# Effects of Different Proteases on Degree of Hydrolysis and Antioxidant Activity of Mung Bean Hydrolysates

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# Effects of Different Proteases on Degree of Hydrolysis and Antioxidant Activity of Mung Bean Hydrolysates

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**Abstract:** *This study evaluated the use of three proteases, namely acid, alkaline, and neutral proteases for production of mung bean hydrolysate (MBH). The acid and alkaline proteases achieved degree of hydrolysis (DH) of 68.81% and 71.96%, respectively, which were much higher than neutral protease (42.04%). The antioxidant activities, assessed through DPPH and ABTS radical scavenging assays, revealed that acid protease hydrolysates showed the highest DPPH activity of 82.32 µg Trolox equivalent (TE)/g, while alkaline protease hydrolysates showed the highest ABTS activity of 6.93 mg TE/g. Two-step enzymatic hydrolysis using acid protease followed by alkaline protease achieved higher DPPH activity of 81.68 µg TE/g, while alkaline protease followed by acid protease resulted in higher ABTS activity of 4.17 mg TE/g. These results highlight the significant impact of enzymatic treatment on the DH and antioxidant efficacy of MBH.*

**Keywords:** Mung bean; enzymatic hydrolysis; antioxidant activities.

## 1. INTRODUCTION

Mung bean (*Vigna radiata L. Wilczek*) popularly known as green gram, believed to be native crop of India, is a tiny circular shaped bean in green color widely cultivated throughout Asia. Mung bean is rich in proteins, carbohydrate, dietary fiber, vitamins, and minerals and contains a low amount of fat. Since it is rich in protein, it can be considered as the meat alternative for vegetarians. Its high nutritional value and its health benefits include antioxidant, anticancerous, anti-inflammatory and hypolipidemic activities. In addition, mung bean has prebiotic and nutraceutical properties. Mung bean contains a notable quantity of oligosaccharides that can promote the growth of beneficial gut bacteria. Researchers have developed functional foods, such as mung bean milk and non-dairy probiotic drinks, using green gram. Additionally, it can serve as a carrier for delivering probiotics to the gut. Green gram is also utilized in cosmetics, land reclamation, and incorporated into various food products like jams, jellies, and noodles [1]. Achieving sustainability requires a holistic approach that are economic, social and environmental sustainability [2]. Mung bean, as an agricultural product, illustrates sustainability dimensions that contribute to both SDG 3 and SDG 11 by enhancing health and well-being while promoting sustainable urban development. Their integration into urban agriculture and food systems supports healthy, resilient, and inclusive communities. By improving nutritional outcomes, fostering community engagement, and supporting sustainable practices, mung beans play a vital role in achieving the intertwined goals of good health and well-being and sustainable cities and communities [3].

Plant proteins are now being proposed as alternatives to animal proteins due to their multiple biological activities and environmental sustainability. Beans are an underutilized pulse that can be used as an excellent source of plant protein having good bioactive potential. Protein hydrolysates are products obtained from the partial hydrolysis of proteins, resulting in the breakdown of larger protein molecules into smaller peptides and individual amino acids. This process can be achieved through enzymatic or chemical methods. In food industry, protein hydrolysates are used as ingredients in various food products to enhance flavor, texture, nutritional value, and antioxidant activity. Protein hydrolysis through chemical methods involves the use of strong acids or bases to break down proteins. This process is typically carried out by heating proteins in the presence of acids or bases, which denature the protein structure and cleave peptide bonds [4].

