ISSN 0511-5728
The West Indian Journal of Engineering
Vol.35, No.1, July 2012, pp.35-40

Amino Acid Profiling and Nucleic Acid Determination of Single Cell Protein

Theresa F. Campbell[a] , Winston A. Mellowes[bΨ] and Carole Lindsay [c]

[a]Department of Chemical Engineering, Faculty of Engineering, The University of the West Indies, St. Augustine, Trinidad, West Indies; E-mail: theresa.campbell@uwimona.edu.jm

[b]Department of Chemical Engineering, Faculty of Engineering, The University of the West Indies, St. Augustine, Trinidad, West Indies; E-mail: wamello@yahoo.com

[c]Department of Biochemistry, Faculty of Medical Sciences, The University of the West Indies, Mona, Jamaica, West Indies; E-mail: carole.lindsay02@uwimona.edu.jm

[Ψ] *Corresponding Author*

(Received 30 September 2011; Revised 8 February 2012; Accepted 08 March 2012)

Abstract: Overripe bananas and plantains - which are rich in carbohydrates - can be utilised in fermentation processes to produce microbial protein. Studies were conducted to determine the protein content, nucleic acid content and essential amino acid profile of the single cell protein (SCP) isolates recovered from the fermented pulps of bananas (Lacatan variety, Musa acuminate) and plantains (French variety, Musa paradisiaca). A standard buffer system of 65 % of a disodium salt ($Na_2HPO_4.2H_2O$) to 35 % of a monosodium salt ($NaH_2PO_4.2H_2O$) was used in the extraction process and the SCP isolates precipitated using alkaline/acid, salting out and ethanol precipitation. The salting out method of precipitation produced the highest protein content (97.1 %), followed by the alkaline/acid method (26.7 %). The nucleic acid content was highest when the alkaline/acid method of extraction and precipitation was utilised (8.5 %) while the salting out and ethanol precipitation methods produced the lowest (6.0 %). The essential amino acid content of the SCP isolates revealed relatively high levels of lysine in all samples (0.074-1.289 g/100 g of SCP). The samples obtained via alkaline/acid extraction and precipitation produced the highest overall yields (0.113-1.659 g essential amino acid/100 g of SCP). Those obtained via ethanol precipitation on the other hand did not contain valine or methionine.

Keywords: *Single cell protein (SCP), nucleic acid and essential amino acids*

1. Introduction

Yeasts use the naturally occurring sugars in the juices of fruits as an energy source. Ripe bananas and plantains have high sugar content and are rich in nutrients needed for the sustained growth of yeasts. As glycolysis occurs there is considerable growth in the number of yeast cells present and therefore, fermentation residue contains a significant amount of these cells. Yeast cells – according to Chae et al. (2001) – contain a large amount of protein and other nutrients. The main focus of this research is therefore to obtain the residual yeast cells and recover protein (single cell protein, SCP) from the mechanically disrupted cells so as to assess the nucleic acid content and essential amino acid profile of the isolates.

The suitability of a protein for application within the food industry is dependent on its composition since its nutritional value depends largely upon the pattern and concentration of essential amino acids that it provides for the synthesis of nitrogen-containing compounds within the body (Henley and Kuster, 1994). It is therefore important that the quantification of essential amino acids present within a protein sample be determined before it is incorporated into foods.

SCP has been found to have numerous applications in animal nutrition since it can be used for the fattening of calves, pigs, poultry and fish and also in foodstuffs as aroma carriers, vitamin carriers and emulsifying aids and also to improve the nutritive value of baked products in soups, ready-to-serve meals and in diet recipes (Nasseri et al., 2011). Supplementation cereals with SCP (especially those from yeast) are also said to be as good as animal protein (Nasseri et al., 2011). Although SCP has high nutritive value however, its nucleic acid content is the main cause for concern regarding its use in food.

Waste bananas and plantains often present disposal problems and as such the utilisation of these fruits to produce value-added products is greatly encouraged (Hammond et al., 1996; Nasseri et al., 2011). Overripe bananas and plantains are ‘waste’ and as such, the feedstock for the fermentation process is available at very low cost. The results of the study will provide a source of protein that can be used to fortify both food and feed. With increase in population and worldwide

protein shortage, the use of microbial biomass as food and feed is encouraged. Essential amino acids must be supplied by the diet and therefore the incorporation of a material possessing these compounds into foods would prove beneficial to consumers.

