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Purification and functional characterization of a novel tyrosinase (diphenolase) inhibitory peptides prepared from *Solunum tuberosum* peels protein via enzymatic hydrolysis

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ABSTRACT

The present study was designed to prepare a novel tyrosinase inhibitory peptides from *Solunum tuberosum* (potato) peels (by-product of potato processing). Isolated protein was hydrolyzed by immobilized lettuce protease at pH 10 to produce a potato hydrolysate (peptides) with the highest tyrosinase inhibitory activity than that of the parent protein. For maximum production of peptides, hydrolysis process including incubation temperature and protein concentration per reaction mixture was optimized. Separation of potato hydrolysate by ultrafiltration provides three fractions (F_1, F_2 and F_3) of which F_1 (< 10 KDa) showed the strongest tyrosinase inhibitory potency (12.07 + 0.0 μg KE/μg peptides). F_1 was further fractionation by three reverse phase-high performance liquid chromatography (RE-HPLC) to a finally single tyrosinase inhibitory peak. Collected peak (potato tyrosinase inhibitor) was further analyzed using HPSEXC chromatography confirmed its purity with molecular weight 485.98 kDa. It had superior tyrosinase (diphenolase) inhibitory potency compared to those of the original protein (990.44 μg KE/μg peptides). The K_m and V_{max} values of tyrosinase activity towards L-DOPA in presence of potato inhibitor decreased with increased the tyrosinase concentrations. The kinetic studies revealed that potato inhibitor defined as uncompetitive type. The results of this study suggested that potato peels are a good source of natural tyrosinase inhibitory peptides which exhibit therapeutic potential for curing or preventing some diseases.

1. Introduction

Tyrosinase (EC 1.14.18.1, polyphenol oxidase) is responsible for melanin biosynthesis and formation of the skin and hair colour. Melanin is produced by conversion of L-tyrosine to dihydroxyphenylalanine (L-DOPA) then oxidized to dopaquinone by tyrosinase (Ai et al., 2014). It is an enzyme widely distributed in nature. In plants, tyrosinase is responsible for browning certain fruits and vegetable during their handling and storage. In mammals, the enzyme is responsible for skin pigmentation and was linked to Parkinson disease and other neurodegenerative diseases (Asanuma et al., 2003).

In recent years, increased interest for the study of tyrosinase inhibitory activity arose because of the significant industrial and economic impact of the tyrosinase inhibitors. Tyrosinase inhibitors contributed to the treatment of the pigmentation of skin diseases and the prevention of enzymatic browning of vegetables. They had become increasingly important in medicinal and cosmetic products. Different inhibitory compounds derived from natural sources or synthetic pathways had been identified (Loizzo et al., 2012). It was reported that mushroom tyrosinase could be inhibited by benzoic acid and pyridine derivatives (Gheibi et al., 2015), flavonol (Kubo et al., 2000), Kojic acid (Saghaie et al., 2013), quercetin (Fan et al., 2017) and some vegetable fruits (Kamkaen et al., 2007).

Actually, only very few tyrosinase inhibitors were used in the industry due to food safety, off-flavours and economic considerations. Sulphite-containing compounds were traditionally used as anti-browning agent, but their application on fresh-cut food products has been banned by the U.S. Food and Drug Administration as in addition to causing off-flavours, they can cause allergies (FDA, U, 1986).

Bioactive peptides had enormous potential as ingredients of pharmaceuticals; for example, blood pressure-lowering capsules (Hartmann and Meisel, 2007). The importance of safety in the food industry directed researchers in a constant quest towards better inhibitors from natural sources as they are largely free of any harmful side effects

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(Loizzo et al., 2012). Many of the known bioactive peptides can exert more than one of therapeutic effects (multifunctional) (Meisel, 2004). Bioactive peptides were enzymatically prepared from food proteins such as: Sardinelle muscle proteins, corn protein, okra and shortfin scad with antioxidant, antibacterial and angiotensin I-converting enzyme inhibitory peptides (Zhou et al.,2012; al., 2012; Ishak and Sarbon, 2017; Jemil et al., 2017; Montilha et al., 2017).

Solanum tuberoum is the fourth largest yielding crop plant in the world, producing nearly 300 million metric tons of tubers per annum (Jones et al., 1986). The potato peels contain 1–4% protein (Akintomide and Antai, 2012). A large amount of potato peels are discarded during processing for hips by many industries. Enzymatic hydrolysis of potato peels protein and production of bioactive peptides is the aim of the present study. Production of tyrosinase inhibitory peptides is of great importance. Up till now, no research on tyrosinase inhibitory peptides from potato peels has been carried out. Therefore, the objective of this study was to prepare novel tyrosinase inhibitory peptides. Protein was extracted from potato peels and was hydrolyzed by previously prepared immobilized protease (Ali et al., 2016). Optimization of potato protein hydrolysis conditions with immobilized lettuce protease was established. Potato peels protein and its hydrolysate were tested for their tyrosinase inhibitor potency. Tyrosinase inhibitor peptides was purified by ultrafiltration followed by Re-HPLC chromatography and its kinetic were studied.

