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REDUCED PROTEIN CARBONYLATION OF CUBE STEAK AND CATFISH FILLET USING ANTIOXIDATIVE COATINGS CONTAINING CHEDDAR WHEY, CASEIN HYDROLYZATE AND OOLONG TEA EXTRACT

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ABSTRACT
Antioxidative efficacy of thermized Cheddar whey-based edible coating containing casein hydrolyzate (CH) and Chinese oolong tea extract (OTE), by themselves or in combination, in in decreasing protein oxidation of Mississippi farm raised retail-cut catfish fillet and cubed beefsteak was investigated. Coatings were prepared by dispersing Cheddar whey powder (5%), sorbitol (2.5%), calcium chloride (0.125%), glucomannan (0.25%) and carboxymethyl cellulose (0.25%) on a weight per volume basis (w/v) in either 0.2 M McIlvaine's iso-ionic buffer (pH 7.0) at 22˚C by itself or in combination with CH (0.25%). The OTEs were prepared by steeping tea leaves (1, 2 and 5%, w/v) for three minutes at 85˚C in the same buffer. Catfish fillet and cubed beefsteak samples of uniform geometry and weight (5g each) were coated with either, (1) the coating with added CH, (2) only OTEs, or (3) coatings containing both CH and OTEs. Protein oxidation was determined by assessing carbonyl content (CC) of the treated samples immediately or after 1, 3, 5 and 7 days of storage at 4OC in polyover-wrapped styrofoam trays. Coating the cubed beefsteak with solution both CH plus OTE (3%, w/v) resulted in the lowest CC content after 7 days of storage compared to all other treatment showing a significant 33% reduction compared to control. Coating catfish with CH plus OTE (5%, w/v) showed significantly reduced protein oxidation at the third and fifth days of storage – indicated by 49 and 45% decreases of CCs, respectively, relative to the control.

Keywords*: refrigerated storage life, polyphenol, carbonyl, sweet whey, muscle food*

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1. INTRODUCTION

Oxidative degradation of protein cause off flavor/color development and significant wastage of muscle foods (Dave and Ghaly 2011). Cube steak, which is a tough cut of beef that is tenderized by pounded with a meat tenderizer or by using mechanical tenderizer, is particularly susceptible (Weerasinghe et al.2013). This is due to substantial increase in surface area of cube steak and enhancement of oxidative environment due to exposure to metallic cutter and thus to metal ions during the cubing process. Metal is known to significantly enhance oxidative degradations of proteins (Dalle-Donne et al., 2003). Moreover, forceful tenderization during cubing releases hemoglobin and muscle myoglobin. These oxygen saturated heme proteins (Ronda et al., 2008) are free to intermingle with other cellular constituents leading to accelerated lipid oxidation resulting in undesirable odor in the stored products (Richards et al. 2010). Retail cut catfish fillet are also highly prone to oxidation and exhibit several types of deterioration in quality, such as development of off-color and odor, decline in nutritive values and accumulation of toxins following the process of oxidative degradation (Thiansilakul et al. 2007). This problem is particularly pronounced in fish due to its high contents of poly-unsaturated fatty acids and results in a considerable decline in shelf life (Coronado et al. 2002).

Several antioxidants, both synthetic and natural, have been used to enhance the storage lives of several muscle food products (Williams et al. 2013). Unfortunately, long term consumption of food treated with synthetic preservatives may potentially result in adverse health effects in the consumers

(Pourmorad et al. 2006). Therefore, natural food products with antioxidative properties have attracted considerable interests in the recent years to be used as potential food preservative agents (Shon and Haque 2007 a,b; Haque et al. 2009; Mukherjee and Haque 2015). Key functional attributes of native (unheated) whey was seen to be significantly enhanced when thermized (heated) (Shon and Haque, 2007c) and this particularly true for Cheddar whey (Weerasinghe et al., 2013). Remarkable antioxidative properties have also been noted in the chymotryptic hydrolyzate of casein (CH). This may be attributed to the highly redox-sensitive thiol groups present in the peptides (Brandes et al. 2009). We have also reported the strong antioxidant efficacy of tea (Sreenivasan and Haque 2013) which results from its polyphenol and flavonoids content (Labbe et al. 2006).