Enzymatic hydrolysis of proteins involves the use of proteolytic enzymes, such as proteases or peptidases to break down proteins into smaller peptides and amino acids. Proteases cleave specific peptide bonds within the protein sequence, while peptidases further break down the resulting fragments into individual amino acids. Enzymatic hydrolysis is often preferred over chemical methods due to its specificity, efficiency, and ability to preserve the nutritional integrity of the protein. It operates under mild conditions, minimizing the risk of protein denaturation and the formation of undesirable by-products [5]. Various proteolytic enzymes have been utilized to generate bioactive peptides that offer numerous health benefits and commercial applications [6]. In the past decade, bioactive peptides derived from food proteins, such as soy protein isolate, chia protein, egg white protein, fish residue, collagen (sea cucumber) and milk protein concentrate (MPC) [7]. However, protein hydrolysis can also affect the sensory properties of the products by producing a bitter taste which inhibits their use as a food ingredient [8]. Antioxidants are compounds that, when present in deficient concentrations in foods or the human body, can delay, control, or prevent oxidative processes in foods and the human body. Various methods and activities are involved in how these antioxidant compounds inhibit oxidation [9].

Mung bean meal protein hydrolysate has been prepared by enzymatic hydrolysis using various

proteases such as Bromelain [10], Alcalase, Neutrase, Protamex, Flavourzyme and Papain with 3000 U/g (enzyme unit/substrate weight) [11]. The degree of hydrolysis was in the range of 5-23%. The ABTS radical scavenging activities and the ferric ion chelating activity of hydrolysates using Alcalase and Protamex were also higher than those using other proteases [11]. The proteolysis was also combined with the use of high-pressure assisted enzymatic proteolysis (300-600 MPa) to improve the antioxidant activity of the bean protein hydrolysate [12]. The enzymatic hydrolysis depends on pH, temperature, sample/buffer ratio, enzyme concentration, and hdrolysis time [13-15].

This study aimed to enzymatically produce mung bean hydrolysate with high antioxidant activity. The effects of different proteases on degree of hydrolysis and antioxidant activity of mung bean hydrolysates were investigated. As each protease has different substrate specificity, it was then expected that the combine use of two enzymes might have synergistic effects and increase both DH and antioxidant activity.

## 2. MATERIALS & METHODOLOGY

### 2.1. Materials

Peeled mung bean was purchased from local market (Hatyai, Thailand). The commercial enzymes, acid protease (80,000 IU/mL), alkaline protease (250,000 IU/mL) and neutral protease (80,000 IU/mL) were purchased from iKnowZyme (Reach Biotechnology, Thailand). All chemicals used in this study were of analytical grade.

### 2.2. One-step hydrolysis using single protease

The procedure for one-step hydrolysis of mung bean using single protease is shown in Fig. 1. The ground mung bean flour (120 mesh) was suspended in deionized water (1 g/5 mL). The obtained mung bean flour dispersion was added with 3000 U/g (enzyme unit/substrate weight). The enzymatic hydrolysis was performed under conditions of pH 4.0, 50 °C for acid protease, pH 9.0, 50 °C for alkaline protease and pH 7.0, 50 °C for neutral protease (Table 1).

**Table 1** Hydrolysis conditions for preparation of protein hydrolysates from Mung bean

| Hydrolysis Conditions | Units | Acid Protease | Alkaline protease | Neutral protease |
|---|---|---|---|---|
| Incubation temperature | °C | 50 | 50 | 50 |
| pH | - | 4 | 9 | 7 |
| Enzyme/Substrate | U/g | 3000 | 3000 | 3000 |
| Mung bean/buffer ratio | g/mL | 1:5 | 1:5 | 1:5 |
| Hydrolysis time | h | 4 | 4 | 4 |
| Inactivation temperature | °C | 100 | 100 | 100 |
| Inactivation time | Min | 10 | 10 | 10 |

