Salivary Ceruloplasmin Activities Ferroxidase & Oxidase in Celiac Patients

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Abstract

Aim of study : of the current study was to evaluate salivary ferroxidase ceruloplasmin activities in celiac patients with different histopathological severity. **Material and methods:**This study included 75 celiac patients with different mean age (18.68± 11.13) year, who had positive screen for celiac antibodies, and who had gastrointestinal symptoms. In order to simplify the comparison with the healthy control group, celiac patients were divided into two groups according to their histopathological severity: severe (marsh Ilia, b,c) & less severe (marsh 0,1) . All these patients have been evaluating for salivary ceruloplasmin (Cp) concentration and Cp ferroxidase activities. To confirm this activity, polyacrylamide gel electrophoresis was carried out and then stained for Cp ferroxidase as well as for Cp oxidase activity. Furthermore, the concentrations of salivary total protein, albumin, and globulin were measured too.**Results:** A significant increase ($p<0.05$) in salivary concentration of ceruloplasmin was found in all above mentioned patients groups in comparison to that of the control group, except for total villous atrophy (marsh IIIc) patients group. Salivary Cp ferroxidase activity revealed statistically significant decrease among the patient groups as well as between them and the control group. As far as salivary total protein and globulin showed signif icant increase ($p<0.05$) in comparison to that of the control groups. While albumin levels indicated non-significant increased.

Key words:

saliva, ceruloplasmin, celiac disease, mucosal histopathological damage, electrophoresis.

Introduction

Proteins play a central role in cell functions and cell structure; they are classified according to their biological functions (Leninger, 2005). The knowledge of body fluid proteins and their alternations in health and disease has grown rapidly, some of the alternations have a genetic origin, and many more reflect physiological or pathological processes (Grizzle et al., 2003). Saliva contains proteins, in concentration of approximately 3% of plasma protein level (Edgar,1992).Human saliva proteins can be informative for disease detection and surveillance of oral health (Hu, et al., 2007). Saliva has been studied for several pathological conditions, such as celiac disease CD(Lanander-Lumikari, 2000) ,Celiac disease (CD)also known as a celiac sprue and gluten-sensitive entropathy, it is an immune-mediated disorder that primarily affects the gastrointestinal tract (GI),

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characterized by chronic inflammation of the small intestinal mucosa causing villous atrophy, and malabsorption of nutrients after the ingestion of gluten and its related peptides, in the form of wheat, barley and rye cereals (Catassi, 2010).The clinical manifestations of CD are changeable in nature and vary markedly with the age of the patient, the duration and extent of disease, and the presence of extraintestinal pathological conditions (Corazza , *et al.* 1995; Cattassi , *et al.* 1997; Richard , *et al.* 2001). In addition, to the classical gastrointestinal form, a variety of other clinical manifestations of the disease has been described, including atypical and asymptomatic forms (Trier , *et al.* 1998). The keystone treatment of CD patients is a lifelong elimination diet in which food products containing gluten are avoided (Alessio , 2001; Al Tintas *et al.*,

2008).Severeal antioxidant maneuvers aim at modifying the oxidative status in CD patients like Ceruloplasmin (Cp) (Ferroxidase; Iron (II):02 oxidoreductase, EC 1.16.3.1); the major blue copper containing glycoprotein (Holmberge, 1948). It is a major enzymatic contributor to the antioxidant defense system of human plasma. It acts as an antioxidant by severeal mechanisms (Zowczak *et al.*, 2001; Cogalgil & Taysi, 2002; Taysi *et ah,* 2002) inhibiting iron-dependant lipid peroxidation and OH formation from hydrogen peroxide by its ferroxidase activity (Zowczak *et al.*, 2001; Shakour-Shahabi, et.al., 2010),reacting and scavenging H202 and superoxide anion, and inhibiting copper-inducedlipid peroxidation by binding copper ions (Zowczak *et al.*, 2001; Taysi *et al.*, 2002). According to the literature over 90% of human copper is associated with Cp as a non dialyzable fraction and the remaining 5-10% of plasma copper is believed to be fairly loosely attached to albumin and histidine, and only traces of copper is present as free Cu++ (Burtis & Ashwood, 1999). Because of its oxidase activity, Cp is also known as copper oxidase and this activity can be used for measurement of Cp. Ceruloplasmin performs its ferro-oxidase activity at the cell surface by binding of iron to transferrin which is the first step in the transformation of Fe++ to Fe+++. Serum Cp level was reported to increase during sport, pregnancy and estrogen supplement, as well as in states such as infections, malignities, Hodgkin's disease and cholangitis. While a decrease in this level was reported in malnutrition and malabsorbtion states, nephrotic syndrome, and primary biliar cirrhosis. (Beshgetoor & Hambidge, 1998; Shakour-Shahabi, et.al., 2010). While salivary Cp level was only reported to increase in oral epithelial tumors (Grant et.al. 1988; Daoud, 2008).The involvement of the oral biochemical changes in CD has not attracted much attention, although the mouth is part of the gastrointestinal system. (Tomasi, et al, 1980).

