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Enzymatic preparation of palm kernel expeller protein hydrolysate (PKEPH)

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Abstract: Utilization of palm kernel expeller (PKE), a palm oil milling by-product, may be diversified through the exploitation of its protein component. The PKE protein could be effectively extracted using an alkaline solution and followed by enzymatic hydrolysis to produce PKE protein hydrolysates or crude PKE peptide. The extraction of PKE protein was successfully carried out using an alkaline solution at pH11, at ratio of 1:10 (g/ml), PKE powder to alkaline solution with continuous shaking, 150 rpm, in a water bath operating at 50°C for 30 min. The extracted protein powder (PKEP) had 68.50±3.08% crude protein, 0.54±0.03% fat and 0.73±0.02% ash. The freeze-dried PKEP was re-suspend in particular buffer and hydrolyzed with proteolytic enzymes (Alcalase® 2.4L, Flavourzyme® 500MG, pepsin or trypsin) to obtain PKEP hydrolysate (PKEPH). The effect of enzyme concentration (0, 2, 4, 6, 8 & 10%) and time of hydrolysis (0, 6, 12, 24, 48 h) was studied to determine the most efficient hydrolytic conditions. Results showed that all enzymes tested were capable of hydrolyzing the PKEP and producing hydrolysates with different degree of hydrolysis (DH%). At 8.0% concentration, Alcalase® 2.4L hydrolyzed PKEP into the highest DH (75.96%) hydrolysate (PKEPH) after 1h hydrolysis. Although only with 2.0% Alcalase 2.4 L concentration, it was sufficient to produce PKEP hydrolysate of 81.35% DH %, but it required 12 h to hydrolyze the protein. Pepsin was relatively the least efficient protease to hydrolyze the PKEP.

Keywords: Palm kernel expeller protein (PKEP), protease, PKEP hydrolysate (PKEPH), degree of hydrolysis (DH)

Introduction

In Malaysia, palm oil milling industries had generates a large amount of palm kernel expeller (PKE) through screw pressing process. PKE can be another great plant protein sources. Its crude protein content (14.5-19.6%) and the nutritive value of palm kernel expeller have made PKE an excellent ingredients in ruminant (Alimon, 2004) and aquaculture (Ng, 2004) diets. However, due to the high crude fiber content, there is a limit for inclusion in animal diet especially for sheep and goats (Dahlan *et al*., 2000). Indirectly, it limits the usage of PKE worldwide. In fact, PKE contains valuable protein which can be extracted and converted into value-added products by further enzymatic hydrolysis. The isolated PKE protein may be utilized in conventional applications, such as protein fortification, emulsification, body formation in baked products (Arifin *et al*., 2009) or even as bioactive peptides in pharmaceutical products. Thus, the market value of PKE may appreciate accordingly.

Due to cost, religious and health issues such as cholesterol content of animal protein, interest in alternative plant protein has grown up in food and non-food industries. This has resulted in increasing numbers of studies on the extraction and modification of plant protein. Extractability determines the amount of the protein available from a particular source such as seed flour (Liu, 1997), thus the extraction efficiency is very important if the procedure is to be adopted for commercial exploitation. The effectiveness of any extraction processes is based on the solubility of the protein. Therefore, to extract or isolate protein successfully, the use of acid, saline or alkaline solution is needed (Eromosele *et al*., 2008). Extensive study has been carried out on protein extractability of soy meal (Bass *et al*., 1997); African yam bean (Eromosele *et al*., 2008) and palm kernel meal (Arifin *et al*., 2009). However different types of protein will favor certain treatment and yield different products. Through non-enzymatic or enzymatic modification, intact protein may change. Peptide bond hydrolysis, by acid or alkaline, may be used to yield smaller protein products with more uniform molecular size (Frederick, 1992). Frederick (1992) reported that food protein with extensive alkaline treatment or hydrolysis may not be readily digested. Therefore, to improve the quality of PKEP, further modification was carried out. Protein structure is generally modified to improve solubility (Jamel, 1992), thereby improving their effective use in food products better suited for human nutritional

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utilization. Different types of proteases (endo- and exo-peptidases) may give different degree of hydrolysis due to their particular targeted amino acid. The objective of this study was to extract protein from PKE and determine the most appropriate protease and its' optimum concentration and hydrolysis time to produce PKE protein hydrolysates.

