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Effect of Chitin Extraction Processes on Residual Antimicrobials in Shrimp Shells

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Abstract

The present study examined the influences of industrial chitin extraction processes on the residual oxytetracycline (OTC) and oxolinic acid (OA) in shrimp carapaces and shells. The drugs were orally administered by catheter to the kuruma shrimp (*Penaeus japonicus*) and vannamei shrimp (*Penaeus vannamei*). The shrimps were sampled at 6-h post-dosing and their carapaces and shells were collected and used as raw material in the chitin extraction. Residual OTC levels in raw materials were 9 – 18 fold higher than the maximum residue limit (MRL) (0.2 μg/g). The residual OTC was reduced 10 – 30% by washing treatment. By the acid treatment with HCl, the OTC residues were reduced >98%. The deproteinized material contained negligible levels of OTC. Residual OA levels in raw materials was much higher (134 – 376 fold) than the MRL (0.03 μg/g). The residual OA was reduced considerably (34 – 68%) by washing treatment. By the demineralization, the residual OA could not be detected in both shrimp carapaces, while the levels of OA residues were determined more than MRL in shells. In the deproteinized materials, no residual OA were detected. Finally, residual OTC and OA could not be detected in the chitin materials for both shrimps.

Keywords: Oxytetracycline, oxolinic acid, shrimp, chitin, drug residues

Introduction

Chitin is a polysaccharide, made of N-acetyl-D-glucosamine units connected by β (1, 4) linkage and it is the most abundant biopolymer on earth next to cellulose. Chitin, especially its main derivative chitosan, has shown potential for numerous applications in the pharmaceutical, cosmetic and food industries (Rinaudo, 2006). There has been a strong demand for chitin and chitosan in Japan and all over the world. Recently, the major industrial sources of raw material for the production of chitin are shells of crustaceans such as crabs and shrimps (Percot *et al*., 2003).

In the Southeast Asia region, the shrimp processing industry has been rapidly growing with the significant increase in cultured shrimp production. As a consequence, a huge amount of bio-waste, such as carapace (head shells) and abdominal (tail) shells, is produced from the industries, because shrimps are normally sold as headless and often peeled of the outer shell. Therefore, chitin producers are attracted to the cultured shrimp wastes (carapaces and shells).

Generally, antimicrobial agents, such as tetracyclines, quinolones and sulfonamides, are used for the prevention and treatment of infectious diseases in shrimp farming (Li and Randak, 2009). Among these drugs, oxytetracycline (OTC) and oxolinic acid (OA) are the most commonly used antimicrobials in Japanese and Thai shrimp culture farms. Our previous studies (Uno *et al*., 2006a, b) showed that OTC and OA were accumulated and retained strongly in shrimp shells for long time, and moreover, both drugs were considerably thermostable in shells. If chitin is made from shrimp shells containing the residual antimicrobials, it is doubtful whether the chitin products are safe as food (particularly, functional health food) materials. In our recent work (Uno *et al*., 2010), the OTC residues in shrimp shells were considerably reduced (>80%) by hydrochloric acid treatments, whereas those were reduced to 30% by alkaline treatment. Currently, the industrial extraction process of chitin from shrimp shells consists of two basic steps: demineralization by acid treatment such as HCl; and deproteinization by alkaline treatment such as NaOH (Percot *et al*., 2003). At present, there is no information concerning the effect of these processes on residual drugs in shrimp shells.

Hence, the objective of the present study was to investigate the influences of industrial chitin extraction processes on the residual OTC and OA in shrimp shells.

Materials and Methods

Chemicals

Oxytetracycline hydrochloride (OTC, purity >95%) and oxolinic acid (OA) were purchased from Sigma (St. Louis, MO, USA). Unless otherwise indicated, chemicals used were of analytical or HPLC grade.

Shrimp

Kuruma shrimp, *Penaeus* (*Marsupenaeus*) *japonicus*, (n = 120, 21.5 ± 2.0 g) were obtained a shrimp farm in Okinawa, Japan. Vannamei shrimp, *Penaeus* (*Litopenaeus*) *vannamei*, (n = 100, 26.4 ± 3.6 g) were obtained from a shrimp farm in Chanthaburi, Thailand. The shrimps were analyzed to confirm the absence of OTC and OA before the experiment. Each shrimp species was randomly separated into two experimental groups (OTC and OA treatment groups). The shrimps were kept in tanks with brackish water at a salinity of 24 ppt. The water temperature was 22 and 27°C for the kuruma shrimp and vannamei shrimp, respectively. The shrimps were fed *ad libitum* with commercial shrimp pellets before administration. The shrimps were starved for one day before administration of the drugs.