2. Materials and Methods

2.1 Alcoholic Fermentation

The crushed pulp of over-ripe bananas and plantains (4.4-6.2 kg) were pasteurised at 90 ºC for 45 mins. Two batches of each fruit were processed and 50 % v/v of water was added to each. They were then cooled to room temperature (25 ºC) and inoculated with 0.1 % w/v of wine yeast (a saccharomyces cerevisiae strain) and 8.5 g (4.3 g/gallon of slurry) of pectolase. They were left to ferment for 1 week after which they were centrifuged at 9000 g for 15 mins. The fermented pulps were stored at 4 ºC.

2.2 Protein Extraction

A buffer system of 65 % of $Na_2HPO_4.2H_2O$ to 35 % of $NaH_2PO_4.2H_2O$ was prepared. 200 g of pulp was blended with 50 % w/w of ice and 200 mL of buffer added. The resulting homogenate was divided into three portions where one was used for salting out, another for ethanol extraction and the final portion for alkaline extraction. All samples were maintained at a temperature of 5 ± 2 ºC. The sample for alkaline extraction was adjusted to a pH of 10.8 using 1 M KOH and left for 30 minutes. All three portions were then centrifuged at 15,000 g for 20 mins at 4 ºC. The pellets were washed with 50 % v/v of buffer and the supernatants stored at 4 ºC for further analysis.

2.3 Protein Precipitation and drying

100 mL of each supernatant was measured in duplicate. The samples to be used in the salting out and the alkaline/acid precipitation methods were kept at a temperature of 4-5 ºC for the duration of the precipitation. Those for ethanol precipitation were kept at ambient temperature (25 ºC) for the same duration.

In the salting out method, protein precipitation was carried out by the careful addition of 16.4 g $(NH_4)_2SO_4$ to 100 mL of supernatant (Zewdie-Bosuner, 1994). For the alkaline/acid method, precipitation was accomplished by carefully adjusting the pH of the solution to a pH of 4 using 1 M H_2SO_4. For the precipitation method with ethanol, 100 mL of 60 % ethanol was added to 100 mL of supernatant at 25 ºC and the pH adjusted to 4.0 with 1 M HCl (Birla, 2004). The mixture was then allowed to sit for 30 mins.

After precipitation all samples were centrifuged at 15,000 g for 20 mins and the protein precipitates stored at -17 ºC. After 24 hours the samples were freeze dried for 24 hours at a condenser temperature of -40 ºC and their masses determined. The moisture content of the SCP obtained from the plantain substrate using the salting out method of precipitation (PSO) was determined.

2.4 Determination of protein, nucleic acid and essential amino acid contents

The use of three extraction procedures and two fruits resulted in six protein isolates. The protein content of the samples was analysed using the Kjeldahl method (Horwitz and Latimer, 2005) while the nucleic acid content was determined using the UV method outlined by Garcia (1990). The essential amino acids analysis was done using a modified method by Gonzalez-Cartro et al. (1997) where 0.5 g of each protein isolate was hydrolyzed with 15 mL of 6 M HCl at 110 ºC for 24 hrs. After hydrolysis, the samples were filtered and diluted with distilled water. A 5 mL aliquot of each extract containing 900 µg of internal standard was prepared. 100 µL of each solution was then removed and derivatised. 20 µL of the derivatised solution was then injected onto the High Performance Liquid Chromatography (HPLC) column. Spiking of the different samples was done so as to positively identify essential amino acids.

