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Research Paper

Antioxidant capacity of soybean protein isolate hydrolyzed with protease produced by *Aspergillus* 3.084 *in vitro*

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Abstract. *Aspergillus*, being known as a protease producing fungi, is used in Chinese and Korean traditional fermentation. In this paper, *Aspergillus* 3.084, one of the most common fungi in China fermentation industry, was employed to produce protease and hydrolyze soybean protein, and then the optimum extraction condition of protease was determined by the antioxidant activities of protease extraction solution. The protease was extracted from the culture medium, and performed in hydrolyzing soybean protein isolation. Extract condition of protease, hydrolysis ability of proteases of *Aspergillus* in hydrolyzing soybean protein isolation and the antioxidant ability of hydrolysate were investigated by detecting enzyme activity, degree of hydrolysis, scavenging activities of $OH\cdot$, $O_2^{\cdot-}$ and $DPPH\cdot$. Maximum protease activity was obtained at 60 min, pH 7.5 and 32°C in extraction step. Optimum protease hydrolyzing time, E/S, temperature and pH for degree of hydrolysis and scavenging activities was 3 h, 6000, 37°C and 7.5, respectively.

Keywords: Soybean protein, protease, *Aspergillus* 3.084, antioxidant activity.

INTRODUCTION

Soybean is a well-known source of protein with high protein and peptide content. Peptides made from soybean exhibit variety of functional properties, including immunomodulatory, antioxidant, anticancer (Lin *et al*., 2013). Fermentation, one of the oldest food-processing techniques, can improve the antioxidant properties of soybean through various biochemical transformations.

Aspergillus 3.084 is a common fungi in Chinese fermentation industry, it secretes amylase, glucoamylase, α-amylase, alkaline protease, acid protease and neutral protease. The most familiar product made by *Aspergillus* is soybean paste, and the antioxidant capacity of soybean paste is given by fermentation process. This process also improved the properties of soybean by biochemical transformations including proteolysis, saccharification, esterification, etc (Rhyu and Kim, 2011). Soybean paste produced generally by two steps is a traditional seasoning in China and Korean, first step was pretreatment by steaming and shaping, fermentation was followed by microorganisms (Lee *et al*., 2014). In the beginning of fermentation processing, starter named qu in China and meju in Korean was added into pretreated soybean, which contained the microorganisms, including *Bacillus*, *Aspergillus*, *Scopulariopsis*, *Cladosporium*, *Mucor*, *Lichtheimia*, *Rhizopus* and *Penicillium* (Jung *et al*., 2014). Microorganisms have been identified in Korean meju, and *Aspergillus* was the main microorganism in china soybean paste (Wardhani *et al*., 2013). Soybean hydrolysates, such as amino acids and peptides, enriched the taste, flavor, even the antioxidant capacity of soybean paste.

The antioxidant capacity test included hydroxyl free radical (OH·) scavenging activity, 1,1-diphenyl-2-picrylhydrazylfree radical (DPPH·) scavenging activity and superoxide anion ($O_2{\cdot}^-$) scavenging activity. Superoxide radical is known to be harmful to cellular components as a precursor of more reactive oxidative species; it is also documented to play an important role in the peroxidation of lipids (Monod *et al*., 1991). Therefore, the scavenging activity on superoxide radicals is a way to investigate the antioxidant activity. Free radical is one of the factors that induce the pathogenesis of a wide range of age-related degenerative diseases through the oxidative modification of DNA, proteins and vital molecules (Malathi and Chakraborty, 1991; Cohen, 1973). DPPH, a stable radical-generating reagent, was used in the present study for primary screening of the antiradical activities (Yokozawa *et al*., 1998; Kalaskar *et al*., 2014). The main antioxidant properties was mainly given by the peptides, which was hydrolyzed by enzyme and acid. Protease secreted by *Aspergillus* 3.084 was employed to hydrolyze the soybean protein isolate, and the antioxidant properties of peptides, the enzymatic hydrolysate, was studied in this paper. Effects of temperature, pH value and ratio of enzyme/soybean protein powder ([E/S]) on the antioxidant properties of peptides hydrolyzed by extracted protease was investigated.

MATERIALS AND METHODS

Materials

Soybean protein powder (protein content of 80.2%) was obtained from the valley of god biological technology group co., LTD (China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and bull serum albumin (BSA) were purchased from Sigma Chemicals Co., LTD (USA).

