Improved extraction of phospholipid-rich oil from Antarctic krill by enzymolysis

Huan Dong

Norwegian University of Life Sciences
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By

Huan Dong

Department of Animal and Aquaculture Sciences

Norwegian University of Life and Sciences

Ås, Norway

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Abstract

The aims of this research project were to identify a functional protease and optimize hydrolysis conditions for this enzyme to facilitate efficient alcohol extraction of phospholipid (PL)-rich oil from Antarctic krill, *Euphausia superba.* The following hydrolysis conditions tested were: frozen krill and water ratio (KWR), hydrolysis temperature (HTe), pH, enzyme dosage (ED), and hydrolysis time (HTi). These conditions were studied by single factor and orthogonal experiments. Finally, the efficiency of various solvents used for extracting the krill oil was compared.

Alcalase was identified as the most suitable enzyme for enzymolysis of Antarctic krill among 5 commonly used enzymes based on the degree of hydrolysis (DH).

The optimal conditions for enzymolysis Antarctic krill with alcalase were: KWR = 2:1, HTe = 55~60ºC, pH = 8.0, ED = 3000 U/g and HTi = 4 h (end pH = 6.5~6.7). Range analysis in orthogonal experiments gave the following ranking of influencing factors: KWR (R = 6.2) > ED (R = 2.3) > pH (R = 0.3) > HTi (R = 0.2). Under the optimal conditions, the DH was 31.1 %. Meanwhile, peptides with an average length of 3.2 and a molecular mass of 352 were obtained.

Extraction was performed from "PEF", defined as freeze dried lipid phase after 75~80℃ for 5 min after hydrolysis, and 600xG centrifugation for 5min. Compared with 5 commonly used solvents, 99.5% ethanol gave the most complete extraction of PL rich krill oil (KO).

Under the conditions of extraction temperature (ETe) = 45 ºC, PEF and 99.5% ethanol ratio (PER) = 1:10 and extraction time (ETi) = 3h, highest KO extractability (m (KO) / m (PEF)) was 74%; PL concentration (m (PL) / m (KO)) was 32.1%; total KO extractability (m (KO) / m (total lipid in PEF)) was 99.4%; total PL extractability (m (PL) / m (total PL in PEF)) was 102.8%.

Under the conditions of ETe = 25º℃, PER = 1:6, ETi = 1h, highest PL concentration was 33.9%; KO extractability was 61%; total PL extractability was 89.5%; total KO extractability was 82.0%.

Under the conditions of ETe = 45º℃, PER = 1:8, ETi = 2h, highest astaxanthin (AX) concentration in the extract was 172.2 ppm; KO extractability was 72%; total AX extractability was 103.9%; total KO extractability was 91.1%.

High acid value (AV) and low PL concentration due to hydrolysis during frozen storage for 6 months, and lipase and phospholipase (PLase) during the time from catch until the material was frozen, thawed and incubated. Under the conditions of ETe = 25º℃, PER = 1:6, ETi = 3h, the minimum acid value was 28.6 mg KOH/g.

In conclusion, this application of alcalase for hydrolysis of Antarctic krill resulted in a high degree of hydrolysis. As a result, lipid and water phase were separated to two layers, in which the upper layer enriched more fat and less protein. Extraction with 99.5% ethanol from this PEF resulted in total krill oil and phospholipid extractabilities at nearly 100%.

Keywords: Enzymolysis, Antarctic krill oil, Phospholipid, Degree of hydrolysis

Contents

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Abbreviations

AA＝ Amino Acid
AV＝ Acid Value
AX＝ Astaxanthin
DH＝ Degree of Hydrolysis
DHA＝ Docosahexaenoic Acid
EAA＝ Essential Amino Acid
ED＝ Enzyme Dosage
EPA＝ Eicosapntemacnioc Acid
ETe＝ Extraction Temperature
ETi＝ Extraction Time
FA＝ Fatty Acid(s)
FAA＝ Flavor Amino Acids
FD＝ Freeze Drying
FM＝ Fish Meal
HTe＝ Hydrolysis Temperature
HTi＝ Hydrolysis Time
HUFA＝ Highly Unsaturated Fatty Acids
KWR＝ Frozen Krill and Water Ratio
NEAA＝ Non-Essential Amino Acid
NFE＝ Nitrogen Free Extract
PC＝ Phosphatidyl Cholines
PE＝ Phosphatidyl Ethanolamines
PEF＝ Phospholipid Enriched Fraction
PER＝ PEF and 99.5% Ethanol Ratio
PL＝ Phospholipid
PLase＝ Phospholipase
PI＝ Phosphatidyl Inositols
PS＝ Phosphatidyl Serines
PUFA＝ Polyunsaturated Fatty Acid
SFE＝ Supercritical Fluid Extraction
TAA＝ Total Amino Acid

1. Introduction

1.1. Background

1.1.1 Antarctic krill biological characteristics and fishery

Table 1. Classification of *Euphausia superba.*

Kingdom	Animalia
Phvlum	*Arthroaoda*
Subphylum	*Crustcia*
Class	*Crustcia*
Superorder	*Eucarida*
Order	*Euphausiacea*
Family	*Euphausidae*
Genus	*Euphausia*
Species	*Euphausia superba*

Aker FDA (2010)

Krill are in the family of pelagic marine planktonic crustaceans, and there are more than 80 species of krill widely distributed throughout the world's oceans. Antarctic krill (*Euphausia superba*) mostly live in the Southern polar seas. It swims in huge swarms that can be as large as six kilometers in length and have a density of up to one million individuals per m^3 (Hamner et al., 1983). Antarctic krill grows to a length of 6 cm, weighs up to 2 g, and can live for up to six years. It is a key species in the Antarctic ecosystem (Stephen & Yoshinari, 1997).

Despite their small size, krill likely has the largest biomass of any multi-cellular animal species on earth. The total annual capture from all fisheries has been approximately 130 million tons (MT) since 2000. By comparison, krill biomass has been estimated at 400–1550 MT with a sustainable harvest at 70–200 MT (Suzuki & Shibata, 1990). However, newer estimates suggest that the krill biomass may be lower (Priddle et al., 1998; Smetacek & Nicol, 2005; Nicol & Foster, 2003) estimated the annual krill capture to be 0.1 MT, making krill an underutilized species. However, due to the role that krill play in marine ecology, an internationally monitored and governed ecosystem approach is a necessity for a long-term sustainability of this fishery (Everson, 2000; Laws, 1985).

Krill fishing began in 1961 by the Union of Soviet Socialist Republics. International studies on krill for processing and utilization began around the same time. Before that, Japan, the United States, Poland, Norway and Chile had successively begun fishing and research of krill (Chen et al., 2009). Thus, when China took part in catching

Antarctic krill in 2009, the era of processing and utilization of Antarctic krill had already begun.

1.1.2 Nutritional components in Antarctic krill

Table 2. Nutritional components in Antarctic krill (capture in May, 2013).

