2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activities of protein hydrolyzed were determined as described by [20] [19] with slight modification. The ABTS solution was prepared with the final concentration of 7 mM ABTS and 2.45 mM potassium persulfate. The mixture was left in the dark at room temperature for 12-16 h before use. Prior to the assay, the ABTS solution was diluted with 0.2 M sodium phosphate buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. Then 40 µl of the sample containing 2.0 mg/ml was added to 4 ml of diluted ABTS solution. The mixture was shaken vigorously for 30 s and left in the dark for 6 min. An equivalent volume of distilled water instead of the sample was used for the blank. The absorbance of the resultant solution was measured at 734 nm. The ABTS scavenging activity was calculated as [(Ac - As)/Ac] x 100%, where Ac and As are the absorbance of the control and sample, respectively.

The scavenging effect of on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical was measured according to the method of Shimada *et al.* [20] with slight modifications. A volume of 1 ml of each sample was added to 2 ml of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. The antioxidant activity was calculated as [(Ac - As)/Ac] x 100%, where Ac and As are the absorbance of the control and sample, respectively.

For the superoxide anion scavenging activity measurement, the autoxidation of a pyrogallol method described by Tang *et al.*, [21] was followed with slight modification. Briefly, 0.1 ml of protein hydrolyzed was mixed with 1.8 ml of 50 mM Tris-HCl buffer (pH 8.2). The mixture was incubated at 25^{o}C for 10 min, and then 0.1 ml of 10 mM pyrogallol (dissolved in 10 mM HCl) was added. The absorbance of the solution at 320 nm was measured up to 4 min. The oxidation rate of pyrogallol for the sample was calculated as the slope of the absorbance line (ΔAs). The autoxidation rate pyrogallol for control was measured with 1.0 ml of double distilled water (ΔA0). The superoxide anion scavenging activity was calculated as [(ΔAo-ΔAs)/Δ Ao]x100%.

The fast protein liquid chromatography (FPLC) of melinjo seed protein isolates and hydrolyzed was performed on a Superdex 200 HR 10/30 column of 7.5 x 300 mm. The column was fitted to an AKTAPURIFIER, FPLC apparatus, equipped with a P-900 pump and UPC-900 model. The eluant was 50 mM phosphate buffer (pH 7.0) 0.15 M NaCl, at a flow rate of 0.5 ml/min. Samples were passed through a 0.45 mm filter and injected through a 500 ul-loop.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in accordance with the method of Laemmli [22]. As the SDS-PAGE gels, use was made of a 15% and the protein was detected with the use of Commassie Brilliant Blue. To roughly estimate the molecular weight and amount of the protein, molecular weight markers (PageRuler™ Low Range Unstained Protein Ladder, Thermo scientific; 5 to 100 kDa and a synthetic peptide at 3.4 kDa) were electrophoresed in such a manner that bands stood for 20 ng.

The analyzed spectra were determined using Fourier transform infrared (FTIR). The sol-gel immobilized alcalase and sol-gel un-immobilized alcalase were taken in the wavelength region between 400 and 4,000/cm at the ambient temperature with a Bruker Alfa FTIR spectrometer (Germany).

The thermal analyses using Differential Scanning Calorimeter (DSC) were performed with Thermoplus EVO, DSC8230, Rigaku, Japan. The calorimeter was calibrated with indium (melting point = 156.7^{o}C) and the reference used was liquid paraffin [23]. The sample (1-3 mg) were weighed in aluminum DSC pans. The pans were sealed and scanned from 50 to 220^{o}C at a heating rate of 10^{o}C/min under nitrogen gas. Onset temperature (To) and peak temperature (Tp) of samples; and the enthalpy values (ΔH) were measured to characterize the thermal properties of samples.

Scanning Electron Microscopy (SEM). SEM photographs were taken with Hitachi TM3000, scanning microscope to examine the morphology, surface structure and inside structure of sol-gel non-immobilized alcalase and sol-gel immobilized alcalase at the required magnification at room temperature. The beads were deposited on brass hold and sputtered with a thin coat of gold under vacuum. Acceleration voltage used was 20 kV with the secondary electron image as a detector.

