Article published online: 2024-05-23

THIEME OPEN ACCESS

Original Article

Modification of Bioactive Properties in Food Protein Hydrolysates by Alcalase and Trypsin

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J Health Allied Sci[NU]

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Abstract

Background Protein hydrolysates are the fragments of proteins that form during the hydrolysis with promised bioactive properties. Enzymatic hydrolysis produces bioactive peptides with specific amino acid profile. Among all the properties, angiotensin-I converting enzyme (ACE-I) inhibitory activity of the protein hydrolysates attained maximum attention to combat life-threatening disease like hypertension.

Methodology In the present study 10 protein-rich food sources (> 20% protein content on dry basis) were used to prepare protein hydrolysates using alcalase and trypsin to investigate the effect of enzymes on ACE-I inhibitory and antioxidant activity.

Results Among all the sources, the highest degree of hydrolysis was observed in sardine (62.89%) followed by soybean (61.24%) when hydrolyzed by alcalase. Kidney pea exhibited highest ACE-I inhibitory activity with IC_{50} value of 0.517 and 0.521 when hydrolyzed using both trypsin and alcalase, respectively. All the sources selected showed nonsignificant changes in ACE-I inhibitory activity between alcalase and trypsin ($p < 0.05$) except Bengal gram and chicken. Antioxidant activity was higher in alcalase hydrolyzed egg yolk (43.08%).

Conclusion Among the sources, all animal proteins hydrolyzed using alcalase exhibited significantly higher ($p < 0.05$) antioxidant activity than trypsin. However, protein hydrolysates prepared from sardine using alcalase would yield bioactive peptides with higher ACE-I inhibitory and antioxidant activity. Therefore, use of alcalase as a substitution for trypsin would be economical for large-scale production. Henceforth, food protein hydrolyzed by alcalase can be used as a functional ingredient for the development of functional or nutraceutical foods to combat lifestyle diseases.

Keywords
- protein hydrolysates
- ACE-I inhibitory activity
- proteolytic enzymes
- food proteins

Introduction

Proteins are large biomolecules comprised of chains of amino acids. It is also an important macronutrient in a diet which is required for overall growth and development of the body. Bioactive peptides are the small fraction of a protein which are composed of 2 to 20 amino acids which are encrypted within the protein and released during hydrolysis of protein.[1] Protein hydrolysates are the biostimulants that can be defined as the fragments of protein which contain enormous quantity of bioactive peptides which are made up of single amino acids to oligo- and polypeptides.[2] Hydrolysis of proteins can be achieved by digestion of proteins using different proteolytic enzymes obtained from natural digestive enzymes such as pepsin, trypsin, and chymotrypsin or from microorganisms such as

DOI https://doi.org/10.1055/s-0044-1782643.
ISSN 2582-4287.

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

alcalase, neutrase, and flavourzyme.[3] Food protein hydrolysates extracted using different proteolytic enzymes are proven to have antimicrobial, antioxidant, anti-inflammatory, anticancer, and antihypertensive properties.[4–8] Therefore, use of such protein hydrolysates from food sources can serve as a natural remedy for the treatment or controlling of lifestyle diseases.

Hypertension is a coronary heart disease which is characterized by the constant raise in blood pressure. Angiotensin-I converting enzyme (ACE-I) plays a vital role in the regulation of blood pressure in the human body. ACE-I regulates regular blood pressure by converting the inactive decapeptide angiotensin-I into an active form angiotensin-II which is a potent vasoconstrictor.[9] The excessive production of ACE-I can lead to raise in blood pressure and can lead to hypertension. Hypertension is a coronary heart disorder condition in which the systolic blood pressure is above 180 mm Hg. Henceforth, inhibition of ACE-I is a preliminary action to control high blood pressure. Synthetic ACE-I inhibitors such as captopril, enalapril, lisinopril, and such others are available in the market. Extensive consumption of synthetic ACE-I inhibitors has proven to cause side effects such as cough, dizziness, headache, taste disturbance, visual disorders, dry mouth, insomnia, tiredness, and depression.[1] Therefore, use of natural compound with minimum or no side effects and high ACE-I inhibitory activity can be an alternative to synthetic drugs. The side effects posed by synthetic ACE-I inhibitors have made people look toward natural remedies as a cure for the disease. Thus, protein hydrolysates extracted by enzymatic hydrolysis can be a suitable alternative for the synthetic drugs as the specificity of enzymes will allow peptide production and simplify the identification of bioactive peptides.[10] Trypsin is a classical proteolytic enzyme chiefly used in protein hydrolysis, but the enzyme may not be ideal for the hydrolysis of all types of protein due to its substrate specificity, presence of inhibitors, high cost, and it is also reported to have allergic and can carry animal-borne diseases. Researchers and industry professionals are looking for an alternative protease with different bioactive properties based on the specific requirements and their applications, the use of which does not comply the requirement for large-scale production of protein hydrolysates.[11] Therefore, there is indeed an alternative for trypsin for industrial production of protein hydrolysates without compromising the bioactive properties of the hydrolysates. The study by Yathisha et al[8] reported that the protein hydrolysates prepared using alcalase exhibited higher bioactive properties than that of the other proteolytic enzymes such as papain and flavourzyme. Therefore, in the present study, two different enzymes, alcalase and trypsin, were used to prepare hydrolysates from protein-rich food sources (plant and animal) available locally. Hydrolysis of plant and animal sources with a proteolytic enzyme can produce protein hydrolysates but the specific amino acid which is responsible for demonstrating the ACE-I inhibitory activity may vary.[1] On the other hand, hydrolysis of plant and animal proteins has its own pros and cons in terms of breakdown of protein during hydrolysis (such as protease inhibitors, lower degree of hydrolysis (DH), and interference of fat), digestibility, and bioavailability of the protein hydrolysates.