Oral Administration

The doses of OTC and OA, individually, were 50 mg/kg b.w. Each drug for oral administration was mixed in a slurry of food. The slurry was orally administered by catheter (40 x 1.2 mm O.D.) to the shrimp. The shrimps were sampled at 6-h post-dosing. The OTC and OA levels almost peaked at 6h in our previous research (unpublished). Twenty shrimp, used as each control group, were given feed without the drugs.

Carapace and Shell Sampling

The shells, including cephalothorax (head) and abdomen, were peeled from the shrimp body and were separated into the carapace and abdominal shell portions. Each of the carapace and shell was collected and pooled.

Processes for the Preparation of Chitin from Carapace and Shell

The chitin extraction processes in this research included the following steps:

Washing: The raw samples were washed thoroughly in distilled water. Samples were filtered to remove excess water from the solids.

Demineralization: The washed samples were soaked in 2.4 M HCl (1:10 solid-to-solvent, w/v) at 40°C for 3 hours, stirring constantly. The demineralized samples were extensively washed with deionized water to neutral pH and filtered to remove excess water from the solids.

Deproteinization: The samples were soaked in 2 M NaOH (1:10 solid-to-solvent, w/v) at 80°C for 5 hours with constant stirring, washed with deionized water to neutrality and filtered to remove excess water from the solids.

Drying: The samples were dried in a forced air oven at 70°C for 5 hours.

A 3-g portion of samples at each step was used for the HPLC analyses. The overall process for the preparation of chitin from carapaces and shells is given in Figure 1.

Analytical Procedures

The HPLC system consisted of a Jasco PU-980 pump, a fluorescence detector FP-2020 (Japan Spectroscopic, Tokyo, Japan), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20-µL loop and a Chromatopac C-R6A integrator (Shimadzu Seisakusho, Kyoto, Japan).

The extraction method of OTC was previously described (Uno *et al*., 2006a; 2010). Briefly, samples (0.5 g) were homogenized with 30 ml of 5% trichloroacetic acid containing 0.5% EDTA and centrifuged for 20 min at 12,000 rpm. To the supernatant, 5 ml of *n*-hexane was added and vortexed. The aqueous layer was poured into a Sep-Pak C18 cartridge column (Waters Corporation, Milford, MA, USA). After the aqueous layer passed through the column, the column was washed with 30 ml of water. The retained OTC was eluted from the column with 20 ml of methanol. The eluate was evaporated to dryness. The residue was dissolved in 1 ml of the HPLC mobile phase and a 20-µL portion of the solution was injected into the HPLC. The operating conditions were as follows: column, Mightysil RP-18 column (150 x 4.6 mm I.D., Kanto Chemical Co., Inc., Tokyo, Japan); mobile phase, 1 M imidazole buffer (pH 7.2) containing 50 mM magnesium acetate and 1 mM EDTA-methanol (75:25, v/v); flow-rate, 0.8 ml/min; column temperature, 40°C ; and detection, FL (Ex 380 nm/Em 520 nm).

The extraction procedures of OA were carried out by our previous method (Uno et al., 2006b). Samples (0.5 g) were homogenized with 30 ml of 0.2% metaphosphoric acid-methanol (7:3) and centrifuged at 12,400 x *g* for 20 min. To the supernatant, 5 ml of *n*-hexane were added and vortexed. The aqueous layer was concentrated to *ca.* 10 ml at 40°C under vacuum. The concentrated solution was poured into a Bond Elut C18 column