Protease preparation

Aspergillus 3.084, obtained from Shandong wife food group co., LTD, Zibo, Shandong, was used in this study. The culture media were prepared with 20 g soybean meal, 20 g wheat bran and 10 g flour in 250 ml erlenmeyer flasks and soaked with 50 g distilled water. Flask was mixed thoroughly and all flasks were autoclaved at 121°C for 20 min, cooled to room temperature, inoculated with 1 ml of conidial inoculums (1×10^9), and then incubated at 32°C for 72 h (Belmessikh *et al*., 2013).

Optimization of extract condition of protease

Fermented substrate (10 g) was milled and mixed with 50 ml 0.1 mol/L pH7.5 phosphate buffer and homogenized by ultrasound for 1 hour at 37°C to extract the enzyme. Extraction condition under different pH, temperature and time was optimized. Protease activity of the enzyme solution was analyzed by the method described by Lowry, enzyme-activity was micrograms of tyrosine produced by hydrolyzing the casein within one minute.

$$Enzyme\ activity(U) = \frac{A}{T} \times N$$

A, tyrosine (μg)
T, time (min)
N, dilution ratio
Total protein content of the extracted solution was measured by using coomassie brilliant blue staining with bovine serum albumin as a protein standard (de Castro and Sato, 2014).

2.4 Peptide preparation

Hydrolysis reaction was carried out under different temperature (27, 32, 37, 42, 47 °C), hydrolysis time (1, 2, 3, 4 and 5 h), pH (3.5, 5.5, 7.2, 9 and 10.5) and ratio of enzyme/soybean protein ([E/S], 1000, 2000, 4000, 6000 and 8000U per gram soybean protein). The original temperature, hydrolysis time, pH and protease activity were set at 37°C, 2 h, 7.2 and 25 U/ml. After the hydrolysis reaction, solution was kept at 95°C for 3 min to inactivate the protease and cooled as the experimental group. Reaction solution (10 ml) was adjusted to pH 8.2 by 10 mol/L NaOH or 10 mol/L HCl and diluted to 20 ml for the determination of the degree of hydrolysis and, 10 reaction solution was adjusted to pH 7.0 and diluted following the same step as above-mentioned for the other tests[14]. Mixture of enzyme and soybean protein was kept at 95°C for 3 min to inactivate the protease, which was unhydrolyzed solution and used for the control group.

Determination of the degree of hydrolysis (DH) [15-17]

Distilled water (10 ml) was mixed with 10 ml reaction solution, and then 20 ml 36.5% formaldehyde solution (adjusted to pH 8.2 with 0.1 mol/L NaOH) was added (Castillo *et al*., 1962; Gould and Frantz, 1945; Mehler, 1957). The mixture solution was titrated to pH 9.2 by 0.1 mol/L NaOH, titration volume was V_1. Unhydrolyzed solution 10 ml (mixture of enzyme and soybean protein) was used for control, titration volume of which was V_2. The degree of protein hydrolysis was calculated as follows

$$Degree\ of\ protein\ hydrolysis\% = [(V_1 - V_2) \times N/(C \times k \times V_R)] \times 100$$

V_1, consumption of NaOH for hydrolysis solution (ml)
V_2, consumption of NaOH for control (ml)

N, concentration of NaOH solution (mol/ml)
C, soybean protein concentration (mg/ml)
k, mol of peptide bonds per gram protein, k = 7.75 × 10^{-6} mol/mg for the soybean protein
V_R, volume of reaction solution

1,1-Diphenyl-2-picrylhydrazylfree radical (DPPH·) scavenging activity

DPPH• scavenging activity was estimated following the procedure, 2 ml hydrolysate was added to 2 ml 2 mmol/L DPPH• (dissolved in absolute ethyl alcohol), after reacting for 30 min in dark, absorbance of the mixture was measured at 517 nm. Also, an equal volume of each sample was mixed with 2 ml absolute ethanol but without DPPH• as control to avoid interference of protein and peptide[18], 2 ml distilled water was mixed with2 ml2 mmol/L DPPH• (dissolved in absolute ethyl alcohol) for the absorbance of DPPH•, and 2 mL unhydrolyzed solution mixed with 2 ml of 2 mmol/L DPPH• (dissolved in absolute ethyl alcohol) for the absorbance of control group. The scavenging percentage of hydrolyzed solution on DPPH• was calculated according to the following equation[19].

$$Scavenging\ percentage\ of\ DPPH\bullet\% = [(A_C - A_H)/A_B] \times 100$$

A_C, absorbance of control group
A_H, absorbance of hydrolysate
A_B, absorbance of DPPH• (blank group)