2.4.1 Derivatisation of Amino Acids

The method used for derivatising amino acids is a modification of that proposed by Gonzalez-Castro et al. (1997). 0.1 mL aliquot of the samples was dried under a stream of nitrogen at 35 ºC. The residue was re-dissolved in 0.1 mL of methanol: water: triethylamine (TEA) (2:2:1) and the solvents removed under nitrogen at 35 ºC. 0.1 mL of methanol: water: triethylamine: phenylisothiocyanate (PITC) in the ratio 7:1:1:1 was then added at room temperature (25 ºC) and the samples left to stand for 20 mins. The solvents were then removed under a nitrogen stream and stored at 4 ºC pending chromatographic analysis. Prior to injection, the amino acids were re-dissolved in 1 mL of Eluent A buffer. Final concentrations for standards injected were 0.5, 2, 4, 6 and 8 µg/mL, each with 18 µg/mL internal standard. In all instances the injected amount was 20 µL.

Essential amino acid standard calibration solutions were prepared in 0.1 M HCl and a fixed volume of internal standard added. These solutions were also derivatised.

2.4.2 Chromatographic Separation of Amino Acids

The analysis was conducted using an Agilent 1100 Series HPLC. The chromatograph was equipped with a 126 programmable solvent module, model 508 auto sampler, a dual head pump and a UV detector set at 254 nm. Separations were carried out on a Waters 250 mm x 4.6 mm reverse phase column, packed with 5 µm spherisorb ODS-2 connected to a 1 cm x 4.6 mm ID guard column of similar packing. Gradient elution was performed using a solvent mixture of Eluent A (0.5 mL/L triethylamine to 0.14 M sodium acetate) at a pH of 6.1and Eluent B (a 60:40 ratio of acetonitrile and water).

The column temperature was maintained at 30 ºC and the gradient system employed had an initial composition of 10 % A and 90 % B for 5 minutes followed by an increase in A to 60 % over 10 minutes and a further increase to 100 % A in 3 minutes. 100 % A was maintained for 2 minutes before returning to the initial setting of 10 % A in 2 minutes.

3. Results and Discussion

3.1 Protein Precipitation

The alkaline/acid and ethanol precipitation processes involved the use of acid to facilitate precipitation. In both cases the solid protein first appeared in the form of primary particles which formed rapidly when the soluble protein molecules of the extract encountered the lower pH of the precipitator. At this point the proteins became insoluble due to altered surface charges (Petenate and Glatz, 1983). As the particles aggregated at a slower rate, the aggregates grew until a limiting factor(s) was encountered.

Ammonium sulphate was used to effect salting out of another batch of protein extract. The method of precipitation was similar to that of alkaline/acid and ethanol precipitation except, in this case, the pH was not adjusted. When precipitating proteins by the salting-out mechanism, the two main parameters are the temperature and ionic strength of the solution (Carpineti and Piazza, 2004). The SCP isolates obtained are shown in Figures 1 and 2.

SCP obtained from the salting out method of precipitation had the largest protein recovery (63-70 %) while those obtained using alkaline/acid and ethanol precipitation had a recovery of 29-31 %. The plantain substrates also produced greater yields (average mass of 1.56 g) than those from the banana substrates (average mass of 0.28 g). Limiting amounts of the SCP isolates for analysis resulted in only that obtained via the salting out method of precipitation from the plantain substrate (PSO) being analysed for its moisture content. This was found to be 6.54 %, indicating efficient drying.

Figure 1. Dried Salting Out, Alkaline/Acid and Ethanol Single Cell Protein Isolates Obtained from Fermented Plantain Pulp

Figure 2. Dried Salting Out, Alkaline/Acid and Ethanol Single Cell Protein Isolates Obtained from Fermented Banana Pulp

3.2 Protein and Nucleic Acid Content of the Single Cell Protein

The SCP product is important because of its high nutritional value and its composition of amino acids, particularly the essential amino acids. These values might however vary since features of the SCP are dependent on the genetic make-up of the species and its growth conditions (Daramwal and Gaur, 2004). The protein and nucleic acid content of the SCP isolates are presented in Table 1. The protein contents of the SCP obtained from the banana substrate (99 and 46 %) were higher than those of the plantain substrate (95 and 7 %). The salting out mechanism produced higher protein content (over 95 %) than the other methods (7-46 %).

The nucleic acid content of the samples ranged from 5.4-8.9 %. These compounds are very essential in the body; however, as stated by Daramwal and Gaur (2004), foods with high nucleic acid content are rendered unfit for human consumption. SCP obtained from any substrate will have higher nucleic acid content than any conventional food source (Daramwal and Gaur, 2004) and it is for this reason they have not been used for direct human consumption (Srivastava and Srivastava, 2003).

