

**Extraction and purification of gliadin from local variety wheat as specific antigen and using it in the enzyme linked immunosorbent assay (ELISA) as serological test diagnosis of celiac disease.**

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***Abstract***

Gliadin was extracted from local variety wheat (Ashuur) in which wheat grains were milled and sieved out and gluten was isolated by hand wash method then gliadin was extracted with alcohol, this crude gliadin was purified by gel filtration chromatography by sephadex G-100, then molecular weight and purity detected by Polyacrylamide gel electrophoresis and the results were four bands with molecular weights: 86245, 50118, 23307 and 12022 D.

Enzyme linked immunosorbent assay (ELISA) was used firstly to determine the optimum concentration of gliadin antigen with test serum by chequar-board, the chosen concentration was 2.5 µg/ml by using calibrators standards, controls and solutions of commercial kit (Pharmacia diagnostic kit).

sephadexG100  
50118 ,23307 ,12022  
IgG antigliadin antibody . 86245  
chequar-board (ELISA)  
1:100 2.5µg/ml

### ***Introduction***

Celiac disease or gluten sensitive enteropathy is an autoimmune inflammatory disease of small intestine (Ciccocioppo *et al.*, 2005; Nelson, 2002; Farell, 2002), the earlier name of this disease were nontropical sprue and idiopathic steatorrhea (Falchuk, 1991). Celiac disease is the end result of three processes that culminate in intestinal mucosal damage: Genetic predisposition, Environmental factor, Immunologically based inflammation (Marsh, 1992).

The autoimmunity involves plasma cells that produce immunoglobulin A (IgA) and immunoglobulin G (IgG) that are directed against a variety of autoantigens including tissue transglutaminase, calreticulin protein, enterocyte cells(Karska *et al.*,1995), endomysial cells( Maki *et al.*, 1991), and the immune process in celiac disease may be because of unmasking cryptic antigen in the small bowel which may share epitopes with other antigens present in other organs particularly in the endocrine glands, or tissue transglutaminase may generate epitopes breaking the immune tolerance (Molberg *et al.*, 1998).

Gluten induced inflammation in genetically susceptible persons and found that the majority of patients have HLA class II DQ2 and the minority has DQ8 (Nelson, 2002; Nikulina *et al.*, 2004; Sollid *et al.*, 2001).

Gluten is a mixture of individual proteins classified in two groups: prolamine and glutenin, the most troublesome component of gluten is prolamine (gliadin) in wheat and comprise 69% of total endosperm protein (Fassano and Hervath, 2000), gliadin is a strong protein, poorly soluble in neutral aqueous solution but soluble in alcohol and dilute ethanoic acid of low ionic strength or in solution of hydrogen bond breaking disrupting agent such as sodium dodecyl sulfate (Sahi, 1981).

Gliadin is extremely heterogeneous mixture of proteins that contain four major groups on the basis of electrophoresis mobility (Jones *et al.*,1959): alpha which was found to be implicated in the pathogenesis of the disease (Dieterich *et al.*,1997), beta, gamma was found that they contain intermolecular disulfide bond which links one part to another but omega

does not contain that, this mean they do not contain cystine or methionin in their primary structure, thus alpha beta and gamma gliadin defined as toxic, they contain these sequences:

-pro-ser-gin-gin- , -gin-gin-gin-pro- (pro:prolin, gin: glutamine, ser: serine), while omega did not contain these sequence (Skerrit *et al.*, 1990).

In study the on Spelta and wheat it was found that , because of large difference between different alpha type gliadin amino acids from common bread classes of gliadin suggest that all classes alpha, beta, gamma and omega are toxic (Shan *et al.*,2002).

Glutamine comprises one of each three amino acids(35%), and prolin is one of each seven amino acids( 20%), and have low amount of essential amino acid especially lysine(Sollid, 2002).

The peptide 33-mer alpha gliadin (56-88) was resistant to further break down by luminal protease and brush border enzymes due to its high prolin content comprise 13 of 33 residue are proline so this peptide is naturally formed by gastrointestinal digestion (Sollid, 2002; Qiao *et al.*, 2004), this gives the fact that this highly immunostimulatory peptide is an end product of the gastrointestinal proteolytic digestion and given its central role in the pathogenesis of celiac disease (Jarmon, 2005).