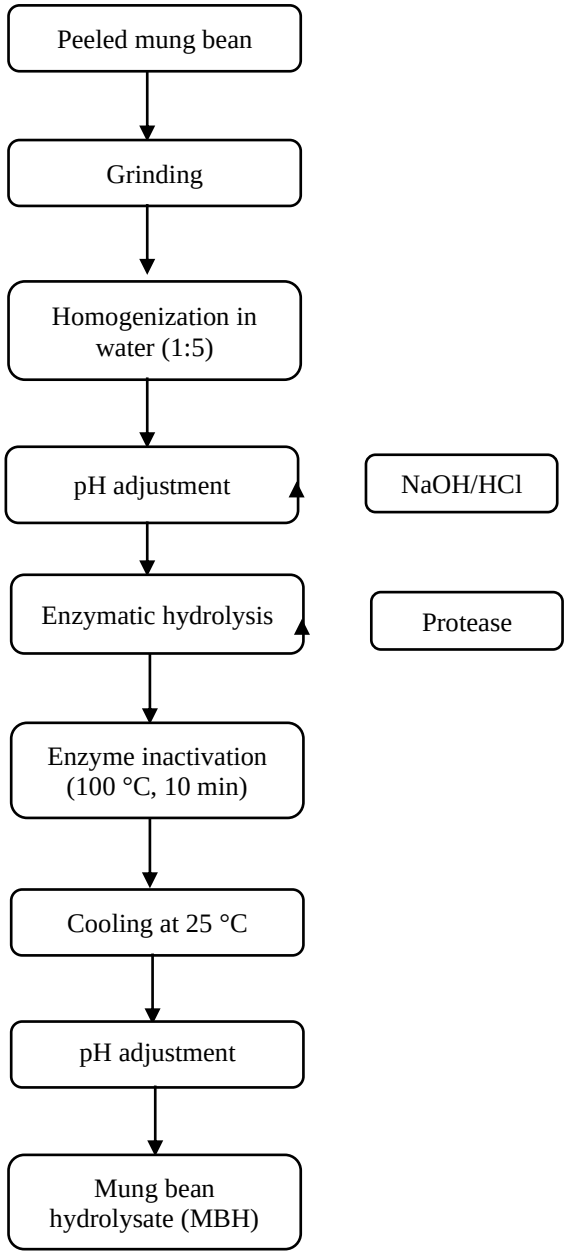


**Fig. 1** Flow diagram for the preparation of mung bean hydrolysate

After hydrolysis, the enzymatic hydrolyzed dispersion was heated at 100 °C for 10 min to inactivate the enzymes, cooled to 25 °C, pH was adjusted to neutral. The MBH was stored at -20 °C for later analysis. The DH and antioxidant activities were compared. The suitable enzyme that gave the maximum antioxidant activity was then selected.

### 2.3. Two-step hydrolysis using different proteases

The obtained mung bean flour dispersion was added with 1500 U/g (enzyme unit/substrate weight) The enzymatic hydrolysis was performed under the conditions for acid protease (pH 4.0, 50 °C) for 60 min and the pH was adjusted to 9.0 and 1,500 U/g of alkaline protease was added, and the hydrolysis was continued for more 60 min. The two-step hydrolysis using alkaline protease followed by acid protease was also performed. After hydrolysis, the enzymatic hydrolyzed dispersion was heated at 100 °C for 10 min to inactivate the enzymes, cooled to 25 °C and protein hydrolysates were stored at -20 °C for

later analysis. The sample abbreviated respectively as AK (Acid-Alkaline protease) and AA (Alkaline-Acid protease).

**2.4. Determination of degree of hydrolysis (DH)**

The o-phthaldialdehyde (OPA) reagent was prepared as follows: 7.620 g disodium tetraborate decahydrate and 200 mg sodium-dodecyl-sulfate (SDS) was dissolved in 150 mL deionized water. The reagents were dissolved homogeneous before continuing. 160 mg 97% OPA was dissolved in 4 mL ethanol. The OPA solution was transferred to the solution. 176 mg dithiothreitol 99% (DTT) was added to the solution and the solution was made up to 200 mL with deionized water. The serine standard was prepared as follows: 50 mg serine was diluted in 500 mL deionized water (0.9516 meqv/L). The sample was dissolved with 5 mL of 7M hydrochloric acid, boiling at 100 °C for 60 min, then centrifuged at 10,000 g for 5 min. The sample 4 g was dissolved in 100 mL of deionized water. After that 400 µL of solution mixed with 3 mL of OPA reagent and it was allowed to stand for 2 min and the absorbance at 340 nm was determined with spectrophotometer [16]. The degree of hydrolysis (DH) of MBP was calculated by Eq. (1)

$$DH\ (\%) = (B/A) * 100 \quad (1)$$

where B is the concentration of serine (mg/mL) of sample; A is the concentration of serine (mg/mL) of total hydrolysate.