The current study looks at the combined efficacy of the above mentioned natural antioxidants. Edible coating based on thermized Cheddar whey protein concentrate containing CH without and with various concentrations of Chinese oolong tea extract to reduce oxidative degradation of protein in stored retail-packed of cube steak and Mississippi grown retail-cut catfish fillet. The level of carbonylation, which has been thoroughly investigated in connection with oxidative degradation of proteins (Dalle-Donne et al., 2003) was used as the marker of protein oxidative stress. The protein carbonyl content (CC) was sensitively detected in the samples. Protein carbonyl content (CC) as stable 2,4-dinitrophenyl (DNP) hydrazone product by derivatization with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990).

2. MATERIALS AND METHODS

Materials

Retail-cut catfish fillet was purchased from a local catfish farm (Superior Catfish Inc., Macon, MS, USA). Freshly prepared cube steak from top round or top sirloin cut of beef was from a local grocery. Fresh milk for Cheddar whey preparation was obtained from the Mississippi State University mixed dairy herd (Mississippi State, MS, USA). The full-leaf Chinese oolong tea was purchased from Teavana (Stratford, CT, USA). Luminal, 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (ABAP), Trolox, trichloroacetic acid (TCA), hydrochloric acid, guanidine hydrochloride, casein hydrolyzate (CH), sodium phosphate dibasic, citric acid and 2,4-dinitrophenylhydrazine (DNPH) were from Sigma-Aldrich Co. (Milwaukee, WI, USA). Sorbitol and carboxymethyl cellulose (CMC) were from Archer Daniels Midland (Decatur, IL, USA) and FMC Biopolymers (Newark, DE, USA), respectively. Calcium chloride was from American International Chemical (Framingham, MA, USA). Ninety six well plates (black-sided, clear bottomed) were from Fisher Scientific (Hanover Park, IL, USA). All other reagents were analytical grade.

Methods

Preparation of thermized Cheddar whey protein concentrate (TCWPC)

Fresh milk was used to manufacture Cheddar cheese at the Mississippi State University Dairy Plant according to Kosikowski (1982). The resultant whey was thermized at 70˚ C for 30 min as described earlier (Haque and Shon, 2007) and processed to produce Cheddar whey powder as detailed before (Ji and Haque, 2003). Concentration of the whey was at low temperature (65-70°C) by vacuum evaporation, using a falling film vacuum-evaporator (APV, Sorborg, Denmark), to obtain 24-29% solids (w/v) as opposed to the starting solids content of 6.8% measured by a hand-held refractometer (Fisher Scientific, 0°-32° Brix range). The concentrated whey was then directly spray-dried using a pilot plant scale spray-drier (APV, Sorborg, Denmark) to obtain the TCWPC.

Preparation of oolong tea extracts (OTE):

Oolong tea extract of three different concentrations were prepared by steeping Chinese oolong tea leaves (1, 3 and 5g/100mL or 1, 2 and 5%, w/v) at 85OC for 3 minutes in 0.2 M McIlvaine's iso-ionic buffer (pH 7).

Preparation of coating solutions

Coatings for forming the edible film were prepared as described before (Weerasinghe et al. 2013) with minor alterations. On a weight per volume basis (w/v), TCWPC (5%), sorbitol (2.5%), calcium chloride (0.125%), glucomannan (0.25%), and CMC (0.25%) were dispersed in 100 mL (final volume) of either of two buffers, (1) 0.2 M McIlvaine's iso-ionic buffer alone (pH 7.0) at 22˚C, or (2) same buffer containing either of the OTEs. The CH containing edible coatings had CH (0.25%, w/v) added these buffers.

