Effects of Production Factors and Egg-Bearing Period on the Antioxidant Activity of Enzymatic Hydrolysates from Shrimp (*Pandalopsis dispar*) Processing Byproducts

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ABSTRACT: The effects of production factors (protease used, percent enzyme, hydrolysis time, and water-to-substrate ratio) on the antioxidant activity of hydrolysates produced from shrimp processing byproducts (SPB) were assessed using Taguchi's L_{16} (4^5) fractional factorial design. SPB hydrolysates showed excellent ABTS radical scavenging activity, metal ion chelating capacity, and inhibition of lipid peroxidation, but weak DPPH radical scavenging activity and ferric ion reducing antioxidant power. The protease used significantly influenced antioxidant activities while hydrolysis time and percent enzyme affected radical scavenging activities and inhibition of lipid peroxidation, respectively. Differences in the lipid and amino acid contents observed between SPB collected early and late in the egg-bearing period may have contributed to the slight variance in antioxidant activities displayed by their hydrolysates. Nevertheless, SPB hydrolysates produced using Alcalase or Protamex had high antioxidant activity regardless of production factors and egg-bearing period.

KEYWORDS: shrimp processing byproducts, enzymatic hydrolysis, Taguchi's L_{16} (4^5) design, antioxidant activity, egg-bearing period, amino acid content

■ INTRODUCTION

Pandalopsis dispar, or sidestripe shrimp, is the larger and higher value of the two main shrimp species[1] harvested by the 3.9 million dollar British Columbia shrimp trawl industry.[2] While the market for higher value sidestripe shrimp remains stable,[3] the heads and shells, which account for half of the shrimp weight,[4] are typically removed after processing and have no further market value.[5] Many studies have examined the potential use of these underutilized materials, termed shrimp processing byproducts (SPB), for generating hydrolysates of a wide variety of bioactive and functional properties that are applicable to industries such as pharmaceuticals, functional food, and nutraceuticals.[6−9] Hydrolysates with antioxidative peptides have been of recent interest for use as natural food ingredients that suppress the lipid peroxidation associated with off-flavors and formation of potentially toxic compounds, which lead to product deterioration, shortened shelf life, and heightened risk of serious diseases. While synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) exhibit strong antioxidant activity, their application in food products is strictly regulated due to concern over potential adverse health effects.

Enzymatic hydrolysis is a common process used to produce bioactive peptides, including those with antioxidant activity. Production factors such as enzyme-to-substrate ratio,[8] protease used,[10] time of hydrolysis,[11] and water-to-substrate ratio[12] have been shown to influence the antioxidant activity of hydrolysates. As well, antioxidative hydrolysates have been produced from shrimp shells,[8] SPB,[13] and prawn muscle.[10] However, to our knowledge, there has not been any report on the generation of antioxidative hydrolysates from the SPB of *P. dispar* or on the congruent effect of the aforementioned production factors on antioxidant activity of the hydrolysates.

After *P. dispar* undergoes protandric hermaphroditism in the third year of life, the egg-bearing period commences when females begin extruding and attaching eggs to their lower abdomen in October, where eggs incubate until hatching in early March.[14] Events of the reproductive cycle including protandry, spawning, and the egg-bearing period have been described to result in physiological changes,[15] potentially altering shrimp composition. Significant changes have also been reported in the amino acid content of European lobster eggs during the course of embryogenesis.[16] Further, it was suggested that greater seasonal variation in amino acid content of the SPB fraction than the meat fraction may be expected due to reproductive changes occurring in the gonads and hepatopancreas located within the cephalothorax.[17] However, the changes in amino acid content of SPB obtained at different times during the egg-bearing period and whether these changes influence the antioxidant activity of their hydrolysates have yet to be studied.

Therefore, the objective of this study was to apply Taguchi's L_{16} (4^5) fractional factorial experimental design to examine the effects of production factors, specifically the protease used, percent enzyme, hydrolysis time, and water-to-substrate ratio, on the antioxidant activity of *P. dispar* SPB hydrolysates. In addition, the amino acid content and antioxidant activity of hydrolysates produced from SPB sampled at two different times during the egg-bearing period of *P. dispar* were investigated.

Table 1. (a) Experimental Conditions for Shrimp Processing Byproducts Hydrolysate Production as Determined by Taguchi's L$_{16}$ (4^5) Fractional Factorial Design, Extent of Hydrolysis of Hydrolyzed Samples, and Antioxidant Activities of Hydrolysates. (b) Results of Analysis of Variance for the Effects of the Four Production Factors from Taguchi's L$_{16}$ (4^5) Fractional Factorial Design on Five Antioxidant Activities of Shrimp Processing Byproducts Hydrolysates

(a)

sample	protease used[a]	percent enzyme (%)	hydrolysis time (h)	water-to-substrate ratio	extent of hydrolysis (meq/g)[b]	ABTS radical scavenging activity (%)	DPPH radical scavenging activity (%)	ferric ion reducing antioxidant power (Abs at 700 nm)	metal ion chelating capacity (%)
A2-1	A	0.2	1	1:1	1.97	83	14	0.05	87
A4-8	A	0.4	8	2.5:1	2.45	88	2	0.06	97
A6-24	A	0.6	24	1.5:1	3.02	87	12	0.07	81
A8-4	A	0.8	4	2:1	2.30	87	2	0.04	98
A8-4 (1)[c]	A	0.8	4	2:1	2.09	87	8	0.09	98
A8-4 (2)[c]	A	0.8	4	2:1	2.64	86	4	0.09	99
B2-24	B	0.2	24	2.5:1	0.67	76	17	0.06	43
B4-4	B	0.4	4	1:1	0.64	68	0	0.06	41
B6-1	B	0.6	1	2:1	0.56	69	3	0.07	51
B8-8	B	0.8	8	1.5:1	0.68	72	4	0.03	43
F2-4	F	0.2	4	1.5:1	1.43	74	6	0.05	70
F4-24	F	0.4	24	2:1	2.69	80	14	0.03	31
F6-8	F	0.6	8	1:1	3.05	76	2	0.00	68
F8-1	F	0.8	1	2.5:1	2.31	78	0	0.03	89
P2-8	P	0.2	8	2:1	1.22	84	16	0.06	88
P4-1	P	0.4	1	1.5:1	1.51	84	16	0.08	94
P6-4	P	0.6	4	2.5:1	1.75	84	16	0.07	94
P8-24	P	0.8	24	1:1	2.22	85	26	0.08	86
C0-4	C	0.0	4	2.5:1	0.31	56	13	0.23	84
standards[d]	-	-	-	-	-	75	39	0.02	87