**2.5. Determination of antioxidant activity**

To determine antioxidant capacity, the sample 1.5 mL was added with 1.5 mL of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10-60 µM. The activity was expressed as µmol Trolox equivalents (TE)/g sample [17]. ABTS radical scavenging activity was determined by ABTS assay. Preparation of ABTS+ radical solution: 50 mL of 2.45 mM potassium persulphate will be mixed with 50 mL of 7 mM ABTS and kept in the dark for 12-16 h. The mixed solution will be diluted with a 0.2 M phosphate buffer solution (pH 7.4) to obtain an absorbance of 0.70 ±0.02 at 734 nm. Fresh ABTS solution was prepared for each assay. Sample (150 µl) will be mixed with 450 µL of ABTS solution and the mixture was left at room temperature for 15 min in dark. The absorbance was then measured at 734 nm using the spectrophotometer. The activity was expressed as µmol Trolox equivalents (TE)/g sample [18].

**3. RESULTS & DISCUSSION**

**3.1 One-step hydrolysis using various single proteases**

**3.1.1 Degree of hydrolysis of mung bean hydrolysate**

Three different proteolytic enzymes, including acid protease, alkaline protease and neutral protease were used to compare the hydrolytic efficiency. The degree of hydrolysis (DH), which represents the percentage of cleaved peptide bonds in a protein hydrolysate [19], were compared. Fig. 2 shows the DH curves of mung bean hydrolysate (MBH) by acid protease, alkaline protease and neutral protease between 0 and 4 h. The DH of MBH with acid protease increased from 27.10% to 68.81% at 4 h. The hydrolysates generated by alkaline protease and neutral protease achieved the highest DH values of 71.96% and 42.04% at 3 h of hydrolysis, respectively. These results showed that the hydrolytic capacity of acid protease and alkaline protease were higher than that of neutral protease. The variation in DH could be attributed to differences in bean varieties and enzymatic hydrolysis conditions, which lead to differing extents of peptide bond cleavage in the protein structure [13].

**3.1.2 Biological properties of mung bean hydrolysate**

**3.1.2.1 DPPH radical scavenging activity**

The increase in antioxidant activity of mung bean hydrolysate may be attributed to the higher concentration of peptides with smaller molecular weights and phenolic compounds [17]. The DPPH free radical scavenging activities of MBH from three enzymes hydrolysates (acid protease, alkaline protease and neutral protease) were presented in Fig. 3. Acid protease achieved the highest DPPH activity of 82.32 µg TE/g at 2 h, followed by alkaline protease (60.11 µg TE/g) at 1.5 h and neutral protease (42.59 µg TE/g) at 1 h. However, DPPH radical scavenging activity of MBH decreased when increasing time. The DPPH radical scavenging activity by acid protease, alkaline protease and neutral protease at 4 h was 61.06 µg TE/g, 37.07 µg TE/g and 31.74 µg TE/g, respectively. Higher DH does not always correlate with increased antioxidant activity. In some cases, it may result in lower antioxidant effectiveness due to the excessive breakdown of peptides into free amino acids, which may have less antioxidant potential than longer peptide chains [20]. These results showed that the hydrolytic capacity of acid protease was higher than that of alkaline protease and neutral protease. The antioxidant activity of protein hydrolysates is influenced by various factors, including DH, the presence of free amino acids (FAAs), nitrogen solubility, and the type of enzyme used in the hydrolysis process [4].