Sample preparation

Cube steak and catfish fillet of uniform geometry and weight (5g) were rinsing with deionized water, draining, and dipped in one of five different types of coatings; (1) buffer alone (control), (2) buffer plus CH for two minutes at 22˚C. The coatings contained either, (1) CH (0.25%, w/v, in 0.2 M McIlvaine's iso-ionic buffer), (2) 100 ml of either of the OTEs (1, 3 or 5 g/100ml buffer), and (3) both CH and OTE.. The control was the test sample dipped only in the buffer under exactly the same conditions. All dipped treatments and control were dried in a stream of nitrogen, packed in polyover-wrapped styrofoam trays typically used commercially for retail display, either analyzed immediately (nil storage treatments) for assessing baseline state of oxidation or stored for 1, 3, 5 and 7 days at 4˚C and analyzed for storage related oxidative degradation of proteins.

Analysis of protein oxidation

Protein oxidation, as indicated by the carbonyl content (CC) of the samples were measured according to the method described by Haque et al. (2009) with a few modifications. Samples were homogenized in 50 mL 0.2 M McIlvaine's iso-ionic buffer (pH 7.0) using a commercial blender (Oster Digital Blender, Sunbeam Products Inc., Boca Raton, FL) for 30 sec at a speed attenuation of 18, 0.5 mL of the homogenate was mixed with 2 mL of 20% (w/v) TCA, centrifuged at 3000×g for ten min at 4°C in a Sorvall TM LegendTM Micro 21R centrifuge (Thermo Scientific, Waltham, MA) to obtain pellets that were redispersed in 2 mL 10 mM DNPH reagent (prepared in 2 N HCl) followed by incubation for 1 hour at 22°C. The pellets were again redispersed in 2 mL of 20% TCA, centrifuged at 3000×g for ten min to obtain pellets that were washed twice with 2 mL of ethanol/ethyl acetate mixture (1:1) to remove residual DNPH, and finally dissolved in 1.5 mL of 6 M guanidine hydrochloride. for measurement of absorbance at 370 nm using a BioMate™ 3 UV-Vis spectrophotometer (Thermo Electron Scientific Instruments Corporation, Middleton, WI, USA).

Measurement of peroxyl and alkoxyl radical quenching efficacy of coatings containing the test antioxidants

Short-term antioxidative efficacy or antioxidative activity (AA) and long term antioxidative efficacy or antioxidative persistence (AP) of the treatments as compared to control (buffer alone) were determined as described earlier (Mukherjee and Haque 2016; Haque et al. 2013 a, b). Peroxyl and alkoxyl radicals were generated in vitro by the pyrolysis of ABAP and concentrations of unquenched radicals, as stoichiometrically reflected by chemiluminescence of added luminol, was determined using a FlexStation 3 microplate reader equipped with the SoftMax® Pro Microplate Data Acquisition and Analysis Software (Molecular Devices, CA). The reaction the mixture consisted of equal volumes (50 µL) of test sample, 10 mM luminol oxygen saturated McIlvaine's iso-ionic buffer without and with Trolox, which is a water-soluble analog of vitamin E, was used as the standard. The reactions were initiated, by injection of 12.5 mM ABAP, referred to as the first challenge (1) to determine AA, in in black-sided (side light impervious)/clear bottomed 96-well assay plates and chemiluminescence (in relative light unit, RLU) was recorded every 1.5 min.

The recorded chemiluminescence (Lu) maxima (Max) of the control (C) (buffer alone) within the first hour following the first injection of 12.5 mM of ABAP into the reaction mixture

(the first challenge) (1) was termed LuMaxC1. Similarly, the chemiluminescence maxima observed for the controls within the next hour following the second injection of ABAP (the second challenge) (2) was termed LuMaxC2. These were the points during pyrolysis when radical production was at a peak under our experimental conditions. The chemiluminescence of each individual test samples following the first and second hour of each challenge and at the time of peak radical production determined from LuMaxC1 and LuMaxC2 were respectively termed LuMax1 and LuMax2. The percent reduction of LuMax1 and LuMax2 as a result of radical quenching compared to the controls, as shown in the expression below, after the first and second challenge respectively gave the AA and AP that was expressed a percentage.