2. Materials and methods

2.1. Plant materials

Dry lettuce seeds (*Lactuca sativa*), family *Asteracea*, were bought from local markets (Cairo, Egypt). Fresh potato peels (*Solanum tuberosum*), family *Solanaceae*, were purchased from local markets (Cairo, Egypt). They were oven dried at 60 °C to inactivate the enzymes present.

2.2. Chemicals

L-Tyrosine, L-dihydroxyphenylalanine (DOPA), tyrosinase, Kojic, Lysozyme, carbonic anhydrase, tyrosine and tripeptides (Hip-His-Leucine) were obtained from Sigma-Aldrich Co. (St. Louis, Mo, USA). All chemicals used in this study were of analytical grade.

2.3. Preparation of potato protein

Proteins were extracted from dried potato peels, by two methods:

2.3.1. First method

Protein was prepared from dry plant wastes by alkaline extraction followed by isoelectric precipitation at pH 4.0 as described by Megias et al. (2009) with minor modifications. Dried plant wastes (30 g) were soaked with 0.1 N sodium hydroxide (250 mL) and were left overnight at 4 °C for non-protein components to precipitates. The precipitates were removed by centrifugation at 3500 g for 10 min followed by filtration on Whatman's filter paper N0 41. The clear solution resulted were acidified to pH 4 using 1 N hydrochloric acid and left overnight at 4 °C. The protein precipitates were collected by centrifugation at 3500 g for 10 min at 4 °C then dialyzed overnight against distilled water. The supernatant was stored at −4 °C for further use as potato protein.

2.3.2. Second method

Protein were extracted from dry plant wastes under neutral pH condition as described by Palupi et al. (2010) with minor modifications. Dried plant wastes (5 g) were soaked with 100 mL distilled water and left overnight at 4 °C. The homogenate was filtered by gauze followed by centrifugation at 3500 g for 10 min. The resulted supernatants were saturated with ammonium sulphate to a concentration of 70% and left overnight at 4 °C. The precipitates were collected by centrifugation at 3500 g for 10 min then dialyzed overnight against distilled water with dialysis bag molecular weight cut off 10–14 kDa. The supernatant was stored at −4 °C for further use as potato protein PII.

2.4. Protein determination

Protein concentration was determined by method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.5. Preparation of the immobilized lettuce protease

Immobilized protease was prepared according to the method of Ali et al. (2016). Crude protease was extracted from dry lettuce seeds with 0.1 M Tris-HCl buffer, pH 10.0. Free protease was precipitated from the prepared crude enzyme by ammonium sulphate at 0–60% saturation. Alginate-glutraldehyde beads were prepared by dropping 2% sodium alginate into 0.2 M CaCl2 solution with continuous stirring and stored at 4 °C for 24 h prior to use. Alginate beads were activated by being added to 6.5% glutraldehyde in 0.2 M Tris-buffer at 25 °C with stirring for 2 h. Two g of calcium alginate activated beads were mixed with 1.5 mL of the free enzyme followed by adding 1.5 mL distilled water to ensure full immersion of beads in enzyme solution. The loading process was performed for 1 h under continuous shaking at 9 °C.

2.6. Production of potato peptides

Production of peptides was accomplished through enzymatic hydrolysis of potato protein, using immobilized lettuce protease. Immobilized protease beads were added to 0.1 mL of potato protein followed by 2 mL 50 mM Tris-HCl buffer, pH 10. The tubes were incubated for 1 h in a water bath at 70 °C. The undigested protein was precipitated with trichloroacetic acids and the amount of protein split products (peptides and amino acids) was separated and stored at −10 °C to be further used.

2.7. Determination of the prepared peptides

The peptides solution was estimated with phenol reagent which gives a blue colour (Fayek and El-Sayed, 1980), using tyrosine as standard. To 5 mL of the supernatant and 5 mL 0.5 mL NaOH after mixed well; 0.5 mL Folin reagent was added drop wise while shaking. After standing for 10 min at room temperature, the absorbance was measured at 660 nm.

2.8. Optimization peptides production

For maximal production of peptides, effect of different protein concentrations on peptides production was studied at different temperatures (60 and 70 °C) and pH 10.0. Affinity of immobilized lettuce protease towards potato protein (apparent K_m) as substrate was investigated.

2.9. Separation of potato peptides by ultrafiltration

The potato protein hydrolysate was ultra filtrated according to the method of Tsomides (1993). The ultrafiltration was performed using ultrafiltration centrifugal units (Amikon, Millipore, USA), with molecular weight cut-off 100 and 10 kDa. The centrifugation was first performed for molecular weight separation of 100 kDa cut-off centrifugal units at 5000 g for 30 min. Two fractions were collected, F_3 with molecular weight above 100 kDa and F with molecular weight below

100 kDa. The last fraction F was ultrafiltered for molecular weight separation by 10 kDa cut-off centrifugal units at 7500 g for 15 min. Two fractions were collected F_2 with molecular weight above 10 kDa and below 100 kDa and F_1 with molecular weight below 10 kDa. Peptides concentration of each fraction was estimated. The three fractions collected F_3, F_2 and F_1 were stored at −4 °C to be further used.