**3.1.2.2 ABTS radical scavenging activity**

Protein hydrolysates prepared using various proteases showed varying ABTS radical scavenging activities. The MBH from three enzymes hydrolysates (acid protease, alkaline protease and neutral protease) were presented in Fig. 4. The highest activity was observed in MBH with alkaline protease (6.93 mg TE/g), followed by acid protease (6.11 mg TE/g) at 2 h of hydrolysis. After 2 h of hydrolysis, it was observed that the antioxidant activity decreased. Excessive hydrolysis can lead to reduced antioxidant effectiveness as very small peptides and free amino acids may not be as effective as slightly larger peptide chains [20]. In addition, the lowest activity was observed in neutral protease. These results demonstrated that alkaline and acid proteases have a higher hydrolytic capacity compared to neutral proteases.

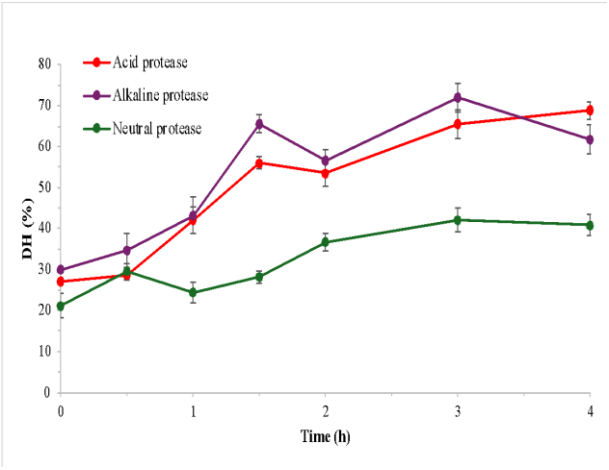


**Fig. 2** Degree of hydrolysis (DH) of mung bean hydrolysate (MBH) hydrolyzed by three enzymes.

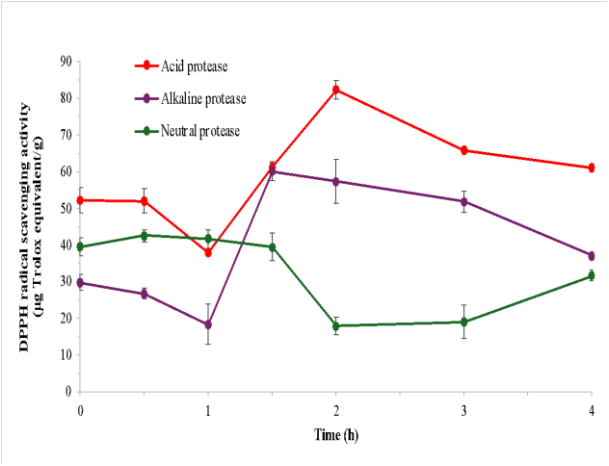


**Fig. 3** DPPH radical scavenging by mung bean hydrolysate (MBH) hydrolyzed by three enzymes.

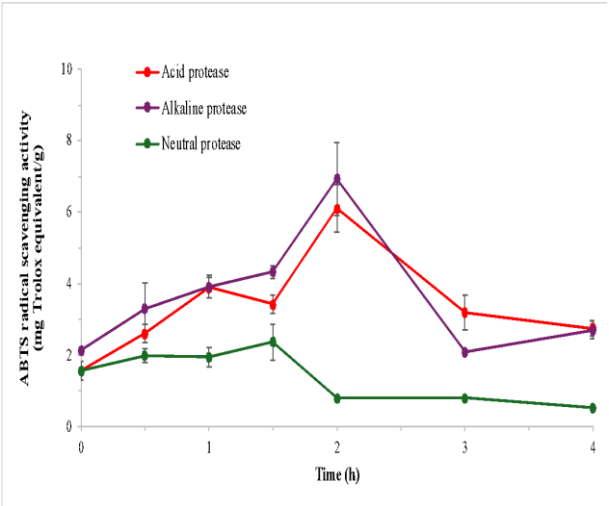


**Fig. 4** ABTS radical scavenging by mung bean hydrolysate (MBH) hydrolyzed by three enzymes.

### 3.2 Two-step hydrolysis using different proteases

#### 3.2.1 Degree of hydrolysis of mung bean hydrolysate (MBH)

It has been reported that sequential hydrolysis can produce peptides with enhanced bioactivity, including antioxidant. This is because different enzymes target specific peptide bonds, generating a diverse range of bioactive peptides [7]. In this study, it was expected that the two-step hydrolysis using different catalytic activities of enzymes might offer several benefits for producing protein hydrolysates with enhanced functional and nutritional properties. To evaluate the combine use of acid and alkaline protease, two successive enzymatic hydrolysis of mung bean were performed. The hydrolysis time was set at 2 h as this time gave the maximum antioxidant activities. The total enzyme activity was set at 3,000 U/g. In the first step, half of enzyme (1,500 U/g) was added and the hydrolysis was performed for 1 h. In the second step, the pH was adjusted and another half of enzyme (1,500 U/g) was added and the hydrolysis was performed for more 1 h.