Materials and Methods

Inclusion criteria: A total of 75 cases with different chief complaints and presentation like chronic diarrhea, bloating, chronic abdominal pain and short

stature or if they were positive for a CDantibody screen were included in this study. These patients attended to the center of Gastroenterology and Hepatology, they were referred from different hospitals in Baghdad and other governorates in Iraq during the period of May 2010 to June 2011.The age of these patients ranged from 2 year to 43 years, all patients were subjected to a personal interview using especially designed questionnaire format full history with detailed information (age, sex, symptoms, autoimmune diseases, gluten diet if intake).The control group consisted of 46 apparently healthy individuals who matched in age and gender with patien

ts, and had no history of any gastro-intestinal problem (from the friends and relatives), which refused to subject to Oesophago-gastro-duodenoscopy (OGD).**Endoscopic Biopsy** A minimum of 3 biopsies were taken from different sites of the distal part of the duodenum, further examination of the duodenum, stomach and Oesophagus were performed. Histological analyses of the biopsies were carried out by two blinded expert pathologists while withdrawing the scope, the biopsies were placed in 10% formalin in a ground glass tube (universal tube) (Richard *et al.* ,2002).The diagnosis of CD was based on the presence of villous atrophy (total, subtotal or partial) with increased intraepithelial lymphocyte (IEL) counts on initial endoscopic biopsies. These histological analyses were scored according to the Marsh 1992 classification (Marsh. 1992) revised in 1997: [Marsh Ilia (partial villous atrophy), Marsh Illb (subtotal villous atrophy), and Marsh IIIc (total villous atrophy)] (Rostami et al. 1997).

Collection and treatment of saliva samples: Unstimulated, whole, mixed-saliva samples of 1 to 5 ml. were collected on ice, under resting conditions in a quiet room between 8.0-9.0 A.M. Patients and healthy were asked to rinse their mouth with normal saline and to generate saliva in their mouth and to spit into a plastic container for 10 minutes. After collection, the saliva was immediately centrifuged at (2000 xg) for 10 minutes at 4°C. The resulting supernatant was stored frozen at - 34°C in eppendrof tubes until assayed.The crude saliva samples were concentrated by using two different methods in order to use it in Cp Ferroxidase activity as well as in the electrophoresis technique. 1-By using dialysis bag: A volume of 27 ml of crude saliva (saliva was pooled separately to each group under study) were concentrated in dialysis bags (MWCO = 8-14 KD) using pure sucrose as a dehydrant. 2-Dry Sephadex G-25 medium(Saul, A. 1984): (0.042gm) of dry Sephadex G-25 was weighed in a 1.5ml Eppendorf tube which was previously pierced the base of the tube carefully by a fine needle, the resulting hole is small enough to ensure that Sephadex powder is unable to leak from the bottom of the tube. The eppendorf tube was mounted on a vial, and both the eppendorf tube and the vial was put into plastic centrifuge tube, 0.15 ml sample was added to the sephadex powder, and left at (4°C) for lOmin allowing the sephadex to swell.

Then the assembly was centrifuged for 2 min at (1000xg). The concentrated saliva was collected in the lower vial.