Therefore, the purpose of the study was to investigate the ability of these enzymes to produce protein hydrolysates with better antihypertensive activity. The outcome of the study can facilitate the economic way of larger scale production of protein hydrolysates for developing nutraceutical products to combat hypertension.

Materials and Methods

Materials

The protein-rich plant sources (> 20% on wet basis) such as green gram (*Vigna radiata*), Bengal gram (*Cicer arietinum*), kidney pea (*Phaseolus vulgaris*), soybean (*Glycine max*), and cashew (*Anacardium occidentale*) and animal sources (> 50% on dry basis) such as chicken (*Gallus g. domesticus*), mackerel (*Scomber scombrus*), and sardine (*Sardina pilchardus*) and chicken egg were purchased from local market of Mangaluru, Karnataka, India.

Reagents

The chemicals required for the study including 1,1-diphenyl-2 picrylhydrazyl (DPPH), ACE-I from rabbit lung (1 U/mL), and the ACE synthetic substrate hippuryl-l-histidyl l-leucine (HHL) and proteolytic enzymes such as alcalase (*Bacillus licheniformis*) and trypsin (bovine pancreas) were purchased from Sigma-Aldrich. All the other chemicals such as sodium hydroxide, sodium chloride, L-ascorbic acid, and other chemicals used in the study were of analytical grade.

Preparation of Protein Hydrolysates

The protein hydrolysates from selected sources were prepared as per the method described by Yathisha et al,[8] with slight modifications. The selected food sources (50 g) in 500 mL distilled water were ground finely using a mixer grinder. Animal sources were ground using meat mincer (Panasonic MK-MG1500) to get a homogenate mixture. The obtained homogenate was heated at 90°C for 20 minutes to inactivate endogenous enzymes followed by rapid cooling.

The homogenized samples were adjusted to pH 8.5 using 0.1 N NaOH and kept in water bath at 55°C followed by the addition of 1% (w/v) alcalase. Similarly, the pH was adjusted to 8.0 and kept in water bath at 55°C with 1% (w/w) of trypsin. Hydrolysis was performed for 4 hours for maximum production of protein hydrolysates. The pH was maintained at 8.5 and 8.0 throughout the hydrolysis using 0.1 N NaOH. After the completion of hydrolysis time, the temperature was raised to 90°C for 20 minutes to terminate the hydrolysis by inactivating the enzymes and cooled to 4°C.

Further, both hydrolysates (alcalase and trypsin) were centrifuged at 8,000 × *g* for 30 minutes at 4°C using cold centrifuge (Eppendorf Germany 5810R). The supernatant collected was lyophilized (ZIRBUS Sublimator VaCo 2) at a pressure of 0.200 mbar. The lyophilized protein hydrolysates were stored at –20°C and reconstituted in distilled water prior to every analysis.

Determination of Degree of Hydrolysis

DH refers to the extent of free peptides released into the solution by cleaving the peptide bond. The DH was measured by estimating the soluble nitrogen using trichloroacetic acid (TCA) method as described by Yathisha et al.[8] Briefly, up to 950 µL of protein hydrolysates (5 mg/mL [50 µl of 10% TCA]) was added and incubated at room temperature for 15 minutes followed by centrifugation at 5,000 revolutions per minute for 10 minutes. The supernatant was estimated for the protein content and the DH was calculated using the formula:

$$DH\% = (10\%\ TCA\ soluble\ protein\ /\ Total\ protein) \times 100$$

Proximate Analysis

Moisture, protein, fat, and ash content of protein hydrolysates from both alcalase and trypsin were estimated using the methods of AOAC.[12] Moisture was estimated gravimetrically using hot air oven (Rotek Instruments-RHO-24HNS-1813) by heating at 105°C. Protein was estimated using total nitrogen by Kjeldahl digestion and distillation unit (Borosil India 100KID000006). Fat was estimated using Soxhlet apparatus (Rotek Instruments- RHMS-60–1807) using petroleum ether and total ash by combustion (at 550°C) using Muffle furnace (Rotek Instruments RMFH-4–1804).

Measurement of ACE-I Inhibitory Activity

ACE-I inhibitory assay was performed to evaluate the effectiveness of alcalase over trypsin. Briefly, 50 µL of protein hydrolysates were added to 150 µL of 5 mM/L HHL in 50 mM of sodium borate buffer (pH 8.3). The mixture was incubated at 37°C for 5 minutes followed by the addition of 50 µL standard ACE-I (0.1 U/mL). The reaction mixture was again incubated at 37°C for 1 hour. The reaction was terminated by adding 250 µL of 1 N HCl. The liberated hippuric acid was extracted by adding 500 µL of ethyl acetate and evaporated. The residue was redissolved in 3 mL of 1 M NaCl and the absorbance was determined at 228 nm using ultraviolet (UV) spectrophotometer (Eppendorf, Model MJEA129893).[13]

$$\text{ACE-I inhibitory activity } (\%) = (A_{sample} - A_{blank}/A_{control}) \times 100$$

Determination of Antioxidant Activity

DPPH radical scavenging activity of the protein hydrolysates was estimated to check the antioxidant potential of the protein hydrolysates. Briefly, 2 mL of 0.16 mM/L of DPPH solution in methanol was added to the test tube containing 100 µL of protein hydrolysates (1 mg/mL concentration) and the mixture was made up to 2 mL with distilled water. The mixture was incubated at room temperature for 30 minutes at dark. The absorbance of the solution was measured at 517 nm using UV spectrophotometer (Eppendorf, Model MJEA129893).[14]

$$\text{Scavenging activity } (\%) = [1-(\{A_{sample} - A_{sample\ blank}\}/A_{control})] \times 100$$

Statistical Analysis

All data represent the mean value ± standard deviation of three independent measurements. The comparison between two groups was performed using two-way analysis of variance. GraphPad prism software version 8.3.0 was used to analyze the differences and statistical significance at $p < 0.05$.

