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Kinetic and Thermodynamic Properties of Immobilized Lettuce Protease and Its Role in Production of Novel Bile Binding and Tyrosinase Inhibitory Peptides from Some Plant Wastes

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Original Research Article

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Abstract: This study was designed to compare the properties, kinetics and stability of the immobilized lettuce protease with the free one. We also aimed to test the use of the immobilized lettuce protease for the production of novel bile binding and tyrosinase-inhibitory peptides from plant food processing wastes (cauliflower midrib and broad bean leaves). Immobilized lettuce protease was prepared previously from *Lactuca sativa* (lettuce) seeds. Free lettuce protease was immobilized on alginate-glutraldehyde beads by covalent bond at optimum conditions with high immobilization efficiency. In the present study, immobilized protease showed optimal activity 221.5 U/mg enzyme protein at pH 10 and 70°C, while the free one showed 22 U/mg enzyme protein at pH 7 and 60°C. K_m values of the immobilized and free protease toward azocasein were 1.143 and 1.442 mg, and V_{max} values were 142.9 and 24.6 U/mg enzyme protein, respectively. Immobilization of lettuce protease resulted in an increase of activation energy (E_a) for azocasein hydrolysis from 9.6 to 23 Kj/mol. Immobilization improved its turnover number K_{cat} (15.96 Kj/mol) and catalytic efficiency K_{cat}/K_m (13.96 Kj/mol) compared to that of the free one (3.09 and 2.143 Kj/mol, respectively). Deactivation energy (E_d) values were 38.76 and 55.41 for the free and immobilized protease, respectively, confirming the enzyme stability by immobilization. Free protease could be stored for 70 days at 4°C with loss of only 20% of its initial activity, while the immobilized one could be stored for 23 and 46 days with retention of 114 and 74.5% of its initial activity at 9°C, respectively. $CaCl_2$ increased the rigidity, stability and activity of the immobilized enzyme by 125%. Cauliflower peptides were enzymatically prepared from cauliflower protein. It had higher bile binding and tyrosinase inhibitor potency than the parent protein. Immobilized lettuce protease and cauliflower peptides could be applied in food and pharmaceutical industries.

Keywords: Immobilized protease, Kinetics, Thermodynamic, Peptides, Bile binding and Tyrosinase Inhibitor.

INTRODUCTION

Alkaline proteases alone constituted approximately 89% of the total protease market due to its wide applications in the food industry, pharmaceuticals and bioremediation [1]. They were also recognized as the most important tool in the hydrolysis of protein to its corresponding peptides and amino acids [2]. Proteases were purified and characterized from some plants [3, 4].

In industry, enzymes were used as tools to catalyze specific and unique operations to yield the production of important applications, although the important use of enzymes, high operation costs, instability, special storage requirements, sensitivity to microenvironment and difficulty for reusability had limited their industry-wide use. Enzyme immobilization allowed these application problems in industry to be overcome [2]. Immobilized enzymes were defined as enzymes physically confined or localized in a certain defined region of space with an increase in catalytic activities, and they could be used repeatedly and continuously. Product purity was usually improved, while effluent handling problems can be minimized by immobilization [2]. Proteases were classified on a number of bases: their pH optima (as acidic, neutral or alkaline); substrate specificity (collagenase, keratinase, elastase, etc.); or their homology to well-studied proteins such as trypsin and pepsin. They also were classified into four categories (Serine, cysteine, aspartic and metalloprotease) based on their mode of action [5].

Many different proteins had been isolated from wheat and soybean proteins, sesame seed, soybeans and nuts [6-8]. Bioactive peptides were fundamental constituents of many products or ingredients marketed as “Functional Foods” or “Nutraceuticals.” They had enormous potential as ingredients of pharmaceuticals;

for example, they could be used in blood pressure-lowering capsules [9]. Many bioactive peptides such as antioxidants, antihypertensive and antimicrobials were derived from food proteins by enzymatic hydrolysis that had been previously carried out [10, 11]. In vitro binding of bile acids and salts by lupine and corn proteins and their hydrolysates was carried out [12, 13]. Tyrosinase was known to be a key enzyme in melanin biosynthesis and was found in plants and mammalian cells. The monophenolase activity of tyrosinase catalyzes the hydroxylation of monophenols to o-diphenols, and diphenolase activity catalyzes the oxidation of o-diphenols to o-quinones. Then, o-quinones are transformed into melanin. Tyrosinase inhibitors, with Monophenolase or diphenolase activities or both, inhibit melanin synthesis and can be used to treat hyperpigmentation in humans [14] and prevention of browning reaction [15].

Food processing wastes from plants represent a disposal problem for the concerned companies regarding pollution or disposal cost. One of the methods of disposal is their re-use in the industry, either as a source for food additives of high quality [16] or as a possible source of functional ingredients [17]. Cauliflower is a vegetable with the highest waste index, contributing to 45–60% of the total weight of the vegetable [18, 19]. This generates a huge amount of solid waste, with urgency for disposal due to the foul odour produced upon decomposition. The white cauliflower by-product wastes (upper stem and leaf midribs) contained a high content of aromatic amino acids (Phenylalanine & tyrosine) in addition to lysine, leucine and valine [16]. Broad bean is considered to be a worldwide crop that is marketed after a simple industrial process of pod removal. The importance of broad bean leaves as a source of protein was reported by Friedman [20].

As a part of our continuous search for preparation of new potent immobilized protease and new natural bioactive peptides, lettuce seeds (*Lactuca sativa*) were chosen as the most suitable source for extraction of the protease enzyme. It was successfully immobilized on calcium alginate with the covalent binding technique, and its immobilization process was previously optimized [21]. In the present work, we investigated the physicochemical properties, kinetics, thermodynamics and enzyme stability of the immobilized lettuce protease and compared them with those of the free one. Storage stability of the immobilized protease were also studied. Its valuable application in the production of bioactive peptides from some plant food wastes was investigated. Therefore, cauliflower midribs and broad bean leaves were chosen as sources of protein. Their hydrolyzed proteins, by immobilized lettuce protease, were tested for their bile-binding capacity and tyrosinase inhibitor potency.

