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Immunochemical identification of the mammalian peptide hormone obestatin from tea plant (C*amellia sinensis*)

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Abstract: This study determines obestatin-like substances from the young shoots of the tea plant [*Camellia sinensis* (L.) O. Kuntze (Theaceae)]. Proteins were extracted from the vegetative tea leaves using the QB (Quick Buffer) buffer as an extraction buffer. Obestatin-like substances in tea extract were investigated using an indirect home-made enzyme-linked immunosorbent assay (ELISA). Human obestatin-like immunoreactive substances from tea extract were isolated and characterized by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) and immunoblotting techniques. Immunochemical results showed that there are strong human obestatin-like immunoreactive substances (0.048 ± 0.0064 ng/mg protein) in vegetative tea leaves. This finding was completely unexpected since this hormone was considered to be present solely in animals. Furthermore, a single obestatin-like immunoreactive protein band of 13 kDa was identified by tricine-SDS-PAGE and Western blotting of extract of vegetative tea leaf proteins. Present investigation is the first report of presence of obestatin-like immunoreactive substances in plants. It is concluded that obestatin-like bioactive peptides derived from plants can affect gastrointestinal tract structures as endogenous obestatin does and hence play a role *in appetite regulation and body weight* gain.

Keywords: Obestatin-like substances, preproghrelin gene-derived peptides, bioactive peptides, appetite control, tricine-SDS-PAGE, Western blotting.

INTRODUCTION

Obestatin is presumed to be cleaved from C- ghrelin or arise independently from distinct ghrelin transcripts (Seim *et al.,* 2010). While comparing preproghrelin sequences of the human ghrelin gene including 117 residues in 11 mammal species, in addition to the mature ghrelin peptide, another peptide containing high-level protected glycine residues and 23 amino acids was identified. C- terminally amidated obestatin peptide identical to residues 76-98 of the C- terminal peptide (C-ghrelin) of preproghrelin that are formed by the post-translational processing of preproghrelin peptide and amidation of obestatin is likely essential for its biological activity as well as acylation of ghrelin (Zhang *et al.,* 2005).

Obestatin is synthesized with ghrelin in all organs producing ghrelin by colocalization method. Contrary to the orexigenic effects of ghrelin, obestatin functions as an anorexigenic hormone repressing food intake and GH secretion induced by ghrelin only when coad ministered with ghrelin, decelerating gastritis discharge and jejunal motility and, reducing weight gain in rodents (Ariyasu *et al*., 2001). The studies have shown that obestatin improves memory (Carlini and Schiöth, 2007), regulates sleep (Szentirmai and Krueger, 20006), affects cell growth (Camina *et al.,* 2007; Pazos *et al*., 2007; Meszarosova *et al*., 2008), increases pancreatic enzyme fluid secretion (*Kapica et al.,* 2007), strengthens the survival of pancreatic β-cells (Granata *et al.,* 2010) and inhibits glucose-induced insulin secretion at high glucose concentrations only (Ren *et al.,* 2008; Egido *et al.,* 2009).

The central administration of amidated obestatin in mice does not have any suppressive effect on eating and drinking. Interestingly, it was reported that obestatin in circulation does not have specific uptake by the blood-brain barrier (BBB) endothelium cells and is rapidly degraded in the circulation (Pan *et al.,* 2006; Vergote *et al.,* 2008). Therefore, it was alternatively proposed that obestatin produces its effects on eating and drinking through direct interactions with the digestive system, by reducing the contractility of muscle strips *in vitro* in jejunum and suppressing the gastric emptying *in vivo* after obestatin treatment. Thus, the inhibition of jejunal contraction could generate an afferent vagal signal to induce satiety in the brain (Zhang *et al.,* 2005).

Some of the recent studies have proven that ghrelin-immunoreactive homologs are also present in plants (Aydın *et al.,* 2006, Aydın *et al.,* 2011; Calapoglu *et al.,* 2018). While the studies on both the gene and protein structure of ghrelin revealed that this hormone has been well-protected in the evolutionary process, the fact that it has high homology among vertebrates empowers the hypothesis that it can be a universal hormone. Although obestatin and ghrelin hormones are coded by the same gene, it is not known whether obestatin hormone has

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immunoreactive homologs in plants with ghrelin hormone.