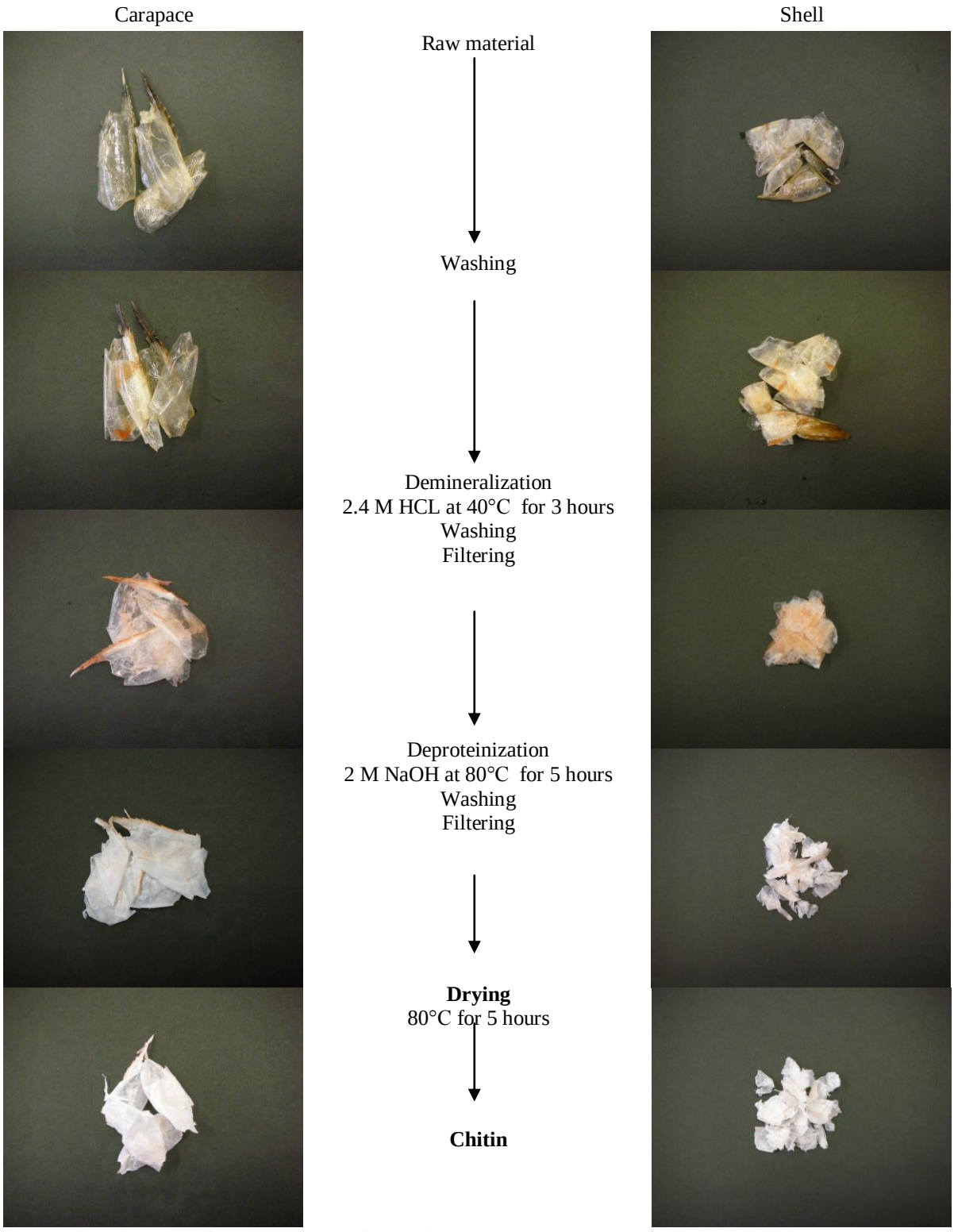

Figure 1. Overall process for the preparation of chitin from carapaces and shells.

(Varian, Harbor City, CA, USA) pre-washed with 5 ml of methanol and 10 ml of water. After the solution passed through the column, the column was washed with 10 ml of 10% methanol. The retained OA was eluted from the column with 10 ml of methanol. The eluate was evaporated to dryness. The residue was dissolved in 1 ml of 30% acetonitrile and a 20-µL portion of the solution was injected into the HPLC. The operating conditions were as follows: column, Hisep column (150 x 4.6 mm I.D., Spelco, Bellefonte, PA, USA); mobile phase, 0.05 M citric acid-0.2 M disodium hydrogenphosphate-acetonitrile (70:15:15, v/v); flow rate, 1.0 ml/min; column temperature, room temperature; and detection, FL (Ex 325 nm/Em 365

nm).

The recoveries of OTC from carapace, shell and chitin spiked at 1.0 µg/g were 82.7 ± 2.8, 83.2 ± 2.4 and 72.4 ± 3.1% (n = 4) for the kuruma shrimp, and 85.1 ± 8.4, 82.3 ± 7.2 and 73.3 ± 1.8% (n = 4) for the vannamei shrimp, respectively. The limits of quantitation were 0.01 µg/g for each. The recoveries of OA from carapace, shell and chitin spiked at 1.0 µg/g were 82.3 ± 1.5, 82.6 ± 1.4 and 78.6 ± 1.6% (n = 4) for the kuruma shrimp, and 81.0 ± 1.3, 79.9 ± 1.5 and 75.7 ± 1.3% (n = 4) for the vannamei shrimp. The limits of quantitation were 0.02 µg/g for each. Typical chromatograms of OTC and OA in shell, chitin and their controls are shown in Figures 2 and 3, respectively. Both the retention times of OTC and OA were 4 minutes. No interfering peaks were observed in the chromatograms of control.