III. Result and Discussion

A. Physicochemical Characteristics of Sol-Gel Immobilized Alcalase

The protein content of immobilized alcalase on sol-gel matrices in washing solutions disclosed that between 69.1 and 72.8% of the enzyme has been immobilized and the degree of immobilization had a standard deviation of 1.31%. The FTIR spectra of sol-gel non-immobilized alcalase and sol-gel immobilized alcalase are shown in Fig. 1.

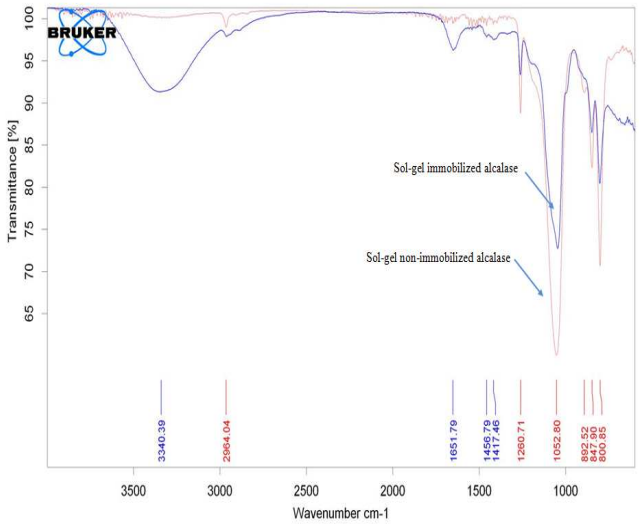

Fig. 1 Infrared spectra of sol-gel non-immobilized alcalase and sol-gel immobilized alcalase

The sol-gel immobilized alcalase showed a broadband spectrum between 3,000 and 3,700/cm and which was attributed to O–H stretching vibrations. The strong band spectrum of sol-gel immobilized alcalase at 1.260.71/cm was indicated to the carbonyl group. Sol-gel immobilized alcalase showed a peak at 1,052.8/cm and 1,294/cm due to C–N stretching, which confirmed the grafting of the monomer [24].

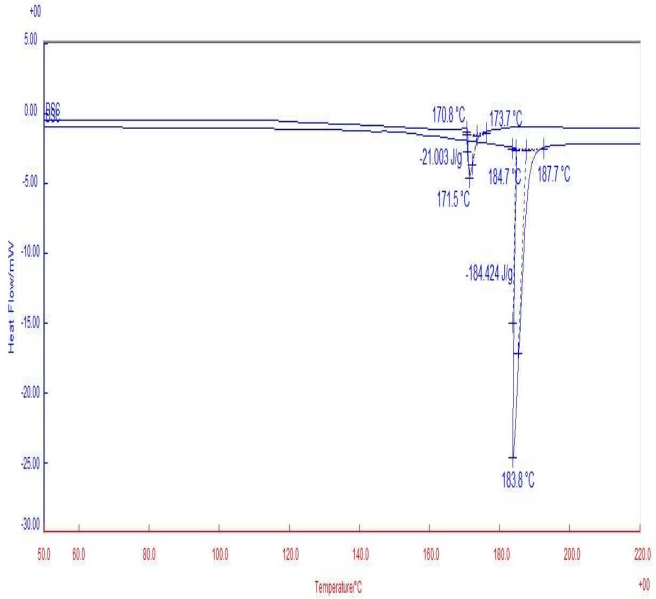

Fig. 2 DSC analysis results of sol-gel non-immobilized alcalase and sol-gel immobilized enzyme

The thermal properties of the sol-gel non-immobilized alcalase and sol-gel immobilized alcalase were performed using DSC analyses, and the representative thermogram results are shown in Fig. 2. Regarding the thermal DSC thermogram in Fig. 2, the temperature of the end points of the Tp shifted to lower temperatures with the grafting of the sol-gel non-immobilized alcalase. The peak temperature value of the sol-gel non-immobilized alcalase was lower than that of sol-gel immobilized alcalase. This is indicated that the grafted chains might act as internal plasticizers. In our data, peak temperature and enthalpy value of the sol-gel non-immobilized alcalase and sol-gel immobilized alcalase were found to be 171 and 184^{o}C; and 21.0 and 184 J/g, respectively. The increasing of Tp and ΔH value was seen when sol-gel interacted with enzymes. This increase could be clarified that polymer matrix is more rigid after interacting. Similar observed were reported by Chauhan *et al*. [24] and Isiklan *et al*.[25].