MATERIALS AND METHODS

Raw materials

Dry lettuce seeds (*Lactuca sativa*), family *Asteracea* were purchased from local markets. *Vicia faba* (broad bean) leaves, family *Leguminosaease* and *Brassica olercea* (cauliflower) midribs, family *Brassicaceae* were acquired fresh from agricultural field. They were washed and oven dried at 60°C to inactivate the enzymes present.

Reagents

L-Tyrosine, L-DOPA and tyrosinase were obtained from Sigma chemical company (USA). Colorimetric enzymatic kit for bile acids determination with standard bile acids reagent was purchased from BEN Biochemical Enterprise (Italy). Azocasein and other reagents used in this study were of the analytical grade.

Preparation of immobilized lettuce protease

Immobilized protease was prepared according to the method of Ali *et al*., [21]. Briefly, crude protease was extracted from dry lettuce seeds with 0.1M 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 0.2M $CaCl_2$ solution with continuous stirring and stored at 4°C for 24 h prior to use. Alginate beads were activated by add to 6.5% glutraldehyde in 0.2M Tris-buffer at 25°C with stirring for 2h. Two g of activated beads were loaded with 1.5 mL of the free enzyme followed by 1.5 mL distilled water to ensure full immersion of beads in enzyme solution. The loading process was performed for 60 min under continuous shaking at 9 °C. Resulted immobilized beads were assayed for their protease activity using azocasein as substrate.

Protein determination

The protein concentration was determined by method of Lowry *et al.,* [22], using bovine serum albumin as a standard.

Determination of protease activity

The activities of free and immobilized proteases were determined according to the method of Cabral *et al*., [23] using azocasein as a substrate. The standard reaction mixture contained 2 mL of 0.25% (w/v) azocasein in 0.05 M Tris-HCl buffer, pH10 and adequate amount of enzyme in a total final volume of 2 mL. The reaction mixture was incubated in a water bath at 40 °C for 60 min. The reaction was stopped by adding 0.5 mL 5% (w/v) trichloroacetic acid and the supernatant was separated by centrifuged at 3,500 rpm for 10 min. for the free protease and by decantation for the immobilized one. To 2 mL of the clear supernatant, 1mL of 1N sodium hydroxide solution was added. The

absorbance of the samples was measured by LKB Biochrom Nova spec II spectrophotometer at 450 nm wave length.

One unit of proteolytic activity was defined as the amount of enzyme that yielded an absorbance change of 0.1 at 450 nm per hour using azocasein as substrate. Specific activity of protease was expressed as units per milligram enzyme protein. The activity of protease was average values of three repeated measurements.

Physicochemical properties of the free and immobilized proteases

Optimum pH and temperature of the free and immobilized proteases were determined. The enzymatic hydrolysis conditions, namely pH of 0.05 M Tris-HCl buffer, from 6.0 to 11.0, incubation temperatures ranged from 40 to 80°C and incubation time from 20 to 120 min were investigated by varying one parameter at a time keeping other parameters unchanged. Relative protease activity was calculated as percentage to the maximum activity. Activation energy (E_a) was calculated using Arrhenius plot as described by Siddiqui *et al*. [24]. The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factory by which the rate increase due to a raised in the temperature by 10°C [25].

$$Q_{10} = \text{antilog}_e (E_a \times 10/RT \times T).$$

Protease activities were estimated at different enzyme concentrations at ranging from 0.02 to 0.15 mg per reaction mixture for free enzyme and range from 0.004 to 0.04 mg per reaction mixture for immobilized enzyme using azocasein as substrate. Effect of different $CaCl_2$ concentrations on activity and stability of the protease enzymes was carried out.

Catalytic constant and thermodynamic parameters for azocasein

The effect of different substrate concentrations on protease activities was estimated by incubating different substrate concentrations ranging from 2.0 to 8.0 mg per reaction mixture with the free and immobilized protease. Catalytic constant; Michaelis-menten constant (K_m), maximum reaction velocity (V_{max}), turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) values of the free and the immobilized enzymes toward azocasein were determined according to Lineweaver and Burk [26]. Thermodynamic parameters; enthalpy of activation (ΔH^*), free energy activation (ΔG^*), entropy of activation (ΔS^*), free energy of substrate binding ($\Delta G^*_{E\text{-}S}$) and free energy for transition state formation ($\Delta G^*_{E\text{-}T}$) for azocasein hydrolysis were calculated using the Eyring's absolute rate equation derived from the transition state theory [27].

$$K_{cat} = (K_b T / h) \times e\,(-\Delta H^*/ RT) \times e\,(\Delta S^*/ R)$$

Where,

K_b Boltzmann's constant (R/N) = 1.38×10^{-23} jK^{-1}

T Absolute temperature (T)

H Planck's constant = 6.626×10^{-34} Js

N Avogadro's number = 6.02×10^{-23} mol^{-1}

R molar Gas constant = 8.314 JK^{-1} mol^{-1}

$\Delta H^* = E_a - RT$....................................eq. (1)

$\Delta G^* = - RT \ln (K_{cat}\, h / K_b \times T)$................eq. (2)

$\Delta S^* = (\Delta H^* - \Delta G^*) / T$........................eq. (3)

$\Delta G^*_{E\text{-}S} = - RT \ln K_a$

$\Delta G^*_{E\text{-}T} = - RT \ln (K_{cat} / K_m)$

Thermostability and thermodynamic properties of the protease enzymes

Small aliquots of the prepared enzymes (free and immobilized enzymes) were pre-heated at different temperatures ranged from 40 to 80°C for varying time intervals 60 to 120 min. In case of immobilized protease, 0.2 M $CaCl_2$ solution was added to increase the resistance and stability of the beads to heat. The remaining enzymes activities were then estimated using the standard assay conditions. Log residual activities were plotted against time at different temperatures used for inactivation. The deactivation rate constant K_d and the heat inactivation E_d for irreversible thermal inactivation were calculated from Arrhenius plot of ln K_d against 1/T in Kelvin. ΔH^*_d, ΔG^*_d, ΔS^*_d were calculated by applying equation 1, 2 and 3, using E_d instead of E_a and K_d instead of K_{cat}. Half-life (t) and D-value were calculated using the relation: Half-life (t) = ln2 / K_d and D-value = ln / K_d [28].

