

Characterisation of Chitosan from Blowfly Larvae and Some Crustacean Species from Kenyan Marine Waters Prepared Under Different Conditions

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Abstract—Isolation of chitosan from cuticles of blue bottlefly larvae *Calliphora erythrocephala*, and shells of crab *Sylla cerrata*, lobster *Panulirus ornatus*, prawn *Paeneaus indicus* was carried out. The yield of chitin was 12.0%, 23.0%, 15.7% and 28.0% respectively. In the same order the yield of chitosan was 66.0%, 74.6% 74.3% and 75.0% from chitin. Ash in the crab and lobster chitosan demineralised with 0.5M HCl was 30.2 and 22.4% respectively. This was reduced to 0.2 % for lobster and 0.4% for crab using 2M HCl for demineralisation and 0.5M HCL was adequate for demineralisation of prawns to bring the ash content to < 1%. The ash content in the blowfly larvae was negligible. The conditions used for chitosan isolation in blowfly larvae were milder requiring no demineralisation step. The time to obtain soluble chitosan in 1% v/v acetic acid was 8 hr for crab and lobster at 100°C deacetylation and 4 hr at 120°C while for prawns it was 6 hr at 100°C and 3 hr at 120°C deacetylation temperature. The average molecular weight (\bar{M}_v) for crabs was 556,000 after 8 hr deacetylation and 148,000 at 140°C deacetylation temperature. With 2M HCl used for demineralisation first, it was 439,000 for a 4 hr period. Crabs, first demineralised then deprotenised the \bar{M}_v was 155,000 for a 3 hr deacetylation at 120°C and 417,000 for 1 hr deacetylation. An 8 hr deacetylation at 100°C for lobsters gave \bar{M}_v of 791,000. It was reduced to 560,000 after 4 hr of deacetylation at 120°C and to 236,000 at 140°C for 3 hr. Prawns had a \bar{M}_v of 507,000 after 6 hr deacetylation at 100°C and reduced to 455,000 after a 3 hr deacetylation. For insect larvae, at 100°C deacetylation for 4 hr the \bar{M}_v was 413,500 while for 1 hr, 2 hr and 2.5 hr deacetylation time at 120°C it was 369,000, 308,500 and 263,000 respectively. The degree of deacetylation (DD) increased with temperature and time of deacetylation. For crab, demineralised then deproteinised, it increased from 72.9% in 1 hr then 81.5% in 3 hr. In prawn chitosan it was 60.0% for the 6 hr deacetylation at 100°C and 69.2% for 3 hr deacetylation at 120°C. The DD of insect larvae was 62.56% after 4 hr of deacetylation at 100°C. When deacetylated at 120°C it was 64.0% after 1 hr, 79.9% after 2 hr and 80.7% after 2.5 hr. The moisture content showed a slight increase with DD. Temperature increase and time of deacetylation caused a decrease in \bar{M}_v and a more conservative increase in DD.

INTRODUCTION

Chitin, poly [β-(1-4) -2-acetamido-2-deoxy-D-glucopyranose] occurs in insects, crustaceans and molluscs as an important constituent of the exoskeleton and in certain fungi as the principal

fibrillar polymer in the cell wall. In nature it is second in abundance to cellulose. The most important derivative of chitin is chitosan: poly [β-(1-4)-2- amino-2-deoxy-D-glucopyranose] and is chemically or enzymatically produced by deacetylation of chitin (Roberts, 1992; Muzzarelli,

1977). Commercial production of chitin and chitosan takes place from waste shells of shrimps, prawns, crabs, lobsters and crayfish. Newer sources for chitin production continue to be explored from fungi (Kuhlmann *et al.*, 1999) and insect larvae (Struszczyk *et al.*, 1999).

There are great variations in the properties of chitin and chitosan that occur depending on procedures used for preparation. This has attracted significant interest in view of the varied proposed novel applications of these functional polymers especially chitosan as readily seen over a broad range of scientific areas including applications in biomedical, food and various chemical industries (Knorr, 1984 a, b; Muzzarelli, 1977, Sanford and Hutchings, 1987).