AA = (LuMaxC1/LuMax1) × 100 (%). ……... (1)
AP = (LuMaxC2/LuMax2) × 100 (%)……. (2)

Statistical analysis

The results were analyzed with Student's t-tests (Student, 1908) to deduce ($\alpha = 0.05$) whether,
(1) CCs of the treated samples significantly differed from the control,
(2) AA and AP of the test samples were significantly different from the control, as well as from each other.

3. RESULTS AND DISCUSSION

Protein oxidation:

The effect of the edible films on reducing protein oxidation of cube steak was evident at both the initial and final periods of the study (Fig. 1).

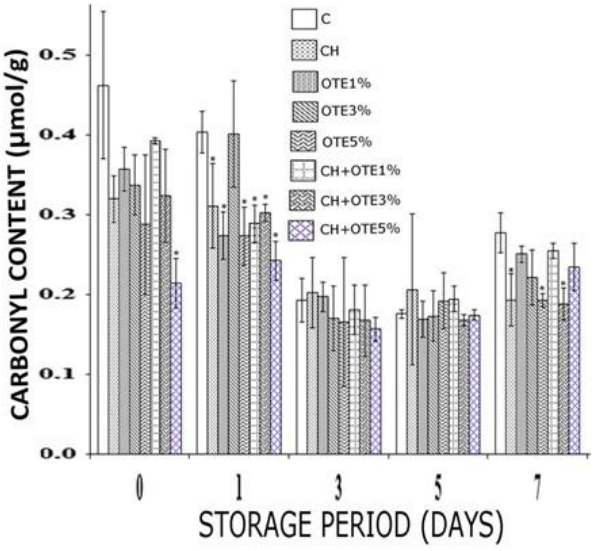

Fig. 1. Mean carbonyl content (CC) of cube steak samples immersed in coating dips containing casein hydrolyzate and various concentrations of oolong tea extract after different periods of refrigerated storage (4˚C).

The x axis represents storage period and y axis denotes CC in samples. Samples dipped only in 0.2 M McIlvaine's iso-ionic buffer were the controls for the different storage periods. Significantly lower ($P<0.05$) average CCs relative to control are identified with asterisks (*). Abbreviations: C, control; CH, casein hydrolyzate; OTE1%, oolong tea extract (1%, w/v); OTE3%, oolong tea extract (3%, w/v); OTE5%, oolong tea extract (5%, w/v).

Treatment containing CH plus 5% (w/v) OTE was best able to dramatically reduce carbonyl contents (CC) in cube steak immediately on dipping and against oxidative degradation on the first two days of the investigation as evident by 54 and 40% reductions in CC, respectively, relative to the control (Table 1).

Table 1. Percent reduction in protein oxidation (indicated by percent decrease in carbonyl content) of cubed beefsteak coated with different concentrations of tea extract, in the presence or absence of casein hydrolyzate, compared to the controls (dipped in only 0.2 M McIlvaine's iso-ionic buffer) at various periods of storage at 4^{O}C.

Storage Period (Days)	Treatments						
	CH	Tea Extract (1%, w/v)	Tea Extract (3%, w/v)	Tea Extract (5%, w/v)	CH+Tea Extract (1%, w/v)	CH+Tea Extract (3%, w/v)	CH+Tea Extract (5%, w/v)
	% Reduction in CC (compared to control)						
0	30.9	22.8	27.1	37.8	14.9	30.0	53.8
1	22.9	32.2	0.5	32.2	28.4	25.1	39.9
3	-4.9	-2.1	11.8	14.6	6.3	13.2	18.8
5	-17.6	3.8	1.5	-9.2	-10.7	4.6	0.8
7	30.4	9.7	20.3	30.4	8.2	32.6	15.5

Abbreviations are as follows: CH: Casein hydrolyzate; w/v: weight/volume, CC: Carbonyl content.

