New Opportunities for Seafood Processing Waste

Appendix 4: Investigation of On Board Strategies to Transform Patagonian Toothfish (Dissostchus eleginoides) Waste

Dr Janet Howieson

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Contents

1. Introduction

Austral Fisheries harvest Patagonian toothfish (*Dissostchus eleginoides*) in waters south of Australia. The average catch is between 5 to 7t/day green weight. On board, the fish is headed, gutted and tailed (HGT), resulting in about one third of harvested product being classified as offal, an average of 1.7 to 2.4 tonne of offal produced per day. On a big catch day, up to 13 tonnes can be processed, equating to 4.5 tonnes of offal. Each trip runs for around 50-60 fishing days, with the season running from 15 April to 14 November.

Discharge of the offal overboard is subject to CCAMLR Conservation Measure 25-03 (2011), due to the risk of seabirds such as albatrosses and petrels being attracted to the offal and striking the trawl warps, consequently getting caught in the trawl nets (Abraham and Thompson 2009; Bull 2007, 2009). Conservation measures relating to this risk to seabirds is listed below:

Conservation Measure 25-03(2011). Minimisation of the incidental mortality of seabirds and marine mammals in the course of trawl fishing in the Convection Area

The Commission,

Noting the need to reduce the incidental mortality of, or injury to, seabirds and marine mammals from fishing operations,

Adopts the following measures to reduce the incidental mortality of, or injury to, seabirds and marine mammals during trawl fishing.

1. The use of net monitor cables on vessels in the CAMLR Convention Area is prohibited.
2. Vessels operating within the Convention Area should at all times arrange the location and level of lighting so as to minimise illumination directed out from the vessel, consistent with the safe operation of the vessel.
3. The discharge of offal[2,3] and discards[4] shall be prohibited during the shooting and hauling of trawl gear.
4. Nets should be cleaned prior to shooting to remove items that might attract birds.
5. Vessels should adopt shooting and hauling procedures that minimise the time that the net is lying on the surface of the water with the meshes slack. Net maintenance should, to the extent possible, not be carried out with the net in the water.
6. Vessels should be encouraged to develop gear configurations that will minimise the chance of birds encountering the parts of the net to which they are most vulnerable. This could include increasing the weighting or decreasing the buoyancy of the net so that it sinks faster, or placing coloured streamers or other devices over particular areas of the net where the mesh sizes create a particular danger to birds.
[1] Except for waters adjacent to the Kerguelen and Crozet Islands
[2] 'Offal' is defined as bait and by-products from the processing of fish and other organisms, including parts or sections of fish or organisms which are by-products of processing.
[3] 'Stick water' is a liquid discharge produced as a by-product of processing of krill and fish. As stick water does not contain a source of food for birds, it is not considered as offal (see footnote 2).

[4] *For the purpose of this conservation measure, 'discards' are defined as whole fish or other organisms, except elasmobranchs and invertebrates where the vessel is fishing north of 60°S, returned to the sea*

A study was undertaken to investigate how particle size of fish waste discharged from trawl vessels impacted on reducing the risk of seabird by catch (Pierre et al., 2012). Three forms of fishery waste were assessed, these included: unprocessed discharge (offal, fish discards), hashed discharge (smaller chunks passed through a hasher pump) and cutter pump discharge (waste passed through a hasher and a cutter pump to further reduce particle size). The results from the study concluded that overall there was a decrease in seabirds when the hashed and cutter pump waste was discharged in comparison to the unprocessed discharge. Mincing trials performed by Abraham (2008) also concluded that full retention or mealing has shown to reduce seabird numbers to a greater extent.

Although the smaller particle sized waste appeared to attract less seabirds further studies suggested that the temporal discharge patterns may also be important. A study which investigated the impact of discharging offal at different time intervals have concluded that discharging the offal in batches, rather than continuously will reduce the number of seabirds behind the vessel (Abraham 2010). Specifically, a 2 hour batch interval results in around a 50% reduction of seabird attendance. Holding the waste for 4 hours significantly reduced the number of seabirds and again after 8 hours, numbers were further reduced (Pierre et al. 2010).

Currently in the Austral Fisheries operation, to mitigate the risk to seabirds the head, gut and tails are minced with water in tank on board before travelling over 200km out of the fishing zone. The mince is then steamed before discarding overboard. This travel to discard the offal comes at considerable costs both in fuel and lost fishing time.

Fish processing waste and offal can be hydrolysed to produce a fish hydrolysate. Following hydrolysis, the components can be separated into solids (insoluble protein), lipids (fats and oils) and aqueous solution (soluble proteins). It was considered that the aqueous solution may meet the criteria for "stickwater" thus perhaps potentiating the disposal of some of the waste legally at the fishing grounds.

CCAMLR allows stick water to be disposed of in the fishing zones. CCAMLR has stated there is "no specification for stick water" and as long as it "does not contain a source of food for birds" (pers comm. CCAMLR 2015) it can be disposed overboard. Table 1 lists the compositional analyses that could potentially quantify stick water composition. On average the suggested total solids is approximately 5% and fat and oil less than 1%.

Table 1 Summary of the varying composition of stick water

Reference	Total Solids	Protein	Ash	Fat/Oil	Water
Lassen 2012	5.6%	3.5%	0.95%	0.6%	NA
Thakur 2006	~5%	NA	NA	<1%	95%

Additionally the oil and solid component of a hydrolysed product could be further processed into by-products for the pharmaceutical, aquaculture and agricultural industries.

2. Objective

Investigate the feasibility of developing an on board hydrolysis waste treatment and discharge regime that meets current C*CAMLR* requirements for protecting seabirds and could potentially result in the development of alternative, economically viable by-product options.

The research was divided into several sections.

Section 3: Composition of toothfish waste.
Section 4: Acid and Enzyme hydrolysis of Toothfish Waste.
Section 5: Commercial Feasibility Viability and Conclusions

In addition, and aligned with the project, a Masters of Food Science student completed a research project "Fish protein extraction from Patagonian toothfish (*Dissostichus eleginoides)*, by-products using the modified endogenous enzymatic method" and this report is attached as Appendix 1.

3. Composition of Toothfish components

To gain preliminary data, Patagonian toothfish frames processed at Catalano's Seafood in December 2013 were despatched for proximate composition analysis (Figure 1). It is noteworthy that the actual on board processing waste is mainly composed of heads, guts and tails rather than frames.

Figure 1 Patagonian toothfish frames sent for proximate composition analysis

Fish frames analysis indicate a high fat and moisture content, as shown below in Table 1.

Table 1 Patagonian toothfish frame average proximate composition

		Average
Ash	g/100g	1.37
Moisture	g/100g	41.97
Protein	g/100g	9.95

Metals		
Calcium	mg/kg	17666
Phosphorus	mg/kg	8900
Zinc	mg/kg	7.83
Fatty Acid Profile		
TOTAL FAT	g/100g	46.57
Saturated Fat	g/100g	8.6
Monounsaturated Fat	g/100g	29
Polyunsaturated Fat	g/100g	8.97
Trans Fat	g/100g	<0.1

Further analysis of Patagonian toothfish flesh by McManus and Hunt (2013) in indicated that approximately 20% of the fillet is fat, with most of the fats monounsaturated (Table 2). The fish frames analysed as part of this project had 46.57% total fat, which is more representative of the on board processing waste than the fillets.

Table 2 Fatty Acid Profile of different components of Patagonian toothfish.

		McManus and Hunt 2013		**This study**
		1 Small fish fillet	**Ave of 3 large fish fillets***	**Ave 3 fish frames**
TOTAL FAT	g/100g	24.9	19.2	46.57
Saturated fat	g/100g	8.5	6.9	8.6
MUFA	g/100g	14.5	11.3	29
PUFA	g/100g	1.8	0.9	8.97
Trans fat	g/100g	0	0	<0.1
Omega 3	g/100g	1.843	0.774	NA
Omega 6	g/100g	0	0.102	NA

**Sample of fillets taken from the anterior or mid dorsal or dorsal and posterior sections combined for fat and moisture results to be representative of the whole fillet.*

The results from Wilson's (2004) research on fatty acid composition of different sections of Patagonian toothfish analyses have indicate a large variability in composition between each section. However, for all sections of the fish, monounsaturated fats represent the highest percentage of fatty acid group. The stomach and ovaries contained the highest percentage of Omega 3 EPA and DHA, while the lowest levels were in the head. The head, stomach, skin and ovaries will form most of the Patagonian toothfish processing waste to be investigated in this study. The stomach and ovaries lipid content is variable depending on maturity, reproductive status, dietary intake and the extnt of digestion of the stomach contents (Wilson 2004). This creates variability in the fatty acid composition of the fish oil.

Results

4 Acid and Enzyme Hydrolysis of Toothfish Waste

4.1 Trial 1

4.1.1 Objective

To assess if Patagonian toothfish waste can be transformed by heating only or acid (phosphoric acid) or enzymatic (Protamex and Alcalase) hydrolysis.

4.1.2 Materials and Methods

Frozen Toothfish heads were supplied by Austral fisheries. The heads were thawed overnight before experimentation.

Heating only trial

1.435 kg of Patagonian toothfish heads were placed into the Sunbeam Sous Vide/ Slow Cooker for 24 hours at 70°C. At 24 hours, 460.6g toothfish waste was centrifuged at 4000rpm for 10 minutes to extract the oil. From this, the oil/ water layer was further centrifuged for 5 minutes at 4000rpm.The remainder of the Patagonian toothfish sample in the cooker was hydrolysed with 3.5% phosphoric acid (30mL).

Acid Hydrolysis Trial

3.016 kg of whole Patagonian toothfish heads were placed in a Sunbeam Sous Vide/ Slow Cooker. 3.5% (105.56mL) by volume of phosphoric acid was added to the fish heads. The fish heads were incubated at 6 hours at 40°C and left at room temperature for approximately 17 hours before increasing the temperature to 50°C for approximately 12 hours. The pH of the waste mixture was monitored and kept between 3.5 and 4. Batches were stirred daily until liquified (end of Day 3).

The liquified fish waste was passed through a colander with 1 mm holes to remove undigested material. Hydrolysate was centrifuged for 10 minutes at 5000rpm to extract the oil.

Enzyme Hydrolysis trials

Protamex

2.009 kg of Patagonian toothfish heads was placed in the Sunbeam Sous Vide/ Slow Cooker, with the frames cut into smaller pieces. 0.2% Protamex (weight of waste) (4 g) was added to 1.6L tap water to dissolve before adding to the fish heads. It was incubated for 2 hours at 55°C, stirring occasionally through the digestion process. The water bath temperature was increased to 95°C for 1 hour to deactivate the enzyme. The hydrolysate was sieved to removed undigested material. Hydrolysate was centrifuged for 10 minutes at 5000rpm to extract the oil.

Alcalase

Two whole toothfish heads (1.504kg) were thawed overnight and placed into the Sunbeam Sous Vide/Slow Cooker with 500mL water and 2% by weight of alcalase (30g). The toothfish was hydrolysed at 60°C for 2 hours. Following hydrolysis, the undigested material was sieved out. The hydrolysate was decanted in a transparent container for 24 hours to observe the separation of the different components.

4.1.3 Results and Discussion

Heating only trial

After 24 hours of heating, 1.7% oil was extracted from the toothfish waste. Heating alone did not hydrolyse the toothfish heads (Figure 2).

Figure 2 Toothfish heads incubated at 70˚C after 24 hours

Acid Hydrolysis

Acid hydrolysis with phosphoric acid required 14 hours to hydrolyse the heads (Figure 3).The temperature fluctuated below the optimum activity temperature of 40-50°C during hydrolysis, which may have affected hydrolysis time. Formic acid could be an alternative for acid for hydrolysis.

Figure 3 Acid hydrolysis with phosphoric acid. Toothfish heads (left); after incubation for 6 hours (middle); and after incubation at 50C for 14 hours (right).

Enzyme hydrolysis

The commercial enzymes, alcalase and protamex were effective in hydrolysing the toothfish waste. The rate of hydrolysis was observably faster with the enzymes, hydrolysing the toothfish in approximately 2-3 hours. However, enzyme hydrolysis requires a higher operating temperature at 55-60°C. Protamex is ~$63/kg and required at a concentration of 0.2% waste product. The hydrolysate had a particle size of 0.6mm.

The alcalase had hydrolysed some of the skin and eyeball before the temperature had reached 60°C, 50 minutes after the machine was turned on (Figure 4). After 2.5 hours, the flesh had detached from the bone and hydrolysed. The structure of the head had broken, but the bones were still solid. After 24 hours, the bones had not hydrolysed.

Figure 4 Enzyme hydrolysis using alcalase. Toothfish heads with enzyme added before heating (left); after 50 minutes with temperature still below 60°C (middle); and after 24 hours hydrolysis (right).

The oil and aqueous components of the filtered hydrolysate from all hydrolysate treatments rapidly separated when left on the bench to decant and by centrifugation. After 24 hours settling the alcalase hydrolysate showed a clear distinction between the aqueous and oil layer (Figure 5). The middle layer was a mixture of the solid, aqueous and oil phase.

Figure 5 Decanted hydrolysate after 24 hours

Table 3 shows the % amount of the different components following centrifugation for the acid and protamex hydrolysis treatments. The oil extracted from hydrolysates using the enzyme was visually clearer. Analysis of oil quality and composition is required in future trials, with due care to preserve the sample.

Table 3 Comparison of results- acid vs enzyme hydrolysis

Method of hydrolysis	Initial weight total (heads) (g)	Final weight total (heads) (g)	Fish Oil (g)	Fish Oil Percentage (%)
Phosphoric Acid	3016	3016	235.90	7.8
Protamex	2009.82	1730.8	101.01	5.8

The trials were small scale, and larger scale experiments would potentially need to include the initial mincing of the toothfish heads which may accelerate hydrolysis. Agitation of mince during hydrolysis should be considered in larger scale trials. Maintenance of optimal temperature may be challenging.

4.1.4 Industry comments on Results

The results from Trial 1 were discussed with Austral Fisheries staff in relation to upscaling trials on board the vessels. The following issues were raised:

- Acid hydrolysis with phosphoric or formic acid would not be possible due to safety issues. It was therefore decided to focus on enzyme hydrolysis.
- The temperature required for enzyme hydrolysis and methods to control temperature on board needs to be investigated. One Austral skipper noted:

"When I was working for Sanford's they too looked at this system of offal treatment. One of the issues we found was the enzymes worked fine when the temperature was above 10 degrees but went into a hibernation state once temp went below this. There were some new vessel designs coming out with a heated offal tank to get around this temperature issue.

