Madras Agric. J., 100 (10-12): 883-886, December 2013
https://doi.org/10.29321/MAJ.10.001427

Physico - chemical Characteristics of Chitosan Extracted from Silkworm Pupae

H.N. Suresh, C.A. Mahalingam and P. Priyadharshini
Department of Sericulture, Centre for Plant Protection Studies,
Tamil Nadu Agricultural University, Coimbatore - 641 003

Chitosan was extracted from silkworm pupae using different methods and the physico - chemical characteristics such as moisture, nitrogen and ash, degree of deacetylation, solubility, viscosity and pH of the final product were analysed. The moisture, nitrogen, ash and pH ranged from 7.09 to 8.57 per cent, 3.32 to 4.12 per cent, 0.36 to 0.41 per cent and 7.14 to 7.4 respectively. The degree of deacetylation was upto 97 per cent. The effect of deacetylation in comparison with different duration was also studied. The results revealed that there was increase in deacetylation per cent of chitin with increase in duration of treatment. The solubility of chitosan at one per cent acetic acid solution was 100 per cent whereas viscosity ranged from 47 to 71.4 cP.

Key words: silkworm pupae, chitosan, degree of deacetylation, solubility, viscosity

Chitin forms elytra and integument of insects and it is a macromolecular linear polymer of anhydrous N-acetyl- D-glucosamine. The monomer units are linked by beta (1-4) glycosidic bonds as in cellulose (Tolaimate *et al*., 2000). It is highly hydrophobic, insoluble in water and most of the organic solvents. The annual production of chitin in nature accounts to approximately 100 billion tonnes.Though it forms the second most abundant natural biopolymer, next to cellulose, it is the most under exploited biomass resources available on earth (MPEDA, 1998) Deacetylation of chitin with strong alkali yields free base 2-amino-2-deoxy-D-glucosamine commonly known as chitosan (Rinaudo, 2006). Its molecular structure is similar to cellulose. It is insoluble in water but soluble in dilute acids (in aqueous solutions). Chitosan is chemically inert, biocompatible, non antigenic, non toxic, biofunctional, less expensive and eco friendly (Malviya *et al.,* 2010 Chitin is obtained in industrial scale from shrimps and crustaceans, in general (Yanga *et al*., 2000). However, the pupae of the silkworm can be an alternative source of chitin and consequently the chitosan (Zhang *et al*., 2000). Silkworm pupae, a by product from the silk industry, is cheaper and easily available (Paulino *et al*., 2006). The silkworm pupae on dry weight basis contain 49-51% of protein, 20% fat, 4.19% ash, 2.71% chitin and 4.82% other components. For comprehensive utilization of pupae, pupal oil and protein can be extracted, and chitin can be extracted from the remaining produce (Ni and Liang, 1999).

Chitosan finds immense application in food industry, cosmetics, agriculture, water treatment, textile, biotechnology, paper industry; wound therapy, drug delivery, cell delivery systems, orthopaedics, ophthamology and bone healing (Chen *et al*., 2002). It exhibits antimicrobial activity against bacteria, fungi, and yeast. It is hypoallergenic, haemostatic, has rapid blood clotting property and acts as fat attractor by binding to dietary lipids.

The success of chitosan in each of the specific applications is directly related to deep research into their physicochemical properties such as moisture, nitrogen, ash, degree of aceetylation, solubility, viscosity and pH respectively. Therefore, this research was undertaken to study the above mentioned characteristics of chitosan extracted from silkworm pupae by different production methods.

Materials and Methods

The chitosan samples A and B were extracted from mulberry silkworm (*Bombyx mori* L.) pupae by chemical method and enzymatic method. The sample C was prepared from eri silkworm (*Samia cynthia ricini)* pupae by chemical method. Finally the chitosan sample D was prepared from de oiled pupal material of mulberry silkworm. The moisture, nitrogen and ash contents of chitosan samples were determined by the method outlined in A.O.A.C (1995). Viscosity was determined by dissolving 0.3 g of chitosan sample in 300ml of 1 per cent acetic acid. The sample was placed under spindle No.1 of Brookfield viscometer (USA) and viscosity was measured at 60 rpm. A quantity of 0.5 g of chitosan was mixed with 50 ml of distilled water and used for measuring the pH using Digital pH meter.