Materials and Methods

Palm kernel expeller (PKE) preparation

Palm kernel expeller (PKE) of species *Elaeisguineenssi* var. *dura* was provided by FELDA Kernel Products Sdn. Bhd., Malaysia. Before proceed on further treatment and analyses the PKE was ground and passed through a sieve of 0.2 mm mesh to obtain a fine powder. The proteases used include Alcalase® 2.4L of *Bacillus licheniformis*, Subtilisin® A (Sigma), pepsin of porcine gastric mucosa (Sigma-Aldrich) and trypsin of porcine pancrease, Type II-S (Sigma), and Flavourzyme® 500MG (Novozymes).

Protein extraction from Palm Kernel Expeller (PKE)

To extract the protein, the fine PKE powder of PKE was suspend in 1.0 N NaOH with the ratio of solid/liquid, 1:10 (PKE flour, g : alkaline solution, ml) and adjusted the suspension to pH 11 using 0.1 N HCl. The extraction was carried out in a water-bath shaker (902/OVT 27-2176, Hotech Instruments Corp.) at 50°C and shaking speed of 150 rpm for 30 min. This was then followed by centrifugation (Kubota Model 2420) at room temperature and speed 4000 rpm for 20 min to obtain the supernatant. To recover the extracted protein in the supernatant, precipitation was carried out using 3.0 N HCl, at pH range between 4.3–4.5. The precipitate obtained was defatted by re-suspending in 95% ethanol, shaken well and centrifuged at 10 000 rpm for 20 min. Finally, the protein pellet (PKEP) was collected and lyophilized in a freeze dryer (Alpha1-4 LD Plus, Model HO27080, Christ, German) at -40ºC for 24 h. The proximate compositions crude protein content, fat and ash content, of the dried powder were than determined (AOAC, 2000).

Preparation of palm kernel expeller protein hydrolysate (PKEPH)

PKEPH was prepared via enzymatic hydrolysis using Alcalase® 2.4 L, Flavourzyme® 500MG, pepsin and trypsin, separately. The enzymatic hydrolysis was carried out under their respective optimal temperature and pH conditions to ensure the enzyme functioning well in the provided condition. The temperature and pH used were 55ºC and pH 8.5 for Alcalase® 2.4 L; 55ºC and pH 7.0 for Flavourzyme® 500MG; 40ºC and pH 2.0 for pepsin and 40ºC and pH 8.5 for trypsin. The buffer solution used were 0.01 M disodium phosphate (1.42 g/L) adjusted with 0.1 M HCl to achieve pH 8.50 and pH 7.0; and 0.2 M KCl adjusted with 0.2 M HCl to achieve pH 2.0.

Approximately, 0.1 g defatted PKEP powder was dissolved into 10ml buffer solution in 25 ml conical flask. To determine the optimum hydrolysis time, the mixture was hydrolyzed for 0, 6, 12, 24, and 48 h by 2% enzyme. Meanwhile, the effect of enzyme concentration on hydrolysis was studied using 0, 2, 4, 6, 8, and 10% of the respective enzymes (Alcalase® 2.4L, Flavourzyme® 500MG, pepsin or trypsin) was carried out in a water-bath shaker (150 rpm) at desirable temperatures as mentioned above for 1 h.

When the hydrolysis completed, the mixtures were then heated at 100ºC for 10 min to inactive the enzymatic activity. Protein hydrolysates were collected by centrifuging the heat-treated mixture at 4000 rpm for 20 min, whereby impurities and denatured enzyme were separated from the PKEPH. The supernatant obtained was used for further analysis.

Determination of nitrogen content in PKEP powder

Nitrogen content was determined by Kjeldahl standard method (AOAC, 2000) using Kjedltec 2200 Analyzer unit (Foss, Denmark). Nitrogen content was calculated by equation (1) below:

$$N\ \% = \frac{(0.01 \times \text{titration volume} \times 1.402 \times 6.25)}{\text{Sample weight} \times 1000} \times 100 \quad (1)$$

Where, N = nitrogen

Determination of degree of hydrolysis (DH %)

The degree of hydrolysis (DH%) of PKEP hydrolysate was determined based on the method described by Sathivel *et al*. (2003). After hydrolysis, 5 ml of aliquoted PKEPH was added to 5 ml of 20 % trichloroacetic acid, (TCA) to produce a TCA-soluble mixture. Each mixture was centrifuged and the TCA-soluble nitrogen of supernatant was measured using a Kjedltec 2200 Analyzer unit (Foss, Denmark) and based on the standard Kjeldhal method (AOAC, 2000). A conversion factor of 6.25 was used to calculate total protein content. The DH % was calculated using equation (2).

$$DH\ \% = \frac{(10\%\ \text{TCA soluble nitrogen in the sample})}{\text{Total nitrogen in sample}} \times 100 \quad (2)$$

Where DH = Degree of hydrolysis; TCA = Trichloroacetic acid

Data analysis

Data was analyzed and all experiments were done in three replications.

