

Hydrolyses of meat and soybean proteins using crude bromelain to produce *halal* peptone as a complex nitrogen source for the growth of lactic acid bacteria

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Abstract

The aim of the present work was to study the effect of temperature and time on the hydrolysis of meat and soybean proteins by crude bromelain to produce *halal* protein hydrolysates as nitrogen source for the growth of lactic acid bacteria. Crude bromelain was extracted from pineapple without any further purification. The hydrolysis of meat and soybean proteins by crude bromelain were carried out at various incubation temperature (30°C, 40°C, 50°C) and time (1, 2, 3 h). The degree of hydrolysis and soluble nitrogen contents of the protein hydrolysate were then measured. Incubation temperature at 50°C yielded the highest degree of hydrolysis for both protein samples. The degree of meat protein hydrolysis significantly increased to 75.30% during the first hour, and reached to more than 95% after three hours with soluble nitrogen content of 201.32 mg/100 mL. Similar profile was also observed in the soybean protein which yielded 85.14% protein hydrolysate with soluble nitrogen content of 260 mg/100 mL. The meat and soybean protein hydrolysates obtained were used as nitrogen sources in media containing sucrose, young coconut water, mung-bean sprout extract and tomato extract for the growth of *Lactobacillus plantarum* Dad 13. The performance of these media for cellular growth was almost as good as the performance of commercial MRS growth medium.

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Keywords

Halal protein hydrolysate

Crude bromelain

Degree of hydrolysis

Nitrogen content

Complex nitrogen source

Introduction

Beside carbon source, bacteria also require nitrogen source for their growth and metabolism. In the laboratory, these sources are provided by the growth media with the nitrogen source usually being the most expensive component. Organic nitrogen, such as peptones, is widely used for the production of biomass and in other biotechnology areas. The term “peptone”, also commonly known as protein hydrolysate, is defined as the partial digestion (hydrolysis) of proteins. The main function of protein hydrolysate in biotechnological applications is to provide complex nitrogen source for the growth of microorganisms at both laboratory and industrial scales (Pasupuleti and Braunverly, 2010).

The raw material proteins for the production of protein hydrolysate can be of animal- or plant-origin. The most commonly used animal-derived protein in biotechnological applications are casein, whey and meat obtained from different organs, while the widely used plant-derived proteins are soy and wheat. However, many protein hydrolysate productions have

come from other raw material such as *Sardinella* spp. (sardines), *Jatropha curcas* (flowering plant) cake, *Selaroides leptolepis* (yellowstripe trevally), *Thunnus albacares* (yellowfin tuna), cockle meat wash water and rice bran (Ghorbel *et al.*, 2005, Apitawatanapiwat *et al.*, 2009, Komplong *et al.*, 2009, Safari *et al.*, 2009, Ovissipour *et al.*, 2010, Haslaniza *et al.*, 2010).

Protein hydrolysate can be made by hydrolysing the protein sources using acid, alkaline or enzyme (which is the predominant hydrolysing agent). A wide variety of proteases are commercially available from animal, plant and microbial fermentations. The most commonly used animal-derived enzymes to hydrolyse protein are pancreatin, trypsin, and pepsin (Bridson, 1998); while papain and bromelain are plant-derived (Haslaniza *et al.*, 2010, Arshad *et al.*, 2014, Utomo *et al.*, 2014) and bacterial proteases are obtained from bacterial fermentations (Ghorbel *et al.*, 2005, Safari *et al.*, 2009, Jisha *et al.*, 2013). Animal-derived enzymes can either be *halal* or *haram* depending on their sources. *Halal* in Islam means permissible and lawful, while *haram* means prohibited. Raw material animal-derived protein is considered *halal* if the

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animal is slaughtered according to Islamic Shari'ah laws which do not include pigs and dogs. Therefore, if the raw material proteins or the enzymes are derived from pigs or dogs, they are considered *haram* and prohibited for example pepsin and protease extracted from pig's stomach (Khattak *et al.*, 2011). Since Islam is the largest religion in Indonesia, the demand for *halal* foods and food products is substantial and of immediate interest and importance.