Scanning electron microscopy photographs of the sol-gel non-immobilized alcalase and sol-gel immobilized alcalase taken at 50 and 1,000 times magnifications were shown in Fig. 3. By comparing the surface morphology of internal structure of sol-gel non-immobilized alcalase (Fig. 3A and B) and sol-gel immobilized alcalase (Fig. 3C and D) were shown that the grafted chains changed drastically the morphology of sol-gel formation. As shown in Fig. 3C and D, sol-gel immobilized alcalase are almost spherical in shape and show roughness on the surface.

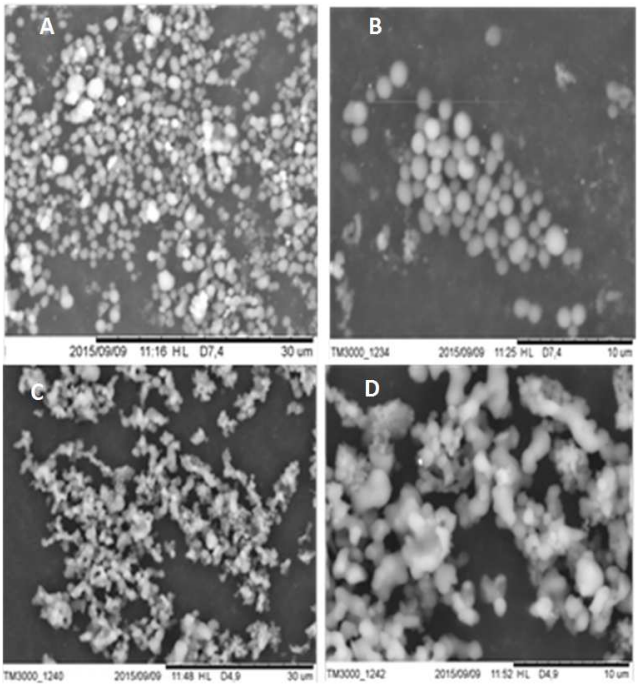

Fig. 3 SEM photographs of cross section of the sol-gel non-immobilized alcalase (A and B) and sol-gel immobilized alcalase (C dan D)

B. Effect of Protein Hydrolyzed Using Sol-Gel Immobilized Alcalase on Antioxidant Activity

Melinjo seed protein isolated hydrolysis reaction under standard conditions that the enzyme and substrate concentration (E/S) was 0.8%; the reaction temperature was 50°C and pH was 8.0. Five parallel experiments which hydrolysis time was 0.5, 1, 2, 6 and 12 h were carried out, respectively. The results were shown in Fig. 4. It can be seen from Fig. 4 that the degree of hydrolysis (DH) of protein hydrolysate was increased rapidly with the increasing of time of hydrolysis in the from 0.5 to 2 h.

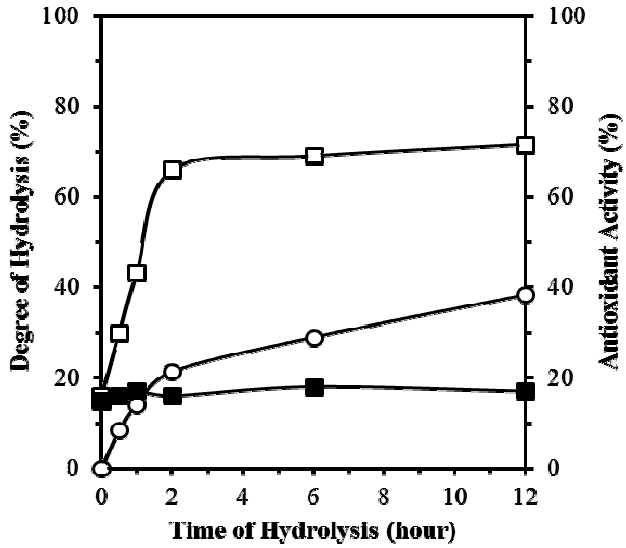

Fig. 4 The degree of hydrolysis and antioxidant activity during hydrolysis of melinjo seed protein isolated with sol-gel immobilized alcalase. The degree of hydrolysis (o), the antioxidant activity of non-hydrolysis protein (■), and antioxidant activity of hydrolysis (□)