Samples dipped in the coating with CH, supplemented with 3%, w/v OTE showed significantly (P<0.05) reduced CC compared to the control (33%) even after seven days of storage. The CC of the samples coated with the same solutions were numerically lower than all the other samples for the same days of storage.
In the catfish fillets too, the combination of CH and different concentrations of OTE tended to show significantly reduced (P<0.05) protein oxidation compared to the controls throughout the different periods of storage (Fig. 2), though their preservative efficacy varied across the storage period depending on the concentration of OTE. Coating with only CH or OTE (5%, w/v) caused 26 and 38% reductions in CC compared to the control on the third day (Table 2). However, a marked augmentation in preservative efficacy of the coating was observed when CH was mutually supplemented with the same concentration of tea. The combination resulted in numerically lower CCs relative to all other samples on the same day of storage (49% reduction relative to the control). Similar tendency was also noted at the fifth day of storage.

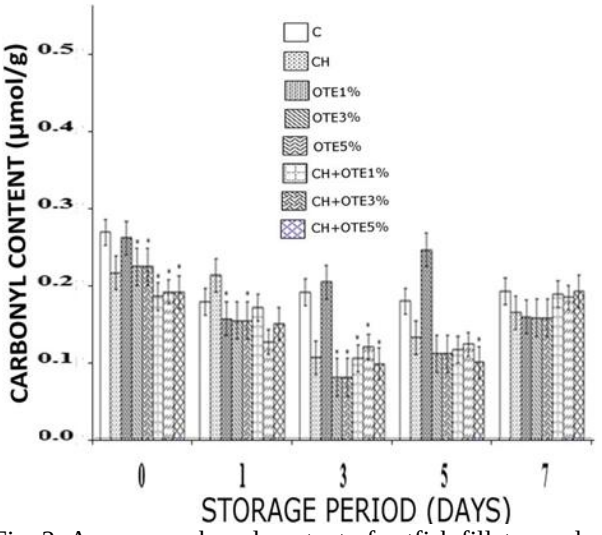

Fig. 2. Average carbonyl content of catfish fillet samples immersed in coating dips containing casein hydrolyzate and various concentrations of oolong tea extract at different periods of refrigerated storage. The descriptions of axes, treatment controls, abbreviations and asterisks are as described in the legend for Fig. 1.

As the storage time progress till five days, the CC in all samples decreased though the decrease was significantly more in the antioxidative coatings (Figs. 1 and 2).
After seven days, there was numeric though not significant increase in the CC of all samples appearing more as if there was a leveling off of the carbonylation and subsequent degradation of CC.
Protein carbonyls are known to partake in the formation of Strecker aldehydes with amino acids like leucine and isoleucine (Estevez et al., 2011). Amide carbonyl-carbonyl interactions may also take place along with interactions with lipid-peroxidation products resulting in the apparent levelling off of the CC (Figs. 1 and 2). The Strecker degradation of amino acids is one of the main reactions leading to the final aroma compounds in the Maillard reaction (Estevez et al., 2011).
Radical-mediated oxidative stress makes protein side chain residues, arginine, proline lysine and threonine particularly prone leading to increase in CC and this is accelerated by transition metals like iron and copper (Estévez, 2011). The intensely forceful beating or shredding during the cubing process will leave iron ions intermingled in the muscle tissue laced with hemoglobin and myoglobin, heme proteins that are saturated with oxygen. Processing particularly with iron or copper based equipment will severely enhance oxidation (Park and Xiong, 2007). In recent times many natural and synthetic antioxidants have been used to thwart oxidative degradation (Finley et al., 2011; Serra et al., 2013). Plant extracts rich in phenolics from herbs and spices, e.g. rosemary, are effective protectors against oxidative decay in a broad variety of food products. Green tea reportedly slows down CC formation in Bologna (Jongberg et al., 2013).

Antioxidant activity and persistence:
All the samples, alone or in combination exhibited significantly and dramatically greater (P<0.05) AA and persistence (AP) relative to the control (Fig. 3).

Table 2. Percent reduction in protein oxidation (indicated by percent decrease in carbonyl content) of Mississippi grown retail-cut catfish fillet coated with different concentrations of tea extract, in the presence or absence of casein hydrolyzate, compared to the controls (dipped in only 0.2 M McIlvaine's iso-ionic buffer) at various periods of storage at 4^{O}C.