Results

Preparation of Protein Hydrolysates

The present study aimed to investigate the application of proteolytic enzyme alcalase for a large-scale production of protein hydrolysates as an alternative to trypsin. A comparative study was performed to prepare protein hydrolysates from different food sources using both trypsin and alcalase to see the action of enzymes on the protein sources. The product yield from all the sources is given in ►**Table 1**.

Table 1 Product yield of plant and animal protein hydrolysates obtained from alcalase and trypsin

Sl. no	Source	Product yield (%)	
	Plant sources	**Trypsin**	**Alcalase**
1	Bengal gram dal	17.2	15.4
2	Green gram	21.2	25.6
3	Soybean	29.6	27.4
4	Cashew	25.2	24.2
5	Kidney pea	26.4	28.8
	Animal sources		
6	Chicken	15.04	13.42
7	Mackerel	14.66	16.53
8	Sardine	9.13	7.72
9	Egg white	12.43	8.03
10	Egg yolk	5.22	4.24

Kidney pea and cashew showed higher product yield when hydrolyzed with both trypsin and alcalase. Similarly, in animal source egg white and mackerel showed higher product yield among others.

Proximate Composition of Protein Hydrolysates

Nutritional analysis of obtained protein hydrolysates was performed to estimate the chemical composition of the hydrolysates. The proximate analysis of protein hydrolysates from both plant and animal sources are given in ►**Table 2**. The moisture content of all the samples were found to be $> 15\%$. Protein content in the hydrolysates of plant and animal sources from alcalase and trypsin hydrolyzed food sources were ranged between 25.81 to 61.28% and 30.69 to 62.19%, respectively. The maximum protein content among the plant sources was found in green gram protein hydrolysate (61.28%) and sardine (65.04%) from animal source using alcalase.

Degree of Hydrolysis Influenced by Proteolytic Enzymes

DH can be defined as the ratio of cleaved peptide bond to the total number of peptide bond in the substance.[13] In the present study, protein hydrolysates extracted from alcalase, and trypsin were analyzed for their DH% (►**Fig. 1A and B**). Among all the protein hydrolysates, alcalase extracted hydrolysates from soybean and sardine showed the highest DH% of 61.24 and 62.89%, respectively. Whereas trypsin extracted hydrolysates from kidney pea and egg white could only exhibit maximum of 42.91 and 54.43% of DH, respectively.

Higher Degree of Hydrolysis Exhibited Improved ACE-I Inhibitory Activity

In the current study, protein hydrolysates obtained from both plant and animal sources using alcalase and trypsin were assessed for its ACE-I inhibitory activity. ►**Table 3** shows the IC_{50} values of protein hydrolysates. The ACE-I inhibitory activity of alcalase hydrolyzed sources ranged between 0.52 and 0.61 mg/mL. Among which kidney pea exhibited 50% inhibitory activity at 0.517 mg/mL followed by egg white and green gram (IC_{50} value 0.5278 and 0.5227 mg/mL, respectively, $p > 0.05$). Similarly, trypsin hydrolyzed kidney pea exhibited 50% inhibitory activity at 0.5214 mg/mL followed by sardine and egg white (IC_{50} value 0.5221 and 0.5265 mg/mL, respectively). However, Bengal gram dal and chicken protein hydrolysates showed significantly different ACE-I inhibitory activity between trypsin and alcalase. In due course, food sources hydrolyzed with alcalase can corroborate with the trypsin in producing protein hydrolysates exhibiting ACE-I inhibitory activity. Similar results were also observed in free radical scavenging activity of protein hydrolysates.

Alcalase-Mediated Protein Hydrolysates in Animal Sources Shows Greater Free Radical Scavenging (DPPH) Activity

Among all the hydrolysates prepared, egg yolk hydrolyzed using alcalase showed higher antioxidant activity (43.08%) followed by chicken (39.48%). Whereas hydrolysates from

Table 2 Proximate composition of plant and animal protein hydrolysates obtained from alcalase and trypsin

Sl. no	Food sources	Moisture (%)		Protein (%)		Fat (%)		Ash (%)		Carbohydrates (by difference)	
		Alcalase	Trypsin	Alcalase	Trypsin	Alcalase	Trypsin	Alcalase	Trypsin	Alcalase	Trypsin
I	Plant sources										
1.	Bengal gram dal	7.09 ± 1.4^a	6.92 ± 1.3^a	52.53 ± 1.9^a	31.25 ± 0.7^b	8.92 ± 0.3^a	11.28 ± 0.6^a	2.26 ± 1.7^a	6.23 ± 1.9^a	29.2 ± 2.3^a	44.32 ± 2.5^b
2.	Green gram	10.10 ± 0.7^a	10.0 ± 00^a	61.28 ± 1.0^a	42.57 ± 1.3^b	5.15 ± 0.7^a	6.08 ± 1.7^a	6.25 ± 0.8^a	5.18 ± 3.2^a	17.22 ± 3.2^a	36.17 ± 1.2^b
3.	Soybean	11.57 ± 2.1^a	6.90 ± 0.3^a	58.36 ± 2.0^a	42.28 ± 1.0^b	15.41 ± 0.8^a	20.71 ± 1.0^b	5.55 ± 0.4^a	6.22 ± 1.1^a	9.11 ± 1.3^a	23.89 ± 3.4^b
4.	Cashew	8.63 ± 1.8^a	6.60 ± 1.3^a	25.81 ± 0.9^a	40.86 ± 2.8^b	13.46 ± 1.4^a	10.48 ± 1.6^a	8.18 ± 0.4^a	2.94 ± 0.3^b	43.85 ± 2.6^a	39.12 ± 1.6^b
5.	Kidney pea	10.65 ± 0.4^a	10.75 ± 0.1^a	32.53 ± 1.6^a	30.69 ± 0.8^a	10.17 ± 0.6^a	10.10 ± 0.1^a	9.10 ± 0.6^a	6.4 ± 2.7^a	37.79 ± 2.84^a	42.06 ± 3.7^b
II	Animal sources										
6.	Chicken	13.56 ± 0.3^a	13.19 ± 0.2^a	60.71 ± 1.7^a	53.71 ± 1.8^b	16.40 ± 0.3^a	15.18 ± 1.6^a	4.85 ± 0.4^a	02.89 ± 0.6^a	3.81 ± 2.9^a	13.53 ± 2.12^b
7.	Mackerel	06.5 ± 0.2^a	15.76 ± 0.1^b	61.77 ± 1.6^a	56.07 ± 00^b	$20.15 \pm 0.1a^a$	18.00 ± 0.2^b	7.84 ± 0.2^a	12.22 ± 1.5^a	0.74 ± 2.1^a	2.45 ± 0.84^a
8.	Sardine	10.8 ± 1.4^a	10.83 ± 1.1^a	65.04 ± 1.5^a	62.19 ± 2.3^a	14.52 ± 1.4^a	12.00 ± 0.0^a	5.62 ± 0.1^a	07.98 ± 0.2^b	4.02 ± 2.4^a	5.4 ± 2.26
9.	Egg white	11.5 ± 0.7^a	09.72 ± 00^a	58.72 ± 00^a	44.6 ± 1.6^b	0.30 ± 0.1^a	01.92 ± 1.3^a	3.65 ± 0.9^a	04.74 ± 0.3^a	26.43 ± 0.9^a	37.72 ± 1.8^b
10.	Egg yolk	8.65 ± 1.0^a	10.45 ± 3.4^a	48.89 ± 1.2^a	35.27 ± 3.3^b	18.35 ± 0.2^a	18.97 ± 0.4^a	4.3 ± 0.4^a	04.00 ± 0.5^a	19.81 ± 2.8^a	28.01 ± 2.6^b

