Scanning Electron Microscope (SEM) (Model No. JSM 6610-LV) at magnification of 1.00K (Majid et al., 2018). This work therefore is designed to evaluate the ability of biochemical modification in enhancing the functional properties, and nutritive quality of Solojo protein. Solojo an underutilized legume commonly grown in the South-West region of Nigeria, will be biochemically modified for its possible industrial application through its functional properties.

II. Experimental

Materials

The raw material investigated in this research study is Solojo Cowpea (Vigna unguiculata L.) which occur in two varieties i.e. Dark-ash solojo (DAS) and Brown Solojo (BS). These two underutilized varieties found in South-West region of Nigeria where they are called 'Solojo' were obtained from Bodija market in Ibadan, Western Nigeria. They were stored in polyethylene bags at room temperature (25-26^{o}C).

Figure 1: Dark-Ash Solojo Cowpea (DAS)

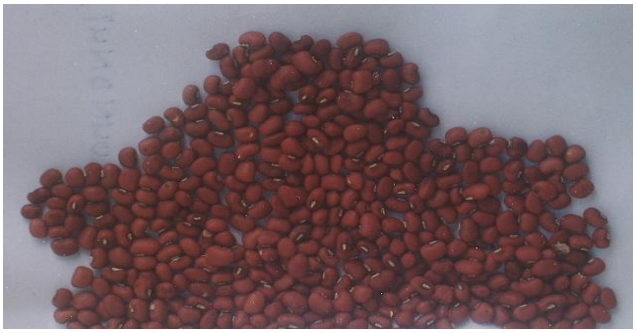

Figure 2: Brown Solojo Cowpea (BS)

III. Methods

The dehulled cowpea seeds were cleaned and screened to get rid of every irrelevant materials and unwholesome seeds. The Solojo seeds (DAS and BS) for germination were sterilized by soaking in 0.07 % Sodium hypochlorite (Rumiyati et al., 2012) for 30 min, then rinsed thoroughly. The Solojo seeds were then immersed for 6 h in distilled water at ambient temperature (1:10 w/v) (~25oC), then placed in a colander and germinated under subdued light in an open laboratory **(Rusydi, 2011)** for 0, 6, 24, 36, 48 and 72hrs. Other treated portions of the Solojo seeds (DAS and BS) were dehusked, dried, milled into flour and defatted. Protein was isolated by isoelectric precipitation method. Proximate, antinutritional analysis and functional properties [Water Absorption Capacity (WAC), Oil Absorption Capacity (OAC)] of the flours and protein isolates were determined by standard methods. Amino acids and molecular weight of the protein isolates were determined by amino acid analyzer and sodium-dodecyl-sulphate-polyacrylamide-gel-electrophoresis.

Surface morphology, functional group and thermal properties were determined for protein isolates by scanning electron microscopy, Fourier Transform Infrared (FTIR) spectrometry and differential scanning calorimetry, respectively. Data were analysed by descriptive statistics and ANOVA at α0.05

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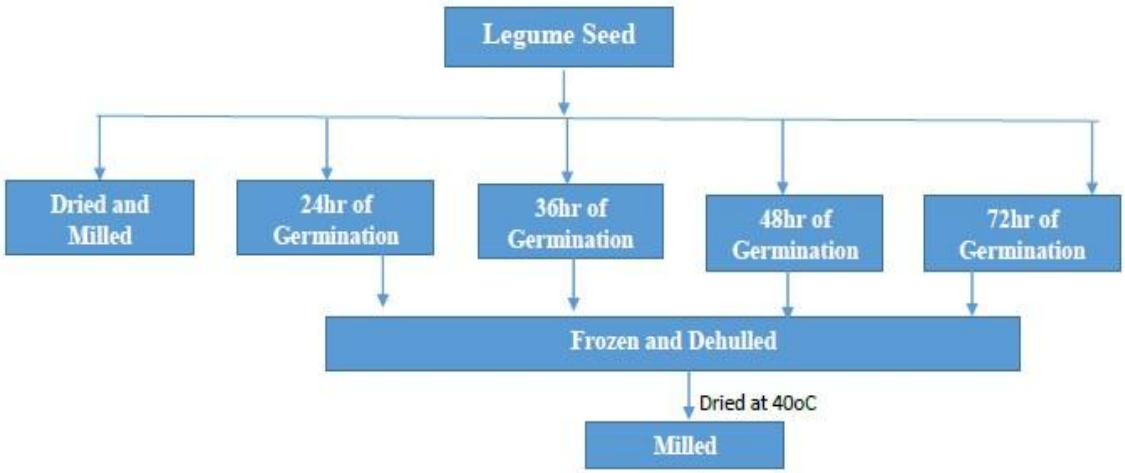