(b)

production factor	p-value[e]				
	ABTS radical scavenging activity	DPPH radical scavenging activity	ferric ion reducing antioxidant power	metal ion chelating capacity	extent of lipid peroxidation[f]
water-to-substrate ratio	0.060	0.969	0.860	0.314	0.075
percent enzyme	0.326	0.255	0.987	0.290	0.041*
hydrolysis time	0.024*	0.024*	0.348	0.096	0.705
protease used	0.000*	0.013*	0.123	0.002*	0.004*

[a]A = Alcalase, B = bromelain, F = Flavourzyme, P = Protamex, C = endogenous proteases. [b]Data from Cheung and Li-Chan.[7] [c]Sample codes followed by (1) or (2) denote sample replicates of the corresponding sample. [d]Concentrations of standards were as follows: 20 μM and 15 μM Trolox (ABTS and DPPH radical scavenging activity, respectively), 15 μM Trolox (ferric ion reducing antioxidant power), and 25 μM EDTA (metal ion chelating capacity). [e]Values with an asterisk (*) denote factors that are statistically significant (p < 0.05). [f]As represented by absorbance readings at 500 nm after 170 h of incubation in a linoleic acid model system.

■ MATERIALS AND METHODS

Materials. The SPB (shells, tails, and cephalothorax recovered from cooked material by hand-peeling) of *P. dispar* used in this study was provided in frozen form by Albion Fisheries Ltd. (Vancouver, BC). Upon receiving, samples were kept overnight at 4 °C, then distributed into 1000 g packages and stored at −25 °C prior to use. SPB were obtained twice: once in November (early in the egg-bearing period or EEB) and once in February (late in the egg-bearing period or LEB).

Food-grade proteases were donated by Neova Technologies Inc. (Abbotsford, BC). Alcalase 2.4 L FG (*Bacillus licheniformis*, 2.4 AU/g), Flavourzyme 1000 L (*Aspergillus oryzae*, 1000 LAPU/g), and Protamex (*Bacillus amyloliquefaciens* and *Bacillus licheniformis*, 1.5 AU/g) were products from Novozymes North America Inc. (Salem, NC) while bromelain (from pineapple stem, 2000 GDU/g) was manufactured by Bio-Logics, Inc. (Montreal, QC).

The following chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON): 2-2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potas-

sium ferricyanide, ferrozine, ferrous chloride, ferric chloride, linoleic acid, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT). Trichloroacetic acid and potassium persulfate were from Fisher Scientific (Fairlawn, NJ) while 2,4,6-trinitrobenzenesulfonic acid (TNBS) was purchased from Thermo Scientific (Rockford, IL) and ethylenediaminetetraacetic acid tetrasodium salt (EDTA) was a product of BDH Inc. (Toronto, ON).

Proximate Analysis. Proximate analysis of SPB was performed according to Cheung and Li-Chan[7] using vacuum oven drying, dry ashing, petroleum ether extraction, and nitrogen combustion methods for moisture, mineral, fat, and protein contents, respectively.

Hydrolysate Production. Hydrolysate samples were prepared as described in Cheung and Li-Chan[7] using the conditions assigned in the Taguchi's L$_{16}$ (4^5) fractional factorial design (Table 1a). Briefly, 1000 g of SPB was blended with distilled deionized water and preheated in a 50 °C water bath. Proteases were added, and samples were incubated for the indicated time with constant stirring, after which they were boiled for 10 min to terminate hydrolysis, sieved through a 2 mm mesh, and centrifuged at 3300g for 20 min. The soluble fraction of the hydrolyzed samples (hereafter referred to as hydrolysates or hydrolysate samples) was recovered for antioxidant
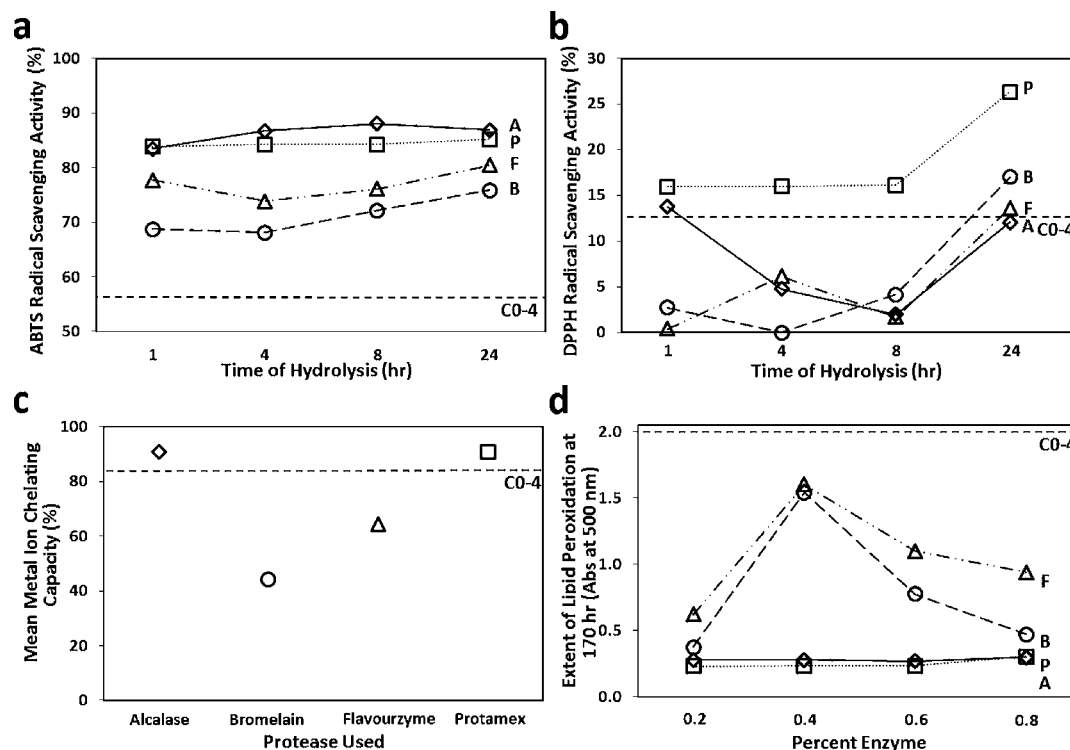
Figure 1. The effects of significant production factors (named in parentheses) on the following antioxidant activities exhibited by shrimp processing byproducts hydrolysates: (a) ABTS radical scavenging activity (protease used and hydrolysis time), (b) DPPH radical scavenging activity (protease used and hydrolysis time), (c) metal ion chelating capacity (protease used), and (d) inhibition of lipid peroxidation after 170 h of incubation (protease used and percent enzyme). Proteases are depicted as follows: Alcalase (◇ or A), bromelain (○ or B), Flavourzyme (△ or F), and Protamex (□ or P). The dashed line (--- or C0-4) in each graph represents the level of activity displayed by the hydrolysate produced without exogenous enzymes.