Prolin directly affected peptide binding by controlling peptide conformation and by acting as one preferred anchor residue into HLA-DQ2 peptide and also prolin control deamidation of glutamine by tissue transglutaminase which acts preferentially on sequences glutenine-x-proline (Sollid, 2002).

Anderson,(2000) detected 50-500 matches within gluten protein and 5-250 in horiden and 8-100 in secalin but non in avenin, this on the base that glutamic acid must be positioned in front of specific anchor position in the pocket of HLA-DQ2 .

Current understanding indicates that different gluten peptide are involved in celiac disease process some are toxic other are immunogenic; toxic peptide is able to induce mucosal damage when added in culture of duodenal mucosal biopsy (Marsh and Crowe, 1995), and in vivo in upper

and basal intestine (Howdel *et al.*, 1981). While immunogenic peptide when it is able to stimulate specific HLA-DQ2 or DQ8 restricted T cell derived from jejunal mucosa or peripheral blood of celiac patients (Ellis and Ciclitira, 2001).

### ***Materials and methods***

#### **Extraction and purification of gliadin:**

##### **Gluten extraction:**

We used variety Ashur of wheat grains were, which were milled by electric miller and sieved out. The flour oil was removed by normal hexane by soxhlet at 45C. Gluten was obtained by hand wash method (A.A.C.C., 1976).

##### **Gliadin extraction:**

Gliadin is alcohol soluble fraction (Chan and Bushuk, 1970), so 1gm of gluten was put in flask and in 10 ml 70% ethanol alcohol was added in flask on magnetic stirrer for 2 hours as, then centrifuged (3000g by Griffin Christ assembled in England). The supernatant was collected and frozen dried.

#### **Purification of gliadin by gel filtration chromatography:**

##### **Preparation of sephadex and column:**

Sephadex G100 of 20gm (Pharmacia Co. made in France) was dispersed in 1 liter of distilled water for three days then degases (Edward high vacuum in Britain) and poured into 2.5 x 80 cm Column and equilibrate with proper solution: 0.1N acetic acid, 3M urea and sodium azide 0.02% to avoid microbial growth (Khan and Bushuk, 1979). Sephadex length was 80 cm.

##### **Filtration process:**

Blue dextran 0.05g was used to count the void volume, 200 mg of gliadin was dissolved in 5ml of eluent (0.1N acetic acid, 3M urea) then filtrated by filter paper no.1 and injected slowly on the column wall, 3ml were collected every 10 minute, 100 tubes were collected and read on 280nm in Ultraviolet spectrophotometer (GBS scientific equipment UV).

**The dialysis:**

The proper peak was dialyzed by dialysis bag (M.W. cut off 12000-14000 fisher sci. co. USA) for 24 hours against distilled water, and then was frozen dried.

**Estimation of protein concentration:**

The protein concentration was estimated by the use of Ultraviolet spectrophotometer (GBS scientific equipment UV) with 280 nm and 260nm and according to the equation:

Protein concentration = (1.55 x absorbance at 280) – (0.77 x absorbance at 260) according to Hudson and Hay, (1989).

**Detecting the molecular weight of gliadin by SDS-polyacrylamide gel electrophoresis:**

Electrophoresis was done by using Disk polyacrylamide gel and sodium dodecyl sulfate according to Lammler, (1970), in which the alcohol soluble protein were dialyzed against distilled water then applied to electrophoresis.

The distance of stain and protein was measured by putting the gel on paper then by measuring the distance from upper surface of gel up to band zone.

Relative mobility (R<sub>m</sub>) = distance of protein moving/distance of bromophenol blue stain moving.

The molecular weight of standard protein and R<sub>m</sub> value was drawn as a relationship between R<sub>m</sub> and Log of molecular weight of standard proteins then R<sub>m</sub> of protein sample, after that molecular weight was measured.

**Enzyme linked immunosorbent assay technique:**

An enzyme linked immunosorbent assay (ELISA) for measuring antibodies responses (IgG antigliadin antibody) was established by checker-board of gliadin antigen which was locally purified in this study and the commercial kit (Pharmacia diagnostic kit made in Germany), the ELISA test was performed according to Stukus, (1997). as following:

A- The purified gliadin antigen by gel filtration and after dialysis, a different concentration (2.5, 5, 10, 15, 20) µg/ml in 100µl coating buffer of carbonate-bicarbonate pH=9.6 (Duguid *et al.*, 1975) was coated in which

100µl in each well of polystyrene microtiter plate (Nunc. intermed. co., Denmark) and incubated over night at 4C.