Figure 3: Preparation of Beans Flour/Schematic representation

IV. Result and Discussion

Structural morphologies of total protein isolate of the two varieties of solojo cowpea were studied by scanning electron microscope (SEM). The morphological properties of raw and germinated protein isolates of DAS and BS are presented in Figures. 4 – 15. Results showed the apparent structural differences between them. The Raw DAS showed plate like structure with relatively large smooth gutter-like cavities having bulky particle sizes and irregular geometry shapes and Raw BS showed flaky structure like that of Albumins. This pattern is similar to that of albumin shapes of the Great Northern Bean, field pea protein and that of *Crotalaria pallida* (**Azagoh *et al*., 2016; Ukil *et al*., 2017**).

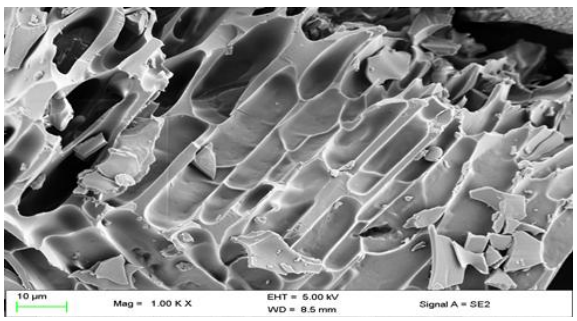

Figure 4. DAS Raw Isolate /Scanning Electron Microgram

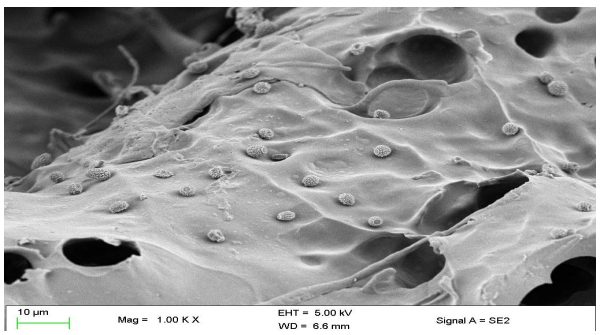

Figure 5. DAS 6 h Isolate/ Scanning Electron Microgram

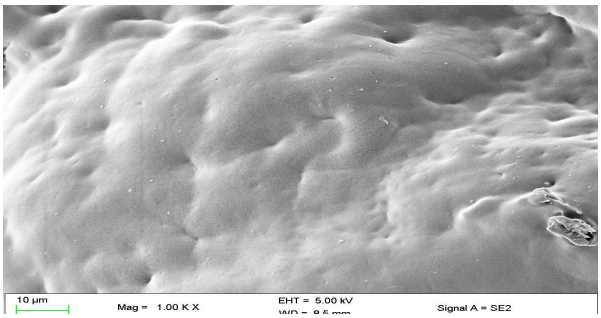

Figure 6. DAS 6 h Isolate/ Scanning Electron Micrograph

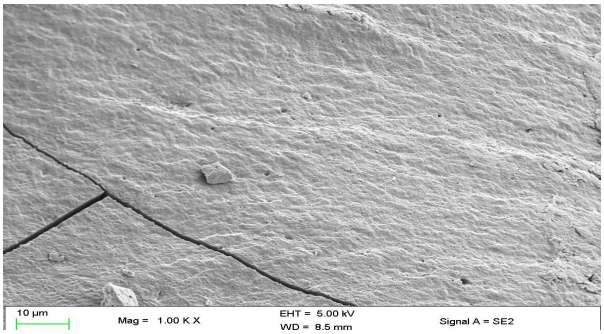

Figure 7. DAS 36 h Isolate /Scanning Electron Micrograph

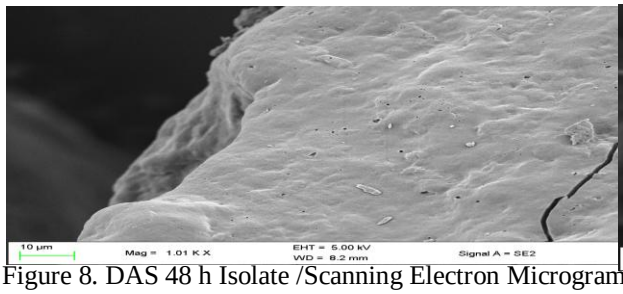

Figure 8. DAS 48 h Isolate /Scanning Electron Micrograph

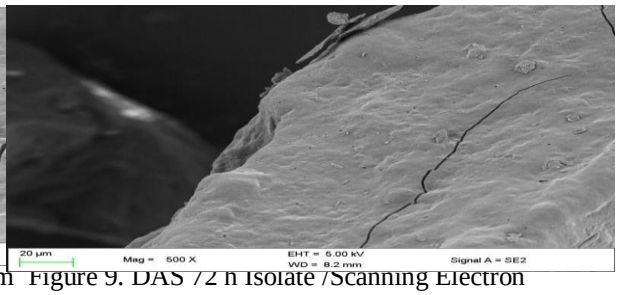

Figure 9. DAS 72 h Isolate /Scanning Electron Micrograph

After six hours of modification, a change in morphology of the original isolate was observed with gradual disappearance of the platelike and flake-like structure and the vacuum space; DAS 24 h protein isolates then exhibited a spongy plate like structure. Rise in the germination period to 36 h led to disappearance of the cloudy mass and formation of smooth non homogeneous mass. Further increase in the germination time to 48 and 72 h led to smoother mass with a few dispersed particles with cracked surface like that observed for bambarra. The observation of the microstructure presentation from 36 h is also comparable to that obtained for bitter vetch (*Vicia ervilia*) protein films strengthened by microbial transglutaminase, in this case, the images of BVPC films containing mTGase clearly indicate a more compact microstructure, with evident continuous zones **(Porta *et al*., 2015 and Ukil et al., 2017).