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Research Article

**EXTRACTION AND PURIFICATION OF POLY-DIGESTIVE  
ENZYMES FROM SPROUTED WHEAT AND *BACILLUS  
CEREUS* AS ALTERNATIVE THERAPY FOR CELIAC  
PATIENTS**Gamal A. Gabr<sup>\*1,2</sup>, Salwa M. El-Sayed<sup>3</sup> and Abd El-Hameed H. M<sup>4</sup><sup>1</sup>Pharmacology Department, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, KSA.<sup>2</sup>Agricultural Genetic Engineering Research Institute (AGERI), Agric. Res. Center, Giza, Egypt.<sup>3</sup>Biochemistry Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt<sup>4</sup>Food Technology Research Institute, Agricultural Research Center, Giza, Egypt**Abstract:**

*Celiac disease (CD) is a disease of the digestive system resulting from the intolerance of autoimmune system against gluten and gliadin proteins in wheat, barley, rye and some varieties of oats. The aim of this work is to extract and purify protease enzymes from sprouted wheat seeds and Bacillus cereus and to evaluate their ability for the hydrolysis of both gluten and gliadin. Data indicate that the highest purity of poly-digestive protease enzymes extracted from sprouted wheat and Bacillus cereus was obtained by 0.2M trichloroacetic acid and acetone 80%, respectively. Our findings showed synergetic effect of the mixture of proteases enzyme extracted from the two sources at 1 mg/ml to hydrolyze gluten and gliadin as compared to their individual activities as well as Digestin, a positive control.*

**Key words:** *Celiac disease, Proteases, sprouted wheat, Bacillus cereus, Gluten, Gliadin.*

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## INTRODUCTION:

Wheat is an important portion of the daily meals, consume in various forms and it has negative effects on some people who have sensitivity to gluten [1]. Celiac disease (CD) is a chronic intestinal malabsorption disease characterized by life-long intolerance to materials such as prolamins in wheat (gliadin), barley (hordein), rye (secalin), and possibly oats (avidins), due to genetic factors [2, 3]. It is characterized by severe immune damage to the intestinal mucosa, typically involves abdominal distension, weight loss, chronic diarrhea, severe malnutrition and osteoporosis [4-6]. CD affects approximately 1% of the world population [7]. Wheat gluten proteins are among the most studied of food proteins. They are divided according to their solubility properties into two major groups; gliadins and glutenins, the first one being involved in the celiac disease [8]. The epitopes of gluten are immune-stimulatory, these immune residues are 33 amino acids ranged from 57 to 89 amino acids sequences of  $\alpha$ -gliadin fraction which contains many repeating units of glutamine and proline residues [9]. Proline makes  $\alpha$ -gliadin residue resistant to the gastrointestinal proteolysis in some individuals with wheat sensitivity leading to CD. There is an increasing number of dietary enzyme supplements on the market that claim to effectively degrade gluten and in this way support gluten digestion in individuals suffering from gluten-related disorders [10, 11]. The mode of action of these enzymes is based on studies that have shown that the immunogenicity of gluten can be reduced by breaking down the gluten protein into smaller peptides [9, 12].

Proteases are produced by various plant, animal and microorganism sources, the latter are most widespread in nature and are preferred owing to their fast growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [13]. Among the bacteria, *Bacillus strains* are the most important producers of commercial extracellular enzymes, including proteases [14]. On the other hands, the plant products with wide safety index are good sources of novel therapeutic agents [15]. Thus, the researchers' attention shifted to use herbal medicine in the treatment of multiple diseases. So, the aim of this work is to extract and purify the protease enzymes from two different sources (germinated wheat seeds, and *Bacillus cereus*) and to evaluate their ability for the digestion of dietary gluten and gliadin.

## MATERIALS AND METHODS:

### Germination of wheat seeds.

Wheat grains were obtained from the local market. The grains were cleaned, dried in the sunlight and stored. Wheat kernels were placed on Whatman No. 3 filter paper in a 90-mm diameter Petri-dish saturated with 5.5 mL of distilled water. The Petri-dishes were covered with a polyethylene bag and kept at 25 °C for 7 days. The germinated seeds were collected and stored at -20 °C [16].