activity assays. The samples were coded for protease (A = Alcalase, B = bromelain, F = Flavourzyme, P = Protamex, and C = no exogenous enzyme added), percent enzyme (2, 4, 6, or 8 for 0.2, 0.4, 0.6, or 0.8% weight enzyme per weight SPB, respectively), and hydrolysis time (1, 4, 8, or 24 h). For example, the sample code A2-1 denotes the hydrolysate produced after 1 h hydrolysis of SPB with 0.2% Alcalase. The fourth factor in the Taguchi design, water-to-substrate ratio, was not included in the coding system since it was not found to significantly affect hydrolysate antioxidant activity.

Extent of Hydrolysis. Extent of hydrolysis (EH) was represented by the α-amino content of hydrolyzed samples, which was determined in triplicate using the TNBS method as described by Cheung and Li-Chan.[7]

Determination of Antioxidant Activity. The ABTS and DPPH radical scavenging activity, ferric ion reducing antioxidant power (FRAP), and metal ion chelating capacity (MCC) assays were carried out following the methods described by Samaranayaka and Li-Chan.[18] The final assay concentration was 1 mg/mL for the DPPH radical scavenging activity and FRAP assays, while 0.067 mg/mL and 0.1 mg/mL were used for ABTS radical scavenging activity and MCC assays, respectively.

Inhibitory Activity in a Linoleic Acid Peroxidation Model System. The inhibition of lipid peroxidation by SPB hydrolysates in a linoleic acid peroxidation model system (LAPS) was determined according to Samaranayaka and Li-Chan[18] with the following modifications. Linoleic acid (50 mM) was prepared in absolute ethanol while hydrolysates were dissolved in sodium phosphate buffer (0.1 M, pH 7.0) to a final assay concentration of 40 μg/mL. Hydrolysate sample (1 mL), linoleic acid (1 mL), and distilled deionized H_2O (0.5 mL) were mixed in capped, glass vials and incubated in the dark at 60 °C in a Maqni Whirl water bath with shaker (Blue M Electric Company; Blue Island, IL). Phosphate buffer replaced the hydrolysate sample in the assay control, while BHT and BHA (at a final assay concentration of 40 μg/mL in 50 mM linoleic

acid) were used as standard or reference antioxidants. The extent of lipid peroxidation in the presence of hydrolysate or standard antioxidant and in the assay control was monitored periodically over 170−195 h using the ferric thiocyanate method as described by Chen, Muramoto, Yamauchi, and Nokihara,[19] and the results were reported as absorbance at 500 nm over time.

Amino Acid Analysis. Amino acid analysis of SPB and selected hydrolysate samples was performed by the Advanced Protein Technology Centre at the Hospital for Sick Children in Toronto, ON (http://www.sickkids.ca/Research/APTC/Amino-Acid-Analysis/index.html). Amino acid content of LEB and EEB samples was determined after precolumn derivatization with phenylisothiocyanate by reversed-phase high performance liquid chromatography and detection at 254 nm. Total amino acid contents were analyzed after first hydrolyzing samples with 6 N HCl and 1% phenol at 110 °C for 48 h while free amino acid contents were obtained by analysis without acid hydrolysis. Both total and free amino acid contents were expressed as grams per 100 g sample (dry basis).

Statistical Analysis. The effect of the four production factors (protease used, percent enzyme, hydrolysis time, and water-to-substrate ratio) on the antioxidant activity of hydrolysates, and the variance in antioxidant activity of hydrolysates produced from SPB obtained at two different times during the egg-bearing period, were analyzed by the General Linear Model Analysis of Variance followed by Tukey's comparison test using Minitab software (version 16, Minitab Inc.; State College, PA). Differences were considered to be statistically significant at the 5% level.

■ RESULTS AND DISCUSSION

Effect of Production Factors on the Antioxidant Activity of SPB Hydrolysates. Sixteen different SPB hydrolysates, produced using unique combinations of production factors according to the fractional factorial design, were

evaluated for the effect of the four production factors on antioxidant activity. Triplicate hydrolysates were prepared for one of these combinations (A8-4) to assess the reproducibility of antioxidant activity of hydrolysates produced under the same conditions. The hydrolysate produced after 4 h hydrolysis of SPB without the addition of exogenous proteases (C0-4) was used to assess the antioxidative potential of SPB hydrolysate generated solely through endogenous proteolytic activity. The extent of hydrolysis and antioxidant activities of these 19 samples are summarized in Table 1a.

