Screening Some Local Egyptian Seeds for Different Proteolytic Enzymes and Their Application to Produce Potent ACE-I Milk Hydrolysates

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Abstract: The aim of this study is to test the possible presence of different proteolytic enzymes with promising industrial application in local low cost plant seeds. The aqueous extracts of 28 dry seeds representing eight families (*Cruciferae, Umbelliferae, Leguminosae, Rosaceae, Asteraceae, Gramineae, Tiliaceae,* and *Cucurbitaceae*) were screened in order to find the most promising source for production of industrially important proteolytic enzymes. The aqueous extracts were tested for the presence of proteolytic enzymes by measuring the proteolytic activity (PE) using soluble casein at three pHs (4.5, 6.5 and 9) to test the presence of acidic, neutral or alkaline proteases. Results of screening experiments indicated that the proteolytic enzymatic activity is family related. The results showed that the family *Cruciferae* tested members were rich in proteolytic activity at acidic, neutral and alkaline pH, followed by the family *Umbelliferae.* The tested seeds members of *Leguminosea* family and *Gramineae* family show poorer availability of proteolytic enzymes. Although the seed *Raphanus sativus* shows the highest proteolytic activity at pH 4.5, it is not producing a milk hydrolysate with the highest Angiotensin Converting Enzyme inhibitory activity as the seed *Apium graveolens* does. The seed *Coriandrum sativum* had higher proteolytic activity at pH 6.5 then *Foeniculum vulgare* but the later produces a milk hydrolysate with higher ACE inhibitory activity then the former. The seed *Raphanus sativus* produces a milk hydrolysate with lesser ACE inhibition then *Petroselinum crispumat* pH 9.

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1. Introduction

Proteases are physiologically necessary for living organisms; they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms (**Rao, *et al.*, 1998**). Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery (**Gupta, *et al.*, 2002**).

Protein digestion by proteolytic enzymes modifies the chemical, physical, biological and immunological properties of proteins. These enzymes have been widely applied in pharmaceutical, medicinal, food, detergent, leather and biotechnological industries (**Nallamestty, *et al.*, 2003** and **Salvador, *et al.*, 2006**). In addition to protein modification, the original proteins are progressively cleaved into a number of peptides, some of which are biologically active (**Qi, *et al.*, 2007**).

Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract (**Erdmann, *et al.*, 2008**). Because of their therapeutic potential for treatment or prevention of diseases, bioactive peptides may be used as components in functional foods or nutraceuticals (**Vercruysse, *et al.*, 2005**). Bioactive peptides have been detected in many different food sources (**Dziuba, *et al.*, 1999**).

Angiotensin converting enzyme (ACE) raises blood pressure by producing the vasoconstrictor angiotensin II and degrading the vasodilator bradykinin (**FitzGerald, *et al.*, 2004**). ACE inhibitor peptides, present as natural ingredients in both animal and vegetable proteins, provide valuable alternatives for the synthetic drugs (**Hermansen, *et al.*, 2000**).

The present study was conducted to investigate the possible presence of proteolytic enzymes with promising industrial application in local low cost plant seeds. Twenty eight plant seeds representing eight families were screened in order to find the most promising source for production of industrially important proteolytic enzymes. The plant families include Cruciferae, Umbelliferae, Gramineae, Rosaceae, Asteraceae, Cucurbitaceae, Tiliaceae and Leguminosae. Digested skim milk by the action of seven plant seed proteolytic fractions shows a potent ACE-inhibitory activity at the different pH's applied for the test

2. Materials and Methods:

2.1. Materials

Twenty eight dry seeds from eight families were used in the present study. They were brought from local markets. Table (1) illustrates the names of the different used screened seeds and their families. Soluble casein and L-Tyrosine were obtained from BDH, England. Hippuryl-L-histidyl-leucine (Hip-His-Leu) substrate and angiotensin I-converting enzyme (ACE, EC 3.4.15.1) from rabbit lung were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All the other chemicals were of analytical grade.