2.10. Reversed phase-high performance liquid chromatography (RE-HPLC)

The peptides fractions from ultrafiltration with high tyrosinase inhibition (for diphenolase) were subject to further purification using semi-preparative HPLC according to Al-Azzouny et al. (2011). The purification was performed on HPLC system model Waters 1500 with binary Pump Model 1525, using reversed phase C_{18} Hypersil™ ODS 10 μm particle size 250 mm (Thermo, USA). The elution was performed using a primary wash run with flow rate 1 mL/min with a gradient from 100% solvent A (water) to 100% solvent B (60% methanol: 10% acetonitrile: 30% water with 0.1% TFA) from 0 to 5 min then isocratic with 100% solvent B for 25 min. The second separation run was performed at flow rate 1 mL/min with a gradient from 100% solvent B (60% methanol: 10% acetonitrile: 30% water with 0.1% TFA) to 100% solvent C (100% methanol) from 0 to 6 min then isocratic with 100% solvent C till end of run at 30 min. Concentration in each peak was estimated. The peaks collected were dried and stored at −4 °C to be further used.

2.11. High performance size exclusion chromatography (HPSEX) of potato tyrosinase inhibitor

Potato tyrosinase inhibitor was subjected to separation by High performance size exclusion chromatography (HPSEX) using the methods stated by Koza and Fountain (2013) with the following condition:, flow rate 1 mL/min; Agilent 1100 series (Waldborn,Germany), quaternary pump (G1311A), Degasser (G1322A), ThermostatedAutosampler (G1329A), variable wave length detector (G1314A); and column: Zorbax 300SB C18 column (Agilent Technologies, USA). Detection was carried out at wave lengths 280, 214 or 195 nm depending on samples content nature. The solvent system consisted of 25 mM sodium phosphate, pH 6.8%, and 30% ACN. The injection was carried out under ambient temperature. Standards peptides (Carbonic anhydrase Mwt 30,000 Da, lysozyme Mwt 14,307 Da, tripeptide (Hip-His-Leucine) Mwt 429.47 Da and tyrosine Mwt 181.19 Da) were assayed for standard molecular weight calibration curve on HPSEX.

2.12. Measurement of tyrosinase inhibitor potency

Potato protein, hydrolysate, ultrafiltration fractions (F_1, F_2 and F_3) and the purified collected peaks from HPLC were assayed for their tyrosinase inhibitory activity. Tyrosinase inhibitory potency was determined as described by Fawole et al. (2012) with some modifications. Kojic also was assayed as a positive control. Assays were carried out in a 96-well micro-titre plate and a Multiskan FC plate reader (Thermo scientific technologies, China) was used. In triplicate, each 50 μL of potato protein, hydrolysate fractions and HPLC peaks were mixed with 30 μL of tyrosinase in phosphate buffer, pH 6.5. After 5 min incubation, 0.1 mL of substrate (1 mM L-tyrosine or 6 mM L-DOPA) was added to the reaction mixtures and incubated further for 10–30 min at room temperature. A blank test was used as each sample that had all the components except L-tyrosine or L-DOPA. Results were compared with a control without sample. Absorbance values of the wells were then determined at 492 nm. The percentage of tyrosinase inhibition was calculated as follows:

$$\text{Inhibition}(\%) = [(\text{A control} - \text{A sample})/\text{A control}] \times 100$$

where A control is the absorbance of tyrosinase activity and A sample is the absorbance of the test reaction mixture containing tyrosinase inhibitors (protein or peptides). Kojic was used as positive control. Tyrosinase inhibitor potency was represented as μg kojic Equivalent per μg protein or peptides. All analyses were at least performed in triplicate.

2.13. UV spectrum analysis

Potato proteins, peptides hydrolysate (H), fractions F1 and the peaks I from semi- preparative HPLC were analyzed for the UV spectrum using UV spectrum Visible Spectrophotometer: Victoria, 3170, Australia.

2.14. Kinetics of potato tyrosinase inhibitor

In presence of potato tyrosinase inhibitor, the kinetic study (apparent K_m and V_{max}) of tyrosinase toward L-DOPA was determined according to the method of Kubo et al. (2003). Different concentrations of potato inhibitor were added to different concentrations of substrate (L-DOPA) from 40 to 100 μg per reaction mixture. Adequate amount of tyrosinase was added and the absorbance was determined at 492 nm after 10 min.

2.15. Statistical analysis

The results were expressed as a mean ± SD (standard deviation) for each analysis. Data was analyzed statistically using Student's *t*-test (2 tailed) by SPSS program.

3. Results

Potato peels (by-product of potato processing) was used for preparation of tyrosinase inhibitor peptides.

3.1. Peptides production

Protein was isolated by two methods I and II. Protein isolated by method II (339.6 ± 2.3 μg protein/g dry potato peels) was lower than that by method I (506.84 ± 17.3 μg protein/g dry potato peels). Both isolated proteins were subjected to hydrolysis by immobilized lettuce protease. Potato protein isolated by method II was hydrolyzed successfully by immobilized lettuce protease, yielded 2707.31 ± 3.3 μg peptides/g dry potato peels, while potato protein isolated by method I failed to be hydrolyzed by the immobilized lettuce protease.