Table 1. Protein and Nucleic Acid Content of the different Single Cell Protein Isolates

Sample	Protein Content (%)	Nucleic Acid Content on a Dry Weight Basis (%)
B(SO)	98.73	5.8
B(Alk)	46.14	8.0
B(OH)	NA*	6.6
P(SO)	95.36	6.3
P(Alk)	7.23	8.9
P(OH)	7.62	5.4

* - Not Available due to insufficient amounts of B(OH) for analysis.
* - SCP obtained from banana (B) and plantain (P) substrates via salting out precipitation (BSO and PSO), alkaline/acid precipitation (BAlk and PAlk) and ethanol precipitation (BOH and POH) respectively.

The quantity of nucleic acid reported in the various protein isolates is relatively low (5.4-8.9 %) compared to those reported by Scrimshaw (1975) which ranged from 8-25 %. According to several clinical studies and the recommendations of the Protein Advisory Group (PAG) of the United Nations, safe daily intake of nucleic acids for most adults is estimated to be approximately 4 g of which 2 g can be derived from SCP (Scrimshaw, 1975). This quantity of nucleic acid corresponds to a nucleic acid content of 3 % (dry wt) in the SCP product if considerable amounts of SCPs are contained within the diet. This indicates that the quantities reported in the protein isolates are still above acceptable levels if considerable amounts are to be consumed.

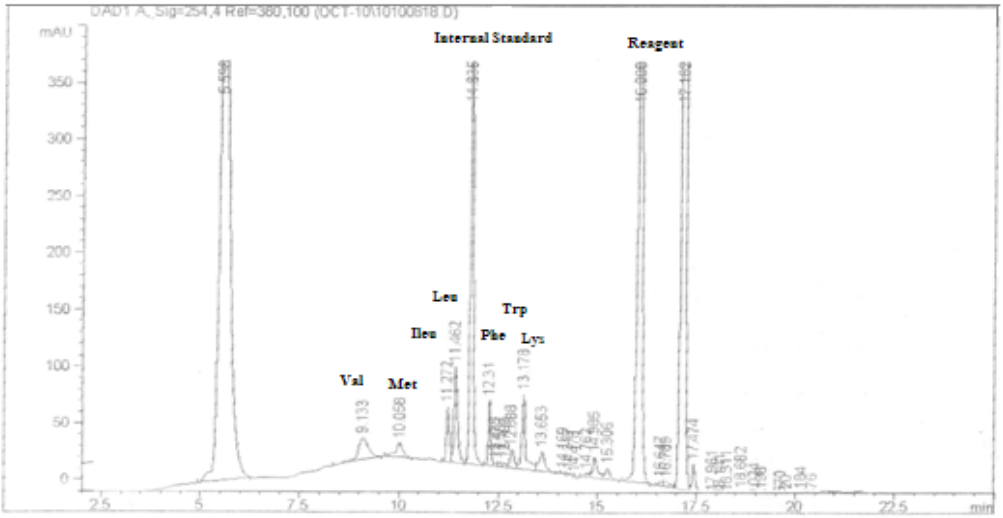
According to Daramwal and Gaur (2004), it is possible that application of advances in the field of biotechnology will be able to help in the production of safe SCP. The alkaline/acid method of extraction and precipitation produced SCP with higher levels of nucleic acids than the samples precipitated via other methods and this might be due to difference in the conditions to which the samples were subjected. An overall reduction of the nucleic acid content of the SCP isolates can be achieved by chemical modification of yeast nucleoproteins with anhydrides and the addition of nucleases (Nasseri et al., 2011).

3.3 Essential Amino Acid Analysis of Protein Isolates

The amino acid composition of the protein isolates was determined using a High Performance Liquid Chromatography (HPLC). Before analysis, the samples were hydrolysed. The protein isolates obtained via ethanol precipitation (from both the banana and plantain substrates) had a deeper orange hue than those obtained using alkaline/acid precipitation. The precipitates resulting from the salting out method were of the palest colour. The colour differences among the different precipitation methods indicate that there are compositional differences between the isolates.

