FINAL REPORT
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Developing and Formulating Novel Peptide-based Antioxidants from Rendering Proteins for Potential Aquaculture and Pet Foods

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Duration of Project: 21 months

Lay Summary:

My funded research project "**Developing value-added peptide-based antioxidants from rendering products for potential aquaculture and pet food market"** demonstrated that the hydrolysates of some commercial rendering products (e.g., fish meal from the by Zeigler Bros., Inc. and poultry meal from Carolina By-products) possessed antioxidant activities, which may enable them to be used as natural antioxidants as substitutes of some commercial antioxidants such as BHT, Vitamin E and ethoxyquin that are widely used in formulated animal feeds. Latest progress showed their antioxidant activities follow different mechanisms. Patent application has been submitted through CU IP committee and is pending in review.

Objective (s): As proposed in my original proposal and discussed in the committee meetings, my research group in the last funded period plus the no-cost extension period aimed to focus the experiments on (1) investigating whether the commercial rendering protein products have antioxidant activities after the chemical and enzymatic hydrolysis treatments; (2) whether these antioxidants follow multiple mechanisms; (3) determine sequence of the isolated antioxidant peptide and the antioxidant synergistic effects of the hydrolysates with some commercial antioxidants (i.e, Petox and Santoquin provided by the Carolina By-products).

Project Overview: My research project is based on our current knowledge on peptide-based antioxidants that can be produced, either chemically or enzymatically, from rendering product proteins and other proteins sources. Since rendering animal co-products are rich of proteins, it is hypothesized that antioxidant peptides can be produced and extracted from rendering meals via different hydrolytic means. The result details are summarized in the followings:

(1) **Samples:** We used three different protein hydrolysate meals that were provided generously by the Zeigler Bros., Inc. and the Carolina By-products (CBP). Two fish meals provided by

the Zeigler Bros., Inc. are labeled as “High antioxidant” and “Low antioxidant”, which were known to be originally added with “high amount of antioxidant” and “low amount of antioxidant”. In contrast, the “Premium pet meal” from and the Carolina By-products is used as a comparison “without added antioxidant”.

Table 1: Independent variables and their coded and actual values used for RSM optimization

Independent variable	Units	Symbol	Coded levels				
			-1	0	1	Axial (-α)	Axial (α)
Temperature	◦C	X_1	25	35	45	10	60
E/S – ratio (ω/ ω)	%	X_2	0.5	1.5	2.5	0.25	6
Hydrolysis time	h	X_3	4	5	6	3	7

Table 2: Three-factor central composite design used for RSM with tested and predicted values of the total antioxidant capacity of the hydrolysates from the Premium pet meal (CBP).

Standard order	Factor1 (X_1) Temperature (˚C)	Factor2 (X_2) E/S ratio (ω / ω %)	Factor 3 (X_3) Hydrolysis time (h)	Response (Y1) TAC (Total antioxidant capacity) (mM)	
				Tests	Predicted
		Corner Points			
1	-1	-1	-1	0.73	0.73
2	1	-1	-1	0.93	0.87
3	-1	1	-1	1.32	1.30
4	1	1	-1	1.43	1.46
5	-1	-1	1	0.66	0.64
6	1	-1	1	1.00	1.04
7	-1	1	1	1.02	1.09
8	1	1	1	1.49	1.50
		Axial Points			
9	-1.68	0	0	0.93	0.92
10	1.68	0	0	1.39	1.38
11	0	-1.68	0	0.52	0.55
12	0	1.68	0	1.46	1.41
13	0	0	-1.68	1.15	1.17
14	0	0	1.68	1.18	1.13
		Center Point Replicates			
15	0	0	0	1.32	1.33
16	0	0	0	1.32	1.33
17	0	0	0	1.36	1.33
18	0	0	0	1.36	1.33
19	0	0	0	1.32	1.33
20	0	0	0	1.29	1.33

(2) **Preparing antioxidant hydrolysates:** To test the antioxidant activities of the hydrolysates, two hydrolytic methods were adopted, including the alkaline hydrolysis by a strong base (0.5N NaOH at 90ºC for 2hr, then neutralized), and an enzymatic hydrolysis by a commercial enzyme Protease (Sigma). The hydrolysis condition was also optimized by the response surface methodology under the condition of pH=7.5, E/S within 0.5—1.5%, temperature within 25-45ºC and hydrolysis time for 4-6 hr (see **Table 1 and 2, and Figure 1**). Our results showed that alkaline hydrolysis produced better antioxidant hydrolysates than the enzymatic hydrolysis, which was selected for further comparison with the commercial antioxidants (**Table 3** and **Figure 2**).

