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Removal of Lipids and Diarrhetic Shellfish Poisoning Toxins from Blue Mussels (*Mytilus edulis*) during Acid and Alkaline Isolation of Proteins

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Diarrhetic shellfish poisoning (DSP) toxins pose a serious health risk for consumers of bivalves and other shellfish, as well as a huge economic burden for the bivalve-producing farmers. In this work, the aim was to utilize a solubilization-based protein-isolation method to produce a low-DSP toxin protein isolate from toxic blue mussels that are unsuitable for the whole shellfish market. A homogenate of whole mussel meat was solubilized at low pH (2.8) or high pH (11.1), followed by centrifugation and reprecipitation of the solubilized mussel proteins at the isoelectric pH. In a second centrifugation, precipitated proteins were collected. These processes resulted in 81 (acid solubilization) and 72% (alkaline solubilization) reduction in the initial DTX-1 toxin content of the mussel meat. No other DSP toxins were found in the protein isolates. Acid processing of mussel meat resulted in 50% reduction in the total lipid content, while alkaline treatment did not significantly affect the lipid content. The effect of citric acid and calcium chloride addition to the mussel meat−water homogenate on lipid and toxin content was also investigated. A poor correlation factor was surprisingly obtained between reductions in DTX-1 toxin and lipids in protein isolates from processed toxic mussels. Results from an analytical mass balance of the DTX-1 toxin during acid processing showed that 61% of this toxin ended up in the aqueous supernatant after the second centrifugation. The present study presents a promising alternative way of utilizing mussels for food production in periods when they are toxic.

KEYWORDS: Mussels; *Mytilus edulis*; toxins; DSP; DTX-1; diarrheic shellfish poisoning; lipids; citric acid; calcium chloride; acid or alkaline solubilization; protein isolation; proteins

INTRODUCTION

The diarrhetic shellfish poisoning (DSP) toxins are lipophilic polyether molecules produced by dinoflagellates of the *Dinophysis* sp. and *Prococentrum* sp. genera, and they accumulate in the digestive system of the bivalves when feeding on those plankton species (*1–3*). In many bivalve-producing areas, they have a far-reaching economic impact, as their appearance leads to the imposition of long bans on the culture harvest (*4, 5*).

There are three parent compounds of DSP: okadaic acid (OA), dinophysistoxin-1 (DTX-1), and dinophysistoxin-2 (DTX-2) (**Figure 1**). The carboxylic group at C1 of OA can be esterified to form DTX-4 and DTX-5, which are water-soluble precursors of OA (*6, 7*). The hydroxyl group at C7 of OA, DTX-1, and DTX-2 can also be esterified with a fatty acid varying in chain length and degree of unsaturation to form 7-*O*-acyl esters, collectively called DTX-3. In general, the lipid solubility of a toxin will predict its propensity to accumulate within cells. OA diol ester has no ionizable group and would be expected to move across cell membranes with relative ease (*8*). It is postulated in the literature that this could prove a significant factor in DSP transfer and toxicity within the food web, including human consumers. This is because DTX-4 is the major DSP toxin found within the dinoflagellate *Prococentrum lima* and it will be hydrolyzed in seconds to OA diol ester but then only slowly to OA, following cell lysis (*9*). In blue mussels (*Mytilus edulis*), the esterified OA did not exceed 50% of the total OA equivalents, while in other seafood species such as cockle, oyster, and razor clam, more than 95% of the total OA was found esterified (*10*).

The European Commission (*11*) has laid down a maximum level of 160 μg OA equiv kg^{-1} of whole shellfish meat. However, this limit is criticized by many research groups, and it is suggested to be lowered by 50% (*12–14*). Current methods that could make mussels suitable for food production in periods when they are toxic are mainly focused on the live mussels and are either not effective enough or economically not feasible. DSP toxins display a remarkable thermoresistance, rendering any thermal treatment of shellfish an ineffective way to reduce the toxicity. Hence, the only current approach to remove toxins from mussels is natural detoxification (i.e., depuration), whereby the mussels are maintained in the sea or in land-based operations for several weeks till the toxic episode has disappeared (*15, 16*). Kroken et al. (*17*) reported a land-based

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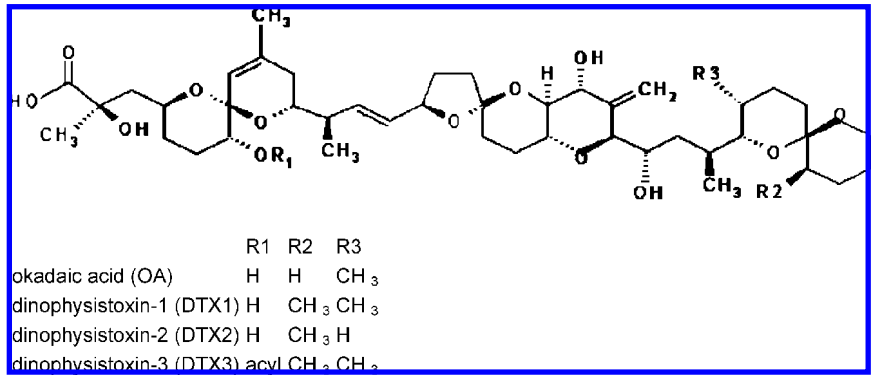

Figure 1. Structure of the DSP toxins adapted from ref *12*.

depuration method exhibiting 1–4 days of half-life depending on the initial load of toxicity. However, both aforementioned methods have questionable feasibility since the process is time-consuming and/or depends on the metabolic activity of the mollusks (*18, 19*).

