Original Research Article

"Silkworm Exuviae: A Rich Source of Chitosan with Unique Properties"

Abstract

This study investigated the chitosan content in various developmental stages of silkworms, specifically focusing on larval cuticle, moult exuviae and larval exuviae. The chitosan percentage varied significantly among different stages, with the highest content observed in bivoltine hybrid larval exuviae (19.37%) and the lowest in the 1^{st} instar larval cuticle (9.93%). Chitosan content showed a consistent increase during moulting stages, reaching its peak in exuviae.Comparisons were made based on chitin weight, revealing that the chitosan content was highest in the 5th day fifth instar bivoltine hybrid larval cuticle (75.95%). The investigation extended to examine properties of chitosan, including moisture content, nitrogen content, ash content, degree of deacetylation (DD), solubility, viscosity and pH. Moisture content ranged from 5.80 to 7.84%, nitrogen content from 5.05 to 6.17%, ash content within 0.50 to 0.80% and DD from 85.10 to 92.71%. Solubility ranged from 92.25 to 98.25%, and viscosity was measured between 47 and 58 cP. The pH values ranged from 6.72 to 7.49.These findings contribute valuable insights into the chitosan characteristics at different developmental stages of silkworms, providing a comprehensive understanding of its variations and potential applications.

1. Introduction

Chitin is a natural polymer, chemically composed of linear chain of acetylglucosamine group [Synowieski et al 2003]. Chitin is usually obtained from shrimps and crustaceans on a large scale in industries. But, an alternative source of chitin is found in insect (beetles, grasshoppers, mealworm, silkworm) and their shed outer layer, is called exuviae. This alternative source provides chitosan, a substance derived from chitin after a specific process called deacetylation (Yang *et al.*, 2000; Zhang *et al.*, 2000; Suresh *et al*., 2012). Chitosan has attracted considerable interest in the field of biomedical applications due to its various advantageous biological attributes, such as its antimicrobial properties, biocompatibility, biodegradability, haemostatic capabilities and wound healing potential. This is primarily due to its distinct physical and chemical characteristics (Tokoro*et al*., 1989; Kobayashi *et al*., 1990; Farkas, 1990).

The insect body wall contains protein, chitin, mineral, oil pigment and other ingredients (Ni and Liang, 1999). In contrast, the cuticle of insects is composed of chitin in a matrix with cuticular proteins, lipids and other compounds (Nation, 2002). The mealworm exuvium and whole body of mealworm larvae valuable source of chitin and chitosan (Song *et al.*, 2018). The chitin content in different stages of *Vespa crabro* was observed to progressively rise as they matured (Kaya *et al.*, 2016). Paulino *et al.* (2006) reported that the silkworm pupal chitin and chitosan showed high purity than chitin and chitosan produced from crustacean shells. Silkworm specially reared for cocoon productionand for that silkworm complete its five instars larval cycle by passing four moults. After each moult, larvaeshedsits skin called exuviae.In sericulture industry,silkwormlarvae, pupae, pupal exuviae and egg shell are the excellent source of chitin and chitosan. [Battampara*et al.* 2020:Heaut*et al*. 2020]. Silkworm pupae exuviae and beetle larval cuticle excellent source Zhang *et al*. (2000).

On the basis of these earlier studies silkworm can be good source of chitosan, so we decide to extract chitosan from moult exuviae (skin after moult) and larval exuviae (after spinning) and analyse its physico-chemical properties of Chitosan to see its feasibility to commercial chitosan.

2. Material and methods

Materials

For this experiment, the 3rd and 4th moult exuviae of cross breed (Kolar gold) silkworms were obtained from farmers silkworm rearing house, Kurburu village, Chintamani (Taluk), Chikkaballapur (Dist.) and exuviae (3rd& 4th moult) of bivoltine hybrid silkworm (FC1×FC2) were collected from commercial silkworm rearing house, College of Sericulture, Chintamani, cleaned and dried for further chitosan extraction. Whereas, larval exuviae (after spinning) collected from grainage, College of Sericulture, Chintamani (Plate 1). The Sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were used to extract the chitin and chitosan.