For maximum production of peptides from potato protein, optimal enzymatic hydrolysis conditions including temperature and protein concentration were investigated. The results showed higher peptides production at 70 °C than that at 60 °C by 1.17 times (Table 1). A linear relationship between potato protein concentration and peptides production was found. It obeys Arrhenius plot low (Fig. 1). K_m and V_{max} values of the immobilized lettuce protease toward potato protein were 38.67 and 333.3 μg/R.M., respectively.

Potato protein and its hydrolysate were tested for both diphenolase and monophenolase inhibition activities. Protein hydrolysate showed

Table 1
Effect of incubation temperatures and substrate concentrations on peptides production from potato peels by immobilized lettuce protease.

Optimization of hydrolysis conditions		Peptides production (μg/R.M.)
Incubation temperatures	70 °C	25.66 ± 0.1
	60 °C	21.93 ± 01
Potato peels protein concentrations/R.M.	3.0 μg	25.67 ± 0.32
	6.0 μg	42.89 ± 3.21
	9.0 μg	67.33 ± 0.64
	12.0 μg	85.67 ± 0.96

R.M. means reaction mixture.

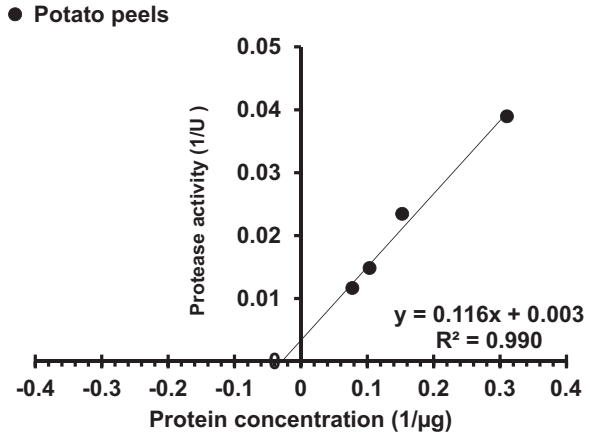

Fig. 1. Lineweaver-burk plot of immobilized lettuce protease toward potato protein. K_m and V_{max}values were 38.67 µg and 333.3 U, respectively.

Table 2a
Diphenolase and monophenolase inhibitor potency of potato peels protein and its hydrolysate.

Samples	Yield	Tyrosinase inhibitor potency	
		Diphenolase inhibitor potency	Monophenolase inhibitor potency
	(µg/g dry peels)	(µg KE/µg)	(µg KE/µg)
Protein	339.6 ± 2.3	0.28 ± 0.01	0.004 ± 0.0
Hydrolysate	2707.3 ± 31.3	0.39 ± 0.01 *	0.13 ± 0.0 *

- Yield was expressed as µg/g dry potato peels.
- Tyrosinase inhibitor potency was represented as µg kojic Equivalent per µg protein or peptides (µg KE/µg).
- Data are expressed as mean ± SD; n = 3.
- Mean assigned with * denotes a statistically significant difference at $p < 0.05$ when tyrosinase inhibition activity of hydrolysate was compared to that of protein.

higher diphenolase and monophenolenase inhibitor potency than their parent protein (Table 2a). Diphenolase inhibitor potency of hydrolysate was higher than that monophenolenase inhibitor potency by 3.0 times.

3.2. Ultrafiltration fractionation of the prepared potato hydrolysate

The prepared peptides were fractionated into F_3, F_2 and F_1 fractions. They were tested for both diphenolase and monophenolase inhibition activities. The diphenolase inhibitor potency was absolutely concentrated in fraction F_1 below 10,000 kDa and its value was 12.07 KE per µg peptides, while the monophenolase activity was localized in F_3 with low monophenolase inhibitor potency (0.53 KE per µg peptides) as illustrated in Table 2b. Fraction F_1 with high diphenolase inhibitor potency was chosen for further purification by reversed phase HPLC.

3.3. Purification of potato peptides F_1 by HPLC chromatography

Purification of potato F_1, using reversed phase HPLC, resulted in some fractions (Fig. 2a). Fractions were collected and assayed for tyrosinase inhibition activity. One fraction showed high diphenolase inhibition activity. It was selected for further fractionation by two successive RP-HPLC (second and third) (Fig. 2b,c). Absorbance was measured at 214 nm. Finally, only single homogenous peak had high diphenolase inhibitor potency (990.44 KE per µg peptides) (Table 3). Potato tyrosinase inhibitor (14.6 µg) was yielded from 1 g dry potato peels with tyrosinase inhibitor potency higher than that of its parent protein by 356 times.

Table 2b
Tyrosinase inhibitor potency of the ultrafiltration fractions.