2.2 Preparation and extraction of the crude enzymes:

Twenty eight dry seeds in the rest states were separately crushed and mixed with dist. water at 9 °C with continuous shaking over a period of 12 hours. This extract was then centrifuged at 3000 r.p.m for 15 minutes, and the supernatant was collected, dialyzed against dist. water and then used as the crude enzyme preparation (milk-clotting enzyme, MCE and proteolytic enzyme, PE)……..

2.3 Determination of the proteolytic activity (PA):

The proteolytic activity of the prepared enzyme solutions was estimated according to Greenberg (1975) using soluble casein as a substrate. The assay used is as follows:

From 1% (w/v) soluble casein solution, 0.5 ml was pipette into a test tube followed by 1.0 ml of 0.05 M phosphate buffer, pH 7.0 then 0.5 ml of the enzyme solution. The test tube was incubated in a water bath at 40 °C for 20 minutes. The reaction was stopped by adding one ml of 15% trichloroacetic acid and the tube was left 30 minutes, and then centrifuged. To 0.5 ml of the clear supernatant, blank and L-tyrosine solution (as standard), was added to 7.5 ml of 0.5 M NaOH followed by 0.5 ml of diluted Folin Ciocalteu's reagent with dist. water (1: 2) with continuous shaking. The absorbance of the samples and blank were read by spectrophotometer at 660 nm wave length against blank. One unit of protease enzyme was defined as the amount of enzyme that could liberate 1.0 µmole of amino acid per min under the conditions described above using L-tyrosine as standard.

The activity of MCE and PE values of samples was average of three repeated measurements.

2.4 Preparation of standard curve of L-tyrosine:

It was prepared according to Greenberg (1957). This standard curve was made for measuring the proteolytic activity (PA). The standard tyrosine was dissolved in 0.2 M HCL solution.

2.5 Estimation of protein:

The protein concentration was determined by Lowry *et al*. (1951) method using bovine serum albumin as a standard.

Procedure:

To 0.5 ml of sample, blank and bovine serum albumin solution as standard, 2.5 ml of reagent was added and left to stand for 10 min at room temperature; 0.25 ml diluted Folin reagent with distilled water (1:2) was added. These ingredients were mixed well and left for 20 min. The absorbance of the samples and the standard were read by LKB Biochrom Nova Spec II spectrophotometer at 750 nm against a blank solution.

Calculations:

$$\text{Protein concentration} = \frac{\text{O.D of test}}{\text{O.D of standard}} \times \text{Conc. of Standard}$$

2.6 Preparation of Angiotensin-1-converting enzyme inhibitor (ACEI).

Preparation of skimmed-milk hydrolysate:

The production of ACE-inhibitors (bioactive peptide/s) from skimmed milk was prepared according to the method of Cushman and Cheung (1971). Five ml of 1% skimmed milk was mixed with 1ml of enzyme solution and pH was adjusted to different pHs (4.5, 6.5 and 9.0) unless otherwise stated. The mixture was then incubated at 37 °C for 3 hr. The resulting mixture was heated at 90 °C for 5 min to inactivate the enzyme and pH was readjusted to 7.0. After centrifugation at 15000 g for 10 min, a clear supernatant was collected and lyophilized. The lyophilized materials were dissolved in 0.5 ml of distilled water and then the resulting solution was centrifuged to remove the insoluble materials. Then the ACE-inhibition activity of the filtrate was measured.

2.7 Determination of the ACE-inhibitory effect of the prepared skimmed-milk hydrolysate at different pHs in vitro:

The ACE-inhibition activity of the filtrate was measured according to Cushman and Cheung (1971) with some modification. Briefly, 200 µl of HHL buffer (5 mM Hip-His-Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) was mixed with 60 µl of borate buffer and pre-incubation with 20 µl of ACE (0.05 units/ml) and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 250 µl of 1 M HCl and mixed with 1.7 ml of ethyl acetate. The mixture was centrifuged. After

removing the ethyl acetate layer, the remaining precipitate was dissolved in 1 ml of distilled water and the absorbance was measured at 228 nm to measure the ACE activity (Figure 1). The extent of inhibition was calculated as follows:

ACE-inhibitor activity (%) = 100- [100x(C-D)/(A-B)]

Where:

A is the absorbance in the presence of ACE and without the ACE- inhibitory component; B is the absorbance without ACE and the ACE-inhibitory component; C is the absorbance with ACE and ACE inhibitory component; D is the absorbance without ACE and with the ACE-inhibitory component.