Superoxide anion ($O_2^{\cdot-}$) scavenging activity

All experiments were performed at 25°C, the assay medium (0.05 mol/L TRIS buffer, pH 8.2) 4.5 ml mixed with 100 µl hydrolyzed solution and 400 µl 2.5 mmol/L pyrogallol, stopped reaction 4 min later with 0.1 ml 10 mol/L HCl and recorded at 325 nm. Also, 100 µl distilled water was used in blank group, and 100 µl unhydrolyzed solution replaced hydrolyzed solution in control group. The scavenging percentage of hydrolyzed solution on $\mathbf{O_2^{\cdot-}}$ was calculated according to the following equation.

$$Scavenging\ percentage\ of\ \boldsymbol{O_2^{\cdot-}}\% = [(A_C - A_H)/A_B] \times 100$$

A_C, absorbance of control group
A_H, absorbance of hydrolysate
A_B, absorbance of $\mathbf{O_2^{\cdot-}}$ (blank group)

Hydroxyl free radical (OH·) scavenging activity

The assay for hydroxyl radical (OH•) was determined using the methods of Wang *et al*. The OH• in aqueous media is generated through the Fenton reaction, reaction mixture contained 0.24 mol/L K-phosphate buffer (pH 7.4), 1.0 mmol/L salicylic acid, 0.3 mM $FeSO_4$/EDTA (4 mmol/L), 0.8 mmol/L H_2O_2, and 100 µl of hydrolysate. Distilled water (100 µl) was used in blank group, and unhydrolyzed solution (100 µl) replaced hydrolysate in control group. The scavenging percentage of hydrolyzed solution on OH• was calculated according to the following equation.

$$Scavenging\ percentage\ of\ OH\bullet\% = [(A_C - A_H)/A_B] \times 100$$

A_C, absorbance of control group
A_H, absorbance of hydrolysate
A_B, absorbance of OH• (blank group)

RESULTS

Protease preparation

Aspergillus 3.084 grew on potato agar medium with yellow mature spores in the middle part of fungi colony (Figure 1A). When *Aspergillus* grow and secreted the enzyme, medium beside the *Aspergillus* became more transparent for culture medium contained 1% casein and enzyme hydrolyzed casein to make this vitrification. Figure 1B was fermented substrate, fungi covered the surface and grow inside the substrate.

Optical density of tyrosine in the enzyme activity detection of extracted solution and the linear trend line of OD and tyrosine concentration (correlation between OD and tyrosine concentration) was Figure 2, the enzyme activity of solution was obtained.

Optimization of extract condition of protease

Fermentation production was extraction with different condition included time, temperature and pH to investigate the optimal extract condition, and 60 min, pH 7.5 and 32°C were performed in this step (Figure 3).

DH and antioxidant activity under different E/S

DH increased following the increase of E/S (decrease of substrate concentration), even the same trend appeared in DPPH• scavenging activity of hydrolyzed soybean protein. DPPH• scavenging activity was less steep than DH, the values were between 13 to 19.5. Small molecular weight proteins and peptides generated following the E/S increase, which had higher DPPH• scavenging activity

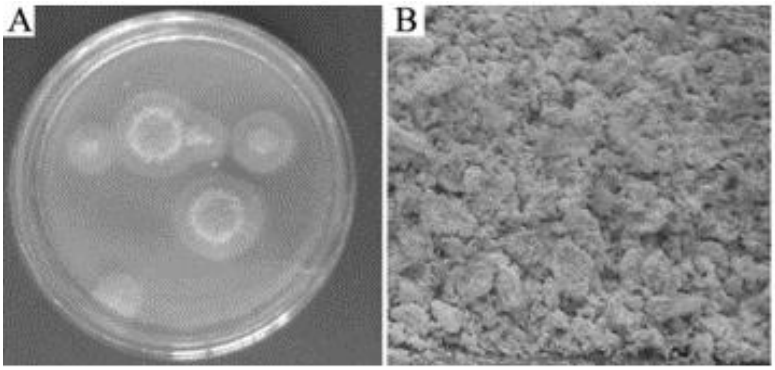

Figure 1. A. *Aspergillus* 3.084 grow on culture dish, B. fermented substrate.

