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PHYSICOCHEMICAL PROPERTIES AND PROFICIENT ACTIVITIES OF CHITIN AND CHITOSAN ISOLATED FROM THE SHELL WASTE OF A NATIVE FRESH WATER CRAB *OZIOTELPHUSA SENEX SENEX*

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ABSTRACT

Chitin and Chitosan are the two important bioactive compounds obtained from the shell waste of a native fresh water crab *Oziotelphusa senex senex* by the chemical method that involves demineralization (DM), deproteinization (DP), decolorization (DC) and deacetylation (DA). The present study was conducted to isolate, purify and determine the physicochemical characteristics of the chitin and chitosan and its proficient activities obtained by altering the sequence of the chemical process based on the time period and particle size under laboratory condition. Two different protocols (DMPAC, DMPA2C) were adopted for two different particle sizes of 0.2 mm and 0.4 mm. The physicochemical characteristics and the % of yield, total proteins, moisture, ash content and carotenoid of chitin and chitosan were estimated. The results showed a significant difference statistically in the yield, total proteins, moisture, ash content and carotenoid of chitin and chitosan in both male and female crabs. The results of the present study also confirmed that the bioactive molecules present in this chitosan (particle size 0.2mm) of a fresh water crab, *Oziotelphusa senex senex* had both antioxidant and antihypertensive activity.

Key Words:- Chitin and Chitosan,

INTRODUCTION

Shell wastes produced during shellfish processing are the source of some important bioactive compounds such as chitin, chitosan and astaxanthin. Chitin and chitosan are the most important nontoxic, biocompatible and biodegradable naturally occurring biopolymer (Ruiz – Herrera, 1978).

These bioactive compounds are found in abundance in invertebrates, fungi etc. but not found in higher plants and animals (Austin *et.al.*, 1981). Annual production of shell fishery is about 100 million pound in which 85 % of waste residue is discharged by dumping in land without pretreatment (Rout 2001).

Crustacean shells are treated with acid to dissolve calcium carbonate, followed by alkaline to solubilize proteins which are present in crustacean shells in high concentrations, followed by a decolorization step which is usually necessary to remove remaining pigments and to obtain a colorless product. These treatments are applied to each chitin source regardless of the nature of starting material. The residual protein and pigments needs to be removed for further utilization, especially for biomedical applications (Rinaudo, 2006). To keep the environment clean it is necessary to reduce, recycle and reuse of the wastes that represents a realistic approach of reutilization

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concept of these bioactive products. Hence the present study was carried out on the following aspects:

1. To isolate and purify the chitin and chitosan by different methods from the shell wastes.
2. To estimate the total yield, total proteins, moisture, ash content and carotenoid of chitin and chitosan of the fresh water crab, *Oziotelphusa senex senex*.
3. To determine the antioxidant activity (DPPH Assay) and antihypertensive (ACE inhibitory Assay) of the soluble chitosan obtained by different methods.

MATERIALS AND METHOD

Collection and Preparation of Materials

The shell wastes of male and female crab of *Oziotelphusa senex senex* were collected separately in a plastic trough. Later the shell was subjected to dechlorinated tap water treatment for the removal of certain attached connective tissues and other impurities. The shells were then dried in the oven at 70ºC for a period of 24 hours. The shells were then grinded finely using a mixer grinder and the sieve size of 0.2 mm and 0.4 mm were used to obtain the respective shell powder sizes. These dried shell powders of male and female crab of *Oziotelphusa senex senex* were stored separately at room temperature in the air tight zip lock pouch.

Protocol for Isolation of Chitin and Chitosan

Isolation of chitin and chitosan were carried out using a standard protocol with slight modification (No *et. al.,* 1995). Two different methods (i.e., DMPAC, $DMPA_2C$) were carried out using two different particle sizes of 0.4 and 0.2mm.