With this study, it has been aimed to perform immunochemical identification of obestatin-like substances from [*Camellia sinensis* (L.) O. Kuntze (Theaceae)] described as a healthy drink due to its high-level bioactive molecules and high antioxidant capacity, based on the hypothesis that similarly to ghrelin hormone, obestatin hormone can also be a universal polypeptide and play a significant role in the bioregulation of living organisms. With the thought that determining obestatin homologs in plants can reveal changes in the scientific understanding about therapeutic function and evolution in living organisms; this study was planned as a "preliminary study".

MATERIALS AND METHODS

Materials

The vegetal material was harvested in Solaklı tea plantation located in the district of Of, Trabzon Province, Turkey. Young shoots of the tea plant in the first shooting periods (May 2014) were collected and stored frozen until the use. A pool of human serum from 10 healthy subjects was aliquoted and frozen at –20°C for use as positive control.

Protein extraction

Proteins from the frozen plant material (*C. sinensis*) were extracted using the QB extraction buffer protocol according to Ni *et al.* (1996) with a minor modification. Firstly, 10 g frozen plant tissue was finely ground into powder in liquid nitrogen using pre-chilled pestle and mortar. Powdered plant material was intermixed with 2 g insoluble polyvinylpolypyrrolidone to remove phenolic compounds. Immediately before thawing, 20 mL of cold QB buffer (1mM EDTA, 100mM KPO_4 (pH 7.4), 10% (v/v) Glycerol, 1% (v/v) Triton X-100, 1mM DTT and 0.0165% (v/v) protease inhibitor) were added and homogenized until the consistency of slightly watery toothpaste at 4°C. The homogenate was transferred into a falcon tube. The pooled homogenate was subjected to centrifugation at 6000 x *g* for 30 min at 4°C to remove cellular debris and the resultant supernatant was further centrifuged at 6000 x *g* for 30 min at 4°C to completely remove any insoluble components. The finally resulting supernatant was stored frozen at -20°C until the use. The total protein concentration of supernatant fluid was determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as standard.

Enzyme-linked immunosorbent assay (ELISA)

Human serum pool sample as a positive control and tea extract were diluted in coating buffer (0.05M carbonate buffer, pH 9.6) to a 1:2 dilution. Each well of a polystyrene microtiter plate (Nalge Nunc International, Denmark) was coated in with 100 µL of the diluted samples in triplicate. A standard curve in the range between 10 and 0.078µg mL^{-1} was prepared using commercial pure standard of human obestatin peptide (Abcam, Cambridge, UK) by two-fold dilutions in coating buffer and it was dispensed in duplicate. The coated plates were incubated overnight at 4°C. after incubation the plate wells were washed three times with PBS-T (137 mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4) plus 0.05% Tween 20 and then blocked with 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) in PBS-T (PTF) for 1h at 37 C. In the next step, each well was washed as before and 100µL of 1:1000 dilution of commercial rabbit anti-obestatin polyclonal antibody (Millipore Corp, Temecula, CA) in PTF were added to the plate and incubated at 37°C for 1 h. After incubation, the wells were washed tree times with PBS-T to remove unbound antibody. Then, 100 µL of a 1:10000 dilution of peroxidase-conjugated anti-rabbit IgG (AnaSpec, Fremont, CA.) in PTF were added to the plates. The plates were than incubated at 37°C for 1 h and washed five times with PBS-T to remove unbound conjugate. The substrate buffer (0.05 M phosphate-citrate buffer, pH 5.0) containing 0.4mg mL^{-1} of ortho-phenylenediamine and 0.04% of 30% hydrogen peroxide was then added (100µL per well). The plates were then incubated at at 37°C for 30 min. In the final step, the reaction was stopped by adding 50µL per well of 3 M sulphuric acid. Mean optical density values were measured at 490 nm, using a microplate reader (BioTek Instruments, Inc.), after 30 min. Sample obestatin concentrations were calculated with five-parameter logistic fit analysis using My Assay Readerfit ELISA software (www.MyAssay.com).