Results and Discussions

PKE is produced after intensive screw pressing of palm kernels which might cause heat damage to the protein component (O'Mara *et al*., 1999) and thus it may reduce the protein extractability. Thus a higher normality (N) of alkaline solution is needed to increase the extractability of PKE protein and the final protein concentration of the PKEP was increased to 68.50 ± 3.08%. On the contrary, Eromosel *et al*. (2008) reported that extractable African yam bean (*Sphenostylishstenocarpa*) protein (%) decreased from 17.8 to 14.9% when they changed the normality of the extracting solution from 0.01 N to 0.10 N NaOH. However, extraction pH>10, which is high normality of alkaline may cause extreme change in the protein environment and be dentrimental to such protein quality. To improve the protein quality, extracted protein need to undergo enzymatic hydrolysis or further modification.

Furthermore by increasing the normality of alkaline solution, the solid/liquid ratio of extraction was reduced to 1:10 which is lower than the solid/liquid ratio of 1:30, in PKM protein extraction suggested by Arifin *et al*. (2009). According to Eromosel *et al*. (2008) study, decreases in extractable protein was observed at higher solid/liquid ratio and it is parallel to Rivers *et al*. (1981) study which also reported a decrease in nitrogen extractability when an large excessive amount of solvent was employed. Therefore, they obtained an optimal solid/liquid ratio of protein extraction at 1:40 or lower. However, due to the differences of protein source, the ratio of solid/liquid for PKE protein extraction is lower. PKE is a by-product in palm oil milling industry which using screw pressed/ expeller technology. The protein content of PKE which undergo this processing is lower than the by-product which is undergoes the solvent extraction. Therefore, a higher concentration or normality of alkaline solution and lower solid/liquid ratio is used to extract the heat-damaged PKE protein.

In addition, Kain *et al*. (2009) reported that protein solubility (%) was depending on pH and showed a gradual decreased in peanut protein extractability from 84 to 74%, as pH increases from 10 to 12. Thus, the extractability of PKEP was reasonably high at pH 11. As pH is increases, the particles come closer, columbic forces between positive and negative charges of the protein residues; van der Waals attraction and hydrogen bonding would then hold the mass together against the forces. Therefore, when the pH increases, the net negative charge increases then the desegregation (solubility) is increased progressively (Boulet *et al*., 2000). Shen *et al*., (2008) concluded that higher temperature would be beneficial for the tea protein extraction. It was in agreement with Ordonze *et al*. (2001) who revealed an increase in protein yield from 13.95 to 17.83% when temperature was increased. Thus 50ºC could be a suitable choice for increasing the extractability of PKEP and avoiding further protein degradation at higher temperature.

To improve the quality of PKE protein, PKEP was subjected to hydrolysis by Alcalase® 2.4L, Flavourzyme® 500MG, pepsin or trypsin. Results showed that hydrolysis was affected by hydrolysis time (Figure 1) and enzyme concentration (Figure 2). Besides, different types of proteases showed differentiability to hydrolyze PKEP.

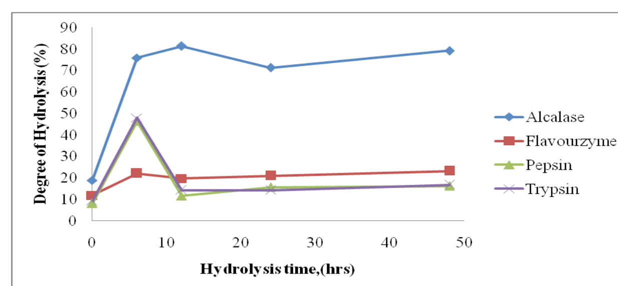

Figure 1. The influence of hydrolysis time on degree of hydrolysis by different proteases

To study the influence of hydrolysis time, PKEP was hydrolyzed with respective proteases for 0, 6, 12, 24 and 48 h. Rapid hydrolysis of the PKEP was observed within the first 6 h by all enzymes studied (Figure 1). Alcalase® 2.4L showed the highest DH % (71.31 – 81.35%) over the entire period of hydrolysis (0 – 48 h) compared to others. Pepsin and trypsin exhibited the same trends which achieved ~46 DH % up to 6h and continue to decrease after that. Flavourzyme® 500MG produced the lowest DH (23.17%) of PKEP hydrolysates among all the proteases. Basically proteolytic enzyme will interact rapidly with insoluble protein particles and loosen the polypeptide chain to the surface and begin to hydrolyze the protein particles slowly. Thus, the more compact core of proteins are cleaved more slowly (Benjakul and Morrisey, 1997). Therefore, if the kinetic reaction of the enzymes and substrate is low, the time for completing the hydrolysis or achieve the optimal hydrolysis condition may increase.