About 30-40% of crustacean shell waste consists of protein, 30-50% calcium carbonate and 20-30% chitin (Johnston and Peniston, 1982). These proportions vary with species and season (Green and Mattick, 1979). The preparation of chitin and chitosan differ with crustacean species and preparation methods (Brine and Austin, 1981). Important characteristics of chitin and chitosan are molecular weight and degree of deacetylation. These vary with process conditions and they influence the functional properties of chitosan (Wu and Bough, 1978; Shimara *et al.*, 1984; Brine and Austin, 1981; No and Meyers, 1997). In Kenya, despite availability of crustacean waste from crabs, lobsters, prawns and crayfish from the local beach hotel industry and some processors, no studies have been conducted on the properties and the functional potential of the shells for chitin and chitosan production. With the above-mentioned physico-chemical variations in chitosan per species, attempts are made in this study to provide some baseline data for chitosan from the species in Kenya namely *Panulirus ornatus* (lobster) *Sylla cerrata* (crab) and *Paenaeus indicus* (prawn). Coupled with exploration of newer sources for this robust biopolymer, attempts are also made to isolate and characterise chitosan from the blue bottlefly larvae *Calliphora erythrocephala*. This provides a chance for the exploitation of knowledge of physico-chemical properties of the polymer in insects which exist in abundance and it is easier to harness for the polymer. Insects can

be looked at as pests on one hand and as a potential economic source of the versatile biopolymers. Scavenger insects like Blowfly family *Calliphoridae* deposit their eggs in garbage and with development period of 10 days to 3 weeks from egg to adult (Smith and Wall, 1997; Wall and Shearer, 1997) is an easier source of raw material.

The aim of this study was to prepare chitosan from insect larval cuticle and some species of crustaceans from Kenyan marine waters under similar and different acid concentrations, time, and temperature regimes with emphasis on molecular weight (M_v) and degree of deacetylation (DD) and ash content.

MATERIALS AND METHODS

Summary of Species/ Sizes

Species:		Carapace length (cm)	Carapace width (cm)
<i>Panulirus ornatus</i>	Lobster	8.1-10.3	4.5 - 7.0
<i>Sylla cerrata</i>	Crab	10.1-10.3	14.5 - 15.8
<i>Paenaeus indicus</i>	Prawn	2.0 - 2.8	0.2 - 0.5
<i>Calliphora erythrocephala</i>	Insect larvae	1.5 - 2.0	0.5 - 0.7

Insect Larvae

Preparation of chitin was according to Struszczyk *et al* (1999) with slight modifications. A total of 10.2 kg of frozen insect larvae of *Calliphora erythrocephala* stored in half kilogram blocks were thawed, washed with distilled water, homogenised using a kitchen blender and filtered in a Büchner funnel. The process was repeated twice. The homogenised sample was mixed with 2.5% w/v NaOH, agitated with a mechanical stirrer for 2 hr at 50°C, filtered and washed with distilled water until neutral. The process was repeated twice. The resulting deproteinised larval chitin sample was washed in 99% ethanol, air dried at room temperature and weighed.

For chitosan preparation the dried larval chitin was deacetylated with 50% w/w NaOH at 100°C with agitation using a mechanical stirrer for 4 hr, filtered, washed to neutrality with distilled water

then with ethanol and left to dry at room temperature. Deacetylation was also carried out at 120°C for 1 hr, 2 hr and 2.5 hr as above.

Crustacean shells

For chitin preparation, approximately 3 kg each of shells of lobsters, crabs and prawns were collected from the Intercontinental, Serena and Reef Hotels as well as the Tamarind restaurant all on the coast north of Mombasa, Kenya. Any remaining flesh was removed and the shells put in black polythene bags and immediately taken to the lab at KMFRI, washed, and then dried in an oven at 100°C overnight. They were crushed to smaller pieces, repacked and 1 kg of each sent by air to the University of Potsdam, Germany and stored at -30°C until analysis.