### Isolation of the microorganisms

Screening of proteases producing bacteria was done by using skimmed agar plate method according to the method of Castro and Cantera [17]. One gram of soil sample was diluted with 100 ml of sterilized distilled water. The agar plate with skimmed milk was inoculated with 0.1 ml of heated diluted soil sample and incubated for 24 hr at 37°C. The colony with clear zone was taken for further identification.

### Identification of protease producing bacteria

Protease producing bacteria was identified by observing the hydrolysis zone around the bacterial colony. One colony was chosen according to its clear zone size and transferred to new nutrient agar plate and incubated at 37°C for 48 hr for further identification on the basis of the morphological and biochemical test.

### Extraction of crude enzyme from sprouted wheat:

Four gram of germinated wheat grain were taken in a mortar and ground into fine powder, then mixed with 50 ml of phosphate-citrate buffer pH 6.9 (extraction buffer). The extract was overnight stirring, then the filtrate was centrifuged for 15 min at 10000 rpm for 15min. The clear supernatant containing enzymes was lyophilized and used as crude enzyme [18].

### Extraction of crude enzyme from *Bacillus cereus*:

The bacterial colony was grown in a medium containing 0.5% (w/w) glucose, 0.75% (w/w) peptone, 0.5% (w/w) MgSO<sub>4</sub>, 0.5% (w/w) KH<sub>2</sub>PO<sub>4</sub> and 0.01% (w/w) FeSO<sub>4</sub> (pH: 8.0) in orbital shaking incubator at 60°C for 72 hr. After incubation, the culture broth was centrifuged at 4°C and 3600 rpm for 30 min. The cell precipitate was discarded and the supernatant contain the enzyme was taken for further purification [19].

### Determination of protein content:

The concentration of total protein, gluten and gliadin were measured by the Bradford method [20]. The Bradford assay relies on the binding of the dye is a rapid Comassie Brilliant Blue G-250 to protein. 10  $\mu$ l

of protein fraction was added to 300 µl of Bradford reagent, incubated for 5 min and measured at 595 nm.

#### Assay of proteolytic enzymes activity:

The enzyme activity was determined according to the method of El-Beltagy et al., [21] by using casein digestion. Tyrosine which absorbs ultraviolet radiation at 280 nm was used as standard curve in the determination of protease activity. One unit of the enzyme activity is defined as the amount of enzyme that releases 1 mg of tyrosine per ml in 1 minute under the assay conditions. The specific activity was reported as U/mg protein and the percentage of recovered activity was calculated by dividing of the total activity of the enzyme fraction over total activity of crude extract multiplying by hundred.

#### Partial purification of protease:

This was achieved by salting out of the crude enzyme extract solution with ammonium sulphate, precipitation, acetone or TCA. The enzyme solution was prepared by dissolving amount of the lyophilized crude enzyme in 100 ml of sterilized distilled water. Solid ammonium sulfate was added slowly to the crude enzyme extract at saturation levels 40, 60, and 80% with gentle stirring in an ice bath overnight. The collected precipitates were dissolved in 10 ml of the extraction buffer (Shaha, 2002). The alkaline protease was collected from the crude extract by precipitation with 40-80% of cold acetone. The precipitate was dissolved in 0.1M phosphate buffer, pH 7.0 after centrifugation at 15,000 rpm for 15 min at 4°C [22]. The crude enzyme solution was treated with 2.5 M of TCA then centrifuged at 15000 rpm for 15 min. The pellet was dissolved in chilled acetone and again centrifuged at 14000 rpm for 5 min. The

obtained pellet was dissolved in 0.1M phosphate buffer at pH 7 [23].

#### Extraction of gliadin:

Gliadin was extracted by treatment of gluten with 70% ethanol and stirred for 45 min in ice bath, then centrifuged at 6000 rpm for 10 min [24].

**Gluten and gliadin hydrolysis:** Add 100 mg of the partially purified extracted protease to 100 ml of each of gluten and gliadin suspension solution, the mixture was incubated at 30°C for 2 h with stirring in ice bath. The hydrolyzed protein solution was heated for 30 second at 100°C to inactivated the protease [16].

