Preparation Protein Hydrolysate From Moringa Oleifera and Its Use as Fortified Vegetarian Food

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KEYWORDS

Moringa oleifera Plants, Protease, protein, antioxidant, purification, FTIR, Amino acid analysis.

ABSTRACT

The moringa oleifera tree is known as a "miracle tree" because of its abundance in certain macro and micronutrients that are crucial to human nutrition. Depending on the cultivar and origin, the chemical makeup of the various Moringa tree sections might change. There are several uses for M. oleifera leaves, seeds, and flowers in food. Protein isolate from seed flour and protease extraction from leaves were prepared for this investigation. The protein was then partially purified using ammonium sulphate with saturated 75% and dialyzed, after which the specific activity (22.161, 65.422, and 115.064 U/mg) was recorded. Protease was used to create the protein hydrolysate through enzymatic hydrolysis, and the DPPH technique was used to calculate the antioxidant activity. Amino acid analysis and FTIR were determined. Lastly, moringa protein hydrolysate was introduced to the product, such as putties, in place of the moringa instead of the meat.

1. Introduction

One of the most well-known medicinal plants, Moringa oleifera is found throughout tropical regions (Stohs, 2015). It has a variety of hydrolytic enzymes, the most important of which are proteases, which have been shown to have pharmacological effect (Satish, 2012). Moringa oleifera is a fast-growing, perennial, angiosperm tree that belongs to the Moringaceae family. At chest height, it can grow to a height of 7 to 15 meters with a diameter of 20 to 40 cm. It is commonly recognized as a high-protein vegetable, a source of cooking oil in underdeveloped nations, and a medicinal plant known by several names, including "medicine chest," "miracle trees," and "man's best friend."

Originally from South Asia, it has recently attracted interest in both medicine and society in tropical and subtropical regions, including tropical Asia, Latin America, the Caribbean, Western, Eastern, and Southern Africa, as well as the Pacific Islands, where it is now widely cultivated and has naturally occurred (Alegbeleye, 2018; Shi, 2018). We made an effort to look into the protease activity of leaf extracts from Moringa oleifera in water. After isolating and purifying the protease from Moringa leaves, we discovered that it functions best at an alkaline pH when it is caseinolytically active. Proteases originating from plants, animals, and microbes are widely used in several industries, such as the food, pharmaceutical, leather, and brewing sectors (Turk, 2006; Li, 2013; López-Otín, 2008). These enzymes account for over 60% of all enzyme sales globally (Li, 2012). Hydrolyzing enzymes, or proteases, are employed in detergents, bioremediation procedures, the food, leather, and pharmaceutical industries, and are regarded as one of the most significant classes of industrial enzymes.

This study aimed to extract, partially purify, and characterize the protease enzyme from Moringa (Moringa oleifera) leaves. Additionally, it sought to determine the impact of the hydrolysate of this enzyme on vegetarian food that has been supplemented..

2. Methodology

Preparation of Moringa oleifera seed defatted flour After sorting, the dry seeds were pulverized in a milling machine that had a 200 µm mesh size fine screen attached to it. According to Mune et al. (2016), the flour was defatted twice using a hexane/ethanol (1:1, v/v) solvent solution and a 1:3 (w/v) solid to liquid ratio. The defatted flour was stored in a polyethylene bag at 4 °C after being oven-dried at 45 °C.

Preparation of Moringa oleifera protein from flour that has been defatted. The defatted flour was suspended at a ratio of 1/20 (w/v) of flour to liquid in a 0.17 M NaCl solution. After that, the protein was extracted twice at pH 2 while being continuously stirred for an hour at 65 °C. It was then recovered at pH 8 by centrifuging at 1290 ×g for 30 minutes. After extracting the protein, it was freeze-dried and cleaned with distilled water (Bakwo, 2022).

Extraction of crude enzyme extract The Biotechnology Research Center at AL-Nahrain University collected mature Moringa Oleifera leaves from a garden. The leaves were crushed, along with 20 mM phosphate buffer (pH 7.8), 0.1% tween 20 detergent, and protease cocktail inhibitor. The mixture was then placed in a refrigerated centrifuge (Selecta/spain) and run for 10 minutes at 4°C at 10,000 rpm.

Enzyme precipitation: The crude extract was mixed with 75% ammonium sulphate to obtain the protein precipitate, which was then dissolved in 20 mM tris buffer for further determined activity assay and protein concentration.