	Yield	Diphenolase inhibitor potency		Monophenolase inhibitor potency	
	(%)	(µgKE/µg)	Relative times	(µgkE/µg)	Relative times
Hydrolysate	100	0.39 ± 0.0 *	1.0	0.13 ± 0.0 *	1.0
F_3	47.1	0.36 ± 0.0	0.92	0.53 ± 0.2 *	4.06
F_2	51.16	0.58 ± 0.0 *	1.5	0.0	–
F_1	1.84	12.07 ± 0.0 **	30.7	0.0	–

- Tyrosinase inhibitor potency was expressed as µg kojic Equivalent per µg protein or peptides (µgKE/µg).
- Relative potency was calculated as tyrosinase inhibitor potency of peptides per that of hydrolysate.
- Data are expressed as mean ± SD n = 3.
- Mean assigned with * denotes a statistically significant difference at $p < 0.05$ respect to tyrosinase inhibition activity of hydrolysate.
- Mean assigned with ** denotes a statistically significant difference at $p < 0.001$ respect to tyrosinase inhibition activity of hydrolysate.

3.4. Determination of potato tyrosinase inhibitor molecular weight

Potato inhibitor was subject to analysis on HPSEX chromatography to confirm its purity and determine its molecular weight. Potato inhibitor was separated at average retention time of 3.889 min (Fig. 3a). Standards peptides [Carbonic anhydrase Mwt 30,000 Da, lysozyme Mwt 14,307 Da, tripeptide (Hip-His-Leucine) Mwt 429.47 Da and tyrosine Mwt 181.19 Da and] were also subjected to separation by HPSEX. The retention times of Carbonic anhydrase, lysozyme, tripeptide (Hip-His-Leucine) and tyrosine were 3.232, 3.232, 3.687 and 4.009 min, respectively. A linear relation between log standard molecular weight and log retention time with a correlation coefficient (R^2) of 0.95 (Fig. 3b). The molecular weight of potato tyrosinase inhibitor was calculated to be 485.98 Da.

3.5. UV spectrum analysis

The UV spectrum analysis was performed for the potato protein (P) and its hydrolysates (H), ultrafiltration fractions F_1 and the collected HPLC purified peaks I. All the samples showed absorption below 300 nm and absorption spectra below 200 nm in the UV for the HPLC purified collected peaks (Fig. 4). Potato peels samples showed a single absorption peak at 268 for H and a single absorption peak at 272 nm for F_1 sample. The disappearance and appearance of new peaks may be due to enzymatic hydrolysis of the potato protein. The UV spectrum of potato peptides peaks I showed a unique absorption peak at 195 nm proving the sole presence of a peptide and the success of the purification method.

3.6. Kinetic of potato tyrosinase inhibitor

We studied the effect of potato tyrosinase inhibitor on the diphenolase activity of the tyrosinase enzyme. Different concentrations of potato inhibitor were used to determine inhibition pattern for tyrosinase diphenolase activity in presence of L-DOPA as tyrosinase substrate (Fig. 5a). The nature of inhibition was evaluated by the double reciprocal Lineweaver-Burk plot in the presence and absence of potato inhibitor. It showed a dose-dependent inhibitory effect, as potato inhibitor concentration increased, tyrosinase activity rapidly decreased. The decrease of the K_m, V_{max} and slopes were decreased with Increase of potato inhibitor concentrations (Fig. 5b,c,d). Potato tyrosinase inhibitor was a uncompetitive type.

