INTERNATIONAL JOURNAL OF INNOVATIVE RESEARCH & DEVELOPMENT

ISSN 2278 – 0211 (Online)

Nutritional Composition and Functional Properties of Acetylated and Maleylated Derivatives of Pigeon Pea (*Cajanus cajan*) Protein Isolate

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Abstract:
Chemical modification has been used as a means of improving the nutritional quality and functional properties of proteins through the alteration of the protein structures. In this present study, effects of acetylation and maleylation on the nutritional and functional properties of pigeon pea protein isolate were evaluated. Pigeon pea protein isolate was modified by acetylation using acetic anhydride and maleylation with maleic anhydride. The result of the proximate composition revealed that modification increased the moisture content while there was no significant difference in the percentage crude protein of the native protein isolate and the modified protein isolates. The ash content and the crude fibre also reduced following modification. The result obtained from the invitro multienzyme protein digestibilities showed that both native and modified protein isolates had high digestibilities. However, a reduction in the digestibilities was observed in the protein isolate following modification (94.20% for native protein isolate), 91.00%, and 80.14% for acetylated and maleylated protein isolates respectively. Acetylation and maleylation increased the protein solubility in both acidic pH range below the isoelectric point and in the alkaline region. Acetylation and maleylation also improved the water absorption capacity, oil absorption capacity, foaming capacity and the emulsion properties of the protein isolate.

Keywords: *Acetylation, Maleylation, Protein isolate, Functional Properties, Pigeon Pea.*

1. Introduction

Proteins compose of amino acids that differ in type, arrangement and quantitative relationship. They are the major structural component of muscles and other tissues in the body. (Jay *et al*, 2004). Proteins are available in different varieties of dietary sources including animals and plant origin. Some of the animal sources of proteins include eggs, milk, meat, fish and poultry while the vegetable sources such as legumes, nuts and soy. Protein isolates from these sources are the most refined form of protein products having 90 – 95% protein content and lowest non – protein compounds. Protein isolates are, nowadays believed to have played a major role in the development of new class of formulated foods. They are the acceptable ingredients for diary application due to their fine particle size and dispersibility. Emulsion capacity and stability, colour and flavour which are critical in diary application. Isolates (especially soy proteins) are being used to fortify all types of pasta products such as macaroni, spaghetti, to improve nutritional value etc. (Sipos, 2013). However, the native protein isolates have limited functionality and this necessitated the need for development of processes to improve the plant protein functional characteristics. Functional properties are the physical and chemical characteristic of the specific protein influencing its behaviour in food system during processing, storage, cooking and consumption.

Advances in science and technology has transformed soybean into diverse products and enhanced its commercialization and marketing systems leading to the neglect of other underutilized legumes such as pigeon pea. Pigeon pea *(Cajanus cajan*) is a perennial legume from the family Fabaceae. Today, pigeon pea is widely cultivated in all tropical and semi-tropical region of both the old and the new worlds. It has been reported to contain 20-22% protein, 1.2% fat, 65% carbohydrate and carbohydrate and 3.8% ash (FAO,1989). The mineral content and amino acid profile compares closely with those of soyabean except in methionine content (Apata and Ologboho 1994, Osagie, 1998). Pigeon pea contains high lysine which is additional asset for its use in food formulations.

The aim of this study is to determine the nutritional composition and functional properties of the acetylated and Maleylated derivatives of pigeon pea protein isolate.

2. Materials and Methods

2.1. Procurement of Samples

The pigeon pea used in this study was purchased from Oja-Oba in Ado-Ekiti, Ekiti State, Nigeria. The samples were authenticated by a curator in the Department of Crop, Soil and Pest management of the Federal University of Technology, Akure, Ondo State,

2.2. Preparation of Sample

The seeds were screened to remove stones, immature and bad ones after which they were dehulled, dried and milled into powder. The powdered sample was stored in screw-capped air-tight container and refrigerated at 4^0C prior to analysis.

2.3. Preparation of Defatted Flour Sample

Defatted sample of the seed flour was prepared by continuous extraction method using n-hexane for nine hours in a soxhlet apparatus. The defatted flour sample was thoroughly air-dried to remove any trace of solvent.