An alternative use of mussels in periods when they are toxic could be to produce a mussel protein isolate (PI). Seafood PIs have successfully been used, for example, in surimi and/or kamaboko products (*20*), in marinades to enhance organoleptic properties of seafood products (*21*), and as emulsifiers (*22*). Between 1999 and 2001, a series of new processes were developed for efficient separation of seafood muscle proteins from lipids, bones, scales, and other impurities (*23, 24*). These processes result in protein-rich, low-lipid surimi-like isolate that could open up new doors for adding value to blue mussels that due to excessive toxin content, small size, and/or damage are otherwise not suitable for the whole shellfish market. It should here be stressed that as much as 1/3 of the total harvested mussels are thrown away or used for animal feed because of too small size or crushed shells.

In brief, the protein isolation processes involve solubilizing the proteins using high (~10.5) or low (~3) pH, followed by centrifugation to remove insoluble matter. Purified soluble muscle proteins are then precipitated at the protein isoelectric point (pH ~5.5) and isolated using a second centrifugation. Using herring light muscle, Undeland et al. (*25*) observed a 68 and 60% reduction in the lipid to protein ratio after isolating proteins with the after acid and alkaline techniques, respectively. Similar observations have been reported for Spanish mackerel, mullet and croaker (*26*), channel catfish muscle (*27*), and bristly sardine (*28*). On the basis of these findings, we hypothesized that the lipid-soluble DSP toxins might get efficiently removed together with the lipids during acid and/or alkaline protein isolation. Because most of the lipid removal normally takes place in the first centrifugation step, we expected the same to be true for the DSP toxins.

The overall aim of this study was thus to investigate the efficiency of the acid and alkaline protein isolation processes in reducing the lipid and DSP toxin contents of minced mussel meat. To elucidate how the pH shifts affected the solubility of the DSP toxins, special attention was paid to the partitioning of the DSP toxins into the different phases of the process and to the relation between lipid and toxin removal. As a strategy to further enhance DSP removal, the effect of adding citric acid and calcium chloride (Cc) before solubilizing the mussel tissue was also investigated. Liang and Hultin (*29*) showed that calcium chloride aided the membrane lipid removal during centrifugation of muscle homogenates that were solubilized at pH 3 in the presence of citric acid or malic acid. Finally, the efficacy of using a low centrifugation force (800*g*) during acid and alkaline processing on lipid and DSP toxin removal was also tested. This was because the high speed centrifugation step normally involved in the protein isolation processes is a highly costly operation.

MATERIALS AND METHODS

Materials. Fresh blue mussels were obtained from Scanfjord AB (Sweden) and transported to the laboratory on ice, where they were immediately processed. Toxic blue mussels were obtained from a mussel farm in Sogndal, Norway. Chemicals and reagents were purchased from Sharlau Chemie S.A. (Sentmenat, Spain). Solvents were purchased from Labscan Ltd. (Dublin, Ireland). All reagents were of ACS grade, and all solvents were of high-performance liquid chromatography (HPLC) grade.

Methods. *Sample Preparation.* Mussel shells were opened by hand, and the meat, including most of the mussel liquor, was manually removed from the shells and collected in a beaker placed in a container filled with crushed ice. The mussel meat (~300–400 g) was then blended for 60 s using a miniprocessor (Ide line, model 905, Wormanco El AB, Skara) precooled at –20 °C so that the temperature was kept low throughout the preparation. Blended mussel meat was then either processed immediately or stored at –80 °C for up to 4 days in plastic zip-lock bags. The blended meat from the toxic blue mussels was stored at –80 °C for up to 5 months.

Acid and Alkaline Processing Using High or Low Centrifugation Speed. Blended mussels were transferred to a beaker placed in a container filled with crushed ice and were homogenized in 6 volumes of cold distilled–deionized water for 40 s at 14000 rpm using an Ultra Turrax T18 Basic homogenizer (IKA, Taquara, RJ, Brazil). The pH of the homogenate was brought down to 2.8 (acid solubilization) using 2 N and/or 0.4 N HCl or to 11.1 (alkaline solubilization) with 2 N and/or 0.4 N NaOH, using a precalibrated Hamilton double pore electrode (Bonaduz, Switzerland) coupled to a pH meter (MeterLab PHM210, Radiometer analytical S.A., Villerbanne Cedex, France). The solubilized homogenate was centrifuged at 8000*g* or 800*g* for 20 min in a Sorvall RC50 Plus centrifuge (DuPont, Newton, CT) set at 4 °C. The supernatant of the first centrifugation was separated from the sediment and the floating top layer through three layers of commercial cheesecloth (AKLA AB, Danderyd, Sweden). The sediment, including the top layer, was kept at –20 °C till used for further toxin, lipid, and protein analysis. The supernatant fraction was readjusted to pH 5.8 when solubilization took place at pH 2.8 or to 5.2 when solubilization took place at pH 11.1 to precipitate the solubilized proteins. Experimental data (not shown) indicated the lowest solubility for the acid-solubilized proteins at pH ~5.8, while the lowest solubility for the alkaline-solubilized proteins was at pH ~5.2. After centrifugation at 8000*g* or 800*g* for 20 min, the resultant sediment, called PI, was collected and analyzed for moisture, lipid, toxin, and protein contents.

To study the possibility of increasing the water removal from the final PI, a centrifugation method was employed. A certain amount (~10 g) of freshly prepared PI with known moisture content (MC) was centrifuged at 20000*g* in the Sorval RC50 centrifuge for 20 min. The water loss from the PI was directly determined by weighing the aqueous phase in the centrifuging bottle.

Cc as Helping Agents in Lipid Removal. Citric acid dissolved in cold distilled–deionized water was stirred into the 1:6 mussel meat in water homogenate to reach a final concentration of 5 mM. After 1 h of incubation on ice, a freshly prepared solution of calcium chloride was also mixed in to reach a final concentration of 10 mM. Then, the homogenate was solubilized either at high or low pH and processed as described in the previous section.