Solubility

An amount of 0.1g of chitosan powder was placed in centrifuge tube and dissolved in 10 ml of 1 per cent acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25 ^{o}C. The solution was then immersed in a boiling water bath for 10 minutes, cooled to room temperature (25^{o}C) and centrifuged

1*Corresponding author email: camahalingam@yahoo.com

at 10,000 rpm for 10 min. The supernatant was decanted. The undissolved particles were washed in distilled water (25 ml) then centrifuged at 10,000 rpm. The supernatant was removed and undissolved pellets were dried at 60₀ C for 12h. The solubility percentage was determined by

$$\text{Solubility}(\%) = \frac{\text{(Initial weight of tube + chitosan) - (Final weight of tube + chitosan)}}{\text{(Initial weight of tube + chitosan) - (Initial weight of tube)}} \times 100$$

Degree of Deacetylation

A quantity of 0.25 g of chitosan was dissolved in 50 ml of 0.1M acetic acid and stirred for 45 minutes for complete dissolution. Then, the solution was diluted to100 ml with distilled water and absorbance was measured at 200 - 204 nm using U-VIS spectrophotometer (Jasco, Japan), degree of deacetylation was calculated using standard graph (Muzzarelli and Rachetti, 1985).

Results and Discussion

The data on moisture percentage, nitrogen per cent, ash content, degree of acetylation, solubitity in acetic acid, viscosity and pH of chitosan extracted by different methods are furnished in Table 1.

Moisture

Moisture content ranged from 7.09 to 8.57 per cent. Among the samples A, B, C, D higher moisture content of 8.57 per cent was observed in B (chitin prepared by enzymatic method of deproteinization), followed by C (eri silkworm pupal chitin prepared by chemical method of deproteinization) (8.29). Lower moisture percentage (7.09) was observed in A (chitin prepared by chemical method of deproteinization). Sandford (1984) reported that moisture content of the chitosan should be less than 10 per cent for its commercial use. Toan (2009) also reported that moisture content of chitosan samples from shrimp shells ranged from 8 to 10 percent.

Nitrogen

The nitrogen content of the chitosan samples ranged between 3.32 and 4.12 per cent but was lower (7.06 to 7.97 %) than that reported by No and Meyers (1995) for chitosan extracted from crab and shrimp

Table 1. Physico - chemical parameters of chitosan extracted by different methods

Parameter	A	B	C	D
Moisture (%)	7.09 ± 0.32	8.57 ± 0.68	8.29 ± 0.354	7.38 ± 0.58
Nitrogen (%)	3.32 ± 0.10	4.04 ± 0.16	4.12 ± 0.26	3.36 ± 0.17
Ash (%)	0.37 ± 0.01	0.41 ± 0.02	0.4 ± 0.03	0.36 ± 0.03
Degree of acetylation (%)	91.12 ± 0.42	46.55 ± 0.61	91.86 ± 1.30	97 ± 1.00
Solubility (%) in 1 % acetic acid	100 ± 0.00	72.2 ± 2.95	100 ± 0.00	100 ± 0.00
Viscosity (cP)	54.89± 0.29	71.4 ±1.03	57.08 ± 0.80	47 ± 1.00
pH	7.4 ± 1.42	7.3 ± 1.15	7.37 ± 0.17	7.14 ± 0.00

A - Chitosan prepared from chemical method of deproteinization from mulberry silkworm pupae
B - Chitosan prepared from enzymatic method of deproteinization from mulberry silkworm pupae
C - Eri-silkworm pupal chitosan prepared from chemical method of deproteinization D - Chitosan prepared from de-oiled pupal material of mulberry silkworm

shell. Nessa *et al.* (2010) observed the nitrogen content of the prawn shell chitosan samples varied between 7.91% and 8.3 per cent. Higher nitrogen content (4.12 %) was recoeded in chitosan prepared from eri silkworm pupae. Low nitrogen content of 3.03 per cent was achieved by chemical method of chitosan preparation.

Ash

The measurement of ash is an indicator of the effectiveness of the demineralization step for removal of calcium carbonate. Demineralization resulted in products having 31 - 36% ash (Bough *et. al.,* 1978). The ash content in chitosan is an important parameter and some residual ash of chitosan samples may affect their solubility, consequently to lower viscosity, or can affect other important characteristics of the final product (Nessa *et al.,* 2010). The decrease of ash content increases the purity of chitosan (Yuan, 2011).