Note: Values are represented as triplicates of mean ± standard deviation (SD). Mean value with different alphabets in the superscript between the columns in the same group indicates significant difference ($p < 0.05$) determined using one-way analysis of variance (ANOVA).

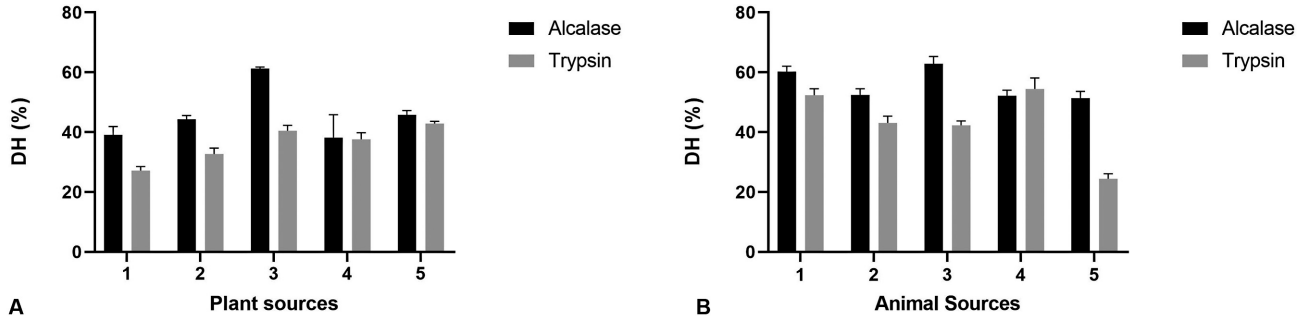

Fig. 1 (A) Degree of hydrolysis of plant sources obtained using alcalase and trypsin. 1. Bengal gram; 2. Green gram; 3. Soybean; 4. Cashew; 5. Kidney pea. (B) Degree of hydrolysis of animal sources obtained using alcalase and trypsin. 1. Chicken; 2. Mackerel; 3. Sardine; 4. Egg white; 5. Egg yolk.

Table 3 ACE-I inhibitory activity of protein hydrolysates extracted using alcalase and trypsin

Sl. no	Source	IC_{50} (mg/mL)	
	Plant source	Trypsin	Alcalase
1	Bengal gram dal	0.89 ± 0.013^{Ac}	0.60 ± 0.024^{Bc}
2	Green gram	0.52 ± 0.004^{Aa}	0.61 ± 0.031^{Ac}
3	Soybean	0.56 ± 0.011^{Ab}	0.56 ± 0.006^{Ab}
4	Cashew	0.52 ± 0.005^{Aa}	0.53 ± 0.003^{Aab}
5	Kidney pea	0.51 ± 0.003^{Aa}	0.52 ± 0.003^{Aa}
	Animal source		
6	Chicken	0.54 ± 0.011^{Aa}	0.63 ± 0.027^{Bc}
7	Mackerel	0.54 ± 0.012^{Aa}	0.53 ± 0.007^{Ba}
8	Sardine	0.54 ± 0.012^{Aa}	0.52 ± 0.006^{Ba}
9	Egg white	0.52 ± 0.002^{Aa}	0.52 ± 0.002^{Ba}
10	Egg yolk	0.55 ± 0.003^{Aa}	0.57 ± 0.010^{Bb}

Abbreviation: ACE-I, angiotensin-I converting enzyme.
Note: Values are represented as triplicates of mean ± standard deviation (SD) ($N = 3$). Values between the enzymes that do not share similar letters in the superscript are significantly different at $p < 0.05$. Values within the sources that do not share similar letters in the superscript are significantly different at $p < 0.05$ as determined using two-way analysis of variance (ANOVA).

green gram using alcalase showed the least (5.96%). On the other hand, higher antioxidant activity was observed in egg white (25.31%) and the least was found in cashew (9.74%) when hydrolyzed with trypsin. The antioxidant activity of all the sources is given in ►**Table 4**. Among the plant sources, antioxidant activity of both alcalase and trypsin hydrolyzed sources were found to be nonsignificant except green gram ($p > 0.05$). Besides, alcalase hydrolyzed animal sources also exhibited significantly higher antioxidant activity than trypsin ($p < 0.05$).