The PITC method of analysis was chosen because of its advantages which include its rapid and simple derivatisation, minimum interference from reagent peaks as a result of the coupling reagent and the solvent being volatile and readily removed by evaporation, and also because of its ability to avoid the instabilities as well as the quantification difficulties associated with fluorescence active coupling reagents.

The more polar, acidic amino acids were eluted first followed by amino acids with aliphatic R groups. The amino acids with hydrophobic aromatic R groups were next and the last amino acid to be eluted was the basic lysine as shown in Figure 3. Identification of amino acids in the isolates was done by comparison of the Relative Retention Times (RRT) of the sample peaks with those of the standards. The addition of 18 µg of internal standard (norleucine) to the samples prior to derivatisation was to monitor the physical and chemical losses and variation during analysis.

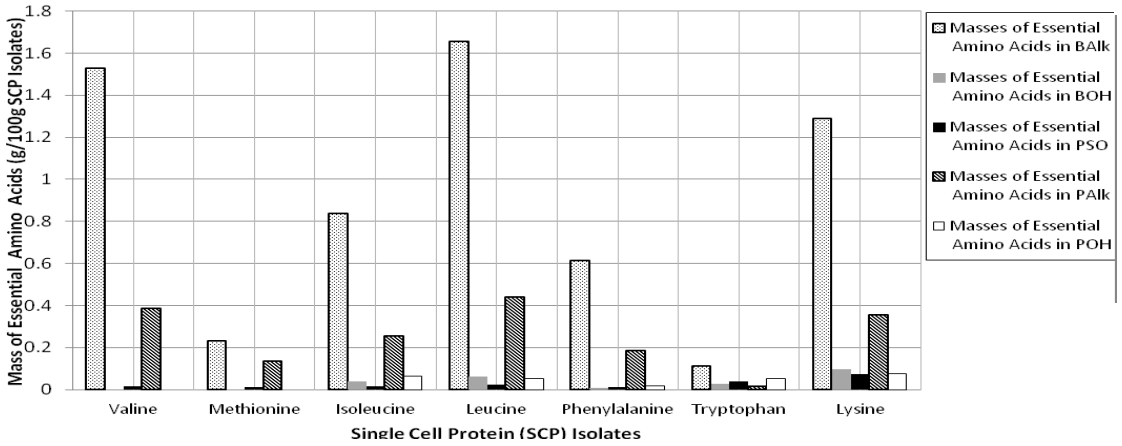


* PAlk - SCP obtained from Plantain Substrate using Alkaline/Acid Extraction and Precipitation

Figure 3. Sample Chromatogram for Total Essential Amino Acids in PAlk

Chromatographic peak areas were identified and quantified using the data analysis system of the machine. A calibration line was used that was prepared from the average values of the RRTs in minutes and areas in absorbance units (AU) of the amino acids in the standard runs. A known amount of each amino acid was analysed and as such, a response factor (m) was calculated. This factor was determined graphically from the calibration curve as the slope of the plot of area vs. amount ratio. Area and amount ratios are the area and amount of each amino acid divided by the area and amount of internal standard (norleucine) added. This response factor was used to calculate the amount of amino acid in the isolates. Spiking of samples was done so as to positively identify specific amino acids and also to evaluate the percent recovery of the amino acids. In all cases the percent recovery was 80 %. Histidine and Threonine (two of the nine essential amino acids) are omitted from the analyses due to difficulties in identification.

The quantities of essential amino acids resulting from the protein isolates were determined and the values reported in Figure 4. A maximum of 1.66 g essential amino acid per 100 g of protein isolate was reported where this represented the quantity of leucine in the SCP obtained from banana pulp using alkaline/acid extraction and precipitation (BAlk).



* - BSO is omitted from the analyses due to difficulties encountered in analysis.
* - SCP obtained from Banana (B) and plantain (P) substrates via salting out precipitation (BSO and PSO), alkaline/acid precipitation (BAlk and PAlk) and ethanol precipitation (BOH and POH), respectively

Figure 4. Masses of Essential Amino Acids Present in Single Cell Protein Isolates

The nutritional value of a protein is determined by its amino acid profile. The amino acid concentrations, specifically the essential amino acids, are the basis for protein quality evaluation. The quantity of lysine in all the protein isolates was relatively high. The resulting concentrations were expected since according to Daramwal and Gaur (2004), lysine is present in SCP at optimal concentrations. This is also supported by Goldberg (1985).