The hydrolytic activity of the sol-gel immobilized alcalase to protein isolated was stronger in the from 1 to 2 h. This increase can be explained by the fact that the DH is a measure of the extent of hydrolytic degradation of a protein and is most widely used indicator for proteolytic processes. Furthermore, the hydrolytic activity of sol-gel immobilized alcalase weakened gradually with time when the reaction was longer than 2 h. This reduction of hydrolysis rate might be due to the limited amount of substrate, as the short chain peptides formed might not be further cleaved by sol-gel immobilized alcalase. The reduction of hydrolysis rate might also hypothetically be attributed to the decrease in peptide bonds available for hydrolysis, the decrease in enzyme activity, or product inhibition [26].

The antioxidant activity of melinjo seed protein isolated hydrolysates were determined using ABTS cation scavenging activity method, result presented in Fig. 4. The radical ABTS is reduced with concomitant conversion to a colorless product in the presence of antioxidants with hydrogen-donating or chain breaking properties and the ABTS radical is relatively stable and is readily reduced by antioxidants [27]. Antioxidant activity of melinjo seed protein hydrolysed using sol-gel immobilized alcalase on ABTS radical scavenging activity assay was slightly increased with hydrolysis time up to 2 hours and tended to be static thereafter. The Fig. 4 also shown that the antioxidant activity of melinjo seed protein hydrolysate (68%) was demonstrated 5 times higher than the non-hydrolyzed (13.6%) at 2 hours hydrolysis. The increased antioxidant activity through hydrolysis seems that this shown to process contributed to antioxidant activity by releasing active peptides from melinjo seed protein isolated.

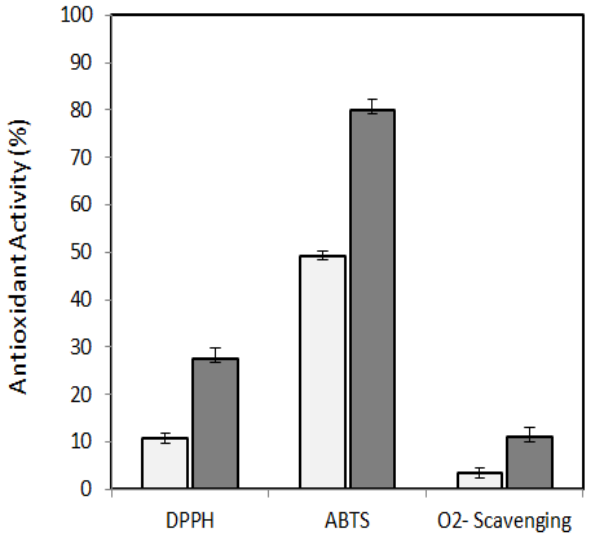

Fig. 5 Comparison of antioxidant capacities of non-hydrolysis (□) and hydrolysis (■) measured by DPPH, ABTS, and superoxide radical scavenging assays

In this study also compared the antioxidant activity of protein hydrolysate using the DPPH and ABTS scavenging methods. The effects of protein hydrolysate on the scavenging activities are shown in Fig. 5. The scavenging ability of the protein hydrolysed using sol-gel immobilized alcalase on DPPH and ABTS radical was higher than that of non-hydrolysis protein. The antioxidant activity for DPPH radical scavenging were 10.77% (non-hydrolyzed) and 27.67% (hydrolyzed) at concentration of 0.25 mg/ml and ABTS radical scavenging activity were 49.27% (non-hydrolyzed) and 80.09% (hydrolyzed) at concentration of 0.04 mg/ml. In addition, the difference between the radical scavenging capacities for DPPH and ABTS could be, in part, due to the difference of the radicals solubility and diffusivity in the reaction medium. Although DPPH scavenging is a widely used method for the assessment of free radical scavenging activity of natural products, it has a notable limitation when used to interpret the role of hydrophilic antioxidants because DPPH can be dissolved only in organic media (especially in alcoholic media), not in aqueous solutions [28], [29]. A further disadvantage is that DPPH serves both as oxidizing substrate and as the reaction indicator molecule; therefore, the assay would easily lead to the problem of spectral interferences [30]. In contrast, ABTS can be solubilized in aqueous as well as organic media; thus, radical scavenging activities of both hydrophilic and lipophilic compounds can be measured [29]. The results suggested that the ABTS method was more sensitive than the DPPH assay for the measurement of the antioxidant activity of water-soluble proteins in an aqueous solution. A similar observation was reported for fermented shrimp bio waste, which exhibited 40% scavenging activity for DPPH at a concentration of 1.0 mg/ml, compared with a 95% activity against ABTS even at a concentration of 0.5 mg/ml [31].