Figure 2 exhibits an increasing trend of DH % with increasing enzyme concentration used (0 – 10%) after 1 h hydrolysis. The result indicates

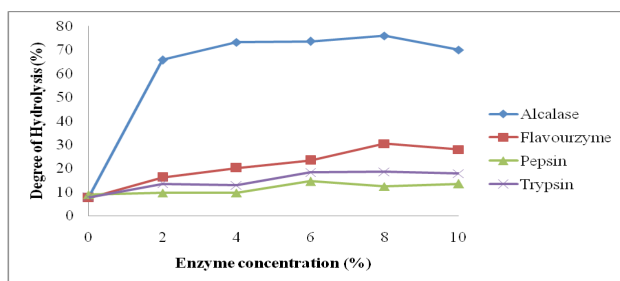

Figure 2. The influence of enzyme concentration on degree of hydrolysis by different proteases

that peptide bonds were more likely cleaved in the presence of a higher amount of enzyme when hydrolysis time (1 h) was limited. Compared to other enzymes, at 8.0%, Alcalase® 2.4L hydrolysed PKEP to the highest degree of hydrolysis (75.96%). This could be due to the different proteolytic ability of the different enzymes. Four of the enzymes, namely Alcalase®, Flavourzyme® 500MG, pepsin and trypsin used in this study were peptidases which act only on peptide bonds. Proteolytic enzymes may be classified broadly under two groups: endopeptidases, which also known as proteinases and exopeptidases (Boyce and Tipton 2007). Alcalase® 2.4L, pepsin and trypsin are endopeptidases and Flavourzyme® 500MG is a mixture of endo- and exopeptidase mixture (Khairi, 2010). As endopeptidases, Alcalase® 2.4 L and trypsin are serine protease, pepsin is an aspartic protease and Flavourzyme® 500MG is an aminopeptidases (Boyce and Tipton, 2007). Each of the enzymes has their own catalytic mechanism. The major interaction of serine protease is with the side chains of amino acids which contribute the carboxyl group to the bond being hydrolyzed and has a very broad range of substrate specifications (Rao *et al*., 1998). Thus as a typical example of serine protease, Alcalase® 2.4 L achieved the highest degree of hydrolysis of the substrate, PKEP.

Although trypsin is a serine peptidase, it cleaves after positively charged residues, which means it cleaves peptides on the C-terminal side of lysine and arginine residues. In other words, trypsin has highly specific targeted cleavage site and the rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site and no cleavage occurs if a proline residue is on the carboxyl side of the cleavage (Sweeney and Walker, 1993). This may explain why as a serine protease, the effect of hydrolysis by Alcalase® 2.4L and trypsin on PKEP was significantly different. Rebeca *et al*. (1991) also reported that alkaline protease (Alcalase® 2.4L and trypsin) exhibits higher hydrolysis activities than the acid protease (pepsin) or neutral protease (Flavourzyme® 500MG).

Figure 1 shows a similar trend of degree of hydrolysis due to trypsin and pepsin. As an aspartic endopeptidase, pepsin has broad specificity with a preference for peptides containing linkages with aromatic or dicarboxylic L-amino acids. It preferentially cleaves the C-terminal with Phe or Leu and to a lesser extent with Glu linkages and does not cleave at Val, Ala or Gly (Sweeney and Walker, 1993). Thus the effect of hydrolysis performed is not compatible to Alcalase® 2.4L. Flavourzyme® only performed well at increasing enzyme concentration. However, the hydrolytic ability showed by the enzyme was significantly less than the Alcalase® 2.4L.

Conclusion

Protein was successfully extracted from PKE using an alkaline extraction solution (pH 11) and prepared as PKE protein (PKEP) powder. The crude protein content of the powder was 68.50 ± 3.08 %. Consequently, the protein powder was subjected to enzymatic hydrolysis to produce PKEP hydrolysate. The hydrolysis time and enzyme concentration significantly affected the degree of hydrolysis of the PKEP. Among the proteolytic enzymes studied, Alcalase® 2.4L at 2% concentration, was the most efficient enzyme to hydrolyse the protein, producing the highest DH % of hydrolysate (81.35%) after 12 h of hydrolysis.

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