** The BS isolates morphology which presented a thin wafer-like structure like that of albumin, possessing wide surface area could in part justify the great solubility of albumins in neutral environment, enabling better access to water molecules. The structure was observed to become more homogeneous and compact, with no cavities as germination proceeded. This is like the image found for the outer and inner depths of chorizo stuffed in natural and synthetic casings before and after 5 days of smoking **(Ledesma *et al.*,2016)** both protein isolate presented irregular, rectangular-shaped particles, which were agglomerated and had a thick mass with limited pores. The likeness between the particles in these micrographs is in agreement with that of loss of albumin proteins from the isolate extracted at pH 9. This isolate is envisioned to have most of the features of globulins **(Azagoh *et al*., 2016).** The patterns with increase in germination time clearly indicate a more compact microstructure, with evident continuous zones. The DAS protein isolates presents micrograph similar to those of white bambarra (WB) and black bambarra (black bambarra) surfaces which presented cracking **(Kaptso *et al*., 2015)**. The micrograph of BS 6 h germinated protein isolate, which presents a flaky plate like structure is comparable to that of commercial textured *Glycine max* protein **(Brishti *et al*., 2017; Dey and Sinhababu, 2018).** Azagoh *et al*. (2016) also observed that the micrograph of freeze-dried pea protein isolate presented an irregular shaped structure, with denser mass and some pores which was observed to form a more compact structure with increase in germination time. The mode of drying was also found to affect the surface morphology as observed in the case of freeze drying and spray drying **(Azagoh *et al*., 2016) (Kaptso et al., 2015).** The observations made in the SEM micrographs of the modified protein isolates have been reported to be attributed to degradation of the stored proteins (synthesised during seed development) to small peptides or amino acids during germination **(Yang and Li, 2010),** the higher molecular weight amino acids being broken down to lower molecular weight amino acids