Discussion

Protein hydrolysates are the mixture of bioactive peptides ranging from poly-, oligo-, tri-, and dipeptides which are proven for its therapeutic application against various lifestyle disorders. Formation of protein hydrolysates comprises many techniques such as alkali, acid, enzymatic, and thermal hydrolysis. Among which enzymatic hydrolysis has its own advantage in terms of producing specific bioactive peptides exhibiting bioactive properties.[1] Each proteolytic enzyme has its specific cleaving point on a polypeptide chain under provided conditions for the hydrolysis such as pH, temperature, time, and enzyme-substrate concentration.[9] Trypsin is a pancreatic serine protease that commonly serves as a digestive enzyme specifically cleaving peptide bonds at the carboxyl side of the arginine and lysine residues.[15] Despite of its high efficiency, trypsin has shown certain negative impact on hydrolysis of protein. Studies have reported that most of the plant source contains trypsin inhibitors.[16–18] The presence of trypsin inhibitor can affect the action on source protein during hydrolysis. On the other hand, usage of digestive enzyme which needs to be extracted from body organs requires additional cost for industrial application. Therefore, researchers are looking for an alternative economical proteolytic enzyme for industrial application.[19]

Alcalase is a serine endopeptidase produced by fermentation by *B. licheniformis*.[20] Alcalase is observed to have high specificity compared with trypsin for aromatic (Phe, Trp, Tyr), sulfur containing (Met), acidic (Glu), basic (Lys),

Table 4 Free radical scavenging activity of protein hydrolysates extracted using alcalase and trypsin

Sl. no	Source	Percentage (%) activity	
	Plant sources	Trypsin	Alcalase
1	Bengal gram dal	10.64 ± 1.0^{Ab}	8.71 ± 1.6^{Ab}
2	Green gram	18.03 ± 0.4^{Ba}	5.96 ± 1.5^{Ab}
3	Soybean	17.34 ± 4.4^{Aa}	18.80 ± 0.9^{Aa}
4	Cashew	9.74 ± 1.3^{Ab}	15.13 ± 1.7^{Aa}
5	Kidney pea	18.25 ± 3.4^{Aa}	16.71 ± 0.9^{Aa}
	Animal sources		
6	Chicken	18.29 ± 2.4^{Ab}	39.48 ± 1.4^{Bd}
7	Mackerel	14.84 ± 0.8^{Ab}	28.64 ± 0.7^{Bb}
8	Sardine	22.41 ± 0.6^{Aa}	33.06 ± 1.3^{Ba}
9	Egg white	25.31 ± 1.7^{Aa}	32.20 ± 1.8^{Bab}
10	Egg yolk	16.33 ± 0.3^{Ab}	43.08 ± 1.8^{Bc}

Note: Values are represented as triplicates of mean ± standard deviation (SD) ($N = 3$). Values between the enzymes that do not share similar letters in the superscript are significantly different at $p < 0.05$. Values within the sources that do not share similar letters in the superscript are significantly different at $p < 0.05$ as determined using two-way analysis of variance (ANOVA).

aliphatic (Leu, Ala), and hydroxyl (Ser) residues at the C-terminal end.[21] A schematic representation of hydrolysis (breakdown) of protein by trypsin and alcalase is shown in ►**Fig. 2**. Therefore, in the present study, protein hydrolysates were prepared with alcalase and trypsin to see the competence of alcalase with trypsin. The total soluble portion separated after hydrolysates are considered as percentage (%) of product yield. However, the actual protein content in the powder