Method I: (DMPCA)

Both male and female crab shell powder of *Oziotelphusa senex senex* was treated separately in ratio of solid to either acidic or alkaline solution of 1:10 throughout the experiment. Initially the shell powder of size 0.4mm was treated with 1N hydrochloric acid for a time period of 5 hours for demineralization. The acid was drained out from samples and thoroughly washed with dechlorinated tap water for 30 minutes. The demineralized shell was then subjected to 5% NaOH solution for 12 hours at room temperature with constant stirring using magnetic stirrer. The filtrate was again washed for 30 minutes and dried in hot air oven. Decolorization was carried out using oxalic acid and potassium permanganate and thoroughly washed. Then it was dried in hot air oven. Deacetylation process was achieved by subjecting the decolorized dried shell powder to vigorous alkaline treatment of 50% NaOH for two hours at 70 ºC water bath.

Method II: ($DMPCA_2$)

The same above procedure of method I was followed in method II but the last step of the protocol (i.e., process of deacetylation) was done for two times. The protocol followed in method I and II was carried out for the male and female crab shell powder size of 0.2mm

Protocol for qualitative analysis of Chitin and Chitosan

The chitin and chitosan of the male and female crabs were taken separately for the qualitative analysis of minerals such as Carbonate, Sulphate, Phosphate and Calcium by standard protocol (AOAC, 1990), Proteins (Gornall, *et al.,* 1949), Chitin and Chitosan (Richards, 1951), moisture (Black, 1965), ash (Reaff, 2009) and Carotenoid (Lopez-Cervantes, *et al.,*2006).

Protocol for quantitative analysis of Chitin and Chitosan

The chitin and chitosan of the male and female crabs were taken separately for the quantitative analysis to estimate the total yield, protein, moisture, ash and carotenoid using standard method.

Total Yield: The total yield of chitin and chitosan were carried out using a standard protocol with slight modification (No *et. al.,* 1995).

Total Protein: The estimation of total protein present in the male and female chitin and chitosan of different particle size was determined by Kjeldahl method (AOAC, 1990 method 984.13) and spectroscopic method (Marion, 1976).

Total Moisture: The moisture content of the male and female chitin and chitosan of different particle size was determined by the gravimetric method. The moisture content was determined measuring the sample after and before drying (Black, 1965).

Total Carotenoid: The carotenoid present in samples were estimated by following a standard protocol using acetone (Lopez Cervantes *et al.,* 2006).

Total Ash: The samples were dried, carbonized and then incinerated at 550 ± 25 °C. After cooling, the mass of the residue was determined for ash (Reaff, 2009).

ACE inhibitory activity assay

The ACE inhibitory activity was measured for chitosan obtained from male and female of different particle size using standard method in which the Angiotensin I-converting enzyme releases hippuric acid

from hippuril-L-histidyl-Lleucine by hydrolytic removal of the dipeptide, His-Leu (Cushman & Cheung, 1971). The percent inhibition of enzyme activity was calculated as follows.

$$\% \text{ of ACE inhibition Activity} = \frac{(\text{O.D of Hipurric acid})_{Control} - (\text{O.D of Hipurric acid})_{Sample} \times 100}{(\text{OD of Hipurric acid})_{Control}}$$

DPPH activity assay

Due to the complete solubility of 0.2mm chitosan particle size in 1% acetic acid the samples were considered for the antioxidant activity. The DPPH free radical scavenging activity was measured for chitosan obtained from male and female of 0.2mm particle size using standard method (Blois, 1958). During the assay, two ml of $6x10^{-5}$ M methanolic solution of DPPH were added to 50 µl of the samples. The mixture was incubated in dark place for 15 min at 25°C, after which the absorbance was recorded at 515 nm using UV spectrophotometer. The percentage of DPPH free radical scavenging activity was calculated using the formula:

$$\% \text{ Scavenging Activity} = [(A_0-A_1/A_0)] \times 100$$

Where, A_0 is the absorbance of Blank and A_1 is the absorbance of Sample.