For *halal*-fermented dairy products such as yogurt and fermented milk, the starter cultures (lactic acid bacteria) used should also be *halal* in the sense that they are grown in *halal* growth media (which contain *halal* plant-based protein hydrolysates). *Halal* protein hydrolysates can be produced using enzymes extracted from plants such as bromelain from pineapple (*Ananas comosus* (L.) Merr.). Bromelain is a crude extract from the fruit or stem of pineapples. Major applications of bromelain including in the baking industry, meat tenderisation and production of protein hydrolysates (Arshad *et al.* 2014).

In the present work, crude bromelain was extracted from pineapples, and then examined for its performance to hydrolyse meat and soybean proteins. Meat and soybean were selected because they are the common raw protein material used in the production of protein hydrolysates. The objective of the present work was therefore to investigate the effect of temperatures and times on the hydrolysis of meat and soybean protein isolates using crude bromelain to produce *halal* peptone as a complex nitrogen source for the growth of lactic acid bacteria.

Materials and methods

Materials

Pineapples and minced *halal* beef were purchased from Pasar Kranggan, Yogyakarta, Indonesia. *Halal* soybean protein isolate powder was purchased from Gushen Biological Technology Group C. Ltd., China. Mung bean sprouts, young coconut water, tomatoes and sugar were also purchased from the local market. The *Lactobacillus plantarum* Dad 13 was obtained from the Food and Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Extraction of crude bromelain

Pineapples were peeled, cleaned, cut into small pieces, and placed in juicer (Cosmos JC-388). The liquid crude bromelain was automatically separated from the solid waste.

Hydrolysis of meat and soybean proteins by crude bromelain at various incubation temperatures and times

For the production of meat protein hydrolysate, minced beef (5.0 g) in an Erlenmeyer flask was added with 20 mL crude bromelain, and incubated in shaker water bath (Memmert WNB45) at 50°C for 1, 2 and 3 h. For the production of soybean protein hydrolysate, soybean protein isolate powder (2 g) was added with 50 mL crude bromelain, and also incubated in shaker water bath at 50°C for 1, 2 and 3 h. The hydrolysis was terminated by placing the mixture in hot water ($\approx 90^\circ\text{C}$) for 15 min to denature the enzyme before the mixture was cooled. The degrees of hydrolysis and nitrogen contents in the protein hydrolysates were then determined. The best incubation time was then chosen.

The production of protein hydrolysate was then conducted at 30, 50 and 70°C for 3 h. Minced beef and soybean proteins were hydrolysed by the crude bromelain with the substrate:enzyme ratio described above. The hydrolysis was terminated by placing the mixture in hot water ($\approx 90^\circ\text{C}$) for 15 min to denature the enzyme before the mixture was cooled. The degrees of hydrolysis and nitrogen contents in the protein hydrolysates were then determined.

The use of meat protein hydrolysate and soybean protein hydrolysate as complex nitrogen sources for the growth of lactic acid bacteria

The medium consisted (in 1 L) of: 30 g sucrose (carbon source), 250 mL protein hydrolysate (each from meat and soybean sources prepared earlier), 400 mL mung bean sprout extract, 300 mL young coconut water, and 20 mL tomato extract.

To prepare the mung bean sprout extract, the mung beans were washed, and added with water at a ratio of 1:2.5. The mung bean sprouts were extracted by boiling for 2 h, and centrifuged at 10,000 rpm for 20 min. The supernatant was stored at -20°C .

To prepare the tomato extract, washed tomatoes were cut into small pieces ($\pm 5 \text{ mm}^3$), and then sterilised at 121°C for 20 min. The liquid was collected using filter paper, and stored at -20°C .

To prepare the coconut water, young coconut water was filtered to remove sediment and debris, and kept at -20°C . Approximately 1% v/v of 24 h-old *L. plantarum* Dad 13 culture was inoculated into the *halal* media. For control, the culture was also incubated in commercial MRS broth. Incubation was performed at 30°C for 24 h, and viable cells were counted using dilution-plating technique.