#### Statistical analysis

The data were presented as means  $\pm$ SD from three replicates. Data were subjected to one-way ANOVA. The means of different treatments were compared using Duncan's multiple range test at  $p \leq 0.05$ . Statistical analyses were performed using SPSS statistical software (IPM SPSS statistical version 20) [25].

#### RESULTS:

Proteases and proteolytic enzymes constitute one of the most important groups of enzymes and are attracting worldwide attention in attempts to exploit their physiological and biotechnological applications. In this study, partial purifications and biochemical characterizations of a protease from sprouted wheat grains and *Bacillus cereus*.

#### Purification of protease enzymes

Results of partial purification of proteases from germinated wheat and *Bacillus cereus* by different concentrations of ammonium sulphate, acetone and TCA were illustrated in Table (1, 2 and 3).

**Table 1: Purification of protease extracted from germinated wheat and *Bacillus cereus* by ammonium sulphate**

	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> saturation (%)	Total Protein (mg)	Recovered protein (%)	Total activity (U)	Recovered activity (%)	Specific activity (U/mg protein)	Purification fold
Sprouted wheat	0	0.68 <sup>a</sup> $\pm$ 0.006	100.00	1430 <sup>a</sup> $\pm$ 3.055	100.00	2103 <sup>d</sup> $\pm$ 23.679	1.00
	40	0.10 <sup>b</sup> $\pm$ 0.009	14.70	285 <sup>d</sup> $\pm$ 1.637	19.93	2850 <sup>c</sup> $\pm$ 261.189	1.35
	60	0.07 <sup>c</sup> $\pm$ 0.003	10.29	345 <sup>c</sup> $\pm$ 1.154	24.13	4928 <sup>b</sup> $\pm$ 210.332	2.34
	80	0.07 <sup>c</sup> $\pm$ 0.001	10.29	390 <sup>b</sup> $\pm$ 0.929	27.27	5571 <sup>a</sup> $\pm$ 139.568	2.65
<i>Bacillus cereus</i>	0	0.34 <sup>a</sup> $\pm$ 0.054	100	1380 <sup>a</sup> $\pm$ 2.025	100	4059 <sup>b</sup> $\pm$ 638.788	1.0
	40	0.07 <sup>b</sup> $\pm$ 0.003	20.29	290 <sup>d</sup> $\pm$ 1.258	21.01	4143 <sup>b</sup> $\pm$ 167.230	1.02
	60	0.07 <sup>b</sup> $\pm$ 0.002	20.59	310 <sup>c</sup> $\pm$ 0.351	22.46	4429 <sup>b</sup> $\pm$ 141.694	1.09
	80	0.05 <sup>b</sup> $\pm$ 0.003	14.71	375 <sup>b</sup> $\pm$ 2.200	27.17	7500 <sup>a</sup> $\pm$ 501.951	1.85

Data represent the means  $\pm$  SE calculated from three replicates. Different letters refer to significant differences at ( $P \leq 0.05$ )

The results clearly indicated that the 80% ammonium sulphate fraction possessed the lowest protein level (0.07 mg), which representing 10.29 % protein recovery and exhibited the highest specific activity (5571 U.mg-1 protein) and 2.65 purification fold respectively. This was followed by the 60% fraction, which possessed 0.07 mg protein representing 10.29 % recovery and specific activity (4928 U.mg-1 protein) with 2.34 purification fold. The 40 % ammonium sulphate fraction had higher protein

contents (0.10 mg) and specific activity (2850 U.mg-1 protein). The highest purification folds were also noticed within 60 and 80 % of  $(\text{NH}_4)_2\text{SO}_4$  saturation, while the lowest purification folds were noticed within 40 %  $(\text{NH}_4)_2\text{SO}_4$  saturation. The crude enzyme extract from *Bacillus cereus* contain 0.34 mg of protein. The fraction precipitated with 80% ammonium sulphate showed the highest protease activity (375 U) which represents 27.17% of the recovered activity.