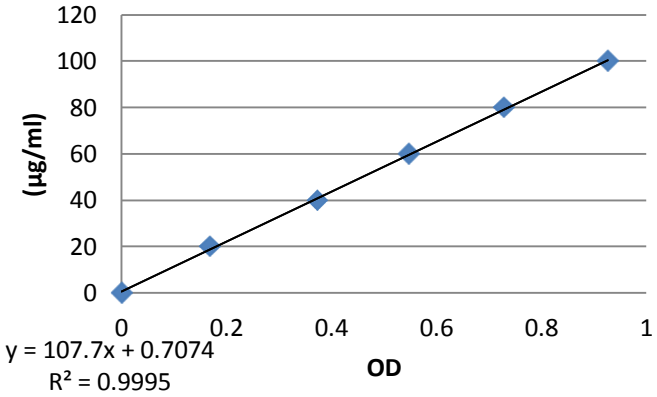

Figure 2. Standard curve of OD and tyrosine concentration.

(Figure 4).

$O_2^{\bullet-}$ and OH• scavenging activities declined, and the trend of $O_2^{\bullet-}$ scavenging activity was slower than OH• scavenging activity. Soybean protein hydrolyzed by protease to small molecular weight protein and peptide, more of that were produced in low substrate concentration (high E/S). This result means that small molecular weight proteins and peptides had stronger $O_2^{\bullet-}$ and OH• scavenging ability (Figure 4).

DH and antioxidant activity under different reaction time

The DH and antioxidant activities of hydrolyzate solution were monitored every 1 h up to 5 h by mixture of soybean protein and proteases solution at 6000 E/S. Curves of DH, DPPH• scavenging activity, $O_2^{\cdot-}$ scavenging activity and OH· scavenging activity showed rising trend following reaction time (Ortiz and An, 2000), which suggested that scavenging activities increased with the DH (Figure 5). B.P. Lamsal depicted that the subunits of β-conglycinins, and acidic and basic subunits of glycinin, almost entirely disappeared hydrolysate at 2% DH of soybean protein isolation (Lamsal *et al*., 2007), and then small molecular weight peptides and amino acids were obtained. Among those hydrolyzed peptides and amino acids, several kinds of them in soybean hydrolysate had antioxidant activity which was obtained from the hydrolyzed bitter peptides.

DH and antioxidant activity under different temperature

Those detected indexes had the same trend following the increase of temperature (Figure 6). Low DH was detected in high temperature for the induction of protease inactivation and protein denaturation, and antioxidant activities were also decreased with the DH. Antioxidant activities were lower in low temperature than high, coincided with trend of DH.

DH and antioxidant activity under different pH

Extracted enzyme solution contained many kinds of proteases with the optimum reaction pH of this solution was 7.5. Antioxidant activities varied following the change of DH, and low values measured at two extreme points (Figure 7). Acid protease included in solution had optimum

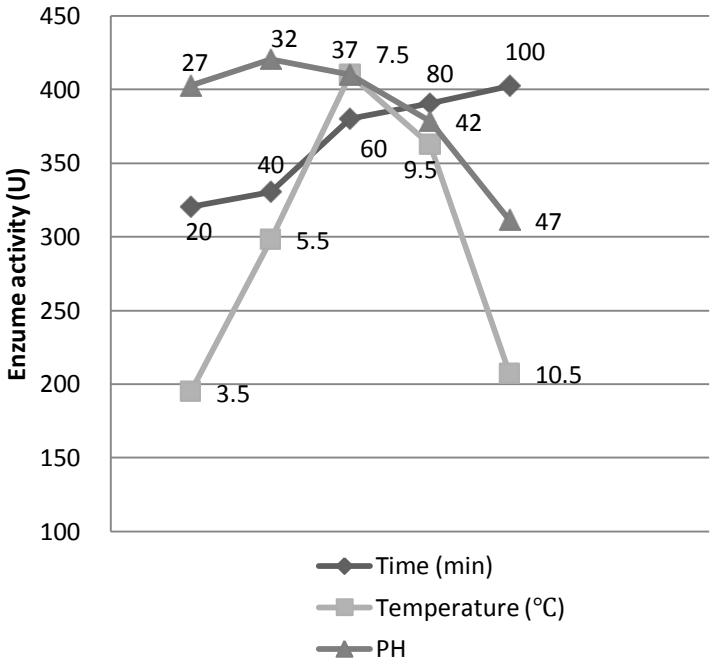

Figure 3. Curves of enzyme activity.