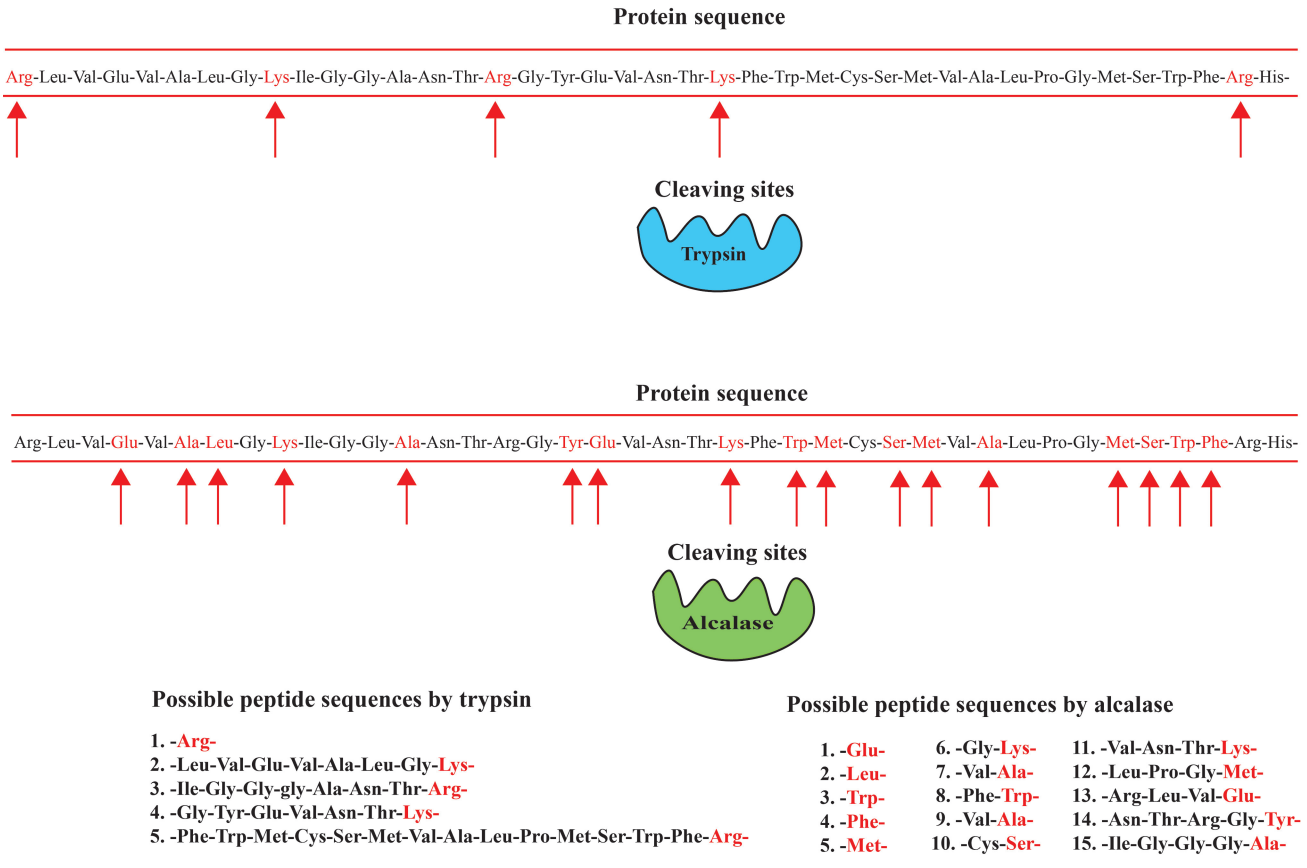

Fig. 2 Schematic representation of cleavage sites and possible peptide formation from an unknown parent protein during hydrolysis process using trypsin and alcalase.

obtained was analyzed further. The higher protein content in the hydrolysates observed could be a result of solubilization of protein during hydrolysis and removal of solid nonprotein residues, chiefly separation of fat layer by centrifugation.[9] However, alcalase hydrolyzed proteins exhibited significantly higher ($p < 0.05$) protein content than trypsin hydrolyzed proteins except for kidney pea and sardine. Similar results were reported earlier by Rawdkuen et al[22] in which change in the protein content was observed when the same source was hydrolyzed using two different proteolytic enzymes (crude papain and Calotropis proteases). However, hydrolysates possessing peptides with specific amino acids and their position makes significant changes in the bioactive properties they possess.[1] The protein content in the hydrolysates is extremely influenced by the extrinsic and intrinsic factors such as type of enzymes, condition during hydrolysis, and type of protein and their amino acid composition, respectively. Additionally, the type of enzyme (exopeptidase or endopeptidase) used for the hydrolysis has an impact on the protein content and DH of the hydrolysates.[1]

The fat content in the raw materials also can interrupt proteolytic hydrolysis so as the bioactive properties. Among the plant protein hydrolysates, soybean possessed higher fat content when hydrolyzed with both trypsin and alcalase (0.71 and 15.41%, respectively). Similarly, among the animal sources mackerel showed 23.15 and 18.0% of fat when hydrolyzed with alcalase and trypsin, respectively. In this study, all the protein hydrolysates possessed higher protein content when the fat content was lower. This could be because higher fat content in the source can restrict the hydrolysis process, thereby resulting in lesser protein content. The presence of fat in the protein hydrolysates is also reported to have negative effect on the shelf life of the protein hydrolysates which described that the protein hydrolysates from Nile tilapia was observed to develop fishy odor, flavor, and lipid oxidation during storage.[23] The action of proteolytic enzymes on the source proteins are key feature during the preparation of protein hydrolysates and they are termed as DH.[24] In the present study, both plant and animal sources exhibited significantly higher ($p < 0.05$) DH% when hydrolyzed with alcalase except egg white. The high DH% also corroborate with the study reported by Daliri et al and Baharuddin et al,[25,26] in which DH of soybean protein hydrolysates and eel protein hydrolysates showed 55 and 69%, respectively. Our results also corroborate with previous studies performed by Yathisha et al and Karimi et al,[8,27] where they found alcalase hydrolyzed protein hydrolysates exhibited higher DH% than the trypsin hydrolyzed proteins. Several other studies also revealed that alcalase hydrolyzed food sources such as okara, egg yolk, and black soldier fly exhibited high DH %.[28–30] Several methods are employed to determine the DH % in protein hydrolysates. But then, TCA methods generally show higher DH for the protein hydrolysates when compared with pH stat and formol titration method.[31] With respect to all the above-mentioned factors essential for an efficient DH%, our study observed a higher efficiency in hydrolysis by alcalase as compared with trypsin. The occurrence of high DH% directly influences the bioactive properties of the protein hydrolysates.[8,32,33]

The excess synthesis of ACE-I is a key enzyme responsible for constant raise in blood pressure. Therefore, inhibition of ACE-I can be a first-line therapy for hypertension. In our study, both plant and animal sources exhibited ACE-I inhibitory activity (►**Table 3**). These results are in line with the findings of Ambigaipalan et al and Hanafi et al.[13,34] In the present study, the ACE-I inhibitory activity of plant protein hydrolysates prepared with both alcalase, and trypsin was found to be nonsignificant ($p > 0.05$) except for Bengal gram and soybean. On the other hand, all the animal sources showed significant difference in the ACE-I inhibitory activity between the two proteolytic enzymes ($p < 0.05$). However, not much difference was observed among the sources hydrolyzed with the same proteolytic enzymes. Previous reports also suggest that protein hydrolysates prepared using alcalase shows higher ACE-I inhibitory and antioxidant activity than trypsin, papain, and flavourzyme.[8,35] These results shed light on the efficiency of alcalase enzymes in producing bioactive peptides with high ACE-I inhibitory activity. The higher ACE-I inhibitory activity shown by alcalase might be because of the wide range of amino acid recognition capability of alcalase than any other enzymes used for hydrolysis.[20] ACE-I inhibitory activity of unhydrolyzed *Moringa oleifera* seed protein hydrolysates showed negative ACE-I inhibitory activity. The study articulates that the enzymatic hydrolysis is responsible for the release of biologically active peptides with potent ACE inhibitory properties.[36] However, the type of source protein used for the hydrolysis plays a key intrinsic factor to generate bioactive peptide with specific amino acid profile exhibiting potent ACE-I inhibitory activity. These results endorse the use of alcalase for the production of protein hydrolysates with high ACE-I inhibitory activity either from plant or animal sources. Protein hydrolysates from plant and animal sources are also assessed for their free radical scavenging activity. Free radical scavenging activity of a biomolecule donates hydrogen atom to exert antioxidant activity. DPPH assay is an extensively employed technique for the quantitative assessment of antioxidant effectiveness of compounds as free radical scavengers or hydrogen donors.[6] In our study, it was observed that alcalase hydrolyzed animal sources exhibited significantly higher antioxidant activity than trypsin. These results are also supported by the study of Shazly et al[37] in which hydrolysates from bovine and buffalo caseins hydrolyzed using alcalase exhibited higher antioxidant activity than trypsin. Other studies demonstrating antioxidant activity by protein hydrolysates used black soldier fly, cricket fly, housefly larvae etc.[30,38,39] The bioactive mechanisms of protein hydrolysates are not well understood and only a few studies have attempted relating to the structure-activity relationship.[38] Nevertheless, to exhibit highest antioxidant activity, the presence of amino acids in the peptides plays an important role in exhibiting antioxidant activity. Henceforth, no single antioxidant mechanism can represent the overall antioxidant activity of the peptides.[37,38] However, the antioxidant activity of protein hydrolysates from both plant and