Determination of protease activity

One millilitre casein solution (1% w/v) in test tube was added with 2 ml Tris-HCl buffer (pH 8.0) and 1 mL crude bromelain, and then covered with aluminium foil before being incubated in water bath (Memmert WNB45) at 30°C for 10 min. The reaction was stopped by adding 2 mL TCA 20%, and left for 30 min at room temperature to precipitate the insoluble protein. The mixture was filtered through Whatman paper No. 41. The filtrate (0.75 mL) was added with 2.5 mL Na₂CO₃ buffer (0.4 M) and 0.5 mL Folin solution (1:2), and incubated for 20 min. Its absorbance was then measured at 760 nm using spectrophotometer (Genesis 10S UV-Vis). The standard curve for tyrosine with concentration ranged from 0 to 40 µg/mL was prepared. The tyrosine solution (0.75 mL) with various concentrations was added with 2.5 mL Na₂CO₃ dan 0.5 mL Folin solution, and incubated for 20 min. Its absorbance was then measured at 760 nm using spectrophotometer (Genesis 10S UV-Vis). A standard curve was constructed with linear regression of $y = 0.0269x + 0.0155$ with R² of 0.9978. As a control, 1 mL 1% casein solution was added with 2 mL Tris-HCl buffer (pH 8.0), and incubated for 10 min in the water bath at 30°C. The mixture was then added with 2 mL 20% TCA prior to addition of 1 mL crude bromelain, and incubated for 30 min. The following process was the same as done for the sample. Unit activity is defined as µmol of tyrosine released per minute per mL of enzyme under assay conditions.

Determination of nitrogen contents in raw materials and in protein hydrolysates

The nitrogen contents in raw materials and in protein hydrolysates were analysed by the Micro-Kjeldahl standard method (AOAC, 1995) using the formula as follows:

$$\% \text{ (w/w) N} = \frac{\text{Titration volume (mL)} \times \text{N HCl} \times 14 \times 100}{\text{Sample weight (mg)}}$$

The soluble nitrogen contents in the protein hydrolysates were determined using the formula as follows:

$$\text{Soluble N (mg/100 mL)} = \frac{\text{Titration volume(mL)} \times \text{N HCl} \times 14 \times \text{dilution factor}}{\text{Sample volume (mL)}}$$

Determination of degree of hydrolysis in protein hydrolysates

Following the hydrolysis of proteins, the protein hydrolysates were added with 20% (w/v) TCA (trichloroacetic acid) with a volume ratio of 1:1 to get 10% TCA soluble material. The mixtures were left to stand for 20 min to allow for precipitation, and then diluted to the volume of 100 mL. Sample (35 mL) was placed in conical tube, and centrifuged for 20 min. The supernatant (1.5 mL) was analysed for protein content using the Micro-Kjeldahl method (AOAC, 1995). The degree of hydrolysis (DH) was calculated using the formula as follows:

$$\text{DH (\%)} = \frac{\text{Soluble N after hydrolysis} \times 100}{\text{Total N in the sample}}$$

Results and discussion

Activity of crude bromelain

The crude bromelain extracted from pineapple yielded 2.74 U/g pineapple. The result showed that the increase in temperature from 30°C to 50°C increased the enzyme activity more than twice, but higher temperature of 70°C resulted in the decrease in the enzyme activity (Table 1). Arshad *et al.* (2014) reported that the optimum temperature for stem bromelain activity were in the range of 40-60°C. Martins *et al.* (2014) found that bromelain remained active even after 60°C with an optimum temperature of 50°C. Enzyme is a globular protein. Therefore, incubation at high temperature can result in the unfolding of protein structure that can destroy the three dimensional conformation of the active site thereby inactivating the enzyme. Temperature affects the activity and stability of enzyme. Usually, the higher the temperature, the shorter the stability. Following incubation at 50°C for 1 h, the remaining activity of bromelain was 83%, while at 40°C the enzyme remained stable (Jutamongkon and Chareonrein, 2010). The optimum temperature is not always used for enzymatic reaction. Depending on the temperature stability, lower temperature can be applied for longer time to obtain certain degree of hydrolysis.