The protease used in enzymatic hydrolysis of SPB significantly affected the ABTS and DPPH radical scavenging activities, MCC, and inhibition of lipid peroxidation after 170 h in the LAPS (Table 1b). Hydrolysis time also significantly influenced the radical scavenging activities of hydrolysates while percent enzyme was a significant factor affecting the inhibition of lipid peroxidation. Water-to-substrate ratios had no significant impact on the antioxidant activity of hydrolysates. SPB hydrolysates in general had very weak FRAP, and hence none of the production factors were found to significantly affect the reducing power of hydrolysates. The results for each of the antioxidant activity assays are discussed in further detail below.

ABTS Radical Scavenging Activity. The antioxidant activity of SPB hydrolysates was determined using the ABTS radical scavenging activity assay, which measures the decrease in absorbance at 734 nm as antioxidants scavenge ABTS radicals and render them colorless.[20] All SPB hydrolysates produced with exogenous proteases had high ABTS radical scavenging activity of 68−88% at 67 μg/mL (Table 1a). In particular, hydrolysates produced by Alcalase or Protamex showed activities reaching 83−88% and 84−85%, respectively, both exceeding the activity of 20 μM Trolox (75%). SPB hydrolysates produced using bromelain or Flavourzyme had lower activities (68−80%) while C0-4 had the weakest activity (56%). High ABTS radical scavenging activity has also been reported for defatted SPB hydrolysates produced using Alcalase (50% at 7.4 μg/mL)[13] and the water-soluble fraction of white shrimp cephalothorax extract (75 μmol of Trolox equivalent/g of sample).[21]

ABTS radical scavenging activity of hydrolysates was significantly affected by the protease used and hydrolysis time (Table 1b). Hydrolysates produced using bromelain or Flavourzyme displayed a decrease in activity from 1 to 4 h of hydrolysis, after which activity gradually increased as hydrolysis time increased up to 24 h (Figure 1a); in contrast, hydrolysates produced using Alcalase or Protamex maintained high activity regardless of the hydrolysis time. Extensive proteolysis leading to the release of shorter peptides and free amino acids has been reported to increase ABTS radical scavenging activity. For example, higher ABTS radical scavenging activity was reported in the lower molecular weight fractions (1.3 kDa) of ornate threadfin bream hydrolysates,[22] and the ABTS radical scavenging activity of red snapper hydrolysates also increased with degree of hydrolysis.[23] These findings were consistent with the results of this study, in which ABTS radical scavenging activity was found to be significantly correlated with EH (r = 0.7126).

DPPH Radical Scavenging Activity. The ABTS and DPPH radical scavenging activity assays share similar principles in quantifying electron-transferring antioxidants in solution, though the former uses an aqueous solution and the latter uses an alcoholic solvent.[20] In contrast to the high scavenging activity for ABTS radicals, low DPPH radical scavenging

activity of 0−26% assayed at 1 mg/mL was observed for all SPB hydrolysates compared to the 39% displayed by 15 μM Trolox (Table 1a). The scavenging activity for DPPH radicals found in this study for *P. dispar* SPB hydrolysates was lower than that reported for fermented *Penaeus mondon* biowaste (40% activity at 1 mg/mL).[24]

DPPH radical scavenging activity was significantly affected by the protease used (Table 1b), with hydrolysates produced using Protamex showing the highest DPPH radical scavenging activity (16−26%) (Table 1a). The higher activity of hydrolysates produced using Protamex in this study differs from the results reported in a study using defatted SPB as the starting material, in which hydrolysates produced using Alcalase showed higher activity than those produced by five other proteases including Protamex and Flavourzyme.[13] Hydrolysis time was also shown to significantly affect DPPH radical scavenging activity in the current study, where a sharp increase of activity was observed for all hydrolysates produced after 24 h of hydrolysis compared to those made after 1, 4, or 8 h (Figure 1b). Enzyme concentration reportedly played a role in optimizing DPPH radical scavenging activity of shrimp shell hydrolysates,[8] but was not found to have a significant impact on the activity of shrimp byproduct hydrolysates in another study using response surface methodology.[9] A water-to-substrate ratio of 1:1 during hydrolysis was also predicted to optimize DPPH radical scavenging activity of cobia skin gelatin hydrolysates.[12] However, neither percent enzyme nor water-to-substrate ratio was a significant production factor in the present study, potentially due to the predominant influence incurred by the type of proteases used.

Ferric Ion Reducing Antioxidant Power. The FRAP assay measures an antioxidant's ability to donate hydrogen ions where higher absorbance values observed at 700 nm reflect stronger reducing activity of samples.[20] As shown in Table 1a, C0-4 had the highest absorbance, almost 10-fold greater than that of 15 μM Trolox, while all hydrolysates produced with exogenous proteases had low activities (abs_{700nm} < 0.10). As such, no significant production factors were detected (Table 1b). Though the FRAP and ABTS radical scavenging activity assays share similar chemical mechanisms, the former occurs in an acidic environment and the latter in a neutral environment.[20] A study on white shrimp cephalothorax extract reported a high correlation between FRAP and ABTS radical scavenging activity (R^2 = 0.97).[21] However, the opposite relationship was observed in the current study where C0-4 had the lowest ABTS radical scavenging activity and highest FRAP while hydrolysates produced with exogenous proteases had high ABTS radical scavenging activity but weak reducing power.