B- After shaking out, the plate was washed and flooded with washing buffer(Phosphate buffer saline PBS containing bovine serum albumine BSA , 0.095% (w/v) sodium azide and detergent) to block uncoated sites and reduce the non specific binding .This procedure was repeated three times for 3-5 minutes.

C- The wells were coated with 62.5µl of blocking buffer: (phosphate buffer saline 0.1M, pH=7.2, with 0.3% tween 20 from(BDH chemical Ltd. Poo, England) for 1 hour.

D- The plate was washed as in stage (B).

E- 100µl of controls (positive and negative) and gliadin IgG antibody calibrators at concentrations of (0, 3,7,16, 40, and 100 U/ml in BSA, 0.095% w/v sodium azide , detergent and human serum) were added to each gliadin concentration for 1 hour.

F- The plate was washed as in stage (B), and then conjugate (anti-human-IgG-horse radish peroxidase) of 100 µl was added in room temperature for 30 minutes.

G- The plate was washed as in stage (B), the enzyme bound to the wells assayed by adding 100 µl of substrate 3, 3', 5', 5'-tetramethyl benzidine in dark place for 30 minutes.

H- The reaction was stopped by the addition of 100 µl of stopping solution: 0.5M H<sub>2</sub>SO<sub>4</sub> then read at 450 nm by (Biokit reader ELX800).

### *Results and discussion*

#### **Extraction and purification of gliadin:**

#### **Purification of gliadin by gel filtration chromatography:**

Crude gliadin was applied to gel filtration in order to purified gliadin, the result was four peaks as seen in figure (3.1), the first peak (I) appear in the void volume, the second peak (II) and the third one (III) is gliadin peak according to Sahi and Moore, (1998), and Sahi, (1981). So (II and III) peaks which were involved tube number 38 to 72 were dialyzed against distilled

water to eliminate eluent buffer. Sephadex G-100 was used in gel filtration according to Beitz and Wall, (1972), and Sahi and Moore, (1998).

#### Estimation of gliadin concentration:

The result of protein concentration was 0.356 mg/ml according to the protein concentration equation.

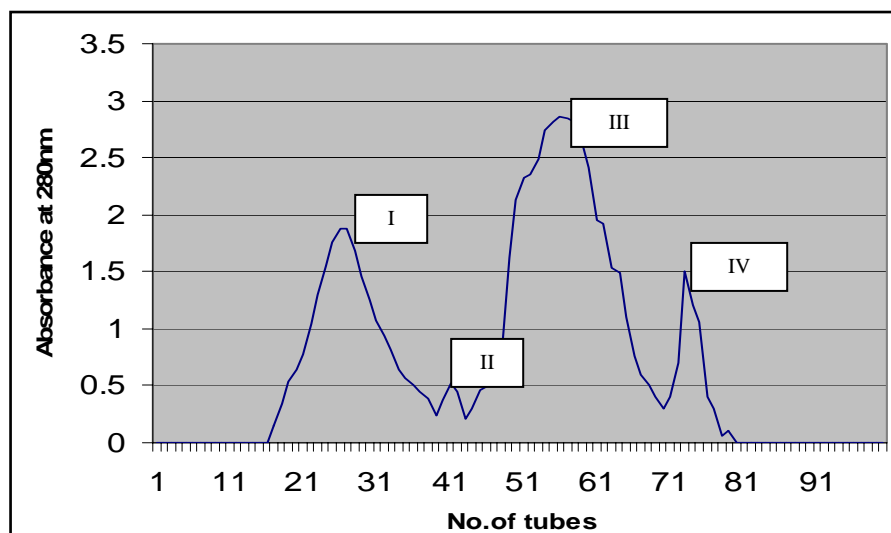
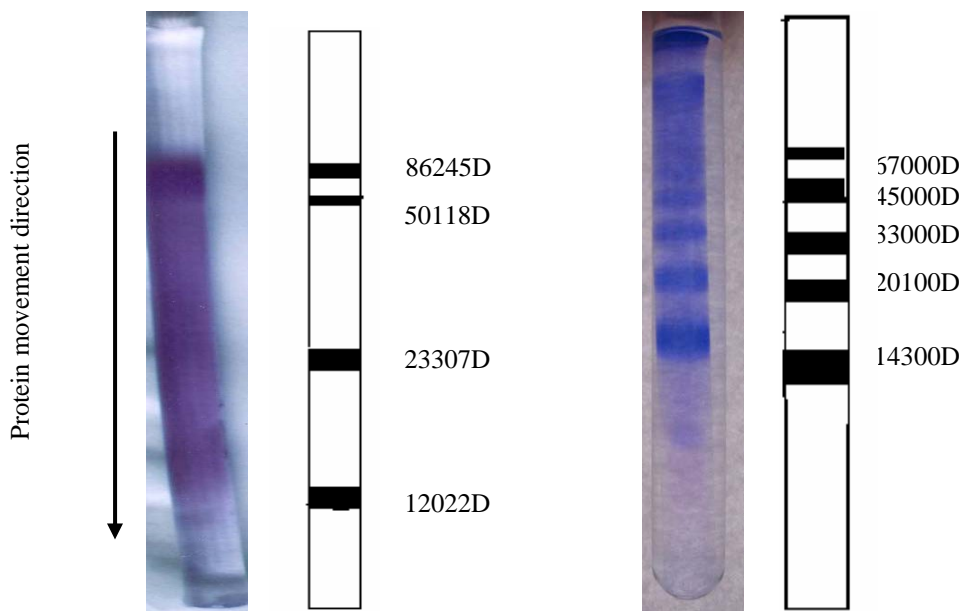


