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Plant protein hydrolysates from soybean and rice grain as a supplement for medium in human skin fibroblast 1184 cell culture.

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ABSTRACT

In cell culture, addition of serum in the medium plays an important role for the growth of the cells. Each medium consists of two main parts which are a basal media and serum. Serum, mainly from animal source is very expensive and possesses higher potential of viral contamination. The alternative for solving this problem is to use a serum-free media with an addition of some supplements. The most important source of supplement is from plants which could reduce the cost. In this study, protein hydrolysates from soybean and rice as a supplement for medium in human skin fibroblast (HSF) 1184 cells to substitute the addition of serum in the medium were investigated. Plant protein hydrolysates from soybean and rice were prepared through enzymatic hydrolysis using two different enzymes; Alcalase and Flavourzyme. These hydrolysates were characterized according to their solubility and peptide size. Different growth behavior was observed when these protein hydrolysates were added in medium with and without Fetal Bovine Serum (FBS). Protein hydrolysates produced using Flavourzyme has no significant effect on cell culture, while hydrolysates from Alcalase were supplementary for HSF 1184 cell culture. Since plant proteins do not contain all the necessary amino acids for HSF 1184 cell culture growth, they cannot be solely substituted with FBS. Depending on the enzyme used, supplementation with hydrolysates corresponding to a high degree of hydrolysis and composition of peptides with small molecular size, led to different maximal cell density. It is whereby degree of hydrolysis for soybean and rice hydrolysed by Alcalaseis slightly higher (31.59% and 36.52% for soybean and rice, respectively) when compared to hydrolysis by Flavourzyme (17.58% and 34.36% for soybean and rice, respectively). Results also demonstrated that preparation procedure for protein isolates were efficient because the protein concentration was about two times greater than primary meal and some undesirable materials for mammalian cell culture were eliminated from meal. From this study, protein hydrolysates from soybean hydrolysed by Alcalase have shown the best performance for supplementation into a medium for HSF 1184 cell growth.

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INTRODUCTION

Protein hydrolysates are thought to act as concentrated balanced nutrient mixtures that may partly or fully replace serum. Alternatives to using serum are of special interest for biopharmaceutical production using intensive cell culture because large-scale production benefits most from reduction of cost [1]. Plants are able to give a highly purified source of soluble amino acids, peptides, vitamins, and essential elements of cell culture. Supplementing media with plant hydrolysates has been shown to improve protein production of engineered animal cells [2].

Different sources of plant for protein hydrolysates has been proposed as a supplement in medium for promoting cell growth which include protein hydrolysates from soybean [3], wheat [4], rice [3, 4] and rapeseed [5, 6]. Soybean protein hydrolysates have been utilized extensively in mammalian cell culture and demonstrated the best result compared to other plants [3].

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Soybean protein hydrolysates have been studied for promoting proliferation of human keratinocytes in serum-free media to find an alternative for bovine pituitary extract (BPE) [7]. Soybean protein is an excellent source of nutrient keratinocytes, although it did not fully substitute the BPE. Rice protein hydrolysates were also reported to slightly enhance cell growth during cultivation in protein-free media inside microcarriers [4]. Rice protein hydrolysates were used to investigate secretion and extracellular proteolysis of recombinant interferon-γ as well as CHO-320 cell growth [8].

Various cells other than Human Skin Fibroblast (HSF) 1184 cells for example mouse hybridoma ME-750 cell [1] and Chinese Hamster Ovary (CHO) cell lines [2, 9] have been used to investigate the ability of plant protein hydrolysates for cells' growth. Several factors have been reported to affect the HSF 1184 cell culture including the amount of protein in plants, types of proteins in plants, preparation of protein isolates before enzymatic hydrolysis, types of enzymes used for enzymatic hydrolysis and activity of enzymes. Protein isolates containing peptide with small molecular size was reported to be useful for cell growth [1, 6]. The types of enzymes used have been reported to affect the degree of hydrolysis and size of peptides produced [6].

Enzymatic hydrolysis is an effective way for production of peptides with small molecular size. The most important factor in the enzymatic hydrolysis is degree of hydrolysis. The degree of hydrolysis has been shown to be the sole parameter needed to control the physicochemical properties and consequently, the functionalities (solubility, foaming and emulsifying properties) which depend on the nature of peptides present in the hydrolysates [10]. In this study, plant protein hydrolysates using Alcalase and Flavourzyme were produced via enzymatic hydrolysis method.