	Moisture content	**Crude protein***	**Total fat***	**PL in total fat (%)**	**Ash**	**Acid Value**
Raw krill	77.8	13.2	4.6	31.1	2.9	18.1
Frozen krill	79.6	12.1	4.2	31.1	2.7	18.1
FD krill	3.6	57.3	20.0	31.1	12.3	18.1

(Analyzed in November, 2013)
FD: freeze dried
PL: phospholipidphospholipid
*Frozen krill = Raw krill+8%water
*Crude protein determined by the Kjeldahl method.
*Total fat determined by Soxhlet extraction

Currently, Chinese research on Antarctic krill is still in an early stage. Evaluation of the nutritional content of Antarctic krill has been comprehensive. Studies on human health functions have started, while research on bioactive components is comprehensive and transparent. Specifically, Antarctic krill has been found to be rich in protein, and the essential animal acid composition is balanced. The lipid composition is especially interesting from an immediate marketing point of view, since the krill is rich in *n-3* PUFA. Analysis of whole Antarctic krill showed a range of 77.9% to 83.1% for moisture, 11.9% to 15.4% for crude protein and 0.5% to 3.6% for total lipids (Chi et al., 2010). Krill total lipids concentration depends on season, sex and maturity. Generally, the highest total lipids concentration reaches during March and June. PL concentration in Female krill is twice as much as than male. PL concentration increases as krill age increase and plunge after ovulation (Saether et al., 1986).

1.1.3 Comparison of amino acids profile

Table 3. Comparison of amino acid profiles in different krill species and in fish meal (g/100g dry weight).

	Euphausia superba	***Fenneropenaeus chinensis***	***Metapenaeus ensis***	**FM**
Asp	4.2	6.4	6.1	5.2
Thr*	1.7	2.4	2.2	2.0
Ser	1.5	2.4	2.1	2.3
Glu	10.9	11.5	10.1	8.1
Gly	2.8	7.1	5.2	3.8
Ala	5.3	6.6	8.8	3.4
Val*	3.1	2.6	3.6	2.6
Met*	2.4	2.1	2.4	1.3
Ile*	3.3	2.6	3.6	2.5
Leu*	5.0	4.8	6.4	4.1
Tyr**	1.6	2.3	2.2	1.7
Phe*	3.7	3.1	3.1	2.2
Lys*	5.9	6.3	4.4	4.2
His	1.0	1.4	1.2	1.3
Arg	1.8	4.0	3.0	3.2
Pro	1.1	1.9	1.9	3.8
Trp*	0.8	0.7	0.7	0.8
Cys**	1.3	0.9	1.2	0.9
EAA%	25.9	24.6	26.4	19.7
NEAA%	31.5	44.5	41.8	33.7
TAA%	57.4	69.1	68.2	53.4
EAA/TAA	45.1	35.6	38.7	36.9
EAA/NEAA	82.2	55.3	63.1	58.5
FAA%	23.2	31.5	30.2	20.5
FAA/TAA	40.4	45.6	44.3	38.4

(Sun, et al., 2008; Zhang, et al., 2008)
*EAA
**Semi-EAA
EAA: essential amino acid
NEAA: non-essential amino acid
TAA: total amino acid
FAA: flavor amino acids
FM: fish meal
FM is imported from Peru
FAA = Asp+Glu+ Gly+Ala

The results in Table 3 show that *Euphausia superba* has lower total amino acid

(57.4%) compared with *Fenneropenaeus chinensis* (69.1%) and *Metapenaeus ensis* (68.2%), higher than fish meal (53.4%). However, *E. superba* has higher EAA/TAA (45.1%) and EAA/NEAA(82.2%) than *F. chinensis* (35.6%, 55.3%), *M. ensis* (38.7%, 63.1%) and fish meal (36.9%, 58.5%).

According to FAO/WHO (1985) recommended protein pattern for human adults and infants, EAA/TAA from good quality protein should above 40%, while EAA/NEAA should be above 60%. As we can see, *E. superba* EAA/TAA is 45.1%, EAA/NEAA is 82.2%. That means *E. superba* is a good amino acid resource, which can meet the human amino acid requirements. In addition to the amino acid content, bioavailability also must be assessed to determine protein quality. Gigliotti et al., (2008) showed *in vivo* analysis of protein quality indicated that protein digestibility corrected for amino acid score and protein efficiency ratio was equal to casein.

Flavor amino acid (FAA) is composed of Asp, Glu, Gly, Ala. The FAA/TAA ratio is an important index for palatability. *E. superba* contains lower (40.4%) than *F. chinensis* (45.6%), *M.ensis* (44.3%), but higher than fish meal (38.4%). Antarctic krill protein is still a suitable resource for making krill hydrolysate and flavoring.

1.1.4 Autolysis and autolytic enzyme

Krill digestive glands have high activity hydrolytic enzymes including proteases, carbohydrases, nucleases and lipases. Autolysis begins post mortem. Hydrolytic enzymes are released into the surrounding tissue which results in rapid tissue liquefication and eventual spoilage. Fluoride from shell will also transfer to krill meat at the same time. Thus, krill should be deshelled or heated to inactivate autolytic enzymes immediately after capture. If Antarctic krill is processed for food, the process should finish within 3h. By contrast, if krill is to be used for feed, the process should finish within 10h (Liu, 2011). Dai et al., (2012) argued that optimized krill autolytic condition as hydrolysis time of 180 min, temperature of 50 ºC, pH value of 7.0, the time for UV radiation of 20 min and Ca^{2+} concentration of 0.28 mol/L. The maximal amino acid nitrogen concentration and the maximal degree of hydrolysis solution at optimized autolytic hydrolyzing condition were 0.875 g/L and 36.18% respectively.

Anheller et al., (1989) and Sjödahl et al., (2002) reported krill has special protein degradation system containing trypsin analog, chymotrypsin analog and carboxypeptidase. Trypsin and chymotrypsin are endoproteases while carboxypeptidase A and B belong to the exoproteases. Therefore, krill autolytic proteases have both endoprotease and exoprotease activity.

Autolysis becomes the biggest obstacle to overcome in the processing and utilization of Antarctic krill. However, krill enzymes have been studied in detail and exploit many new industrial applications because of its low temperature resistant and high

activity. Proteases cleave different specific peptide bonds can find application in detergents, leather industry, pharmaceutical industry and bioremediation processes.

1.1.5 Fluoride concentration and defluorination

Low doses of fluoride have positive effects on human dental health, while high doses of fluoride cause skeletal fluorosis. Soevik & Braekkan (1979) first described fluorine levels in Antarctic krill reaching 2400 ppm / DW and fluorine concentration were mainly distributed in the shell. Pan et al., (2000) reported fluoride distribution: cephalothorax (1962 ppm, 49% F) > venter shell (3558 ppm, 30.7% F) > anal leg (2177 ppm, 13.5% F) > muscle (250 ppm, 6.8% F) >> sea water (1.3 ppm F). Pan et. al., (2000) also pointed that fluoride contributes in various cycles related to krill molting.

Removal of the exoskeleton is important not only for decrease fluoride concentration, but also influent feed utilization. Ikegamie et al., (1990) argued that indigestible polysaccharides such as chitin impede digestion and absorption. But the results were not conclusive because animal species and life stage difference.