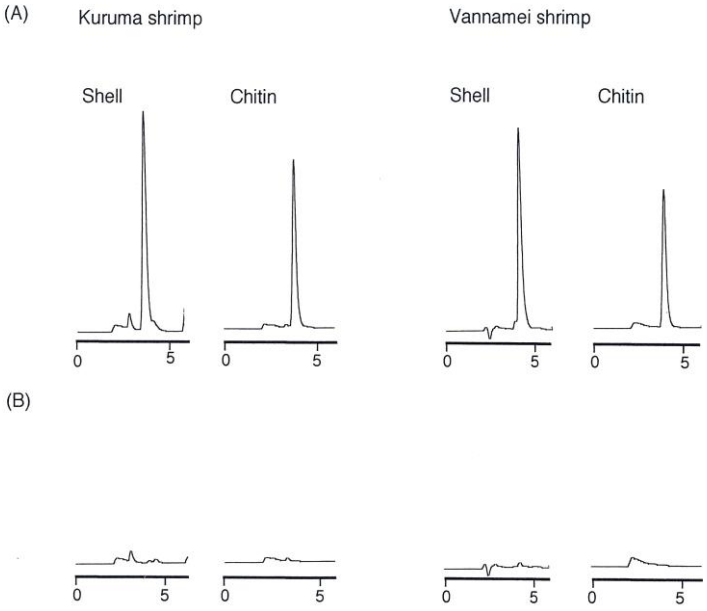

Figure 2. Typical chromatograms of oxytetracycline in shell and chitin,(A) shell and chitin samples spiked at 1.0 µg/g oxytetracycline ; (B) control (without the drug).

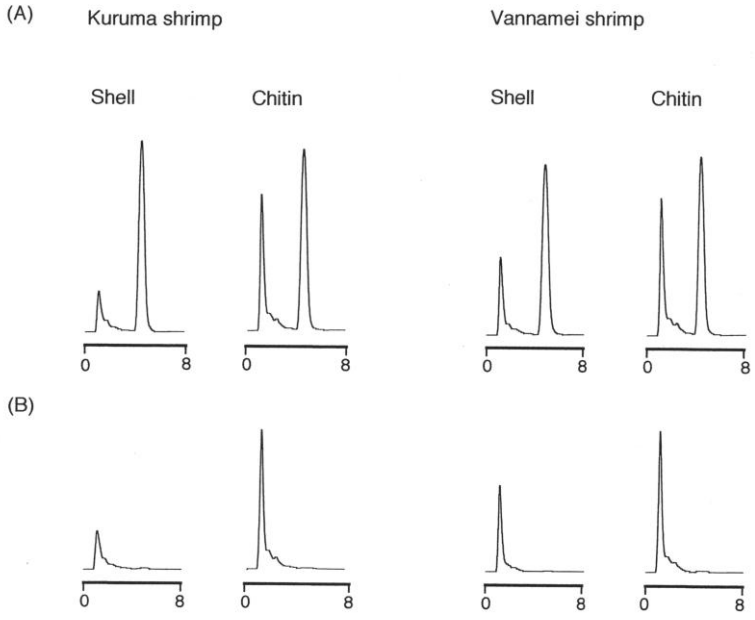

Figure 3. Typical chromatograms of oxolinic acid in shell and chitin, (A) shell and chitin samples spiked at 1.0 µg/g oxolinic acid ; (B) control (without the drug).

Results and Discussion

Chitin Yields from Carapace and Shell

The yields of all extraction steps are given in Table 1. The demineralization with HCl solubilized calciumcarbonate and other salts from carapaces and shells resulted in reduction of their raw materials weight by 8 – 9% for the kuruma shrimp and 13 – 15% for the vannamei shrimp. The large weight losses occurred during the deproteinization step. The deproteinization with NaOH caused even more solubilization, yielding 44 – 48% of the raw materials for both species. The final chitin yield was 13.0 and 8.9% for the kuruma shrimp and 15.3 and 9.5% for the vannamei shrimp from carapaces and shells, respectively. The present processing conditions resulted in higher chitin yield from carapaces than shells for both shrimp species. Chitin yield in conventional extractions generally ranges from 5 to 8% (Kjartansson *et al.*, 2006).