The objective of this study was to investigate the plant protein hydrolysates from soybean and rice, hydrolysed by Alcalase and Flavourzyme, as supplement for medium in Human Skin Fibroblast 1184 cells for promoting cells growth. It is hypothesized that different enzymes used for different plant affected the degree of hydrolysis of soybean and rice, the properties of protein hydrolysates produced and thus, their ability to promote cells' growth. Our previous studies on the production and biological properties of plant hydrolysates suggested the possibility that this material might represent an inexpensive and safe alternative to serum for cell culture.

MATERIALS AND METHODS

Materials:

Soybean was purchased from the local market in Johor Bahru, Malaysia. Alcalase was bought from Sigma-Aldrich (Kuala Lumpur, Malaysia) with activity 2.4 U/g. Flavourzyme was bought from Sigma-Aldrich (Kuala Lumpur, Malaysia) with activity 500 U/g. Human skin fibroblasts cell line 1184 was obtained from ECACC United Kingdom. All other chemicals were of analytical grade.

Preparation of extracts and protein isolates:

Extraction methods for oil seeds (soybean) and cereal grain (rice) were used for the two different samples, respectively. These methods were a mixture extraction by solvents such as alcohol and acetic solvents, and membranes dialysis. For soybean extraction, soybean meal was vigorously mixed for 1 hour at ambient temperature with n-hexane at a meal to solvent ratio 50% (w/v). Actually, n-hexane has been used for defatting. After drying of extracts in the oven at 60 ºC, the dry extracts were grounded again. After that, the extracts were vigorously mixed for 30 minutes at ambient temperature with a solvent mixture of methanol/deionized water (65/35) at an extract to solvent ratio of 20% (w/v) for removal polyphenols. After centrifugation at 10000g for 5minutes, the supernatant was discarded while the meal was recovered. The extracted meal was extracted with s solvent mixture of ethanol/deionized water (65/35) in room temperature for 30 minutes at a meal to solvent ratio of 40% (w/v) under vigorous stirring for removal of phytic acid. After centrifugation at 10000g for 5 minutes, the supernatant was discarded and the meal was recovered for next extractions. The extract was dried in the oven overnight in 60ºC. The pretreated meal was then extracted with NaOH 1N for 1hour at dry meal to solvent ratio of 20% (w/v) with constant mechanical stirring at room temperature. The slurry was centrifuged at 10000g and 5 minutes and the supernatant was recovered, adjusted pH based on isoelectric point of each seed (pH 4.5 is the isoelectric point of the major fraction of Soybean proteins) with HCl 1N solution and centrifuged at 11000g for 10 minutes. Precipitated proteins were recovered, solubilized in deionized water and placed into Slide-A-Lyzer® Dialysis Cassettes (PIERCE Co.) with a molecular size cut-off of 20000 Da. The cassettes were submerged for 72 hours at 37ºC in a stirred tank containing deionized water. Dialysate was replaced after 24 hours. The protein preparation was then frozen at -20ºC and later freeze-dried (HetoPowerDry®LL1500). After freeze drying, the protein isolates were analyzed by chemical analyzing methods. On the other hand, for rice extraction, the method of isolates preparation is similar to soybean and the only difference is at the first stage of extraction. Indeed, after grinding, rice meals were vigorously mixed for 1 hour at ambient temperature with acetone at a meal to solvent ratio 50% (w/v). The remaining approaches were similar to soybean extraction.

Enzymatic hydrolysis of protein isolates:

Hydrolysis of protein isolates samples were carried out in a 120 ml beaker that it has been put in a 500 ml beaker as hot bath for adjusting the temperature. The samples were heated and stirred using FAVORIT Stirring Hotplate (Iramac, Perak, Malaysia). The pH-Stat method was used for measurement the degree of hydrolysis (DH) [11]. Enzyme preparations, Alcalase and Flavourzyme, adjusted to pH 7.5, were added to the substrate at an enzyme/protein ratio 2.5%. The total time of experiment was 180 minutes. Each experiment was repeated three times. A control experiment was also performed without enzyme addition. Reaction was stopped by heat treatment at 95ºC for 15 minutes, assuring a total inactivation of protease.