Table 1. Effect of temperatures on the activity of crude bromelain

Enzyme	Temperature (°C)	Enzyme Activity (U/mL)
Crude bromelain	30°C	2.14 ± 0.11
	50°C	4.68 ± 0.10
	70°C	4.42 ± 0.32

Hydrolysis of meat and soybean proteins by crude bromelain

Hydrolysis of meat and soybean proteins by crude bromelain were conducted in the substrate:enzyme ratio of 5 g:20 mL and 2 g:50 mL, respectively. Enzymatic reaction was carried out at 50°C for different incubation times which were 1, 2 and 3 h. The result showed that in the first hour of incubation, the degree of hydrolysis increased significantly, reaching to 75.34% and 71.71% DH for meat protein and soybean protein, respectively (Table 2). The DH continued to increase until 3 h of incubation. Similar patterns were obtained for the soluble protein content in protein hydrolysates. This might indicate that the crude bromelain could hydrolyse protein in meat and soybean proteins. The longer the incubation time, the higher the soluble nitrogen concentrations. This result is in agreement with those reported by Haslaniza *et al.* (2010) and Ovissipour *et al.* (2010) in which as the incubation time increased, the DH also increased. Most of the other studies used commercial protease to produce protein hydrolysate, such as alcalase, protamex or food-grade bromelain (Ghorbel *et al.*, 2005, Haslaniza *et al.*, 2010 and Ovissipour *et al.*, 2010). However, the present work used crude bromelain from pineapple fruits. It seemed that crude bromelain could in fact be used to hydrolyse protein sources and produce protein hydrolysates with high DH to be used as component in bacterial growth media.

Table 2. The degrees of hydrolysis and soluble nitrogen contents of protein hydrolysates of meat and soybean protein isolates at various incubation times using crude bromelain at 50°C

Incubation time (h)	Meat protein hydrolysate		Soybean protein hydrolysate	
	Degree of hydrolysis (%)	Soluble nitrogen (g/100 mL)	Degree of hydrolysis (%)	Soluble nitrogen (g/100 mL)
0	16.64 ± 1.33	32.03 ± 2.66	5.85 ± 0.01	17.82 ± 0.0
1	75.34 ± 2.72	152.33 ± 13.32	71.71 ± 1.27	224.21 ± 2.66
2	95.79 ± 0.99	193.77 ± 7.99	79.11 ± 2.31	241.16 ± 5.33
3	99.09 ± 1.57	201.31 ± 7.99	85.14 ± 0.76	260.00 ± 0.0

Another factor that markedly influences the performance of enzymatic hydrolysis of protein materials is temperature. Hydrolysis of meat and soybean proteins by crude bromelain were carried at 30°C, 50°C and 70°C for 3 h. The increase in temperature from 30°C to 50°C increased the DH and soluble protein content in both types of protein

hydrolysate (Table 3). However, when protein hydrolysis was carried out at 70°C, the performance decreased, as indicated by the lower DH and soluble protein content in the protein hydrolysates. Based on the results, the optimum temperature of hydrolysis of protein materials using crude bromelain was 50°C. A study done by Haslaniza *et al.* (2010) showed that optimum temperature of hydrolysis of protein precipitate from cockle meat wash water using food-grade bromelain was 45°C. The thermal enzyme stability, incubation time and enzyme concentration should be considered in determining the temperature of incubation. As reported by Jutamongkon and Charoenrein (2010), incubation at 40°C showed no loss of fruit bromelain activity up to 60 min, whereas at 50°C for 1 h the remaining activity was approximately 83%. Therefore, hydrolysis of protein at lower temperature such as 30°C could be applied but need longer incubation time to achieve the desired DH. However, incubation at room temperature faces the risk of microbial contamination. So, higher incubation temperature for a short time using higher concentration of enzyme could be applied to prevent microbial contamination.

Table 3. The degrees of hydrolysis and soluble nitrogen contents of protein hydrolysates of meat and soybean protein isolates at various incubation temperatures for 3 h using crude bromelain.