All chitosan samples obtained from the different production methods had less than one per cent of ash content. Higher ash content of 0.41 per cent was observed in B (chitin prepared by enzymatic method of deproteinization), followed by C (eri silkworm pupal chitin prepared by chemical method of deproteinization) (0.40 %). Lower ash content (0.36 %) was observed in D (Chitin prepared from de-oiled pupae). These findings are in agreement with (No and Meyers, 1995) who reported that 1 per cent of ash with high quality grade chitosan was obtained from crab and Toan (2009) recorded less than 1 per cent ash content in shrimp shell materials.

Degree of Deacetylation (DD)

An important parameter in characterizing the chitosan is the degree of deacetylation, i.e. the ratio of *N*-acetyl-D-glucosamine to D-glucosamine structural units. It also had an impact on the extent of moisture absorption, charge distribution, intrinsic viscosity and chitosan solubility in aqueous solutions (Cho *et al.,* 2000; Kumar, 2000; Singla and Chawla. 2001 ; Schatz *et al.,* 2003). The degree of deacetylation (DD) of silkworm pupae extracted chitosan samples ranged from 46.5 to 97 per cent. According to No and Meyers (1995), DD of chitosan ranged from 56 per cent to 99 per cent. Sample D had the highest DD (97%), followed by C, A and B (91.86%, 91.12%, and 46.55%, respectively). The solubility of sample B was lower (72.2 %) due to incomplete degree of deacetylation (46.55 %).

Higher DD is very important characteristic of chitosan which is widely used for drug delivery, wound healing, enzyme immobilization, dietary ingredient, food preservative, waste water treatment, molecular imprinting antitumor, haemostatic, hypocholesterolemic, antimicrobial, and antioxidant and metal reduction (Muzzarelli and Muzzarelli, 2005; Jian *et. al.,* 2008).

Deacetylation v/s Time

There was significant difference between the deacetylation percentage and duration of the

Table 2. Duration of deacetylation of chitin

Duration (h)	Deacetylation (%)		Mean (%)
	Mulberry silkworm chitin	Eri silkworm chitin	
D1 (2.0)	60.08	59.81	59.94
D2 (2.5)	72.30	74.96	73.63
D3 (3.0)	85.46	88.54	87.00
D4 (3.5)	90.82	91.96	91.39
D5(4.0)	98.21	96.08	97.14
Mean	81.37	82.27	81.8187
	SE d	CD (0.05)	
B	0.069	0.144**	
D	0.109	0.227**	
B x D	0.154	0.322**	

** - Significant at 1%

ANOVA: **B** (Silkworm), **D** (Duration)

treatment. The data on deacetylation of chitin with different duration of treatment are furnished in Table 2. Higher deacetylation per cent was observed in D5 (4 h of treatment) (97.14 %) which was followed by D4 (3.5 h of treatment) (91.39 %). Lower deacetylation per cent was observed in D1 (2 h of treatment) (59.24 %). Deaectylation of chitin increased with increased duration of treatment. Higher deacetylation of 98.21 per cent was achieved in 4 h of treatment. Mima *et al.* (1983) observed that increase in reaction time increased the percentage of deacetylation.

Knaul *et al.* (1998) prepared 70.8 per cent DD chitosan with 50% NaOH for 1 h under nitrogen atmosphere. These results indicated that DD of chitosan are mainly affected by NaOH concentration, reaction time, and temperature. Paulino *et al,* (2006) reported that the chitosan prepared from silkworm pupal chitin showed an average deaectylation per cent of 83 when chitin was treated with 40 per cent NaOH. Tsaih and Chen (2003) used 50% NaOH to de-acetylate chitin for 1 to 9 h and the results showed that the DD of the resulting chitosan increased with reaction time.

Solubility

Among the samples, A,C and D were found to have excellent solubility of 100 per cent with no significant difference except sample B which showed comparatively lower solubility (72.2 %) and this may be due to lower degree of deacetylation (DD). Brine and Austin (1981) noted that lower solubility suggested incomplete removal of protein and acetyl group. Since solubility of chitosan depends on the removal of acetyl group from chitin, low DD value and the presence of protein contaminants remaining in the sample during the process of analysis could adversely interfere with the results.