3. Results and Discussion

3.1. Screening for Milk-clotting enzyme (MCE) and proteolytic enzyme (PE) in different dry seeds:

The aqueous extracts of 28 dry seeds representing eight families (*Cruciferae, Umbelliferae, Leguminosae, Rosaceae, Asteraceae, Gramineae, Tiliaceae, and Cucurbitaceae*) were incubated with soluble casein dissolved in 0.1 M acetate pH 4.5, 0.1 M phosphate pH 6.5 and 0.1 M borate buffers pH 9.0 in order to assess the proteolytic activity (digesting activity). Measuring the proteolytic activity (PE) at three pH (4.5, 6.5 and 9) test the presence of acidic, neutral or alkaline proteases.Table (2) represent the results of screening experiments for the existence of proteolytic activities (PA) of the extract of 28 dry seeds at different pHs. Table (2) shows that the extract of seeds number 2, 7, 8, 11, 13, 20 and 22 had higher PA at pH 4.5 (ranging from 1.73 to 6.00 µ/g dry seed) and the extract of seeds number 4, 6, 13, 19 and 20 had higher PA at pH 6.5 (ranging from 2.1 to 20.46 µ/g dry seed) while the extract of seeds number 2, 9 and 22 had PA at pH 9.0 (ranging from 2.55 to 4.25 µ/g dry seed). The 5 members tested from *Cruciferae* family (seeds *Brassica napus*, *Raphanus sativus*, *Eruca sativa*, *Brassica alba*, *Brassica nigra*) appear abundant in acidic proteases, alkaline proteases and neutral proteases. while the 5 members of the family *Umbelliferae* (*Coriandrum sativum* , *Anethum graveolens* , *Apium graveolens* , *Petroselinum crispum* , *Foeniculum vulgare*) appears abundant in neutral proteases followed by acidic then alkaline proteases with *Coriandrum sativum* seeds having the highest proteolytic activity value for both acidic and neutral pH. The eight seeds members of *Leguminosea* family show poorer availability of proteolytic enzymes except for seeds *Trigonella foenum-graecum* followed by *Phaseolus vulgaris* and *Vigna unguiculata*. The 2 members of *Gramineae* family are poor in proteolytic activity. The seeds *Corchorus olitorius* (*Tiliaceae* member) and seeds *Lactuca sativa* (*Asteraceae* member) appear surprisingly rich for different proteolytic activity with milk clotting ability especially seeds *Corchorus olitorius*. From table 2 it appears that the family *Cruciferae* is rich in proteolytic activity at acidic, neutral and alkaline pH, followed by the family *Umbelliferae*. we can conclude that a number of low cost local seeds in Egypt could be considered a mine source for proteolytic enzymes to be used in different applications

3.3. Proteolytic enzyme (PE):

The aqueous extracts of eight dry seeds were chosen to digest skimmed-milk at different pHs (Table 3). They are used for production of Angiotensin-1-converting enzyme inhibitor (ACEI). Their choice was made according to their PA.

3.3.1. Production of Angiotensin-1-converting enzyme inhibitor (ACEI):

Skimmed-milk protein was hydrolyzed by proteases extracted from the above eight seeds for 3 hours at three pHs (4.5, 6.5 and 9.0). The resulted hydrolysates were used as ACEI *in vitro* (Figure 2).