Figure (1): Gel filtration result for alcohol soluble fraction.

#### SDS-polyacrylamide gel electrophoresis:

The result of electrophoresis of gliadin was four bands as appeared in figure (2A) their molecular weight: 86245, 50118, 23307 and 12022 D this was estimated by using standard proteins in the electrophoresis as in figure (2B). Figure (3) show the result of standard proteins as a relationship between Log of molecular weight and relative mobility.

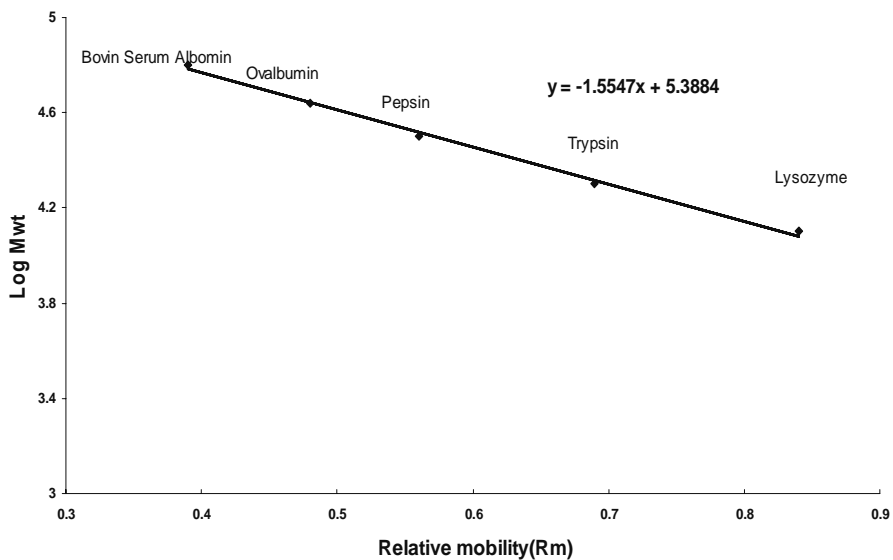
The result of molecular weight agrees with the molecular weight gained by Sahi and Moor, (1998) between 67000-100000D, and agrees with Jankiewicz and Pomeranz, (1965) between 15000-150000D. Electrophoresis was used to find out the molecular weight and gliadin purity and the results were confirmed this.



A- Gliadin protein

B- Standard proteins

**Figure (2): The electrophoresis result.**



**Figure (3): The electrophoresis result of standard proteins as a relationship between of Log of molecular weight and relative mobility (y=-1.5547 X + 5.3884).**



**Enzyme linked immunosorbent assay (ELISA):****Chequer-board:**

Different gliadin concentrations were used (2.5, 5, 10, 15, and 20)  $\mu\text{g/ml}$  against calibrators and controls of commercial kit for measuring IgG antibodies response and was read on 450 nm.