Fig. 5 shows the DH of the hydrolysates of mung bean using two-step hydrolysis. The DH of MBH by acid protease for 1 h followed by alkaline protease for 1 h (AK) increased from 18.29% to 62.50%. While the MBH generated by alkaline protease followed by acid protease (AA) gave lower maximum DH value of 34.96%. It should be noted that the amount of protease used in each step was only half of those used in the hydrolysis using single protease. Only the hydrolysis using acid protease followed by alkaline protease showed the further increase in DH. It was possible that in the first stage, the strong hydrolysis ability of acid protease hydrolyzed the protein into peptides, and in the second stage the peptides were further hydrolyzed under the action of alkaline protease, and the DH of the protein continued to increase.

#### 3.2.2 Biological properties of mung bean hydrolysate

##### 3.2.2.1 DPPH radical scavenging activity

The DPPH free radical scavenging activities of MBH by AK and AA are presented in Fig. 6. In the first stage, acid protease achieved the highest DPPH radical scavenging activity of 81.68 μg TE/g at 60 min. This activity was higher than using single protease despite in the first step using only half amount of enzyme (1,500 U/g). In the second stage, the protein was hydrolyzed under the action of alkaline protease and it was found that the DPPH radical scavenging activity decreased. While when alkaline protease was used in the first step, the DPPH radical scavenging activity was much lower than using single protease possibly due to lower amount of protease used. In the second step using acid protease, the DPPH radical scavenging activity drastically increased. However, the final DPPH radical scavenging activity by AA was lower than that by AK. These results suggested that the single acid protease at lower amount (1,500 U/g) was more suitable for producing MBH with high DPPH radical scavenging activity.

##### 3.2.2.2 ABTS radical scavenging activity

Protein hydrolysates prepared using two-step hydrolysis showed varying ABTS radical scavenging activities. The ABTS radical scavenging activities of MBH by AK and AA are presented in Fig. 7. The highest activity was observed in MBH by AA (4.17 mg TE/g) at 120 min, followed by AK (3.35 mg TE/g) at 60 min of hydrolysis. In the first stage, the use of both alkaline and acid proteases for 60 min gave comparable ABTS radical scavenging activity. In the second stage, when the MBH was further hydrolyzed by acid protease the ABTS radical scavenging activity further increased. However, when using alkaline protease in the second step the ABTS radical scavenging activity decreased. It should be noted that the single protease was more suitable for the

ABTS radical scavenging activity.