3.3.3 The Angiotensin-1-converting enzyme (ACE) inhibitory effect of skimmed-milk hydrolysate at different pHs:

Results in Table (4) show that seeds no 8 (celery) hydrolysate showed strong ACEI activity more than 95% *in vitro* at pH 4.5 and seeds no. 6 (coriander) and 10 (fennel) hydrolysate showed strong ACEI activity more than 95% *in vitro* at pH 6.5. Results illustrated also that seeds no. 2 (Radish), 7 (Dill) and 26 (Corchorus) hydrolysate showed moderate ACEI activity (72 - 88%) *in vitro* at pH 4.5 and seeds no. 2 (Radish) and 9 (Parsley) showed ACEI activity (74-82%) at pHs 9.0.

Although the general rule states that the higher the proteolysis, the higher the chance for the presence of an inhibitory peptide/s.. The results showed that the ACE inhibitory activity of the hydrolysate is not related to the extent of hydrolysis produced by the enzyme extract but rather on the specificity of the enzyme and the type of peptide released. .....

Developed countries like USA, Japan and France are marketing functional food products or nutraceuticals containing biologically active peptide with different health claims. In the present experiment, the seeds of radish show the highest values for angiotensin converting enzyme inhibitory peptide at pH 4.5 while coriander shows the highest at pH 6.5. Both seeds have a history in the Egyptian market as beverage intake at the fifties for the first

one and in folk remedies for the second one..... This makes the possibilities for consumer acceptance of a nutraceuticals to protect and treat hypertension based on both seeds simple after passing the required tests successfully.

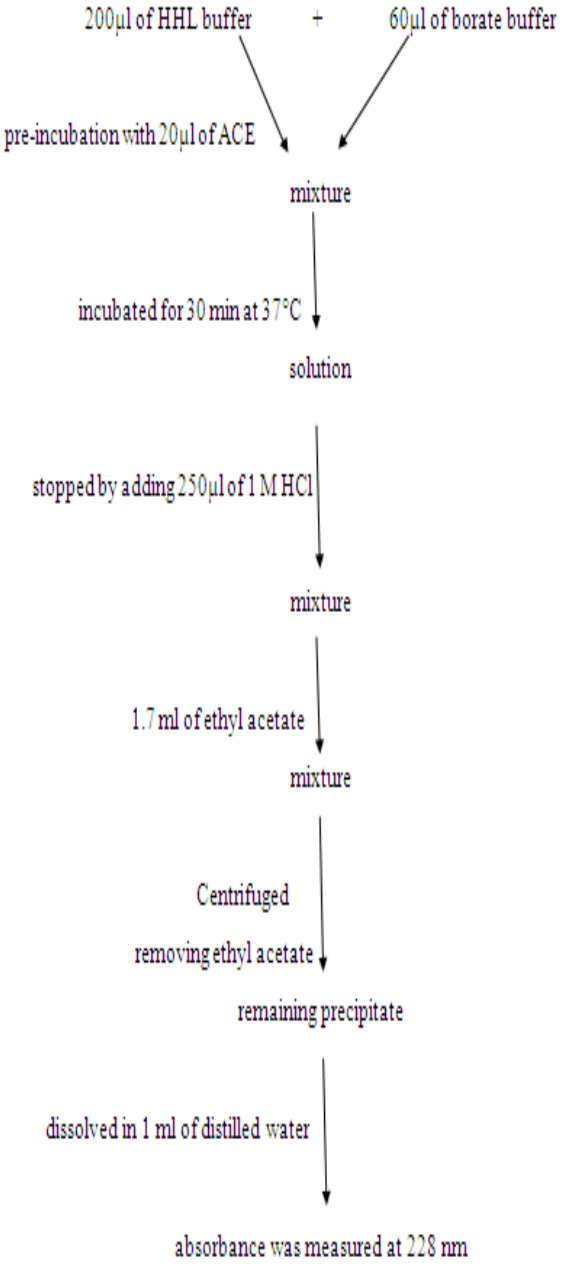

Figure 1: Schematic presentation of the ACE-inhibitory effect determination for the prepared skimmed-milk hydrolysate at different pHs *in vitro*.