Methods

Chitosan extraction

The chitin and chitosan extraction involved mainly three steps *viz*., Deproteinization, Demineralization and Deacetylation (Suresh *et al.*, 2012).

Deproteinization

Dried exuviae/cuticle was treated for 4 h with 4 % NaOH at 70 °C with 1:10 ratio (material to liquid).

Demineralization

Deproteinized material was treated with 3 % HCL (1:10; material to liquid ratio) heated at 25 °C to remove the mineral. After demineralization got the product chitin.

Deacetylation

Chitin was boiled with 45 % aqueous NaOH (1:12 ratio) at 90-95°C for 3 h to remove acetyl group resulting chitosan.

Physicochemical properties of silkworm chitosan

Moisture Content (%)

Moisture content of the chitosan was determined by the gravimetric method (Black, 1965).

$$\text{Moisture content (\%)} = \frac{\text{Wet weight(g)} - \text{Dry weight(g)}}{\text{Wet weight(g)}} \times 100$$

Ash (%)

Two grams of chitosan were put into a clean crucible and heated in a furnace at 500°C for 2 hours. After cooling, the crucible and its contents were weighed A.O.A.C. (1990).

$$\textbf{Ash}\ (\%) = \frac{\text{Weight of residue (g)}}{\text{Sample weight (g)}} \times 100$$

Viscosity (cp)

Chitosan viscosity was measured using an Ostwald viscometer. 0.5g of chitosan was dissolved in a mix of 10ml 0.5M acetic acid and 20ml 0.25M sodium chloride, then stirred for 10 mins in a vortex mixer. (Chen and Tsaih, 1998). A vertical viscometer held on a stand filled with solution up to mark A. Solution flow time from mark A to B was measured thrice. Then, compared the flow time of the test liquid with a known viscosity liquid.

$$\text{Viscosity (cp)} = \frac{f_1\, t_1}{f_2\, t_2} \times \eta_2$$

Where,

f_1=Density of chitosan solution

t_1 =Time of flow of chitosan liquid

f_2 =Density of standard liquid

t_2 =Time of flow of standard liquid

η_2=Viscosity of standard liquid

Solubility (%)

Chitosan powder (0.1g) dissolved in 10ml of 1% acetic acid for 30 mins at 25°C using an incubator shaker (240 rpm). The solution was boiled for 10 mins, cooled and centrifuged at 10,000 rpm for 10 mins. Supernatant was removed. Undissolved particles were washed with 25ml distilled water, centrifuged again at 10,000 rpm and dried at 60°C for 12h (Tammineni*et al.*, 2014).

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

Determination of degree of deacetylation (DD)

Potentiometric titration assessed to measure DD (Renata *et al.*, 2012). Chitosan (200 mg) dissolved in 20 ml of 0.1 M hydrochloric acid was mixed with 25 ml of distilled water and stirred for 30 min. Then, another 25 ml of water was added and stirring continued for another 30 min until complete dissolution. The resulting solution was titrated against 0.1 M sodium hydroxide.

$$\text{DD (\%)} = 2.03\frac{V_2 - V_1}{m + 0.0042(V_2 - V_1)}$$

Where,

m - Weight of the sample

V_1, V_2 - Initial and final burette reading.

2.03 - Coefficient resulting from the molecular weight of chitin monomer unit

0.0042 - Coefficient resulting from the difference between molecular weights of chitin and chitosan monomer unit

Nitrogen (%)

Nitrogen content was determined using Micro-kjeldhal method AOAC (1995).

pH

Chitosan of 0.5g was dissolved with 50 ml of distilled water and used to measuring the pH by using a Digital pH meter.

Result and discussion

4.3 CHITIN AND CHITOSAN YIELD OF LARVAL INTEGUMENT AND MOULT EXUVIAE

The data pertaining to the per cent chitin and chitosan yield over silkworm cuticle/exuviae and per cent chitosan yield over chitin among cross breed and bivoltine hybrid are presented in Table 1 and Fig. 1. The yield of chitin and chitosan extracted from silkworm cuticle and exuviae were measured in terms of percentage.