Currently, there are many reliable and safe methods to defluoride. Wang & Yin (2012) used calcium phosphate ($Ca_3(PO_4)_2$) and chitin as defluoridation agents. The results suggested that calcium phosphate has a far greater capability than chitin for the removal of fluoride (F) from water under similar reaction conditions. Li (2011) studied three different methods, which were nanofiltration, electrodialysis and calcium chloride precipitation, aimed at removing excessive fluoride from Antarctic krill hydrolysate. Calcium chloride precipitation was recommended because it is simple, short time-consuming and effective. Fluoride concentration from Antarctic krill hydrolysate decreased from 70 ppm to 2 ppm. The loss of amino nitrogen was 6.8% and the total nitrogen loss was 9.6%, by the same procedure.

1.2 Chemistry and biological activity

1.2.1 *n-3* PUFA

Table 4. Krill oil fatty acid profile comparison with various fish oils (%).

Fatty acid	Anchovy oil	Menhaden oil	Salmon oil	Krill oil
C14:0	7.4	7.3	3.7	7.7
C16:0	17.4	19.0	10.2	15.4
C18:0	4.0	4.2	4.7	0.9
C16:1	10.5	9.0	8.7	4.9
C18:1	11.6	13.2	18.6	12.1
C18:2 *n-6*	1.2	1.3	1.2	1.2
C18:3 *n-3*	0.8	0.3	0.6	1.4
C18:4 *n-3*	3.0	2.8	2.1	0.1
C20:1	1.6	2.0	8.4	0.9
C20:4 *n-6*	0.1	0.2	0.9	0.4
C20:5 *n-3*(EPA)	1.6	11.0	12.0	14.7
C22:1 *n-9*	1.2	0.6	5.5	0.7
C22:5 *n-3*	1.6	1.9	2.9	0.3
C22:6 *n-3*(DHA)	8.8	9.1	13.8	6.2
∑ n-6	1.3	1.5	2.1	1.9
∑ n-3	15.8	25.1	30.9	24.0
n-3:n-6	12.2	16.7	14.7	12.6

Aker FDA (2010)

Table 4 shows that krill oil contains 14.7% EPA and 6.2% DHA, with a ratio of (*n-3*: *n-6*) at 12.6. *n-3* polyunsaturated fatty acids (*n-3*PUFA) belong to an essential fatty acid family characterized by their first double bond at carbon atom number 3 counted from the methyl end of the carbon chain constituting the backbone of fatty acids.

There are two subgroups of *n-3* fatty acids. One, α-linolenic acid (ALA), derived from plant oils such as canola oil, rapeseed oil and linseed oil, is composed of 18 carbon atoms with three double bonds (18:3). The other group is derived from seafood, and the major marine *n-3* fatty acids are EPA (20:5) and DHA (22:6). In humans, ALA can, to a limited extent, be elongated and desaturated to EPA and DHA. Otherwise, EPA and DHA are mainly acquired from seafood.

Calder (2006) discussed the biological role and mechanism of action of long-chain *n-3* fatty acids. It is well known that the *n-6* fatty acid, arachidonic acid, gives rise to the eicosanoid family of mediators (prostaglandins, thromboxanes, leukotrienes, and related metabolites). These mediators have inflammatory actions in their own right and also regulate the production of other mediators including inflammatory cytokines.

An *n-3* index below 4% was associated with an increased risk for cardiovascular disease, whereas an *n-3* index above 4% reduces the risk for heart disease (Pottala et al., 2010). In the same context, a study investigating patients with stable coronary artery disease found that those with an *n-3* index above 4% had a 27% lowered risk of death, compared to those with an *n-3* index below 4%.

1.2.2 *n-3* PUFA Phospholipid

Marine animals have high amounts of *n-3* polyunsaturated fatty acids (PUFA) such as EPA and DHA. Unlike fish oil that most of o *n-3* fatty acids in corporate into triglyceride, the krill oil majority of EPA and DHA are contained in the phospholipid. The percent of phospholipid in oil extracted from krill is typically 40 to 45%. Clinical and pre-clinical studies that compare krill oil and fish oil suggested that krill oil may be more effective than fish oil in terms of bioavailability for tissue cells. Graf et al., (2010) studied that in older rats, the incorporation of DHA was more than twice as high from phospholipid than from triglycerides in e.g. brain, liver and kidney. In the brain, DHA uptake was significantly higher in 11 out of 14 brain regions after phospholipid administration compared to triglyceride administration. Numerous studies in humans, such as study the study by Schaefer et al., (2006), had linked low DHA levels in blood plasma to brain-related disorders like Alzheimer's disease, and several different protective roles of DHA in brain had been suggested.

N-3 PUFA phospholipid not only exists in krill, but also found in shellfish and fish roe, especially the pollock roe and squid eggs. Wang (2008) extracted squid egg to get phospholipid product (more than 85% of phospholipid) contained over 60% of PC in weight and over 30 % of DHA in fatty acids by ethanol assisted SFE.

Kolakowska (1986) proved that phospholipid were very sensitive in frozen krill stored in-22ºC. Meanwhile, triglyceride was quite stable. Frozen process itself was only slightly affected by the lipid composition. However, after 30 days of storage, free fatty acid concentration almost doubled. Storing the -22ºC, 6 months, 70% of the phospholipid were decomposed, and free fatty acids amount increased to 6-20 fold. Monoglyceride was absence in fresh krill, but it appeared after several months frozen storage.

Transesterification of soybean phospholipid and fish oil catalyzed by immobilized phospholipase A1 in a solvent free system to synthesize phospholipid enriched with *n-3* polyunsaturated fatty acids is successful. The optimal conditions were as follows: 20% immobilized phospholipase A1 (basis of the phospholipid mass), mass ratio of fish oil to soybean phospholipid 8∶1, the total mass of the substrate 510 g, water dosage 55μL, temperature 55 ºC, time 12 h. Under the optimal conditions, the concentration of EPA and DHA in the finished product was 8.0% and 17. 8% (Sun et al., 2010).

1.2.3 Astaxanthin

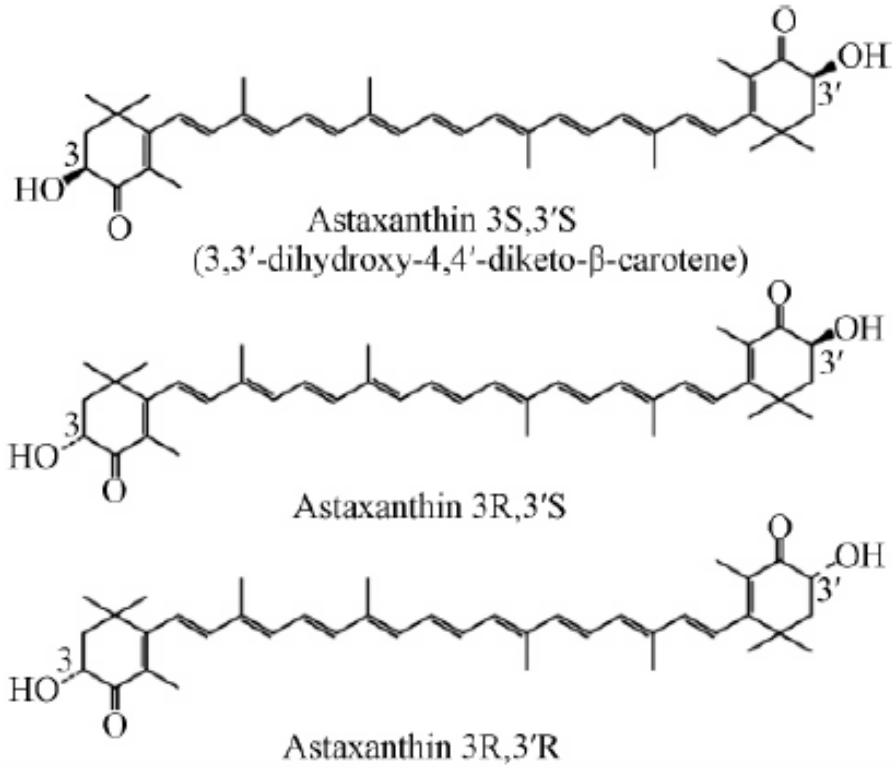


Figure 1. Chemical structures of astaxantin stereo isomers.