**Table 2: Purification of protease extracted from sprouted wheat and *Bacillus cereus* by acetone.**

	Acetone saturation (%)	Total Protein (mg)	Recovered protein (%)	Total activity (U)	Recovered activity (%)	Specific activity (U/mg protein)	Purification fold
Sprouted wheat	0	0.68 <sup>a</sup> ± 0.016	100	1430 <sup>a</sup> ± 3.055	100	2103 <sup>c</sup> ± 23.679	1.00
	40	0.07 <sup>b</sup> ± 0.002	10.29	260 <sup>de</sup> ± 2.081	18.18	3714 <sup>d</sup> ± 126.108	1.77
	60	0.06 <sup>b</sup> ± 0.010	8.82	224 <sup>c</sup> ± 2.227	15.66	3400 <sup>d</sup> ± 558.711	1.78
	80	0.06 <sup>b</sup> ± 0.001	8.82	474 <sup>b</sup> ± 2.565	33.15	7900 <sup>a</sup> ± 28.465	3.76
<i>Bacillus cereus</i>	0	0.34 <sup>a</sup> ± 0.054	100	1380 <sup>a</sup> ± 2.025	100	4059 <sup>b</sup> ± 638.788	1.00
	40	0.07 <sup>b</sup> ± 0.002	20.59	215 <sup>d</sup> ± 2.775	15.58	3071 <sup>c</sup> ± 101.181	0.76
	60	0.07 <sup>b</sup> ± 0.003	20.59	305 <sup>c</sup> ± 0.850	22.10	4357 <sup>b</sup> ± 257.828	1.07
	80	0.05 <sup>b</sup> ± 0.001	14.71	405 <sup>b</sup> ± 2.740	29.35	8100 <sup>a</sup> ± 386.193	2.00

Data represent the means ± SE calculated from three replicates. Different letters refer to significant differences at (P≤0.05)

The results show that, there were no significant changes among protein contents of the precipitated fractions with acetone at all concentrations. The 80% acetone fraction showed the highest total activity of protease enzymes (474 U) followed by 40% fraction (260 U). Also, the highest specific activity was recorded at 80% acetone precipitation (7900 U/mg protein) which represent 3.76 of purification fold. Also, the highest total activity of protease extracted from *Bacillus cereus* was 405 U which represents

29.35% of recovered activity and specific activity (8100 U/mg protein) at precipitation with 80% acetone which represent 2.0 of purification fold. acetone at 60 % (4357 U. mg-1 protein) was no significant with crude enzyme (4059 U. mg-1 protein). The purification with acetone at 40 % (3071 U. mg-1 protein) was significant decrease compared with crude enzyme (4059 U. mg-1 protein). While at 80% acetone fraction (8100 U. mg-1 protein) was the highest significantly increased.

**Table 3: Purification of protease extracted from germinated wheat and *Bacillus cereus* by TCA.**

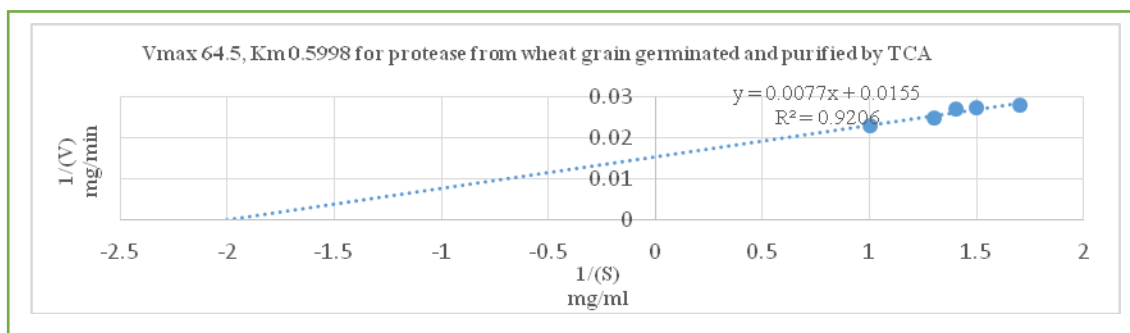
	TCA (M)	Total Protein (mg)	Recovered protein (%)	Total activity (U)	Recovered activity (%)	Specific activity (U/mg protein)	Purification fold
Sprouted wheat	0	0.68 <sup>a</sup> ± 0.016	100	1430 <sup>a</sup> ± 3.055	100	2103 <sup>c</sup> ± 23.679	1.00
	0.2	0.04 <sup>b</sup> ± 0.002	5.88	385 <sup>b</sup> ± 2.081	26.92	9625 <sup>a</sup> ± 217.206	4.58
<i>Bacillus cereus</i>	0	0.34 <sup>a</sup> ± 0.054	100	1380 <sup>a</sup> ± 2.025	100	4059 <sup>b</sup> ± 638.788	1.00
	0.2	0.05 <sup>b</sup> ± 0.005	14.71	325 <sup>b</sup> ± 2.651	23.55	6500 <sup>a</sup> ± 593.571	1.60