	Treatments						
	CH	Tea Extract (1%, w/v)	Tea Extract (3%, w/v)	Tea Extract (5%, w/v)	CH+Tea Extract (1%, w/v)	CH+Tea Extract (3%, w/v)	CH+Tea Extract (5%, w/v)
Storage Period (Days)	% Reduction in CC (compared to control)						
0	19.6	2.5	23.6	16.6	30.9	28.8	28.9
1	-19.8	12.2	23.6	13.5	3.9	29.4	16.0
3	44.5	-6.9	41.5	58.2	45.3	37.3	48.9
5	26.3	-37.4	13.7	38.3	35.0	31.3	44.6
7	14.5	17.5	-5.4	18.0	1.9	4.2	0.0

Abbreviations are as described in the footnote of Table 1.

Addition of OTE, even at its lowest concentration of 1% or CH (0.25% w/v) resulted in augmentation of AA by 180 and 638% respectively. Moreover, AA was enhancement was even more pronounce, being 401 and 917% at the same level of addition. In combination at the same concentration, the increase in the AA and AP were intense being 889 and a huge 1,388%, respectively.

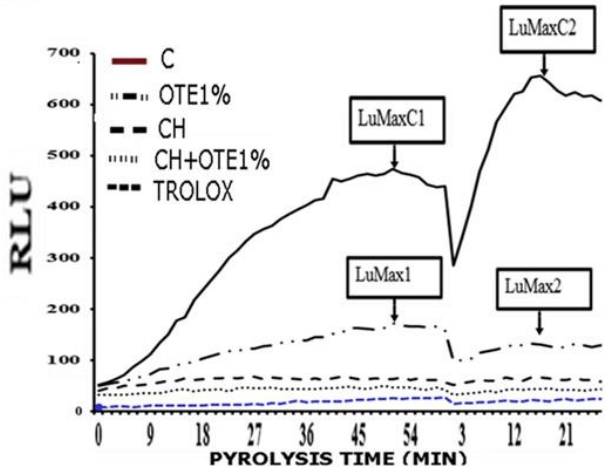

Fig. 3. Short (antioxidative activity) (AA) and long term (antioxidative persistence) (AP) radical quenching efficacy of casein hydrolyzate (0.25% w/v) (CH), oolong tea extract (1% w/v) (OTE) and CH+OTE reflected by reductions in relative light units (RLU) due to scavenging of peroxyl and alkoxyl radicals in vitro generated by pyrolysis of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP). The X and Y axes respectively represent pyrolysis time and luminol emitted chemiluminescence stoichiometrically reflecting concentration of unquenched radicals. The terms, LuMaxC1 and LuMaxC2, represent the points during pyrolysis when chemiluminescence maxima representing maximal radical generation was reached during the assessment of AA and AP, respectively, for the control (C). Whereas LuMax1 and LuMax2 represent reduced chemiluminescence maxima of the coating mixes at the same point in pyrolysis during their respective determination of AA and AP. Details are given in the materials and methods.

Tea is rich in polyphenolic compounds including catechins, theaflavins, tannins, and flavonoids and these have been known to be antioxidative (Khan and Mukhtar, 2013). Milk derived peptides have been reported to be antioxidative (Power et al., 2013). Novel antioxidative peptide have been derived from buffalo casein (Shanmugam et al., 2015), ovomucin (Chang et al., 2013), porcine myofibrillar protein (Saiga et al., 2003) and other foods (Kitts and Weiler, 2003). We have also reported that the same CH used for this study is antimicrobial (Haque et al., 2016). However, use in combination to reduce CC in muscle foods has not been elucidated.

4. CONCLUSION

The investigation showed not only the efficacy of CH and/or OTE to substantially reduce protein oxidation of muscle food products, but also indicated the fact that the optimum preservative efficacy of such agents may vary with their concentration or combination depending on specific food products. The study exhibited the significantly beneficial use of GRAS status edible substances to enhance the shelf-lives of oxidation susceptible muscle

foods that could lead to considerable economic benefit to the retailers while reducing the need for use of synthetic antioxidants that may pose health hazards following long term usage.

5. ACKNOWLEDGEMENTS

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