Metal Ion Chelating Capacity. Metal ion chelators act as antioxidants by binding with pro-oxidative metal ions to prevent their radical-forming reaction with hydrogen peroxide.[20] All SPB hydrolysates in this study displayed high MCC ranging from 31 to 99% when assayed at 0.1 mg/mL (Table 1a), with all of the hydrolysates produced using Alcalase or Protamex, except for A6-24, having MCC similar to or higher than that exhibited by 25 μM EDTA. The MCCs shown by SPB hydrolysates were comparable to or surpassed those reported for Pacific hake and silver carp hydrolysates, the highest of which were 46% at 5 mg/mL[18] and 93% at 1 mg/mL, respectively.[11]

The protease used was the only production factor significantly affecting MCC, the effects of which are illustrated in Figure 1c. While hydrolysates made using Alcalase or
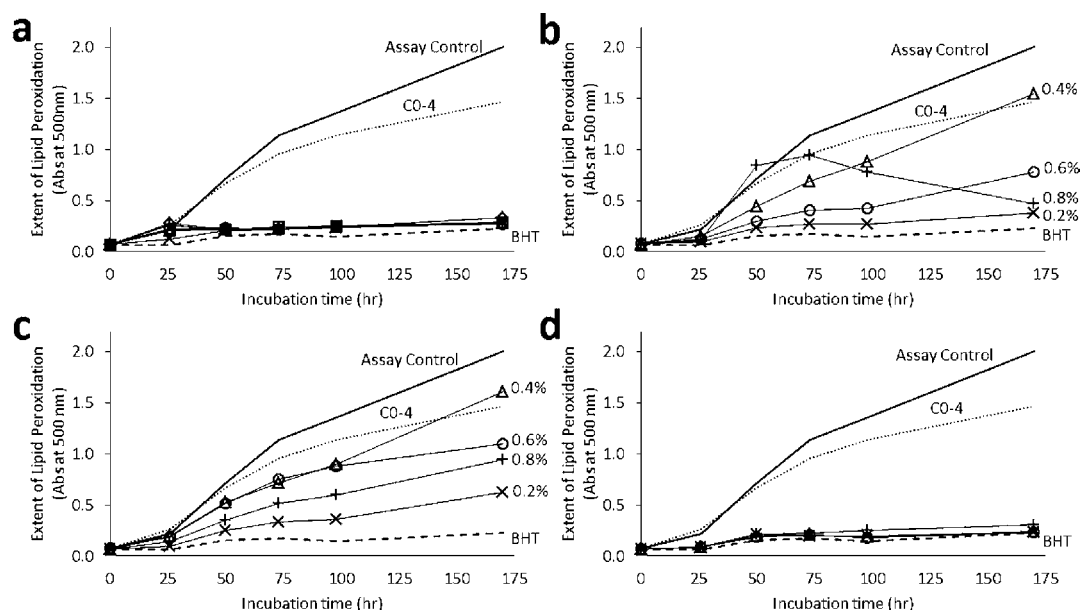
Figure 2. The extent of lipid peroxidation in a linoleic acid model system as influenced by shrimp processing byproducts hydrolysates produced using (a) Alcalase, (b) bromelain, (c) Flavourzyme, and (d) Protamex at enzyme concentrations of 0.2% (✕), 0.4% (△), 0.6% (○), and 0.8% (+). Assay control (—), C0-4 (···), and BHT (---) are included in each graph while sample replicates A8-4(1) (□) and A8-4(2) (◇) are also shown in panel a. Data points are averaged values of assay triplicates.

Protamex had high MCC, those prepared with bromelain had significantly weaker MCC while Flavourzyme-produced hydrolysates displayed a wide range of activities (Table 1a and Figure 1c). Though hydrolysis time was not a significant factor ($p = 0.096$), the lowest MCC was observed in samples made with the longest hydrolysis time among hydrolysates produced using Alcalase, Flavourzyme, or Protamex (Table 1a). Conversely, the hydrolysates produced using bromelain, Flavourzyme, or Protamex had the highest MCC when hydrolyzed for the shortest time. This observation agrees with that reported for barley hordein hydrolysates where prolonged hydrolysis time was associated with a decrease in MCC, which was postulated to be due to the degradation of larger, cage-forming peptides that entrapped ions as proteolysis is extended.[25] On the contrary, a positive correlation between MCC and degree of hydrolysis has also been reported.[11,26] Thus, the relationship between MCC and degree of hydrolysis may differ depending on starting materials and production factors. As suggested in the current study, other production factors may have minor influences on MCC despite not being significant. For example, F8-1 produced after 1 h hydrolysis using 0.8% Flavourzyme exhibited high activity (89%) whereas F4-24, having been hydrolyzed with only 0.4% Flavourzyme but for 24 h, had much lower MCC of 31%. However, one can only speculate on the relationship between production factors as the fractional factorial experimental design currently employed does not allow for analysis of potential interactions among factors.

Inhibition of Lipid Peroxidation. The ability of hydrolysates to inhibit lipid peroxidation in the LAPS was quantified by monitoring hydroperoxide production as indicated by the absorbance at 500 nm using the ferric thiocyanate assay.[19] All SPB hydrolysates exhibited inhibitory activity, with the protease used and percent enzyme significantly affecting inhibitory activity after 170 h incubation (Table 1b, Figure 1d). Hydrolysates produced using Alcalase or Protamex had excellent inhibition of lipid peroxidation with effectiveness comparable to BHT (Figures 2a and 2d) and BHA (data not

shown) regardless of the other production factors employed. Weaker and more variable inhibitory activity was displayed by hydrolysates produced using bromelain or Flavourzyme (Figures 2b and 2c); in fact, the sample containing B8-8 had a higher absorbance (greater peroxidation) than the assay control after 50 h of incubation, suggesting the presence of pro-oxidants. Of the four enzyme levels tested for hydrolysate production, hydrolysates produced using 0.2% enzyme had the lowest absorbance after 170 h (Figure 1d). Further increases in percent enzyme either had little influence on activity (e.g., hydrolysates produced using Alcalase or Protamex) or resulted in a sharp decrease in activity at 0.4% percent enzyme, followed by an increase in activity as percent enzyme increased from 0.4% to 0.8% (e.g., hydrolysates produced using bromelain or Flavourzyme). The hydrolysate C0-4, which was produced without adding any exogenous enzyme, had the lowest inhibitory activity among SPB hydrolysates, but did nevertheless suppress the extent of lipid peroxidation relative to the assay control.