Electrophoresis

Immunochemical determination of obestatin-like substances from the tea extract was analyzed in tricine-SDS-PAGE followed by the Western blotting. The Tricine–SDS–PAGE was performed as standard method (Schagger, 2006) with a 4% stacking gel (33:1, 3% C, 49.5% T) and 10% polyacrylamide separating gel (33:1, 3% C, 49.5% T). The electrophoresis buffers were as follows: anode buffer (0.1 M Tris, pH 8.0), cathode buffer (0.1% SDS, 0.1 M Tricine, 0.1M Tris and pH 8.25) and gel buffer (0.1% SDS, 1.0 M Tris and pH 8.45). The samples were mixed at a ratio of 1:1 with the buffer sample containing 1% (w/v) SDS, 24% (w/v) glycerol, 4% (v/v) β-mercaptoethanol, 100 mM Tris-HCl (pH 6.8), 0.02% (w/v), Coomassie Brilliant Blue R250 and heated for 5 min in a bath water at 95 C. The samples (20 ml of loading volume per well) were loaded to wells with a micro syringe. The electrophoresis was performed in a Mini-PROTEAN® 3 cell (Bio-Rad, Hercules, CA, USA). The voltage was kept constant (50 V and 200 mA) until the samples completely entering the stacking gel, and then the voltage was set to 100 V and 300 mA. The voltage was maintained constant until the end of the chromatography.

Table 1: Obestatin levels in tea and human plasma samples

Origin	Types of sample	Types of assay	Antibody/specificity	Processing method	Obs. Con.(ng/mg prot.) and Ref.
Alpco	Plasma	Competitive (ELISA)	Anti-obestatin-NH_2 (Monoclonal)	EDTA + protease inhibitor	0.052±0.005 (Calapoglu *et al.*, 2016)
Alpco	*Camellia sinensis* (L.) Kuntze	Competitive (ELISA)	Anti-obestatin-NH_2 (Monoclonal)	TCA/acetone recipitation/phenol extraction + protease nhibitor	0.859±0.072 (Calapoglu *et al.*, 2016)
Home-made	Plasma	Direct (ELISA)	Anti-obestatin-NH_2 (Polyclonal)	EDTA protease inhibitor	1.70±0.20 (This study)
Home-made	*Camellia sinensis* (L.) Kuntze	Direct (ELISA)	Anti-obestatin-NH_2 (Polyclonal)	QB extraction + protease inhibitor	48.00±6.40 (This study)

Immunoblotting

Size-separated proteins were electro-plotted onto a Immobilon-P Membrane, PVDF, 0.45µm (Merck Millipore, USA) with Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) using transfer buffer (0.025M Tris, 0.192M glycine, 20% (v/v) methanol, pH 8.3) and 95 V/350mA for 1h. Then, they were blocked for 30 min with blocking buffer (PTF, which contained 3% BSA) for 1h and subsequently incubated at 4°C overnight with 1:1000 dilution of anti-obestatin antibody (Millipore Corp, Temecula, CA) in PTF. The blots were washed 3x10min with PBS-T. The blots were then incubated in peroxidase-labeled monoclonal antibody (AnaSpec, Fremont, CA) in diluted 1:5000 in PTF for 1h at room temperature. The blots were washed 3x10 min with PBS-T. The immune complex was developed by BCIP/NBT (Sigma-Aldrich, St Louis, MO, USA). Immunoblots were imaged using a computerized image analysis system (Kodak MM 2000 Image Station, USA).

Obestatin-like substances isolation from polyacrylamide gels

The isolation of obestatin-like substances from tea extract was performed by indirect extraction from an electrophoretic gel according to the method described by Calapoglu et al. (Calapoglu *et al.,* 2018). The supernatant of gel pieces were examined for the presence of obestatin immunoreactivity with ELISA as described above. The obestatin immunoreactive fractions were pooled, concentrated and applied to tricine-SDS-PAGE. The electrophoretic process was again the same as described above. Proteins bands were stained by silver nitrate method (Blum *et al.,* 1987).

STATISTICAL ANALYSIS

Statistical analyses were performed using a SPSS 20.0 software program (IBM Corporation, Chicago, IL, USA). Plasma obestatin and tissue human obestatin-like immunoreactive substance levels were compared by Mann-Whitney U test. Each value represents the mean± SD of five independent experiments. Differences were considered significant at $p<0.05$.

RESULTS

In this study, obestatin-like substances in tea shoots were revealed by the indirect ELISA using a rabbit polyclonal antibody against the human obestatin peptide. ELISA analyses demonstrated that the human obestatin-like immunoreactive substances in fresh tea shoot extract was present at concentration of 0.048±0.0064ng/mg protein. The serum obestatin level in healthy human subjects (0.0017±0.0002ng/mg protein) was found to be significantly lower than that of the tea shoots extract ($p<0.001$) (fig. 1).