So if they could do the test in at least a refrigerated system to see if these guys can get around this issue would be good".

- Another consideration is the volume of enzyme required and storage capacity on the boat. For the two current test products Protamex and Alcalase, the suggested addition rate is 2g/kg. The volume of water to be added to dissolve the enzyme also needs consideration in a large scale context.
- The enzyme product is listed as hazardous to aquatic organisms. In most other commercial circumstances the enzyme is inactivated by heating to 95 C for 10 minutes but this may not be possible on board. Verification and validation that the enzymes in the hydrolysate has been inactivated is required prior to discharge into the marine environment.
- The separation of the oil/aqueous components on standing may represent an opportunity to have a decanting phase post hydrolysis, settling of the larger particle component, and then "siphon" off the aqueous component and discard overboard, thereby reducing the waste remaining to be stored. This would require approval that the hydrolysis by-product meets the definition of "stickwater" and evidence of enzyme deactivation/lack of toxicity. However it is worth noting that interpretation of the term "stickwater" is about acting as an attractant to birds etc than around size of particles and it was a considered opinion that there is no easy way to test this apart from in the real marine situation.

4.1.5 Summary

Hydrolysis of Patagonian toothfish waste yields high ratios of fats which could be extracted and used in other applications. Enzymatic hydrolysis was more efficient at hydrolyzing Patagonian toothfish heads than acid hydrolysis, although the current limitation on board is heat is required to catalyse reactions and to deactivate the enzymes.

4.1.6 Next Steps

a. Small scale trials with offal directly from the vessel and hydrolyse at different incubation temperatures with alcalase to determine effectiveness and rate of hydrolysis.

b. Research and trial other commercially available enzymes with lower optimum temperature.
c. Analyse different components of the hydrolysate (oil, protein/solid, aqueous):
    i. Volume proportions of each phase
    ii. Proximate composition and quality
        - Protein
        - Oil: fatty acid profile, peroxide value, free fatty acids
    iii. Particle size
    iv. Enzyme activity/concentration in hydrolysate
    v. pH

4.2 Trial 2

4.2.1 Background

Trial 1 (Section 3.1) explored the effectiveness of different methods of hydrolysis- acid and enzymatic, on Patagonian toothfish heads. At the end of both methods of hydrolysis, the bone and eyes were the only undigested materials. Acid hydrolysis with 3.5% Phosphoric Acid at 40-50C required 1 days to complete and does not require deactivation. However, this process is not feasible on board as holding the large quantities of phosphoric acid poses a safety issue. Enzymatic hydrolysis using proteolytic enzymes Protamex (0.1% at 55C) and Alcalase (2% at 60C) digested the Patagonian toothfish head in 2 hours. Water was added to aid the even distribution of the enzymes; however the hydrolysate produced was too watery. Water is necessary to dissolve Protamex as it comes in a powdered form, but Alcalase is in liquid form; more suitable for commercial on board processing.

After discussing the initial results with Austral Fisheries, the next steps of the project include:

- Repeat trials using actual toothfish waste produced on board (rather than heads only)
- Focus on enzymatic hydrolysis. The hydrolysate must be a neutral pH and enzymes deactivated/diluted so it would not pose a risk to aquatic organisms if discarded overboard. Liquid forms of commercial enzymes (eg alcalase and multifect) are preferred.
- Run the enzymatic hydrolysis at variable temperatures as the maximum on board temperature is 10°C.
- Trial enzymatic hydrolysis without water. If this is successful water would not have to be carried on board to aid process.
- Undertake analysis of different components produced after hydrolysis.

Two enzymes available in liquid form were tested in Trial 2. Technical summaries are shown below in Table 4.

Table 4: Information on enzymes used in Trial 2.

Enzyme	Optimum Temperature	Optimum pH	Recommended Dosage	Inactivation

Alcalase 2.4L FG	55C (Muzaifa, Safriani, and Zakaria 2012) 55-60C (Novozymes)		2%(Muzaifa, Safriani, and Zakaria 2012) 0.05%- 0.1% (Novozymes)	90C for 15 mins (Muzaifa, Safriani, and Zakaria 2012) 85C for 20 mins (Novozymes)
Multifect	60C (range 25-70C)	9.5 (range 7-10)	0.25-1% w/w	- 5-10 min @80-85C at operational pH; - pH < 4 for 30 min @50C; -pH >10 for 1hr @ 60C

4.2.2 Objective

To further investigate hydrolysis of toothfish waste, focussing on liquid enzymes and variation in temperature range. Actual processing waste from on board was used in the trials.

4.2.3 Materials and Methods

Toothfish Samples

Frozen heads and guts of Patagonian Toothfish taken directly off the trawler and delivered to Catalano's were used in the trial. Waste was defrosted in the refrigerator two days before use.

Experimental Treatments

Table 5 shows the list of treatments.

Table 5: Treatments for Trial 2

Treatment	Offal treatment	Hydrolysis treatment	Hydrolysis Temperature
1	Minced and on the bone	none	Heated to 55°C
2	Minced	Alcalase (2% w/w)	Refrigerated at 4-8°C
3	On the bone	Alcalase (2% w/w)	Heated to 55°C
4	Minced	Multifect (1% w/w)	Refrigerated at 4-8°C
5	On the bone	Multifect (1% w/w)	Heated to 55°C

For the treatments where the offal was left 'on the bone', the head and gut was cut into pieces to fit into the Sous Vide machine (Figure 6, Figure 7, Figure 8).

Figure 6 Patagonian Toothfish head

Figure 7 Patagonian Toothfish Guts

Figure 8 On the bone hydrolysis, before enzymes were added

The treatments with 'minced' offal involved removing the meat, skin and guts from the bone and using a stick blender to mince up the components (Figure 9). The bone was added back in and used in the hydrolysis process.

Figure 9 Patagonian toothfish waste blended into a mince

Methods

Nil Hydrolysis, Heating only

The weight of the prepared offal was recorded and the offal placed into the Sunbeam Duos Sous Vide and Slow Cooker. The machine was set to 55°C and monitored for 24 hours.

Heated Enzyme Hydrolysis

The weight of the prepared offal was recorded and the offal placed into the Sunbeam Duos Sous Vide and Slow Cooker. The calculated concentration of enzyme (alcalase 2% and multifect 1%) was measured and added to the offal before setting the machine to 55°C to commence the hydrolysis process. The hydrolysis process was monitored and stirred every 30 minutes until the offal was converted to a hydrolysate. It is noteworthy that no additional water was required to aid hydrolysis.

Refrigerated Enzyme Hydrolysis

The mince was weighed into plastic containers before the calculated concentration of enzyme (alcalase and multifect) was added to the mince and stirred until thoroughly mixed and placed into a refrigerator. The sample was monitored and stirred every four hours during the hydrolysis process. It is noteworthy that no additional water was required to aid hydrolysis.

Analysis

The following analyses were conducted after the experiments.

1. Calculation of Proportion of Different Components.

Following the experimental treatments, the bones were removed by passing through a sieve to remove all undigested material and weighed to calculate the hydrolysate percentage recovery. With the hydrolysate component two different methods were used to measure the volume of each "layer" of the final product.

a. Centrifuge: Liquid was homogenised and placed into 50mL centrifuge tubes and centrifuged 5 minutes at 4000rpm at 20C to determine the volume of each component. Following noting the volume the centrifuged components were placed into different containers and frozen for further analysis.

b. Settle: The remaining hydrolysate was placed into glass measuring cylinders to settle for 24 hours before recording the volume of each component.

2. Compositional Analysis

The insoluble protein (solid) and soluble protein (aqueous) components were sent to a NATA accredited laboratory for analysis of the following:

- Proximate analysis
- Total Fat, Total protein

The centrifuged oil component of the hydrolysate was sent to a NATA accredited laboratory for analysis of the following:

- Oil Quality: Peroxide Value, Free Fatty Acid
- Fatty Acid Composition: Fatty Acid Methyl Esters (FAME)

4.2.4 Results and Discussion

Hydrolysis of the toothfish waste at 55°C without the addition of commercial enzymes was not very effective at breaking down the waste product. After three and a half hours of heating there was some liquid visible and some of the skin had dissolved. After 24 hours, there was still flesh, cartilage, bone, fish eye casing and skin remaining; the experiment was ended (Figure 10). The hydrolysis conversion rate was only 43.85% (Table 6). After allowing the hydrolysate to stand for 10 minutes, the different layers were not observable. The hydrolysate was a very light cream colour.

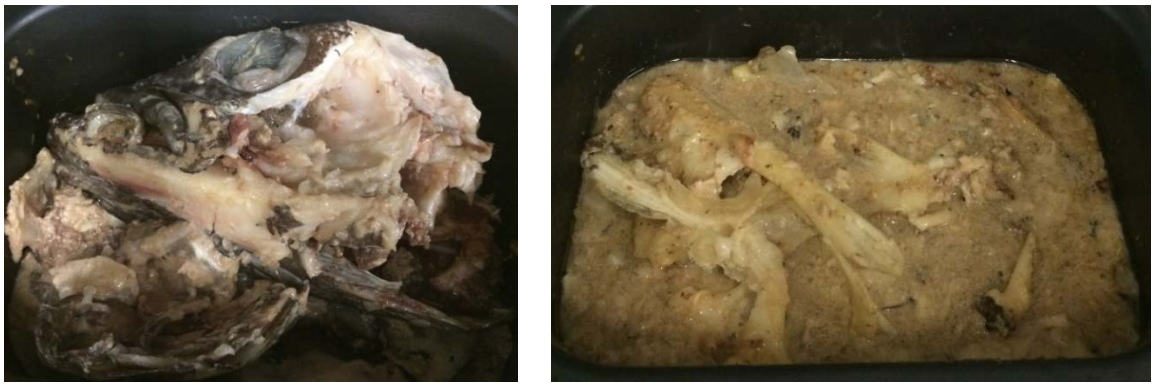


Figure 10 Hydrolysis at 55C with no enzymes. Left: before heat treatment; Right: After 24 hours at 55C

The use of the proteolytic enzymes mutlifect at 1% and alcalase at 2% in refrigerated conditions did not hydrolyse the toothfish. After one week of hydrolysis the mince was a slightly thinner consistency than initially (Figure 11). This experiment confirmed that the multifect and alcalase are relatively inactive at refrigerated temperatures.

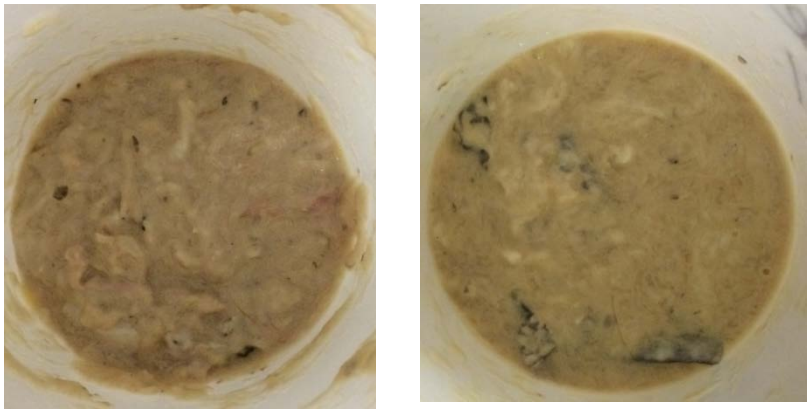


Figure 10 Patagonian toothfish after Alcalase initially added (left) and after 1 week hydrolysis with Alcalase at 4-8C (right)

The use of the enzymes in conjunction with heat was effective at hydrolysing the toothfish waste, with only the bones, cartilage and eyes remaining as solid material after less than 4 hours (Figure 12 and 13). The hydrolysis conversion rates were similar for both treatments are shown in Table 6).

Table 6 Summary of heated hydrolysis conversion rates

Treatment	Hydrolysis Time (hours)	Before hydrolysis		After Hydrolysis		% hydrolysis Conversion	
		Initial Weight(kg)	Weight enzyme	Weight undigested	Weight hydrolysate(kg)	Exc. bones	Inc. bones

			added (g)	material(kg)			
No enzyme	>24 hours	3.178	0	1.785	1.393	-	43.85%
Alcalase 2%	2:54	3.042	60.84	0.358	2.683	97.78%	86.47%
Multifect 1%	3:20	1.948	19.47	0.375	1.572	98.78%	79.93%

Results must be interpreted with consideration of the difficulty standardising the waste to be treated (with components of head, guts etc). Both the multifect and alcalase worked similarly, with the skin dissolving first, followed by the organs, flesh and gills. After one hour of hydrolysis, a large portion of the toothfish waste was liquid and the bones were more flexible. At the end hydrolysis the hydrolysate was light brown in colour a very runny consistency (Figure 13). Visually, the hydrolysate had a high oil content. After leaving the sample for 5 minutes to settle the hydrolysate started to separate..

Figure 11 Unhydrolysed components of the Patagonian Toothfish waste after enzyme hydrolysis

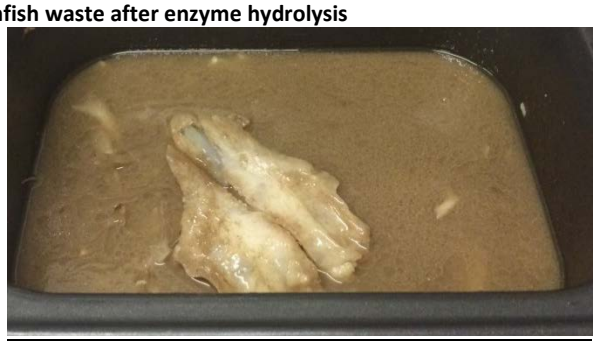


Figure 12 End of hydrolysis. L-R: Alcalase 2%; Multifect 1%

Centrifuging the samples was most effective at separating the different physical components in the different treatments with a clear distinction between the solid (insoluble protein), aqueous (soluble protein) and oil phase (Figure 14). The top and lightest layer was oil that was a light yellow/straw colour with a very mild fishy odour. The second layer was fats which are usually solid at room temperature. The third layer was stick water and solids were at the bottom.