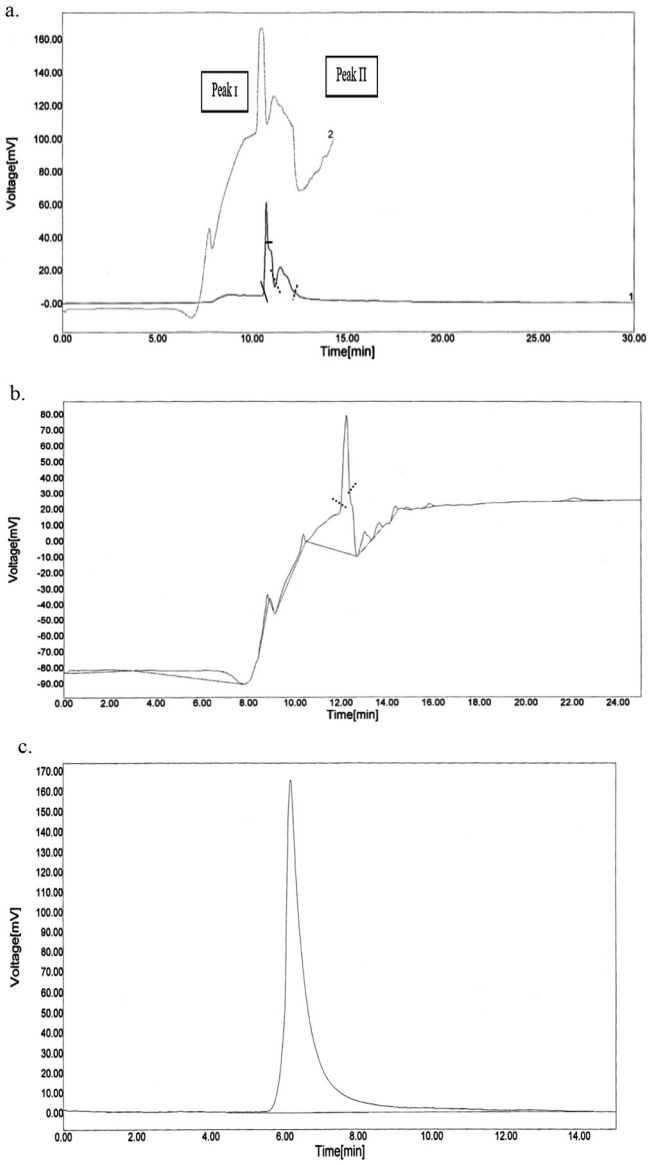

Fig. 2. a. First HPLC purification of F_1 : (1) is for UV acquisition at 280 nm and (2) is for 214 nm. The line - represents where peak I was collected and line …….. where peak II was collected. **b.** Second HPLC purification of peak I with UV acquisition at 214 nm. The line …. represents where the purified peak was collected. **c.** Third HPLC purification of recollected peak with UV acquisition at 214 nm.

Table 3
Tyrosinase (diphenolase) inhibitor potency of HPLC collected peaks.

Samples	Relative peptides concentration	Diphenolase inhibitor potency	
	(%)	(μg KE/μg)	(Times)
F_1	100	12.07	1.0
Peak I	29.45	990.44	8203
Peak II	25.89	0.0	0.0

Diphenolase inhibitor potency was expressed as μg kojic Equivalent per μg peptides (μg KE/μg).

4. Discussion

Tyrosinase inhibitory peptides were prepared from potato peels using immobilized lettuce protease. We chose potato peels (by-product of potato processing), for peptides production, as they are of low cost,

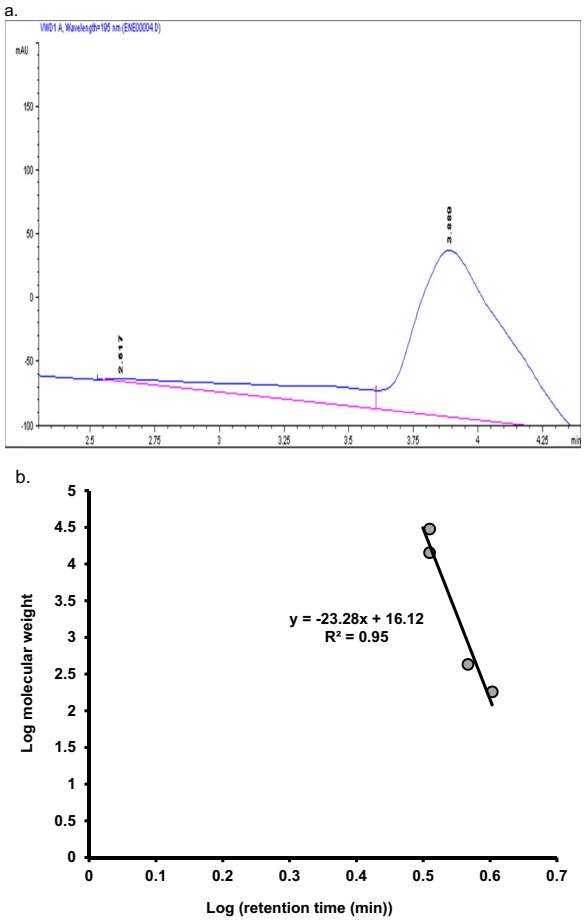

Fig. 3. a. HPSEX chromatography of pure potato tyrosinase inhibitor peptides. **b.** Calibration curve of the standard molecular weight protein elution on HPSEXC.

contain protein, available and with continuous regeneration. Increasing interest in the recycling of plant wastes was reflected by the number of reports for their re-utilization.

4.1. Peptides production

Potato protein isolated by method II was vulnerable to enzymatic hydrolysis by immobilized lettuce protease, while protein isolated by method I (isoelectric focusing) showed resistance to hydrolysis by the immobilized protease. This is may be due to the drawback of using high alkaline pH for protein extraction. Martínez-Maqueda et al. (2013) reported that the alkaline pH applied on protein may result in altering the protein quality to undesirable reactions. Examples of such reactions include, formation of toxic compounds, racemization of amino acids and reduction of digestibility. Method II avoided the harmful effects of irreversible protein denaturation as previously reported (Whitford, 2013).

Maximum production of peptides from potato protein, was obtained at 70 °C. This was similar to that reported by Adebiyi et al. (2008) from rice bran. Immobilized lettuce protease had satisfactory affinity toward potato protein (K_m and V_{max} values were 38.67 and 333.3 μg/R.M., respectively). The relative amount of peptides released was related to the enzyme substrate ratio and to the affinity of the enzyme towards potato protein. Enzyme/substrate ratio for high yield production of peptides was an important step to judge industrial application (Guerard et al., 2002).