Astaxanthin (AX) is a carotenoid widely used in salmonoid and crustacean aquaculture to provide the pink color. Like many other carotenoids, AX is a lipid-soluble pigment which has a conjugated double bond chain at the centre of compound. Common sources of natural AX are the green algae *Haematococcus pluvialis*, the red yeast, *Phaffia rhodozyma*, as well as crustacean byproducts.

AX has two chiral centers, at the 3- and 3′-positions. Therefore, there are three stereo isomers; (3R,3′R), (3R,3′S) (meso), and (3S,3′S). Synthetic AX contains a mixture of the three, in approximately 1:2:1 proportions. AX from *P. rhodozyma* is (3R,3′R) isomer; AX from crustacean aquaculture and *H. pluvialis* is mainly (3S,3′S) isomer which has better bioactivity for human.

AX is claimed to an antioxidant. It can protect the human body against neurodegenerative conditions, UV-light effects, cancer and improve its immune system. It can reach into every part of the cell, so it works in each part of human body, organs and skin (Higuera-Ciapara et al., 2006).

1.3. Manufacturing Process

1.3.1 Different process comparison

Table 5. Advantages and disadvantages compared by different patented process.

Name of company	Critical process	Advantages	Disadvantages	Patent
Aker	Cooking, air drying, ethanol extraction	Easy, cheap, large capacity	Poor KO quality and extractability	Aker FDA 2010
Olympic	Enzymolysis, vacuum drying, SFE	High KO quality and extractability,	Expansive, small capacity	US20130225794
Neptune	Acetone dehydration, ethyl acetate extraction	High Astaxanthin concentration	Solvent residue, explosive	EP01417211B1
Chile	Cooking, squeezing, Centrifugation	Not apply solvent extraction	Poor phospholipid extractability	CN 102311868A
Extrusion	Extrusion, air drying, ethanol extraction	High extractability	Oxidation, Poor KO quality	CN 102952625A

KO: krill oil
SFE: supercritical fluid extraction

There are several process combinations using krill meal to obtain krill oil. However, extraction methods differ. MSO^{TM} applied by Enzymotec (an Israely Company) is a multi-stage solvent extraction process exclusively developed and designed for krill oil extraction and purification, with improved physical properties include decreased odor, increased fluidity and low TVN level. Patent EP2612672 (by Aker Biomarine AS, Norway) shows that krill oils can be obtained from krill meal using supercritical fluid extraction in a two stage process. Stage 1 removes the neutral lipid by extracting with neat supercritical CO_2 or CO_2 plus approximately 5% of a cosolvent. Stage 2 extract the actual krill oils by using supercritical CO_2 in combination with approximately 20% ethanol.

1.3.2 SUPERBA™ Krill Oil Process

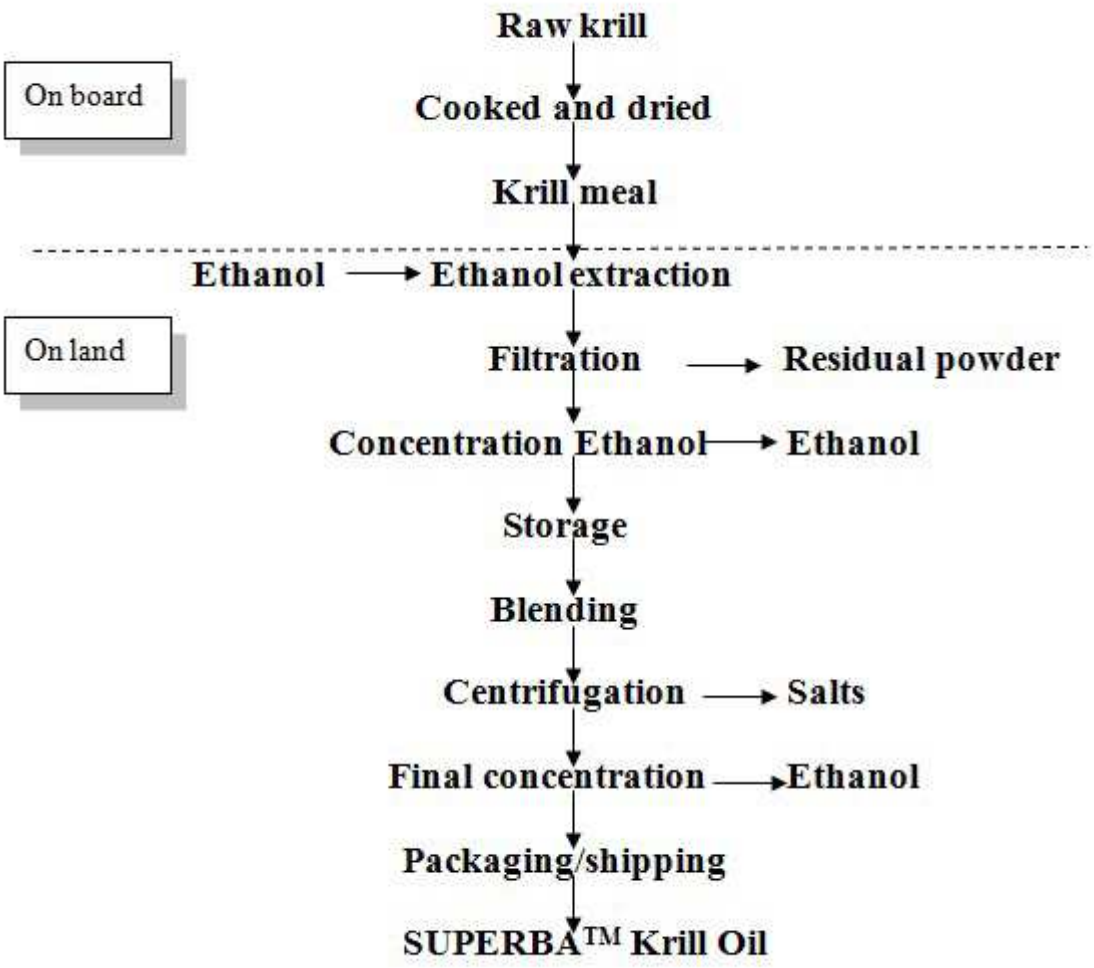


Figure 2. Manufacturing process diagram of SUPERBA™ Krill Oil

Manufacturing process

The Superba™ method employed by Aker Biomarine AS, is described in Fig. 2. The harvested Antarctic krill is cooked and dried on the vessel to prepare krill meal. The raw material that is cooked and dried, krill meal, is a biomass composed of lipids, carbohydrates, and proteins. Krill meal is carefully packed and ship to land. By using food-grade ethanol extraction, the proteins and free carbohydrates are removed.