Data represent the means ± SE calculated from three replicates. Different letters refer to significant differences at (P≤0.05)

A precipitated fraction of protease extracted from *Bacillus cereus* and obtained by TCA contained low protein content (0.05 mg) compared with the crude enzyme (0.34 mg) which represented 14.71% protein recovery of the original protein, the recovered activity by 23.55% of the original activity, and high specific activity (6500 U.mg<sup>-1</sup> protein) compared to those of crude enzyme extract. Specific activity 0.2 M TCA (6500 U.mg<sup>-1</sup> protein) was significant increase compared with crude enzyme (4059U.mg<sup>-1</sup> protein).

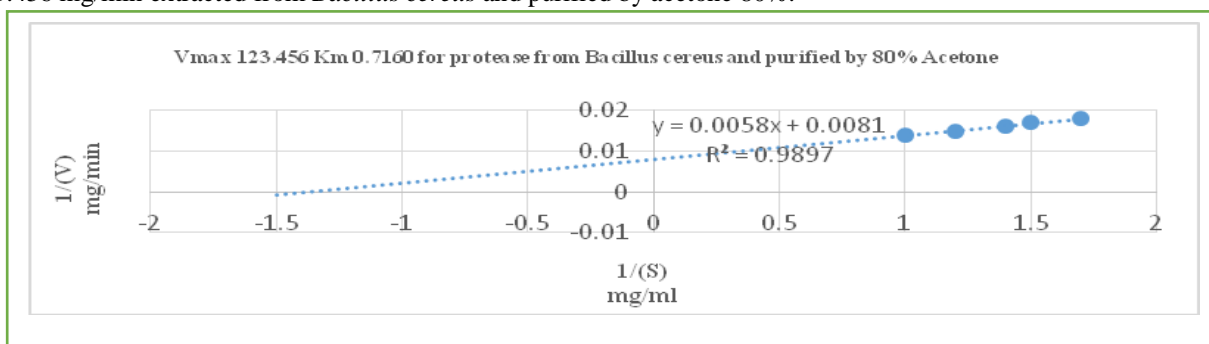
#### Kinetic studies on proteases which partially purified from different sources.

The data in Figs. 1 and 2 show the effect of substrate concentration on the enzyme activity for partial purified enzyme to estimate the kinetic constants, Km (Michaelis constant) and Vmax (the maximum reaction velocity) using the substrate bovine serum albumin (BSA). This was accomplished by the construction of Lineweaver- Burk plot. The Lineweaver- Burk plot declared that the Km value of protease enzyme was 0.5998 mg/ml and the Vmax was 64.5 mg/min extracted from wheat grains 7 day and purified by TCA.



**Fig. 1. Lineweaver- Burk plot show kinetic constants (Km and Vmax) of protease enzyme extracted from wheat grains germinated for 7 days and purified by TCA.**

The Lineweaver- Burk plot declared that the Km value of proteases enzyme was 0.7160 mg/ml and the Vmax was 123.456 mg/min extracted from *Bacillus cereus* and purified by acetone 80%.



**Fig. 2. Lineweaver- Burk plot show kinetic constants (Km and Vmax) of protease enzyme extracted from *Bacillus cereus* and purified by acetone 80%.**

#### Gluten hydrolysis with two proteases from sprouted wheat and *Bacillus cereus*.

The data in Table 4 shows the effect of the two different protease sources with concentration of 1 mg on gluten hydrolysis. The mixture of protease extracts from germinated wheat grains and *Bacillus*

*cereus* purified by acetone 80% gave the same percentage of protein hydrolyzed (35%) compared to that with the positive control Digestin. This might be ascribed to the combination of different types of protease enzymes which are cysteine and serine proteases.