Gliadin concentration 20  $\mu\text{g/ml}$  was given a linear shape relationship between absorbance and concentration with calibrators (figure 4:E) and a high absorbance reading with calibrator (6) as seen in table (1) which was (1.266) and was gave a differential reading between negative (0.370) and positive (0.757) controls, the concentration 15  $\mu\text{g/ml}$  of gliadin gave a differential reading between negative (0.358) and positive controls (0.831) and was given a high reading with calibrator (6) as appeared in table 1 which was (1.306) and a linear relationship between absorbance and concentration with calibrators (figure 4:D), also 10  $\mu\text{g/ml}$  of gliadin concentration gave a differential reading between negative (0.308) and positive controls (0.941) and was given a high reading with calibrator (6) which was (1.329) as illustrate in table (1) and gave a linear shape as relationship between absorbance and concentration with calibrators (figure 4:C), with 5  $\mu\text{g/ml}$  of gliadin concentration was given a differential reading between negative (0.305) and positive (0.976) controls as illustrate in table 1 and was given a high reading with calibrator 6 and was given a linear shape as relationship between absorbance and concentration with calibrators (figure 4: B).

The concentration 2.5  $\mu\text{g/ml}$  of gliadin was gave the most discriminative reading between negative (0.269) and positive (1.068) controls as illustrate in table (1) and was gave the highest reading with calibrator 6 among other concentrations of gliadin and was given a standard linear shape as a relationship between absorbance and concentration with calibrators as seen in figure 4:A which most similar the standard linear relationship between absorbance and result with calibrators that is given by commercial kit (figure 4: F) than other concentrations of gliadin. So gliadin concentration 2.5  $\mu\text{g/ml}$  was the most suitable concentration for ELISA.

The absorbance was become lower with increasing of gliadin concentration and this because unsuitable coating of gliadin which make a competition of antibodies on the antigen.

ELISA regard as one of the best tests according to immunity response and sensitivity in evaluating the antibodies in nanogram (Chart *et al.*, 1995), is generally more cost effective by subjectivity in visual detection of immunoflorescence (Garcia-Carega and Kerner, 2004). And the scientific researchers which deal with ELISA look after the lower concentration which gives a high reading with test sera by using chequar- board to find the optimum concentration of the antigen to use in ELISA such as Issa, (1997) and Al-Tememy, (2001), so this concentration of 2.5 µg/ml appeared to be enough to coat and saturate the wells of ELISA microtiter plate, and this concentration has economic benefit according to lower antigen concentration.

Mesurement of antigliadin antibodies has been well standardized as compared to current assays for antitissue transglutaminase and antiendomysial antibodies because the false positive tests of tissue transglutaminase antibody may be seen in patient with autoimmune liver disease, however its less specific (www.mayo.edu., 2001). Also IgA antigliadin, IgA antiendomysial is negative in patients with IgA deficiency which is associated with celiac disease (Brown *et al.*, 1995), and the IgA antiendomysial antibodies disappears within several months after institution of gluten free diet, and IgA antigliadin antibodies more persistent than IgA antiendomysial; may persist for 6 months after gluten free diet (Nelsen, 2002).

But IgG antigliadin antibody most persistent may be detectable up to 12 months after institution of gluten free diet but the false positive cases could be seen in patients with Croh'ns disease and food allergy(Mosses, 2002). ELISA with antigliadin antibody is important because the changes in small intestine could be due to protracted viral enteritis or slow regression of virus which induces immune reaction so endoscopic diagnosis may give false results (Goldstein, 2004).

**Table (1): ELISA absorbance results of controls and calibrators against different concentrations of gliadin.**

Controls	Gliadin concentration				
	2.5µg/ml	5µg/ml	10µg/ml	15µg/ml	20µg/ml
Cal.0	0.204	0.216	0.243	0.224	0.254
Cal.3	0.286	0.286	0.342	0.406	0.314
Cal.7	0.377	0.312	0.341	0.319	0.328
Cal.16	0.632	0.527	0.551	0.413	0.468
Cal.40	0.916	0.742	0.746	0.74	0.769
Cal.100	1.649	1.361	1.329	1.306	1.266
Negative	0.269	0.305	0.308	0.358	0.370
Positive	1.068	0.976	0.941	0.831	0.757