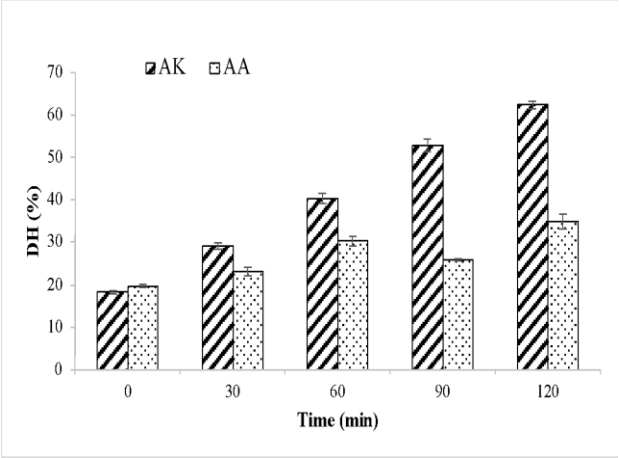


**Fig. 5** Degree of hydrolysis (DH) of mung bean hydrolysate (MBH) using two-step hydrolysis. AK (Acid-Alkaline protease) and AA (Alkaline-Acid protease).

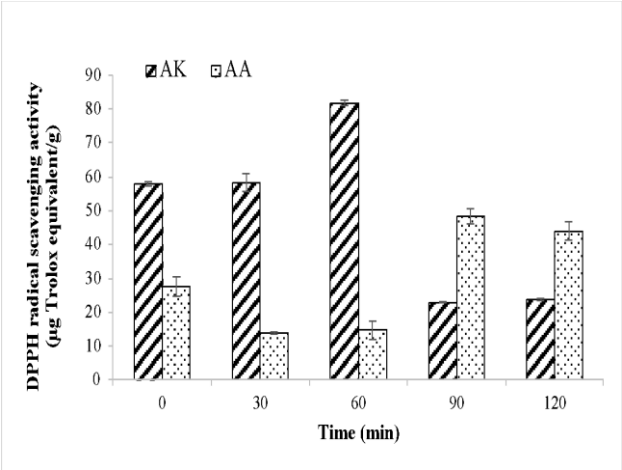


**Fig. 6** DPPH radical scavenging by mung bean hydrolysate (MBH). AK (Acid-Alkaline protease) and AA (Alkaline-Acid protease).

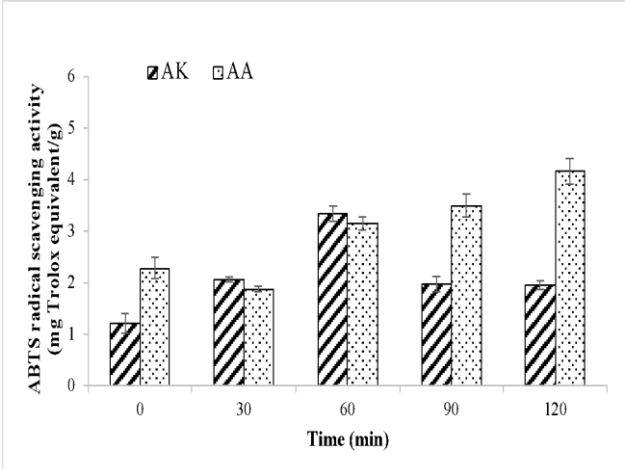


**Fig. 7** ABTS radical scavenging by mung bean hydrolysate (MBH). AK (Acid-Alkaline protease) and AA (Alkaline-Acid protease).

## 4. CONCLUSIONS

MBH prepared by using different proteases, exhibited different DH and antioxidant activities. The DH of MBH with alkaline protease was highest at 71.96% followed by acid protease (68.81%) and neutral protease (42.04%). The acid protease gave much higher DPPH radical scavenging activities of 61.06 µg TE/g compared to those of acidic protease (37.07 µg TE/g) and neutral protease (31.74 µg TE/g). While the highest ABTS radical scavenging activity was observed when using alkaline protease (6.93 mg TE/g), followed by acid protease (6.11 mg TE/g). The combine use of two enzymes in two-step enzymatic hydrolysis slightly increased DH but did not significantly improve the antioxidant activity. This study has shown that different functional properties of MBH could be obtained by using different proteases. The MBH prepared by enzymatic hydrolysis may serve as natural antioxidants and can be applied in functional food industry.

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