Following extraction, the defatted krill meal and the ethanol oil solution are separated, The ethanol-oil solution is then concentrated by evaporation and stored. The ethanol-oil solution is analyzed for ethanol, neutral and polar lipids, and astaxanthin

concentration. Several batches are blended and the ethanol-oil solution is clarified by centrifugation. The ethanol is then evaporated from the oil solution and the final product is analyzed to verify the conformity with product specifications. The final product is filled into suitable containers and stored at 2-8°C and can be shipped by land, air, or boat.

1.3.3 RIMFROST™ Krill Oil Process

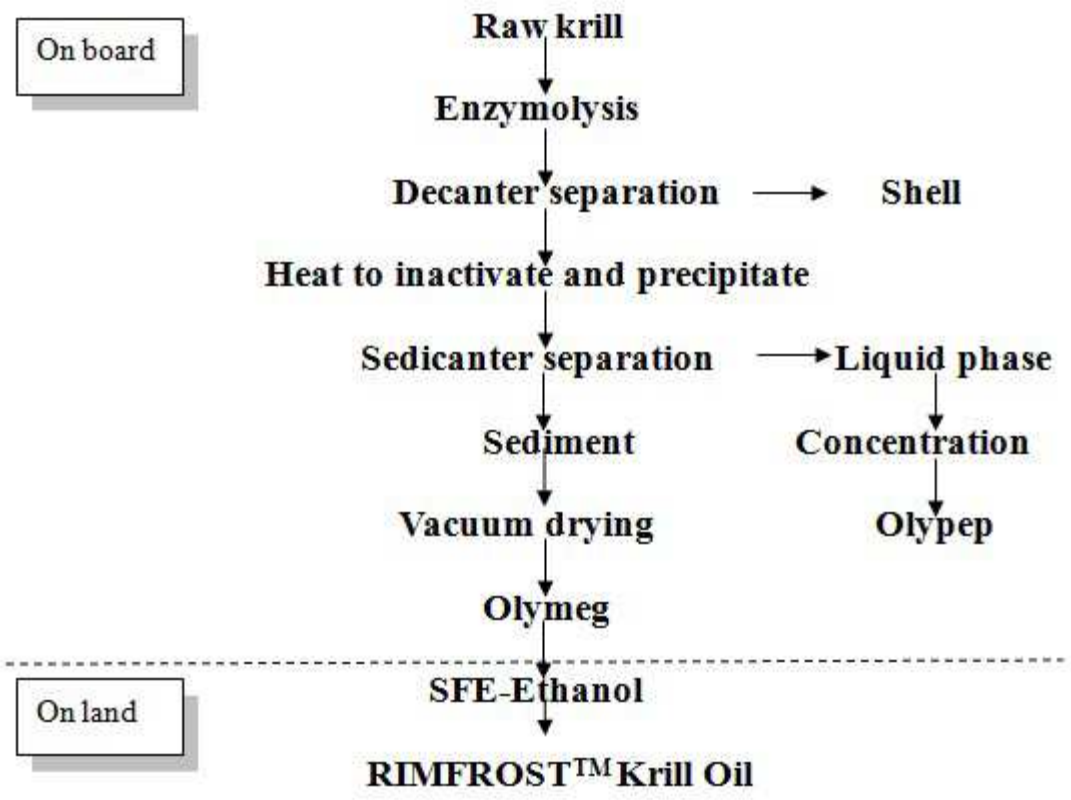


Figure 3.Manufacturing process diagram of of RIMFROST™ Krill Oil

The Rimfrost™ method employed by Olympic is described in Fig. 3. As soon as Antarctic krill is fished, protease is added together with autolytic enzyme for efficient hydrolysis. Antarctic krill is liquefied and shell is separated. Since fluoride mainly accumulated in exoskeleton, de-shelling will decrease fluoride level in products obtained further down the processing line. Then the material is heats to inactivate enzymes including lipase and PLase. Moreover, high temperature contributes to lipid-protein precipitation. After that, sedicanter is applied to form three layers: neutral oil phase, peptide phase and lipid-protein sediment. Lipid- protein sediment dries by vacuum drying afterwards. The dried product enriches krill lipids, named "Olymeg" or PPC, is preserved below 0 °C. Concentrated peptide phase, named "Olypep"or CHF, contains 55-70% dry matter.

"Olymeg" is thawed before it is subject to ethanol assisted SFE. Enzymolysis combined with SFE will increase krill oil extractability and have high comprehensive

utilization.

1.4 Aim of study

Oil extraction based on enzymolysis is a promising new technology as a result of its high extractability, mild conditions and environment friendly. Enzymolysis applied in krill oil extraction will get three products: krill hydrolysate (peptide and salt), krill oil and krill protein. In this case, oil extraction based on enzymolysis will have better comprehensive utilization and result in higher added value.

Krill PL enriched EPA and DHA account for more than 40% (w/w) in krill oil. However, PL in vegetable oil normally less than 1%, therefore common extraction methods (pressing or hexane extraction) is not effective for extract PL rich krill oil.

To increase PL rich krill oil extractability, enzymolysis was applied for degraded protein and lipid complex structure. Enzymolysis is affected by enzyme species, krill and water ratio, temperature, pH, enzyme dosage, and time. This study targeted maximi9zed degree of hydrolysis (DH) by optimizing these affecting factors.

Extraction parameters (solvent species, ratio, temperature, time and frequency) were also optimized by orthogonal experiment targeting high extractability, PL concentration, AX concentration and low acid value.

To get high quality and high extractability PL rich krill oil, anti-oxidation, defluorination and inactivation of lipase are also under consideration.

2. Materials and methods

2.1 Materials and Chemicals

The Antarctic krill was supplied by a Chinese factory vessel. After catch, the krill were drained and placed in molds for quick-freezing at about -30°C on board the ship after harvest. The resulting 13 kg blocks of Antarctic krill were individually wrapped in plastic film and are packaged two per carton. The packaged Antarctic krill are maintained at temperatures around -20°C.

The following proteases were used:
Neutral protease: from *Bacillus subtilis*, Nanning Pangbo biotech company, Guangxi, China. Actual activity: 368 U/mg
Alkaline Protease: Trade name: Alcalase, from *Bacillus licheniformis*, Nanning Pangbo biotech company, Guangxi, China. Actual activity: 383 U/mg
Papain: from *Carieapapaya*, Nanning Pangbo biotech company, Guangxi, China. Actual activity: 220 U/mg
Flavorzyme: from *Aspergillus oryzae*, Nanning Pangbo biotech company, Guangxi, China. Actual activity: 35 U/mg
Trypsin: from porcine pancreas, Nanning Pangbo biotech company, Guangxi, China. Actual activity: 244 U/mg

95.5% ethanol was food grade, other reagents were analytical grade.