Table 1: Chitin and chitosan yield from silkworm cuticle and exuviae.

Samples	Chitin (%) produced over dry wt. of larval cuticle/exuviae	Chitosan (%) produced over dry wt. of larval cuticle/exuviae	Chitosan (%) produced over dry wt. of chitin
S_1: Chitosan from 1st instar larval cuticle of bivoltine hybrid	13.25^{f}	9.93^{e}	74.92^{a}
S_2: Chitosan from 1st instar larval cuticle of cross breed	13.57^{f}	10.21^{e}	75.12^{a}
S_3: Chitosan from 2nd instar larval cuticle of bivoltine hybrid	15.00^{e}	11.15^{d}	74.47^{ab}
S_4: Chitosan from 2nd instar larval cuticle of cross breed	15.14^{e}	11.30^{d}	74.90^{ab}
S_5: Chitosan from 3rd moult exuviae of bivoltine hybrid	19.76^{d}	14.38^{c}	73.44^{b}
S_6: Chitosan from 3rd moult exuviae of cross breed	20.66^{d}	15.01^{c}	73.48^{b}
S_7: Chitosan from 4th moult exuviae of bivoltine hybrid	23.83^{c}	17.83^{b}	74.56^{ab}
S_8: Chitosan from 4th moult exuviae of cross breed	23.63^{c}	17.59^{b}	74.46^{ab}
S_9: Chitosan from 5th instar larval cuticle of bivoltine hybrid	24.81^{b}	18.84^{a}	75.95^{a}
S_{10}: Chitosan from 5th instar larval cuticle of cross breed	25.48^{ab}	19.25^{a}	75.42^{a}
S_{11}: Chitosan from larvalexuviae of bivoltine hybrid (After spinning)	26.02^{a}	19.37^{a}	74.47^{ab}
S_{12}: Chitosan from larvalexuviae of cross breed (After spinning)	25.89^{a}	19.28^{a}	74.49^{ab}
F - test	*	*	*
SEm ±	0.318	0.231	0.452
CD @ 1%	0.905	0.658	1.289
CV%	3.457	3.361	1.354

1. Chitin yield (%)

The chitin content varied significantly among different larval stages and exuviae of *Bombyx mori*, ranging from 13.25% to 26.02%. Bivoltine hybrid larval exuviae exhibited the highest chitin percentage (26.02%), while the lowest was observed in the 1st instar larval cuticle of bivoltine hybrid (13.25%). Notably, the chitin content increased during the pupal stage, reaching its peak in larval exuviae. This aligns with Kaya *et al*. (2016) findings, showing a threefold increase in chitin storage during the larva-to-pupa transition. Marei *et al*. (2016) was reported that, chitin content was 22.5 per cent in Locust (*Schistocerca gregaria*F.). Kim *et al.* (2017) reported that, chitin content was about 20.9-23.30 per cent in cricket (*G. bimaculatus*D.). Antonov *et al.* (2019) reported that, chitin content was 21.30 per cent in dead moths of *Hermetiaillucens*(L.). Shin *et al*. (2019) reported that the chitin content was 10.50, 12.70 and 14.20 per cent in larvae, pupae and adult of rhinoceros beetle, respectively. Soetemans*et al.* (2020) reported that, the chitin content in black soldier fly exuviae varied from 23 to 31 per cent. The variation in chitin content emphasizes its dynamic role in insect development.