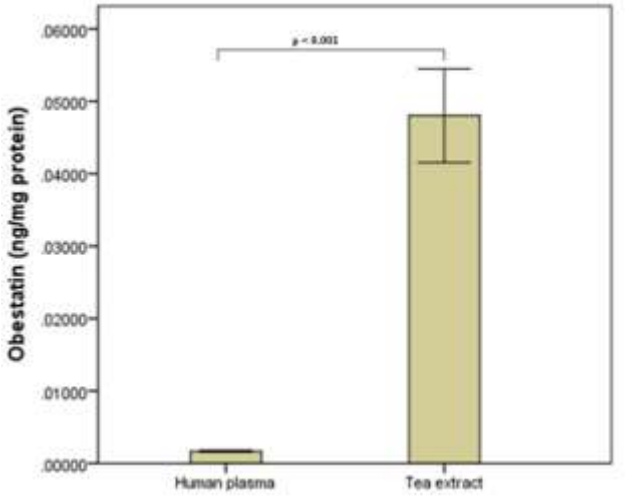

Fig. 1: Quantitative analysis of obestatin (ng/mg protein) in human serum and young shoots of tea (*C. Sinensis* (L.) O. Kuntze). Values are an average of five individual measurements (S.D. shown by error bars).

The identity of the obestatin-like immunoreactive substances in the tea extract was further confirmed by immunodetection using the Western blot analysis. The specificity of the obestatin antibodies was also evaluated by Western blot analysis (fig. 2). After performing the

Western blot analysis, a strong band corresponding to the human obestatin peptide was obtained the expected molecular weight of 2.545 kDa. The Western blot analysis with polyclonal antibody aganist the human obestatin peptide showed positive band only ~13 kDa for tea shoots extract (fig. 2).

In this study, to determine which peptide bands contain actually obestatin immunoreactivity, the polyacrylamide gels were sliced into segments after electrophoresis. Peptides extracted from the segments by passive diffusion were then tested by the ELISA using polyclonal anti-obestatin antibodies. The ELISA positive fractions were pooled and assessed by tricine-SDS-PAGE (fig. 3). The tricine-SDS-PAGE profile of the ELISA positive fractions (fig. 3, lane 3) shows a band centred at about 13 kDa, which is entirely consistent with the Western blot analysis (fig. 2, lane 1).

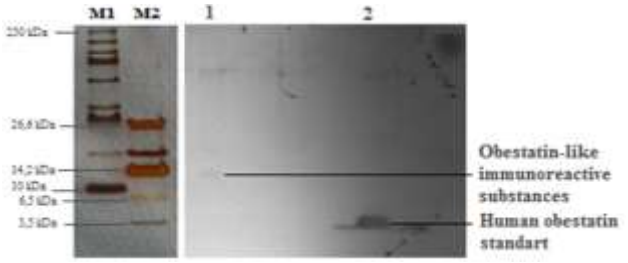

Fig. 2: Tricine-SDS-PAGE and Western-blot analyses of human obestatin and tea extract. M1: Molecular weight standard in kDa (10-250 kDa), M2: Ultra-low range molecular weight standard (1.060-26.600 kDa), Line 1: Tea extract (30µg/mL), Line 2: Human obestatin standard (10µg/mL).

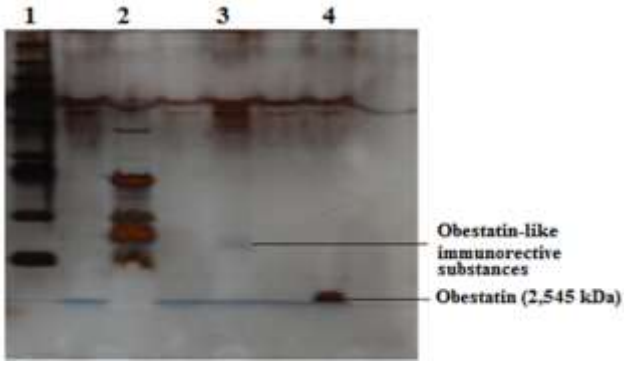

Fig. 3: The tricine SDS-PAGE band pattern of the obestatin-like immunoreactive substances extracted from *C. sinensis*. Line 1: Molecular weight standard in kDa (10-250 kDa), Line 2: Ultra-low range molecular weight standard (1.060-26.600 kDa), Line 3: ELISA positive fraction, Line 4: Human obestatin standard (MW: 2.545 kDa).