RESULT AND DISCUSSION

The physicochemical analysis was recorded for both the male and female chitin and chitosan separately (Table 1). The parameters such as total yield, total protein, moisture, ash and carotenoid were quantified in both chitin and chitosan obtained from male and female crabs are shown separately (Table 2) (Table 3). The quantitative analysis of chitin showed higher percentage of total yield, moisture and ash in male of particle size 0.2mm, where as the proteins, was recorded to be highest in male particle size 0.4mm. The quantitative analysis of chitosan showed higher percentage of total yield, total protein, moisture and ash in male of particle size 0.2mm. A similar work was carried out in Krill in which 14 % yield of chitosan (Brzeski ,1982) 18.3% from crawfish shell (Sun-ok Fernandez Kim, 2004) and 18.6% from prawn waste (Alimuniar and Zainuddin, 1992) was obtained. The protein content was higher in the male than that of female. In comparison to the mud crab *Scylla serrata* the protein content found in *Oziotelphusa senex senex was* slightly lower (Sujeetha, *et al.,* 2015).

Table 1. Physicochemical analysis of chitin and chitosan in a fresh water crab shell *Oziotelphusa senex senex*.

S. No.	Parameters	Chitin		Chitosan	
		Male	Female	Male	Female
1.	Test for Minerals (CO_3, SO_4, PO_4, Ca)	-	-	-	-
2.	Test for Proteins	+	+	+	+
3.	Test for Chitin	+	+	+	+
4.	Test for Chitosan	+	+	+	+
5.	Test for Ash	+	+	+	+
6.	Test for Carotenoid	+	+	+	+

Table 2. Quantitative analysis of Chitin in a fresh water crab shell *Oziotelphusa senex senex*.

S.No	Parameters Expressed in %	Particle Size – 0.4 mm				Particle Size – 0.2 mm			
		Method - I		Method - II		Method - I		Method - II	
		Male	Female	Male	Female	Male	Female	Male	Female
1.	Total Yield	33.77±0.35	32.12±0.33	33.25±0.62	31.83±0.28	36.27±0.41*	34.14±0.25	36.34±0.17*	33.81±0.77
2.	Total Protein	12.62±0.47	10.31±0.89	12.81±0.52*	10.14±0.43	12.11±0.42	10.09±0.57	12.35±0.39*	10.03±0.74
3.	Moisture	42.34±0.32	41.57±0.93	42.11±0.69	41.32±0.54	48.10±0.79*	47.44±0.56	47.93±0.57*	47.45±0.67
4.	Ash	1.58±0.04	1.68±0.06	1.55±0.03	1.66±0.05	1.78±0.03*	1.21±0.08	1.81±0.02*	1.85±0.02
5.	Carotenoid	1.23±0.17*	1.12±0.11	1.11±0.13	1.07±0.15	1.03±0.14	0.96±0.07	1.06±0.11	0.91±0.06

Each value represents the percentage Mean ± S.D.

*indicates the significant difference ($p \leq 0.05$) between male and female chitin of different particle size.

Table 3. Quantitative analysis of Chitosan in a fresh water crab shell *Oziotelphusa senex senex*.

S.NO.	Parameters Expressed In %	Particle size – 0.4 mm				Particle size – 0.2 mm			
		Method - I		Method - II		Method - I		Method - II	
		Male	Female	Male	Female	Male	Female	Male	Female
1.	Total Yield	24.99±0.	21.64±0.	22.39±0.	20.19±0.	24.75±0.4	22.57±0.	20.89±0.6	19.55±0.
2.	Total Protein	9.67±0.7	7.56±0.2	8.87±0.2	6.57±0.3	11.45±0.2	9.56±0.8	10.12±0.5	8.37±0.4
3.	Moisture	21.13±0.	20.89±0.	19.01±0.	18.32±0.	28.19±0.8	27.64±0.	34.37±0.4	31.98±0.
4.	Ash	1.58±0.0	1.68±0.0	1.55±0.0	1.66±0.0	1.78±0.03	1.21±0.0	1.81±0.02	1.85±0.0
5.	Carotenoid	1.23±0.1	1.12±0.1	1.11±0.1	1.07±0.1	1.03±0.14	0.96±0.0	1.06±0.11	0.91±0.0

Each value represents the percentage Mean ± S.D.
*indicates the significant difference ($p \leq 0.05$) between male and female chitosan of different particle size.