The ability of proteases to produce hydrolysates exhibiting high inhibitory activity in the LAPS varies with different starting materials. Bullfrog skin hydrolysates produced using Alcalase had the highest inhibitory activity (assayed at 0.05 mg/mL) after 7 days of incubation when compared to five other proteases[27] while wheat germ protein hydrolysates produced using Alcalase only had moderate activity ($abs_{500nm} > 0.500$ assayed at 0.4 mg/mL) after 5 days of incubation.[28] As well, Protamex-digested microalgae had higher inhibitory activity than those digested with Alcalase or Flavourzyme,[29] whereas Alcalase-digested brown seaweed had higher inhibitory activity than those digested with Protamex.[30] Though there is a paucity of research on the inhibitory activity of SPB hydrolysates in the LAPS, hydrolysates of SPB (mixed shrimp species) produced by Alcalase were recently reported to significantly suppress lipid peroxidation and its induced color change in croaker fish muscle.[6] The current study showed that SPB hydrolysates produced using either Alcalase or Protamex displayed strong

Table 2. (a) Total Amino Acid Content[a] and (b) Free Amino Acid Content[b] of Shrimp Processing Byproducts (SPB) Obtained Early in the Egg-Bearing Period (EEB) and Late in the Egg-Bearing Period (LEB) and the Resulting Hydrolysates Produced Using Endogenous Enzymes (C0-4), Alcalase (A8-4), Bromelain (B4-4), Flavourzyme (F2-4), and Protamex (P6-4)

(a)

amino acid	total amino acid content (g/100 g sample, dry basis)											
	SPB		C0-4		A8-4		B4-4		F2-4		P6-4	
	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]
Asx[d]	1.75	3.82	1.87	3.34	5.38	5.64	4.95	7.00	4.74	5.82	5.47	5.81
Glx[e]	4.71	5.88	3.99	5.97	8.59	9.02	8.00	11.16	7.91	9.56	9.05	9.48
Ser	1.93	1.71	1.09	1.58	2.92	2.80	2.17	2.91	2.22	2.51	2.82	2.82
Gly	2.20	2.08	6.50	4.77	3.85	3.29	5.84	4.32	4.94	3.56	4.10	3.52
His	1.36	1.01	0.83	0.83	2.07	1.78	1.57	1.75	1.63	1.56	2.02	1.78
Arg	3.93	3.46	5.48	4.51	6.08	5.66	6.41	6.62	6.09	5.46	6.20	5.71
Thr	1.84	1.48	0.97	1.51	2.81	2.83	1.95	2.73	2.03	2.33	2.79	2.88
Ala	2.37	2.27	2.16	2.74	3.61	3.79	3.46	4.40	3.38	3.84	3.61	3.91
Pro	1.95	1.79	3.13	3.14	3.15	2.74	3.59	3.36	3.25	2.77	3.17	2.93
Tyr	2.14	1.94	0.93	1.34	3.06	3.10	1.83	2.30	1.92	2.15	2.82	3.01
Val	2.54	2.15	1.18	1.95	3.74	3.61	2.49	3.37	2.57	3.01	3.51	3.59
Met	1.44	1.20	0.58	0.94	2.16	2.04	1.20	1.76	1.34	1.60	2.06	2.02
Ile	2.35	2.11	0.82	1.59	3.24	3.50	1.81	2.80	2.04	2.68	3.09	3.40
Leu	3.29	3.06	1.42	2.83	4.62	5.14	3.11	5.03	3.49	4.71	4.55	5.06
Phe	2.19	1.93	0.82	1.52	3.13	3.37	1.69	2.61	1.98	2.47	2.98	3.30
Lys	3.08	3.20	1.44	3.34	5.04	6.18	3.14	6.30	4.11	5.90	4.80	6.11
∑TAA[f]	39.07	39.09	33.21	41.90	63.45	64.49	53.21	68.42	53.64	59.93	63.04	65.33

(b)

amino acid	free amino acid content (g/100 g sample, dry basis)											
	SPB		C0-4		A8-4		B4-4		F2-4		P6-4	
	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]	EEB	LEB[c]
Asx[d]	0.01	0.05	0.15	0.30	0.14	0.23	0.10	1.34	0.94	1.42	0.08	0.18
Glx[e]	0.03	0.10	0.68	0.47	0.42	0.38	0.38	1.88	1.76	1.97	0.23	0.23
Ser	0.01	0.03	0.19	0.18	0.18	0.22	0.11	0.82	0.84	0.87	0.10	0.15
Gly	0.23	0.43	4.80	2.22	1.55	0.61	3.11	1.27	3.41	1.34	1.61	0.72
His	0.01	0.02	0.15	0.10	0.12	0.12	0.08	0.42	0.49	0.43	0.08	0.08
Arg	0.55	0.43	8.47	1.88	2.92	0.74	4.13	2.38	6.84	2.58	2.93	0.76
Thr	0.01	0.05	0.15	0.23	0.24	0.36	0.08	0.62	0.54	0.64	0.15	0.28
Ala	0.06	0.16	1.06	0.78	0.54	0.55	0.71	1.47	1.69	1.56	0.40	0.43
Pro	0.15	0.33	1.94	1.53	0.65	0.43	1.43	0.72	1.33	0.72	0.74	0.47
Tyr	0.01	0.05	0.21	0.34	0.18	0.31	0.12	0.94	0.83	0.95	0.10	0.22
Val	0.02	0.08	0.26	0.38	0.34	0.32	0.18	1.05	1.02	1.04	0.19	0.25
Met	0.01	0.01	0.18	0.20	0.25	0.48	0.12	0.66	0.65	0.61	0.12	0.32
Ile	0.01	0.05	0.20	0.28	0.31	0.15	0.14	0.85	0.73	0.83	0.23	0.17
Leu	0.03	0.09	0.32	0.49	0.24	0.48	0.23	2.47	2.08	2.43	0.23	0.53
Phe	0.02	0.06	0.22	0.31	0.26	0.35	0.16	1.12	1.27	1.09	0.18	0.37
Trp	0.02	0.10	0.14	0.50	0.11	0.27	0.07	1.35	0.30	1.34	0.07	0.24
Lys	0.00	0.02	0.34	0.15	0.19	0.05	0.18	0.34	1.56	0.37	0.13	0.05
∑FAA[g]	1.18	2.06	19.46	10.34	8.64	6.05	11.33	19.70	26.28	20.19	7.57	5.45

[a]Determined after acid hydrolysis. [b]Determined without acid hydrolysis. [c]Data from Cheung and Li-Chan.[7] [d]Asx represents both aspartic acid and asparagine. [e]Glx represents both glutamic acid and glutamine. [f]Sum of total amino acids. [g]Sum of free amino acids.

inhibitory activity in the LAPS, warranting further investigation of both proteases for generating antioxidative SPB hydrolysates for use in food systems.