Dialysis: The pellet dissolved in Tris buffer as obtained above was then dialyzed in 3.5cm/ml dialysis tubing overnight in a magnetic stirrer by immersing the tubing in a buffer containing Tris (pH 7.8) and phenyl-methyl-sulfonyl fluoride (PMSF), which was repeated thrice for complete exchange of buffer (Swarnali,2018).

Determination of Protease Activity

Using azo-casein as a substrate, the Erlanger et al. (1961) method was slightly altered to measure protease activity. Each purification step was performed by adding 50µl of buffer containing 20 mM Tris-HCl and 2 mM CaCl2 (pH 8), to 50µl of sample. After 10 minutes of incubation at 25°C, 100 µl of 30% tri acetic acid (v/v) was added to stop the reaction. The reaction mixture's absorbance was measured at 410 nm.

Protein Measurement

The protein content of the crude extract was assessed using the biuret method (Gornall, 1949) with bovine serum albumin (Sigma Chemical Co.) serving as the reference. The mg/ml unit of measurement for protein was used.

Moringa protein hydrolysate (MPH) was achieved by hydrolyzing the protein, which was done with some modification utilizing protease (37 °C, pH 7.8) and all of the enzyme and reaction conditions as previously reported (He R., Alashi, 2013). To the partially purified protein, trypsin was introduced.After three hours of digestion (with the pH being kept constant with the addition of 1 M NaOH or 1 M HCl), the mixture was heated to 95 °C for fifteen minutes in a water bath, and it quickly cooled in an ice bath. After centrifuging the undigested proteins at 8000×g for one hour at 4 °C, the supernatant containing the target peptides was freeze-dried to produce the corresponding enzyme, MPH.

Degree of Hydrolysis (DH) Determination

According to Yue et al. (2013), the formaldehyde titration method was used to determine the DH in

the following ways: After dilution with 60 milliliters of distilled water with magnetic stirring, five milliliters of hydrolysates were titrated to pH 8.2 using 0.05M NaOH (standard titration solution), and the volume was noted. The beaker was then filled with 10 ml (14% formaldehyde) and titrated with 0.05 M NaOH until pH 9.2 was reached. Additionally, the amount of NaOH consumed was noted. The following equation was used to compute the value of DH:

$$DH = \frac{C \times (V1 - V2) \times V/5}{m \times \text{percentage of protein in raw material} \times 8} \times 100$$

C: the concentration of standard titration solution of (0.05M) NaOH.

V1: the consumed volume of (0.05 M) NaOH titrating up to pH 9.2.

V2: the consumed volume of (0.05 M) NaOH titrating up to pH 8.2 .

V: the total volume of collagen hydrolysate.

m: the mass of the raw material.

Determination of amino acid composition

High-performance liquid chromatography (HPLC, Shimadzu, Japan) was used to examine the amino acid contents of five µg/ml of freeze-dried collagen samples that had been dissolved in CH3COOH 0.5 M solution. O-phthalaldehyde (OPA) is at least ten times more sensitive than ninhydrin in the production of a fluorescent derivative for primary amino acids (Ersser et al., 1991).Utilizing mobile phases A and B (Me-OH:THF0.02M:odium acetate PH 5.9 (80:2.5:17.5) and UV, respectively, and methanol (Me-OH): Tetrahydrofuran (THF) 0.02M:Sodium acetate PH 5.9 (20: 2.5: 77.5). detector in the ministry of technology and science ,the reaction takes place rapidly at room temperature. Unfortunately, there is a low response to cysteine and none to proline and hydroxyproline. To calculate the amino acid concentration , the following equation is followed :-

$$\text{Concentration of sample (µg/ml)} = \frac{\text{Area of sample X Conce. of St.}}{\text{Area of St.}}$$

Conce. : Concentration ,St. : standard

And the residue equation:

$$\text{Residue/1000} = \frac{\text{\% of amino acid} \times 10}{\text{No. of amino acid}}$$

Determination of DPPH radical- scavenging activity:

Using the Salman et al., 2019 approach, the scavenging effect on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical was assessed. 0.1 mM DPPH in 100 ml of 95% ethanol, or 0.0039 g of weight. To 1.5 ml of DPPH, 1.5 ml of sample solution was added. After shaking, the mixture was allowed to sit at room temperature in a dark area for half an hour. The resultant solution's absorbance was measured at 517 nm. Higher DPPH scavenging activity is indicated by a low absorbance reading, which can be written as:

Scavenging activity (%) = [(blank A_{517}—sample A_{517}) / blank A_{517}] × 100%.