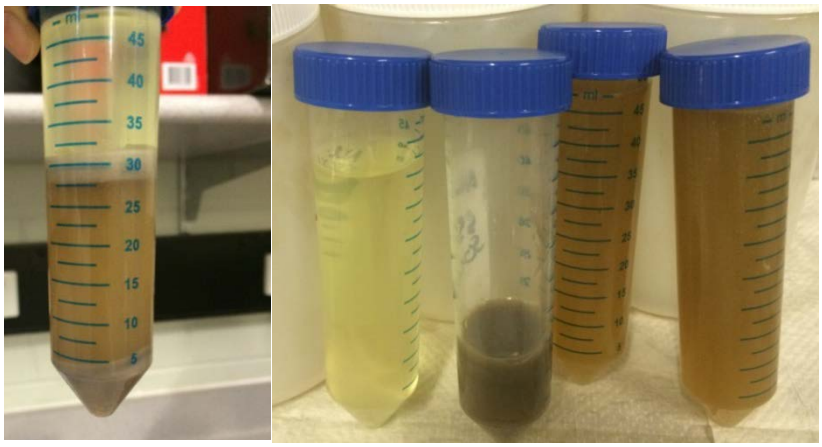


Figure 13 Left: Sample centrifuged with the 4 distinct layers. Right (L-R): Oil, solids and aqueous solution separated after centrifuging heat and enzyme treated toothfish hydrolysate.

The nil enzyme treatment had the smallest percentage of fats & oils (26.79%) and the highest percentage of solids (22.36%) (Table 7). After centrifuging the oil colour was similar to the enzyme treated hydrolysates, however the stick water and solids were a pale cream colour (Figure 15).

Figure 14 Centrifuged hydrolysate with no enzymes

Table 7 Average percentage composition of heat treated hydrolysates after centrifuging

Treatment	% Composition		
	Fats and Oils	Aqueous solution	Solid
No enzyme	26.79%	50.85%	22.36%
Alcalase 2%	38.02%	47.79%	14.18%
Multifect 1%	35.57%	51.81%	12.62%

The percentage of the different components of the hydrolysates produced using alcalase and multifect were very similar with 35.57-38.02% fats and oils and 47.79-51.81% aqueous solution. The physical characteristics of all the components were similar for the two treatments hydrolysed with enzymes and heat treated.

Allowing the hydrolysate to settle for 24 hours in the glass cylinder, there were only 3 distinct layers (Figure 16). The top layer was oil, which had the same appearance as the centrifuged oil. There were

small pieces of white sediment at the bottom of cylinder that was undigested meat and bone small enough to pass the sieve. The middle layer was the largest layer of the settled hydrolysate containing all four components, confirmed by later centrifuging this section (Table 8). The hydrolysate produced with alcalase had a lower percentage of oil than the multifect hydrolysate. This could be attributed to the freezing and defrosting of the hydrolysate before allowing the sample to settle. The Multifect treated hydrolysate was poured directly into the glass cylinders after hydrolysis and the warmer temperature would have aided the separation of the oil from the middle layer. The different layers in the hydrolysate produced with no enzyme were not visually apparent.

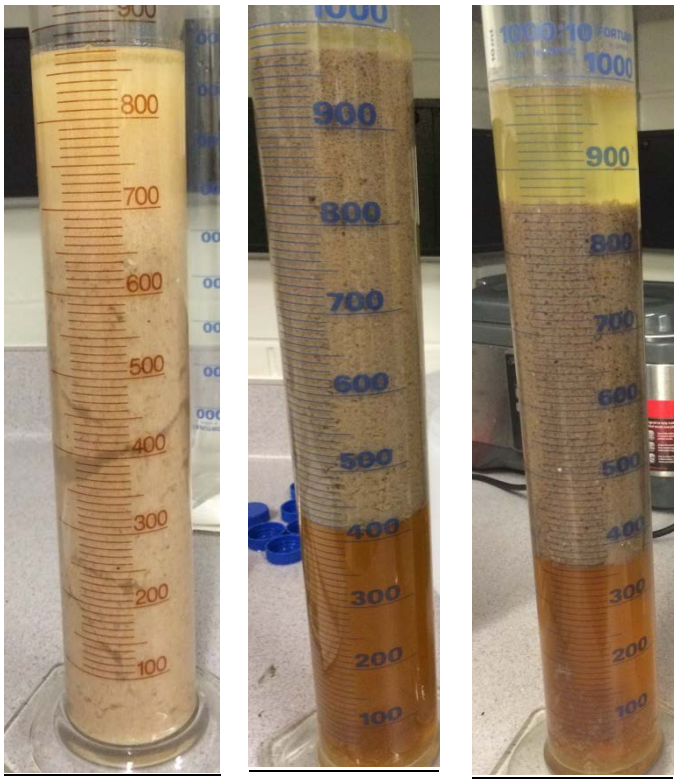


Figure 15 Hydrolysate left to settle for 24 hours (L-R): No Enzyme; Alcalase 2%; Multifect 1%

Table 8 Percentage composition of heat treated hydrolysates left to settle for 24 hours

Treatment	% Composition		
	Oil	Matrix of all components (middle layer)	Aqueous phase
No enzyme	1.15%	98.85%	-
Alcalase 2%	5.93%	58.46%	35.60%
Multifect 1%	11.15%	53.96%	34.89%

Based on the results, the on board hydrolysis of the Patagonian Toothfish heads and guts has the potential to decrease waste by 34- 50%, depending on the method used to separate the different components of the hydrolysate and the ability to discard the aqueous “stickwater” if it complies with the CCALMR regulations. The solids, fats and oils could be frozen and further treated onshore. The commercial application of centrifuging would only be feasible if there was an on board centrifuging machine able to process the volume of hydrolysate and agreement to significant investment in infrastructure. Settling the hydrolysate may separate the components with the aqueous solution at the bottom. If there is a tap at the bottom of the hydrolysate holding tank, the stick water

component could be drained off into the ocean, leaving ~65% of the hydrolysate by products. An issue with thisprocess (assuming meeting CCALMR) is the amount of movement experienced on board. If the weather is rough the conditions would hinder the settling process.

Chemical Analysis

Analysis of the aqueous solution is in Table 10. The protein levels are higher than indicated in the proposed "stickwater" definition (Table 1).

Table 2 Proximate composition of the aqueous solution hydrolysed with alcalase

	Unit	Aqueous solution
pH		6.3
Ash	g/100g	1.4
Moisture	g/100g	81.8
Fat, total	g/100g	0.3
Protein, total	g/100g	14.0
Nitrogen, Kjeldahl	g/100g	2.24
Calcium	mg/kg	170
Potassium	mg/kg	2000
Phosphorus	mg/kg	1700
Selenium	mg/kg	1.1

The quality and composition of the oil extracted from the toothfish hydrolysate with enzyme hydrolysis (Table 11) were within the quality guidelines for crude fish oil noted by Bimbo (1998), and listed in Table 12. The temperature in the southern waters where the toofish is fished may be advantageous in maintaining the quality of the fish oil.

Table 3 Summary of the oil quality and composition of the oils extracted from enzyme hydrolysate

		Oil: Alcalase 2%	Oil: Multifect 1%
Fatty Acid Profile (NATA Lab 1)	Saturated	19.1%	18.9%
	Monounsaturated	55.1%	57.0%
	Polyunsaturated	25.8%	24.0%
	Trans	0.6%	<0.1%
Fatty Acid Profile (NATA Lab 2)	Saturated	17.7%	17.5%
	Monounsaturated	58.2%	59.9%
	Polyunsaturated	10.8%	9.6%
	Trans	1.3	1.2%
Oil Quality (Lab 1)	FFA (as oleic acid)	2.65%	2.17%
	Acid value (mgKOH/g)	5.27	4.32
	Peroxide Value (meq/kg)	2.8	4.4

Table 4 Quality guidelines for crude fish oil. Source: (Bimbo 1998)

Moisture and impurities %	0.5-1% max
FFA, % oleic acid	Range 1-7%, usually 2-5%
Peroxide meq/kg	3-20
Anisidine Value	4-60

Iron	0.5-0.7 ppm
Copper	<0.3 ppm
Phosphorus	5-100 ppm

Both oils contained over 50% monounsaturated fats and less than 20% saturated fats. Two sets of each oil sample were sent for Fatty Acid Profile analysis. The first NATA laboratory results were conducted locally, whereas NATA laboratory 2 had to be sent to the eastern states frozen but may have been exposed to temperature variation during transport. This could explain the lower polyunsaturated fats % and higher monounsaturated fats % in the analysis at laboratory 2 than laboratory 1. As the peroxide value and FFA were not conducted at laboratory 2, it cannot be confirmed.

The FAME analysis indicated that the level of omega 3 and 6 fatty acids is slightly higher for the oil extracted from alcalase hydrolysate (Table 13). The detailed fatty acid composition is in Appendix 2.

Table 5 Summary of Omega 3 and Omega 6 levels found in the extracted Patagonian toothfish

Fatty Acid (Lab 2)	**% Composition (of total fats)**	
	Oil: Alcalase 2%	**Oil: Multifect 1%**
C18:3w3 alpha-Linolenic (ALA)	0.5	0.4
C20:5w3 Eicosapentaenoic (EPA)	3.4	2.8
C22:6w3 Docosahexaenoic (DHA)	4.3	3.7
Omega 3 Fatty Acids	8.9	7.6
Omega 6 Fatty Acids	2.4	2.3

The levels of omega 3 EPA and DHA of the extracted oils using both enzymes were slightly higher than the amount found in the head, mid section, tail section, skin and a whole Patagonian toothfish in a previous study (Wilson 2004) (Table 14). The stomach and ovaries of the toothfish have the highest level of EPA and DHA and, as this would be included in the on board processing offal; this may increase the omega 3 levels.

Table 6 Comparison of EPA and DHA levels in the extracted oil vs different sections of the Patagonian toothfish

		% EPA	**%DHA**
Trial 2	Alcalase 2%- extracted oil	3.4	4.3
	Multifect 1%- extracted oil	2.8	3.7
Wilson (2004)	Head	1.6	2.2
	Mid Section	2.2	3.9
	Tail Section	2.0	3.4
	Skin	2.0	2.3
	Stomach	5.1	8.8
	Ovaries	9.1	10.0
	Whole Fish	2.2	3.5

In comparison to some other fish oils and hydrolysates, the omega 3 EPA and DHA levels in the oil extracted from Patagonian toothfish is observably lower (Table 15).

Table 7 Omega 3 EPA and DHA levels in other commercially available marine oils

Source	Species	% EPA	%DHA
Trial 2- Patagonian Toothfish offal	Alcalase 2%- extracted oil	3.4	4.3
	Multifect 1%- extracted oil	2.8	3.7
SAMPI (tuna hydrolysate)	SAMPI fish hydrolysate	11.7	19.7
Bimbo (1998) for some marine oils commercially available	Anchovy	22	9
	Jack mackerel	13	15
	Menhaden	14	8
	Sardine/Pilchard	16	9
	Capelin	8	6
	Herring	6	6
	Mackerel	7	8
	Norway Pout	9	14
	Sand eel	11	11
	Sprat	6	9
	Tuna	6	22

Vegetable based alternatives are being used in aquaculture feed due to a shortage of fish oils being available. With just under 40% fat composition in the hydrolysate, this oil may be a potential good source of oil for use in aquaculture feed. The oil may also help as an attractant and could also potentially be used as a biofuel.

4.2.5 Industry comments on Results

On the 3rd November 2015, a meeting was set up with Austral Fisheries to discuss the results from the trial and next steps. The key outcomes from the meeting include:

- Feasibility: is it possible to install a heated hydrolysis unit on board the vessel. The ideal unit for hydrolysing should be capable to heat to 60˚C and have a stirrer/agitator
- Enzyme: would prefer enzyme in powdered form and that can operate at lower temperature than 55˚C. Although the enzymes in the hydrolysate have not been deactivated, the low concentration of enzymes (1-2%) in the subantarctic ocean were thought to be too dilute to be active once mixed with the seawater.
- Separating the different components of the hydrolysate cannot be done by 'settling' due to rough sea conditions.
- CCAMRL: Issue of disposal, would the "aqueous" component meet the definition of "stickwater", especially considering high protein levels. Interaction with whales would also need to be investigated.

- Hydrolysate by products potential outcomes:
    - Oil: extracted and used to run the ship, reducing the fuel required. Currently the fuel used on board is centrifuged. Use as fuel depends on the combustion properties of the oil.
    - Aqueous solution: could dispose overboard 1 tonne at a time. Quality will determine end use- its disposal overboard accounts for 50% of the waste

    - Solids: could be reduced and sold. A fish mealer could be installed on board. Depends on quality of product
    - Bones will remain but can be stored/discarded. Permissions etc.

- Extraction and use of enzymes from toothfish guts may be an option to deliver low temperature hydrolysis.

Further comment from Austral engineers in regard to the use of fish oil in the engines is below:

- *The only thing that worries me is that the Main Engineers and Auxiliary Eng are using the same fuel and as they are not high horse power the tolerances are smaller larger engines have a better ability to handle mixtures.*
- *If we had larger engines same as the Cove it may not be an issue. So can we burn Fish oil we would have to check to see what the residual after burn it leaves behind to ensure it didn't create any problem down the track.*

What would be involved in getting a tank that could heat the mix to 55 degrees (maybe half a day's worth of offal) for a few hours?

- *On the Champion we had 4 fish oil tanks just for'd of the Steering gear compartment. About 60t which we converted 30t into Shit tanks.*
- *I wouldn't want to use any of the current offal tanks on any of the vessels as they all have prior jobs apart from Offal – Fuel, ballast, trim.*
- *If we worked on a 10t day GW average we would have about 3.4t of offal. So I think a 2t Stainless tank would have to be fabricated in which we could use hot water coils or electrical elements to control the heat. This tank could be situated in the Factories as it would say 2mtrs by 1 mtrs. If we are looking at a time line of 3 hours for the breakdown of the offal etc we could then drop the stick water, mash the bones and discharge the fish oil it would reduce the amount of offal stored. C/B would the vessel to try this on if any.*

What sort of centrifuges could we deploy to extract all the oil (around 35%) from the final mixture?