2.4. Preparation of Protein Isolates

In preparation of the protein isolate, defatted seed flour, was dispersed in distilled water at a meal ratio of 1:20 w/v (flour/water). The mixture was stirred with a magnetic stirrer for 30 minutes after which the pH of the slurry was adjusted to the pH at which the protein in the flour is most soluble (pre-determined for sample) using 0.1M HCL or 0.1M NaOH dropwisely. The solution was further stirred for 2 hours at 30±2ºC using a Gallenhamp magnetic stirrer to enhance high degree of protein solubility. The slurry was centrifuged at 4,000rpm for 30 minutes at 4ºC. The residue obtained after decanting the supernatant was re-extracted with half the volume of the same solvent under similar conditions. The pH of the combined supernatants was adjusted to the pH at which the protein in the flour is least soluble (the isoelectric point which has been predetermined) with 0.1M HCl to precipitate the protein. The isolate was recovered by centrifugation for 30 minutes at 4^0 C after which it was dispersed in distilled water, poured in dialysis tube and dialyzed against distilled water for 18 hours. The dialyzing water was replaced at intervals of 3 hours during the period of dialysis. The dialysate was freeze dried and then stored in air-tight container in the deep freezer for further analysis

2.5. Acylation of Protein Isolate

Acylation was carried out according to the Method described by Groninger, 1973. Protein isolate was made into a slurry of 25% w/v with distilled water. The pH of the solution was adjusted to 9.0 using 1 mol/L NaOH and cooled in an ice bath to reduce the temperature to 4-5^0 C. Maleic anhydride/Acetic Anhydride was added to the solution at the levels of 0.5 g/g of protein. The pH of the solution was maintained at about 8.5-9.0 with constant stirring. The reaction was completed when the pH of the protein solution stabilized.

2.6. Determination of Degree of Modification

The degree of acetylation/maleylation of free amino acid groups was determined according to the trinitrobenzene sulphonic acid TNBS by Habeeb (1966) as described by Adebowale*et al*, 2009. Protein solution (1 mL) was added to 1 mL of 0.1% TNBS and 1 ml of 4% $NaHCO_3$. The solution was allowed to react at 40°C for 2 h. Then 1 ml of 10% dodecyl sodium sulphate was added to solubilize the protein followed by addition of 0.5 ml of 1 M HCl. The absorbance was then recorded at 335 nm using spectrophotometer against a reagent blank. The absorbance of the control protein isolate was set to 100% free amino groups and the degree of acylation of the modified samples was calculated based on the decrease in absorbance because fewer amino groups will be able to react with the TNBS reagent.

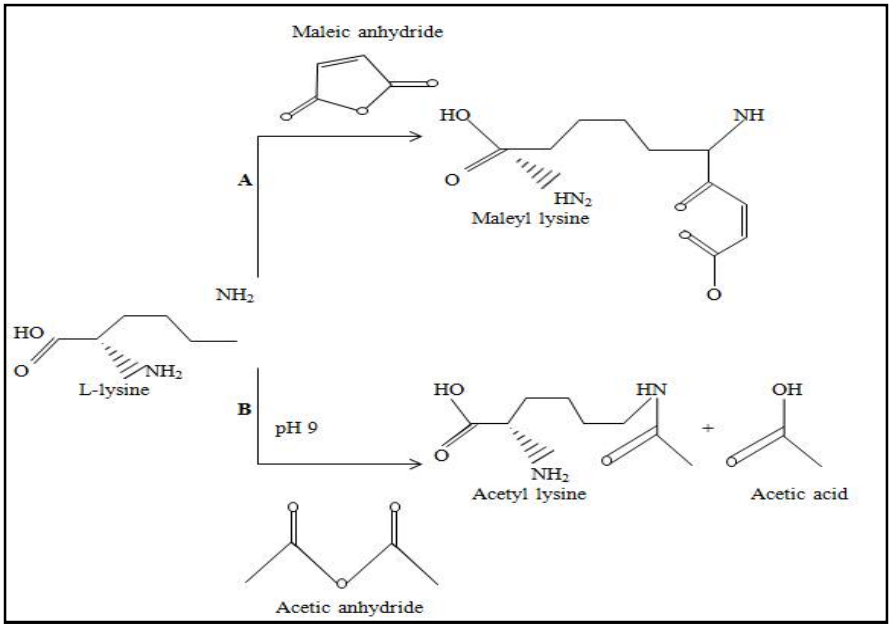

Figure 1: Acetylation and maleylation diagram

2.7. Determination of Proximate Composition

The protein and the modified protein isolates were analysed for their proximate composition (crude fat, total ash, moisture and crude fibre) according to the standard method of AOAC (2005). Carbohydrate was obtained by difference.