FT-IR Analysis

Using a model equipment from SHIMADZU (Japan), FTIR was carried out using potassium bromide KBr pellets that were created from discs containing 2-mg moringa samples in about 200 mg of potassium bromide (KBr) under dry circumstances with a wavelength of 400–4000 cm-1 (Muyonga,

2004).

Preparation moringa discs: There was a control group that did not contain any moringa, and two concentrations of moringa discs were made: one at a weight of 100 grams per gram and another at a weight of 50 grams per gram. This means that the first ratio is 1:1, and the second is 0.5: 1 contains 50% of the moringa leaves and 25% of the leaves, 10% is refined vegetable oil (soybean oil), 3% is refined wheat flour, 3% is condiment mix (10% for each of the chopped onion, shredded carrot, mashed boiled potatoes, tomato, and pumpkin), 0.5% is refined common salt. After that, the discs were shaped and deep-fried in sunflower vegetable oil.It was offered in order to conduct a sensory evaluation.

Sensory attributes: A 5-point scoring system was used to evaluate the sensory qualities of the moringa discs (color, flavor, tenderness, juiciness, and overall acceptability) in separate booths with regulated light, temperature, and humidity. According to Rahman et al. (2014), the sensory scores were 5 for excellent, 4 for very good, 3 for good, 2 for fair, and 1 for bad. Petri dishes held all of the samples.

3. Results and discussion

Purification of protease

Currently, there is a wide range of herbal products that are considerable importance in food and pharmaceutical industries including dental care. Among these herbal products, plant-based proteolytic enzymes, which are mainly proteases have received special attention due to their properties (Sebastián,2018) . The protein concentration from mature Moringa oleifera leaves at various stages of purification is shown in Table 1.

One of the most common salts used in TPP is ammonium sulphate as it is responsible for protein-protein interaction and precipitation by salting-out mechanism[Chew,2019].The purification fold and activity recovery are extremely improved up to 3.74 and 88.35 respectively when the ammonium sulphate is increased to 35% (w/v). Similar results were obtained by (Niphadkar,2015) (Demir,2017).

Table (1): Purification steps of protease from moringa leaves.

Purification steps	Vol.(ml)	Activity (u/ml)	Protein conc. (mg/ml)	Specific activity (u/mg)	Total activity	Total protein (mg)	Yield%	No.of fold
Crude	250	25.73	1.161	22.161	6432.5	290.25	100	1
Precipitate 75%	55	60.45	0.924	65.422	3324.75	50.82	51.686	2.952
Dialysis	30	89.75	0.78	115.064	2692.5	23.4	41.857	5.192

Degree of Hydrolysis

Degree of hydrolysis of moringa protein which was hydrolyzed by protease, the degrees of hydrolysis (DH) was 24% with hydrolysis time 1.5 hour at 4 °C without bitterness.

Free Radical Scavenging Activity: Table 2 shows the antioxidant activity of the protein from moringa leaves determined using the DPPH assay. DPPH generates free radicals; the assay measures the ability of various solvent extracts to scavenge these free radicals. The lower the concentration of DPPH free radicals in solution, the higher is the ability of that particular extract to scavenge free radicals. In the results shown in Table 2, it can be observed that the order of scavenging activity (from low to high) was crude ≥ precipitate ≥ dialyzed, and≤ moringa hydrolysate. Therefore the crude showed higher scavenging activity was 73.200% than the other stages 21.68% , 30.02 % , 23.200 % respectively. Hence, moringa displayed good antioxidant activity (Vusumzi Pakade,2013).

Table (2): The antioxidant activity assay on different stages of moringa protein

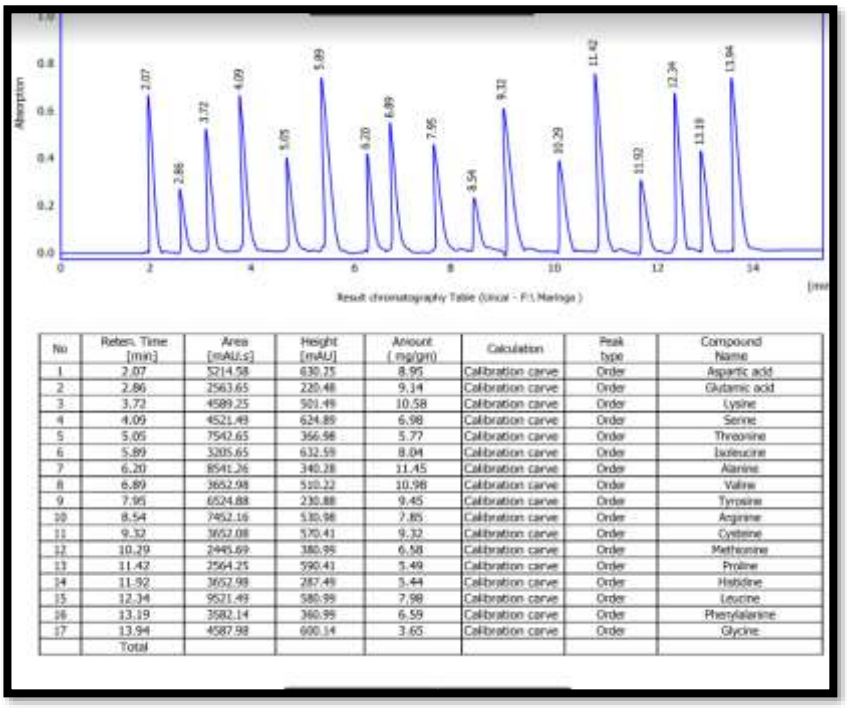
Sample	Abs. at 516 nm	DPPH %
Blank	1.056	-