Certain trends were observed in the concentrations of the various amino acids in the different SCP isolates. It was found that there were similarities between the products of the various precipitation methods regardless of the fruit substrate. The protein isolates obtained via the alkaline/acid method of precipitation had high levels of valine (1.530 g and 0.389 g from the banana and plantain substrate respectively) and leucine (1.659 g and 0.440 g from the banana and plantain substrate respectively) along with lysine (1.289 g and 0.355 g from the banana and plantain substrate respectively) but low values of tryptophan (0.113 g and 0.017 g from the banana and plantain substrate respectively). Amino acids such as tryptophan are partially destroyed by acid hydrolysis (Ninfa and Ballou, 1998) and this could be the reason for the low values obtained. The alkaline/acid samples reported the highest levels of essential amino acids (0.113-1.659 g from the banana substrate and 0.137-0.440 g from the plantain substrate).

The protein isolates obtained via the ethanol method of precipitation had low levels of phenylalanine (0.007 g and 0.016 g from the banana and plantain substrates, respectively). These samples also failed to produce any reading for the amino acids valine and methionine. The peaks characteristic to these compounds were absent from the chromatograms of the samples, and it can therefore be inferred that the ethanol used in the precipitation methodology interfered with the compounds.

The quantities of essential amino acids present in the protein isolates obtained via the salting out

mechanism were of the lowest quantities. The salting out and ethanol methods of precipitation produced the lowest quantities of amino acids; thereby indicating that the method utilised impacts the quality of the resulting protein. The quantities of threonine and histidine were not determined due to interfering peaks in the chromatograms. This was also the reason the amino acids in the SCP obtained from the banana substrate using the salting out method of precipitation (BSO) were not quantified. In order for such determinations to be made, further purification would be required. It is however safe to assume that the amino acids were present in the isolates since SCP contains all essential amino acids (Srivastava and Srivastava, 2003).

Potential areas for further work would include assessing the quality of protein obtained. This could be done by determining the Protein Efficiency Ratio (PER) which is expressed in terms of weight gained per unit of protein consumed by the test animal in short-term feeding trials (Nasseri et al., 2011). Evaluation of the Biological Value (BV) and the Digestibility Coefficient (DC) could also be done, where the former is a measure of the nitrogen retained for growth and maintenance. Another parameter which could be assessed is the Net Protein Utilisation (NPU) which is equivalent to the calculation BV x DC and is a measure of the digestibility of the protein and the biological value of the amino acids absorbed from the food (Nasseri et al., 2011).

4. Conclusion

The protein content of the SCP isolates was highest for those obtained from the banana substrates and for those obtained via the salting out method of precipitation. The nucleic acid content of the alkaline/acid isolates was highest.

The quantities of essential amino acids were highest for the banana and plantain protein isolates obtained via the alkaline/acid method of precipitation. Isolates obtained via ethanol precipitation did not produce valine or methionine and the isolates resulting after precipitation using ammonium sulphate (salting out) produced the overall lowest quantities of essential amino acids. Since essential amino acid requirements vary for infants, children and adults, the SCP isolates which resulted can be considered for use for the different age groups.

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Authors' Biographical Notes:

Theresa F. Campbell *is presently pursuing a PhD in Chemistry at The University of the West Indies, Mona. Her area of interest is nutraceuticals where the bioactivity of compounds isolated from the fruits and leaves of locally grown raspberries (Rubus rosifolius) are being analysed.*

Winston A. Mellowes *(Professor Emeritus) is presently a part-time lecturer in the Departments of Chemical Engineering at The University of the West Indies, St Augustine, where he lectures in Food Science and Technology.*

Carole Lindsay *is presently a lecturer in the Department of Basic Medical Sciences at the University of the West Indies, Mona. Her area of research includes amino acid and fatty acid profiling of traditional Jamaican plants.*

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