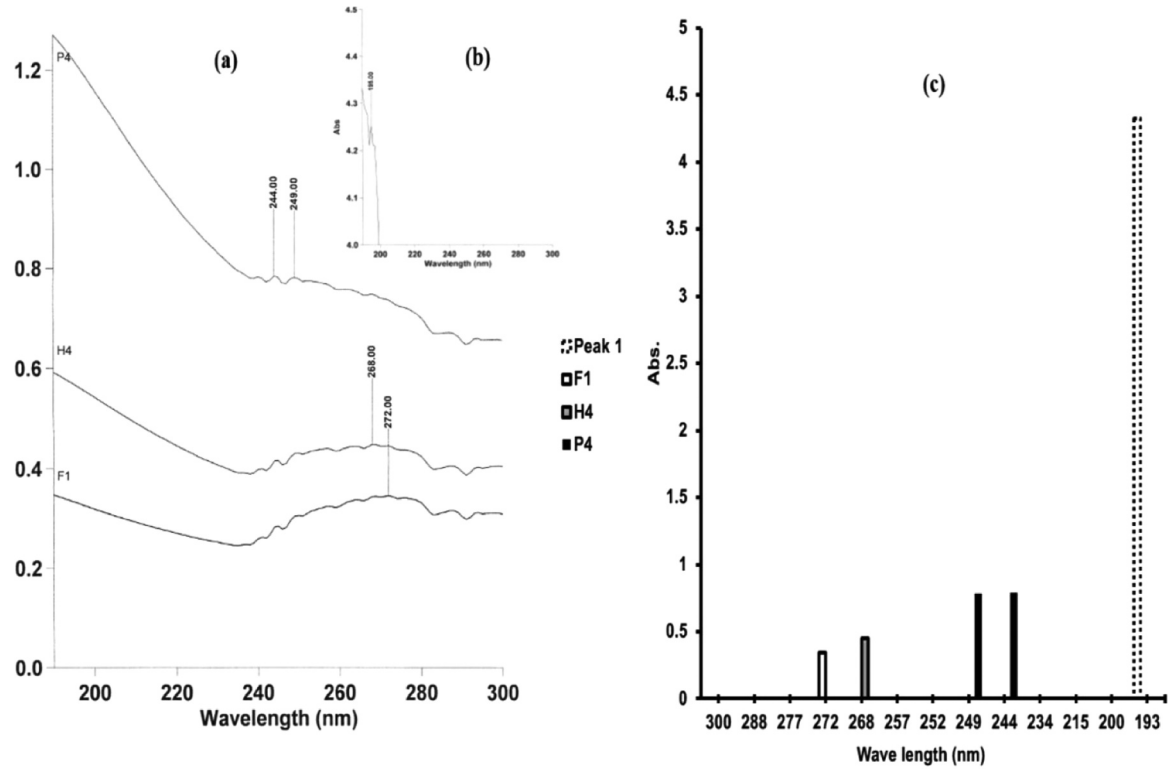

Fig. 4. UV spectrum analysis for potato peels samples. (a) UV spectrum of protein, hydrolysate and ultrafiltered fraction samples; (b) UV spectrum of HPLC collected peak and (c) schematic representation of the UV spectrum (a) and (b).

4.2. Purification of potato hydrolysate (tyrosinase inhibitory peptides)

Potato protein and its hydrolysate (peptides) could catalyze hydroxylation of L-tyrosine to L-DOPA (monophenolase activity) and also oxidation of L-DOPA to dopaquinone (diphenolase activity). They showed both diphenolase and monophenolenase inhibitor potency higher than their parent protein. Some isolated natural products were reported for mixed type inhibition of tyrosinase (diphenolase and