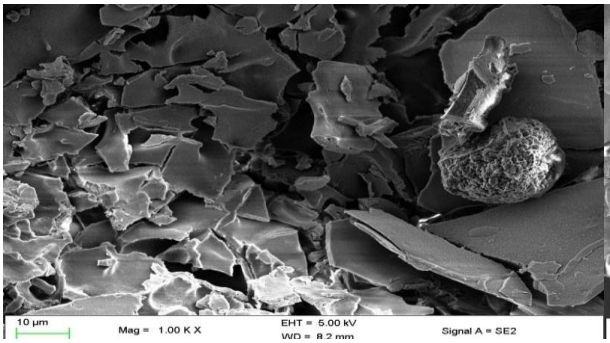

Figure 10. BS Raw Isolate/SEM

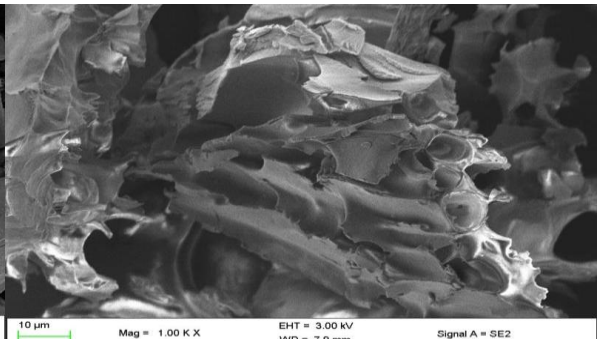

Figure 11. BS 6 h Isolate/SEM

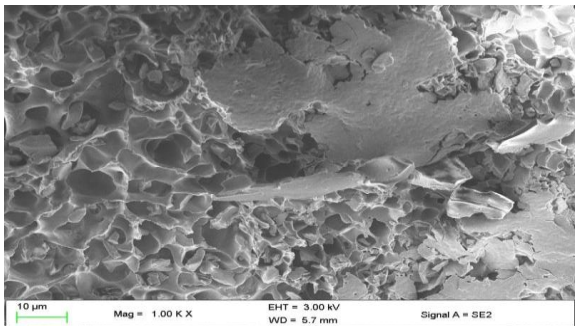

Figure 12. BS 24 h Isolate/SEM

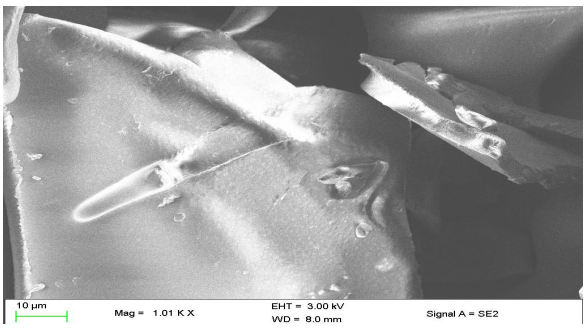

Figure 13. BS 36 h Isolate /SEM

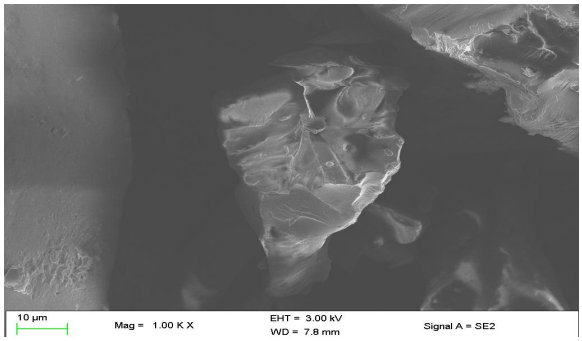

Figure 14. BS 48 h Isolate /SEM

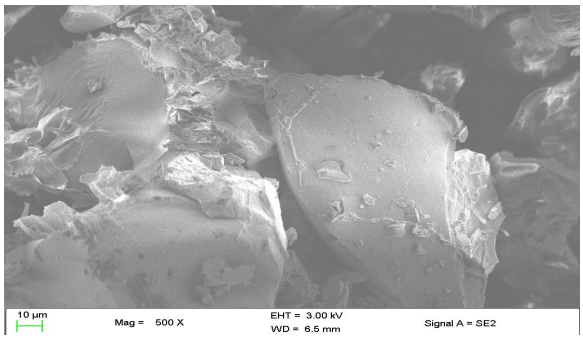

Figure 15. BS 72 h Isolate/SEM

V. Conclusion and Recommendation

Biochemical modification which involves the activation of the intrinsic enzymes of the Solojo cowpea seed itself by germination was carried out for different hours for the two varieties, i.e. the Dark-Ash and the Brown Solojo beans. Proximate analysis, revealed that biochemical modification improved the nutritional quality of the Solojo beans better, when compared with the obtained result of Chemical modification in the literature. Which means that, biochemically modified flour and protein isolates will do better in food processing than chemically modified isolates. Germination also brought about reduction in indigestible dietary fibre that causes flatulence and discourages people from wanting to consume legumes, as a result of the increase in α-amylase activity which brings about the breakdown of complex carbohydrate, this is majorly taken care off. All these make consumption of germinated seed to be healthier than the ungerminated seed.