2.2 Instrumentation

The following instruments were used for anlyasis

Automatic potentiometer	Mettler Toledo	T50
Kjeldahl protein analyzer	Shengsheng (China)	K06
Automatic fat analyzer	Alvaro (China)	SE-A6 (Soxhlet extraction)
UV-Vis spectrophotometer	Youke (China)	UV759 CRT
GC system	Agilent	7820A

(Chromatographic column 1m × 4mm (id), filled with 80~100 mesh supporter coated with 10% DEGS/Chromosorb W DMCS, FID detector)
High speed freezing centrifuge Xiangyi (China) GL-21M

2.3 Analytical methods

Routineous analyses were carried out in accordance with China National Food Safety Standard Determination Methods, indicated by GB.

Ash content: heat ignition method (GB 5009.4-2010)
Weighed 3.0~5.0g sample in crucible that dried to constant weight. First used electromagnetic oven to carbonize until no smoke was detected. Then the samples were transferred to muffle furnace burning 4h in 550°C. When cooled below 200°C, samples were transferred to a desiccator, and weighed again when room temperature was reached.

Acid value: KOH-EtOH titration method (GB/T 5530-2005)
Weighed 0.2~0.3g krill oil sample in plastic cup, added 50mL 99.5% ethanol, dispersed uniformly by ultrasonication. The cup was then transferred to an Automatic potentiometer. Titrated to pH=7 with continuous KOH-EtOH titration, indicated acid value.

Astaxanthin concentration: Dimethyl sulfoxide (DMSO) extraction (SN/T 2327)
Weighed 0.5g sample in centrifuge tube, added 2mL DMSO and water bath 70°C, 5min, then centrifuged in 3500 rpm/ min, 5min. Repeated DMSO extraction again. Transfered all upper layer to 10mL measuring flask, diluted it to10mL, mixed well. Sucked 1mL, diluted 10 times, measured 489nm absorbance by UV-Vis spectrophotometer.

Crude protein concentration: Kjeldahl method (GB 5009.5-2010)
Weighed 2.0~5.0g sample in tube, add 0.2g $CuSO_4 \cdot 5H_2O$, 6g K_2SO_4 and 20mL 98% H_2SO_4. Heated carefully until contents turned to blue and green. Cooled down and installed tube to Kjeldahl protein analyzer. Analyzer dropped NaOH (aq) and bubbled steam, then NH_3 was absorbed by boric acid in titration cup. At last, used standard HCl (aq) titration.

Crude fat concentration: Soxhlet extraction (GB/T 14772-2008)
Weighed 4.0~5.0g sample, added petroleum ether to extracton tube volume 2/3, refluxed 6h in Automatic fat analyzer , cooled down and evaporated petroleum ether, weighed again.

DH: formol titration method (GB/T 5009.39-2003)
Amino acid nitrogen determination:
Sucked 5.0mL sample, diluted it to 100mL, mixed well. Sucked 20mL mixed with 60mL water, dropped 0.05 mol/L NaOH (aq) until pH = 8.2, then added 10mL formol solution (36% HCHO) to fix NH_2, dropped 0.05 mol/L NaOH (aq) until pH = 9.2.
DH = Amino acid nitrogen / Total nitrogen (determined by Kjeldahl method)

DHA and EPA concentration: methanol esterified - GC method (GB/T 5009.168-2003)
Weighed 1.0~2.0g sample, dissolved by 10mL n-Hexane, sucked 1.00~5.00mL to another tube, added 2mol/L NaOH (methanol solution), 60°C water bath for methyl esterification. Standard solution also esterified by methanol.

Chromatographic column 1m × 4mm (id), filled with 80~100 mesh supporter coated with 10% DEGS/Chromosorb W DMCS.
Gas: N_2 50mL/min, H_2 70mL/min, air 100mL/min.
Temperature: Chromatographic column 185°C, inlet 210°C, detector 210°C.

Enzyme activity: Folin-phenol method (GB/T 23527-2009)
Weighed 1.00g enzyme, diluted 10,000 times with buffer, suck 1mL, added 1mL casein (aq), water bath 40°C, 10min, and then added 2mL trichloroacetic acid (aq) to terminate reaction. After filtration, added Na_2CO_3 (aq) and Folin reagent, measured 680nm absorbance by UV-Vis spectrophotometer.

Fluoride concentration: fluoride electrode method (GB/T 5009.18)
Weighed 1.0g crushed sample, added 10 mL HCl (1+1, aq), and extracted 1h. Combined fluoride electrode and calomel electrode to measure fluoride concentration.

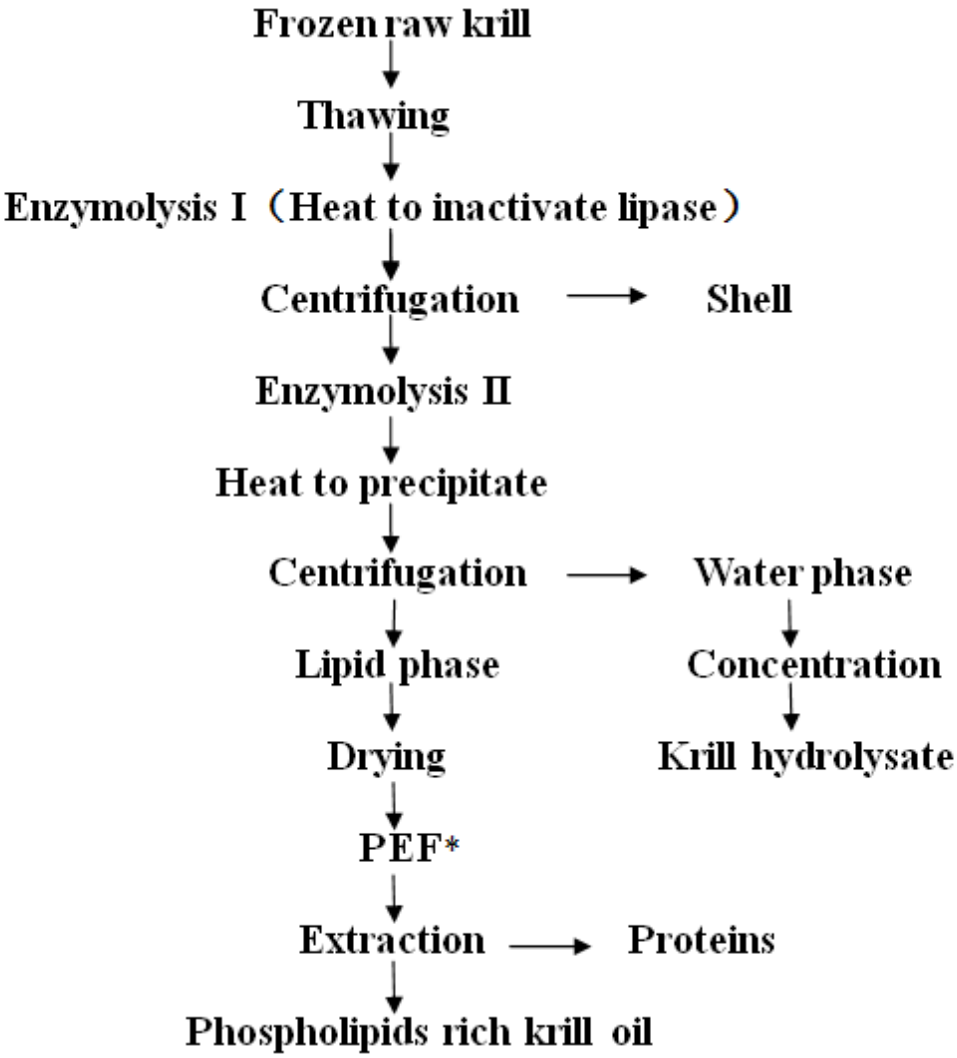
Moisture content: oven drying method (GB 5009.3-2010)
Weighed 5.00~10.00g sample in weighing bottle, put it in 95~105°C drier for 2~4 h, weighed again.