Chemical composition analysis:

Chemical composition of meal and protein isolate were determined. Ash and total fat contents were determined according to AOAC method [11]. Total crude protein content was obtained using the BCA method [12]. The total Nitrogen Free Extracts (NFE) content was calculated by subtraction between the total wet weight and the weight of other compounds. Moisture content was calculated by moisture analyzer (Model ST-LSC 60, Sastec, Selangor, Malaysia).

Nitrogen recovery:

Nitrogen recovery was calculated as the protein content of hydrolysate relative to protein content in protein isolate before enzymatic hydrolysis [13].

Molecular weight distribution by Size Exclusion Chromatography:

Molecular size distribution of peptides for each hydrolysate was analyzed using a High Performance Liquid Chromatography (HPLC) gel filtration system which consisted of a Perkin Elmer Series 200 Auto-sampler (PerkinElmer, Waltham Massachusetts, USA) as liquid chromatography system and a BioSep-SECS2000 column (Phenomenex, Torrance, USA) as size exclusion chromatography column. Elution was performed isocritically at 0.35 ml/min with solvent mixture of deionized water/Acetonitrile (60/40: v/v). Absorbance was monitored at 220 nm. The samples were filtered through 0.22 µm after enzymatic hydrolysis. Data analysis software (Perkin Elmer, Waltham Massachusetts, USA) was used to integrate chromatograms. The chromatogram was divided in five fractions which correspond to the following apparent molecular weight ranges: > 150 kDa, 40 to 150 kDa, 20 to 40kDa, 4 to 20 kDa and< 4 kDa. The proportion (%) of each fraction was expressed as the area of the fraction relative to the total chromatogram area.

Cell growth analysis:

HSF cell propagation was cultivated in 75 cm^2T -flasks in basal medium composed of DMEM supplemented with 10% v/v FBS and 1% Penicillin Streptomycin. T-flasks were incubated at 37ºC in a humidified atmosphere containing 5 % CO_2in an incubator (Thermo Scientific, Melrose, USA). The cells bank was developed during two months. In addition, cell culture was performed three times for finding the adequate initial cell density. According to these triplicate, the adequate initial cell density for the growth in 24-wells was determined as 2×10^5cells/ml. After propagation, the cells were sub-cultured in 24-wells plates. Ten 24-wells plates were used for experiment involving two control samples consist of normal medium for human fibroblast skin cells (DMEM+FBS (10%) + Pen Strep (1%)) as a positive control and normal medium without FBS (DMEM +Pen Strep (1%)) as a negative control. This approach was repeated for each plant samples. Another eight medium consist of four standard medium and plant sample with fewer FBS (DMEM + FBS (9%) + Pen Strep (1%) + Plant extract (1%)) and four standard medium without FBS and with plant extract (DMEM + Pen Strep (1%) + Plant extract (1%)) were prepared. Three wells were considered for each day for counting and calculating of growth curve. HSF cell growth was examined in eight days.

RESULTS AND DISCUSSION

Chemical composition:

The chemical composition of meal and protein isolates from soybean and rice is shown in Table 1. Amount of protein isolate samples is about two times more of amount of protein in primary samples (in meal). Meanwhile, amount of nitrogen free extracts has decreased in the isolate protein (Table 1). Since in the last step, dialysis cassettes have been used, this isolate protein is free from undesirable small molecule such as minerals.

Table 1: Chemical composition of hydrolysates

Plant source	Soy (M)	Soy (PI)	Rice (M)	Rice (PI)
Moisture	5.43	0	10.4	0
Protein	40.8	84.5	5.47	20.3
Oil	18.5	11.3	1.23	0.83
Total ash	7.2	1.4	0.63	0.56
Nitrogen free	28.1	2.74	82.3	78.3
(M) meal; (PI) protein isolate				

Degree of hydrolysis:

Degree of hydrolysis and nitrogen recovery of each enzyme is shown in Table 2. The result shows that hydrolysis using Flavourzyme provides moderate degree of hydrolysis (17.58% and 34.36% for soybean and rice, respectively). Hydrolysis by Alcalase produced higher DH compared to Flavourzyme (>20% higher for soybean; >5% higher for rice). Moreover, hydrolysis using Alcalase produced 1.8 times higher degree of hydrolysis compared to Flavourzyme for soybean protein but no significant difference for rice protein.