Storage stability of free and immobilized protease

This experiment was conducted to determine the storage stability of the free and immobilized proteases in distilled water at - 4°C for 70 days.

Immobilized beads without pre-treatment

The immobilized protease beads was stored in distilled water, 0.2 M $CaCl_2$ and 0.1M Tris-HCl buffer, pH 10 separately at 9°C during storage for about 24 h.

Immobilized beads with pre-treatment

For optimizing of immobilized beads storage at 9°C and preventing microbial contamination, the immobilized beads were preheated before storage at 40°C for 50 min. Then, stored in 0.2 M $CaCl_2$ for 32 and 64 days at 9°C.

The residual activities of the free and the immobilized enzymes after storage were expressed as a percentage of their residual activities compared to the corresponding initial activity.

Preparation of isolated protein from bean broad and cauliflower midrib leaves

Protein was extracted from the cauliflower midribs and broad bean leaves (non-edible part) by alkaline extraction followed by isoelectric precipitation at pH 4.0 as described by Megias *et al*., [29] with minor modifications. Dried samples were extracted separately with 0.1 N sodium hydroxide and were left overnight at 4°C for non-protein components to precipitate. The precipitates were removed by centrifugation at 3,500 g for 10 min followed by filtration on Whatman's filter paper No 41. The clear solution resulted was acidified to pH 4 using 1 N hydrochloric acid and left overnight at 4°C. The protein precipitates were collected by centrifugation at 3,500 g for 10 min at 4°C and used as cauliflower midribs and broad bean proteins.

Peptides production from the isolated proteins by free and immobilized lettuce protease

Adequate amount of free and of immobilized enzyme were added separately to 0.1 mL of protein isolate followed by 2 mL 0.05 M Tris-HCl buffer, pH 10. The tubes were incubated for 60 min in water bath at 60° and 70 °C for free and immobilized, respectively. The reaction was stopped by centrifugation or decantation and the hydrolysate (peptides) solution was collected and stored at -10 °C to be used further. It was determined as µg tyrosine [30].

Determination of Bile binding capacity of the isolated proteins and its hydrolysate

In vitro, bile binding capacity of broad bean and cauliflower midrib proteins and their corresponding peptides were determined according to the method of Kahlon and Woodruff [31]. Cholestyramine resin also was used as potent bile binding standard. It had bile acid binding capacity and considered as cholesterol-lowering drug. Aliquots amount of samples in 0.1 M sodium phosphate buffer at pH 7 was added to 0.9 mL of 1.5 mM standard bile acids solution in the same buffer as the samples. The reaction mixtures were incubated at 37 °C for 60 min. The amounts of bile acids unbound to the samples were analyzed spectrophotometrically at 405 nm using Ben bile-acids analysis kit. The intensity of the colour at the reaction conditions was directly proportional to the bile acids concentration in the sample. The amount of bile bound by samples was calculated and expressed as µg cholestyramine equivalent resin per µg protein or peptides (µg EC/µg protein or peptides). All analyses were at least performed in triplicate.

Determination of Tyrosinase inhibitor potency of the isolated protein and its hydrolysate

Tyrosinase inhibitor potency was determined as described by Fawole *et al*. [32] with some modifications. Kojic acid was used as standard tyrosinase inhibitor. 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 protein isolates and hydrolysate 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 to 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: Inhibition (%) = (A control–A sample) / A control x 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). The amount of tyrosinase inhibition by samples was calculated and expressed as µg Kojic equivalent per µg protein or peptides. All analyses were at least performed in triplicate.

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.

RESUTS AND DISCUSSION

Physicochemical properties of the free and immobilized proteases

Proteases are a unique group of enzymes that have wide applications in pharmaceutical industries. The plant proteases had been reported by Tripathi *et al*., [4] to be stable under extreme experimental conditions. Alginates are widely used in food industry and pharmaceutical applications due to their GRAS (Generally Recognized as Safe) status [33]. The physicochemical properties of the free and immobilized lettuce protease were investigated. Optimum pH was determined by stepwise increasing the pH values of the 0.05 M Tri-HCl buffer from 6 to 11 in the reaction mixture (Fig-1). The free protease showed 2 peaks for activity, one major at neutral pH 7.0 and the second minor at an alkaline pH range of 9 to 11. Optimum pH of the immobilized protease was major in the alkaline range at pH 11 and the second one at pH 6.0. Similarly, a shift in optimum pH by 0.7 units toward the alkaline side was previously reported for the alkaline protease papain, which was immobilized on an enteric polymer [34]. A shift by 1 unit toward the alkaline side was reported by Silva *et al*., [35] for esterase covalently immobilized on Eudragit S-100, respectively.

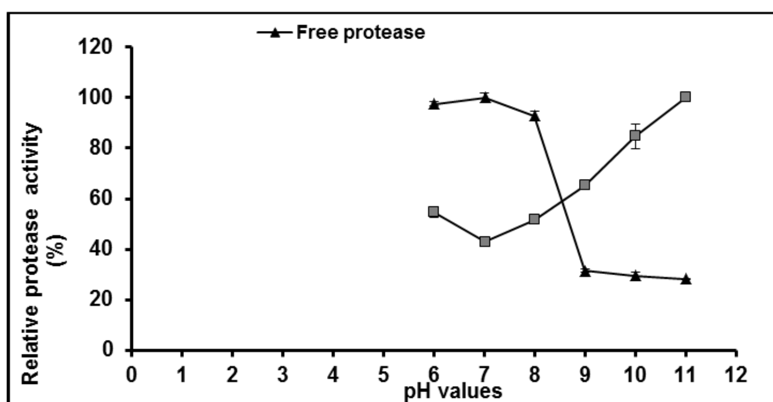

Fig-1: Effect of different pH's values on the activity of the free and immobilized proteases. Relative protease activity was calculated as percentage to the maximum activity. Results were mean ± SD of triplicate data.