Chitin Isolation was done according to Hackman (1954) with some modifications. The shells were milled to fine particles using a rotary mill (Analysemühle A No.9679.1 from ROTH) and 20 g of finely ground shells of each species was used for each analysis. This was mixed with 2M NaOH in a quickfit 1 litre flask fitted with a condenser at a solid to liquid ratio of 1:20 and deproteinised at 100°C for 6 hr with constant stirring. A thermometer probe was used to constantly monitor the temperature in the mix. Filtration of the mixture was done under vacuum using a glass filter (Schott No. 2) and washed to neutral pH with distilled water, then with ethanol and left to dry at room temperature. 6 g of the deproteinised shells were placed in 0.5M HCl in a beaker overnight with constant stirring at a solid to liquid ratio of 1:20. For the crab and lobster shells, the acid strength was altered to 2M and on some occasions crab demineralisation preceded deproteinisation. The final product in each case was washed with distilled water to neutral pH, followed by an ethanol wash and left to dry. The chitin was then decolorized overnight with a 1:1 mixture of acetone/ethanol at a solid:liquid ratio of 1:10. The mixture was stirred constantly, finally washed with ethanol, dried then weighed.

Chitosan preparation: 4 g of chitin was deacetylated with 50% w/w NaOH at a solid to liquid ratio of 1:20 and temperature regimes of 100°C, 120°C and 140°C and in each case time

taken to obtain soluble chitosan product in 1% acetic acid was noted. The chitosan was then filtered through a glass filter (Schott No.2) washed with distilled water to neutral then with ethanol, left to dry at room temperature and weighed.

Characterisation

The percentage yield for chitin was calculated from the weight of chitin produced as a percentage of starting dry raw materials. The percentage yield of chitosan was calculated from the weight of chitosan obtained as a percentage of the chitin weight before deacetylation. Moisture and ash content content of chitosan was determined according to AOAC (1980) methods. Average molecular weight by viscometry was done using an Ubbelohde viscometer (Capillary viscometer type 531No. 10 from Schott) according to Terbojevich and Cosani (1997). The degree of deacetylation (DD) was determined by potentiometric titration according to Bodek (1995).

RESULTS

The yield of chitin from prawn shells treated with 0.5M HCl was 28.4%. Crab and lobster shells were treated with 2M HCl and yields of 23.3% and 15.7% were realized respectively. The yield of chitin from insect larvae was 12.2% without any demineralisation step (Table 1). The yield of chitosan from prawns, crabs, lobsters and insect larvae was 75.1%, 74.6%, 74.3% and 66.7% respectively (Table 2). The initial ash content in the prawn shells was 26.3%. Crab and lobster shells had 45.2% and 35.8% respectively (Table 3). The ash content for insect larvae was much lower than 1% even without demineralisation and was not considered further. Upon treatment of the three crustacean shells with 0.5M HCl for 12 hours, the ash in the prawn shells was reduced to 0.8% while for the crab and the lobster shells it was 30.5% and 22.4% respectively (Table 3). Treatment of crab and lobster shells during demineralisation with 2M HCl reduced the ash content to below 1% i.e. 0.4 % for lobsters and 0.2% for crabs (Table 4).

The time for deacetylation of lobster and crab shells to obtain soluble chitosan in 1% acetic acid using 0.5 M HCl for demineralisation was 8 hr at

Table 1. Percentage Yield of Chitin from Lobster, Prawn and Crab shells. sd. = standard deviation

Source	HCl concentration (M)	% Yield	sd.
Prawns	0.5	28.4	0.1
Crabs	2.0	23.0	0.7
Lobsters	2.0	15.7	1.0
Insect Larvae	-	12.2	0.1

*Based on dry weight basis (crustaceans shells were kept at 100°C over night before storage).