- *We first to decide at what p/gravity we wish to achieve, and then ask Alfa Laval what's available & suitable – but there are plenty of options. Then you have to find a place to store the oil if the mixture is not ok for our engines due to horse power.*

4.2.6 Summary

The study has shown that commercial proteolytic enzymes with increased temperature accelerates the hydrolysis of patagonian toothfish on board processing waste. Both alcalase and multifect can hydrolyse all the waste except for the bone. The "stickwater" component makes up ~50% of the hydrolysate that potentially can be disposed of safely overboard and with ~35-38% oil and fat content, this component can be frozen and further treated on shore into a valuable by product for other industries. However issues include the feasibility of on board hydrolysing, especially when heating is required, the "grey" definition of "stickwater", difficulties in using biofuels, and the EHA/DHA levels in the toothfish oil which would restrict viable commercial uses onshore.

The next steps of the project could include:

- Find out more information about the enzymes including: cost, format, storage conditions and sale size
- Determine the particle size of the hydrolysate
- Run further hydrolysis trials using enzymes to determine minimum effective operating temperature to hydrolyse protein in a reasonable time frame.
- Once compositional data is provided request advice from experts if oil is a suitable source of biodiesel.

4.3 Trial 3

4.3.1 Background

In the previous trials, enzyme hydrolysis at 55°C was effective in hydrolysing Patagonian toothfish waste, yielding 80% hydrolysate (including bones) in less than 3 hours. Both alcalase and multifect are food grade liquids that require refrigerated storage. After meeting with Austral Fisheries, the company was considering the use of enzymatic hydrolysis and could see possibilities of installing a heating tank on board. However, it would be preferable if the enzyme was in powdered form, had a lower optimal activity temperature and did not require specific storage conditions.

As a result a new enzyme, Effectenz, available as a powder was trialled (see Table 16).

Table 8 Summary of some commercially available proteolytic enzymes

Enzyme	Format	Storage conditions	Sold in:	cost
Alcalase 2.4DX	Liquid	Refrigerated	30kg jerry can	$26/kg
Multifect PR 6L	Liquid	Refrigerated	18kg drum	$48.16/kg + GST
Effectenz P100	Liquid	Refrigerated	25kg drum	$29.03/kg + GST
Effectenz P2000	Powder	Ambient	20kg box	$32.63/kg + GST

Effectenz P2000 is a high alkaline protease that has an optimal activity at a broad range of temperatures and pH. It can operate at pH 8, with the optimal pH 10-11. The optimal activity temperature is 60°C; however it is reported to be active at lower temperatures. The enzyme comes in a powdered form and does not require special conditions for storage.

4.3.2 Objective

Determine the parameters for hydrolysing Patagonian toothfish on board processing waste using enzyme Effectenz P2000.

4.3.3 Materials and Methods

Frozen Patagonian toothfish on board processing waste was supplied by Austral Fisheries. The waste including head and guts, was defrosted overnight before use.

Three separate treatments were conducted. Two samples of Patagonian toothfish waste, with the heads and guts kept whole, were placed into the Sunbeam Duos Sous Vide Machine 1% w/w Effectenz 2000 added. They were incubated at 40°C and 50°C for 3 hours, or until protein was liquidised. In the third treatment Patagonian toothfish waste was cut into small pieces and placed

into a bench mixer. Effectenz 2000 was added at 1% by weight into the mixer. Mixing speed was set at low (minimum speed setting) for 5 hours at ambient room temperature (~25°C).

Following the experimental treatments, the bones were removed by passing through a sieve to remove all undigested material and weighed to calculate the hydrolysate percentage recovery. The remaining liquid was homogenised and placed into 50mL centrifuge tubes and centrifuged 5 minutes at 4000rpm at 20C to determine the volume of each component. Following noting the volume the centrifuged components were placed into different containers and frozen for further analysis.

4.3.4 Results and Discussion

The treatment at ambient temperature (24-25°C) did not hydrolyse the toothfish after 5 hours (Figure 16). The continuous agitation physically broke the flesh to a very thick paste.

Figure 16 Toothfish hydrolysed at ambient conditions and 1% enzyme after 5 hour incubation time

The heated treatments at 40°C and 50°C with 1% effectenz hydrolysed the toothfish; incubating at 50°C was more efficient. Incubation at 40°C for approximately 3 hours 30 minutes was required to liquidise the toothfish waste. After sieving the hydrolysate, there were small pieces of flesh and fish skin observed in the final product as shown in Figure 18, indicating the incubation time was not sufficient.

Figure 17 Sieved hydrolysate incubated at to 40°C

The toothfish waste hydrolysed at 50°C was completely liquidised (except the bone) in just over 2 hours of incubation. Sieving the hydrolysate removed the bones and cartilage. The remaining liquid hydrolysate was a thin consistency, light brown/tan colour and no observable pieces of unhydrolysed skin and flesh (Figure 19).

Figure 18 Sieved hydrolysate incubated at 50°C

The highest yield of fish hydrolysate was 74.01% when incubated at 50°C and lowest at ambient temperate at 37.49% (Table 17).

Table 9 Results from hydrolysing toothfish waste with 1% Effectenz at different incubation temperatures

Incubation Temperature	Hydrolysis Time (hours)	% recovery	Comments
Ambient (24-25°C)	>5	37.49%	Lightest colour and thickest consistency
40°C	3:22	66.91%	Skin had been broken down into smaller pieces but not completely hydrolysed, darker brown colour
50°C	2:13	74.01%	Hydrolysed liquid

Centrifuging the hydrolysates had varying results with each treatment (Table 18). The solid and aqueous phase of the ambient temperature hydrolysate after centrifuging was mixed, and was thick, light cream brown colour (Figure 20). Only the oil layer had separated at ambient temperature. The distinction between the solid and aqueous phase of the hydrolysate incubated at 40°C was faint, but visible enough to distinguish. Some solid matter was suspended within the aqueous phase, giving it an opaque brown appearance (Figure 21). The distinction between the solid, aqueous and oil layers was clearest in the hydrolysate incubated at 50°C (Figure 22). The ability to efficiently centrifuge the hydrolysate into each of the different components is important if each component is to be used for other applications in the future.

Figure 19 Before (left) and after (right) centrifuging hydrolysate incubated at ambient temperature

Figure 20 Before (left) and after (right) centrifuging hydrolysate incubated at 40°C

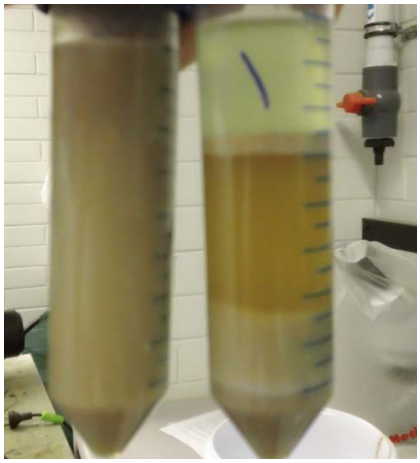


Figure 21 Before (left) and after (right) centrifuging hydrolysate incubated at 50°C

Table 18 Physical composition of hydrolysate treated with 1% effectenz at different incubation temperatures

Incubation temperature	% Composition		
	Oils	Aqueous solution	Solid

Ambient (24-25°C)	32.75%	68%	0%
40°C	36.84%	47.37%	15.79%
50°C	32.75%	39.96%	27.29%

At the bottom of the centrifuge tubes for each treatment, there was undissolved enzymes present (Figure 23). This may indicate the concentration of enzymes used is too high and should be decreased to minimise wastage and costs. The ratio of enzymes to substrate should be calculated using the total protein content of the toothfish waste for future trials.

Figure 22 The bottom of the centrifuge tube where the white part is the enzymes clumped together

4.3.4 Summary

Effectenz 2000 was effective at hydrolysing Patagonian toothfish waste when incubated at heated temperatures; however 50°C is the optimum. The toothfish waste was sufficiently hydrolysed to enable physical separation of each component by weight potentially by using centrifugation. The concentration of enzyme used was too high and investigation into the optimum concentration is required before commercial trials. The enzymes were not deactivated in the trial and further investigation will be required to determine if this stage is required.

4.3.5 Next Steps

- Preliminary Economic and feasibility assessment of the various treatment options for discussion with Austral.
- Optimise enzyme concentration
- Extraction of toothfish gut enzymes to potentially trial at lower temperatures.

5 Conclusions

Following the final trials it was decided to cease the project as there were a number of barriers to commercial feasibility. These included on board processing issues such as heating and ability to

settle into different layers, the “grey” definition of “stickwater”, difficulties in using biofuels, and the EHA/DHA levels in the toothfish oil which would restrict viable commercial uses onshore.

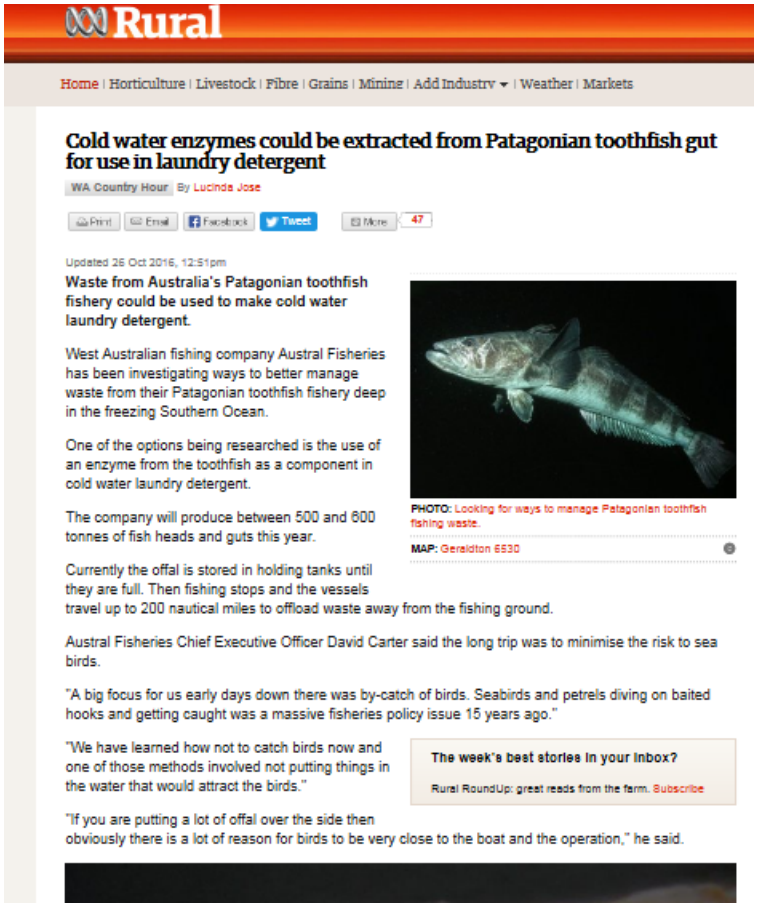
However two further outcomes of the project are discussed below:

1. Masters of Food Science student Ahmad Jauhari completed a study on the toothfish offal: Fish protein extraction from Patagonian toothfish (*Dissostichus eleginoides)* by-products using the modified endogenous enzymatic method. The abstract is below and the full paper is shown in Appendix 2.

   The study and experiment were conducted to evaluate the possibilities of a modified protein extraction method using the endogenous enzyme in Patagonian toothfish by-products. Two modified treatments were constructed and assessed in terms of fish protein extraction abilitiy. One control treatment was used to compare between the two treatments. Treatment 1 used the endogenous enzyme while treatment 3 used the same enzyme but with added citric acid. Treatment 2, as the control of this study used a specific extraction enzyme (alcalase enzyme).

   There were six similar steps (crushing and homogenisation, mixing, hydrolysis, stirring, centrifugation, and freeze-dried) involved in the three treatments. Layer 3 from treatment 2 and 3 obtained high protein contents of 53.50% and 49.14% respectively. The two values did not have a statistically significant difference ($P<0.05$), while treatment 1 had the lowest protein content (33.63%).

   Protein content from treatment 2 and 3 was transformed into a dry shape using a freeze-dryer and assessed regarding the six food functional properties (water-holding-capacity/WHC, oil-holding-capacity/OHC, emulsifying ability/EA and emulsifying stability/ES, and foaming ability/FA and foaming stability/FS). Eggs were used as a control for those assessments. Samples from treatments 2 and 3 had zero value in WHC, EA, and ES while the control sample had positive values (6%, 58% and 23% respectively). Treatment 3 had a higher value at OHC, FC and FS compared to treatment 2, and also higher values at FC and FS than the control. Because of those excellent values, fish protein extraction from treatment 3 can be used as fat and flavour binding, and foaming ingredients in a food system.
2. Media interest in the project followed an article in the FRDC FISH magazine (Figure 24), and as a result Curtin University was approached by the detergent company Proctor and Gamble about testing the toothfish enzymes for cold water activity with a view to possible inclusion in cold water detergents. This enzyme extraction work is continuing with post graduate students.



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Cold water enzymes could be extracted from Patagonian toothfish gut for use in laundry detergent

WA Country Hour By Lucinda Jose

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Waste from Australia's Patagonian toothfish fishery could be used to make cold water laundry detergent.

West Australian fishing company Austral Fisheries has been investigating ways to better manage waste from their Patagonian toothfish fishery deep in the freezing Southern Ocean.

One of the options being researched is the use of an enzyme from the toothfish as a component in cold water laundry detergent.

The company will produce between 500 and 600 tonnes of fish heads and guts this year.

PHOTO: Looking for ways to manage Patagonian toothfish fishing waste.

MAP: Geraldton 6530

Currently the offal is stored in holding tanks until they are full. Then fishing stops and the vessels travel up to 200 nautical miles to offload waste away from the fishing ground.

Austral Fisheries Chief Executive Officer David Carter said the long trip was to minimise the risk to sea birds.

"A big focus for us early days down there was by-catch of birds. Seabirds and petrels diving on baited hooks and getting caught was a massive fisheries policy issue 15 years ago."

"We have learned how not to catch birds now and one of those methods involved not putting things in the water that would attract the birds."

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"If you are putting a lot of offal over the side then obviously there is a lot of reason for birds to be very close to the boat and the operation," he said.

Figure 24 Media interest in toothfish enzymes.

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Appendix 1

Fatty Acid Composition of oil extracted from Patagonian Toothfish hydrolysate using enzyme hydrolysis in Trial 2 using FAME analysis.