**Table 4: Gluten hydrolysis after incubation with different protease sources.**

Protease Sources	Protein remaining (mg/ml)	Protein remaining (%)	Protein hydrolyzed (mg/ml)	Protein hydrolyzed (%)
Sprouted wheat purified by TCA [A]	3.292 <sup>a</sup> ± 0.035	82	0.712 <sup>a</sup> ± 0.035	18
<i>Bacillus cereus</i> purified by acetone 80% [B]	3.356 <sup>a</sup> ± 0.046	84	0.648 <sup>a</sup> ± 0.046	16
[A] + [B]	2.614 <sup>c</sup> ± 0.076	65	1.390 <sup>c</sup> ± 0.076	35
Digestin	2.602 <sup>c</sup> ± 0.034	65	1.402 <sup>c</sup> ± 0.034	35

\*Data represent the means ± SE calculated from three replicates. The name letters refer to significant differences at ( $P \leq 0.05$ ).

\*\*Gluten concentration before hydrolysis was 4.004 mg/ml.

#### Glialin hydrolysis with two proteases from sprouted wheat and *Bacillus cereus*.

The data in Table 5 shows gliadin hydrolysis after 2 hours of treatment with different protease sources with concentration of 1 mg. The mixture of protease extract from *Bacillus cereus* purified by acetone 80% and sprouted wheat grains purified with TCA gave

protein hydrolyzed (19 %) high than each individual enzyme which gave 10 and 9 % respectively. It could be observed that using of these binary mixtures of protease enzymes from different sources at enzyme concentration 1 mg/ml exhibit marked increasing of hydrolyzed gliadin than the value of the positive control Digestin.

**Table 5: Gliadin hydrolysis after incubation with different proteases.**

Protease Sources	Protein remaining (mg/ml)	Protein remaining (%)	Protein hydrolyzed (mg/ml)	Protein hydrolyzed (%)
Sprouted wheat purified by TCA [A]	2.274 <sup>a</sup> ± 0.013	90	0.256 <sup>a</sup> ± 0.050	10
<i>Bacillus cereus</i> purified by acetone 80% [B]	2.310 <sup>a</sup> ± 0.035	91	0.220 <sup>a</sup> ± 0.035	9
[A] + [B]	2.056 <sup>cd</sup> ± 0.005	81	0.474 <sup>cd</sup> ± 0.005	19
Digestin	2.128 <sup>c</sup> ± 0.001	84	0.402 <sup>c</sup> ± 0.001	16

\*Data represent the means ± SE calculated from three replicates. The name letters refer to significant differences at ( $P \leq 0.05$ ).

\*\*Gliadin concentration before hydrolysis was 2.53 mg/ml.

#### Free amino acids concentration after gluten and gliadin hydrolysis:

The data in Table 6 show the free amino acid concentration after 2 hours of treatment different protease sources with concentration 1 mg. The mixture of protease extract from *Bacillus cereus* purified by Acetone 80% and wheat grains germinated for 7 days purified by TCA gave almost

the same percentage of free amino acid concentration (0.020 and 0.022 mg/ml respectively), compared to that with Digestin. This mean the mixture of the two enzymes from wheat grains germinated for 7 days and purified by TCA, and *Bacillus cereus* purified by acetone 80% were hydrolyzed gluten to free amino acid. While Digestin was hydrolyzed gluten to peptides and free amino acids.

**Table 6: Free amino acid concentration in gluten hydrolyzed after 2 hours of treatment with different protease sources with concentration 1 mg/ml enzyme.**

Protease Sources	Free Amino acids (mg/ml)
Wheat grains germinated for 7 days and purified by TCA [A]	0.006 <sup>d</sup> ± 0.001
<i>Bacillus cereus</i> purified by Acetone 80% [B]	0.005 <sup>d</sup> ± 0.002
[A] + [B]	0.020 <sup>c</sup> ± 0.000
Digestin	0.022 <sup>b</sup> ± 0.001

\*Data represent the means ± SE calculated from three replicates. Different letters refer to significant differences at ( $P \leq 0.05$ ).