Table 2: Degree of hydrolysis (DH) and nitrogen recovery (NR) (%)

	Soy		**Rice**	
Enzyme	**DH (%)**	**NR (%)**	**DH (%)**	**NR (%)**
Alcalase	31.59	93.93	36.52	96.72
Flavourzyme	17.58	78.04	34.36	76.94

Nitrogen recovery:

The nitrogen recovery increased with degree of hydrolysis for all samples (Table 2). From this study, it has been revealed that the nitrogen recovery from hydrolysates produced using Alcalase were higher 20-25% compared to hydrolysatesproduced by Flavourzyme. These results indicate that other than amount of protein in the samples, the types of protein and types of enzyme and its ability for protein separation have significant effect on the nitrogen recovery.

Size distribution:

The result from size exclusion chromatography demonstrates an ability of different enzymes for producing peptides with different sizes. Flavourzyme that is an exopeptidase, produced the lowest amount of peptide with small molecular size (15.4% and 19.8% of soybean and rice, respectively), whereas Alcalase as the strongest endopeptidase in this study can provide the most amount of peptides with small sizes (<4 kDa) (Figure 1). From previous work, size of peptides has the most significant effect in using plants proteins in mammalian cell culture [9]. The largest molecular sizes >150 kDa which account for less than 5% in all samples, while fractions 4-20 kDa was produced in the range of 50-66% for Flavourzyme and 12-16% for Alcalase. The fractions 20-40 kDa was produced at a lower amount of about 6-25% for both enzymes. These results showed that Alcalase which is an endopeptidases can produce higher amount of peptides with smallest molecular size compared to Flavourzyme which is an exopeptidases.

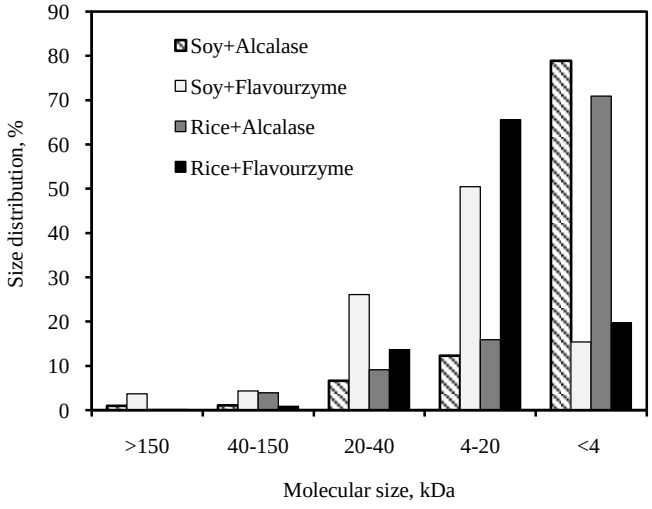

Fig. 1: Soluble peptides molecular size distribution (%)

Human Skin Fibroblast cell culture by using plant derived protein:

The growth curves of HSF in different media were shown in Figure 2. These results demonstrate that plant protein was not enough for mammalian cell culture. Although, the plant proteins had positive effect on cell culture used in a DMEM without FBS, however, their performance was not the same as FBS (the positive control). According to these results, apparently, the plant derived proteins could not act as good as FBS alone. These results indicate that plant derived protein did not have enough factors for HSF cell culture. When normal medium without FBS was used as negative control, the cells died after 3 days (Figure 2). Addition of plant protein hydrolysates into the medium without FBS had shown a slight improvement in the cell growth (cell died after 4 to 5 days) with hydrolysates from Alcalase for both soybean and rice hydrolysates (Figure 2A and 2B).The protein extracts by Flavourzyme as an exopeptidase did not have significant effect on cell culture.

Further investigation with addition of FBS, soybean protein hydrolysates had promoted a higher growth on cell culture as compared to rice protein hydrolysates (Figure 2A and 2B). This behavior describes the effect of different types of protein in different plants, for example soybean isolate has higher protein content compared to rice and thus, its ability for cell growth was better. According to these results, the best protein isolates for mammalian cell culture is an isolate with more than 85% protein content. For both soybean and rice protein hydrolysed by Alcalase the cell growth improved up to day 7 and seems to reduce after day 8. Protein hydrolysates produced by Flavourzyme did not perform as excellent as the protein hydrolysates produced by Alcalase. The cell was able to grow up to day 6 for rice protein hydrolysate (Figure 2B), while soybean protein hydrolysatehydrolysed by Flavourzyme could improve the cell growth up to day 7 but at a lower cell concentration as compared to the hydrolysates from Alcalase (Figure 2A). The result obtained indicates that the higher amount of small molecular sizes of peptides in plant protein hydrolysates help in the cell growth performance, as the hydrolysis by Alcalase produced smaller size peptides in soybean and rice protein hydrolysates (Figure 1 and Figure 2).The finding supports previous study on the effect of peptide sizes of the substrate on the cell growth [1, 6].