		% Composition (of total fats)	
	Fatty Acid	**Multifect 1%**	**Alcalase 2%**
Saturated Fatty Acids	C4:0 Butyric	<0.1	<0.1
	C6:0 Caproic	<0.1	<0.1
	C8:0 Caprylic	<0.1	<0.1
	C10:0 Capric	<0.1	<0.1
	C12:0 Lauric	<0.1	<0.1
	C14:0 Myristic	3.8	3.8
	C15:0 Pentadecanoic	0.3	0.3
	C16:0 Palmitic	10.8	11.5
	C17:0 Margaric	0.1	0.1
	C18:0 Stearic	2.9	2.5
	C20:0 Arachidic	0.2	0.2
	C22:0 Behenic	0.1	0.1
	C24:0 Lignoceric	<0.1	<0.1
	Total Saturated	18.3	18.5
Mono-unsaturated Fatty Acids	C14:1 Myristoleic	0.2	0.1
	C16:1 Palmitoleic	9.5	9.6
	C17:1 Heptadecenoic	<0.1	<0.1
	C18:1 Oleic	36.5	33.8
	C20:1 Eicosenic	11.6	12.1
	C22:1 Docosenoic	3.2	3.4
	C24:1 Nervonic	1.8	1.9
	Total Mono-unsaturated	62.7	60.9
Poly-unsaturated Fatty Acids	C18:2w6 Linoleic	1.3	1.3
	C18:3w6 gamma-Linolenic	<0.1	<0.1
	C18:3w3 alpha-Linolenic (ALA)	0.4	0.5
	C20:2w6 Eicosadienoic	0.3	0.3
	C20:3w6 Eicosatrienoic	0.1	0.1
	C20:3w3 Eicosatrienoic	0.2	0.2
	C20:4w6 Arachidonic (AA)	0.4	0.5
	C20:5w3 Eicosapentaenoic (EPA)	2.8	3.4
	C22:2w6 Docosadienoic	<0.1	<0.1
	Omega 3 Fatty Acids	7.6	8.9
	Omega 6 Fatty Acids	2.3	2.4
	C22:4w6 Docosatetraenoic	<0.1	<0.1
	C22:5w3 Docosapentaenoic (DPA)	0.4	0.5
	C22:6w3 Docosahexaenoic (DHA)	3.7	4.3
Total	Total Poly-unsaturated	10	11.3
	Total Mono Trans Fatty Acids	<0.1	<0.1
	Total Poly Trans Fatty Acids	1.3	1.4
	P:M:S Ratio	0.5:3.4:1	0.6:3.3:1

Appendix 2

CURTIN UNIVERSITY OF TECHNOLOGY

FACULTY OF HEALTH SCIENCES, SCHOOL OF PUBLIC HEALTH

Fish protein extraction from Patagonian toothfish (*Dissostichus eleginoides)*, by-products using the modified endogenous enzymatic method

This Research Project/Research Dissertation Is Presented for The Degree Of

Masters of Science

Curtin University

Student:

AHMAD JAUHARI

Supervisor:

DR. RANIL COOREY

Fish protein extraction from Patagonian toothfish (*Dissostichus eleginoides)* by-products using the modified endogenous enzymatic method

Abstract

The study and experiment were conducted to evaluate the possibilities of a modified protein extraction method using the endogenous enzyme in Patagonian toothfish by-products. Two modified treatments were constructed and assessed in terms of fish protein extraction abilitiy. One control treatment was used to compare between the two treatments. Treatment 1 used the endogenous enzyme while treatment 3 used the same enzyme but with added citric acid. Treatment 2, as the control of this study used a specific extraction enzyme (alcalase enzyme).

There were six similar steps (crushing and homogenisation, mixing, hydrolysis, stirring, centrifugation, and freeze-dried) involved in the three treatments. Layer 3 from treatment 2 and 3 obtained high protein contents of 53.50% and 49.14% respectively. The two values did not have a statistically significant difference ($P<0.05$), while treatment 1 had the lowest protein content (33.63%).

Protein content from treatment 2 and 3 was transformed into a dry shape using a freeze-dryer and assessed regarding the six food functional properties (water-holding-capacity/WHC, oil-holding-capacity/OHC, emulsifying ability/EA and emulsifying stability/ES, and foaming ability/FA and foaming stability/FS). Eggs were used as a control for those assessments. Samples from treatments 2 and 3 had zero value in WHC, EA, and ES while the control sample had positive values (6%, 58% and 23% respectively). Treatment 3

had a higher value at OHC, FC and FS compared to treatment 2, and also higher values at FC and FS than the control. Because of those excellent values, fish protein extraction from treatment 3 can be used as fat and flavour binding, and foaming ingredients in a food system.

Keywords: Patagonian toothfish by-products, fish protein extraction, treatments, The endogenous enzyme, alcalase enzyme, citric acid, functional properties.

1.0 Introduction

Protein is an essential part of humans, animals and plants. In the human body, protein is crucial in terms of structure and function. Not all proteins can be synthesised by the human body, which need to be acquired through the diet. The animal proteins are from meat, poultry, egg, and fish, while the plant proteins come mainly from legumes, grains, and nuts. Animal tissue is a complete source of protein that contains all of the essential amino acids. The amino acids in sufficient quantities and proportions are required by the human body for its life processors. Fish and fish products are a vital resource of protein; an individual fish can contain 15-24% protein embedded in the meat, muscles and other parts of the body.

Based on the Food and Agriculture Organization (FAO) of the United Nations's report in 2014, there were 158 million tonnes of fish produced in 2012, which consisted of 91.3 million tonnes from capture fisheries and 66.6 million from aquaculture (FAO 2014). Although Australia was not one of the big five fish exporter countries, they exported a high-value of seafood equivalent to 21.019 million tonnes of fish and fish products at a value of $524 million. Edible fisheries products such as tuna and salmon were shipped to Hongkong, Vietnam, Japan, China and Singapore. Western Australia is the largest contributor to those exporting figures (ABARES 2014).

Exporter countries can sell their fish with or without processing. They have to change fish into several seafood products If they do not want to sell or export fish as whole shape directly to a market. A processing plant does not use all fish parts to produce a product, and there will be a significant portion of the fish considered inedible and therefore

discarded. The industry can process only the white meat of fish (tuna) while the red meat is considered to be waste and usually ends up as pet food or in landfills (Raghunath 1993; Herpandi and others 2012; Herpandi and others 2011). For instance, to produce a Patagonian toothfish fillet, around half of the raw material is discarded. The discarded materials (head, bones, tail and viscera) may have high protein content due to the attached fish muscle. This waste usually ends up as fish meals in aquaculture or fish feed for animals (Batista 1999). Based on the same data above, humans consumed 136.2 million tonnes in 2012 or around 84.1% and about 21.7 million tonnes or 15.9%, was for non-food uses (FAO 2014). Fish processing and aquaculture industries also sometimes throw fish waste into the ocean. This situation can generate significant pollution (Ramakrishnan and others 2013).

Many poorer members of the global community cannot get access to good quality foods that contain sufficient essential amino acid. This is due to the economic problem (high price of food), demography (far from the food source), natural disaster and war. The FAO claimed significant future challenges in feeding people around the world while maintaining natural resources (FAO 2014).

It is clear that the world faces two opposite problems: the global abundance of fish waste from industries, and the chronic malnourishment affecting hundreds of millions of people. Currently, solutions to overcome the problems were dumped inland or ocean, composted or used as fertiliser, fish meal and fat, and silage (Zdzislaw and others 1994). These solutions just answered the problems partially so still require more efforts to answer it thoroughly. Furthermore, throwing away fish waste not only affects the environment negatively but is also costly regarding shipping and disposal. Some research has been done to utilise the fish waste, make it more economically, and reduce the adverse environmental impacts. A good example is Australia, which is one of the biggest tuna waste fertiliser producers through South Australian Marine Products Pty Ltd (SAMPI 2015).

Based on the above outlined world challenges (malnutrition and environmental impacts) and the fact that fish is a rich source of protein, researchers are investigating how to extract protein from fish by-products and put it into human food. Several extraction

methods were produced to extract proteins from fish waste such as repeated water washing and refining, isolating fish proteins using the pH shift method, solvent extraction, heating method, enzyme/acid hydrolysis, a combination of various methods, and alkaline extraction (Batista 1999; Reza 2013). All the methods above have some advantages. However, almost all the methods still use chemicals in the extraction process, and there is a concern this will have unfavourable effects on the consumer (Mazorra and others 2012). The protein extraction using enzyme seems to be more appropriate than other methods as no chemicals are used. Using external enzymes is also beneficial because it can extend the processing to more than just one type of fish or fish by-product. However, an extraction using external enzymes is uneconomical due to the higher price of an enzyme used, for instance alcalase enzyme (Hordur and Rasco 2000). The cost-effective method would be to utilise endogenous enzymes from the fish itself to assist in the extraction of the protein.

Fish are able to break down its muscle into several components such as amino acids, peptides and polypeptides by using digestive enzymes, in a process called autolysis. Fish also have the endogenous enzyme, which is a group of native enzymes in their body that supports proteolytic activity (Hordur and Rasco 2000).

Employing autolysis and the endogenous enzyme to extract protein from fish waste is a great challenge. The endogenous enzyme comprises of mixing enzymes with different physical, chemical and biological characteristics, which may be difficult to organise in the extraction process. Thus, the end product characteristics will vary in molecular profiles. The industry requires a protein extraction method that can be controlled and produce a uniform end product constantly. Furthermore, external enzymes and other chemical treatments need less time than the endogenous enzyme that has slowly broken down protein inside the fish muscle and flesh (Hordur and Rasco 2000). However, when an extraction protein involves the same raw material, such as the discarded material from Patagonian tootfish, and uses the same pH and an appropriate temperature, the process of extraction and the final product can achieve the desired results, which no previous published research has studied.

Due to the reasons outlined above, the aim and objective of this study is to develop a protein extraction method from Patagonian toothfish (*Dissostichus eleginoides)* by-products using the endogenous enzyme, which generates high protein content and has important food functional properties. It is hypothesised that there will be no significant difference in protein content of the treatment with the endogenous enzyme and the treatment using a commercial enzyme.

2.0 Methodology (Materials and methods)

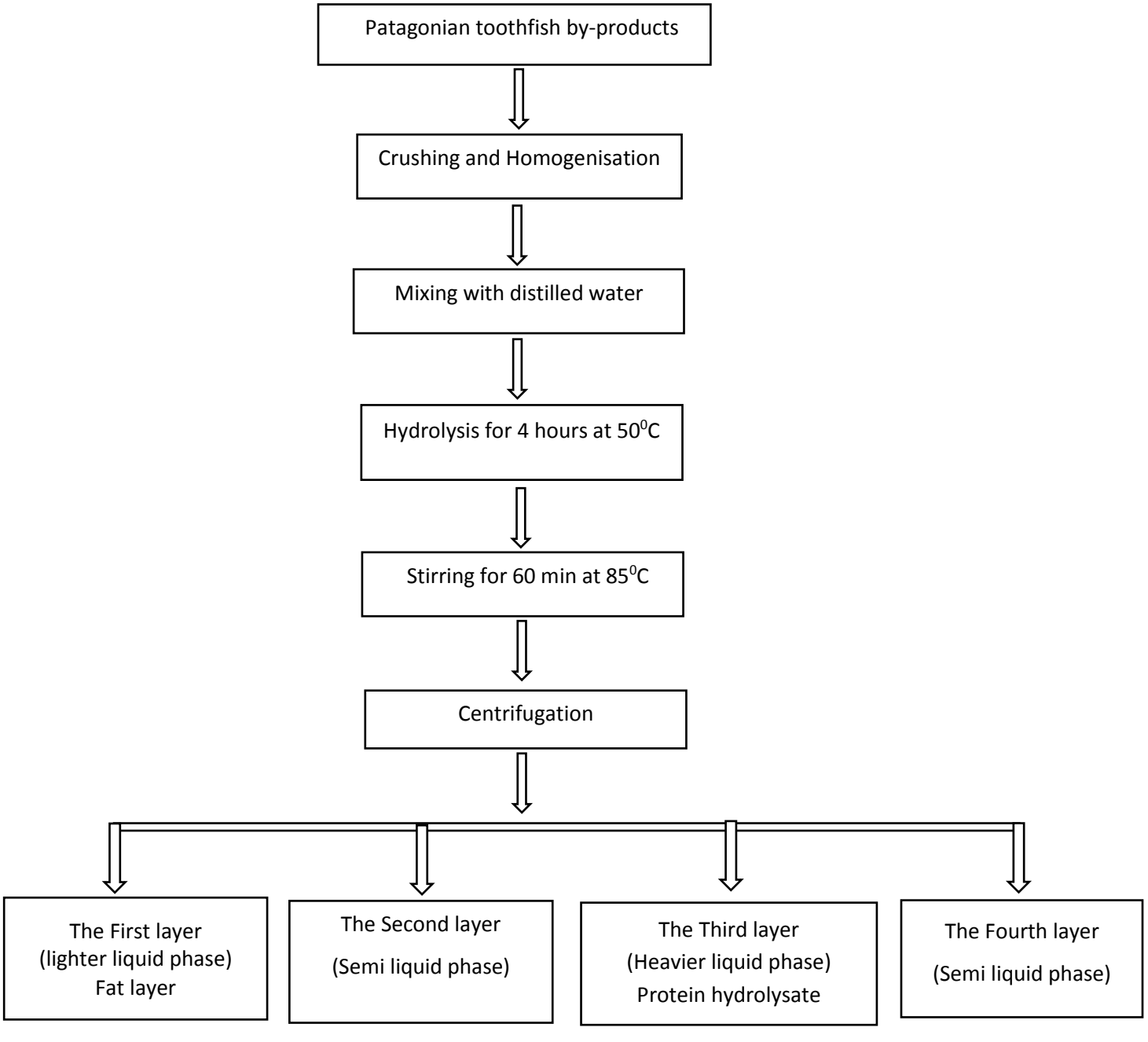
2.1 Materials

Raw materials for this study were fish processing by-products from Patagonian toothfish (*Dissostichus eleginoides*) such as the head, frame and tail, which were supplied by the industry partner (Perth, Western Australia). The materials were transported to Curtin University at 4^0C in ice boxes. All other chemicals, e.g., Alcalase 2.4L® and analytical grade reagents were obtained from Sigma-Aldrich (New South Wales, Australia). Eggs were obtained from a local supermarket (Coles supermarket, Perth, Western Australia). All analysis was performed in triplicate.