Hydrolysates of Shrimp Processing Byproducts from Different Times of the Egg-Bearing Period. As the antioxidant activity of hydrolysates is affected by the amino acid sequence and composition of their constituent peptides, the changes in SPB during different times of the egg-bearing period may potentially affect the antioxidant activity of hydrolysates produced. Thus, SPB were obtained early in the egg-bearing period (EEB) and late in the egg-bearing period (LEB) to assess potential differences in amino acid content and

their influence on antioxidant activity of hydrolysates. Hydrolysis time, percent enzyme, and water-to-substrate ratios during SPB hydrolysis were found in the preceding experiments to have less influence than the protease used on antioxidant activity of the resulting hydrolysates. Thus, the potential effects of the egg-bearing period were investigated by examining only samples produced by the four exogenous proteases and the endogenous enzymes at the hydrolysis time of 4 h (i.e., A8-4, B4-4, F2-4, P6-4, and C0-4).

Proximate Analysis of SPB Acquired Early and Late in the Egg-Bearing Period. The most apparent difference between SPB collected early in the egg-bearing period and late in the

Table 3. Comparison of the Extent of Hydrolysis of Hydrolyzed Samples and Antioxidant Activity of Hydrolysates Produced from Shrimp Processing Byproducts Obtained Early in the Egg-Bearing Period (EEB) and Late in the Egg-Bearing period (LEB)[a]

	extent of hydrolysis (meq/g)		ABTS radical scavenging activity (%)		DPPH radical scavenging activity (%)		ferric ion reducing antioxidant power (Abs at 700 nm)		metal ion chelating capacity (%)	
sample	EEB	LEB[b]	EEB	LEB	EEB	LEB	EEB	LEB	EEB	LEB
A8-4	1.13 x	2.30 y	85 x	87 y	8 y	1 x	0.066 y	0.026 x	94 x	98 x
B4-4	0.36 x	0.64 y	63 x	68 y	3 y	0 x	0.180 y	0.061 x	60 y	41 x
F2-4	0.71 x	1.43 y	71 x	74 y	8 x	6 x	0.162 y	0.048 x	85 y	70 x
P6-4	1.26 x	1.75 y	79 x	84 y	9 x	16 y	0.070 x	0.072 x	89 x	94 y
C0-4	0.41 x	0.31 x	49 x	56 y	10 x	13 x	0.267 y	0.229 x	97 y	84 x

[a]Different letters (x, y) within a sample indicate significant difference in the extent of hydrolysis or antioxidant activity between egg-bearing periods at $p < 0.05$. [b]Data from Cheung and Li-Chan.[7]

egg-bearing period (hereafter referred to as SPB_{EEB} and SPB_{LEB}, respectively) was the presence of eggs in the former. Proximate analysis of SPB_{EEB} showed 74.5 ± 1.6% moisture, and on a dry basis (db) 41.4 ± 1.3% protein, 24.7 ± 2.0% ash, and 5.3 ± 0.3% lipid. The carbohydrate content estimated by difference was 28.7%. The moisture, protein, and ash contents of SPB_{LEB} were 75.1 ± 0.9%, 44.6 ± 0.5% (db), and 21.9 ± 0.6% (db), respectively, similar to those of SPB_{EEB}. However, the lipid content of SPB_{LEB} was much higher at 20.0 ± 0.4%, and the carbohydrate content was therefore estimated to be 13.4%. The protein content of SPB_{EEB} and SPB_{LEB} was higher than that reported for shrimp shells while the ash content was lower.[8,31] The lower lipid content of SPB_{EEB} resembled that of black tiger shrimp shells[31] and redspotted shrimp residue.[32] Relatively higher lipid content observed in SPB_{LEB} may be the result of lipid accumulation within the ovaries throughout the egg-bearing season for yolk production.[33]

Amino Acid Content of SPB_{EEB} and SPB_{LEB} Hydrolysates. The total amino acid content ($\sum$TAA) was virtually the same for SPB_{EEB} and SPB_{LEB} (Table 2a) while the free amino acid content ($\sum$FAA) of SPB_{LEB} was almost twice that of SPB_{EEB} (Table 2b). Conversely, SPB_{LEB} hydrolysates had higher $\sum$TAA and, with the exception of B4-4, lower $\sum$FAA than SPB_{EEB} hydrolysates. Furthermore, these differences were greater in B4-4, C0-4, and F2-4 hydrolysates than A8-4 and P6-4 hydrolysates.