animal sources used in this study are lousy when compared with other naturally occurring antioxidant agents. However, further evaluation of its potential in combating oxidation was not performed. Nevertheless, use of alcalase for the preparation of protein hydrolysates can yield hydrolysates with higher antioxidant activity than trypsin.

Conclusion

Protein hydrolysates obtained from food sources showed ACE-I inhibitory and antioxidant activity. The ACE-I inhibitory activity of alcalase and trypsin hydrolyzed food sources were found to be nonsignificant except for Bengal gram. Whereas antioxidant activity of animal sources hydrolyzed using alcalase exhibited significantly higher antioxidant activity. The results suggest that good protein source like kidney pea or sardine fish will be a better choice for the production of protein hydrolysates. The selection of alcalase enzymes over trypsin would be economically beneficial for the production of protein hydrolysates with high ACE-I inhibitory activity for a large-scale production. However, modification in the hydrolysis process to eliminate fat will be a noble way to prepare protein hydrolysates with high DH% and bioactive property. Therefore, protein hydrolysates from kidney pea or sardine fish prepared using alcalase will be a suitable option for the development of nutraceutical and functional foods with antihypertensive activity. However, the ability of protein hydrolysates to resist gastrointestinal digestion and reaching the targeted organ to exhibit expected bioactive properties is still a challenge.

Protein hydrolysates obtained from food sources showed ACE-I inhibitory and antioxidant activity. The ACE-I inhibitory activity of alcalase and trypsin hydrolyzed food sources were found to be nonsignificant except for Bengal gram. Whereas antioxidant activity of animal sources hydrolyzed using alcalase exhibited significantly higher antioxidant activity. The results suggest that good protein source like kidney pea or sardine fish will be a better choice for the production of protein hydrolysates with bioactive properties. The preference of alcalase over trypsin can generate low molecular weight peptides which intend to have high ACE-I inhibitory activity and can also be economically beneficial for the production of protein hydrolysates on a large scale. However, the ability of protein hydrolysates to resist gastrointestinal digestion and reaching the targeted organ to exhibit expected bioactive properties is still a challenge.

Conflict of Interest

None declared.

References

1 Jogi N, Yathisha UG, Bhat I, Mamatha BS. Antihypertensive activity of orally consumed ACE-I inhibitory peptides. Crit Rev Food Sci Nutr 2022;62(32):8986–8999

2 Schaafsma G. Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. Eur J Clin Nutr 2009;63 (10):1161–1168

3 da Silva RR. Bacterial and fungal proteolytic enzymes: production, catalysis and potential applications. Appl Biochem Biotechnol 2017;183(01):1–19

4 Kimatu BM, Zhao L, Biao Y, et al. Antioxidant potential of edible mushroom (Agaricus bisporus) protein hydrolysates and their ultrafiltration fractions. Food Chem 2017;230:58–67

5 Liu Q-B, Huang X-X, Bai M, et al. Antioxidant and anti-inflammatory active dihydrobenzofuran neolignans from the seeds of Prunus tomentosa. J Agric Food Chem 2014;62(31):7796–7803

6 Verma AK, Chatli MK, Kumar P, Mehta N. Antioxidant and antimicrobial activity of protein hydrolysate extracted from porcine liver. Indian J Anim Sci 2017;87(06):711–717

7 Jahanbani R, Ghaffari SM, Salami M, et al. Antioxidant and anticancer activities of walnut (Juglans regia L.) protein hydrolysates using different proteases. Plant Foods Hum Nutr 2016;71(04):402–409

8 Yathisha UG, Karunasagar I. BS M. Bioactivity and functional properties of protein hydrolysate from muscle and visceral waste of ribbon fish (Lepturacanthus savala) extracted by three different proteolytic enzymes. J. Biol. Act. Prod. Nat 2021;11(04):363–379

9 Sila A, Haddar A, Martinez-Alvarez O, Bougatef A. Angiotensin-Iconverting enzyme inhibitory and antioxidant activities of protein hydrolysate from muscle of barbel (Barbus callensis). J Chem 2013:1–6

10 Montone CM, Capriotti AL, Cavaliere C, et al. Characterization of antioxidant and angiotensin-converting enzyme inhibitory peptides derived from cauliflower by-products by multidimensional liquid chromatography and bioinformatics. J Funct Foods 2018;44(34): 40–47

11 Krishnan A, Woodard SL. Trypzean^{TM}: an animal-free alternative to bovine trypsin. Biotechnol Agric For 2014;68:43–63