Moisture Content Determination. Moisture determination was done according to the official AOAC method 985.14 using an Electrolux 939 oven (Electrolux, Stockholm, Sweden).

Total Lipid Determination. The Lee et al. (*30*) method as modified by Undeland et al. (*22*) was employed for the determination of total lipids in the samples. When analyzing mussel meat and PI samples, the solvent consisted of chloroform and methanol at a 2:1 ratio. In the case of analyzing the supernatant of the second centrifugation for lipid content, the ratio was 1:2 chloroform to methanol, since this sample was expected to contain a very small amount of lipid. Results were expressed both on a dry weight basis and as a lipid/protein ratio to normalize the obtained values and avoid interference from materials like byssus threads and shell pieces that might have been left in the blended mussel samples.

Protein Analysis. The protein content of the samples was measured according to the method of Markwell et al. (*31*). The protein concentration was determined spectrophotometrically at 660 nm using a Cary50 BIO UV–visible spectrophotometer (Varian Australia Pty Ltd., Victoria, Australia). Bovine serum albumin was used to obtain a protein standard curve in the range of 0–100 μg protein/mL.

Determination of Paralytic Shellfish Poisoning (PSP), OA, DTX-1, and DTX-2. Samples taken out for toxin analysis were stored in plastic zip-lock bags at –80 °C until analysis. The samples were homogenized with an Ultra Turrax T25 homogenizer, extracted with 80% methanol, and filtered through a NANOSEP MF GHP 0.45 μm centrifugal filter (PALL Co., East Hills, United States). Then, the methanol extract was subjected to HPLC using as eluents methanol:ammonium acetate (80/20, v/v, pH 5.8) (*1032*). The column used was a YMC-Pack, hydrosphere C18, 150 mm × 2.1 mm i.d. (YMC Co., Ltd., Kyoto, Japan). The toxins were detected with mass spectroscopy using an MS1100MSD electrospray interface detector with negative ionization (Agilent Technologies, Inc., United States). The DTX-1 content was expressed both on a wet weight basis and as a DTX-1/protein ratio. The latter was for comparison reasons and to avoid interference from materials like byssus threads and shell pieces that might have been left in the blended mussel samples.

Statistical Analysis. Data were analyzed by Excel (Microsoft Corp., United States) and analysis of variance using SAS 9.1 (SAS Institute Inc., Cary, NC). Differences between treatment means at the 5% level were determined using Duncan's multiple range test. The correlation factor was determined using the Pearson product-moment correlation. Each treatment was repeated at least three times ($n = 3$), unless otherwise noted. For each data point used in further calculations, the average of at least two analytical readings was taken. In the case of toxin quantification, samples were analyzed at least three times ($n = 3$). In cases where an analysis was performed only twice ($n = 2$), the average ± (max − min)/2 is reported.

RESULTS

Lipid Reduction. Blended fresh mussels were solubilized at high or low pH followed by centrifugation at 8000*g*. The supernatant was then adjusted to the isoelectric pH of the proteins and centrifuged to collect the PI. The lipid content, before and after the treatment, was determined. As can be seen from **Table 1**, the acid treatment resulted in ∼50% reduction in the lipid to protein ratio, while the alkaline treatment had no effect.

Effect of Citric Acid/Calcium Chloride and Centrifugation Force on Lipid/Protein Ratio. To further improve lipid removal, Cc at final concentrations of 5 and 10 mM, respectively, were added to the 1:6 homogenate of blended mussels prior to acid or alkaline solubilization. The samples were then treated as previously, at either high or low pH, and the PIs were analyzed for moisture, protein, and lipid contents.

Table 1. Lipid/Protein Ratio of Mussel Meat after the Acid or Alkaline Solubilization Process (Mean ± SD)[a]

sample	% moisture	% lipid (dry weight)	% protein (dry weight)	lipid/protein ratio
blended mussel meat ($n = 11$)	80.1 ± 1.8	13.5 ± 4.1	45.7 ± 8.5	0.3 ± 0.08 a
acid PI ($n = 9$)	90.0 ± 1.6	11.0 ± 7.0	76.3 ± 15.1	0.15 ± 0.1 b
alkaline PI ($n = 9$)	86.7 ± 2.2	18.8 ± 8.3	63.9 ± 13.8	0.3 ± 0.14 a

[a] Acid or alkaline PI: Blended mussels were homogenized in 6 volumes of water and were adjusted to either pH 2.8 or pH 11.1 and centrifuged at 8000*g* for 20 min, followed by precipitation of the solubilized proteins at pH 5.2 for the alkaline-solubilized proteins or pH 5.8 for the acid-solubilized proteins, and finally a second centrifugation at 8000*g*. Means with the same letter are not significantly different. Blended mussel meat: Mussel meat was blended for 60 s using a miniprocessor precooled at –20 °C.