Glx, Arg, Leu, and Lys were the most abundant amino acids present in SPB (Table 2a) while Arg, Gly, and Pro were the most abundant free amino acids (Table 2b). This is consistent with the essential amino acid content of *Pandalus borealis* processing discards, in which Leu, Lys, and Arg were the most abundant,[34] while Pro and Arg were the most abundant free amino acids in shrimp waste.[5] The most abundant amino acids in SPB hydrolysates were Glx, Arg, Asx, Lys, and Gly, while Arg, Gly, Pro, Leu, and Ala were the most abundant free amino acids. High contents of Glu, Arg, Leu, Lys, Ala, and Gly were previously reported in shrimp waste hydrolysates.[35,36] As well, high total Arg, Glu, Asp, Leu, Lys, and Gly content[17,37] and free Pro, Arg, and Ala content have been reported in the edible fraction of various shrimp species.[38] Arg, Gly, and Ala were also among the most abundant free amino acids in lobster eggs during embryogenesis due to their important roles in anaerobic glycolysis.[16] This may partly explain the consistently higher free Arg content in SPB_{EEB}, where eggs were observed, and its subsequent hydrolysates than SPB_{LEB}. However, further investigation on the amino acid profile of SPB throughout the course of the year is needed to determine if such drastic

changes in amino acid composition are mediated solely by the events of the egg-bearing period.

Differences in Antioxidant Activity of SPB_{EEB} and SPB_{LEB} Hydrolysates. Significant differences were found in antioxidant activity of SPB_{EEB} and SPB_{LEB} hydrolysates, as shown in Table 3. Whereas the ABTS radical scavenging activity was generally higher and FRAP was generally lower in SPB_{LEB}, the differences observed in DPPH radical scavenging activity and MCC between SPB_{EEB} and SPB_{LEB} hydrolysates appeared to be protease-dependent. Evidently, some antioxidant activities of hydrolysates were influenced by the differences in the starting material obtained at different times during the egg-bearing period, the effect of which was mediated by the protease used. However, SPB hydrolysates maintained their overall high ABTS radical scavenging activity and MCC while DPPH radical scavenging activity and FRAP remained low regardless of variances observed between SPB_{EEB} and SPB_{LEB} hydrolysate samples. As well, hydrolysates produced using Alcalase or Protamex consistently had higher antioxidant activity than those produced using bromelain, Flavourzyme, or endogenous proteases despite differences in the starting material used. Thus, the variances observed in antioxidant activities between SPB_{EEB} and SPB_{LEB} hydrolysates were not large enough to alter the overall antioxidative properties of hydrolysates. In fact, no significant differences were detected between hydrolysates prepared from SPB_{EEB} or SPB_{LEB} in their ability to inhibit lipid peroxidation during 195 h incubation in the LAPS (data not shown).

Compositional differences between SPB_{EEB} and SPB_{LEB} noted in the proximate analysis may have contributed to the differences in antioxidant activity of hydrolysates produced from them. The association between lipid content of the starting material and antioxidant activity of their hydrolysates was suggested when a prehydrolysis wash of brownstripe snapper muscle reduced the lipid content of the starting material and increased the ABTS and DPPH radical scavenging activities, FRAP, and MCC of hydrolysates.[23] This may partly explain the lower FRAP, DPPH radical scavenging activity, and MCC observed in some SPB_{LEB} hydrolysates, which were produced from SPB with a higher lipid content. However, changes in lipid content could not be the sole factor differentiating antioxidant activity between SPB_{EEB} and SPB_{LEB} hydrolysates since the latter had higher ABTS radical scavenging activity. Rather, the higher ABTS radical scavenging activity may be related to the higher EH observed in SPB_{LEB} hydrolysates. Changes in the protein content during the egg-bearing period that alter the availability of cleavage sites

accessible to different proteases may account for the differences in EH of SPB$_{EEB}$ and SPB$_{LEB}$ hydrolysates.

Amino acid content differences between SPB$_{EEB}$ and SPB$_{LEB}$ hydrolysates may also influence hydrolysate antioxidant activities since certain amino acids have been shown to be more antioxidative than others. For example, His is commonly associated with antioxidant activity due to its hydrogen-donating and radical-trapping imidazole ring.[19,39,40] Other amino acids such as Tyr,[41] Pro, Trp,[42] Lys,[10] Cys, Met, and Phe have also been associated with antioxidant activity.[39] As well, hydrophobic amino acids have been postulated to increase peptide solubility in a lipid system and aid in peptide inhibition of lipid peroxidation.[40] Indeed, a generally higher total Phe, Tyr, and Lys content and free Trp, Phe, Met, and Lys content was found in SPB$_{LEB}$ hydrolysates, and their ABTS radical scavenging activity was significantly correlated with contents of free Met ($r = 0.7106$) and free Trp ($r = 0.7024$). However, variances in amino acid contents between SPB$_{EEB}$ and SPB$_{LEB}$ hydrolysates were not reflected in the inhibition of lipid peroxidation or overall hydrolysate antioxidant activity.

In summary, the protease used during enzymatic hydrolysis of shrimp processing byproducts was found to significantly affect the antioxidant activity of the resulting hydrolysates while hydrolysis time and percent enzyme showed some influence but to a lesser extent. Among the proteases investigated, Alcalase and Protamex were shown to produce shrimp processing byproducts hydrolysates with consistently high ABTS radical scavenging activity, metal ion chelating capacity, and inhibition of lipid peroxidation comparable to standard antioxidants. Hydrolysates produced from shrimp processing byproducts acquired at the beginning versus the end of the egg-bearing period showed some differences in radical scavenging activity, reducing power, and metal ion chelating capacity, but did not differ in ability to inhibit lipid peroxidation. Despite these differences, which may have been related to changes observed in lipid and amino acid contents of the starting material, the overall trends in hydrolysate antioxidant activity remained similar between SPB$_{EEB}$ and SPB$_{LEB}$. In conclusion, this study revealed that the protease used for hydrolysis of shrimp processing byproducts was the main production factor dictating hydrolysate antioxidative activity and, moreover, that hydrolysates with consistently high antioxidant activity could be produced with the selected proteases, despite variable composition of the substrate shrimp processing byproducts acquired at different times during the egg-bearing period.

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Notes
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■ ABBREVIATIONS USED

ABTS, 2-2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; db, dry basis; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; EEB, early egg-bearing period; EH, extent of hydrolysis; FRAP, ferric ion reducing antioxidant power; LAPS, linoleic acid peroxidation model system; LEB, late egg-bearing period; MCC, metal ion chelating capacity; SPB, shrimp processing byproducts; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid

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