Phospholipid concentration: molybdenum blue colorimetric method (GB/T 5537-2008)
Weighed 2.0~3.0g krill oil in crucible, added ZnO 0.5g, used electromagnetic oven to carbonize until no smoke. Then put crucible to muffle furnace burning 4h in 550°C. After cooled down, added HCl (aq) to free phosphorous. At last, added hydrazine sulfate and sodium molybdate to create blue colour, measured 650nm absorbance by UV-Vis spectrophotometer.

Peroxide value: Fe^{2+} oxidation colorimetric method (GB/T 5009.37-2003)
Weighed 0.1~1.0g sample, add $FeCl_2$(aq). Fe^{2+} was oxidized to Fe^{3+}. $Fe^{3+}$and SCN^- created red colour, measured 500nm absorbance by UV-Vis spectrophotometer.

2.4 Process of extraction krill oil by enzymolysis

The scheme for hydrolysis and extraction followed in the experiments is presented in Fig. 4.



*PEF was defined as freeze dried lipid phase in this study.

Figure 4. Scheme for hydrolysis and extraction

Due to lacking availability of fresh krill, frozen raw krill stored in -20°C was used for this study. Krill hydrolysate was similar to “Olypep” produced by Olympic, but contains a higher proportion of small peptides. Concentration aimed at decrease water activity, so krill hydrolysate can be stored in room temperature.

2.5 Orthogonal design and analysis

Several of the experiments were designed and analyzed by orthogonal collocation, in accordance with Ferguson & Finlayson (1970).

2.6 Individual experiments

The following experiments were conducted (Table 6):

Table 6. List of experiments

Experiment	Aim
Enzymolysis I	To identify the most suitable protease and enzyme concentration for separation of krill shell
Lipase inactivation	To ensure krill oil quality, keep low acid value and high PL concentration in krill oil.
Enzymolysis II	To identify the most suitable protease and optimize conditions for decomposing complex of protein and lipid
Centrifugation	To separate lipid and water phase without oil float
Drying	To reduce weight and stability, and to facilitate high krill oil quality
Extraction	To extract PL rich krill oil as completely as possible and maintain good quality of the krill PL

Expt. Enzymolysis I:
As stated in introduction, krill shell concentrated most of fluoride, therefore, exoskeleton removal is important for decreasing fluoride concentration. An experiment was set up to compare the efficiency of neutral protease, alcalase, papain, flavorzyme and trypsin for shell removal during Enzymolysis I. Incubation took place under the conditions of HTe = 55~60ºC, KWR = 2:1, HTi = 15min (with sufficient stirring). Enzyme doses of 0 (endogenous enzyme activity only), 750 and 1,500 U/g were tested. The evaluation criterion used was visually detectable shell after centrifugation.

Expt. Lipase inactivation:
Lipase (including PLase) has adverse effect for krill oil manufacturing that will decompose triglyceride and phospholipid, increase acid value. Like pasteurization, enzyme inactivation depended on both temperature and time. Both high temperature, short time and low temperature, long time will inactivate lipase. This experiment was

fixed time in 10 min, five temperature zone in every 10ºC was investigated: 50~55ºC, 60~65ºC, 70~75ºC, 80~85ºC, 90~95ºC. Both acid value and PL concentration in krill oil data was used for evaluating lipase activity.

Expt. Enzymolysis II:
Six single factor experiments and one orthogonal experiment were conducted in enzymolysis II. Single factor experiment aimed at finding control range of orthogonal experiment parameters, while orthogonal experiment aimed at optimizing these parameters. These single factor experiments were: KWR with DH (no water, 2:1, 1:1, 1:2); enzyme species with DH (neutral protease, alcalase, papain, flavorzyme, trypsin); ED with DH (1000U/g, 2000 U/g, 3000 U/g, 4000 U/g), hydrolysis pH (7.0, 7.5, 8.0 8.5); HTe with DH (45ºC, 50ºC, 55ºC, 60ºC); HTi with DH (0h, 2h, 4h, 6h, 8h). Chosen alcalase and HTe = 55ºC, orthogonal experiment $L_9(3^4)$ KWR, pH, ED, HTi were conducted.

Expt. Centrifugation:
Centrifugation was applied after heating for precipitation. Time was fixed at 5 min, speed 266G (2000 rpm), 600G (3000 rpm), 1066G (4000 rpm) was investigated by high speed freezing centrifuge. The evaluation criterion used was PEF solidity and whether oil float by vision.

Expt. Drying:
Lipid phase contained around 50% (w/w) water. As I studied in extraction procedure, too much water resulted in krill oil bad fluidity and high acid value. Therefore, drying was not only to shrink weight, but also guarantee krill oil quality. Air drying and freeze drying was compared by PEF state and acid value. Afterwards, PEF was compared by Olymeg in chemical components aspects.

Expt. Extraction:
Conducted orthogonal experiment $L_9(3^3)$ PER (1:6, 1:8, 1:10), ETe (25ºC, 35ºC, 45ºC), ETi (1h, 2h, 3h) and measured following results: extractability, PL concentration, AX concentration, acid value. Each result set was analyzed respectively to optimize conditions.

3. Results and discussion

3.1 Enzymolysis I

Only autolytic protease (no enzyme added) was found ineffective, while all added enzymes added at either concentration were efficient for deshelling during 55~60ºC, 15 min in sufficient stirring (Table 7). Insufficient enzymolysis lead to hydrolysate viscosity increasing which can limit separation by centrifugation.

Table 7. Visual observation of the effect of enzymolysis I and enzyme dosage on separation of shells from the krill meat fraction.

Dosage of neutral protease or alcalase, U/g	**0 (endogenous enzyme only)**	**750**	**1500**
Meat residual in shell	Visible	Not visible	Not visible

Alcalase was cheaper than neutral protease, and both enzymes were effective for enzymolysis. Since industrial application was the final aim for this research, alcalase was chosen for further experiments.

3.2 Lipase inactivation temperature

Table 8. Acid value and PL concentration in krill oil, obtained by 10 min incubation with alcalase at different temperatures.

(10min)	**50~55ºC**	**60~65ºC**	**70~75ºC**	**80~85ºC**	**90~95ºC**
Acid Value	33.2	26.3	26.1	26.5	27.2
PL in KO (%)	31.3	30.5	30.3	30.3	30.1

PL: phospholipid
KO: krill oil

Table 8 shows that during 50~55ºC (10min), the acid value was higher than for the other incubation temperatures. This indicates that lipases were active. When HTe > 60ºC, the acid value was reduced, and both acid value and PL concentration varied little. This indicates that at HTe > 60ºC, the lipases were inactivated within 10min. In this situation (60ºC, 10min), Alcalase was still active (table 7). This temperature regime is also within the temperature range which proteases in krill are active, according to Dai et al.,（2012), who found that krill autolytic protease had high activity in 50~60ºC, and that maximum DH was achieved at HTe = 55ºC. The autolytic proteases had both endoprotease and exoprotease activity. Thus, autolytic protease combined with neutral protease or alcalase can save enzyme dosage, still

obtaining a high DH.