The effect of different incubation temperatures (40–80°C) on the free and immobilized protease activities was investigated. The optimum reaction temperature was 60°C for the free protease and 70°C for the immobilized one (Fig-2A). Upon heating the immobilized enzyme at a wide range of temperature, it covalently attached to cross linked glutraldehyde and was stable [36]. The increase in optimum temperature can be explained by alterations of the physical and chemical properties of the enzyme upon immobilization, which led to an improvement in protease stability. The increase in the optimum temperature for the immobilized protease was previously reported by Ortega *et al.*, [37].They reported a shift in optimum temperature from 50 to 60°C. The same was reported for alkaline protease from *Conidiobolus macro* sporus covalently immobilized on polyamide, in which a shift of optimum temperature from 40 to 50°C was observed [38]. The variations in the optimum temperature value also may depend on the types of support materials and immobilization methods.

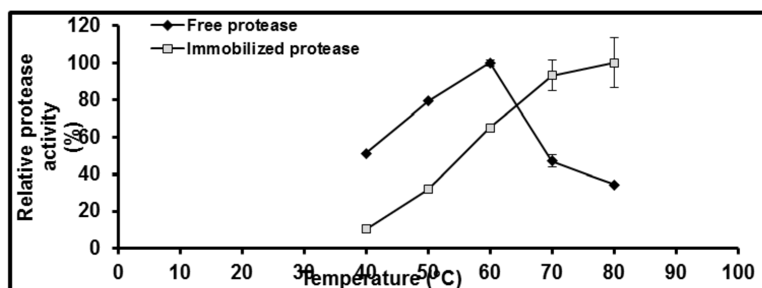

Fig-2A: Effect of temperatures on the free and immobilized lettuce proteases activity. Relative protease activity was calculated as percentage to the maximum activity. Results were mean ± SD of triplicate data.

An Arrhenius plot was done, and the activation energy E_a was calculated. Free enzyme showed diphasic (an increase up to 60°C with E_a 9.69 Kj / mol, whereas over 60°C, it showed a decrease) with E_a -17.41 Kj / mol (Fig-2B), while the immobilized enzyme showed a linear relationship with E_a 18.46 Kj / mol (Fig-2C). Increase E_a of the protease after immobilization confirmed stabilization by immobilization [24]. The temperature Q_{10} values for both enzymes was 1.0. It indicated that the catalytic reaction is temperature dependent [39].

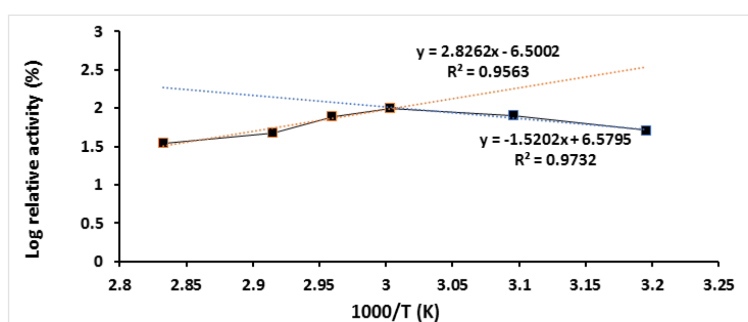

Fig-2B: Arrhenius plots to calculate the activation energy of the free lettuce protease. Using azocasein as substrate. P-value = 0.022 with R Square = 0.9563 and P-value = 0.10 with R Square = 0.9732.

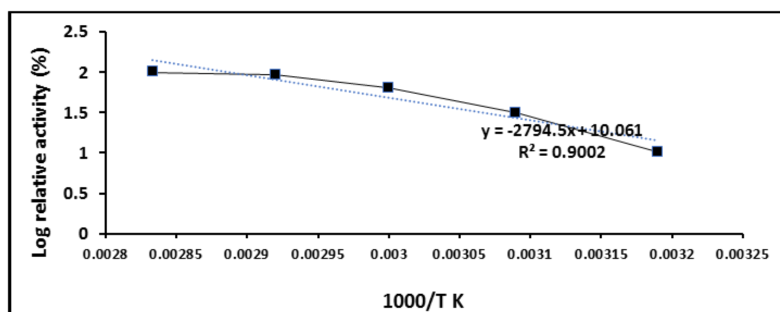

Fig-2C: Arrhenius plots to calculate the activation energy of the immobilized lettuce protease. Using azocasein as substrate. P-value = 0.013 with R Square = 0.90015

The activity of the free protease increased proportionally in a linear relationship with the increase in incubation time up to 120 min, while for the immobilized protease, the activity increased in a linear relationship up to 60 min. Thus, the immobilized protease showed a more rapid reaction rate than the free one, and it reached saturation in a shorter time. This might indicate the easy access of the substrate toward the immobilized protease and the successful immobilization technique.

The activity of the free protease increased proportionally in a linear relationship with the increase in enzyme concentration from 0.02 up to 0.15 mg protein/ reaction mixture (Fig-3A), while the activity of the immobilized protease increased in linear relation with the increase in the immobilized protease concentration from 0.004 up to 0.021 mg (Fig-3B). Thus, immobilized protease activity also reached maximum enzyme at a lower concentration than the free one, which confirms the success and efficiency of immobilization. However, Jarzebski *et al.*, [40] reported the lower reaction rate of immobilized protease toward casein. They suggested the inaccessibility of the bulk substrate toward the immobilized protease.

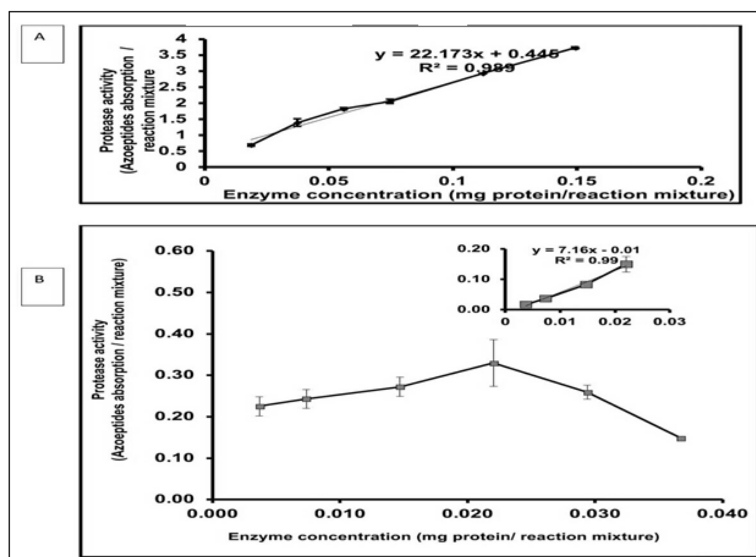

Fig-3: Effect of different enzyme concentrations on the activity of the free P-value = 0.00048 R Square = 0.989 (A) and immobilized P-value = 0.0131 and R Square = 0.99. (B) Relative protease activity was calculated as percentage to the maximum activity. Results were mean ± SD of triplicate data.

