PLANT PROTEIN HYDROLYSATES FROM SOY BEAN 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 development of the cells. Serum, mainly from animal sources and very expensive, has many disadvantages such as viral contaminations. One of the alternatives for solving this problem is to use a serum-free media with addition of some supplements. In this study, protein hydrolysates from plant sources as supplement for medium of human skin fibroblast (HSF) 1184 cells to substitute the addition of serum in the medium was investigated. Plant protein hydrolysates from soy and rice were prepared through enzymatic hydrolysis using two different enzymes namely 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). Hydrolysates from Flavourzyme gave negative effect on HSF 1184 cell culture, while hydrolysates from Alcalase were supplementary for HSF 1184 cell culture. Depending on the enzyme used, the supplementation with hydrolysates corresponding to a high degree of hydrolysis and composition of peptides with small molecular size, led to different maximal cell density. From this study, the protein hydrolysates from soy hydrolysed by Alcalase has shown the best performance for supplementation into medium for HSF 1184 growth.

Keywords: protein, hydrolysates, soy, rice, cell culture

1. Introduction

Protein hydrolysates are generally thought to act as concentrated balanced nutrient mixtures that may partly or fully replace serum. Various acidic or enzymatic protein hydrolysates have been proposed to replace fetal bovine serum in mammalian and insect cell cultures (Farges-Haddani, 2006). Plants provide a highly pure source of soluble amino acids, peptides, vitamins, and essential elements for cell culture. Supplementing media with plant hydrolysates has been shown to improve protein production from engineered animal cells (Zhao et al., 2008). Soy protein hydrolysates have been used widely in mammalian cell culture by different researchers. Soy has shown the best result than other plants (Heidemann and Zhang, 2000). Rice protein hydrolysates were also strong enhancers of γ-IFN production and, to a lesser extent, of cell growth during cultivation in protein-free media inside microcarriers (Ballez et al., 2004). The objective of this study is to investigate the plant protein hydrolysates from soy and rice as supplement for medium in human skin cells for promoting cells growth. It is hypothesized that different enzymatic hydrolysis used for different plant affected the properties of protein hydrolysates produced.

2. Materials and Methods

Preparation of extracts and protein isolates
Extraction methods for oil seeds (soy) and cereal grain (rice) were used for the two different samples, respectively. These methods were a mixture extraction by solvents such as alcoholic and acetic solvents and membranes
dialysis.

Enzymatic hydrolysis of protein isolates
Hydrolysis of protein isolates samples were carried out in a 120 ml beaker that 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 HS0707V2. The pH-Stat method was used for measurement the degree of hydrolysis (DH). Enzyme preparations, Alcalase and Flavourzyme, adjusted to pH 7.5, were added to the substrate at an enzyme/protein ratio 2.5%. The 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
Chemical composition of meal and protein isolate were determined. Ash and total fat contents were determined according to AOAC method (Horwitz, 2000). Total crude protein content was obtained using the BCA method (Smith et al., 1985). 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 Company).

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

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-SEC-S2000 column (Phenomenex, Torrance, USA) as size exclusion chromatography column. Elution was performed isocratically 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 40 kDa, 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 cm² T-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 % CO₂ (Incubator Barnstead - Lab-line, model 397-1, Thermo Scientific, Melrose, USA). The cells bank was developed during two months. The first cell was from passage 3 and 24 vials from passage 6 were produced for final experiments. Cell culture was done three times for finding the adequate initial cell density. According to these three experiments, the adequate initial cell density for the growth in 24-wells was determined as 2 × 10^5 cells/ml. After propagation, the cells were sub-cultured in 24-wells plates. Fifty two 24-wells plates were used for experiment. Two control samples were used as control-plus and control-minus. Three wells were considered for each day for counting and calculating of growth curve. HSF cell growth was examined in eight days.

3. Results and Discussion

Chemical composition
The chemical composition of meal and protein isolates from soy 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

<table>
<thead>
<tr>
<th>Plant source</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Oil (%)</th>
<th>Total ash (%)</th>
<th>Nitrogen free extracts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy (M)</td>
<td>5.43</td>
<td>40.8</td>
<td>18.5</td>
<td>7.2</td>
<td>28.1</td>
</tr>
<tr>
<td>Soy (PI)</td>
<td>0</td>
<td>84.5</td>
<td>11.3</td>
<td>1.4</td>
<td>2.74</td>
</tr>
<tr>
<td>Rice (M)</td>
<td>10.4</td>
<td>5.47</td>
<td>1.23</td>
<td>0.63</td>
<td>82.3</td>
</tr>
<tr>
<td>Rice (PI)</td>
<td>0</td>
<td>20.3</td>
<td>0.83</td>
<td>0.56</td>
<td>78.3</td>
</tr>
</tbody>
</table>

(M) meal; (PI) protein isolate

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

Nitrogen recovery
Nitrogen recovery from hydrolysates produced using Alcalase were higher 20-25% compared to hydrolysates produced by Flavourzyme. These results indicated 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.

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Soy DH (%)</th>
<th>Soy NR (%)</th>
<th>Rice DH (%)</th>
<th>Rice NR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcalase</td>
<td>31.59</td>
<td>93.93</td>
<td>36.52</td>
<td>96.72</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>17.58</td>
<td>78.04</td>
<td>34.36</td>
<td>76.94</td>
</tr>
</tbody>
</table>

Size distribution
Flavourzyme that is an exopeptidase, produced the lowest amount of peptide with small molecular size, 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. These results showed that endopeptidases can produce higher amount of peptides with smallest molecular size compared to exopeptidases.

Cell growth analysis
The growth curves of HSF in different media were demonstrated in Figure 2. These results demonstrated that plant protein is not enough for mammalian cell culture. Although the plant proteins have positive effect on cell culture used in a DMEM without FBS but their performance was not the same as FBS when compared to the control plus. According to these results, it can be seen that the plant derived proteins cannot act as good as FBS alone. These results demonstrated that plant derived protein do not have enough factors for HSF cell culture. When normal medium without FBS was used as control minus the cells died after 3 days. However, the extracts protein by Alcalase could improve the cells growth. The extracts protein by Flavourzyme as an exopeptidase does not have significant effect on cell culture.

Soy hydrolysate has the best action on cell growth while rice extract has some adverse effects on the growth. This behavior describes the effect of different types of protein in different plants, for example soy isolate has higher protein content compared to rice and thus its ability for cell growth was better. This result showed that preparation of protein isolates is really important in using of plants derived protein in cells culture. According to these results, the best protein isolate for
mammalian cell culture is an isolate with more than 85% protein content.

![Graph](image)

Figure 2 Cell concentration in medium for plant extracts hydrolysed by (A) Alcalase; (B) Flavourzyme, with and without FBS. S Soy; R Rice; AL Alcalase; FL Flavourzyme (F) FBS

4. Conclusions

From this study, it has been shown that soy extracts by Alcalase were the best choice for HSF 1184 cell growth. Unlike animal serums, plant proteins may not contain all the essential amino acids in the necessary proportions for HSF 1184 cell culture but they can improve cell growth.

Acknowledgement

The authors would like to acknowledge Universiti Teknologi Malaysia and Ministry of Higher Education (MOHE) for the financial support under Research University Grant Scheme (Q.J130000.2644.08J12).

References