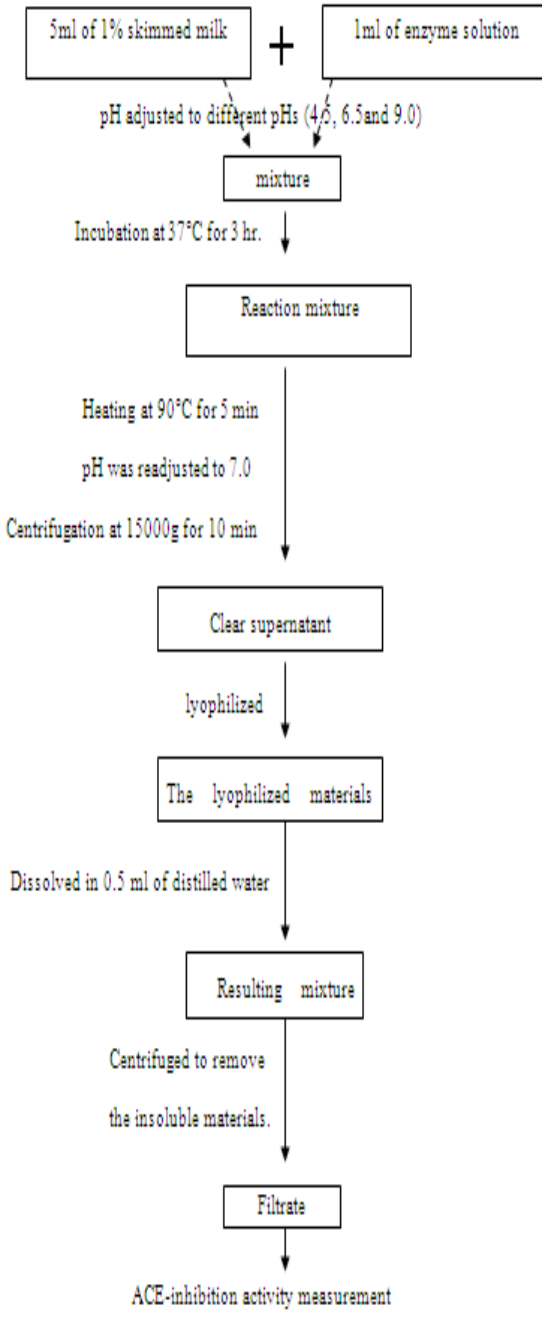

Figure 2: Schematic presentation of skimmed-milk hydrolysate preparation

Table 1: Name of the different screened seeds and their families.

No	Scientific name	English name	Family
1	*Brassica napus*	Rape	*Cruciferae*
2	*Raphanus sativus*	Radish	*Cruciferae*
3	*Eruca sativa*	Arugula	*Cruciferae*
4	*Brassica alba*	White mustard	*Cruciferae*
5	*Brassica nigra*	Black mustard	*Cruciferae*
6	*Coriandrum sativum*	Coriander	*Umbelliferae*
7	*Anethum graveolens*	Dill	*Umbelliferae*
8	*Apium graveolens*	Celery	*Umbelliferae*
9	*Petroselinum crispum*	Parsley	*Umbelliferae*
10	*Foeniculum vulgare*	Fennel	*Umbelliferae*
11	*Vicia faba*	Beans	*Leguminosae*
12	*Vigna unguiculata*	Cowpea	*Leguminosae*
13	*Trigonella foenum-graecum*	Fenugreek	*Leguminosae*
14	*Phaseolus vulgaris*	White bean	*Leguminosae*
15	*Lupines luteus*	Yellow lupin	*Leguminosae*
16	*Trifoluim L.*	Clover	*Leguminosae*
17	*Glycin max*	Soya bean	*Leguminosae*
18	*Tamarindus indica*	Tamarind	*Leguminosae*
19	*Lens culinaris*	lentil	*Leguminosae*
20	*Prunus armeniaca*	Apricot	*Rosaceae*
21	*Prunus domestica*	Prune	*Rosaceae*
22	*Lactuca sativa*	Lettuce	*Asteraceae*
23	*Triticum vulgare*	Wheat	*Gramineae*
24	*Echinochloa crusgalli*	Barnyard grass	*Gramineae*
25	*Panicum repens L.*	Torpedo grass	*Gramineae*
26	*Corchorus olitorius*	Corchorus	*Tiliaceae*
27	*Cucumis melo-flexuosus*	Snake cucumber	*Cucurbitaceae*
28	*Luffa aegyptiaca*	Egyptian Luffa	*Cucurbitaceae*