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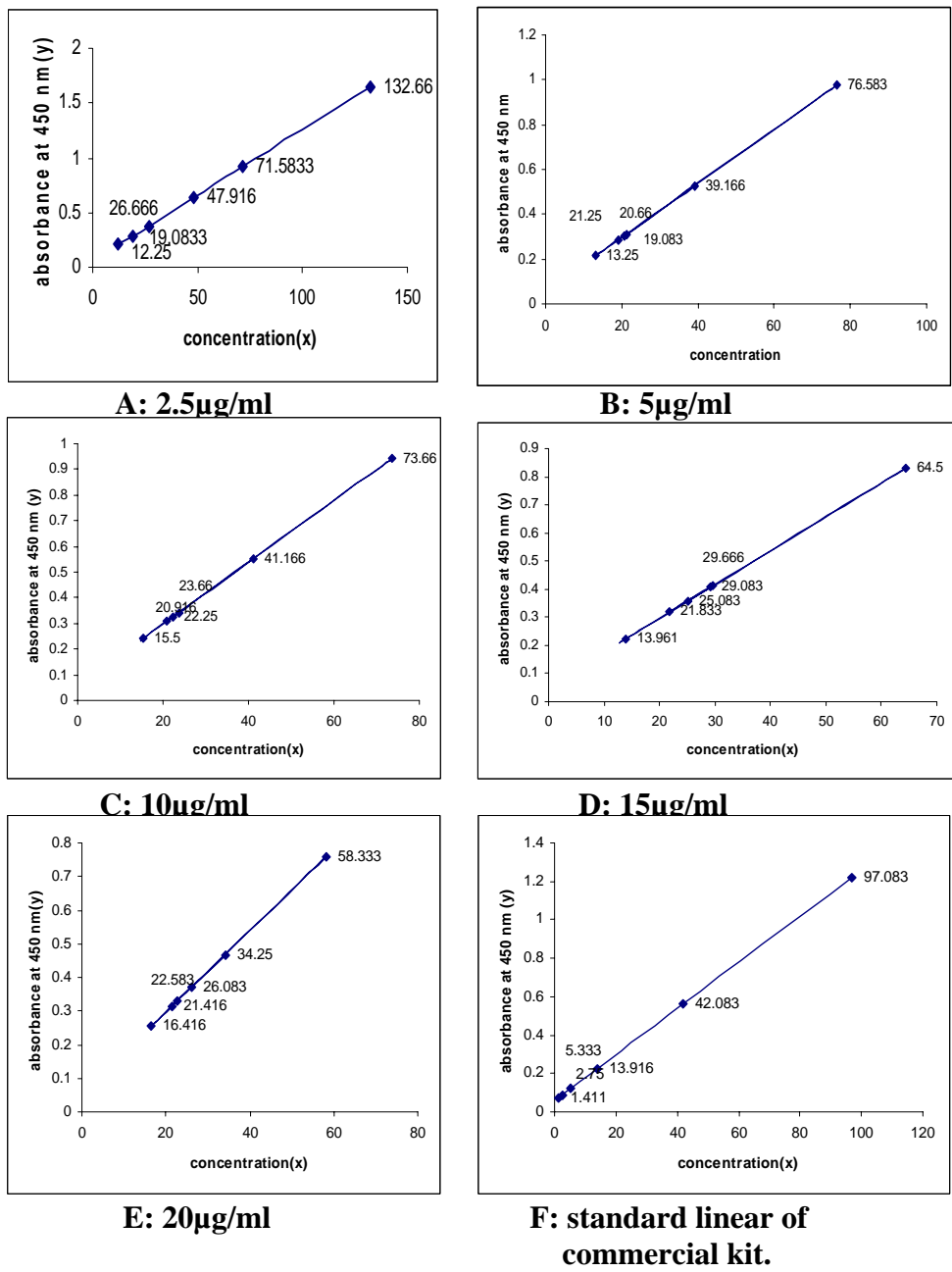


Figure (4): The effect of locally purified gliadin concentration coated the wells of microtiter plate on calibrators as a relationship between absorbance and concentration according to the equation  $y=ax + b$ ;  $a=0.012$ ,  $b= 0.057$ ,  $R-Sqr: 0.9976$ ,  $y$ : absorbance,  $x$ : concentration, (A: 2.5µg/ml, B: 5µg/ml, C: 10µg/ml, D: 15µg/ml, E: 20µg/ml, F: standard linear shape of commercial kit).