2.2 Methods

The experiments were divided into three treatments; the first treatment was the extraction of the protein using the endogenous enzyme, the second treatment was with the alcalase enzyme, and the third treatment used the endogenous enzyme with added citric acid. All other steps of the protein extraction process were similar between the three treatments. The patagonian toothfish by-products were firstly crushed using a manual meat mincer (Dorkert 22, Czech Republic). The sample was then homogenised and mixed with distilled water (1:1 w/v) and blended in a Waring Commercial Blender, model: Blender B011 32BLB0 (New Hartford Conn, USA). Afterwards, the content was incubated for 4 hours at 50^0C in a

water bath (Grant, OLS 200, shepreth, England). The optimum temperature range that can activate proteinase to get rapid hydrolysis of myosin and other muscle proteins is 50–65^{0}C (Fereidoon and Botta 1994). The resulting mixture was then stirred for 60 minutes at 85^{0}C and centrifuged at 12000rpm 20^{0}C for 20 minutes (Eppendorf 5810R centrifuge; Eppendorf, Hamburg, Germany). The steps were modified for the different treatments as described in figure 1 and table 1 below. The different layers formed at the end of the centrifugation step were carefully withdrawn for further analysis and functional property determined.



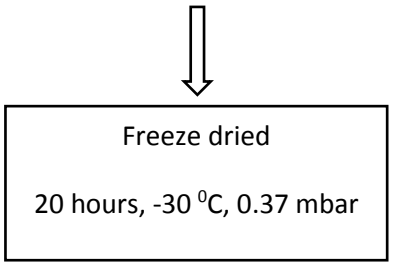


Figure 1. The flow chart of fish protein extraction from Patagonian toothfish (*Dissostichus eleginoides*) by the modified endogenous enzymatic method.

Table 1. Formulation of trials and control of fish protein extraction

Steps	**Trials**		
	1 Endogenous enzymes	**2 Alcalase enzymes (control)**	**3 Endogenous enzymes and Citric Acid**
Crushing and Homogenisation	10 min	10 min	10 min
Mixing	5 min	5 min	5 min
Hydrolysis Time	4 hours	4 hours	4 hours
Temperature	50^{0}C	50^{0}C	50^{0}C
Stirring Time	60 minutes	60 minutes	60 minutes
Temperature	85^{0}C	85^{0}C	85^{0}C
Centrifugation	12000 rpm	12000 rpm	7000 rpm
Freeze dryer	20 hours -30^{0}C 0.37 mbar	20 hours -30^{0}C 0.37 mbar	20 hours -30^{0}C 0.37 mbar

2.3 Chemical analysis

Every layer formed at the end of centrifuging by the three treatments was withdrawn and prepared for the proximate analysis. Samples were homogenised and randomly selected for the proximate composition analysis.

Moisture Content

The Association of Official Analytical Chemists (AOAC) official method 950.46 (AOAC, 2008) in meat moisture content was used to analyse the moisture content of a sample. Approximately 10g of each sample was weighed accurately into previously dried and tared aluminium dishes and dried in the 105^0C air oven (Contherm, digital series, oven, Lower Hutt, New Zealand) until constant weight. Before reweighing and moisture content measured by difference, the samples were cooled in a desiccator. The moisture content was determined by weight difference between before and after the drying process.

Ash

The ash content determination was conducted based on the AOAC official method 938.08 (AOAC, 2005). Approximately 5g was accurately weighed into pre-dried and cooled crucibles. Samples were ashed at 550^0C in a Thermolyne muffle furnace model 48000 Furnace (Thermo Fisher Scientific Inc, Iowa, USA) until constant weight (around 18 hours). Percentage of ash was calculated by the following equation:

$$\% \text{ Ash} = \frac{(\text{ashed weight} - \text{crucible weight})}{(\text{pre-ashed weight} - \text{crucible weight})} \times 100\ \%$$

Protein

Protein content was measured by using the Kjeldahl method according to the AOAC Official Method 955.04 (AOAC 2005). Approximately 1g of each ground sample was weighed and then put it into digestion tubes containing 1 Kjeldahl catalyst tablet (contains 1g Na_2SO_4 and 0.01g Selenium) and 2 or 3 glass beads to which the 8ml digestion acid (100 parts conc H_2SO_4 and 5 parts conc H_3PO_4) and 4ml of 35% hydrogen peroxide was added. The sample was then digested in a Tecator 2020 Digester (Högänas, Sweden) at 420^0C until a clear straw colour was reached. Into the digest 50ml of 40% sodium hydroxide was added and steam distilled in a Kjeltec system 1002 distilling unit, (Foss Tecator, Högänas, Sweden). The distillate was captured into a flask containing 25ml of boric acid as an indicator (80g of boric acid, 20ml of bromocresol green solution and 14ml of methyl red solution and diluted to 2L with deionised water). The distillate was titration against 0.1 M

Hydrochloric acid. One gram of sucrose was used as a blank. The percentage of protein in the samples was calculated using the following equation:

$$\% \text{ Protein} = \frac{(\text{sample titre mL} - \text{blank titre mL})}{(\text{mg sample})} \times 0.1 \text{ M HCL} \times 14.1 \times f \times 100\ \%$$

The conversion factor (f) was 6.25, which is the general factor used for meat and fish products.

Fat

The method for crude fat determination followed the AOAC official method 960.39 for meat (AOAC 2005). Approximately 1.5 g dried sample was ground, weighed and put into a thimble recorded as weight 1. An extraction cup, which is a specific glass beaker in which the fat will collect containing a glass bead, was weighed and recorded as weight 2. The fat was extracted in a Soxhlet Buchi fat extraction unit (Model E-816, Buchi Labortechnik AG, Flawil, Switzerland) over ten cycles or a one-hour period, with petroleum ether (boiling point range 40^0C–60^0C) as the extraction solvent. After extraction, the extraction cup was dried in the 105^0C air oven (Contherm, digital series, oven, Lower Hutt, New Zealand) until it reached a constant weight, and was then cooled in a desiccator. Crude fat was calculated as per the equation below:

$$\% \text{ Crude fat} = \frac{(\text{Wt of extraction cup containing fat} - \text{Wt of empty cup})}{(\text{Wt of thimble and sample} - \text{Wt of thimble})} \times 100\ \%$$

2.4 Functional properties of the extracted protein

Based on the chemical analysis results, the third layers from treatment 2 and 3, which contained the highest protein contents, were analysed for their function properties. Eggs were used as the control for the functional property determination analysis as they are considered the gold standard for food functional properties and used in the food industry for this purpose. All the samples (the layer 3 treatment 2, the layer 3 treatment 3 and eggs) were freeze dried in a CHRIST ALPHA 1-2 LD plus freeze drier (Martin Chris, Osterode, Germany) for 20 hours at 30^0C and 0.37 mbar. The functional properties analysed were: the water and fat-holding capacity, the emulsifying ability and stability, and foaming capacity and stability.

Water-holding capacity (WHC)

The samples (0.2 gram) were mixed with 45mL of distilled water and shaken for 1 hour at 25^0C in a shaking water bath (Grant, OLS200, shepreth, England). This blend was put into a 50ml centrifuge tube and centrifugated at 2500 g (g-force) for 30 minutes at 25^0C in the Eppendorf centrifuge model 5810R (Eppendorf, Hamburg, Germany). After that, the water layer was removed, and the retained sample and water were weighed. The water-holding capacity was expressed as the mass (g) of retained water per mass (g) of the sample (Coorey and others 2013).

Oil-Holding-Capacity (OHC)

The samples (0.2 gram) were mixed with 45mL of Coles brand canola oil (purchased from Coles Supermarkets within the Perth Metropolitan area) then shaken for 1-hour at 25^0C in the shaking water bath (Grant, OLS200, shepreth, England). This then followed the same method as per water-holding capacity method. The oil-holding capacity was expressed as the mass (g) of fat per mass (g) of the sample (Coorey and others 2013).

Emulsifying Ability (EA)

Emulsifying ability (EA) was performed by following Coorey and others (2013) method where 1g of sample was mixed with 100ml deionised water. By adding 0.1 N NaOH or 0.1 N HCl, the pH of the solution was adjusted to 6.0 then stirred and maintained for 10 minutes. The solution then was transferred to a 250ml measuring cylinder. To this solution, 100ml of canola oil was added and homogenised for 10 minutes at high speed (24000 rpm) using a specific homogeniser (IKA T18 Basic Ultra Turrax, staufen, Germany). Afterwards, the emulsion was centrifuged at 3000 rpm for 15 minutes using universal 16 centrifuge (Hettih Instrument, Tuttlingen, Germany). The volume of the emulsified layer was recorded, and the result of EA was calculated using the following formula:

$$EA\ (\%) = \frac{\text{The total volume of emulsified layer in ml}}{\text{The total volume of suspension in ml}} \times 100\ \%$$

Emulsifying Stability (ES)

Emulsifying Stability (ES) was determined as per the method used by Coorey and others (2013). The sample preparations were performed with the same method used for emulsifying ability. The solution was homogenised (IKA T18 Basic Ultra Turrax, staufen, Germany) and heated to 85^0C for 30 minutes at medium speed in the shaking water bath (Grant, OLS200, shepreth, England). After that, the solution was cooled to 20^0C using running water and centrifuged at 3000 rpm for 15 minutes using universal 16 centrifuge (Hettih Instrument, Tuttlingen, Germany). The volume of the emulsified layer was measured and the ES calculated by using the formula:

$$ES\ (\%) = \frac{\text{The total volume of emulsified layer in ml}}{\text{The total volume of suspension in ml}} \times 100\ \%$$

Foaming Capacity (FC)

The method explained by Coorey and others (2013) was used to determine Foaming Capacity (FC) of the samples. A sample was weighed properly at around 1g and then whipped with 100ml of deionised water. While stirring, the pH was adjusted to 6 by adding 0.1 N NaOH or 0.1 N HCl and maintained for 10 minutes. The solution was then moved to a mixing bowl and beaten in a cake mixer (Speedie V/75721/FM1, Japan) for 5 minutes at the highest speed level (speed setting 5 out of 5). The volume was measured by transferring the solution containing foam to a measuring cylinder. The Foaming Capacity (FC) was calculated according to the following equation:

$$FC\ (\%) = \frac{\text{The volume of foam in ml}}{\text{The total volume of suspension in ml}} \times 100\ \%$$

Foaming Stability (FS)

The Foaming Stability (FS) method of the samples was explained by using Coorey and others (2013). Foaming stability was demonstrated as the difference in volume of foam every 30 minutes for 2 hours, and there was recorded four times (FS30, FS60, FS90 and FS120). The foam was collected and measured in the measuring cylinder as per the foam capacity treatment at the given time intervals.

2.5 Data analysis

One-way analysis of variance (ANOVA) with the significance level at P≤0.05 was used to determine the differences in extraction treatments, the protein content and the functional properties. The data analysis and the statistical software STATA 14 (StataCorp LP, Texas, USA) were used to support all of the statistical calculations.

3. RESULTS AND DISCUSSION

The starting material was the patagonian toothfish processing by-products (head and viscera materials from patagonian tooth fish/*Dissostichus eleginoides*), Figure 2. The raw material was first blended (Figure 3) and its composition determined prior to the start of the experimentation, Table 2.

Figure 2. Raw materials used for experiments (heads and viscera from the toothfish)

Table 2. Raw material proximate compositions

Patagonian toothfish	Moisture (%)	Ash (%)	Protein (%)	Fat (%)
Experiment's result (heads and viscera from the Patagonian toothfish)	64.27	7.2	34.16	57.15
Moon and others (2011b) research				
Muscle	63.6	2.9	35.16	22.3
Liver	49.8	2.03	17.33	35.3

Research about the proximate composition of toothfish, especially species from *Dissostichus eleginoides*, is still limited. From Table 2, the current experiment contained higher moisture, ash and fat contents of 64.27%, 7.2% and 57.15% respectively than that of Moon and others (2011a). Moon and others (2011a) found the highest protein from the fish muscle of 35.16%, and that the fish liver had lower values on proximate contents except fat 35.3%. Although the current research sample was the same species of fish (*Dissostichus eleginoides*) as the previous research, in general, the proximate values between them were quite different.

The main reasons for the different results were because they were harvested from a different area. The area where they lived would influence their food source, which in turn would significantly influence the different body composition values within. Another potential reason for the differences in values between them was the difference of samples obtained. Different parts of the fish would differ in its composition, for instance, the ash content varies between the results, Table 2 (head and viscera, muscle and liver) as per the Moon and others (2011b) study. Protein content between the samples was also different. The muscle fish contained more protein than other edible parts of the fish. It also contained essential amino acids and high biological values. This is because myofibrillar proteins are embedded more in muscle fish than others part of the fish body (Hordur and Rasco 2000). The distinction of fat content from viscera, muscle and the liver of the fish can be clearly seen from Table 2. The fat content of the fish not only varies in terms of quantity and fatty acid composition but also is not equally distributed in the fish body. The difference of the fat content not only depends on the biological state of maturity but also on several factors such as age, catching area, season and nutrition (Rehbein and Oehlenschläger 2009).

Figure 3. The raw material after grinding

Treatment 1

Table 3. Chemical composition of fish protein extraction using endogenous enzyme, or, Treatment 1

Layer	Moisture content (%)	Ash content (%)	Protein content (%)	Crude fat content (%)
1	91.24 ± 1.95[a]	2.92 ± 1.80[a]	16.87 ± 2.93[a]	94.17 ± 1.18[a]
2	40.60 ± 2.55[b]	1 ± 0.19[a]	6.47 ± 0.85[b]	56.77 ± 0.99[b]
3	96.28 ± 0.03[a]	13.90 ± 0.58[b]	33.63 ± 0.23[c]	11.31 ± 8.13[c]
4	72.35 ± 0.32[c]	16.16 ± 0.52[b]	10.29 ± 0.32[d]	38.23 ± 1.95[d]

*Data expressed as mean value ± standard deviation
Values sharing the same letter in the same column are not significantly different (P<0.05)

Protein extraction using the natural enzyme (endogenous enzyme) in Treatment 1 produced four different layers. There were 2.8% in layer 1; 27.6% in layer 2; 64.3% in layer 3 and 6.6% in layer 4 in a 50ml tube after centrifugation, Figure 4.