Determination of the different enzymatic activities of ceruloplasmin:- The oxidase activity of Cp was determined using the modified Rice method (Rice, 1962) where Cp catalyzes the oxidation of p-phenylenediamine (which was used as a substrate) to give blue-violet color that measured at X = 540 nm. Salivary Cp ferroxidase activities were determined, in term of the decrease in the concentration of the substrate (ferrous ion) upon its incubation with the enzyme & as described by Erel, 1998.

Evidence for oxidase and ferroxidase ceruloplasmin activities in saliva: 1- Since 1.0 mM azide inhibits Cp more than 98%. The Cp activity of each form has been evaluated by conducting the oxidase and ferroxidase assays in the presence and absence of 1 mM azide on the conventional polyacrylamide gel-electrophoresis. 2- Visible absorption spectrum: Visible absorption spectra of Cp ferroxidase in 0.05 M acetate buffer, pH = 5.5, at 30". One milliliter of the concentrated saliva (specific activity = 0.36) was placed in a 1.5-ml quartz cuvette and the visible absorption spectrum recorded. A second spectrum was recorded with the enzyme solution containing 1.0 mM sodium azide. A third spectrum was recorded with the enzyme solution containing only 19 uM ferrous ammonium sulphate. A fourth spectrum was recorded with the enzyme solution containing 19 uM ferrous ammonium sulphate then 1.0 mM sodium azide. A fifth spectrum was recorded with the enzyme solution containing 1.0 mM sodium azide then 19 uM ferrous ammonium sulphate (Topham and Friden, 1970).

Protein Determination: The salivary total protein concentrations were determined by using modified Lowery method by Hartree (Hartree. 1972). Bbovine serum albumin (BSA) was used as standard& the Protein concentration was expressed in g/1. while salivary albumin concentrations were estimated by the method employing bromocresol green as described by Doumas *et al.* (1971).

Polyacrylamide Gel-Electrophoresis (PAGE): Contintinuous Polyacrylamide Gel-Electrophoresis was performed on 7.5% polyacrylamide separating gel by using LKB Electrophoresis (LKB power supply 2197, LKB multiphor 2117 & multitemp 2209) (Amersham, 1999) to separate proteins and glycoproteins. While Discontintinuous Polya crylamide Gel-Electrophoresis (Lammeli) was used for Cp ferroxidase activity determination by using Pharmacia Gel Electrophoresis Apparatus GE-2/4 LS. Laemmli's gel system without SDS has been used for native PAGE.

The polyacrylamide gel was stained for proteins, using Coomassie Brillant Blue G-250 (CBB) by the method that described by [Neuhoff et al., 1988], stained for Cp oxidase activity using PPD (P-phenylene diamine) substrate (Schen et al,. 1966), stained for Cp ferroxidase activity using Fe (II) as substrate (Kim et al,. 2001 and Chen et al,. 2004).

Statistical Analysis:

The data were analyzed by Duncan's multiple range test at ($p < 0.05$) was accepted as statistically significant, and highly significant when ($p < 0.001$), using the SPSS software. All the analyses were repeated three times.

Results:

The mean ages of the patients included in the current study were 14.58 ± 9.77 year for more severe histopathological celiac group (marsh Ilia, b, c), 17.807±11.707 year for non & less severe histopathological celiac group (marsh 0,1), and 15.80 ±10.32 for the control group,

The sex distribution of patients shows a statistical difference between the female (61%) and the male (3 9%). Mean while only 26.6% of all patients were in gluten free diet (GFD), It is worth to mention that most celiac patients included in the present study were found to be at stage III (82%) ,with the higher ratio of 38% in marsh Illb , then 31% in marsh Ilia, and 13% in marsh IIIc; while the percentage of marsh I and 0 were 7% and 11% respectively.Table 1 showed the mean value of total protein concentration in saliva samples, and revealed a significant increase ($p = 0.035$). Among the groups, in the same table, the levels of salivary albumin showed non- significant increase ($p>0.05$). While salivary globulin indicated a significant increase ($p=0.00$). Table 2a revealed a significant increase in the mean value of salivary Cp concentration (mg/dl) for patients groups in comparison to the control group. While Table 2b that illustrate the Cp concentrations in patient subgroups indicate a significant increase for partial and subtotal villous atrophy celiac patients (marsh Ilia, b) and less histopathological mucosal change marsh (0.1) patients group, and non significant differences for total villous atrophy (marsh IIIc) celiac patients group.In order to detect the differences in total protein present in the studied groups, conventional polyacrylamide gel electrophoresis (PAGE) was carried out for control and celiac patient groups (each pooled saliva samples contributed equally to the protein content 2.0 mg/ml) as in Figure 1.In Table 3 both of the volumes and protein concentrations of the initial crude and final concentrated saliva were obvious in order to utilize in Cp ferroxidase activity determination as obvious in Tables (4).