(3) **Determining antioxidant activities:** The hydrolysates were compared with their antioxidant activities in terms of their power of scavenging free radicals of DPPH, ABTS, ORAC, and the their inhibitive activity against the lipophilic β-carotene linoleic acid. Their antioxidant capacity (**Table 3)** and was also measured in terms of the Oxidative Stability Index (OSI) by a Metrohm Rancimat® apparatus (743 Rancimat, Brinkmann Instruments, Inc., Westbury, NY, USA) (**Figures 2 and 3**).

Table 3: Power of Scavenging DPPH, $ABTS^{+}$, ORAC Radicals and the β-CLAMS inhibition by Antioxidant Hydrolysates Made from Rendering Proteins through NaOH Hydrolysis

Materials	Sample Concentra-tion	DPPH inhibition (%)	ABTS inhibition (%)		ORAC (μmol of TE/g of sample)	(β-CLAMS μmol of TE/g of sample)
High antioxidant	HW_0 (1.8%)	58.85±0.15	52.56±0.05	HW_0 (1.0%)	363.65±1.23	73.28±3.52
	HW_1 (1.0%)	39.78±0.01	41.38±1.12	HW_1 (0.5%)	371.96±2.36	81.06±6.74
	HW_2 (0.5%)	19.61±0.03	24.60±0.06	HW_2 (0.2%)	476.45±7.82	109.2±5.32
Low antioxidant	LW_0 (1.8%)	43.00±0.13	46.72±0.42	LW_0 (1.0%)	201.28±12.37	82.72±5.67
	LW_1 (1.0%)	21.32±1.02	28.15±0.13	LW_1 (0.5%)	256.21±15.35	75.16±7.20
	LW_2 (0.5%)	15.06±0.08	18.34±0.12	LW_2 (0.2%)	290.85±3.31	99.07±11.32
Premium	PW_0 (1.8%)	80.56±2.32	86.60±1.24	PW_0 (1.0%)	190.14±12.43	
	PW_1 (1.0%)	64.28±1.20	69.45±1.05	PW_1 (0.5%)	245.62±18.56	
	PW_2 (0.5%)	41.27±0.03	46.00±0.07	PW_2 (0.2%)	353.52±13.27	
HW, LW and PW: are three different hydrolysates obtained from high antioxidant sample, low antioxidant sample, and premium sample, which were dissolved in water in three concentrations at 1.8, 1.0 and 0.5% for the DPPH and ABTS tests, and at 1.0, 0.5 and 0.2% for the ORAC and β-CLAMS test.						

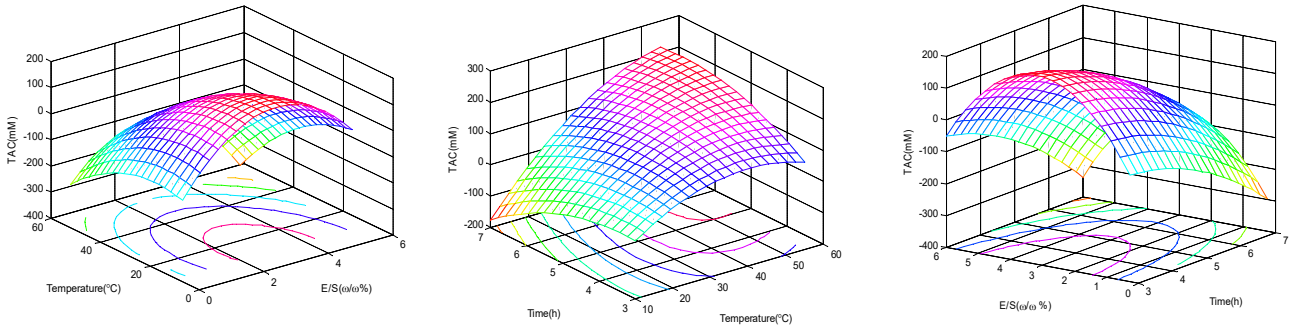

Fig.1. Response surface plot showing the combined effect of temperature and E/S (a), time and temperature (b), E/S and time (c) on the total antioxidant capacity of hydrolysis solution.

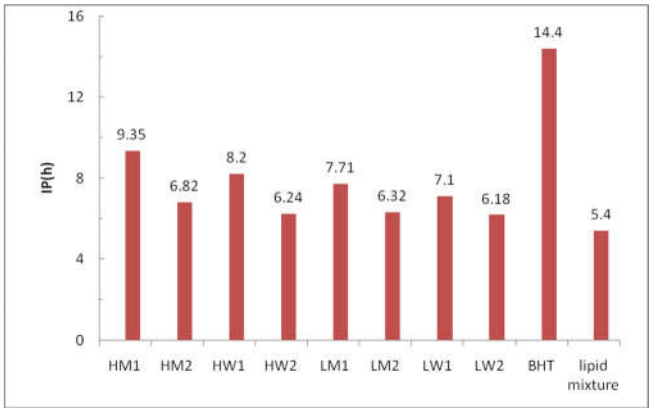

Fig. 2. Comparison of antioxidant power of the protein hydrolysates and BHT (both at 300 ppm) in their Oxidative Stability Index (OSI) in term of the induction point (minutes) of lipids oxidation measured at 110ºC, 20L/h. BHT was used as a comparison control group. HM and HW referred to the hydrolysates dissolved in methanol and water, respectively.