2.8. Determination of In-vitro Multienzyme Protein Digestibility (IVPD)

The in-vitro multienzyme protein digestibility of the isolates was determined following the procedure of Hsu *et al* 1977 as reported by Fagbemi 2004. The enzymes used are procine pancreatic trypsin, bovine pancreatic chymotrypsin and porcine protease type xxiv peptidase all from sigma USA. The activity of the enzymes was initially determined before use by using them to digest casein. The protein isolate and modified protein isolates were ground into fine powder. Each of the samples was dissolved in 50ml distilled water to give a sample suspension of 6.25mg protein per ml. the pH of each sample suspension was adjusted to pH8.0 with 0.1M HCl and 0.1M NaOH and incubated in water bath at 37^0C with constant stirring. Fresh multienzyme solution was prepared to contain 1.6mg trypsin, 3.1mg chymotrypsin and 1.3mg peptidase per ml. the pH of the multienzyme solution was also maintained at pH 8.0. 5ml of the multienzyme solution was added to each sample suspension with constant stirring at 37ºC. The pH of each sample suspension was recorded at10mins after the addition of the enzyme solution. The IVPD was calculated using the equation following regression equation.

Y = 210.464 – 18.103x

Y = in- vitro protein digestibility (%)

X = pH of sample suspension after 10mins.

2.9. Determination of water and oil absorption capacities (WAC and OAC)

The WAC was determined using the procedure of Sathe *et al*., 1982. 10ml of water was added to 1.0g of each sample in a beaker, the suspension was stirred using magnetic stirrer which was later transferred into centrifuge tubes and centrifuged at 3500rpm for 30mins. The volume of the supernatant obtained was measured. The density was assumed to be 1g/ml. the water absorption was calculated as the difference between the initial water used and the volume of the supernatant obtained after centrifuging. The OAC was determined by replacing the distilled water with king's vegetable oil. The water and oil absorption capacities were expressed in g/ml.

2.10. Determination of foaming capacity (FC) and stability (FS)

2.00g of sample was whipped with 100cm^3distilled water in a warring blender and the mixture was immediately poured into a 100cm^3 graduated measuring cylinders. The foaming capacity was calculated as percentage volume increase due to whipping. The foam stability (FS) was determined as foaming capacity after 2hrs.

2.11. Determination of least gelation concentration (LGC)

2-20% sample suspensions of the samples were prepared in 5ml distilled water. The test tubes containing these suspensions were then heated for one hour in a boiling water bath followed by rapid cooling under running cold water and further cooling at 4^0C for 2 hours. The LGC was determined as the concentration at which there was no slipping of the sample contents when inverted (Fagbemi, 2004)

2.12. Determination of emulsion capacity (EC) and stability (ES)

To 0.5g of each sample, 5ml of distilled water and 5ml of king's vegetable oil was added and the mixture was homogenized for 5mins. The emulsion so obtained was centrifuged at 3,500rpm for 5mins. The height of the emulsion layer was noted in the graduated centrifuge tube. The emulsion capacity was expressed as ml of oil emulsified per gram of sample and was expressed as percentage (Nwosu, 2010).

Emulsion stability was determined in a similar manner as above but it involved heating the emulsion at 80^0C in a water bath for 30mins before centrifuging. It was then kept for cooling under running tap for 15min. the emulsion stability was then expressed as the percentage of the emulsifying activity remaining after heating (Nwosu, 2010).

2.13. Protein solubility (PS)

Mixture of 5% protein isolate/distilled water was homogenized and left to solubilize at ambient temperature for about 5 minutes. The pH of the mixture was adjusted to pH 2 – 11 with the aid of 0.1MHCl and 0.1M NaOH and then centrifuged at 3,500rpm for 30 minutes (Adebowale, 2006), The supernatant was decanted and the soluble protein determined using Kjeldhal's method (AOAC, 2005). The value was expressed as the percentage of the protein content of each flour sample and plotted against pH.

→ Statistical Analysis

The analyses were conducted in triplicates and the data generated from all the results obtained were subjected to statistical analysis of variance (ANOVA) using SPSS17 computer package and the mean values separated by Duncan's multiple range test.

3. Results and Discussion

3.1. Degree of Modification

The extent of the reaction of TNBS with the free lysine presents in the native and acylated protein isolate was used to determine the extent of modification. The result showed that the rate of modification with acetic anhydride was greater than maleic anhydride. Thus, acetic anhydride may be a better acylating agent for pigeon pea, this observation is consistent with that observed for acylated mung bean protein isolate reported by El-Adawy, 2000. Chemical modification like acetylation of proteins of leguminous plant seeds lead first to a change in the charge and hydrophobicity of proteins (Krause *et al*., 2001).