Table 2. Percentage Yield of Chitosan from Chitin

Source	% Yield	sd.
Prawns	75.1	2.8
Crabs	74.6	2.8
Lobsters	74.3	1.3
Insect Larvae	66.7	1.0

Table 3. Ash content for prawns, crabs and lobsters demineralised with 0.5M HCl for 12 hr

Source	Initial Ash (%)	sd	Final Ash (%)	sd
Prawns	26.3	0.1	0.8	0.01
Crab	45.2	0.5	30.2	0.03
Lobster	35.8	0.2	22.4	0.80

Table 4. Ash content for crab and lobster demineralised with 2M HCl

Source	Initial Ash (%)	Final Ash (%)	sd
Crab	35.8	0.2	0.01
Lobster	45.2	0.4	0.02

100°C, 4 hr at 120°C and 3 hr at 140°C. It took 6 hr at 100°C for prawns and 3 hr at 120°C (Table 5). Trials to demineralize lobster and crab shells using 2M HCl then deacetylating at 120°C resulted in the soluble chitosan product being obtained after 4 hr (Table 6). Ash content that determines the quality of chitosan was high for the 0.5M HCl demineralised crab and lobster shells (Table 7) but not for prawns (Table 10). When 2M HCl was used to demineralize, the ash content was much lower (Table 7 and 9). Insects do not have calcium carbonate in their cuticle hence no demineralization was done (Table 11). Prawns could be closer to insects in the evolutionary scale and probably

explain also their lower ash content requiring milder demineralisation conditions. The crabs and lobsters used in this experiment were large in size, had thick shells therefore large CaCO₃ amounts deposited in support structures. This would then require stronger acid concentration for effective demineralization. With deacetylation time of 8 hours for crabs at 100°C using 0.5M HCl, the ash content was 31.6 % and \bar{M}_v was 556,000. However at a deacetylation temperature of 140°C the \bar{M}_v was reduced to 148,000 though the ash content was still 21.2% (Table 7.). When the acid concentration for demineralisation was increased to 2M the ash content was reduced to <1% the deacetylation temperature being 120°C and \bar{M}_v was reduced to 439,000 suggesting that increase in temperature caused higher degradation effect.

An attempt was made to demineralise the crab shells with 2M HCl before deprotenisation with deacetylation being carried out in 1 and 3 hours (Table 8.). The ash content was less than 1 %. The 3 hr deacetylation produced chitosan of 155,000 \bar{M}_v with DD of 81.5% while 1 hour deacetylation produced chitosan of \bar{M}_v 417,000 and a DD of

Table 5. Time and deacetylation temperature for lobster, crab, prawn shells demineralised using 0.5 M HCl. (Time indicates when soluble chitosan product in 1% acetic acid is obtained)

Source	Temperature of deacetylation (°C)	Time of deacetylation (Hours)
Lobsters	100	8
	120	4
	140	3
Crabs	100	8
	120	4
	140	3
Prawns	100	6
	120	3

Table 6. Time and deacetylation temperatures for crab and lobster shells demineralised using 2 M HCl. (Time indicates when soluble chitosan product in 1% acetic acid is obtained)

Source	Temperature of deacetylation (°C)	Time of deacetylation (Hours)
Crab	120	4
Lobster	120	4

Table 7. Molecular weight and degree of deacetylation for crab at different temperatures and time

Time of deacetylation (hr)	Temp. of deacetylation (°C)	Ash (%)	sd	Moisture %	sd	MV (DA)	sd	DD %	Sd
8	100	31.6	0.5	7.3	0.2	556,000	4,200	-	-
4	140	21.2	0.3	10.1	0.4	148,000	2,800	-	-
*4	120	0.4	0.0	13.1	0.1	439,000	11,300	70.8	3.7

*Demineralised in 2M HCl

Table 8. Molecular weight and degree of deacetylation for crab demineralised first then deprotenised

Source	Time of deacetylation (hr)	Moisture (%)	sd	MV (Da)	sd	DD (%)	Sd
Crabs	3	13.7	0.2	155,000	9,100	81.5	0.7
	1	13.0	0.1	417,000	5,600	72.9	3.8

*Demineralised in 2M HCl, Ash<1%

*Temp 120°C

Table 9. Molecular weight and degree of deacetylation for lobster chitosan at different temperatures and time

Time of deacetylation (hr)	Temp. of deacetylation (°C)	Ash	sd	Moisture (%)	sd	MV (Da)	sd	DD (%)	Sd
8	100	17.6	0.5	8.8	0.2	791,000	13,400	-	-
4	120	27.3	0.4	7.4	0.3	560,000	2,800	-	-
4	140	18.8	0.2	12.1	0.3	236,000	1,400	-	-
*4	120	0.2	0.01	14.4	0.1	376,500	4,900	82.2	0.8