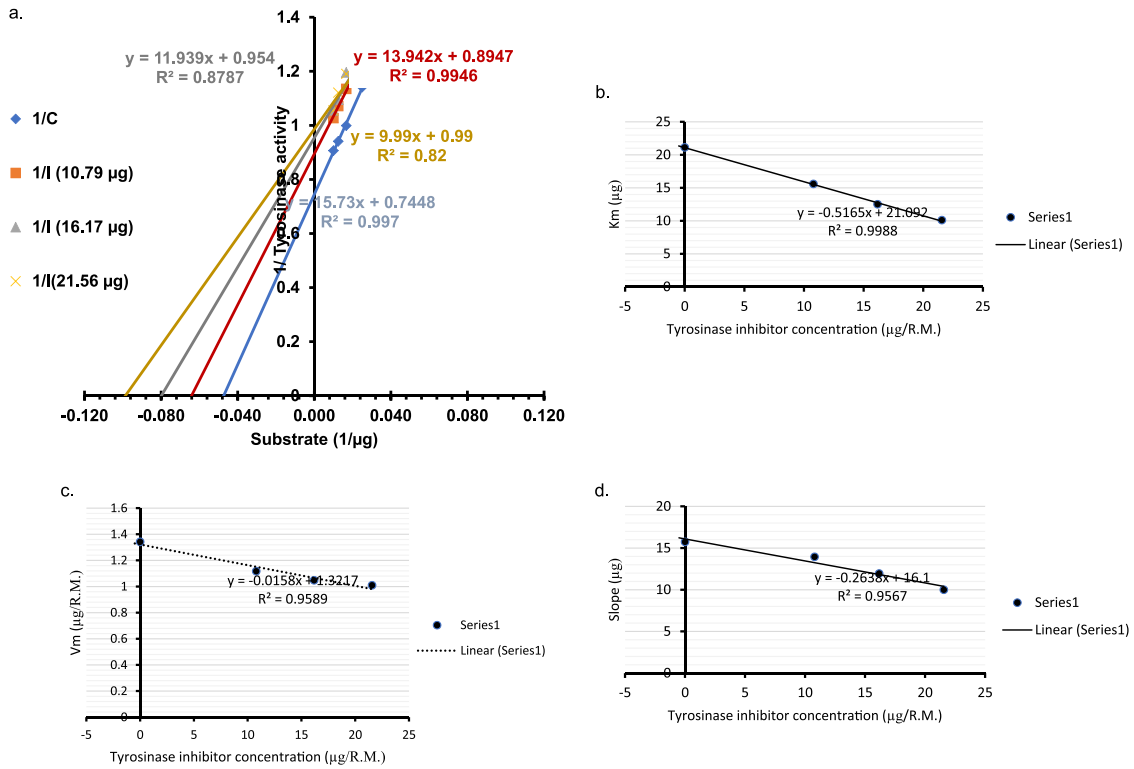

Fig. 5. a. Lineweaver –Burk plot for the kinetic pattern of tyrosinase diphenolase inhibitory peptides. **b.** The dependence of the Km on tyrosinase inhibitory concentration. **c.** The dependence of the Vmax on tyrosinase inhibitory concentration.**d.** The dependence of the slope on tyrosinase inhibitory concentration.

monophenolenase) as pedalitin isolated from *Rabdosiaserra*, mimosine isolated from pacific white *Litopenaeus vannamei and* polysaccharides from *Sargassum fusiforme* (Lin et al., 2011; Nirmal and Benjakul, 2011; Chen et al., 2016).

Ultrafiltration is one of the important purification steps to separate bioactive peptides based on their molecular weights. The prepared peptides were fractionated into F_3, F_2 and F_1 fractions. Fraction F_3 (above 100 kDa peptides) was expressed as a mild hydrolysis product. Fraction F_2 was for peptides with molecular weight ranging from 100 to 10 kDa, which could be expressed as a moderate hydrolysis product. Fraction F_1 was for peptides with molecular weight ranging below 10 kDa, which could be expressed as an extensive hydrolysis product. The differences in the fractions quantity may be related to the susceptible bonds within the amino acid sequences. They possibly depended on the catalytic mechanism of the immobilized enzyme, the preference of breaking point within protein sequence and also on the composition of protein isolates. The diphenolase inhibitor potency was absolutely concentrated in fraction F_1 below 10,000 kDa and while the monophenolase activity was localized in F_3 with low potency. Fraction F_1 with high diphenolase inhibitor potency was chosen for further purification by reversed phase HPLC.

Purification of potato F_1, using reversed phase HPLC, resulted in some fractions. One fraction showed high diphenolase inhibition activity. It was selected for further fractionation by two successive RP-HPLC. Absorbance was measured at 214 nm. Finally, only single homogenous peak had high diphenolase inhibitor potency was obtained. HPSEX chromatography elution profile confirmed potato tyrosinase inhibitory peptides purity with molecular weight of 485.98 Da. We obtained 14.6 mg with high tyrosinase inhibitor potency than both its parent protein and kojic acids from 1 kg dry waste.

4.3. Kinetic of potato tyrosinase inhibitor

We studied the effect of potato tyrosinase inhibitor on the diphenolase activity of the tyrosinase enzyme. It showed a dose-dependent inhibitory effect, as potato inhibitor concentration increased, tyrosinase activity rapidly decreased. This result was consistent with previously published work (Chen and Kubo, 2002). Potato tyrosinase inhibitor was an uncompetitive type. The decrease of the K_m, $V_{max}$and slopes were decreased with increase of potato inhibitor concentrations. Uncompetitive inhibitor, also known as anticompetitive inhibitor. It binds only to the complex formed between the enzyme substrate-complex (E-S complex). Isoflavene “glabrene” extracted from liquorice roots showed uncompetitive inhibition type (Nerya et al., 2003). The extracted from *Cinnamomum zeylanicum* fruit had uncompetitive inhibitory activity against the diphenolase activity of tyrosinase and reducting its catalytic capacity, while the extracted from *citrus grandis fruit had* potent competitive inhibitor type. It competed against L-DOPA to bind to the active site of tyrosinase (Aumeeruddy-Elalfi et al., 2016).

5. Conclusion

Potato proteins were extracted from potato peels (by-product of potato processing) by simple and inexpensive method. Immobilized lettuce protease was successfully used to hydrolyzed potato protein yield tyrosinase inhibitory hydrolysate (peptides) from potato protein. Hydrolysate was fractionated, based on their molecular weight, by ultrafiltration technique. Fraction F_1 (< 10 KDa) showed high tyrosine inhibitor potency and novelty in terms of tyrosinase diphenolase inhibitory peptides. F_1 was further purified to one active peak (potato tyrosinase inhibitor) by reversed phase HPLC. Its homogeneity was confirmed by UV-spectrum analysis and HPSEX with molecular weight 485.98 kDa. It had 990.44 KE per μg peptides. It is recommended to future evaluate its tyrosinase inhibitor activity in vivo for direct applications in industry.

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Conflict of interest

There are no conflicts of interest.

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