Table 2. Effect of Citric Acid/Calcium Chloride and Low Centrifugation Force on Lipid Reduction of PIs (Mean ± SD)[a]

sample	% moisture	% lipids (dry weight)	% protein (dry weight)	lipid/protein ratio
blended mussel meat ($n = 11$)	80.1 ± 1.8	13.5 ± 4.1	45.7 ± 8.5	0.30 ± 0.08 a
Cc acid, 8000*g* ($n = 6$)	92.4 ± 2.4	7.3 ± 7.7	81.8 ± 15.5	0.08 ± 0.08 c
Cc alkaline, 8000*g* ($n = 7$)	88.3 ± 2.7	6.3 ± 4.6	68 ± 8.3	0.09 ± 0.06 c
acid, 800*g* ($n = 3$)	95.3 ± 0.5	23.9 ± 11.0	82.5 ± 13.5	0.27 ± 0.08 a
alkaline, 800*g* ($n = 3$)	88.1 ± 0.2	17.7 ± 1.4	55.8 ± 4.7	0.31 ± 0.006 a
Cc alkaline, 800*g* ($n = 5$)	91.3 ± 3.0	11 ± 7.9	75 ± 7.5	0.15 ± 0.12 b,c

[a] Cc acid or alkaline, 800*g* or 8000*g*: Concentrations of 5 mM citric acid and 10 mM calcium chloride were added prior to solubilization; samples were then solubilized at pH 2.8 or 11.1, and a centrifugation force of 800*g* or 8000*g* was used for both centrifugation steps. Acid or alkaline 800*g*: Samples were solubilized at pH 2.8 or 11.1, and a centrifugation force of 800*g* was used for both centrifugation steps. Means with different letters indicate significant differences ($p < 0.001$).

In a separate set of experiments, the effect of reducing the centrifugation force from 8000*g* to 800*g* during the acid or alkaline process, with or without Cc addition, was also studied. A lower centrifugation force would be more appealing to an industrial application. Results are again expressed as lipid to protein ratios (**Table 2**). It can be seen that Cc significantly ($p < 0.001$) reduced the lipid content of the PIs as compared to the untreated blended mussels and to the respective treatments without citric acid/calcium chloride. No significant difference was found between PIs from the acid- or alkaline-solubilized mussels when Cc were used. Comparing the results from **Tables 1** and **2**, it can also be observed that the lower centrifugation force (800*g*) did not affect the lipid/protein ratio in the alkaline-produced samples, while with the acid process the lipid/protein ratio reduction was less efficient. However, when Cc was used in the alkaline solubilization combined with the low centrifugation *g*-force, the decrease in the lipid/protein ratio was still significant as compared to the untreated blended mussels. Thus, the lipid/protein ratio in the various samples analyzed decreased in the following order: blended mussels ≥ alkaline process, 800*g* ≥ alkaline process, 8000*g* ≥ acid process, 800*g* ≥ acid process, 8000*g* ≥ Cc-alkaline process, 800*g* ≥ Cc-acid process, 8000*g* Cc-alkaline process, 8000*g*.

Table 3. Initial Lipid and Toxin Contents of Three Batches (A−C) of Toxic Blue Mussels[a]

batch	% moisture	% lipids (wet weight)	DTX-1 (μg/kg wet weight)	DTX-1/ protein	OA (μg/kg wet weight)
A	80.5 ± 0.20 (n = 2)	2.5 ± 0.3 (n = 2)	800	8.99	46
B	79.5 ± 0.25 (n = 2)	2.6 ± 0.4 (n = 2)	806	10.01	43
C	79.0 ± 0.25 (n = 2)	2.9 ± 0.2 (n = 2)	1143	15.52	143

[a] For % moisture and % lipids, the average ± (max − min)/2 is reported.

Lipid Content of the Second Supernatant of the Cc Treated Mussel Meat Solubilized at pH 2.8. To study whether the second centrifugation step also contributed to lipid/toxin removal from the Cc-acid, 8000*g* sample, the supernatant fraction of the second centrifugation was extracted with $CHCl_3$:MeOH (1:2, v/v) for total lipid determination. It was found that only 0.034 ± 0.001 g (±SD, n = 3) of lipids was present in the supernatant, which represents 1.8% of the total initial lipids of the mussel meat.

Reduction of Water in PIs. As can be seen in **Tables 1** and **2**, PIs have a high MC ranging from ∼87 to 95%. It was of interest to study whether the water content of these isolates could be reduced. PI (10.2 g) from acid-solubilized mussel meat with a MC of 90% was therefore centrifuged at 20000*g* for 20 min. The water loss was 5.8 ± 0.05 g (n = 2), which represents 58% of the total PI mass and resulted in a new MC of 76.8%.

Toxin Reduction in the PIs Obtained from Acid or Alkaline Processing of Toxic Mussels. Three batches of very toxic blue mussels from different locations of the same mussel farm in Sogndal, Norway, were obtained in December 2006 and analyzed for lipid content, PSP, OA, DTX-1, and DTX-2 toxins. PSP and DTX-2 toxins were below the detection limit (<10 μg/kg). The rest of the results together with data on total lipids are summarized in **Table 3**.

All three batches had very high levels of DTX-1, ranging from 800 to 1143 μg/kg of wet muscle meat. OA was found at levels from 43 to 143μg/kg wet mussel meat.

Contaminated blended mussel meat was subjected to acid and/or alkaline process, and the produced PIs were analyzed for toxin content. OA was not detected in any of the mussel PIs. For comparison reasons, toxin removal is expressed as the % decrease in the ratio of DTX-1 toxin content over protein content of the sample. DTX-1 toxin content in the PI samples is expressed on an 80% MC, which is the MC of the untreated blended (toxic) mussel meat and thus the basis for the European Commission regulations (**Table 4**). It was observed that the highest removal of DTX-1 toxin was obtained with the acid solubilization process, when the high (8000*g*) centrifugation force was used. Using this process, the isolates exhibited on average 85 ± 10% lower toxin/protein ratio (n = 3, ±SD) than the blended mussel meat. However, citric acid/calcium chloride exhibited a negative effect on the toxin removal (70 ± 3%, n = 3, ±SD) when the acid solubilization process was employed. The alkaline process with 8000*g* produced isolates with an average of 81 ± 1% (n = 2) lower toxin/protein content. Cc did not affect the toxin/protein ratio in the case of the alkaline solubilization process combined with 8000*g* centrifugation force. Finally, low centrifugation force (800*g*) during acid or alkaline solubilization was applied to three samples. Results showed that it was not as effective as 8000*g* in reducing the toxin/protein ratio from the solubilized mussels (**Table 4**).