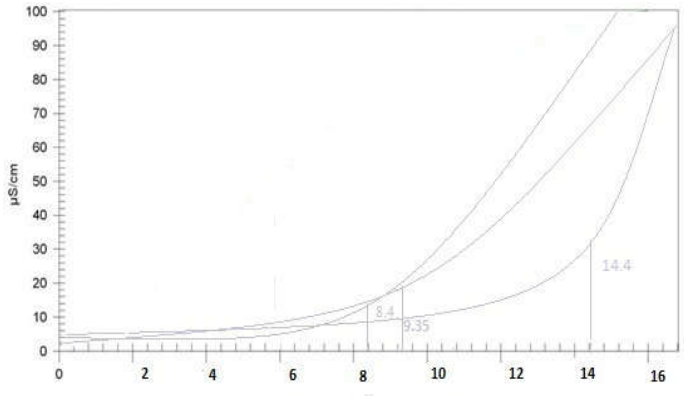

Figure 3: Comparison of IP values of 300 ppm BHT (14.4 h), 100 ppm BHT(8.4 h) and 300 ppm HW_1 (9.35h), which were measured by the equipment Rancimat 743.

(4) **Identification of peptide by LC-MS**: Sequences of two antioxidant peptides derived from the premium pet meal from the UFF separation (MW< 1000) were analyzed by Agilent 6100 quadrupole mass spectrometer (Agilent Technologies,Santa Clara, CA) connected with an electrospray ionization detector. Their amino acid sequences were temporarily identified as Leu-Thr-Cys or Iso-Thr-Cys and His-Cys.

From the above results, it is concluded that the alkaline hydrolysates from the different commercial sources (rendering products) possess stronger hydrophilic antioxidant activities against the different free radicals such as DPPH, ABTS, ORAC radicals, as well as lipophilic antioxidant activity against the oxidation of β-carotene linoleic acid. In details:

1. Both enzymatic and alkaline hydrolyses will produce antioxidant peptides, but the latter method is more desirable in terms of the commercial practice and antioxidant activity;
2. Antioxidant power of the hydrolysates against the free radicals depended on their concentrations, higher the concentration, stronger the antioxidant power;
3. Regarding the hydrolysate samples that were dissolved either in water or methanol, the methanol solution generally gave higher antioxidant capacity (Fig 2), but not significant;
4. Hydrolysates had comparable antioxidant activity to BHT in the DPPH test (previous report) and in OSI test. Also, more importantly, the blank control "premium" poultry meal (without antioxidant addition) also showed high antioxidant activities during the DPPH and ABTS assays. This indicated the possible utilization of the hydrolysates as a (partial) antioxidant substitute;
5. The antioxidant peptides may contain essential amino acids such as Thr and Cys.

Summary: the antioxidant hydrolysates from the rendering proteins can be prepared via base hydrolysis. The obtained hydrolysate exhibited strong antioxidant activity comparable to BHT via different assays.

Impacts and Significance: Rendered animal co-products have been demonstrated to be an excellent source for producing strong antioxidant hydrolysates that are suitable and feasible to be developed into safe, non-toxic and possible substitute of current commercial antioxidants used in rendering industries. It is expected the novel peptide-based antioxidants will significantly reduce rendering industries' financial pressure relying on some natural (i.e., Vit E) and synthetic antioxidants (such as BHT, BHA, and ethoxyquin). Moreover, the novel peptide antioxidants from the rendering products can also be developed into value-added feed ingredients for a huge benefit (profits) because of the huge nutraceutical market for pet and aquacultural functional foods. In fact, some companies (like Kemin Industries in Ames, Iowa, and the Protein Products Inc. in Gainssville, GA) have shown commercial interests on its potential applications.

Intellectual Property Development: According the positive results and potential market application, patent disclosure has been file through the CU intellectual properties office. It is under committee review.

Publications:

1. Bo Li, Feng Chen, Xi Wang, Baoping Ji, Yonnie Wu. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. *Food Chemistry*. 2007, 102:1135-1143;
2. Qing Kong, Feng Chen, Xi Wang, Jing Li, Tao Bi, Bin Guan. Optimization of conditions for enzymatic production of ACE inhibitor peptides from collagen. *Food and Bioprocess Technology*. 2011,4:1205-1211.

Future Work: Within the next funding we will focus on the following objective: (1) Investigate the synergistic effects of antioxidants; (2) determine the sequences of more antioxidant peptides. (3) making protein meal powder mixed with antioxidants in different formula.

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