DISCUSSION

The studies from 2005 until today revealed that obestatin hormone shows an adverse effect on the metabolic and physiologic effects of ghrelin hormone that is an oroxigenic hormone and is coded by the same gene in vertebrates; also, it has regulating effects on food intake and use, energy use and appetite with ghrelin hormone (Zhang *et al.,* 2005). Both the physiology and the relation of obestatin with diseases have been examined since its discovery. Particularly, it is the main subject the relation of which is investigated with diabetes, psychological eating disorder along with obesity.

In this study, obestatin immunoreactivity was examined in the green fresh shoots of tea plant**.** The results in which we obtained home-made from human plasma and tea plant and commercial mass used in our previous studies and the features of the method used are shown in table 1.

According to the data in table 1, the results of human plasma and tea obestatin, in which commercial mass has been obtained, are lower than the results obtained with the home-made method. It is considered that the reason for the difference in the results can result from the immunological method used and differences in sample processing. TCA/acetone precipitation/phenol extraction and total protein extraction include more process stages compared to the QB extraction and during the extraction process, protein losses are possible. Using polyclonal antibody instead of monoclonal antibody in the assay method may lead to a reaction with not only obestatin being a ghrelin gene product but also other ghrelin gene products (proghrelin).

No international standard has been present yet for biologically active peptides produced in the hypothalamus or gastrointestinal tract. Obestatin is also one of these biologically active peptides. To date, there is no international standard of obestatin, probably because of their extreme instability. Consequently, results obtained from different laboratories show the difference to a large extent. Especially, differences can be observed between laboratories even in the results obtained from the measurements performed with kits based on the immunologic assay principle and produced by the same commercial firm.

It has been proven in experimental studies that ghrelin and obestatin hormones have roles in the weight control in vertebrates. As a product of the same ghrelin gene with obestatin in plants, ghrelin hormone was detected in the parenchymal tissues of plum and mulberry plants with the immunohistochemical method. The findings were confirmed with the Western blot, RIA and HPLC methods. Although the molecular weight of ghrelin and amino acid sequence in plants are still not known, the function is considered to be related to growth (Aydın *et al.*, 2006).

In this study, the characterization of peptide causing obestatin immunoreactivity in the extract that shows

obestatin immunoreactivity with the indirect ELISA method in green shoots of tea plant was performed with the tricine-SDS-PAGE and Western Blot. Obestatin immunoreactivity was scanned in gel parts obtained by transecting plant proteins separated with 10% tricine-SDS-PAGE. Obestatin positive fractions were processed and taken to 10% tricine-SDS-PAGE again. The fraction showing a positive reaction with human-anti-obestatin antibodies according to the ELISA technique was observed as one band in the tricine-SDS-PAGE (fig. 3). The weight of the obtained band is equal to approximately 13 kDa when compared to the protein molecular weight standards. When the obtained band pattern is compared to the human obestatin standard (MW: 2.545 kDa), the antibodies of human anti-obestatin are observed to react with the protein with higher molecular weight than human obestatin. This situation indicates that either there is another protein with the same sequence with obestatin in plants or proghrelin peptide reacts with the uncleaved form to obestatin and ghrelin products. Because human proghrelin peptide is a peptide with approximately 13 kDa molecular weight.

The characterization of obestatin-like immunoreactive compounds in tea extract was performed with the Western blot technique (fig. 2). As a result of the Western blot analysis, obestatin-like immunoreactive compounds were observed to be present in the extract obtained with QB buffer. Furthermore, the band image obtained in the Western blot analysis is consistent with the band image of the tricine-SDS-PAGE and ELISA positive obestatin-like immunoreactive compounds.

CONCLUSION

In conclusion, obestatin immunoreactivity was observed in the protein extract obtained from fresh shoots of the tea plant and also characterized by the Western blot and tricine-SDS-PAGE analyses. It is necessary to determine the structural features of characterized obestatin-like immunoreactive substances with protein sequence studies and further studies such as cell culture or animal experiments must be conducted to reveal its physiologic effects. Only when these studies are performed, it will be possible to discuss the effects of prevalently-consumed tea related to obestatin.

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