Germination also improved the bio-availability of the important minerals, this is as a result of breakdown of phytate and other antinutrients which chelates to the divalent metals, most especially Fe, Zn and Ca, making them unavailable. The decrease in value of the phytate and other antinutrients and increase in value of the micro-nutrients shows that germination actually brought about this break down. With the breakdown of the antinutrients, the minerals are made more available for use both in the food product and when consumed.

The amino acid was also found to increase with germination, this is attributed to the breakdown of the higher molecular weight storage protein which are broken down to lower molecular weight proteins, thus making it more available to the body when used in production. Germination has also been found to increase the amount of the limiting sulphur amino acids which makes legume protein to be termed as incomplete. This research work shows that germination had a profound and significant ($p<0.05$) effect on the nutritional

composition of Solojo beans, revealing great improvement and thus making Solojo a potential substitute to other important legumes such as soya beans.

In food formulation, the high bulk density of germinated flours and protein isolate, shows that the flour and protein isolate will be very useful for infant food and geriatric food formulation for this will allow for higher ease of dispersion and also reduce paste thickness, which is a very important attribute in this class of food product. Better protein solubility at higher and lower pH was also observed with germination, this is important because protein solubility is a useful guide for the conduct of protein in the food system. Solubility of a protein is one of the crucial functional properties needed by the food industry, because it greatly affects other properties such as emulsification, gelation and foaming, indicating that Solojo germinated flour and protein isolate can be utilised in various food type. Water absorption capacity is another important functional attribute in foods, such as sausages, custards and doughs, germination brought about the improvement in water absorption capacity of the flour and protein isolate. The addition of a pinch of salt brought about greater protein solubility and therefore increased the desired water absorption properties for food formulation. The increase in OAC with germination means that the flavour retention and mouth feel of foods will be greatly enhanced if used in food formulation.

Germination was also found to improve foaming capacity of flour and the protein isolate of the seed. The improvement brought to foaming capacity as a result of the addition of salt, further reiterate improvement in their ability to be useful for the production of cakes and pastries among other food. The capability of protein to form gels and produce a structure matrix able to hold water, flavours, sugars and food ingredients is important in food applications and in the development of new products, thereby contributing an added magnitude to protein functionality. The low gelation concentration noticed may be an advantage in the use of these flours for the formation of cord or as an added ingredient to other gel forming materials in food products as little quantity will produce the desired gelation needed and this help in product acceptability.

The SDS-PAGE electrophograms revealed that there was disintegration of high molecular weight polypeptides from 97.4KDa until 45KDa and below, making available more easily digestible and assimilable amino acids. This was confirmed by the pattern observed for the Structural Morphological Examination by SEM, of the raw and germinated protein isolate. The raw had a more tightly packed structure with large cavities, which smoothens out with breakdown of the higher molecular weight polypeptides occasioned by germination, this is important because, lower molecular weight proteins are more useful than the higher molecular weight proteins. The thermal stability showed that the dark ash Solojo sample had a better thermal resistivity than the brown variety, although they both still had values better than other legumes. The FTIR analysis did not show the creation of new functional groups with germination, it only showed the increase in intensity, which also confirms the bioavailability of important protein with germination.

This research work shows that biochemical modification (Germination/Malting/ Sprouting) had an enourmous impact on the nutritional composition, functional properties, mineral bioavailability, anti-nutrient content and amino assay of Solojo bean, thus, it could be used as protein supplement in infant, young children and geriatric foods.

Efforts should be increased to promote the cultivation, encourage the consumption and industrial application of this under-utilized legume by the Government, especially in the south-western region where it can survive the rain fall level. Large scale production of this legume which is gradually going into extinction should be encouraged in order to fight the menace of malnutrition in developing countries where animal protein price is exorbitant; This will ensure food security and also creation of jobs, because people can engage in different aspects of the production process and thereby reducing the rate of unemployment.

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