Effect of calcium chlorides on activity and stability of the lettuce protease enzymes

The effect of calcium chloride on protease activity was more effective (increased activity) on the immobilized enzyme than in the free one (Table-1). This could be explained by the effect of calcium on the active site of alkaline protease and on the charge on the alginate beads surface, and both factors could contribute to altered conformation of the immobilized protease that affected its activity.

The addition of 0.2M calcium chloride in the reaction mixture increased the rigidity and strength of the alginates beads, and it enabled the alginate beads to withstand harsh temperatures or alkaline experimental conditions. Fig-4 showed that beads heated to 70°C without 0.2 M $CaCl_2$ were soft and lost their shapes.

Table-1: Effect of different calcium chloride concentrations on the specific activity of the free and immobilized proteases.

Calcium chloride concentration (µmol/RM)	Relative free protease specific activity(Time)	Relative immobilized specific activity (Time)
0.0	1.0	1.0
10	0.72	1.10
20	0.87	1.12
30	0.77	1.10
40	0.83	1.30
50	0.88	1.37
100	0.87	1.24
150	0.88	0.92
200	0.58	0.90

Relative protease specific activity was calculated as the specific activity of enzyme at different calcium chlorides concentrations / specific activity of enzyme in absent of calcium chlorides.

Without calcium chloride With calcium chloride

Fig-4: Effect of calcium chloride solution on beads shape and stability. Beads were heated at 70°C with and without 0.2 M calcium chloride solution for 60 min. (using a digital camera).

Kinetic and thermodynamic parameters for azocasein hydrolysis

A direct relationship was observed between the concentration of azocasein and the activity of free and immobilized protease up to 5 mg / reaction mixture. The K_m and V_{max} values were 1.44 and 24.57 U / mg for the free protease (Fig-5A) and 1.143 and 142.86 for the immobilized one, respectively (Fig-5B). K_m is related to the affinity of the enzyme for the substrate and is converted to product. Small K_m means tight binding, and large K_m means weak binding. Small K_m achieves maximal catalytic efficiency V_{max} at low-substrate concentration. Determination of kinetic constant K_m, V_{max}, K_{cat} and K_{cat} / K_m suggested a higher affinity of immobilized protease for azocasein than the free one (Table-2). Turnover number K_{cat} (catalytic constant) described the fast convert enzyme substrate complex to enzyme and product per second. Lower and negative values of ΔS* and $\Delta G_{E\text{-}T}$ of the immobilized protease indicated the stabilization and efficient transition state of the enzyme–substrate complex. Low $\Delta G_{E\text{-}T}$ led to a disappearance of substrate and an increase of product formation and more enzyme activity. The low value $\Delta G_{E\text{-}S}$ indicates that the conversion of the enzyme substrate complex into product is more spontaneous. The higher K_{cat} / K_m of the immobilized protease is due to the stability of the transition state. Catalytic efficiency (K_{cat} /K_m) explains the binding of the enzyme and substrate and represents the velocity to convert substrate to product.

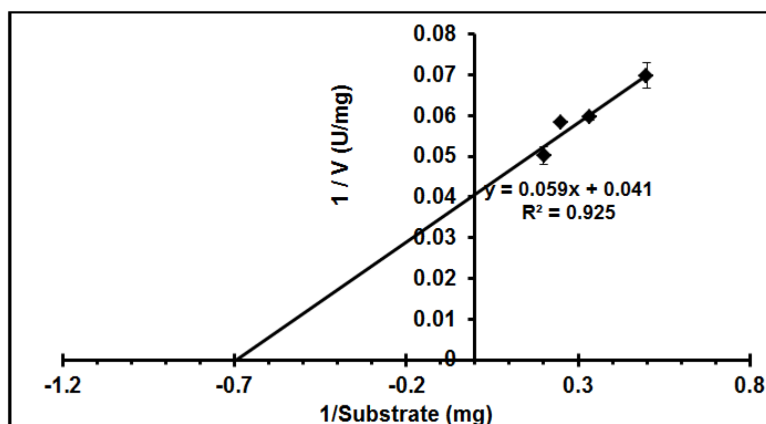

Fig-5A: Lineweaver-Burk plot for free protease using azocasein as substrate

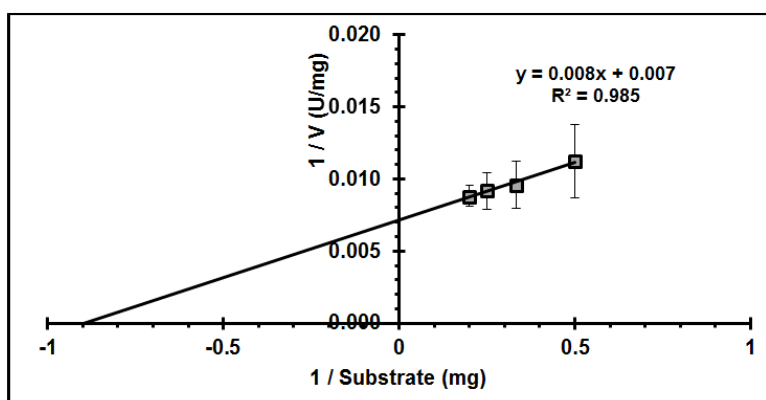

Fig-5B: Lineweaver-Burk plot for immobilized protease using azocasein as substrate

Table-2: Kinetic and thermodynamic parameters for substrate catalysis by the free and immobilized lettuce proteases at optimum conditions (pH and temperature) for each enzyme.