12 AOAC International: AOAC. Official Methods of Analysis of AOAC International. Vol 1. 17th ed. Horwitz W, ed. AOAC International; 2000

13 Ambigaipalan P, Al-Khalifa AS, Shahidi F. Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using alcalase, flavourzyme and thermolysin. J Funct Foods 2015;18:1125–1137

14 Rai AK, Sanjukta S, Chourasia R, Bhat I, Bhardwaj PK, Sahoo D. Production of bioactive hydrolysate using protease, β-glucosidase and α-amylase of Bacillus spp. isolated from kinema. Bioresour Technol 2017;235:358–365

15 Hedstrom L. Serine protease mechanism and specificity. Chem Rev 2002;102(12):4501–4524

16 Sarwar Gilani G, Wu Xiao C, Cockell KA. Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. Br J Nutr 2012;108(Suppl 2):S315–S332

17 Friedman M, Brandon DL. Nutritional and health benefits of soy proteins. J Agric Food Chem 2001;49(03):1069–1086

18 Kaur J, Singh PK. Trypsin detection strategies: a review. Crit Rev Anal Chem 2020;0(00):1–19

19 Lapeña D, Vuoristo KS, Kosa G, Horn SJ, Eijsink VGH. Comparative assessment of enzymatic hydrolysis for valorization of different protein-rich industrial byproducts. J Agric Food Chem 2018;66 (37):9738–9749

20 Tacias-Pascacio VG, Morellon-Sterling R, Siar EH, Tavano O, Berenguer-Murcia Á, Fernandez-Lafuente R. Use of alcalase in the production of bioactive peptides: a review. Int J Biol Macromol 2020;165(Pt B):2143–2196

21 Valencia P, Pinto M, Almonacid S. Identification of the key mechanisms involved in the hydrolysis of fish protein by alcalase. Process Biochem 2014;49(02):258–264

22 Rawdkuen S, Rodzi N, Pinijsuwan S. Characterization of sacha inchi protein hydrolysates produced by crude papain and Calotropis proteases. Lebensm Wiss Technol 2018;98:18–24

23 Yarnpakdee S, Benjakul S, Nalinanon S, Kristinsson HG. Lipid oxidation and fishy odour development in protein hydrolysate from Nile tilapia (Oreochromis niloticus) muscle as affected by freshness and antioxidants. Food Chem 2012;132(04):1781–1788

24 Balti R, Bougatef A, El-Hadj Ali N, Zekri D, Barkia A, Nasri M. Influence of degree of hydrolysis on functional properties and angiotensin I-converting enzyme-inhibitory activity of protein hydrolysates from cuttlefish (Sepia officinalis) by-products. J Sci Food Agric 2010;90(12):2006–2014
25 Daliri EBM, Ofosu FK, Chelliah R, Park MH, Kim JH, Oh DH. Development of a soy protein hydrolysate with an antihypertensive effect. Int J Mol Sci 2019;20(06):1496
26 Baharuddin NA, Halim NRA, Sarbon NM. Effect of degree of hydrolysis (DH) on the functional properties and angiotensin I-converting enzyme (ACE) inhibitory activity of eel (Monopterus sp.) protein hydrolysate. Int Food Res J 2016;23(04):1424–1431
27 Karimi A, Azizi MH, Ahmadi Gavlighi H. Frationation of hydrolysate from corn germ protein by ultrafiltration: In vitro antidiabetic and antioxidant activity. Food Sci Nutr 2020;8(05):2395–2405
28 Sbroggio MF, Montilha MS, de Figueiredo VRG, Georgetti SR, Kurozawa LE. Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. Food Sci Technol 2016;36(02):375–381
29 Bao Z-J, Zhao Y, Wang X-Y, Chi YJ. Effects of degree of hydrolysis (DH) on the functional properties of egg yolk hydrolysate with alcalase. J Food Sci Technol 2017;54(03):669–678
30 Batish I, Brits D, Valencia P, et al. Effects of enzymatic hydrolysis on the functional properties, antioxidant activity and protein structure of black soldier fly (Hermetia illucens) protein. Insects 2020;11(12):1–12
31 Rutherfurd SM. Methodology for determining degree of hydrolysis of proteins in Hydrolysates: a review. J AOAC Int 2010;93(05):1515–1522
32 Sousa P, Borges S, Pintado M. Enzymatic hydrolysis of insect Alphitobius diaperinus towards the development of bioactive peptide hydrolysates. Food Funct 2020;11(04):3539–3548
33 Yathisha UG, Vaidya S, Sheshappa MB. Functional properties of protein hydrolyzate from ribbon fish (Lepturacanthus Savala) as prepared by enzymatic hydrolysis. Int J Food Prop 2022;25(01):187–203
34 Hanafi MA, Hashim SN, Chay SY, et al. High angiotensin-I converting enzyme (ACE) inhibitory activity of Alcalase-digested green soybean (Glycine max) hydrolysates. Food Res Int 2018;106:589–597
35 Choonpicharn S, Jaturasitha S, Rakariyatham N, Suree N, Niamsup H. Antioxidant and antihypertensive activity of gelatin hydrolysate from Nile tilapia skin. J Food Sci Technol 2015;52(05):3134–3139
36 Aderinola TA, Fagbemi TN, Enujiugha VN, Alashi AM, Aluko RE. Amino acid composition and antioxidant properties of *Moringa oleifera* seed protein isolate and enzymatic hydrolysates. Heliyon 2018;4(10):e00877
37 Shazly AB, Mu H, Liu Z, et al. Release of antioxidant peptides from buffalo and bovine caseins: Influence of proteases on antioxidant capacities. Food Chem 2019;274:261–267
38 Wang J, Wang Y, Dang X, Zheng X, Zhang W. Housefly larvae hydrolysate: orthogonal optimization of hydrolysis, antioxidant activity, amino acid composition and functional properties. BMC Res Notes 2013;6(01):197
39 Hall F, Johnson PE, Liceaga A. Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. Food Chem 2018;262:39–47