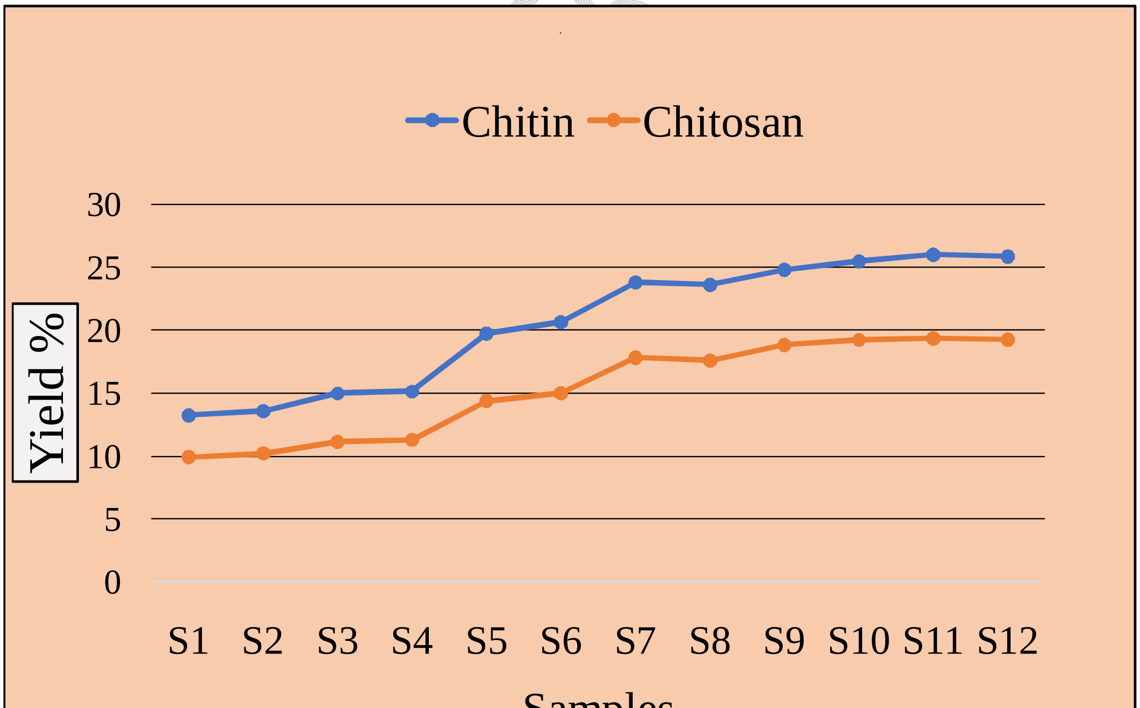

Fig 1: Chitin and chitosan yield from larval cuticle

2. Chitosan yield (%)

Chitosan content varied significantly among larval instars and exuviae, with the highest percentage in bivoltine hybrid larval exuviae (19.37%). The chitosan content ranged from 9.93% to 19.28%, exhibiting a similar trend to chitin, with a notable increase in exuviae. Chitin content gradually rose during the larva-to-pupa transition, peaking in larval exuviae. Chitosan percentage based on chitin weight was highest in 5th day fifth instar bivoltine hybrid larval cuticle (S_9) (75.95 %), followed by 5th day fifth instar cross breed larval integument (S_{10}) (75.42 %) and lower value was observed in 3rd moult bivoltine hybrid exuviae (S_5) (73.44 %). The findings align with Kaya *et al*. (2015) who noted 15 and 12% chitosan content in barbarian grasshopper and European locust, respectively. Kim *et al*. (2017) found 16-20% chitosan in field cricket cuticle, and Song *et al*. (2018) reported 16.21% in *Tenebrio molitor* larval exuviae. Luo *et al*. (2019) observed 28.20% chitosan content in cicada slough shell. These results underscore the potential of insect-derived chitosan for various applications.

In between moults, the larva increases in size and its new exoskeleton was initially soft and pliable. To accommodate the growing body, the exoskeleton must be larger and thicker, which means it contains more chitin. As the larva grows, its metabolic rate increases to support its larger body and energy demands. Therefore, the increased chitin content in the exuviae at every moult may be associated with the larva metabolic needs during growth.

4.6 PHYSICOCHEMICAL PROPERTIES OF CHITOSAN EXTRACTED FROM SILKWORM LARVAL CUTICLE AND EXUVIAE

The results with respect to the physicochemical properties of chitosan extracted from larval cuticle and exuviae of cross breed (PM × CSR2) and bivoltine hybrid are presented in Table 2.