Sample	% Modification
Acetylated protein isolate	85
Maleylated protein isolate	78

Table 1: Extent of Modification

Sample	Moisture content %	%Ash	%fat	%crude protein	%crude fibre	% carbohydrate
Native protein isolate	3.69^{a}±0.04	2.56^{b}±0.05	0.50^{a}±0.01	92.34^{c}±0.02	0.91^{a}±0.02	ND
Acetylated protein isolate	5.00^{c}±0.01	2.54^{b}±0.05	0.50^{ab}±0.02	91.08^{a}±0.01	0.88^{a}±0.01	ND
Maleylated protein isolate	4.73^{b}±0.04	2.28^{a}±0.04	0.54^{b}±0.02	91.59^{b}±0.30	0.86^{a}±0.01	ND

Table 2: Proximate Composition of Native, Acetylated and Maleylated pigeon pea protein isolate
ND = Not Determined

3.2. Values with Different Superscript on the Same Column Are Significant ($P \leq 0.05$)

The chemical composition of native and acylated(Acetylated and maleylated) pigeon pea protein isolate is presented in Table 2. There was a significant difference in the moisture content of the native and acylated protein isolate with the acetylated protein isolate having the highest value. However, the ash content reduced significantly following maleylation while there was no significant difference between the native and the acetylated protein isolate.

Crude fibre is low. The protein content of the proteins ranged between 91.08-92.34%. This result is comparable with the result obtained from acetylated and succinylated African yam bean protein isolate reported by Adebowale *et al* 2009 and for blue lupin isolate reported by Butt and Batool. 2010.

The invitro multienzyme protein digestibility of the native and acylated protein isolates is shown in Table 3. It was observed that the native and acylated proteins have high digestibilities ranging from 80.14-94.20%. However, there was a slight reduction in the digestibility following acylation.The result is consistent with those observed in other legume protein isolates like lupin protein isolates 86.3-93.9% (Iquari *et al*, 2002), 90-94% for chickpea protein isolate(Sanchez-vioque *et al*, 1997). The high digestibilities of these proteins, suggests that they could be used in new food formulations.

Sample	Digestibility (%)
Native protein isolate	94.20^{c}±0.01
Acetylated protein isolate	91.00^{b}±0.20
Maleylated protein isolate	80.14^{a}±0.10

Table 3: Invitro Multienyme Protein Digestibility.

3.3. Values with Different Superscript on the Same Column are Significant ($p \leq 0.05$)

Table 4 presents the functional properties ofthe native and acylated protein isolates. There was a significant increase in the water/oil absorption capacities of acylated protein isolates with the acetylated having a betterWAC/ OAC than the Maleylated protein isolates. Dua *et al*, 1996 reported an increase in OAC of rapeseed meal after acetylation. Oil absorption capacity is the binding of fat to the non-polar side chain of proteins and several factors may affect it such as protein content, surface area, hydropobicity, the charge, liquidity of oil and the method used. (EL-Adawy,2000). This may also be due to the degree of denaturation following modification. The result showed that the protein isolates will be potentially useful in flavour retention and improvement of palatability.

Acylation improved the foaming capacity. The foaming capacity of the acetylated protein is higher than Maleylated protein isolate, this result is consistent with previous report on increase in foaming capacity after acylation reported for jackbean (lawal and Adebowale, 2009), mung pea protein isolate(EL-Adawy,2000). It should be noted that foaming capacity depends on several factors such as pH, type of protein, processing method, temperature, presenceor absence of calcium ion(Fernema,1996, Townsend and Nakal, 1983). Foaming stability reduced following modification because of negative charges imparted during modification causing the protein molecules to unfold and the increase in charge density which prevents protein-protein interaction in foam resulting in destabilization and poor stability (Dua *et al*, 1996). The emulsification properties of acylated pigeon peaprotein was significantly increased ($p \leq 0.05$), this observed increase may be due to the unfolding of protein chains, which causes the hydrophilic residue on the peptides to be exposed leading to an improvement in the emulsification properties. However, Maleylated protein isolate has better emulsification properties. The pigeon pea protein isolate has a least gelation concentration of 14% which is comparable with cashew nut protein isolate reported by Ogunwolu*et al*, 2009.However, acylation improved the gelation properties since the lower the value of the LGC, the better the gelation property of the protein ingredient. The isolate may find use in formulation of curd or as an additive to other gel forming materials in food products.