* Demineralised in 2M

Table 10. Molecular weight and degree of deacetylation for prawn chitosan at different temperatures

Time of deacetylation (hr)	Temp. of deacetylation (°C)	Moisture (%)	Sd	MV (Da)	sd	DD (%)	Sd
6	100	10.9	0.1	507,000	14,100	60.0	2.8
3	120	9.9	0.1	455,500	16,200	69.2	1.5

* Ash <1% , 0.5M HCl demineralisation,

Table 11. Molecular weight and degree of deacetylation for insect larvae for chitosan at different temperatures and time

Time of deacetylation (hr)	Temp. of deacetylation (°C)	Moisture (%)	sd	MV (Da)	sd	DD (%)	Sd
4	100	12.0	0.1	413,500	2,100	62.6	5.0
2.5	120	12.3	0.4	263,000	1,400	80.8	0.8
2	120	12.1	0.1	308,500	707	79.9	1.4
1	120	11.0	0.1	369,000	1,400	64.8	0.7

72.9%. This gave the impression that chitosan behaves differently under different preparation conditions. Temperature and time are influential factors in changing \bar{M}_v and DD values.

An 8 hr deacetylation time for lobsters at 100°C produced chitosan of \bar{M}_v 791,000. After 4 hr deacetylation at 120°C \bar{M}_v was reduced to 560,000 (see Table 9), an indication that temperature and time of deacetylation may influence \bar{M}_v probably by chain degradation. The ash content was however still high. Deacetylation at 140°C for 3 hours reduced the \bar{M}_v to 236,000. When the lobster shell was demineralised using 2M HCl the ash content was reduced to <1% and the \bar{M}_v was 376,500 with a DD of 82.2%.

For prawns with 0.5M HCl demineralisation already with <1% ash content, the time of deacetylation to obtain soluble product was 6 hr at 100°C and the \bar{M}_v was 507,000 (Table 10). When the deacetylation temperature was increased to 120°C the soluble product was obtained after 3 hr and \bar{M}_v was reduced to 455,500. The DD was 60.0% for the treatment at 100°C and about 70% at 120°C. For the insect larvae, at 100°C deacetylation for 4 hr the \bar{M}_v was 413,500 (Table 11). After 2.5 hr, deacetylation at 120°C the \bar{M}_v was reduced to 264,000 with a DD of 81.0%. After 2 hr deacetylation periods, the \bar{M}_v was 309,000 with a DD of 79.9% and 1 hr deacetylation the \bar{M}_v was 369,000 and a DD of 64.8%. The moisture content in the species where DD was determined was in the range of 7 to 13 % mostly indicating a slight increase with increase in DD (Tables 7, 8, 9 and 11).

DISCUSSION

Different workers have reported that chitin and chitosan yields differ between species. Brzeski (1982) and Anderson *et al.*, (1978) reported yields of 70% and 90% respectively from krill. Anderson *et al.*, (1978) reported 60% yield from crab chitin and Aluminiar and Zainuddin (1992) have reported 80% yield from prawn shells. In this study, the yields range from 74.3% for lobster, 74.6% for crab and 75.1% for prawn. This appears to be within the range earlier reported. Chitin yields of 14-27% have been reported by Ashford *et al.* (1977) for shrimp and in the present study 28% was measured