1. Moisture content (%)

The moisture content varied significantly among samples, with the highest in bivoltine 1st instar silkworm cuticle (7.84%), by bivoltine hybrid fifth instar 5th day larval cuticle 7.50% (S_{10}) and the lowest in commercial chitosan (5.80%). Sandford (1984) stressed that chitosan's moisture content should not surpass 10% for commercial suitability. The current results align with Suresh *et al.* (2012), who found moisture content of 7.09% and 8.57% in chitosan from mulberry silkworm pupae and eri silkworm pupae, respectively. Fini and Orienti (2003) noted that commercial chitosan typically has moisture content ranging from 7 to 11%.

Table 2:Physicochemical properties of chitosan extracted from larval cuticle and exuviae.

Samples	Moisture (%)	N (%)	Ash (%)	DD (%)	Solubility (%)	Viscosity (cp)	pH
S_1: Chitosan extraction from bivoltine hybrid 1^{st} instar silkworm cuticle	7.84^{a}	5.05^{i}	0.70^{bcd}	85.10^{g}	92.25^{e}	50.07^{gh}	6.89^{fgh}
S_2: Chitosan extraction from cross breed 1^{st} instar silkworm cuticle	7.76^{ab}	5.12^{h}	0.75^{bc}	85.96^{g}	92.75^{de}	49.85^{h}	6.87^{gh}
S_3: Chitosan extraction from bivoltine hybrid 2^{nd} instar silkworm cuticle	7.76^{ab}	5.29^{g}	0.76^{b}	85.80^{g}	93.75^{bcd}	47.91^{i}	6.81^{hi}
S_4: Chitosan extraction from cross breed 2^{nd} instar silkworm cuticle	7.55^{bc}	5.16^{h}	0.80^{b}	86.13^{g}	92.75^{de}	49.10^{hi}	6.93^{efg}
S_5: Chitosan extraction from 3^{rd} moult exuviae of bivoltine hybrid	7.32^{cd}	6.10^{c}	0.55^{cd}	90.7^{cd}	98.00^{a}	54.78^{e}	7.49^{a}
S_6: Chitosan extraction from 3^{rd} moult exuviae of cross breed	7.30^{cd}	6.00^{d}	0.54^{d}	91.37^{bc}	97.75^{a}	54.73^{e}	7.48^{ab}
S_7: Chitosan extraction from 4^{th} moult exuviae of bivoltine hybrid	7.24^{d}	6.17^{b}	0.52^{d}	92.04^{ab}	98.25^{a}	56.11^{cd}	7.38^{b}
S_8: Chitosan extraction from 4^{th} moult exuviae of cross breed	7.21^{d}	6.11^{c}	0.50^{d}	92.71^{a}	98.00^{a}	55.30^{e}	7.43^{ab}
S_9: Chitosan extraction from bivoltine hybrid 5^{th} instar silkworm cuticle	7.55^{bc}	5.95^{def}	0.60^{bcd}	89.20^{ef}	94.50^{bc}	50.99^{fg}	7.14^{d}
S_{10}: Chitosan extraction from cross breed 5^{th} instar silkworm cuticle	7.50^{c}	5.94^{ef}	0.65^{bcd}	89.71^{def}	93.50^{cde}	51.98^{f}	6.99^{e}
S_{11}: Chitosan extraction from larvalexuviae of bivoltinehybrid	7.46^{cd}	5.93^{f}	0.66^{bcd}	88.68^{f}	94.75^{bc}	57.08^{c}	6.98^{ef}
S_{12}: Chitosan extraction from larvalexuviae of cross breed	7.45^{cd}	5.99^{de}	0.65^{bcd}	90.37^{cde}	95.00^{b}	58.13^{b}	7.27^{c}
S_{13}: Commercial chitosan (Control)	5.80^{e}	6.86^{a}	1.16^{a}	92.88^{a}	94.25^{bc}	160.02^{a}	6.72^{i}
F - test	*	*	*	*	*	*	*
SEm ±	0.08	0.016	0.063	0.384	0.408	0.36	0.034
CD at 1 %	0.23	0.045	0.181	1.104	1.172	1.033	0.098
CV	2.178	0.540	18.524	0.862	0.862	1.175	0.961