The strongest free radicals of superoxide anion radical in cellular oxidation reactions were produced various kinds of cell-damaging free radicals and oxidizing agents [32]. The activity of superoxide anion radical scavenging was monitored by determining the inhibition of pyrogallol autoxidation that is catalyzed by the superoxide radical. In Fig. 5 also showed, the superoxide anion radical scavenging activity of protein hydrolyzed using immobilized alcalase exhibited the higher activity, about 10.96% at a concentration of 0.06 mg/ml compared with non-hydrolyzed (3.39%). There were great variations between an intact molecule of protein non-hydrolyzed and hydrolyzed. Intact protein non-hydrolyzed exhibited only a weak superoxide anion scavenging activity than the protein hydrolyzed. Furthermore, inhibitors of protein non- and hydrolyzed using sol-gel immobilized alcalase on pyrogallol autooxidation showed different activities, indicating that hydrolysis of proteins with sol-gel immobilized alcalase will alter the antioxidant potential of the product. It might be due in part to the action of a sol-gel immobilized enzyme that cleaves peptide bonds at specific amino acids and produces a sequence of amino acids with higher antioxidant effects.

C. Effect of Protein Hydrolyzed Using Sol-Gel Immobilized Alcalase on Synthetic Peptides Profile

Figs. 6A and 6B show the chromatographic and electrophoretic profiles of the hydrolysates melinjo seed protein isolated during hydrolysis. The clearest change observed in hydrolysates with respect to the melinjo seed protein isolated is the reduction in the molecular weight of proteins. The chromatogram profile of the melinjo seed protein isolated on FPLC using gel filtration shows two major peaks around at 32.80 and 41.89 min. The distribution

of peptide size in protein hydrolysates using sol-gel immobilized alcalase was shown in Fig. 5A. When melinjo seed protein isolated was hydrolyzed, the peak at 32.80 disappeared after 2 h, and the resulting hydrolysate in FPLC profile was characterized by protein hydrolyzed or peptides that eluted in the range of 162.32 to 174.32 min. This protein or peptides profile was retained until the 2 h of the sol-gel immobilized alcalase hydrolysis at 50°C, it's suggesting that no more peptide bonds are hydrolyzed. It is approved by the hydrolysis curves showed that after 2 h, the degree of hydrolysis remained practically unchanged (Fig. 4).

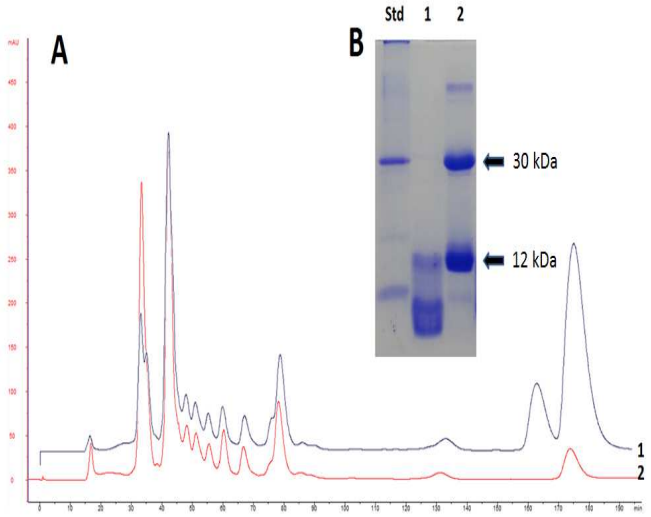

Fig. 6 Effect of hydrolysis of melinjo seed protein isolated using sol-gel immobilized alcalase on the FPLC (A) and SDS-PAGE (B) profile. Melinjo Seed protein hydrolyzed (line 1), and non-hydrolyzed (line 2)