Figure 2 showed stained Cp ferroxidase activity using Fe (II) as substrate in the saliva of the studied groups by using tubular polyacrylamide gel electrophoresis apparatus (Pharmacia Gel Electrophoresis Apparatus GE-2/4 LS) after assembly constant protein content (4.12 mg/ml) in each tube. As an evidence for oxidase ceruloplasmin activities in saliva Figure (3a) shows the electrogram of the ceruloplasmin oxidase activity when the staining of the gel were carried out by using PPD as a substrate (13.8 mM) in the presence of 1 mM sodium azide, while Figure (3b) shows when the staining were carried out in the absence of sodium azide. From the comparison between the activity bands in the zymogram of the studied groups, it is clear that one purple band was demonstrated in each crude saliva samples of the studied groups. While no band was detected when the staining was carried out in the presence of sodium azide. As obvious in Figure 4 the visible absorption spectra emphasized the present of Cp ferroxidase in saliva and no other enzyme have the same affinity to ferrous substrate because sodium azide binds copper in ceruloplasmin and inhibits it's ferroxidase activity completely (95%-99%) (Topham and Frieden, 1970; Erel, 1998).

Table 1: Mean laboratory values for salivary total protein, albumin and globulin with standard deviations in patients and control groups.

		Celiac Patients				Control group	P Value
		Marsh III A	Marsh III B	Marsh III C	Marsh0,1		
Saliva	Protein g/l(±SD)	2.34±0.75	2.3U0.71	2.04±0.77	2.26±0.88	2.00±0.68	0.035
	Alb. g/l(±SD)	0.33±0.18	0.32±0.15	0.28±0.08	0.28±0.19	0.28±0.12	0.186
	Glo. g/l(±SD	2.03±0.55	1.99*0.61	1.79±0.50	1.98±0.40	1.72±0.48	0.30

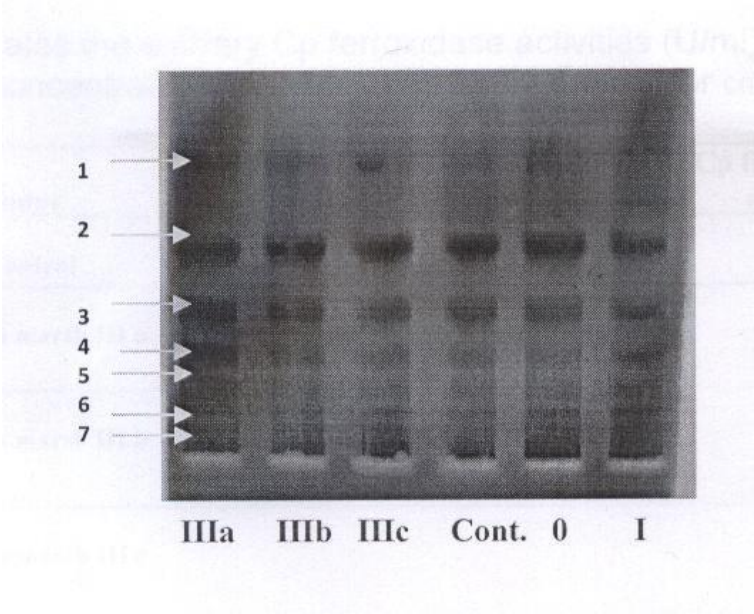
Table 2a: Mean value of salivary Cp concentration (mg/dl) in control and patient groups

group	No.	Age(year) (MeaniSD)	(Mean±SD)mg/dl (Range)
Control	46	17.805±7.324 (5-32)	0.738±0.433 (0.5-0.995)
Patients marsh III	61	14.58±9.772 (2-43)	0.832±0.27 (0.431-1.068)
Patients marsh 0,1	13	17.807±11.707 (4.5-38)	0.839±0.222 (0.621-0.986)