It was of interest to evaluate whether there was a correlation between the lipid and the toxin removal. This correlation was based on the reduction of the DTX-1/protein ratio and of the lipid/protein in the PIs before and after each version of the process (**Figure 2**). A poor correlation coefficient was obtained with a value of 0.22 as calculated by the Pearson product-moment correlation.

Table 4. DTX-1 Toxin Reduction in the PIs Produced from Acid or Alkaline Processing of Toxic Mussels[a]

sample	batch	% moisture	DTX-1 (μg/kg PI)	DTX-1/ protein	% lipids of PIs (wet weight)	DTX-1 (μg/kg) based on 80% moisture
acid 8000g^1	A	90	<10	0.1	1.1	8.9*
acid 8000g^2	B	88	111	2.5	2	100.9*
acid 8000g^3	A	90.6	130	1.8	0.96	114.8*
alkaline 8000g^1	A	87	153	1.9	2.3	140.7*
alkaline 8000g^2	B	88	143	1.6	2.7	130.0*
Cc acid 8000g^2	A	94	120	2.5	1.2	102.1*
Cc acid 8000g^3	A	95	117	2.4	1.1	98.5*
Cc acid 8000g^3	C	90	237	5.3	0.15	210.7
Cc alkaline 8000g^2	A	90	108	1.6	1.4	96.0*
Cc alkaline 8000g^3	A	90	181	2.0	0.57	160.9
Cc alkaline 800g^2	A	93.6	86	1.9	1.07	73.5*
Cc alkaline 800g^3	C	93.4	206	4.0	0.17	176.4
acid 800g^2	A	94.7	122	2.5	1.86	103.1*

[a] OA was not detected in any of the PIs (detection limit, 10 μg/kg). Cc, acid or alkaline: protein isolates obtained from acid- or alkaline-solubilized toxic mussels, with or without addition of Cc prior to adjustment to low or high pH. The centrifugation force was either 8000*g* or 800*g*. Key: 1, PIs prepared from blended fresh mussel meat; 2, PIs prepared from blended mussel meat stored for 2 months at –80 °C; and 3, PIs prepared from blended mussel meat stored for 5 months at –80 °C. An asterisk denotes that the values are below the EU safety levels (160 μg/kg).

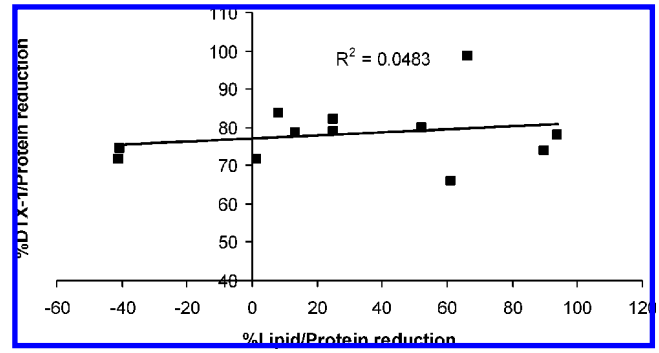

Figure 2. Correlation between DTX-1 toxin and lipid removal from PIs produced from acid- or alkaline-processed toxic blue mussels. The correlation factor between the reduction in the DTX-1/protein ratio and the lipid/protein ratio was obtained using the Pearson product-moment function. r = 0.22 with a critical value of 0.526 for 10 degrees of freedom and a 0.05 level of confidence.

DTX-1 Toxin Analytical Mass Balance. To gain a better understanding of the effect of the acid solubilization process with the addition of Cc on the DTX-1 toxin, a complete mass balance of the DTX-1 toxin during the different steps of the process was performed (**Figure 3**). It was found that on average 14 ± 6% (n = 3, ±SD) of the initial total DTX-1 ended up in the sediment of the first centrifugation and 72 ± 7.6% (n = 3, ±SD) remained in the aqueous phase. From the complete mass balance, it was noted that the overall toxin recovery from the first centrifugation step was 80 ± 5% (n = 3, ±SD). The rest were either destroyed during the solubilization process or transformed to other forms that were not detected by the HPLC analysis. As shown in **Figure 3**, only 5.6 ± 3% (n = 3, ±SD) of the initial DTX-1 content ended up in the PI after the second

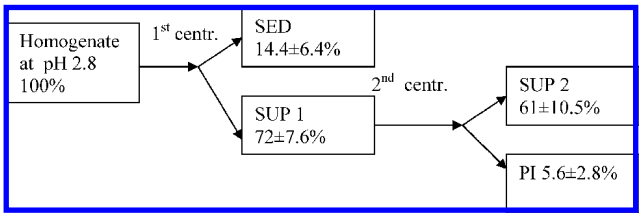

Figure 3. DTX-1 mass balance during production of PI from acid-solubilized toxic blue mussel meat using Cc. PI was obtained from blended toxic mussels treated with citric acid/calcium chloride prior to acid solubilization; both centrifugation steps were performed at 8000*g*. Results are expressed as mean percentages ± SD of the initial DTX-1 toxin in the homogenate. Proper amounts of the homogenate, the sediment (SED) of the first centrifugation, the first supernatant (SUP 1), the PI, and the second supernatant (SUP 2) were used for DTX-1 determination.

centrifugation. At this step, the overall recovery was 89 ± 9.6% (n = 3, ±SD). Surprisingly, 84 ± 8.5% (n = 3, ±SD) of the recovered toxin after the second centrifugation step ended up in the aqueous supernatant, which also contained a small amount of lipids. As mentioned earlier, this amount represented the 1.8% of the total initial lipids of the blended mussel meat. To investigate the possibility that this little amount of lipids is enough to keep the DTX-1 toxin in the aqueous phase of the supernatant, a preliminary trial was conducted, where the lipids of the supernatant fraction were extracted with $CHCl_3$:MeOH (1:2, v/v) and the extract was analyzed for DTX-1 toxin. Results showed that the $CHCl_3$:MeOH extract containing the lipids and/or other hydrophobic compounds also contained 98% of the DTX-1 toxin detected in the second supernatant fraction.