Table 4. Antioxidant Activity of chitosan in a fresh water crab *Oziotelphusa senex senex*

S. No.	Sample	Mean O.D ± S.D.		DPPH %	
		Male	Female	Male	Female
1.	Reagent (blank)	0.631± 0.009	0.631± 0.009	0	0
2.	Chitosan – III	0.308± 0.003	0.340± 0.008	51.19± 0.92*	46.12± 0.32
3.	Chitosan - IV	0.317 ± 0.002	0.364 ± 0.005	49.76 ± 0.93*	43.32 ± 1.09

Each value represents the percentage Mean ± S.D.
*indicates that there is significant difference ($p \leq 0.05$) between male and female.

Table 5. *In Vitro* analysis of ACE inhibition using crude extracts of chitosan in a freshwater crab *Oziotelphusa senex senex*

S.No.	Sample	Mean Optical Density ±S.D		Mean ace inhibition (%) ±S.D	
		Male	Female	Male	Female
1.	Control	0.571±0.008	0.571±0.008	-	-
2.	Chitosan - I	0.365±0.005	0.379±0.009	36.188±0.659	33.741±0.583
3.	Chitosan - II	0.395±0.004	0.407±0.004	30.944±0.091	28.846±0.553
4.	Chitosan - III	0.336±0.005	0.346±0.011	43.006±0.408*	41.084±0.371
5.	Chitosan - IV	0.326±0.005	0.337±0.002	41.433±0.691	39.685±0.942

Each value represents the percentage Mean ± S.D on dry weight basis.
*indicates the significant difference ($p \leq 0.05$) between male and female chitosan.

Antioxidant and ACE inhibitory activity of soluble chitosan obtained from male and female by method II is shown (Table 4). The statistical analysis (t-test) showed a significant difference in total yield, total protein in chitin and chitosan obtained from female and male crab shell of different sizes. The soluble chitosan obtained from the male shell of particle size 0.2mm (method II) showed highest antioxidant and antihypertensive activity. There was a significant difference in male than that of female chitosan in both antihypertensive and antioxidant activity. The chitosan content extracted from the fresh water crab shell waste was similar to the results obtained in shrimp waste (Ornum, 1992) and crawfish shell waste (Johnson, *et al.*, 1978). According to species and seasons the proportion of the chitosan varies (Green, *et al., 1984*). A similar work of antioxidant activity of chitin and chitosan was reported in marine crab *Scylla serrata* (Sujeetha, *et al.*, 2015). Park, Je, and Kim (2004) suggested that chitosan may eliminate various free radicals by the action of nitrogen that is present on the C-2 position of the chitosan. Antioxidants available in synthetic forms are used in actively in food industries because of their, low cost, efficacy and high stability. Related to their toxicological aspects in human very less care is taken. Such artificial antioxidants are used in food industries under strict regulation that caused potential damage to health by such compounds (Chan *et al.*, 2007).

The chitosan III obtained from method II showed highest ACE inhibitory activity which may be due to the antihypertensive protein present in them. An ample evidences exists that bioactive peptides obtained from the natural sources have gained much attention because of their numerous health beneficial effects (Sinead *et.al*., 2011). Hence these peptides exhibits various biological activities such as antioxidant, anti-hypertensive, anti-proliferative, anticoagulant, calcium-binding, anti-obesity and anti-diabetic activities (Dai-Hung Ngo *et al*., 2012). Hence, it can be suggested that bioactive food proteins are alternative sources for synthetic ingredients that can contribute, as a part of functional foods, pharmaceuticals and cosmetics (Se-Kwon and Eresha, 2006). Hence the present study of chitosan obtained from freshwater crab shell waste exhibited the properties such as free radical scavenging and antihypertensive proves to be the hallmark in food industry in which the naturals has emerged to replace synthetic ones.

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CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

REFERENCES

Alimuniar and Zainuddin. An economical technique for producing chitosan. In Advances in Chitin and Chitosan, C.J. Brine, P.A. Sanford, and J.P. Zikakis (Ed.), Elsevier Applied Science, Essex, UK.1992; 627.

AOAC. Official Methods of Analysis, 15th edition, Assoc. Official Analytical Chemist, Washington, D.C, 1990.