Temperature (°C)	Meat protein hydrolysate		Soybean protein hydrolysate	
	Degree of hydrolysis (%)	Soluble nitrogen (g/100 mL)	Degree of hydrolysis (%)	Soluble nitrogen (g/100 mL)
30	80.66 ± 2.40	158.26 ± 0.0	73.31 ± 5.36	224.71 ± 13.32
50	99.33 ± 0.80	209.13 ± 2.66	82.68 ± 2.47	252.47 ± 5.33
70	81.57 ± 1.12	159.20 ± 1.33	70.41 ± 1.24	214.78 ± 5.33

Crude bromelain is a mixture of protein-digestion enzymes. It is a mixture of closely related protease such as different thiol-endopeptidases and other components. During the hydrolysis of protein, some peptides were released, and they were hydrolysed into smaller peptides and amino acids that provided nitrogen source for the bacterial growth. It is promising that the hydrolysis of *halal* meat protein and *halal* soybean protein isolate by crude bromelain can be used to produce *halal* protein hydrolysates that meet the regulatory requirement in the production of *halal* products.

The use of meat and soybean proteins as complex nitrogen for the growth of Lactobacillus plantarum Dad 13

Lactobacillus plantarum Dad 13 is an indigenous lactic acid bacteria isolated from traditional fermented buffalo milk in West Sumatera, Indonesia. This culture met the basic requirements as a probiotic and had some functional properties (Sumaryati *et al.*, 2009, Fitrotin *et al.*, 2015, Rahayu *et al.*, 2016). This culture showed good performance as a starter culture for milk fermentation (Utami *et al.*, 2016). The culture was grown in medium containing either meat protein hydrolysate or soybean protein hydrolysate as a complex nitrogen source. Beside protein hydrolysate, the growth media also consisted of sucrose as a carbon source, and young coconut water, mung bean sprout extract and tomato extract as vitamins, minerals and growth factors sources, respectively. For control, the culture was also grown in commercial MRS medium. Table 4 shows that the growth of *L. plantarum* Dad 13 in *halal* media containing meat or soybean protein hydrolysates were only slightly lower than that in MRS. This means that these hydrolysate proteins could provide comparable, if not better, nitrogen source for the growth of *L. plantarum* Dad 13. The nitrogen source of MRS medium is not only from peptone, but also from Lab-Lemco powder and yeast extract. Young coconut water, mung bean sprout extract and tomato extract in *halal* media provide vitamins, minerals and growth factor for the growth of lactic acid bacteria (Ray, 1996). Lactic acid bacteria are fastidious bacteria, and MRS serves as an excellent commercial growth medium for them. Aspmo *et al.* (2005) also reported that the performance of growth medium containing protein hydrolysate from Atlantic cod (*Gadus morhua*) viscera was almost as good as that of commercial MRS medium for the growth of lactic acid bacteria.

Table 4. The growth of *Lactobacillus plantarum* Dad 13 using different nitrogen sources following incubation at 37°C for 24 h.

Nitrogen source	Viable cells (CFU/mL)
Soybean protein hydrolysate (<i>halal</i> medium)	2.8×10^9
Meat protein hydrolysate (<i>halal</i> medium)	4.4×10^9
Commercial peptone (MRS media)	5.4×10^9

Conclusion

Crude bromelain extracted from pineapple fruits showed its highest activity at 50°C. Crude bromelain could be used to hydrolyse meat and soybean proteins with the degree of hydrolysis of more than 80% at 50°C for 3 h with soluble nitrogen content in the range of 209 -252 mg/100 mL. Crude bromelain juice could be obtained using simple extraction method and showed good performance to produce the protein hydrolysates. Meat and soybean hydrolysate proteins obtained can be used as complex nitrogen source for the growth of lactic acid bacteria. The performance of the *halal* media containing these protein hydrolysates was almost as good as the performance of commercial MRS for the growth of lactic acid bacteria tested. This indicates that these protein hydrolysates are promising complex nitrogen sources for the preparation of lactic acid bacteria starter culture in the production of *halal* fermented food products.

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