DISCUSSION

Protein isolation techniques based on acid or alkaline solubilization have been shown to aid in the removal of lipids from solubilized muscles (*25–27*). Therefore, it was of great interest to study whether these processes could also aid in the removal of the DSP toxins from blue mussels, which are considered to be lipophilic. However, in this study, it was found that only the process based on acid solubilization of whole mussel meat was effective in lipid removal, as expressed by the lipid/protein ratio (**Table 1**). Reduction in the lipid/protein ratio could be further improved for both the acid and the alkaline processes with the use of Cc (**Table 2**). Liang and Hultin (*29*) suggested that Cc might disconnect the linkage between membranes and cytoskeletal proteins. Citric acid might play a role as a binding agent to the basic amino acid residues of cytoskeletal proteins competing with the acidic phospholipids of membranes (*33, 34*). The membranes released from the cytoskeletal proteins could then aggregate or fuse due to the final low pH achieved by the addition of citric acid (pH ∼4.9–5.3) in the homogenate (*35*). Cross-linking of the membrane components by calcium ions could also have taken place, especially at high pH or at physiological pH after alkaline treatment, where calcium ions might interact with the negative charges on the soluble membrane components and induce sedimentation. Vareltzis and Hultin (*36*) showed that after alkaline pH treatment of cod white muscle, only 12% of the recovered membrane proteins and 11% of the recovered phospholipids sedimented at low centrifugation speeds at pH 7.2. Therefore, in the presence of calcium chloride, the suspended membranes could interact with the calcium ions and form bigger aggregates. This would lead to the decrease in total lipids in the PIs, since the membrane phospholipids will be removed in the sediment of the first centrifugation step. Part of the lipophilic toxins that are bound to membrane lipids is therefore also expected to be removed in the sediment.

Most of the results from this study were obtained using a high centrifugation force (8000*g*). However, this is not always feasible to achieve, especially in an industrial scale; therefore, lower forces are preferred. In this study, 800*g* was successful in significantly reducing the total lipid content of the starting material only when Cc were used (**Table 2**). This observation strengthens the hypothesis made by Liang and Hultin (*35*), because aggregation and/or cross-linking of membranes causes the formation of heavier and bigger in size vesicles; therefore, less force is required for sedimentation.

It was expected that we would obtain an enhanced removal of the lipophilic DTX-1 toxin from the samples that were lowered in lipids, that is, the acid-processed samples with or without citric acid/calcium chloride and the alkaline-processed samples with citric acid/calcium chloride. However, surprisingly, the alkaline-processed samples without citric acid/calcium chloride also had a reduced DTX-1 level as compared to the starting toxic material (**Table 4**), even though there was no reduction in the lipid/protein ratio of this PI. This suggested that the DTX-1 toxin removal was independent from the lipid removal. In fact, most of the acid and/or alkaline-treated samples were below the EU regulatory limits (*11*). This suggestion was further strengthened by the poor correlation factor obtained between the reduction in the DTX-1/protein ratio and the lipid/protein ratio (**Figure 2**). In addition, it was found that 61% of the initial DTX-1 remained in the aqueous supernatant after the second centrifugation step (**Figure 3**). From the DTX-1 analytical mass balance, it was observed that only ∼20% of the total initial DTX-1 was recovered in the sediment of the first centrifugation plus the PI after the second centrifugation. This indicates that only a small part of the DTX-1 toxin was actually removed *together* with the lipids and suggests that the acid and/or alkaline process, with or without the addition of citric acid/calcium chloride, allow the DTX-1 toxin to stay in the aqueous fraction. From this result, it can be assumed that by decreasing the MC of the PI, for example, by an extra centrifugation step, the toxin content would also be further decreased. Along with this, the fact that samples produced with citric acid/calcium chloride had higher toxin contents on a wet weight basis could possibly be due to the higher MC of these samples. There is, of course, the possibility that even a small amount of lipids, like 1.8% of the initial total lipids of the starting material (i.e., ∼34 mg) could be enough to keep the majority of the initial DTX-1 toxin (i.e., ∼70 μg) in the aqueous phase of the supernatant. A preliminary trial to test this hypothesis was conducted, where the lipids of the supernatant fraction were extracted with $CHCl_3$:MeOH (1:2, v/v) and the extract was analyzed for DTX-1 toxin. Results indicated that the majority (98%) of the DTX-1 toxin in the supernatant fraction after the second centrifugation step of the process were present in this extract. This clearly shows the propensity of DTX-1 molecules for the $CHCl_3$ and suggests that the DTX-1 toxin could be associated with the small amount of lipids and/or other hydrophobic compounds of the supernatant. Kroken et al. (*17*) suggest that surface-active phospholipids can bind to the lipophilic DSP toxins and in the presence of proteins form an emulsion. Furthermore, even though OA and DTX-1 are considered lipid-soluble substances, they still possess a carboxylic group and hydroxyl groups that under certain pH conditions can be ionized and therefore become more polar. Thus, more work is needed to confirm the hypothesis that

DTX-1 preferably stays in the aqueous phase during the acid and/or alkaline solubilization procedure.

It should be noted that current official DSP toxin methods do not include analysis of the esterified OA, DTX-1, and DTX-2 toxins. Therefore, the existence of any diol esters or DTX-3 products in the toxic mussels was not determined. However, in the case of blue mussels, it has been reported that the esterified OA did not exceed 50% of the total OA equivalents in the worst case, while DTX-1 and DTX-2 esterification percentages were much lower (*37, 38*).