Table 2b: Mean value of salivary Cp concentration (mg/dl) in control and patient subgroups

Group	No.	Age(year) (MeaniSD)	(MeaniSD)mg/dl (Range)
Control	46	15.805±10.324 (5-32)	0.738i0.433 (0.5-0.995)
Patients marsh Ilia	23	13.956±9.479 (2-43)	0.893i0.334* (0.621-1.142)
Patients marsh Illb	28	14.144±9.565 (2.5-33)	0.857i0.320 (0.431-1.068)
Patients marsh III c	10	19.400±12.130 (5-39)	0.746i0.269 (0.599-1.021)
Patients marsh 0,1	13	17.807±11.707 (4-38)	0.839i0.222 (0.621-0.986)

Significant difference in comparison to control at (P < 0.05): Sig. for (marsh Ilia, b, 0,1) patients gp. and non. Sig. for



Figure"!: Conventional poly acrylamide gel electrophoresis (PAGE) 7.5%, using Tris- glycin buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4 C by using a constant current of 40 mA & voltage of 15v/cm. The gel was stained for protein. The samples applied were as follows: 1 pooled crude saliva (mas
h Ilia), 2 pooled crude saliva (mash Illb), 3 pooled crude saliva (mash IIIc), 4 pooled crude saliva (control), 5 pooled crude saliva (mash 0), 6 pooled crude saliva (mash I)

Table 3: Crude and concentrated salivary total protein concentrations (g/1) for celiac patients And control groups.

Salivary Total protein (g/l)						
Steps	Volume of saliva (ml)	Celiac Patients				Control group
		Marsh IIIa	Marsh IIIc	Marsh IIIb	Marsh 0,1	
Crude saliva	27	1.699	1.706	1.583	1.435	1.432
Concentrated by dialysis	2.5	6.00	6.204	5.63	4.500	4.213

Table 4: illustrates the salivary Cp ferroxidase activities (U/ml) and specific activities in determined concentrations of proteins (5.2- 6.2 mg/ml) for control and patients groups.

Groups	Cp Ferroxidase activity (U/ml)	Cp Ferroxidase specific activity (U/mg)
Control	0.212	0.040
Patients marsh III a	0.0958	0.0159
Patients marsh III b	0.097	0.0172
Patients marsh III c	0.1667	0.0267
Patients marsh 0,1	0.1687	0.0298

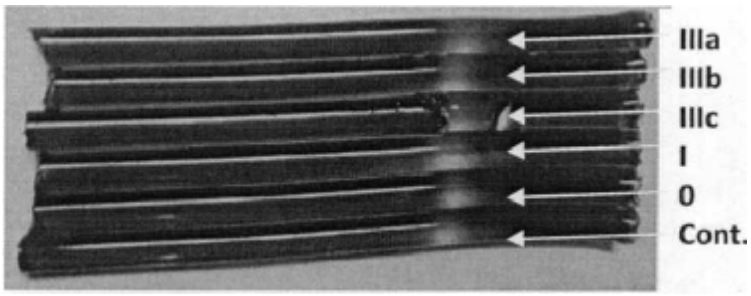


Figure 2: Discontintinuous Polyacrylamide Gel-Electrophoresis (Lammeli) by using Pharmacia Gel Electrophoresis Apparatus GE-2/4 LS. Laemmli's gel system without SDS has been used for native PAGE 7.5% separating gel and 4% stacking gel, using Tris - glycin buffer, pH 8.2 as electrode buffer. Electrophoresis was carried out for 2.5 hours at 4 C by using a constant current of 38 mA & volta ge of 15v/cm. The gel was stained for CP ferroxidase activity. The samples applied were as follows from up to down: pooled crude saliva (mash Ilia), 2 pooled crude saliva (mash Illb), 3 pooled crude saliva (mash IIIc), 4 pooled crude saliva (mash I), 5 pooled crude saliva (mash 0), 6 pooled crude saliva (control).