Effect of Chitin Extraction Processes on Residual Oxytetracycline

Table 2 shows the effect of chitin extraction processes on residual OTC. The levels of residual OTC in raw materials were 2.211 μg/g in carapaces and 2.461 μg/g in shells for the kuruma shrimp, and 1.747 μg/g in carapaces and 3.613 μg/g in shells for the vannamei shrimp. In Japan and the USA, legislation stipulates maximum residue limits (MRLs) for OTC used in the treatment of fish and shellfish. The current MRL for OTC is 0.2 μg/g. Residual OTC levels in raw materials were 9 – 18 fold higher than the MRL. By washing treatment, the residual OTC was reduced 20 – 30% for the kuruma shrimp and 10 – 20% for the vannamei shrimp. The largest reduction occurred during the demineralization step. The OTC residues were reduced >98% by the acid treatment with HCl. It is known that OTC forms complexes with di-cation, such as Ca^{2+} and Mg^{2+}, in hard tissues of fish (Grondel *et al*., 1987; Jacobsen, 1989; Rogstad *et al*., 1991; Namdari *et al*., 1996; Malvisi *et al*., 1996; Maruyama and Uno, 1997). In our previous works, Ca-tetracycline chelates accumulated in shrimp shells and were shown to be considerably stable (Uno *et al*., 2006a). Therefore, it is probable that the Ca-tetracycline chelates were almost all released from carapaces and shells into the hydrochloric acid solution. The deproteinized material contained negligible levels of OTC. In the final chitin (dried) material, residual OTC could not be detected.

Effect of Chitin Extraction Processes on Residual Oxolinic Acid

Table 3 shows the effect of chitin extraction processes on residual OA. The levels of residual OA in raw shell materials were higher than carapaces for both shrimp species. In Japan the current MRL for OA is 0.03 μg/g in shrimp tissues, whereas it is not allocated by the Joint FAO/WHO Expert Committee on Food Additives. Residual OA levels in raw materials was much higher (134 – 376 fold) than the MRL. By the first washing treatment, the residual OA was reduced considerably. Their reductions in carapaces and shells were 34 and 63% for the kuruma shrimp, and 68 and 66% for the vannamei shrimp. By the following demineralization with HCl, the residual OA could not be detected in both shrimp carapaces, while the levels of OA residues were determined more than MRL in shells. No residual OA were detected in the deproteinized and dried materials for the kuruma and vannamei shrimps.

In conclusion, our results showed that the demineralization process with HCl contributed to greater removal of residual OTC and OA in shrimp

Table 1. Yield of samples from each treatment*

	Kuruma shrimp		Vannamei shrimp	
Treatment	Carapace	Shell	Carapace	Shell
Raw material	100	100	100	100
Washed	95.6	94.5	91.2	86.2
Demineralized	91.9	91.5	87.4	84.8
Deproteinized	46.3	47.9	44.3	44.8
Dried (Chitin)	13.0	8.9	15.3	9.5

* Yield expressed as % of weight of raw material.

Table 2. Effect of chitin extraction processes on residual oxytetracycline in shrimp carapaces and shells

	Kuruma shrimp		Vannamei shrimp	
Treatment	Carapace	Shell	Carapace	Shell
Raw material	2.211 ± 0.019*[1]	2.461 ± 0.318	1.747 ± 0.080	3.613 ± 0.322
Washed	1.835 ± 0.233	1.724 ± 0.101	1.522 ± 0.079	2.802 ± 0.181
Demineralized	tr*[2]	0.040 ± 0.003	tr	0.031 ± 0.008
Deproteinized	nd*[3]	nd	nd	tr
Dried (Chitin)	nd	nd	nd	nd

*[1] Values (μg/g) represent the mean of four determinations ± standard deviation. *[2] tr < 0.01. *[3] Not detected.

Table 3. Effect of chitin extraction processes on residual oxolinic acid in shrimp carapaces and shells

	Kuruma shrimp		Vannamei shrimp	
Treatment	Carapace	Shell	Carapace	Shell
Raw material	4.019 ± 0.093*[1]	9.159 ± 0.591	8.268 ± 0.380	11.299 ± 0.470
Washed	2.665 ± 0.569	3.350 ± 0.254	2.619 ± 0.309	3.808 ± 0.097
Demineralized	nd*[2]	0.103 ± 0.017	nd	0.112 ± 0.021
Deproteinized	nd	nd	nd	nd
Dried (Chitin)	nd	nd	nd	nd

*[1] Values (µg/g) represent the mean of four determinations ± standard deviation. *[2] Not detected

carapaces and shells. Therefore, chitin products made by the industrial processes, including the demineralization with HCl and deproteinization with NaOH, may contain no residues, even if shrimp carapaces and shells as sources of chitin contain the residual OTC and OA.

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