Among the different treatments, the acid solubilization process exhibited the highest DTX-1 toxin reduction (**Table 4**). The values thus achieved in acid-produced PIs were well below EU regulations, even though the mussels had a very high initial load of toxicity. There is no valid explanation as to why the acid-processed mussels exhibited higher toxin removal as compared to the rest of the processes. This could be attributed to the amount and type of lipids present in the supernatant after the second centrifugation, as well as to changes in the chemical properties of DTX-1 toxin. However, it should be noted that a relatively high variability in DTX-1 reduction was observed even when the same process version was applied, especially in the acid-processed mussels (**Table 4**). This might be due to the fact that the first acid-processed sample was prepared from fresh mussel meat, while the other two samples were prepared from blended mussels stored at –80 °C for 2 and 5 months. Therefore, more experiments are needed to elucidate the mechanism of DTX-1 removal from the PIs and to determine the effect of different processing parameters on DTX-1 toxin removal.

Nevertheless, the described method in this work for detoxifying mussel meat presents a great opportunity for the mussel industry, especially since preliminary data have shown that it can also be applied on whole crushed mussels, that is, without the need to first remove the shells. The most promising version of the method appears to be the acid process combined with high *g*-force. In laboratory scale, it takes only 1−2.5 h to complete the process, depending on whether citric acid/calcium chloride is used. On an industrial scale, this time can be drastically decreased, because the process can be run continuously. Therefore, the proposed method is convenient and fast. It should be stressed although that the acid and alkaline solubilization methods lead to the production of a protein paste from mussel meat, while land- or sea-based depuration methods do not affect the appearance of the mussel. However, this novel material opens up new doors for product development and is thought to be a highly appealing alternative in periods when farmed mussels have high toxin levels. Also, acid and alkaline solubilization techniques are promising methods to be applied on small or crushed mussels that cannot be used for regular human consumption.

ABBREVIATIONS USED

DSP, diarrhetic shellfish poisoning; OA, okadaic acid; DTX-1, dinophysistoxin-1; DTX-2, dinophysistoxin-2; PSP, paralytic shellfish poisoning; Cc, citric acid and calcium chloride; PI, protein isolate; TL, total lipids; MC, moisture content.

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LITERATURE CITED

(1) Yasumoto, T.; Oshima, Y.; Yamaguchi, M. Occurrence of a new type of shellfish poisoning in the Tohoku district. *Bull. Jpn. Soc. Sci. Fish.* **1978**, *44*, 1249–1255.

(2) Yasumoto, T.; Oshima, Y.; Sugawara, W.; Fukuyo, Y.; Oguri, H.; Igarashi, T.; Fujita, N. Identification of *Dinophysis* fortii as the causative organism of diarrhetic shellfish poisoning. *Bull. Jpn. Soc. Sci. Fish.* **1980**, *46*, 1405–1411.

(3) Murata, M.; Shimatano, M.; Sugitani, H.; Oshima, Y.; Yasumoto, T. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 549–552.

(4) Masselin, P.; Lassus, P; Bardouil, M. High performance liquid chromatography analysis of diarrhetic toxins in *Dinophysis* spp. from French coast. *J. Appl. Phycol.* **1992**, *4*, 385–389.

(5) Reguera, B.; Bravo, I.; Marcaillou-Le Baut, C.; Masselin, P.; Fernandez, M. L.; Miguez, A.; Martinez, A. Monitoring of *Dinophysis* spp. and vertical distribution of okadaic acid on mussel rafts in Ria de Pontevedra (NW Spain). In *Toxic Phytoplankton Blooms in the Sea*; Smayda, T. J., Shimizu, Y., Eds.; Elsevier Science Publishers BV: New York, 1993; pp 553–558.

(6) Hu, T.; Curtis, J. M.; Walter, J. A.; Wright, J. L. C. Identification of DTX-4, a new-water soluble phosphatase inhibition from the toxic dinoflagellates *Prorocentrum lima*. *J. Chem. Soc. Chem. Commun.* **1995a**, *597*, 599.

(7) Hu, T.; Curtis, J. M.; Walter, J. A.; Wright, J. L. C. Two new water-soluble derivatives from the dinoflagellates Prorocentrum maculosum: possible storage and excretion products. *Tetrahedron Lett.* **1995b**, *36*, 9273–9276.

(8) Vieites, J. M.; Fontal, O. I.; Leira, F.; Baptista de Souza, J. M. V.; Botana, L. M. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal. Biochem.* **1997**, *248*, 258–264.

(9) Quillam, M. A.; Ross, N. W. Analysis of diarrhetic shellfish poisoning toxins and metabolites in plankton and shellfish by ion-spray liquid chromatography-mass spectrometry. In *Biological and Biotechnological Applications of ESI-MS*; Snyder, P., Eds.; American Chemical Society: Washington, DC, 1996; pp 351–364.

(10) Vale, P.; Sampayo, M. A. M. Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determined by LC-MS- a widespread phenomenon. *Toxicon* **2002**, *40*, 33–42.

(11) European Commission. Commission decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve mollusks, echinoderms, tunicates and marine gastropods; 2002.

(12) Jorgensen, K.; Jensen, S. Distribution of diarrhetic shellfish poisoning toxins in consignments of blue mussels. *Food Addit. Contam.* **2004**, *21*, 341–347.

(13) Aune, T. Risk assessment of toxins associated with DSP, PSP and ASP in seafood. In *Mycotoxins and Phycotoxins in Perspective at the Turn of Millennium*; Koe, W. J., Samson, R. A., Van Egmond, H. P., Gilbert, J., Sabino, M.; Eds.; Wageningen: Ponsen and Looyen, 2001; pp 515–526.