while No and Meyers (1995) reported yields of 13-26% for crab and this does not vary from this study where the yield is 23%. It is generally accepted that 20-30% of crustacean waste is chitin (Johnston and Peniston, 1982). These proportions vary with species and season of harvest (Green and Kramer, 1984). Ash content for lobsters and crabs are normally higher than in shrimps and reducing their levels is important for ensuring good quality chitosan. Ash contents as high as 63% have been reported in crayfish (No *et al.*, 1989), lobsters *Linuparus trigonus* 54.7% (Ahn and Lee, 1992), crab *Callinectes sapidus* 50% (Muralidhara and Maggin, 1985), prawn *Paenaeus monodon* 29% (Benjankul and Sophanodora, 1993). The initial ash content for the crab *Sylla cerrata*, the lobster *Panulirus ornatus* and prawn *Paenaeus indicus* compare well at 45%, 35% and 27% respectively (Tables 3&4). The reduction in ash is achieved by increasing the acid concentration though there could be a risk of \bar{M}_v reduction due to hydrolysis of glycosidic bonds (Roberts, 1992). No *et al.*, (1989) using crayfish, reduced ash content with 1N HCl from 63% to 0.3 %. Hackmann (1954) used 2M HCl to bring the ash content in the crab to nil. The use of HCl in this study at somewhat higher concentrations was intended to reduce the ash content to less than 1%. Preparation of chitosan under heterogeneous conditions of course produces a product with varying properties. In this study the most important factors that played a role was probably temperature and time of deacetylation, as the alkali concentration remained constant at 50% w/w. These two conditions seemed to affect the \bar{M}_v and DD. This is because higher temperatures cause breakdown of the molecule and prolonged exposure to alkali also causes the chitin to be rapidly deacetylated to an extent sufficient to render it acid soluble and any continuation of the treatment can only reduce the molecular weight. Numerous studies exist (e.g. Peniston and Johnson, 1980; Lusena and Rose, 1953; Wu and Bough, 1978; Bough *et al.*, 1978; Aluminiar and Zainuddin, 1992; and Moorjani *et al.*, 1975) on the effect of temperature and time on deacetylation, alkali concentration, viscosity and \bar{M}_v .

In this study, the effect of time and temperature of deacetylation is seen in all the species tested. The time to obtain soluble product in 1% acetic

acid was longer when the temperature was lower (Table 5.). Thus we see at 100°C, the time to obtain soluble product for crabs and lobsters is 8 hr and for prawns 6 hr. When the temperature was increased to 120°C the time to obtain soluble product was reduced to about 4 hours (Table 5). The effect of time and temperature on \bar{M}_v and DD is shown in Tables 7 to 9. When the temperature and time are increased, the \bar{M}_v is reduced and the DD increases. The DD however increases only up to a particular extent and it is not as elaborate as the decrease in \bar{M}_v .

Lusena and Rose (1953) and Mima *et al.*, (1983) carried out similar studies. They showed that higher temperatures increase the percentage of deacetylation and reduces molecular size and that deacetylation proceeds rapidly to about 68% during the first hour of alkali treatment (in 50% NaOH) and with the reaction progressing, deacetylation reaches only 78% in 5 hr. Thus alkali treatment beyond 2 hours does not deacetylate significantly rather it only serves to degrade the molecular chain. The effect of time of deacetylation on viscosity and \bar{M}_v indicate that chitosan deacetylated for shorter times have high viscosities hence high \bar{M}_v than those deacetylated for longer times (Lusena and Rose 1953; Mima *et al.*, 1983). This is probably what happened in the case of crab, which was demineralised then deacetylated for 1 hr and 3 hr. The 1 hr deacetylation produced chitosan of high \bar{M}_v with a DD of 72.9% while the 3 hr deacetylation produced chitosan of a much lower \bar{M}_v with a high DD. The \bar{M}_v is seen to reduce at a much faster rate than DD increase (Tables 8-11). The milder treatment conditions for insect larva *Calliphora encycophalla* could suggest that it is still possible to obtain soluble chitosan products at much lower temperatures (100°C) at shorter times (4 hr) with fairly high \bar{M}_v (Table 11). Similar studies and results have been given by Struszczyk *et al.*, (1999). The increase in moisture content with DD is probably due to increased hygroscopic nature of chitosan with more hydrogen bonds being available (Roberts, 1992; Chandit *et al.*, 1998).

It can be concluded that insect larval chitosan can be obtained from *Calliphora encycophalla* under fairly mild conditions compared to harsher chemical conditions for the crustacean species

analysed. The yield of chitin and chitosan from the crustacean fall within the ranges earlier reported. Increase in temperature and time of deacetylation reduce viscometric molecular weight and increase DD. Crab and lobster shells require stronger HCl concentration for demineralisation to reduce the ash in the final chitosan product.

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