Viscosity

The viscosity of chitosan samples ranged from 47 to 71.4 cP. Higher viscosity of 71.4 cP was observed in B (chitin prepared by enzymatic method of deproteinization); it was followed by 57.08 cP in C (eri silkworm pupal chitin prepared by chemical method of deproteinization). Lower value of 47 cP was observed in D (Chitin prepared from de-oiled pupae).

The viscosity of chitosan solutions reported in the literature generally ranged from 60 to 780 cP (Alimuniar and Zainuddin, 1992). These ranges of viscosity were also observed by Cho *et. al.* (1998) for five commercially available chitosans.

Bough *et. al.* (1978) stated that viscosity of chitosans varied considerably from 60 to 5,110 cP depending on the species. Sample D had the lowest viscosity (47 cP) comparable to that of other samples as their lower solubility may be due to incomplete deacetylation of chitin whereas B had a high viscosity (71.4 cP). Sample A showed lower viscosity than sample C, though the difference was not significant possibly due to longer duration of deacetylation which might have degraded chitosan.

It is suggested that duration of deacetylation is an important step which might affect the quality of the product. There are some other factors affecting viscosity during the production of chitosan such as the molecular weight, concentration, ionic strength, pH and temperature (Moorjani *et. al*., 1975; Bough *et. al.*, 1978). Chitosan with low viscosity has additional benefits over high viscous chitosan for use in food and pharmaceutical industry.

pH

Higher pH of 7.4 was observed in A (chitin prepared by chemical method of deproteinization) which was followed by B (chitin prepared by enzymatic method of deproteinization) (7.37). Lower pH of 7.14 was in D (Chitin prepared from de-oiled pupae). Chitosan is considered as a strong base as its primary amino groups (pKa is **6.3),** easily form quaternary nitrogen salts at low pH. Thus, in acidic solutions chitosan has high anion capacity. At higher pH, it is a weak base because the primary amino groups are not protonated and therefore do not interact with anions and do not dissociate neutral salts. This is a peculiar feature of chitosan, in so far as it would be classified as a strongly basic anion exchanger with no dissociation capacity for neutral salts.

From this study it is concluded that the silkworm pupae could also be used as a raw material for production of chitosan. Physico - chemical characteristics of chitosan samples prepared by different methods from different sources may help in determining its suitability in various applications.

References

Alimuniar. and Zainuddin. 1992. An economical technique for producing chitosan. In Advances in Chitin and Chitosan, Brine, C.J., Sanford, P.A. and Zikakis J.P. (Ed.), Elsevier Applied Science, Essex, UK. pp.627.dn. Association of official analytical chemists, Washington D.C., 245 p.

AOAC, 1995. Bough, W.A., Salter, W.L., Wu, A.C.M. and Perkins, B.E. 1978. Influence of manufacturing variables on the characteristics and effectiveness of chitosan products. Chemical composition, viscosity and molecular weight distribution of chitosan products. *Biotechnol Bioeng*, **20**: 1931-1943.

Brine, C.I. and Austin, P.R. 1981. Chitin variability with species and method of preparation. *Comparative Biochemistry and Physiol.*, **69**: 283-286.

Chen, L.Y., Du, Y.M., Wu, H.Q. and Xiao. L. 2002. Relationship between molecular structure and moisture-retention ability of carboxymethyl chitin and chitosan. *J. Appl. Polymer Sci.*, **83**: 1233.

Cho, Y.I., No, H.K. and Meyers, S.P. 1998. Physicochemical characteristics and functional properties of various commercial chitin and chitosan products. *J. Agric. Food Chem.,* **46**: 3839-3843.

Cho, Y.W., Jang, J., Park, C.R. and Ko, S.W. 2000. Preparation and solubility in acid and water of partially jasmine deacetylated chitins. *Biomacromolecules,* **1**: 609-614.

Jasmine *et al* ., Jian, Y., Feng, T., Zheng, W., Qing, W., YanJun, Z. and ShiQian, C. 2008. Effect of chitosan molecular weight and deacetylation degree on hemostasis. *J Biomed. Mater. Res.,* **84**: 131-7.

Knaul, J.Z., Kasaai, M.R., Bui, V.T. and Creber, K.A.M. 1998. Characterization of deacetylated chitosan and chitosan molecular weight review. *Can. J. Chem.*, *76*, 1699-1706.