Crude	0.773	73.200%
Precipitate	0.827	21.68 %
Dialysis	0.739	30.02 %
Moringa hydrolysate	0.45	57.38 %

Amino acid Composition

According to Malomo & Aluko (2014), antioxidant properties of peptides are greatly influenced by the amino acid composition of the peptide. Amino acid compositions of the M. oleifera seed protein isolate and hydrolysates are shown in figure 1. Overall, the enzymatic hydrolysis process did not have any measurable effect on the constituent amino acids. Slight changes were observed in the amino acid composition of the protein hydrolysates when compared to the precursor protein (ISO). A similar trend was also observed for hemp seed protein isolate and its protein meal (Malomo & Aluko ,2014). Generally, the isolate and the hydrolysates showed high contents of glutamic acid + glutamine (Glx), glycine and arginine. The apparently low content of cysteine for trypsin hydrolysate (TIH) sample when compared to others might be due to the specificity of the trypsin for peptide bonds involving mainly lysine and arginine.

The combined summary of the compositions reveals the presence of more hydrophobic amino acids (HAA) and the negatively charged amino acids (NCAA) in the samples. Some of these amino acids, especially Tyr, Met, His and Lys have been shown to play specific roles in improving antioxidant properties of peptides (Samaranayaka,2011; Udenigwe,2012). Besides, aromatic amino acids with a large side group such as His (imidazole group) and Trp (indolic group) contribute to the antioxidant potency of peptides because they act as hydrogen donors (Nam,2008) According to (Sarmadi Sarmadi,2010), the interaction of peptides with lipids or entry into target organs can be enhanced by the hydrophobic properties, which helps in promoting the antioxidant effects of peptides. Furthermore, hydrophobicity of peptides, which helps to improve their solubility in lipid medium, has been reported to also improve their antioxidant potentials (Rajapakse,2005; Shimada,1992). The ISO and hydrolysates also show high contents of essential amino acids, which is an indication of their high nutritional values.



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Amount [mg/gm]	Calculation	Peak type	Compound Name
1	2.07	5214.58	630.75	8.95	Calibration curve	Order	Aspartic acid
2	2.86	2563.65	220.48	9.14	Calibration curve	Order	Glutamic acid
3	3.72	4589.25	501.49	10.58	Calibration curve	Order	Lysine
4	4.09	4521.49	624.89	6.98	Calibration curve	Order	Serine
5	5.05	7542.65	366.98	5.77	Calibration curve	Order	Threonine
6	5.89	3205.65	632.59	8.04	Calibration curve	Order	Isoleucine
7	6.20	8541.26	340.28	11.45	Calibration curve	Order	Alanine
8	6.89	3652.98	510.22	10.98	Calibration curve	Order	Valine
9	7.95	6524.88	230.88	9.45	Calibration curve	Order	Tyrosine
10	8.54	7452.16	530.98	7.85	Calibration curve	Order	Arginine
11	9.32	3652.08	570.41	9.32	Calibration curve	Order	Cysteine
12	10.29	2445.69	380.99	6.58	Calibration curve	Order	Methionine
13	11.42	2564.25	590.41	5.49	Calibration curve	Order	Proline
14	11.92	3652.98	287.49	5.44	Calibration curve	Order	Histidine
15	12.34	9521.49	580.99	7.98	Calibration curve	Order	Leucine
16	13.19	3582.14	360.99	6.59	Calibration curve	Order	Phenylalanine
17	13.94	4587.98	600.14	3.65	Calibration curve	Order	Glycine
	Total						