(14) Bundesgesetzblat Teil I Nr.27, ausgegeben zu Bonn am 23. Juni 2000. Verordnung uber die hygieneschen Anfrderungen an Fishereirzeugnisse und lebende Muscheln; 2000.

(15) Gestal-Otero, J. J. In *Epidemiological Impact of Toxic Episodes: Nonneurotoxic Toxins*; Botana, L. M., Ed.; Dekker: New York, 2000; pp 714–760.

(16) Kolberg, S. *Besktivning av Den Svenska Musselnäringen*; Vattenbrukarnas Riskförbund: 1999; p 68.

(17) Kroken, G.; Loken, J. P.; Myklebust, B. International Publication Number WO098640 A1, 2006.

(18) Shumway, S. E.; Vangmond, H. P.; Hurst, J. W.; Bean, L. L. Management of shellfish resources. In *Manual on Harmful Marine Microalgae*; Hallegraef, G. M., Anderson, D. M., Cembella, A., Eds.; UNESCO: Paris, 2003; pp 433–462.

(19) Gonzalez, J. C.; Fontal, O. I.; Vieytes, M. R.; Vieites, J. M.; Botana, L. M. Basis for a new procedure to eliminate diarrheic

shellfish toxins from a contaminated matrix. *J. Agric. Food Chem.* **2002**, *50*, 400–405.
(20) Yongsawatdigul, J.; Park, J. W. Effects of alkali and acid solubilization on gelation characteristics of rockfish muscle proteins. *J. Food Sci.* **2004**, *69*, 499–505.
(21) Mireles DeWitt, C. A.; Nabors, R. L.; Kleinholz, C. W. Pilot plant scale production of protein from catfish treated by acid solubilization/isoelectric precipitation. *J. Food Sci.* **2007**, *72*, E351−E355.
(22) Petursson, S.; Decker, E. A.; McClements, D. J. Stabilization of oil-in-water emulsions by cod protein extracts. *J. Agric. Food Chem.* **2004**, *52*, 3996–4001.
(23) Hultin, H. O.; Kelleher, S. D. Protein composition isolate from a muscle source. U.S. Patent 6,005,073, December 21, 1999.
(24) Hultin, H. O.; Kelleher, S. D. High efficiency alkaline protein extraction. U.S. Patent 6,136,959, October 24, 2000.
(25) Undeland, I.; Kelleher, S. D.; Hultin, H. O. Recovery of functional proteins from herring (*Clupea harengus*) light muscle by an acid or alkaline solubilization process. *J. Agric. Food Chem.* **2002**, *50*, 7371–7379.
(26) Kristinsson, H.; Demir, N. Functional fish protein ingredients from fish species of warm and temperate waters: Comparison of acid- and alkali-aided processing vs. conventional Surimi processing. Advances in seafood byproducts; Conference proceedings, Alaska Sea Grant College Program, 2003.
(27) Kristinsson, H.; Theodore, A. E.; Damir, N.; Ingadottir, B. A comparative study between acid- and alkali-aided processing and Surimi processing for the recovery of proteins from channel catfish muscle. *J. Food Sci.* **2005**, *70*, C298–C306.
(28) Cortes-Ruis, J. A.; Pachero-Aguilar, R.; Garcia-Sanchez, G.; Lugo-Sanches, M. E. Functional characterization of a protein concentrate from bristly sardine made under acidic conditions. *J. Aquat. Food Prod. Technol.* **2001**, *10*, 5–23.
(29) Liang, Y.; Hultin, H. O. Separation of membranes from acid-solubilized fish muscle proteins with the aid of calcium ions and organic acids. *J. Agric. Food Chem.* **2005a**, *53*, 3008–3016.
(30) Lee, C. M.; Trevino, B.; Chaiyawat, M. A simple and rapid solvent extraction method for determining total lipids in fish tissue. *J. AOAC Int.* **1996**, *79*, 487–492.
(31) Markwell, M. A.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87*, 206–210.
(32) Yasumoto, T.; Takizawa, A. Fluorometric measurement of yessotoxins in shellfish by high-pressure liquid chromatography. *Biosci., Biotechnol. Biochem.* **1997**, *61*, 1775–1777.
(33) Liepina, I.; Czaplewski, C.; Janmey, P.; Liwo, A. Molecular dynamics study of gelsolin-derived peptide binding to a lipid bilayer containing phosphatidylinositol 4,5-biphosphate. *Pept. Sci.* **2003**, *71*, 49–70.
(34) Haleva, E.; Ben-Tal, N.; Diamant, H. Increased concentration of polyvalent phospholipids in the adsorption domain of a charged protein. *Biophys. J.* **2004**, *86*, 2165–2178.
(35) Liang, Y.; Hultin, H. O. Effect of pH on sedimentation of cod muscle membranes. *J. Food Sci.* **2005b**, *70*, 164–172.
(36) Vareltzis, P.; Hultin, H. O. Effect of low pH on the susceptibility of isolated cod (*Gadus morhua*) microsomes to lipid oxidation. *J. Agric. Food Chem.* **2007**, *55*, 9859–9867.
(37) Suzuki, T.; Yasumoto, T. Liquid chromatography-electrospray ionization mass spectrometry of the diarrhetic shellfish-poisoning toxins okadaic acid, dinophysistoxin-1 and pectenotoxins-6 in bivalves. *J. Chromatogr.* **2000**, *874*, 199–206.
(38) Jorgensen, K.; Scanlon, S.; Jensen, L. B. Diarrhetic shellfish poisoning toxin esters in Danish blue mussels and surf clams. *Food Addit. Contam.* **2005**, *22*, 743–751.

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