Parameters	Definition	Free lettuce protease	Immobilized lettuce protease
E_a(Kj/mol)	Activation energy	9.69	18.46
V_{max} (U/mg)	Maximum reaction velocity	24.57	142.86
K_m (mg/ml)	Michaelis-menten constant	1.442	1.143
K_{cat}(1/s)	Turnover number	3.09	15.96
K_{cat}/K_m	Catalytic efficiency	2.143	13.963
Δ H* (Kj/mol)	Enthalpy of activation	6.92	15.5
Δ G* (Kj/mo)	Free energy activation	78.75	78.82
ΔS*(J/mol)1/K	Entropy of activation	-215.7	-234.9
ΔG^*_{E-T} (Kj/mol)	Free energy for transition state formation	-2.11	-7.74
ΔG^*_{E-S} (Kj/mol)	Free energy of substrate binding	1.015	0.392
V_{max}/K_m	Specificity constant	17.04	124.99
Q_{10}	Temperature quotient	1.0	1.0

The immobilization of protease enzyme on alginate gel beads showed a marked decrease in K_m and a greater increase in V_{max}, K_{cat} and K_{cat} / K_m than the free one. Decreased K_m indicated the high enzyme affinity toward the substrate. Literatures varied in the reported K_m value results; Ferreira *et al.* [41] and Ibrahim *et al.,* [42] reported a decrease in K_m from 25.8 µM and 0.109 mM for the free one to 20.6 µM and 0.07 mM for the immobilized alkaline protease, respectively, while Ortego *et al.* [37] reported the increase in K_m from 2.4 mg/mL for the free to 6.4 mg/mL for the immobilized, respectively. An increase in V_{max}, K_{cat} and K_{cat} / K_m was reported previously for protease immobilized by being entrapped in calcium alginate beads [43] and alkaline protease immobilized with alginate [42]. The increase of V_{max}, K_{cat} and K_{cat} / K_m may be due to a more-efficient conformation of immobilized protease on alginate beads in respect to the free one [42]. Thus, high

affinity toward substrate and catalytic activity of lettuce protease immobilized with alginate beads indicates the efficiency and effectiveness of the applied support and immobilization approach.

Thermostability of the free and immobilized protease

In the absence of substrate, preheat treatment of the free and immobilized protease with different temperatures (40 to 80°C) for 60 to 120 min and in the presence of 0.2 M $CaCl_2$ were investigated. The free protease was stable at 40°C when heated up to 90 min without any loss of activity (Fig-6A). The free protease lost 47% of the original activity after pre-heating at 60°C for 120 min, while it lost 77% of the original activity after heating at 80°C for 120 min. The immobilized protease showed high heat stability when heated up to 80°C for 60 min without loss of activity (Fig-6B). When he immobilized beads were heated at 80°C for 120 min, it lost 63% of the original activity. Thus, the immobilized protease showed greater thermal stability than the free one. This is similar to the results reported by Ferreira *et al.,* [41] and Ortega *et al*., [37] for covalently immobilized alkaline protease. Improved thermal stability could be explained by the conformational limitation and fixation of the immobilized beads on the surface that protects the enzyme from conformational change caused by heat denaturation, as suggested by Trevan [44].

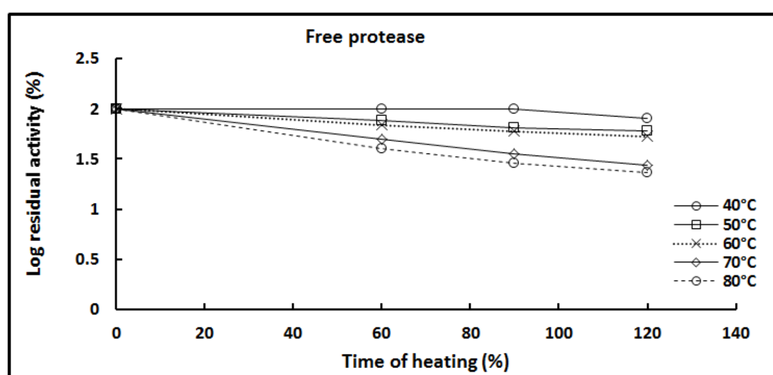

Fig-6A: Log residual protease activity as a function of time.

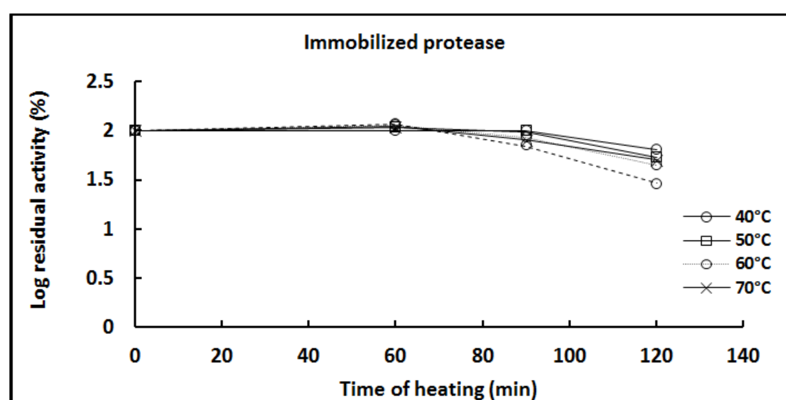

Fig-6B: Log residual immobilized protease activity as a function of time.

Results in Table-3 and Fig-6C showed that large E_d of immobilized protease indicated that more energy is required for deactivation, and this means that it was more thermostable (i.e., the higher the energy needed for denaturing) [37]. The half -life ($t_{1/2}$) is the time required for the enzyme activity to decrease to 50 % of its original activity. The higher its value, the higher the enzyme's thermostability. The immobilized enzyme needed more time to be denatured in comparison to the free one. The large ΔH value of the immobilized protease should be associated with high enzyme thermostability, as reported by Saqib *et al.,* [45]. This confirmed the results of covalent binding enhancing thermal stability, which represented valuable tools for a number of biotechnological processes. $CaCl_2$ solution was added to increase the rigidity and stability of the beads at high temperatures. The addition of 0.2 M calcium chloride solution to the enzyme solution increased its stability.