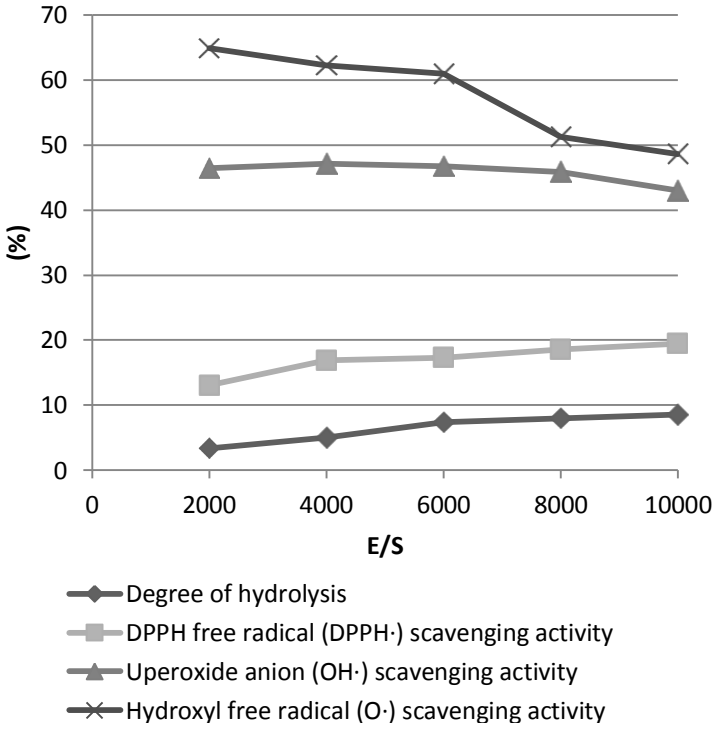

Figure 4. Trends of DH and DPPH•, O_2•$^-$, OH• scavenging activities at different E/S.

action pH at or nearly 3 with its lower hydrolysis ability than alkaline protease at its optimum pH 10.5. Middle protease also had enzyme activity at pH 9 and 5.5, which contributed to the high antioxidant activities. Even the DH at pH 3 was more than double times at pH 7.5 and 9 nearly double times at pH 5.5, there was no so much difference between the oxidant activities at pH 3 and pH 5.5, 7.5 and 9.

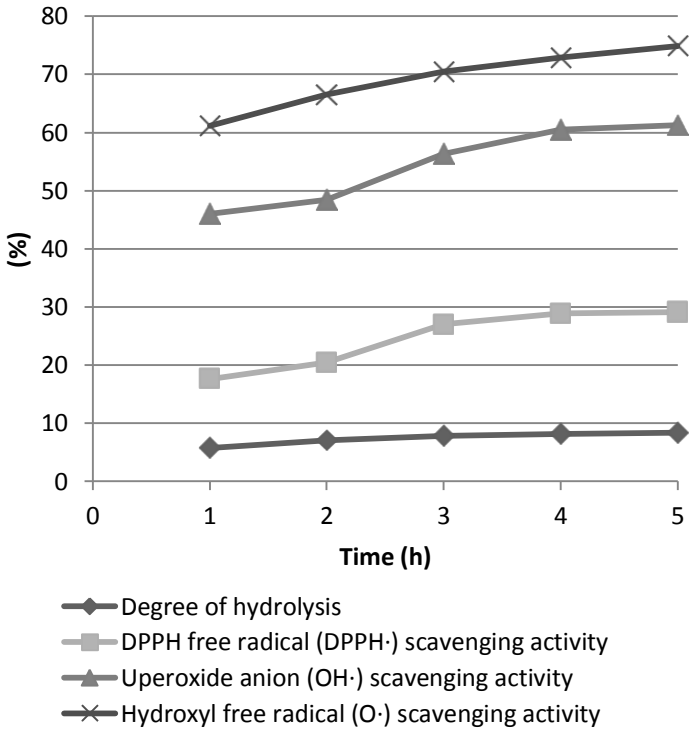

Figure 5. Trends of DH and DPPH•, O_2•$^-$, OH• scavenging activities under different reaction time.