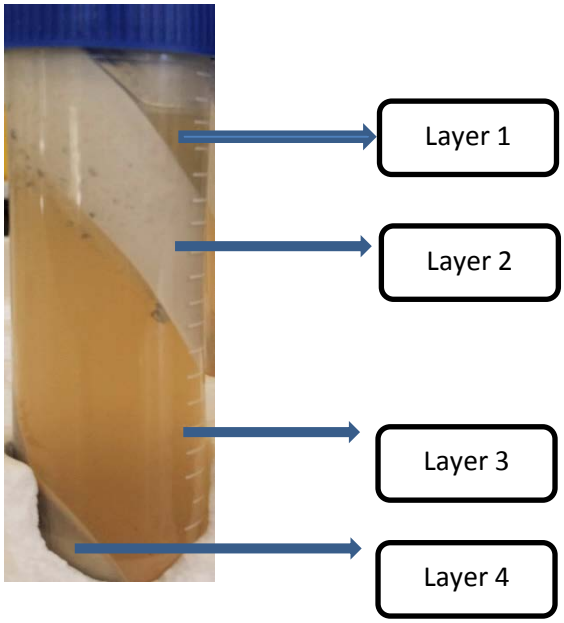


Figure 4. A 50ml tube with fish extraction using Treatment 1 inside.

Layer 1 was oil combining liquid base that can be recognised by the high amount of crude fat and moisture contents of 94.17% and 91.24% respectively, which were significantly lower than in the other layers, Table 3. Layer 2 (Figure 4) had a semisolid texture and its MC, AC and PC were lower than the other layers (40.60% moisture, 1% ash, 6.47% protein

content respectively). Layer 3, which was liquid base, had the highest mean value for moisture and protein contents of around 96.28% and 33.6% respectively. The last layer (Layer 4, Figure 4) had the highest mean value of ash content at 16.16% but it is not significantly different ($P<0.50$) from layer 3 (13.90 %).

Layer 1 and layer 2 were interesting because of the sharp discrepancy between their moisture contents and the different positions in the tube after centrifugation. Layer 1 should be located in the layer 2 position and vice versa because layer 1 contained more moisture content, which influenced the high density of the layer. The higher the density, the lower the layer position (nearer to the bottom). But in this study, the moisture content was not the only factor that impacted on the layer position. The density was also affected by the amount of crude fat content, and the fat density was lighter than water. Although layer 1 contained more water (91.24%), at the same time it contained more fat (94.17%). The combination of moisture and fat content in layer 1 generated the lowest density in this treatment (Sigma-Aldrich 2011). Layer 4 on the other hand, lay on the bottom of the tube because it had the highest value of density. The density originated from having the highest ash content in that layer which was caused by containing bones, skin and cartilage from a fish body. A centrifugal force from centrifugation separated all the components based on their density and particle size; the lower the density, the higher the layer position and the bigger the particles, the lower the layer position (Sigma-Aldrich 2011; Galanakis 2015).

Treatment 2

Table 4. Chemical composition of fish protein extraction using alcalase enzyme or Treatment 2

Layer	Moisture content (%)	Ash content (%)	Protein content (%)	Crude fat content (%)
1	-0.24 ± 0.5[a]	0.02 ± 0.01[a]	0.15 ± 0.01[a]	99.92 ± 0.14[a]
2	53.36 ± 3.47[b]	1.88 ± 0.31[a]	9.02 ± 0.95[b]	81.60 ± 0.91[b]
3	93.29 ± 0.08[c]	7.28 ± 0.37[b]	53.50 ± 6.03[c]	19.17 ± 1.24[c]
4	73.69 ± 0.32[d]	52.05 ± 2.94[c]	28.19 ± 2.56[d]	22.40 ± 0.22[d]

*Data expressed as mean value ± standard deviation
Values sharing the same letter in the same column are not significantly different (P<0.05).

In treatment 2, which included the addition of alcalase enzyme to extract the protein, four layers were formed after conducting protein extraction, Figure 5. Each of the layers were 22.66% layer 1, 6.54% layer 2, 66.66% layer 3 and 3.86% layer 4 (Table 4).

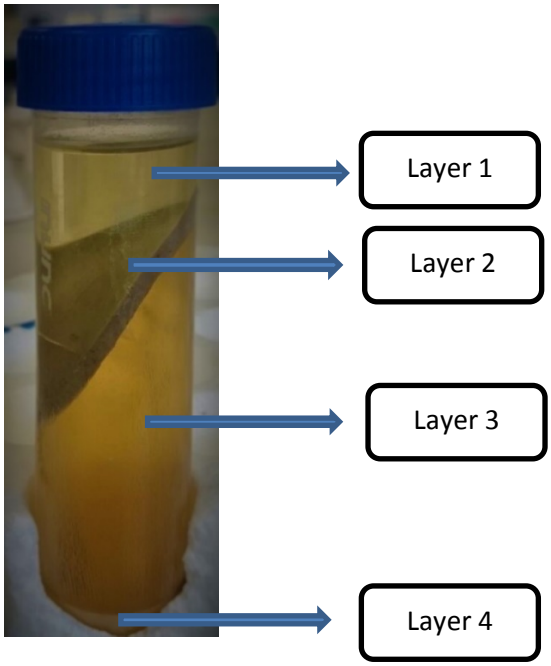


Figure 5. A 50ml tube with fish extraction using treatment 2 inside.

The first layer was the oil layer because it contained almost a hundred percent fat on dry basis which was the highest for this treatment of protein extraction and a little moisture,

ash and protein (less than 0.5%), Table 4. Oil, being lower in density, would be expected to rise to the surface. The minus value of moisture content in this layer would be caused by the loss of other volatile fatty acid components. The second layer had a lower value in fat content (81.60%) than the first layer, but the rest of the proximate results were higher than the first one. The third layer had the highest protein and moisture contents at 50% and 90% respectively. The last layer, layer 4, consisted of 73.43% moisture, 44.51% Ash, 28.19% protein, and 22.40% crude fat content respectively. This layer contained more minerals than other layers, which once again could be due to the bone material, as expected, having the highest density and therefore would settle at the bottom.

The chemical results from Table 4 were unique because each layer contained one compositional material which was higher than the others. They were also significantly different from other values ($P<0.05$). For instance, layer 1 had the highest content of fat, and layer 3 had the highest moisture and protein contents, while the highest ash content was in layer 4. These proximate results and their position on the layer clearly demonstrated excellent outcomes using alcalase enzyme and centrifugation. The alcalase enzyme worked very well when it extracted protein from the complex fish. The enzyme hydrolysed or broke down fish proteins in two phases: rapid and stationary phase. In rapid phase, the enzyme was absorbed into the protein molecule surface. After that, it broke down the fish polypeptide chains into the small peptide chains. The stationary phase occurred when almost all fish protein body was digested (Hordur and Rasco 2000). The centrifugation function was mainly for separation and purification. In this treatment, it supported the hydrolysis enzyme treatment to separate and divide all particles, including protein particles, based on their particle size, density and solubility. At the end, the extraction generated four main layers from the bottom to the top (Sigma-Aldrich 2011; Galanakis 2015).

Treatment 3

Table 5. Chemical composition of fish protein extraction using citric acid or Treatment 3

Layer	Moisture content (%)	Ash content (%)	Protein content (%)	Crude fat content (%)
1a	-0.53 ± 0.38^{a}	0.03 ± 0.02^{a}	0.14 ± 0.01^{a}	91.98 ± 11.34^{a}
1b	87.26 ± 2.63^{b}	1.02 ± 0.03^{a}	24.06 ± 13.38^{b}	62.99 ± 4.32^{b}
2	45.50 ± 1.13^{c}	1.77 ± 0.07^{a}	20.48 ± 3.44^{b}	36.58 ± 2.13^{c}
3	91.17 ± 0.11^{b}	1.19 ± 0.01^{a}	49.14 ± 0.25^{c}	0.93 ± 0.14^{d}
4	81.36 ± 2.63^{d}	13.37 ± 4.86^{b}	34.50 ± 0.61^{bc}	12.62 ± 1.04^{e}

*Data expressed as mean value ± standard deviation
Values sharing the same letter in the same column are not significantly different (P<0.05)

In this treatment, treatment 3, citric acid was added to extract the protein. The citric acid was used to reduce the solution's pH and support the endogenous enzyme to hydrolyse fish protein. The treatment produced five layers, and these made up 6.45% of layer 1a, 3.19% of layer 1b, 17.94% of layer 2, 64.4% of layer 3 and 6.78% layer 4, (Figure 6) and their composition is given in Table 5.

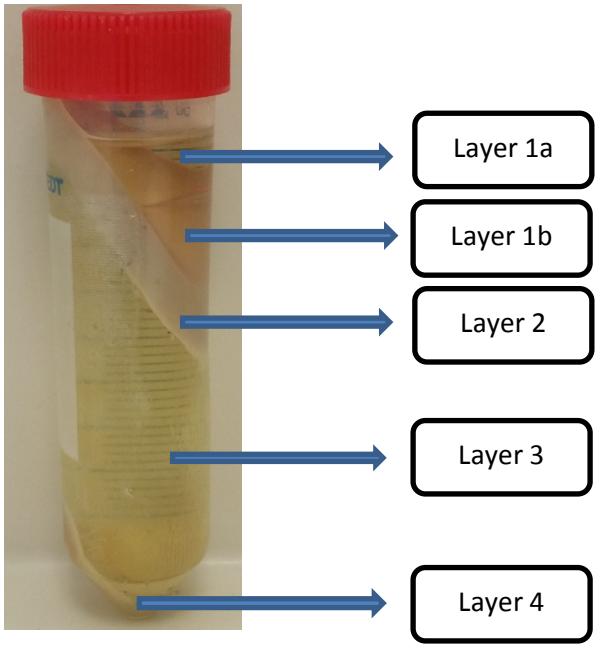


Figure 6. A 50ml tube with fish extraction using treatment 3 (Citric acid) inside.

The proximate analysis results for treatment 3 (Table 5) were very different to the previous treatments (Table 3 and 4) as this treatment contained five layers. Layer 1 was an oil layer with a high value of crude fat content (91.98%) and contained virtually nothing else (less than 1% for all other components). The minus value of moisture content may be due to the loss of other volatile fatty acid components in that layer. Layer 1b and 2 had similar values of ash and protein content (around 1% and 20% respectively), but layer 1b had twice the amount of moisture and crude fat content. The difference in quantity of moisture and fat impacted on the form of the layer where layer 1b was a liquid form with a light orange colour, while layer 2 was more like a semisolid form with a light grey colour. It also leads to the different positions of the two layers where layer 1b was higher than layer 2. The potential reason was the combination of layer 1b (the high amount of moisture and fat content) made its density lighter than layer 2. Layer 1b occurred as a rare phenomenon after centrifugation. Previous research described that commonly the fractions obtained after recovering soluble fish protein hydrolysates divided into four main parts: Sludge and heavy lipid-protein in the first layer (in the bottom), aqueous/soluble protein hydrolysate in the middle, light lipid-protein, and an oil layer on the top (Hordur and Rasco 2000; Ramakrishnan and others 2013; Galanakis 2015).

Layer 3 had the highest mean values of protein and water contents with 50% and over 90% respectively. On the other hand, ash and fat were around 1%. The high content of extracted protein on the supernatant layer proved that the endogenous enzyme run protein extracted well at pH 3 (acid). This means that the endogenous enzyme's activity from the patagonian toothfish by-products increases as the pH is reduced (Maron and Undeland 2010). Similar to treatment 1 and 2, the last layer had the highest content of ash (13.37%). The high content of ash on layer 4 showed that almost all minerals from bone, skins, scales and spines were taken out from the solubilised protein. These materials, known as insoluble fractions, can be used as animal feed (Hordur and Rasco 2000).

Table 6. Chemical composition compilation of fish protein extraction using treatment 1, 2 and 3.

	Moisture			Ash			Protein			Fat		
Layer	**T 1 (%)**	**T 2 (%)**	**T 3 (%)**	**T 1 (%)**	**T 2 (%)**	**T 3 (%)**	**T 1 (%)**	**T 2 (%)**	**T 3 (%)**	**T 1 (%)**	**T 2 (%)**	**T 3 (%)**
1	91.24± 1.95^{a}	-0.24± 0.5^{b}	-0.53± 0.38^{b}	2.92± 1.80^{j}	0.02± 0.01^{k}	0.03± 0.02^{k}	16.87± 2.93^{s}	0.15± 0.01^{t}	0.14± 0.01^{t}	94.17± 1.18^{d}	99.92± 0.14^{d}	91.98± 11.34^{d}
1B			87.26± 2.63			1.02± 0.03			24.06± 13.38			62.99± 4.32
2	40.60± 2.55^{c}	53.36± 3.47^{d}	45.50± 1.13^{c}	1± 0.19^{l}	1.88± 0.31^{m}	1.77± 0.07^{m}	6.47± 0.85^{v}	9.02± 0.95^{v}	20.48± 3.44^{w}	56.77± 0.99^{e}	81.60± 0.91^{f}	36.58± 2.13^{g}
3	96.28± 0.03^{e}	93.29± 0.08^{f}	91.17± 0.11^{g}	13.90± 0.58^{n}	7.28± 0.37^{o}	1.19± 0.01^{p}	33.63± 0.23^{x}	53.50± 6.03^{y}	49.14± 0.25^{y}	11.31± 8.13^{h}	19.17± 1.24^{h}	0.93± 0.14^{i}
4	72.35± 0.32^{h}	73.69± 0.32^{h}	81.36± 2.63^{i}	16.16± 0.52^{q}	52.05± 2.94^{r}	13.37± 4.86^{q}	10.29± 0.32^{a}	28.19± 2.56^{b}	34.50± 0.61^{c}	38.23± 1.95^{k}	22.40± 0.22^{l}	12.62± 1.04^{m}

*Data expressed as mean value± standard deviation

*T 1 =Treatment 1; T 2 = Treatment 2; T 3 = Treatment 3

Values sharing the same letter in the same row in the same chemical composition are not significantly different (P<0.05)

The chemical composition on the dry basis of fish extraction using treatment 1, treatment 2 and treatment 3 are presented in Table 6. The highest moisture content was in treatment 1 layer 3 (96.28%), and the lowest were in treatments 2 and 3, layer 1 (-0.24% and -0.53% respectively). All of the layers 3 in the three treatments had high moisture contents because this layer contained more water than other layers. Interestingly, the combination of water and protein generated soluble protein caused layer 3 to have a high value in protein content. Different from layer 3, layer 1 in all treatments contained more fat but less water except in treatment 1. Layer 4, which is at the bottom, showed high values in ash content. The separate layer can be assumed because of centrifugal movement after centrifuge 12000 rpm and different density (Hordur and Rasco 2000; Sigma-Aldrich 2011; Galanakis 2015).