Austin PR, Brine CJ, Castle JE and Zikakis JP. Chitin: New facets of research. *Science*. 1981; 212: 749.

Black CA. Methods of Soil Analysis: Part I Physical and mineralogical properties. American Society of Agronomy, Madison, Wisconsin, USA; 1965.

Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature*. 1958; 29: 1199 1200.

Brzeski MM. Concept of chitin/chitosan isolation from Antarctic Krill (*Euphausia superba*) shells on a technique scale. In Proceedings of the Second International Conference on Chitin and Chitosan*;* S. Hirano and S. Tokura (Ed.), 1982; 15. The Japan Society of Chitin and Chitosan, Sapporo, Japan.

Chan EWC, Lim YY, Omar M. Antioxidant and antibacterial activity of leaves of *Etlingera* species (Zingiberaceae) in Peninsular Malaysia. *Food Chem*. 2007; 104: 1586-1593.

Cushman DW and Cheung HS. Concentrations of angiotensin-converting enzyme in tissues of the rat. *Biochim. Biophys. Acta*, 1971; 250: 261–26.

Dai-Hung Ngo, Thanh-Sang Vo, Dai-Nghiep Ngo, Isuru Wijesekara and Se-Kwon Kim,. Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *International Journal of Biological Macromolecules*. 2012; 51: 378– 383.

Gornall, A.G, Bardawill, C. J. and David, M. M.. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem*. 1949; 177: 751-66.

Green JH and Kramer A. Food Processing Waste Management. p. 214. AVI publishing Co., Westport, CT; 1984.

Johnson EL and Peniston QP. The production of chitin and chitosan. In Proceedings of the First International Conference on Chitin/Chitosan; Muzzarelli, R.A.A. and Pariser. E.R. (Eds.), 1978; 80-87.

Lopez-Cervantes J, Sanchez-Machado DI, Gutierrez-Coronado MA and Ríos- Vazquez NJ. Quantification of astaxanthin in shrimp waste hydrolysate by HPLC. *Biomedical chromatography*. 2006; 20(10): 981–984.

Marion M Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 1976; 72: 248-254.

No HK, Meyers SP. Preparation and characterization of chitin and chitosan-A Review. *Journal of Aquatic Food Product Technology*. 1995; 4(2): 27-52.

Ornum JV. Shrimp waste. *Info-fish International*, 1992; 6/92:48-51.

Park PJ, Je JYand Kim SW. Free radical scavenging activities of different deacetylated chitosans using an ESR spectrometer. *Carbohydrate Polymer*, 2004; 55: 17–22.

Reaff. Reference: IS 5960 Part 1 : 1996 DGHS Manual- Fish and fish products; 2009.

Richards A Glenn. The integument of arthropods. Univ. Minnesota Press, Minneapolis; 1951.

Rinaudo M. Chitin and Chitosan: Properties and applications, *Progress in Polymer Science*. 2006; 603-632.

Rout SK. Physicochemical, Functional, and Spectroscopic analysis of crawfish chitin and chitosan as affected by process modification. Dissertation, 2001.

Ruiz-Herrera J. The distribution and quantitative importance of chitin in fungi. In Proceedings of the First International Conference on Chitin/ Chitosan; Muzzarrelli, R.A.A. and Pariser, E.R. (Eds.), MIT Sea Grant Program, Cambridge, MA. 1978, 11.

Se-Kwon Kim and Eresha Mendis. Bioactive compounds from marine processing byproducts – A review. *Food Research International*. 2006: 39: 383–393.

Sinéad Lordan R, Paul Ross and Catherine Stanton. Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. *Mar. Drugs*. 2011; 9: 1056-1100.

Sujeetha M, Sharmila S, Jayanthi J and Ragunathan MG. Quantitative and Qualitative analysis of chitin and chitosan from the shell of the mud crab, *Scylla serrata* (Forskal, 1775). *International Journal of Pharmacy & Therapeutics*, 2015; 6(2): 69-72.

Sujeetha M, Sharmila S, Jayanthi J and Ragunathan, MG. Antioxidant property of some extract derived from the mud crab, *Scylla serrata*. *International Journal of Phytopharmacology*. 2015; 6(2): 111-113.