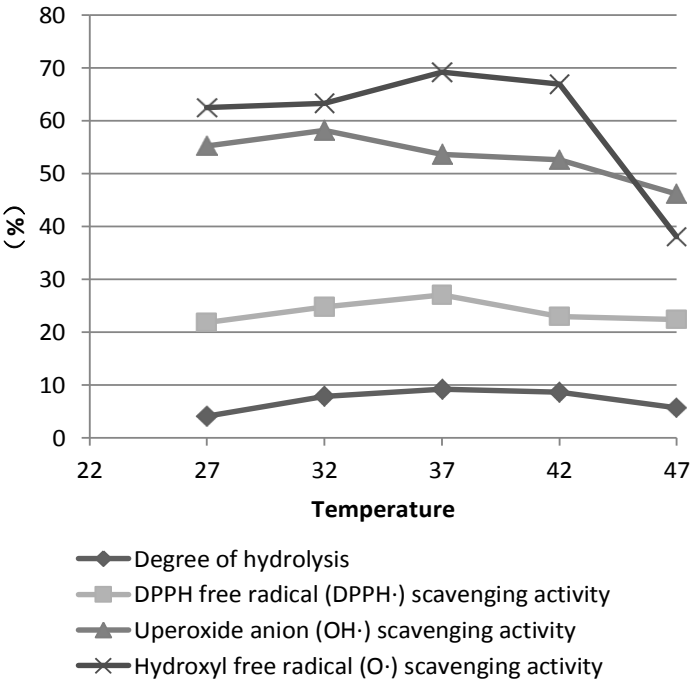

Figure 6. Trends of DH and DPPH•, O_2•$^-$, OH• scavenging activities at different temperature.

Enzyme produced by *Aspergillus* 3.084 contained multiple kinds of proteases, classifying as alkaline, neutral and acid protease, they had their own optimal pH. Ph 7.5 indicated that neutral protease activity was higher than

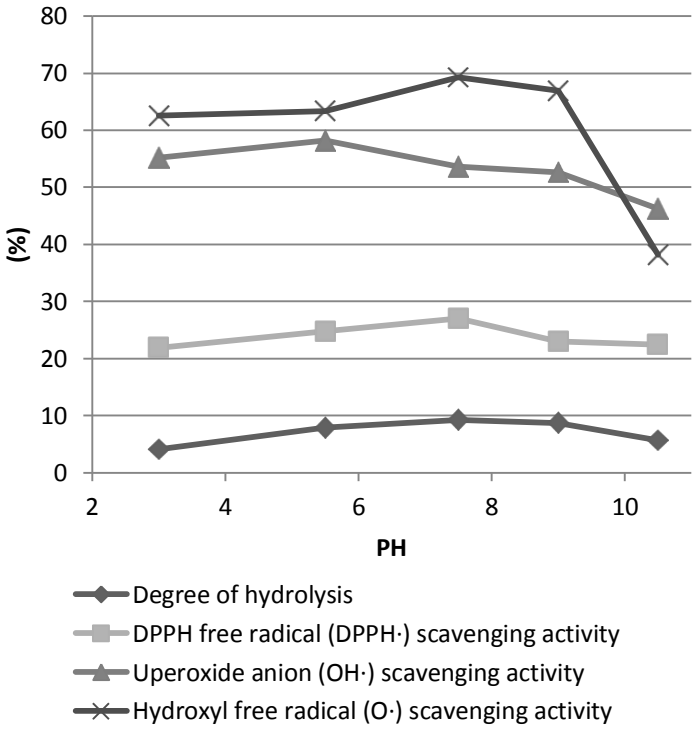

Figure 7. Trends of DH and DPPH•, $O_2^{\bullet -}$, OH• scavenging activities at different pH.

other two, alkaline and acid protease could not reach the same hydrolysis ability even in the optimal pH.

CONCLUSIONS

Action of different kind of protease on soybean protein was different[20], Aspergillus production contained many kinds of proteases which had different extracting properties, hydrolyzing abilities[10]. Longer extracting time give the solution higher enzyme activity following the increasing dissolved enzyme, and enzyme activity reached the top point at pH 7.5 and 37°C, This result indicated that the middle protease played the most important part in enzyme activity of this solution.

Optimum reaction time, E/S, temperature and pH was 3 h, 6000, 37°C and 7.5, following the DH, antioxidant activities of this solution reached the maximum point at the same reaction conditions. Soybean proteins hydrolyzed by limited or controlled enzyme could provide ingredients with desired peptides and functionalities (Lamsal *et al*., 2007), *Aspergillus* 3.084 with multifarious proteases used in traditional fermentation technology contributed to the peculiar flavor of production composed by different hydrolyzed ingredients (Han *et al*., 2001; Zhao *et al*., 2006; Zhao *et al*., 2009). Rising trend of DH was slower after 3 h hydrolysis than before. It reached the top point at 5 h within given time interval, and longer time enhanced the DH and facilitated the peptides production. Higher DH and antioxidant properties were obtained in industrial manufacture for it had longer fermentation time.

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