Although 33.36% protein content in layer 3 was the highest protein value in treatment 1, this value was the lowest protein content when compared to protein contents in layer 3 of treatments 2 and 3 (53.50% and 49.14% respectively). Treatment 1 clearly only used the endogenous enzyme to extract protein from fish by-products, pH 6.7-6.9 at temperature 50^0C without adding anything. This value confirmed the fact that the fish itself contains an enzyme that is able to extract or digest its body to produce protein. This value is also higher than Mazorra and others (2012) research (28.9 ± 0.7% protein content) which used muscle as a sample and added NaOH to adjust the pH 7.

The highest value of protein content in layer 3 treatment 2 (53.5%) is to be expected as the protein extraction used the commercial enzyme (Alcalase enzyme). This result is also similar to Shahidi and others (1995) that the alcalase enzyme is one of the best enzymes to extract protein from fish viscera. Herpandi and others (2013) research supported the result by giving an information about the optimum of using alcalase enzyme that needed 2% Alcalase®2.4L at pH 8.86 and 65.4^0C for 5.74 h. Interestingly, this experiment did not put the sample in the high temperature at preparation step to stop the endogenous enzyme. This condition is different from other previous studies which assume the endogenous enzyme as an inhibitor substance for the commercial enzyme such as alcalase enzyme in the extraction process (Herpandi and others 2012; Herpandi and others 2013; Gildberg 1993).

The second position of protein content was in layer 3 of treatment 3 (49.14%). Although the difference value from the highest content 3–4 %, the two values did not statistically show significant differences ($P<0.05$). This means that the two values are essentially the same. The idea of using citric acid in this formula was to investigate the impact of low pH (3) on the protein extraction by the endogenous enzyme. The citric acid was chosen because this substance had a food grade level substance based on Standard 1.3.1 Food Additives, Food Standards Australia New Zealand (FSANZ 2015). The protein content result from this experiment was unpredictable which is similar to the experiment using the best commercial enzyme (Alcalase enzyme) and almost twice as high as using the endogenous enzyme in pH around 7 (compared to treatment 1 protein content 33.36%). The reduced pH level to 3 was also against that of the previous paper which showed that the optimum pH to extract protein from fish using the endogenous enzyme was 7. That is why Mazorra's treatment always used 1–4 M NaOH to adjust the pH to 7 (Mazorra and others 2012).

Functional properties of the extracted protein

The soluble fraction from layer 3 in treatment 2 and layer 3 in treatment 3 was selected for functional property determination. They were chosen for the subsequent analyses because they had the highest content of protein hydrolysate than the others between 3 different treatments and layers. The primary goal for this determination was to identify the functional properties from the layers which had high protein content. These analyses involved water and oil-holding capacity, emulsifying ability and stability, foaming capacity and stability – the functional properties that are required for the application of extracted protein in food product development to impart required sensory characteristics. The extracted protein was stabilised by freeze drying (figure 7).

Treatment 2 Treatment 3 Control

Figure 7. Freeze-dried form of soluble fraction from treatment 2, 3 and the control

Water holding capacity (WHC) and Oil-Holding-Capacity (OHC)

The amount of water which was absorbed and held by a dried sample after receiving an external force is described as Water Holding Capacity (WHC). Similar to WHC but using oil as the solution absorbed by a sample is the Oil Holding Capacity (OHC) (Coorey and others 2014). The water holding capacity and oil holding capacity from treatment 2 and 3 were measured by comparing against eggs as the control which are a natural food ingredient that have good water and oil capacity and are considered to be the industry standard. The water and holding capacity from treatment 2, treatment 3 and the eggs are shown in Table 7.

Table 7. Water and oil holding capacity of treatment 1, treatment 2 and control samples

No	Sample	Water-holding capacity (g of water retained/ g of sample)	Oil-holding capacity (g of water retained/ g of sample)
1	Treatment 2	0^{a}	1.69 ± 0.21^{a}
2	Treatment 3	0^{a}	7.36 ± 0.74^{b}
3	Control	5.86 ± 3.44^{b}	12.11 ± 1.34^{c}

*Data expressed as mean value ± standard deviation
Values sharing the same letter in the same column are not significantly different ($P<0.05$)

The extracted fish protein did not have any water holding capacity (WHC) (zero) (Table 7), while the control's (eggs) water holding capacity was almost 6 grams/sample. These results

explain that the fish protein extracted from the two treatments do not have the ability to absorb and hold water. The reason for this could be due to several factors such as denaturation of the protein configuration after several extraction processes, which could alter the water interaction and the number of hydrations position in the molecule (Galla and Dubasi 2010). Another explanation was the isoelectric solubilisation/precipitation from the fish protein extracted which changed to a positive charge. The positive charge value was caused by adding acid to the solution, and it impacted on the weakening of hydrogen bonds of protein-water interaction (both hydrogen and protein have positive charges) (Galanakis 2015). The eggs' success to absorb and trap water is because the egg whites generated the formation of a solid gel network, that had not undergone any extraction process that could have denatured its configuration (Coultate 2009).

Different from the water holding capacity, the extracted fish protein was able to hold some oil, and there were significant differences between the samples and the control ($P<0.05$). The eggs had the highest mean value for oil holding capacity (12.1%) while treatment 3 was the second highest (slightly more than 7%) and treatment 2 was the lowest (less than 2%). Interestingly, the differences between the results were almost 5 grams between the three samples and the treatment 3 result was higher than the other treatments' value. The ability of fish protein hydrolysate to bind oil is influenced by three main factors: the protein density, the degree of hydrolysis and enzyme substrate specificity (Sathivel and others 2003; Kristinsson and Rasco 2000). The capability to trap and hold oil also can be used potentially as fat and flavour binding that involves hydrophobic bonding and entrapment as the mechanism in food systems such as simulated meats, baked goods and doughnuts (Vickie and Christian 2014; Damodaran 1996).

Emulsifying Ability (EA) and Emulsifying Stability (ES)

Emulsifying ability (EA) and emulsifying stability are part of the important functional properties of a protein in food products. Emulsifying ability (EA) can be defined as the ability of an emulsifying substance to generate a dispersion material (water-in-oil) while the emulsifying stability is the capacity to retain the stability of the emulsion over the application of force (Coorey and others 2014).

Table 8. Emulsifying ability and emulsifying stability

No	Sample	Emulsifying ability (%)	Emulsifying stability (%)
1	Treatment 2	0^{a}	0^{a}
2	Treatment 3	0^{a}	0^{a}
3	Control	58.38 ± 3.44^{b}	22.58 ± 5.93^{b}

*Data expressed as mean value ± standard deviation
Values sharing the same letter in the same column are not significantly different ($P<0.05$)

There was no significant difference between samples from treatment 2 and treatment 3 regarding emulsifying ability and stability. These results were caused by no Emulsifying Ability (EA) and Emulsifying Stability (ES) results for both fish protein samples (zero results). The control eggs had almost 60% ability to emulsify activity and stability of emulsifying from eggs was around 23%.

Several aspects can influence the emulsifying properties from protein such as absorption rate at the oil-water interface, the amount of protein absorbed, conformational rearrangement at the interface, reduction in interfacial tension and the ability to produce a film that is cohesive and viscous (Schwenke 1996). The inability to emulsify oil-water emulsions and retain its stability could be due to the loss of one or more of these factors. Another possible explanation was the low-quality level of myofibril content from the raw material. As stated previously, the raw material came from the frozen form of patagonian toothfish. The frozen material of fish that was stored for a long time in cold storage or a freezer may influence protein denaturation and aggregation that can lead to a decrease of emulsifying ability and stability. A denaturation affected by a low temperature gives a negative contribution to protein stability, while the increasing the temperature has positive impacts (Schwenke 1996). A similar result was carried out in a study regarding protein solubility and emulsifying capacity in frozen stored fish mince. This study explains that there was a significant difference in the emulsifying capacity of fish meat from fresh meat to frozen meat and also the duration of freezing (Srikar and Reddy 1991). Structure of protein also can impact on its functional properties. The extraction process by enzymes not only separated protein from other substances, but also changed or reduced the primary protein structure (denaturation). Changing of the protein structure can cause

a significant loss of emulsifying properties because the primary structure can become smaller and contain less hydrophobic and hydrophilic functional groups that have a significant role in determining emulsifying properties (Hordur and Rasco 2000).

Foaming Capacity (FC) and Foaming Stability (FS)

Foaming capacity (FC) is the capability to produce foam or gas encapsulation, whereas foaming stability (FS) is the ability to stabilise its foam over a time period or the life time of the foam (Mazorra and others 2012).

No	Sample	Foaming capacity (%)
1	Treatment 2	100.33 ± 1.53^{b}
2	Treatment 3	150.33 ± 7.07^{a}
3	Control	149 ± 1.4^{a}

*Values sharing the same letter in the same column are not significantly different ($P<0.05$)

Table 9. Foaming capacity (FC) at pH 6

The foaming capacities at pH 6 of fish protein extraction treatments 2, 3 and also the control eggs are shown in Table 9. The foaming capacity for treatment 2 was 100% while treatment 3 and the control samples were 150.33% and 149% respectively. These values were interesting because treatment 3 produced a protein extract that had a foaming capacity similar to eggs as the sample control.

In general, the ability of a protein to generate good foam is influenced by several factors: First, protein should be easy to permeate into the air-water interface. Second, the protein should be easy to conformational change and rearrange at the air-water interface. The protein also should be able to reduce the surface tension. Third, protein should have a great stability regarding hydrophobicity (in surface and molecular), net charge and its distribution between all factors. Variations of amino acid sequence in a protein and protein structure, especially the tertiary structure, may also have an important impact on the protein foaming properties (Damodaran 1997). From the present research results (Table 9), it can be

concluded that if the protein from sample treatment 3 had the highest level of all those factors to produce good foam similar to eggs and better than the protein from treatment 2. It means that this fish protein can be used in suitable food products to develop foam in place of egg protein.

The foaming stabilities of samples from the two treatments and the control are presented in Table 10. The symbol of FS30, FS60, FS90 and FS120 are the abbreviations from foaming stability and the time measured at the interval 30, 60, 90 and 120 minutes.

No	Sample	FS30 (%)	FS60 (%)	FS90 (%)	FS120 (%)
1	Treatment 2	$97,67\pm 0.58^{aA}$	$97,67\pm 0.58^{cA}$	97.67 ± 0.58^{fA}	97.67 ± 0.58^{iA}
2	Treatment 3	133.33 ± 1.15^{bB}	132.67 ± 0.58^{dBC}	131.50 ± 0^{gC}	131.33 ± 0.28^{jC}
3	Control	131.67 ± 7.63^{bD}	125 ± 5^{dD}	114 ± 5.29^{hE}	110 ± 5^{kE}

*Values sharing the same letter in the same column are not significantly different (P<0.05)

*Values sharing the same capital letter in the same row are not significantly different (P<0.05)

Table 10. Foaming stability (FS) at pH 6

*

The foaming stability of the sample from treatment 3 was the highest and the best at 120 minutes, which indicates that the foam would be stable for a longer period of time when applied to a food product, even better than the egg protein. Although the results of the foaming stability between the sample from treatment 3 and the control sample were not significantly different (P<0.05) at the 30 and 60 minutes' time intervals, afterwards the protein from treatment 3 was significantly better. The value of the control sample at FS60, FS90 and FS120 decreased significantly from 125% to 110% while the values from the sample from treatment 3 remained the same. The contrast in results was presented by the sample from treatment 2 that had lower but stable foaming values. It can be clearly seen from Table 9 that there were no changing results from FS30 to FS120 (97.67%).

The foaming stability is dependent on some aspects such as pH; protein solubility; and protein molecule size, weight and its interaction between them (Pacheco-Aguilar and others

2008). The sample from treatment 3 had more stable foam than the other samples because their protein fulfilled all of those aspects to stabilise its foam. The samples from treatment 2 and the eggs may have had the same protein molecular, but their size, solubility or interaction was lower than the sample from treatment 3.

The high quality of foaming capacity and stability from treatment 3 gives a choice of another potential substance to be used in a food system as a foaming ingredient which has interfacial absorption and film formation. Example products that can be incorporated with this foaming substance are whipped toppings, ice cream, cakes and desserts (Hordur and Rasco 2000).

3.5 Conclusion

In conclusion, the results of the present study show that fish protein extraction from patagonian toothfish *(Dissostichus eleginoides)* by-products using the modified endogenous enzymatic treatment presented a good result. Two treatments and one control treatment were used to investigate the best treatment for fish protein extraction. These treatments involved 6 (six) steps (Crushing and homogenisation, mixing, hydrolysis, stirring, centrifugation, freeze-dried) but different enzymes and pH level. Treatments 1 and 3 used the endogenous enzyme, which comes from the Patagonian toothfish (heads and viscera), but the pH for the treatment 3 was made lower than the first by adding citric acid. Treatment 2 as the control used the alcalase enzyme. Protein contents from layer 3 in treatments 2 and 3 were higher than the treatment 1, and both of them were not significantly different statistically ($P<0.05$) and this value answered the hypothesis.

The samples from treatments 2 and 3 then were assessed regarding the six food functional properties containing water-holding-capacity (WHC), oil-holding-capacity (OHC), emulsifying ability (EA) and stability (ES), and foaming ability (FA) and stability (FS). These assessments used eggs as the control. The samples from treatments 2 and 3 did not have any values at WHC, EA and ES while the control had all the values (6%, 58% and 23% respectively). Treatment 3 had higher values of FC and FS than the values from treatment 2 and the control.

Based on the results, the fish protein extraction from treatment 3 has potential to be used in the food industry as fat and flavour binding, and also foaming ingredients. Further research is required to analyse the amino acids contained and to evaluate the quality of protein when it put into the real food products.

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