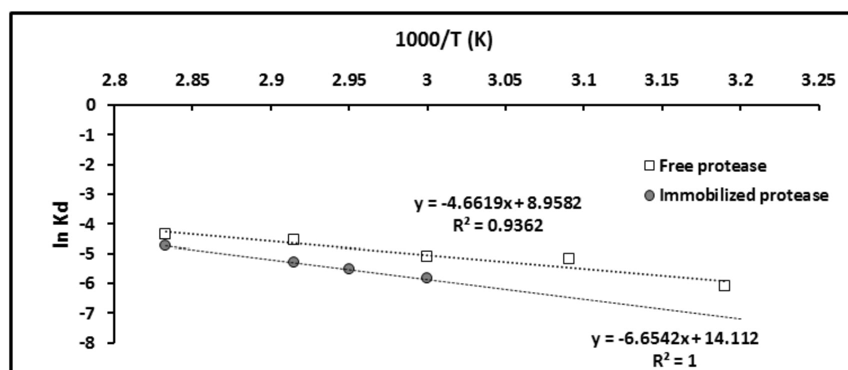

Fig-6C: Ln K_d against reciprocal of temperature.

E_d value for the free lettuce protease was 38.76 Kj/mol and E_d value for the immobilized lettuce protease was 55.41 Kj/mol. P-value of free protease = 0.0069 and R Square = 0.9362 and P-value of immobilized protease = 0.024 and R Square = 1.0.

Table-3: Kinetic and thermodynamic parameters for the irreversible thermal deactivation of the free and immobilized lettuce proteases.

Temp (°C)	Temp (K)	K_d (1/h)	T½ (1/h)	T_D (1/h)	ΔH* (Kj/mol)	ΔG* (Kj/mol)	ΔS* (J/mol)
Free lettuce protease							
40	313	0.0023	Stable up to 90 min		36.158	46.488	-33.0
50	323	0.0055	2.09	6.95	36.075	45.622	-29.56
60	333	0.006	1.93	6.41	35.991	46.813	-32.5
70	343	0.0108	1.07	3.55	35.908	46.530	-30.97
80	353	0.0128	0.9	2.98	35.825	47.373	-32.71
Immobilization lettuce protease							
40	313	0.0029	Stable up to 90 min		52.808	45.805	22.37
50	323	0.0041	Stable up to 90 min		52.725	46.395	19.6
60	333	0.0058	2.01	6.67	52.641	46.921	17.18
70	343	0.0051	2.28	7.58	52.558	48.695	11.26
80	353	0.0087	132	4.39	52.475	48.511	11.23

*Deactivation energy value for the free protease = 38.76 Kj/mol and for the immobilized protease = 55.41 Kj/mol.

Storage stability of free and immobilized protease

Free and immobilized proteases were stored in the distilled water at -4°C for 70 days. The residual activity of free protease reached 80 % of its initial one, while the immobilized beads shrank and completely lost their activities (Table-4).

When immobilized, protease was stored in different solvents of distilled water, 0.1 M Tris-HCl buffer, pH 10 and 0.2 M $CaCl_2$ solution, at 9°C for one day. Beads stored in 0.2 M $CaCl_2$ solvents showed the highest protease activity (125%). Calcium chloride was used to decrease autolysis of the immobilized enzyme. Similarly, Rocha *et al.,* [46] reported a lower loss in the activity of alkaline protease (trypsin immobilized on spent grains) upon storage for 40 days, from 90% loss in Tris-HCl buffer, pH 8 without calcium to only 40 % loss when calcium was added.

Heating the immobilized beads to 40°C for 50 min in a 0.2 M $CaCl_2$ solution yielded stable beads that could be stored for 32 and 46 days at 9°C with 114 and 74% recovery of their initial activity, respectively. This increase in enzyme stability and activity may be explained by the presence of some protein impurities could non-covalently couple to the surface of beads; they may have caused steric hindrance for the substrate binding. So, upon storage in calcium chloride solution after heat treatment, the excess weakly bound impure proteins (non-covalent bond) were released, leading to increased enzyme activity. Upon heating of the immobilized enzyme at a wide range of temperatures, it covalently attached to cross-linked glutraldehyde and was stable [36]. However, impure proteins attached by non-covalent bond or physically adsorbed are easily inactivated due to conformational change or denaturation [47]. The same was noticed when applying pre-heat of beads after enzyme loading at 40°C for 50 min; this resulted in only a 7.2% loss in activity after storage for 46 days, which may be explained by the presence of weakly non-covalently bound excess or impure proteins that interact with better protease activity [46]. Immobilized glucoamylase was stored for 30, 60 and 90 days with 100, 90 and 75 % retention of its activity, respectively [48].

Table-4: Storage stability of the free and immobilized proteases in different solvents (with or without preheated before storage).

Sl. No.	Storage in different solvent	storage time (day)	Immobilized protease activity (U/bead)	Residual protease activity (%)
1	Free protease in distilled water at 4°C.	70	----	80± 0.0
2	Immobilized protease in distilled water at 4 °C.	1	Immobilized beads was shrinking and completely lost their activity.	0.0
3	Immobilized protease in distilled water at 9 °C.	0.0	2.487 ± 0.05	100
4	Immobilized protease in distilled water at 9°C.	1	1.371 ± 0.4	55.1
5	Immobilized protease in 0.1 M Tris-HCl buffer pH 10 at 9°C	1	1.7889 ± 0.16	71.93
6	Immobilized protease in 0.2M $CaCl_2$	1	3.101 ± 0.3	125
7	Immobilized protease in 0.2M $CaCl_2$ at 9°C after preheated at 40°C for 50 min.	0.0	3.101 ± 0.54	100
8	Immobilized protease in 0.2M $CaCl_2$ at 9°C after preheated at 40°C for 50 min.	32	3.562 ± 0.54	114
9	Immobilized protease in 0.2M $CaCl_2$ at 9°C after preheated at 40°C for 50 min.	46	2.309 ± 0.13	74.5

Beads were infected after storage case number 4,5&6 for 32 days. Residual activity was expressed as a percentage of the initial activity before storage. Results were mean ± SD of triplicate data.

Bioactive protein and its hydrolysate

Proteases were used as a tool for liberation of bioactive peptides from cauliflower midrib and broad bean leaves protein. Cauliflower midrib and broad bean leaves were chosen based on their low cost and availability in large amounts and food plant wastes. They contain appreciable amounts of proteins [16, 49]. The production of peptides was accomplished through enzymatic hydrolysis of the isolated proteins by free and immobilized lettuce protease. Differences in the relative amount of peptides released was related to the affinity of the enzyme toward the different proteins (Table 5A). The immobilized enzyme successfully hydrolyzed both isolated proteins, while the free one failed to hydrolyze the cauliflower midrib protein. Production of peptides from bovine casein, soy protein, whey, okora and sardinelle muscle were reported previously by Vaze *et al*., [2], Jemil *et al*., [11] and Rocha *et al*., [46].