\*\*Gluten concentration before hydrolysis was 4.004 mg/ml.

**Table 7: Free amino acid concentration in gliadin hydrolyzed after 2 hours of treatment with different protease sources with concentration 1 mg/ml enzyme.**

Protease Sources	Free Amino acids (mg/ml)
Wheat grains germinated for 7 days and purified by TCA [A]	0.020 <sup>d</sup> ± 0.000
<i>Bacillus cereus</i> purified by Acetone 80% [B]	0.018 <sup>c</sup> ± 0.001
[A] + [B]	0.022 <sup>c</sup> ± 0.001
Digestin	0.025 <sup>b</sup> ± 0.001

\*Data represent the means ± SE calculated from three replicates. Different letters refer to significant differences at (P ≤ 0.05).

\*\*Gliadin concentration before hydrolysis was 2.53 mg/ml

### DISCUSSION:

The purification of protease enzymes extracted from wheat grains germinated for 7 days by ammonium sulfate at all saturation levels (40, 60 & 80 %) has significantly decreasing in protein content of enzyme extracts compared to that of crude enzyme extract and no significant differences among them in their protein contents. The highest specific activity was obtained in the 80% ammonium sulfate fraction (3500 U/mg protein) which represents 1.71 of purification fold. On the other hands, the protease enzyme extracted from *Bacillus cereus* and purified by 80% ammonium sulfate showed high protease activity (375 U) which represents 27.17% of recovered activity. These results are in agreement with Nadeemullah & Mulazhtar, [22] and Kumar et al., [26] who purified the crude extracellular alkaline proteases by fractional precipitation with ammonium sulfate. Similar observations was noticed by El-Safey and Abdul-Raouf [27] who purified the protease enzymes by ammonium sulfate at 80% saturation. In addition to, the protease enzyme extracted from the germinated wheat grains give the highest value of total activity (309 U) which represents 22.15 % of recovered activity at purification by 80% acetone and specific activity (7900 U/mg protein) which represents 3.76 of purification fold. These results are in accordance with the previously reported findings of Nadeemullah and Mukhtar [22] who found that the maximum specific activity of protease enzymes extracted from germinated wheat grains occurs with purification by acetone at 80% concentration. The highest total activity of protease enzymes extracted from *Bacillus cereus* was (405 U) which represents 29.35 % of recovered activity that was noticed with purification by acetone at 80%. The highest specific activity of protease enzymes extracted from *Bacillus cereus* was obtained in the 80% acetone fraction (8100 U/mg protein) which represents 2.00 of purification fold. These results are supported by previously conducted studies by Thengam & Rajkumar [28] who reported that acetone at 80%

concentration is the best agent to purify serine proteases extracted from larvae of red palm weevil because precipitation by acetone does not affect the enzymatic activity of serine proteases and does not cause denaturation of protease enzymes [29].

The specific activity of protease extracted from sprouted wheat and purified by TCA was 9623 U/mg protein and similar findings were published by Shrawan et al., [30] who found that the protease extracted with 0.1M phosphate buffer at pH 7 and precipitated with 0.2 M of TCA show good recovery of enzyme activity. Another study by Michalcová et al., [31] show that the germination process of wheat grains is accompanied by increasing of hydrolytic enzyme activities which includes cysteine proteases. Moreover, the specific activity of protease enzymes extracted from *Bacillus cereus* and purified by TCA was (6500 U/mg protein) which is higher than that of crude enzyme extract (4059 U/mg protein). These results are in accordance with Shrawan et al., [30] who found that the protease enzymes extracted with 0.1M phosphate buffer at pH 7 and then precipitated with TCA 0.2M show good recovery of their activity. From the previous results it can be concluded that the best sources of protease enzymes were wheat grains germinated for 7 days purified by TCA (0.2 M), and *Bacillus cereus* purified by acetone (80%). It is important to note that protease enzymes extracted from germinated wheat grains included cysteine proteases [31] while protease enzymes extracted from *Bacillus cereus* included serine proteases [28, 29]. It can be noticed that protease enzymes extracted from germinated wheat grains which included cysteine proteases were found purified by TCA (0.2 M) while protease enzymes extracted from *Bacillus cereus* which included serine proteases were found purified by acetone at 80% concentration.