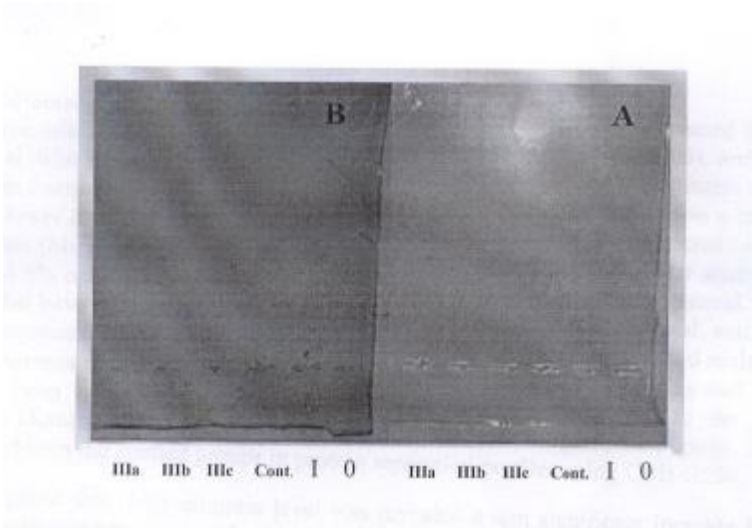


Figure 3: Conventional poly acrylamide gel electrophoresis (PAGE) 7.5%, using Tris- glycin buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4 C by using a constant current of 40 mA & voltage of 15v/cm. The gel was stained for CP oxidase activity. The samples applied were as follows: (a) with sodium azide and (b) without sodium azide: 1 pooled crude saliva (control), 2 pooled crude saliva (mash Ilia), 3 pooled crude saliva (mash lllb), 4 pooled crude

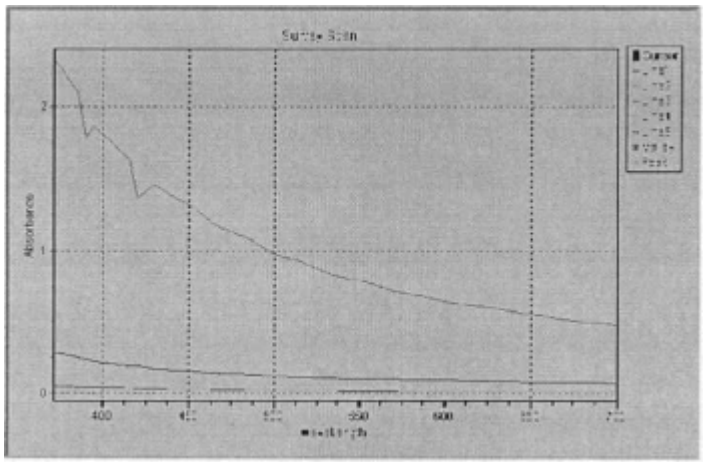


Figure 4: Visible absorption spectra of Cp ferroxidase in 0. 05 M acetate buffer, pH = 5.5, at 30". One milliliter of the concentrated saliva (specific activity = 0.36) was placed in a 1.5 -ml quartz cuvette and the visible absorption spectrum recorded. A second spectrum was recorded with the enzyme solution containi ng 1.0 mM sodium azide. A third spectrum was recorded with the enzyme solution containing only 19 uM ferrous ammonium sulphate. A fourth spectrum was recorded with the enzyme solution containing 19 uM ferrous ammonium sulphate then 1.0 mM sodium azide. A f ifth spectrum was recorded with the enzyme solution containing 1.0 mM sodium azide then 19 uM ferrous ammonium sulphate.