Table-5A: Protein isolates and its hydrolysate (peptides) production from cauliflower midrib and broad bean leaves by free and immobilized lettuce protease.

Waste name	Protein isolates	Peptides production (by free protease)	Peptides production (by immobilized protease)
	(µg/g dry waste)	(µg /g dry waste)	(µg /g dry waste)
Cauliflower midribs	14 ± 0.002	nd	1.0 ± 0.0
Broad bean leaves	447 ± 0.011	2120 ± 0.0	570 ± 0.0

Proteins and peptides yields were calculated asµg peptides/mg dry waste. nd: not detected. Values were given as the mean ± SD (n=3).

Bile binding potency of the prepared proteins and peptides

Bile acids binders help reduce blood cholesterol levels. In vitro, a test for bile acid binding was adopted in this work. We used a human bile acids mixture in the test. This was recommended by Kongo-Dia-Moukola *et al*., [13], who described it as a suitable natural compound for bile acid binding experiments, as it simulates the condition in the human body. This gave our results reliability and offered a final better choice for future in-vivo testing. Cholestyramine was used as a positive control, and the results were expressed relative to the cholestyramine equivalent (µg CE) as µg EC per µg protein or peptides. The bile-binding capacity of the broad bean was almost equal to peptides, while cauliflower hydrolysate had a higher bile binding capacity than its parent protein by 126 times (Table-5B). This was in agreement with the findings previously reported by Yoshie-Stark and Wasche [8] and Corrons *et al.,* [50] for lupin, buck wheat and lentil. The difference between different protein and hydrolysates bioactivities could thus be related to the amino acid contents. Kahlon and Woodruff [31] reported that the variability in the bile acid binding between various treatments (soy protein, pinto beans, black beans and wheat gluten) may relate to differences in anionic,

cationic, physical and chemical structure. Hydrophobic amino acids had been reported by Kongo-Dia-Moukola *et al.,* [13] to strongly bind bile acids via hydrophobic reactions with lipids (bile acids, cholesterol, other lipids and other sterols).

Table-5B: Bile binding capacity of isolated proteins and their hydrolysates (peptides) produce by free and immobilized protease.

Waste name	Protein isolates	Peptides production (by free protease)	Peptides production (by immobilized protease)
	(µg CE/µg)	(µg CE/µg)	(µg CE/ µg)
Cauliflower midribs	1.52 ± 0.0	------	192 ± 0.02
Broad bean leaves	14.7 ± 0.0	1.72 ± 0.0	11 ± 0.01

Cholestyramine resin was tested for bile binding capacity (used as standard). Bile binding capacity was expressed as µg cholestyramine resin equivalent per µg protein or peptides. All analyses were at least performed in triplicate. Values were given as the mean ± SD (n=3).

Tyrosinase inhibition of the prepared proteins and peptides

At present, tyrosinase inhibitors such as kojic acid were used as skin-whitening components in cosmetic industry [51]. Safety considerations increased interest toward the use of proteins and peptides as natural tyrosinase inhibitors. From our results, the hydrolysate of cauliflower protein showed higher tyrosinase inhibitor potency (diphenolase) than its parent proteins by 296 times (Table-5C). It inhibited hydrolysis of L-DOPA by tyrosinase, which is important for formation melanin synthesis (o-diphenolase to o-quinones).The difference in the inhibition potency of the prepared hydrolysate could be related to the type of the parent protein and consequently to the peptides amino acids sequence, as reported previously by Schurink *et al.,* [52]. Tyrosinase inhibition of protein isolated from broad bean was almost similar to its hydrolysate. Both hydrolysates showed no tyrosinase (monophenolase) inhibitor potency. The inhibition of tyrosinase was reported for synthetic inhibitors [51, 52]. Natural isolated isoflavene “glabrene” from licorice roots and phenolic compound from Sundance medicinal plants showed tyrosinase inhibition [53, 54], Thus, peptides generated from cauliflower midrib leaves are good nutritional supplements as bile-binding and tyrosinase-inhibitory peptides.

Table-5C: Tyrosinase inhibitor potency of isolated proteins and their hydrolysates (peptides) produce by free and immobilized protease.

Waste name	Protein isolates	Tyrosinase inhibitor potency of peptides	
		(Prepared by free protease)	(Prepared by immobilized protease)
	(µg KE/µg)	(µg KE/µg)	(µg KE/µg)
Cauliflower midribs	0.028 ± 0.0	-----	8.41 ±0.02
Broad bean leaves	0.049 ± 0.0	0.041 ± 0.0	0.56 ±0.02

Kojic acid was tested for tyrosinase inhibitor potency(used as standard). Tyrosinase inhibitor potency was expressed as µg kojic equivalent per µg protein or peptides. All analyses were at least performed in triplicate. Values were given as the mean ± SD (n=3).

CONCLUSION

Immobilized lettuce protease showed better properties (i.e., thermostable and storage stability) than the free one. The immobilized protease showed high specific activity (221.5 U/mg) at pH 10.0 and 70°C, while the free one showed specific activity 22.0 U/mg protein at pH 7.0 and 60°C. Kinetically and thermodynamically, results confirmed that immobilized protease exhibited better catalytic properties and higher thermal stability than the free one. It can be stored in 0.2 M calcium chlorides at normal refrigerator temperature (at 9°C) up to 32 days without autolysis or infection. Proteins were extracted from cauliflower midribs and broad bean leaves by a simple and inexpensive method, and we produced peptides by using immobilized lettuce protease. Cauliflower midrib peptides had higher bile-binding capacity and tyrosinase-inhibitor potency than its protein by 126 and 296 times, respectively. Thus, the prepared immobilized protease may be suitable for pharmaceutical and food industry applications. It also could be used to produce novel potent bile-binding and tyrosinase-inhibitor peptides.

CONFLIT OF INTEREST

There is no conflict of interest among the authors in carrying out the work.

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