Kumar, M.N.V.R**.** 2000. A review of chitin and chitosan applications. *React. Funct. Polym.,* **46**: 1-27.

Malviya, R., Srivastava, P., Bansal, M. and Sharma, P.K. 2010. Preparation and evaluation of disintegrating properties of *cucurbita maxima* pulp powder. *Int. J. Pharmaceutical Sci.,* **2**: 395-399.

Mima, S., Miya, M., Iwamoto, R., Yoshikawa, S. 1983. Highly deacetylated chitosan and its properties. *J. Appl. Polym. Sci.*, **28**: 1909-1917.

Moorjani, M.N., Achutha, V. and Khasim, D.I. 1975. Parameters affecting the viscosity of chitosan from prawn waste. *J. Food Sci. Technol.,* **12**: 187-189.

MPEDA. 1998. Chitin and Chitosan. Marine Product Export and Development Authority. pp. 16-17.

Muzzarelli, R.A.A. and Muzzarelli, C. 2005. Chitosan chemistry: Relevance to the biomedical sciences. *Adv Polym. Sci.,* **186**: 151-209.

Muzzarelli, R.A.A. and Racchetti, R. 1985. The determination of the degree of hitosn by spectrophotometry.**In**:Chitin in nature and technology. Muzzarelli, R., Jeuniaux, C. and Goddy, G.W. (Eds.).Plenum press, New York. pp. 385-389.

Nessa, F., Masumb, S.M.D., Asaduzzamana, M., Roya, S.K., Hossaina, M.M. and Jahanc, M.S. 2010. A process for the preparation of chitin and chitosan from prawn shell waste. *Bangladesh J. Sci. Ind. Res.,* **45**: 323-330.

Ni, C. and Liang, H.A. 1999. Study on the chemical components of silkworm pupae crust and its microstructure. Faculty of life science, Hubei University, Wuhan. China, pp. 1000-2375.

No, H.K. and Meyers, S.P. 1995. Isolation and characterization of chitin from crayfish shell waste. *J. Agric Food Chem*., **37**: 575-579.

Paulino, T.A., Simromato, J.I., Garcia, J.C. and Nozaki, J. 2006. Characterization of chitosan and Chitin produced from silkworm crysalids. *Carbohydrate polymers*, **64**: 98-103.

Rinaudo, M. 2006 'Chitin and chitosan: Properties and applications', *Progress in Polymer Science*, pp. 603-632.

Sandford, P. 1984. Chitosan - Commercial uses and Potential applications. Skjak, B.K. (Ed). In: Chitin and chitosan: Sources chemistry, Biochemistry, Physical properties and Applications. London: Elsevier Applied Science pp 51-69.

Schatz, C., Viton, C., Delair, T., Pichot, C. and Domard. A. 2003. Typical physicochemical behaviors of chitosan in aqueous solution*. Biomacromolecules*, **4**: 641-648.

Singla, A.K. and Chawla, M. 2001. Chitosan: Some pharmaceutical and biological aspects-an update. *J. Pharm. Pharmacol.,* **53**: 1047-1067.

Toan, N.V. 2009. Production of chitin and chitosan from partially autolyzed shrimp shell materials, *The Open Biomaterials J.,* **1:** 21-24.

Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendam, M. and Votteri, P. 2000. On the influence of the deacetylation process on the physico-chemical characteristics of chitosan from squid chitin. *Polymer*, **41**: 2463-2469.

Tsaih, M.L. and Chen, R.H. 2003. Effect of degree of deacetylation of chitosan on the kinetics of ultrasonic degradation of chitosan, *J. Appl. Polymer Sci.,* **90:** 3526 – 3531.

Yanga, Jen-Kuo, Shihb, Ing-Lung, Tzengc, Yew-Min, Wang. and San-Lang. 2000. Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme and Micro. Technol.*, **26**: 406-413.

Yuan, Y., Chesnutt, B.M., Haggard, W.O., and Joel, D. and Bumgardner. 2011. Deacetylation of chitosan: Material characterization and *in vitro* evaluation via albumin adsorption and pre-osteoblastic cell cultures. *Materials,* **4**: 1399-1416.

Zhang, M., Haga, A., Sekiguchi, H. and Hirano, S. 2000. Structure of insect chitin isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia. *International J. Biological Macromolecules*, **27:** 99-105.

Received: July 20, 2013; Accepted: November 22, 2013