Figure (1): Amino acid analysis of protein hydrolysate from Moringa

The FTIR spectra

The FTIR spectrum were recorded between 4000 and 400 cm−1 Figure 2 on FTIR spectrometer (Schimadzu, Japan). The spectra of protein hydrolysate is indicating that tyrosine is one of the major amino acid released by the action of hydrolysate from Moringa olifera. The assignments are based on previously published FTIR studies of proteins and peptides (Böcker,2007 ; barth,2017). Some of these bands have been shown to be important for monitoring proteolytic reactions. These include bands such as the amide I (~1700–1600 cm−1) and II (~1590–1520 cm−1), the N-terminal (NH3+, ~1510 cm−1) and the C-terminal (COO−, ~1400 cm−1), but changes can also be observed in other FTIR bands. Figure 2 also shows the FTIR spectrum of the purified protease sample t. When comparing the purified protease spectrum to the standard, some of the features of the amide I (~1700–1600 cm−1) and II (~1590–1520 cm−1) have been preserved whereas the COO− stretches from the peptides and the free amino acids have disappeared altogether due to protonation. New bands from the peptide complexes are also clearly visible and the most dominant are the C = O (~1677 cm−1), C–F (~1250–1100 cm−1) and OCO (~950–700 cm−1) stretching bands. Protonation of secondary amides are known to affect the amide II (~1590–1520 cm−1) FTIR band, as it changes the C-N and N-H bond length of the corresponding amide group (Wu,2009). The C-N bond becomes shorter when the carbonyl oxygen is protonated (Murthy,1970). This gives the C-N bond a stronger double bond nature and the nitrogen will have a positive charge which is stabilized by the protein hydrolysate− counter ion. The protein hydrolysate COO− interaction with secondary amide has previously been assigned to an absorption band in the ~1620 cm−1 region of the FTIR spectrum. This band has been referred to as diagnostic for the presence of CF3COO−, and in this study the band is tentatively assigned to an absorption at ~1627 cm−1 (Tam,1973).

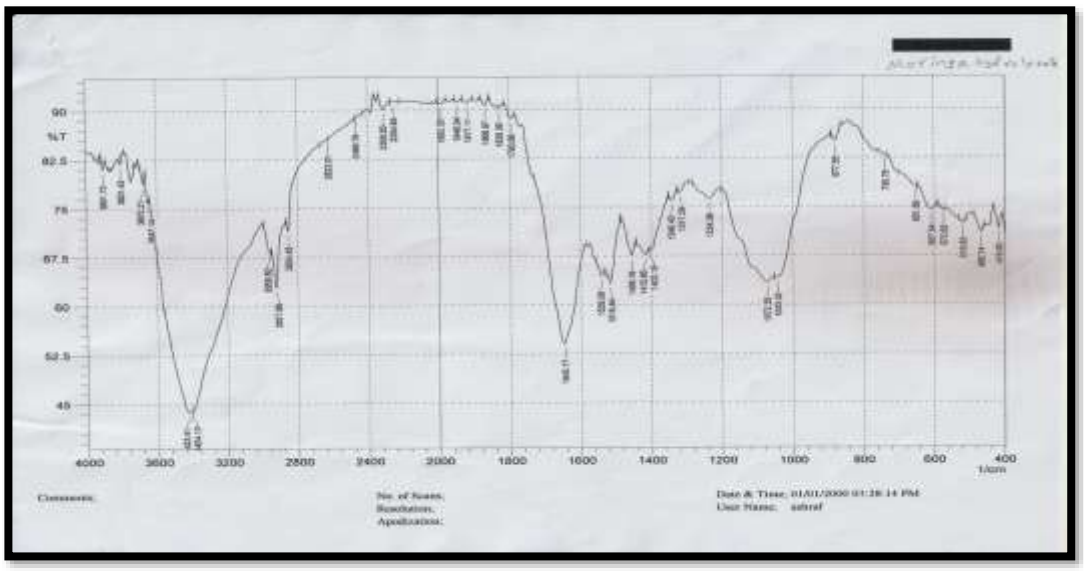


Figure (2): FTIR of protein hydrolysate from Moringa

4. Conclusion and future scope

The plant-derived proteolytic enzymes are finding increasing clinical and industrial applications. To conclude, it can be stated that protease enzyme can be considered as a promising agent, cheap, and safe source which is suitable for using in various industries. We could extract, purify and characterize the enzymatic activity of proteases from the leaves of Moringa oleifera. Further physiochemical properties, substrate specificity and activity of the extracted protease are required for determining its suitability as a protein hydrolysate for application.

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