Sample	Water absorption Capacity (ml/g)	Oil Absorption capacity (ml/g)	Foaming Capacity (%)	Foaming stability (%)	Emulsion capacity (%)	Emulsion stability (%)	Least gelation concentration
Native protein isolate	3.13^{a}±0.12	2.90^{a}±0.06	16.40^{a}±0.08	34.00^{c}±0.15	58.92^{a}±0.01	40.00^{a}±0.01	14.00^{c}±0.01
Acetylated protein isolate	5.30^{c}±0.04	3.80^{c}±0.01	24.00^{c}±0.01	16.00^{a}±0.19	64.36^{b}±0.12	64.36^{b}±0.15	10.00^{a}±0.05
Maleylated protein isolate	3.20^{b}±0.02	3.40^{b}±0.01	20.38^{b}±0.12	26.09^{b}±0.30	68.17^{c}±0.18	68.17^{c}±0.18	12.00^{b}±0.05

Table 4: Functional Properties of Native, Acetylated and Maleylated protein isolates.

3.4. Values with Different Superscript on the Same Column are Significant ($p \leq 0.05$)

3.4.1. Protein Solubility

The result for variation of protein solubility with pH for protein isolates and the modified protein isolates of pigeon pea is presented in Fig 2. It was found that the isoelectric point of the proteins is 5.0 which was in agreement with most plant proteins (Vani and Zayas,1995). It was also observed that the solubility reduced as the pH increases until it reaches the isoelectric point. This was followed by a progressive increase in solubility with further increase in pH. The high solubility profile indicated at alkalinefor all the isolates implies that the legume can best be extracted alkaline extraction followed by precipitationby acid at their isoelectric pH. The high solubility in both acid and alkaline region indicate that they could have promising food applications, Protein solubility is a useful indicator for the performance of protein isolatesincorporated in the food system and also the extent of protein denaturation because of heat andchemical treatment at different pH (Horax*et al*, 2004).

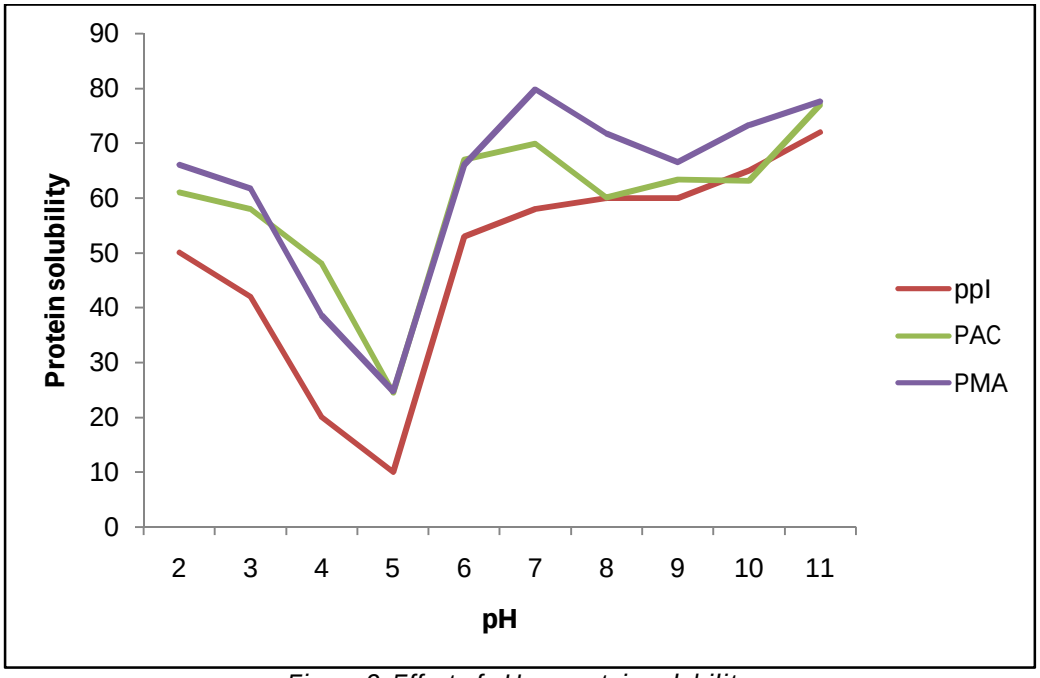

Figure 2: Effect of pH on protein solubility

PPI – pigeon pea protein isolate
PAC- acetylated protein isolate
PMA- maleylated protein isolate

4. Conclusion

The result of the chemical composition revealed that they are good sources of proteinsthe protein isolates and the modified protein isolates have good digestibilities. The high-water absorption capacity obtained in both protein isolate and acylated protein suggests that they may find use in the formulation of some foods such as sausages, doughs, processed cheese and soup.Good emulsifying and foaming properties suggest that these modified protein isolates will find good application as additives for food products such as cakes, breads, whipped toppings and desert in food industries.The enhanced solubility of the protein isolates in both acid and alkaline region indicates that they may be useful in the formulation of both acid and alkaline foods.

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