2. Nitrogen (%)

Significant nitrogen content variations were observed in chitosan extracted from different silkworm stages. The highest nitrogen content (6.17%) was in bivoltine hybrid silkworm (S7), slightly less than commercial chitosan (6.86%). Nitrogen ranged from 5.05% to 6.17%. Similarities were found with previous studies on chitosan from various insect sources, [Kaya *et al*., 2014; Kaya *et al*., 2015; Kaya *et al*., 2016] showed nitrogen content ranging from 6.62% to 6.85%.

3. **Ash (%)**

The ash content varied among chitosan samples, ranging within 1%. Notably, chitosan from 2nd instar silkworm cuticle had 0.75%, surpassing others. Nessa *et al*. (2010) emphasized that premium-grade chitosan should less than 1% ash content. Various sources showed diverse ash contents in earlier studies: silkworms (0.05%), grasshoppers (0.89%), housefly larvae (0.13%), house crickets (1.0%), cicada slough (0.87%) (Sajomsang and Gonil, 2010; Purkayastha and Sarkar, 2020).

4. Degree of deacetylation (DD) (%)

The deacetylation degree varied significantly among samples. The highest was in 4th moult exuviae of crossbreed silkworm at 92.71%, akin to commercial chitosan (S13) at 92.88%. Findings align with Suresh *et al*. (2012) and No and Meyers (1995), showing chitosan's diverse DD, ranging from 46.5% to 97%. Knaul *et al*. (1998) synthesized 70.8% DD chitosan. Paulino *et al.* (2006) observed 83% DD in silkworm pupal chitosan. Song *et al.* (2013) noted 87.90% DD in blowfly larva chitosan. Marei *et al*. (2016) reported chitosan DD percentages from shrimp, beetles, honey bees and locusts as 74%, 95%, 96% and 98%, respectively.

5. Solubility (%)

The solubility of silkwormderived chitosan was highest in 4th moult exuviae of bivoltine hybrid silkworm (S_7) at 98.25%, comparable to other silkworm samples. These findings align with Suresh *et al.* (2012). Chitosan solubility from insect and sources varied, with silkworm chrysalis showing 98.7%, consistent with Luo *et al*. (2019).

6. Viscosity (cP)

Chitosan viscosity varied significantly among samples, ranging from 47 to 58 cP, lower than commercial chitosan (160 cP). Highest viscosity was in chitosan from cross breed larval exuviae (58.13 cP), followed by bivoltine hybrid larval exuviae (57.08 cP). Results align with Bough *et al.* (1978) showing chitosan viscosity disparities. Lower viscosity chitosan has advantages in food and pharmaceutical industries. Kim *et al*. (2016) found chitosan from M. domestica pupal shells had a viscosity of 33.6 cP, while Song *et al*. (2018) noted *T. molitor* chitosan viscosity ranged from 48.0 to 54.0 cP.

7. pH

The range of pH value of current study varied between 6.8 to 7.5. These results are in conformity with those of Suresh *et al.* (2012) who observed that pH value of silkworm pupa chitosan was about 7.3. Paul *et al*. (2014) noted that the pH range of commercial chitosan typically falls within 6.2 to 8.0 range.

Conclusion

In conclusion, the study investigated the chitosan content, yield and various physicochemical properties in different developmental stages of silkworms, focusing on larval cuticle/exuviae and pupal exuviae. Chitosan content exhibited a significant variation among stages, with the highest percentages found in bivoltine hybrid larval exuviae. The chitosan content showed a dramatic increase from larva to pupa. Moisture content, nitrogen content, ash content and degree of deacetylation also varied across samples. Notably, solubility and viscosity were influenced by the source of chitosan, with pupal exuviae displaying the highest solubility and viscosity values. The study contributes valuable insights into the chitosan characteristics of silkworms, demonstrating potential applications in diverse industries.

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