The Michaelis constant  $K_m$  characterizes the affinity of the enzyme for a substrate. It corresponds to the substrate concentration at which  $V$  reaches half of

*V<sub>max</sub>*. A high affinity of the enzyme for a substrate leads to a low *K<sub>m</sub>* value, and vice versa [32]. A high *K<sub>m</sub>* means a lot of substrate must be present to saturate the enzyme, meaning the enzyme has low affinity for a substrate. On the other hand, a low *K<sub>m</sub>* means only a small amount of substrate is needed to saturate the enzyme, indicating a high affinity of the enzyme for a substrate [32]. The Lineweaver-Burk plot show that the *K<sub>m</sub>* and *V<sub>max</sub>* values of protease enzymes extracted from wheat grains germinated for 7 days and purified by TCA (0.2 M) were 0.599 mg/ml and 64.5 mg/min, respectively. While, the Lineweaver-Burk plot declared that the *K<sub>m</sub>* and *V<sub>max</sub>* values of protease enzymes extracted from *Bacillus cereus* and purified by acetone 80% were 0.716 mg/ml and 123.456 mg/min, respectively. These results are in agreement with Kezie et al., [32] who found that the *K<sub>m</sub>* and *V<sub>max</sub>* values of protease enzymes extracted from *Bacillus cereus* are 0.761 mg/ml and 2582 U/min, respectively. The kinetics and constants of protease enzymes extracted from the two sources demonstrate that the best source of protease enzymes was *Bacillus cereus* which exhibited the highest enzyme efficiency as indicated by the highest maximum velocity and the lowest Michaelis constant.

*Bacillus cereus* and germinated wheat grains showed percentages of gluten hydrolysis which is accounted by 16 and 18% respectively. This may be attributed to the different types of protease enzymes extracted from different sources whereas the type of protease enzymes extracted from wheat grains was cysteine proteases while the type of protease enzymes extracted from *Bacillus cereus* was serine proteases [33]. It can be noticed that increasing of gluten hydrolysis in case of protease enzymes extracted from germinated wheat grains (18%), while slight decrease in gluten hydrolysis in case of protease enzymes extracted from *Bacillus cereus* (16%). Using combined mixture of protease enzymes of the above mentioned sources for gluten hydrolysis at enzyme concentration of 1 mg/ml led to marked increase in hydrolyzed gluten (35%) which are comparable with hydrolysis observed with positive control, Digestin.

Protease enzymes extracted from the two sources were also examined to hydrolyze gliadin which is responsible for celiac disease. It is polypeptide consists of 33 amino acids with known sequence rich in proline and glutamine. These amino acids were reported to be responsible for resistance of this polypeptide for digestion by protease enzymes such as pepsin, trypsin and chemotrypsin [34].

At 1 mg/ml of proteases extracted from *Bacillus cereus* and germinated wheat grains, the hydrolyzed gliadin was less than Digestin but their combination produced higher percentage of gliadin hydrolysis. Our findings are also supported by the results of Scherf et al., [35] who found that the cysteine proteases represent the bulk of the activity of the wheat grains germinated for 7 days and is able to hydrolyze the gliadin to small peptides.

The hydrolysis of gluten and gliadin by protease enzymes produces only little amounts of free amino acids because most of these enzymes were reported to be endopeptidases [16]. It means that a lot of breakdown of peptide bonds by action of protease enzymes does not produce free amino acids and consequently cannot be measured by Ninhydrin method. Therefore, Ninhydrin method does not show the true efficiency of protease enzymes for protein degradation in contrary with Bradford method. The free amino acids liberated from gluten and gliadin by incubation with mixture of the two enzymes confirms their complete hydrolysis.

#### CONCLUSION:

It can be concluded that the mixture of proteolytic enzymes extracted from wheat grains and *Bacillus cereus* are able to hydrolyze gluten and gliadin with higher affinity as compared to their effect alone, hence can be developed for celiac disease patients in future.

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