The amount of nitrogen recovery indicates how much nitrogen has been recovered after hydrolysis and it has different concept with degree of hydrolysis. Degree of hydrolysis is defined based on amount of cleaved peptide. In other words, degree of hydrolysis demonstrates influence of enzyme on the substrate, whereas nitrogen recovery exhibits the percent of protein in substrate that has been recovered by enzymes. The nitrogen recovery increases with degree of hydrolysis. It is a predictable behavior based on their definition. It is obvious that different enzymes have different effect on samples and it is observable from degree of hydrolysis and nitrogen recovery. In fact, there are several factors which contribute to the effectiveness of degree of hydrolysis and nitrogen recovery and one of the most important factors is the amount of protein in the substrate. For example, rice has the highest degree of hydrolysis and nitrogen recovery; however, it possesses the least amount of protein. Thus, types of protein in samples and ability of enzymes for separation are also significant.

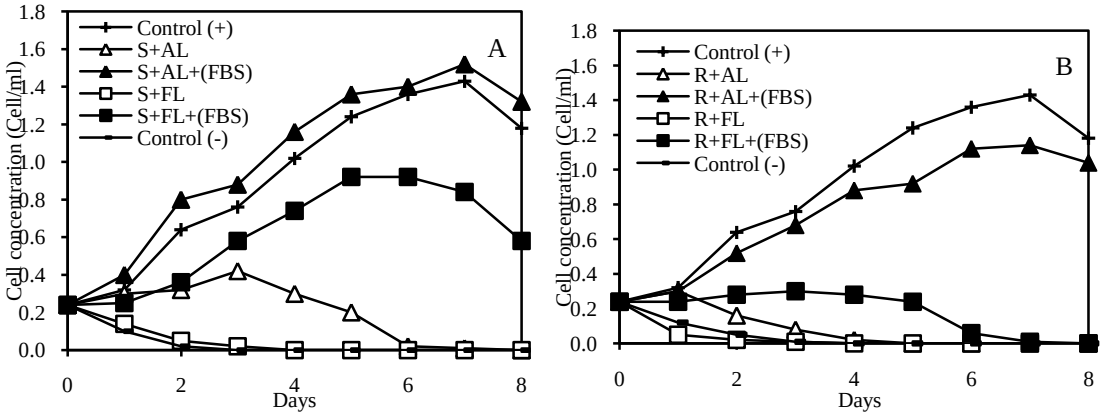

Fig. 2: Cell concentration in medium for plant extracts hydrolysed by (A) Alcalase;(B) Flavourzyme, with and without FBS.S Soybean; R Rice; AL Alcalase; FL Flavourzyme; FBS Fetal Bovine Serum

Conclusions:

It can be concluded that soybean hydrolysates have the potential to substitute the addition of animal-derived serum in the medium for Human Skin Fibroblast (HSF) 1184 cells. The influence of enzyme on the degree of hydrolysis of different plants and the sizes of peptides play an important role in the properties of hydrolysates produced. Hydrolysis with Alcalase produced higher degree of hydrolysis of plant isolates, higher nitrogen recovery and smaller molecular size peptides compared to hydrolysis with Flavourzyme. The result from this study shows that soybean hydrolysed by Alcalase provides the highest growth performance for the Human Skin Fibroblast 1184 cell culture. The production of plant protein hydrolysates offers essential information to

facilitate the choice and amount of proteins in a plant to substitute the animal-derived serum which is harmful such as Fetal Bovine Serum (FBS).It is suggested that the future studies should be done on determining the influence of the molecular size and the nature of peptides on transport over the cell membrane, on cell metabolism and finally, to purify those exhibiting promoting growth effects in mammalian cell culture. It is significant since finding from this research will validate the growth effect in soybean hydrolysates for serum replacement in cell culture. Consequently, even though plant proteins may not contain all the essential amino acids in the necessary proportions for Human Skin Fibroblast 1184 cell culture as compared to animal serum, yet, they are able to improve cell growth.

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