The SDS-PAGE patterns of the sol-gel immobilized alcalase-hydrolyzed isolated melinjo seed protein are shown in Fig. 6B. Non-hydrolyzed melinjo seed protein was composed largely of two polypeptides (line 2). These protein components matched the molecular weight of those reported in the literature [14], that is 30 kDa for a band I and 12 kD for a band II. Hydrolysates generated with sol-gel immobilized alcalase were described by the observation of protein bands II that were resistant to the action of this sol-gel immobilized alcalase (Fig. 6B, line 1). Moreover, protein band I with a molecular weight around 30 kDa, gradually invisible during the incubation with sol-gel immobilized alcalase. This immobilized enzyme was hydrolyzed quickly the peptide bonds of the proteins and protein bands practically invisible after 2 h of incubation. Instead upon loading a high amount of protein only a band II with about 12 kDa of molecular weight that remained resistant to hydrolysis by sol-gel immobilized alcalase was visible (Fig. 6B).

D. Reusability of Sol-Gel Immobilized Alcalase

Reusability is another crucial feature in practical applications of biocatalysts. Leaching and enzyme inactivation is the most prominent drawbacks for large-scale use of sol-gel immobilized enzymes. To investigate reusability, sol-gel immobilized alcalase were reused in hydrolysis system at 50°C for 2 h. The sol-gel immobilized alcalase beads were washed with phosphate buffer and were added to a fresh hydrolysis subtract (E/S, 0.8%). The concentration of peptide was determined in the solution mixture after each hydrolysis. The results of reusability are shown in Fig. 7. In this figure show that the synthesis of the peptide was a sharp decline in the second hydrolysis process around 50%, furthermore in the third and four hydrolysis process did not show a significant change.

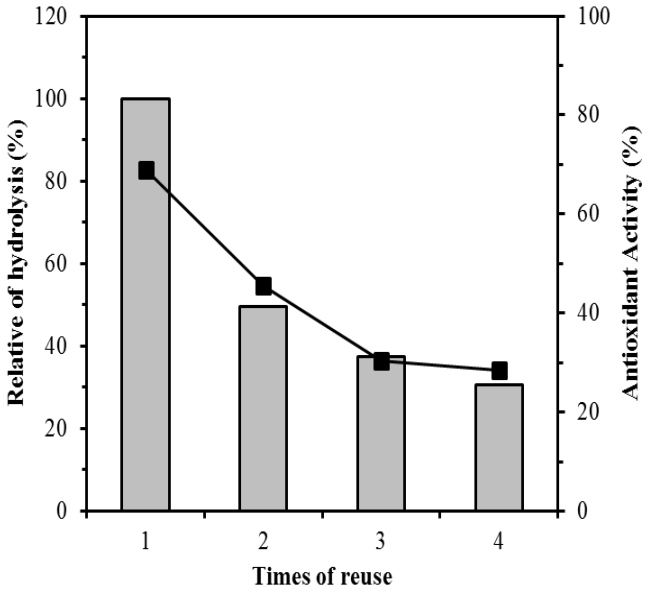

Fig. 7 Reusability of sol-gel immobilized alcalase and antioxidant activity of melinjo seed protein hydrolyzed. Relative to hydrolysis (■), and antioxidant activity (■)

The sol-gel immobilized enzymes may be reused four times with low losing their activity. This loss of proteolytic activity may be caused due to many reasons such as protein denaturation, and or lost enzyme during washing. Similar results have also been reported by Huu *et al*. [33].

IV. Conclusions

The obtained results showed that the hydrolytic activity of the sol-gel immobilized alcalase to melinjo seed protein weakened gradually with time after 2 h. The fast protein liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis profile indicated the sol-gel immobilized alcalase was produced of peptides with low molecular weight from melinjo seed protein isolated. The sol-gel immobilized alcalase retained the initial activity of more than 4 cycles of repeated use in a routine reaction at 50°C. The ABTS assay, the hydrolysate presented a more than 5 times greater activity than non-hydrolyzed melinjo seed protein isolated. It is confirming the potential use of sol-gel immobilized alcalase in synthesis antioxidant peptides from melinjo seed protein isolated.

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