p-phenylenediamine (which was used as a substrate), and by using Erel method (Erel, 1998) for its ferroxidase activity where Cp catalyzes the oxidation of ferrous ion to ferric (which was used as a substrate), the difficulties in salivary Cp ferroxidase activity estimation were concluded in pseudo activities in the normal crude samples which to be compelled to concentrated the saliva (as in table 3)and choice the appropriate protein concentration that give the decent activity for each patients and control groups and a significant differences was obtained between patients groups (as in tables 4). In order to investigate these activities, stained polyacrylamide gel electrophoresis was carried out for salivary Cp ferroxidase and oxidase as in Figures 2 and 3.In Figure 3 oxidase activity was staining in the absence of sodium azide. One clear band was obtained without sodium azide, while no band was detected when the staining was carried out using sodium azide, because sodium azide binds copper in ceruloplasmin and inhibits it's ferroxidase and oxidase activities completely (95%-99%) (Topham and Frieden, 1970; Erel, 1998). Which is considering as an evidence for the presence of salivary Cp oxidase activity. Daoud, 2008 reported distinct differences in the salivary zymograms of stained gel for CP oxidase activity in oral tumorpatients. From the comparison between the activity bands in the zymogram of our studied groups no differences were obtained as in Figure 3b.

Topham and Friden, in 1970 provided visible absorption method to prove the presence of non-Cp ferroxidase II in human serum. Since 1.0 mM azide inhibits Cp and does not inhibit ferroxidase II. The results in Figure 4 (line 5) illustrate the inhibition effect of 1.0 mM sodium azide on the salivary Cp ferroxidase.

Conclusion

To our knowledge no report is available in the literatures concerning studying the enzymatic activities of salivary cp in patients with CD. Our present study highlights the relationship between this disease at its different stages and cp different enzymatic activities; further study is carried out in our laboratories to investigate this relationship more deeply in these patients.

Acknowledgment

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Discussion

In the current study, Salivary total protein levels were markedly increased (p=0.035). This result is agree with Lenander-Lumikari et al. (Lenander-Lumikari et al. 2000), and disagree with Mina, S. et al. who reported a significant reduction not increased in the total protein levels of celiac patients when compared with the control group argue that CD patients who follow a strict gluten-free diet secrete lower levels of amylase, myeloperoxidase, IgA and IgM in stimulated saliva relative to control groups (Mina, et al. 2008). Since the percentage of our patients under study who were in GFD were 26.6% only, the increase may explained on the basis that saliva in general, contain arrays of proteins that have distinct biological function, most of them have antibacterial, antimicrobial, and antibodies properties defend the oral environment against any noxious agents, and such proteins were reported to increase in case of inflammation and tumors (Kashmoola, 2000; Hay and Bowen 1996). Also it has been reported that many serum-derived proteins transferred to the saliva during inflammation (Kaufman and Lamster 2002). The electrozymogram in Figures 1 showed the differences between the studied groups in protein separation profiles using CBB-G250.Throughout this study albumin level was revealed a non significant increase for celiac and marsh (0,1) patients when compared with that of the control group (Table 1). The cause of that may be due to the role of albumin as one of the extracellular antioxidants where albumin constitutes up to 49% of total plasma antioxidant status (Emerson T.E. 1989). Meanwhile albumin acts as sacrificial antioxidant by inhibiting the generation of free radicals through an immediate attacks of albumin molecule itself, so the radical reaction continue on albumin surface and cause damage to albumin molecule (Gutteridge and Wikins 1983; Marx and Chevion 1985)such damage is probably biologically insignificant, due to that the albumin is present in plasma in high concentration (Halliwell and Gutteridge 1986), this result is disagree with Lenander-Lumikari et al. (Lenander-Lumikari et al. 2000).Salivary Cp concentration showed statistically significant increased levels for patients groups (Table 2a). In our previous study on serum Cp concentration in celiac patients a significant increase was obtained in the studied groups in comparison to the controls (Hathama et.al. 2012). So the salivary results could be explained on the basis that many molecules including Cp are capable of penetrating the gingival tissue through the intercellular spaces of the junctional epithelium to the saliva (Grant, et al. 1988). To our knowledge, no written abstract was detected in Medline with salivary ceruloplasmin concentration or activity in celiac patients ups to now.

Throughout this study the estimation of salivary Cp in both its enzymatic activities (ferroxidase and oxidase) has been done by using the modified Rice method (Rice, 1962) for its oxidase activity where Cp catalyzes the oxidation of

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