Table 2: Proteolytic activities (PA) in the extract of 28 dry seeds at different pH's.

			Proteolytic activity (PA)		
			in 0.1M acetate buffer, pH 4.5	in 0.1M phosphate buffer, pH 6.5	in 0.1M borate buffer, pH 9.0
No	English name	Family	(U/g dry seeds)	(U/g dry seeds)	(U/g dry seeds)
1	Rape	*Cruciferae*	0	0.53	0.34
2	Radish	*Cruciferae*	2.52	0	4.25
3	Arugula	Cruciferae	1.07	0.8	0.7
4	White mustard	*Cruciferae*	0	3.65	0
5	Black mustard	*Cruciferae*	0	0	0.54
6	Coriander	*Umbelliferae*	0.72	20.46	0
7	Dill	*Umbelliferae*	6	0	0
8	Celery	*Umbelliferae*	1.74	0	0.33
9	Parsley	*Umbelliferae*	0	1.11	2.55
10	Fennel	*Umbelliferae*	0	1.83	0

11	Beans	*Leguminosae*	2.71	0	0
12	Cowpea	*Leguminosae*	0	0.96	0.06
13	Fenugreek	*Leguminosae*	2.71	5.48	0
14	White bean	*Leguminosae*	0	0.96	0.17
15	Yellow lupin	*Leguminosae*	0	0	0
16	Clover	*Leguminosae*	0	0	0
17	Soya bean	*Leguminosae*	0	0	0
18	Tamarind	*Leguminosae*	0	0	0
19	Lentil	*Leguminosae*	0	2.1	0
20	Apricot	*Rosaceae*	1.73	2.11	0.88
21	Prune	*Rosaceae*	0	0	0
22	Lettuce	*Asteraceae*	4.17	0	3.37
23	Wheat	*Gramineae*	0.26	0	0
24	Barnyard grass	*Gramineae*	0.54	0.41	0.06
25	Torpedo grass	*Poaceae*	0	0.53	0
26	Corchorus	*Tiliaceae*	3	1.12	0
27	Snake cucumber	*Cucurbiaceae*	0	0	0
28	Egyptian luffa	*Cucurbiaceae*	0	0	0

Table 3: PA of the chosen eight dry seeds

PA								
No	At pH 4.5		No	At pH 6.5		No	At pH 9.0	
	English name	(U/g)		English name	(U/g)		English Name	(U/g)
2	Radish	2.52				2	Radish	4.25
3	Arugula	1.07						
			6	Coriander	20.46			
8	Celery	1.74						
						9	Parsley	2.55
			10	Fennel	1.83			
26	Corchorus	3.0	26	Corchorus	1.12			

Table 4: The ACE inhibitory effect of skimmed-milk hydrolysate at different pHs.

Seeds No.	English name	Inhibition activity of ACE (%) at pH 4.5	Inhibition activity of ACE (%) at pH 6.5	Inhibition activity of ACE (%) at pH 9.0
2	Radish	72	0.0	74
3	Arugula	0.0	0.0	0.0
6	Coriander	4	96	0.0
7	Dill	83	0.0	0.0
8	Celery	100	0.0	0.0
9	Parsley	0.0	0.0	82
10	Fennel	0.0	100	0.0
26	Corchorus	88	0.0	0.0

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