Thus, HTe at 60~65ºC seemed to be optimal with respect to avoiding free fatty acid liberated from the lipids, efficient extraction facilitated by proteolysis, and moderate heating to minimize heat-induced destruction of the PL oil.

3.3 Enzymolysis II

3.3.1 Frozen krill and water ratio

Table 9. Degree of hydrolysis obtained by different frozen krill to water ratios.

KWR	Protein concentration %	DH %
1:0	13.1	21.9
2:1	7.8	31.1
1:1	5.8	24.3
1:2	3.7	22.7

DH: degree of hydrolysis
KWR: frozen krill and water ratio

Table 9 shows that when no water added, in conditions of HTe = 55ºC, pH = 8.0, HTi = 6h, alcalase 3000U/g, initial DH=20.7%, DH was only 21.9%. DH first increased, and then decreased as the water content increased. Enzymatic reactions are often related to the concentration of substrate. If the protein concentration is too high, protease wrapped by protein will decrease protease diffusion. Hydrolysis will be inhibited. When the water content rose, protein dilution can release hydrolysis inhibition. However, too high water content will decrease the proteolysis, and DH will consequently decrease.

The Picture 3 in Appendix III also indicates that the more water the hydrolysate contained, the more effective was the separation. In accordance with Stoke's law, particle free settlement velocity increases with increasing viscosity. Viscosity decreases with increasing water (Jones & Talley, 1933).Therefore, increasing water content resulted in better separation. However, too much water may limit water phase evaporation, energy consumption will increase.

Thus, KWR = 2:1 seemed optimal as it gave the highest DH (DH=31.1%), and required only moderate amounts of water to be added.

3.3.2 Enzyme selection

Table 10. Degree of hydrolysis obtained by different enzymes in its best conditions.

Protease species	Enzyme activity U/mg	HTe °C	pH	DH %
Neutral protease	368	50	7.0	29.9
Alcalase	383	55	8.0	31.1
Papain	220	50	7.0	25.8
Flavorzyme	35	50	7.0	41.7
Trypsin	244	45	7.5	27.0

DH: degree of hydrolysis
ED: enzyme dosage
HTe: hydrolysis temperature

Table 10 shows that the use of flavorzyme resulted in the highest DH (DH=41.7%) in conditions of KWR = 2:1, ED = 3000U/g, HTi = 6h, initial DH = 20.7%. It worth to note that flavorzyme is an exoprotease that can hydrolyse protein or peptide one by one amino acid at the time. In this case, amino acid content in flavorzyme hydrolysate was quite high. The aim of this study was to decompose protein-lipid complex, not to obtain free amino acids. Alcalase was the endopeptidase giving the highest DH (DH=31.1%). Alcalase was also heat stabile, at 60ºC (DH=31.4%, data from 3.3.5), while other enzymes were partially or completely inactivate during these conditions. Alcalase was also the cheapest among these 5 proteases. Thus, this enzyme was chosen for the subsequent experiments.

3.3.3 Enzyme dosage

Table 11. Degree of hydrolysis obtained by different alcalase dosages.

ED U/g	DH %
1000	27.6
2000	29.4
3000	31.1
4000	31.7

DH: degree of hydrolysis
ED: enzyme dosage

According to Table 11, in conditions of KWR = 2:1, HTe = 55ºC, pH = 8.0, HTi = 6h, Alcalase, initial DH=20.7%, DH increased linearly by dosage increased within 1000U/g~3000U/g. When the dosage reached 3000~4000 U/g, increased velocity per unit enzyme added decelerated. In consideration of enzyme cost and efficiency, 3000 U/g seemed to be optimal enzyme dosage.

3.3.4 Hydrolysis pH

Table12. Degree of hydrolysis obtained by different hydrolysis pH.

Hydrolysis pH	**pH_{55^oC} after 6 h**	**pH_{25^oC} after 6 h**	**DH %**
7.0	6.8	7.3	25.8
7.5	7.1	7.8	27.6
8.0	7.5	8.2	31.1
8.5	8.0	8.7	24.3

DH: degree of hydrolysis

Alcalase is an alkaline protease, meaning that it works best at pH>7. This was confirmed by the results in Table 12, showing that in conditions of KWR = 2:1, HTe = 55ºC, HTi = 6h, Alcalase 3000U/g, initial DH=20.7%, the pH optimum for alcalase was 8.0 (DH=31.1%). Lower or higher pH decreased DH.

Enzymes are proteins. Enzyme activity is affected by pH due to pH difference can change protein spatial (tertiary and quaternary) structures, both being essential for enzyme activity.

3.3.5 Hydrolysis temperature

Table 13. Degree of hydrolysis obtained by different hydrolysis temperature.

Hydrolysis temperature °C	**DH %**
45	29.4
50	30.2
55	31.1
60	31.4

DH: degree of hydrolysis

Beside pH, hydrolysis temperature also affect protein space structure, low temperatures will result in low hydrolysis rate and low DH. Overheating will irreversible denature protein and cause oxidation, and thereby inactivate the enzymes.

In conditions of KWR = 2:1, HTi = 6h, pH = 8.0, alcalase 3000U/g, initial DH=20.7%, DH rose with temperature increasing from 45 to 60ºC (Table 13). Rising the temperature from 55ºC (DH=31.1%) to 60ºC (DH=31.4%) had marginal effect. Taking the increased energy consumption into consideration, 55ºC was considered as optimal hydrolysis temperature.

3.3.6 Hydrolysis time

Table 14. Degree of hydrolysis and pH obtained by different hydrolysis time.

Hydrolysis time h	pH	DH %
0	8.0	20.7
2	7.6	26.6
4	7.5	30.4
6	7.5	31.1
8	7.4	31.9

DH: degree of hydrolysis

Table 14 shows that DH increased over time in conditions of KWR = 2:1, HTe = 55ºC, pH = 8.0, alcalase 3000U/g, initial DH=20.7%. The hydrolysis seemed to have two stages: 0~4 hours, fast hydrolysis, since DH increased quickly by time; 4~8 hours, slower hydrolysis, since DH increased slowly by time. Cui (2005) argued that the slowdown of hydrolysis rate was due to decrease in specific peptide bonds available for enzyme action.

3.3.7 Orthogonal experiment（$L_9(3^4)$ KWR, pH, ED, HTi）

Table 15. Degree of hydrolysis obtained by orthogonal experiment ($L_9(3^4)$ KWR, pH, ED, HTi).

	KWR	pH	ED	HTi	DH %
1	1:0	7.0	2000	4	21.4
2	1:0	7.5	3000	6	23.3
3	1:0	8.0	4000	8	24.0
4	2:1	7.0	3000	8	29.5
5	2:1	7.5	4000	4	30.0
6	2:1	8.0	2000	6	27.7
7	1:1	7.0	4000	6	25.9
8	1:1	7.5	2000	8	23.7
9	1:1	8.0	3000	4	26.0
$\overline{K1}$	22.9	25.6	24.3	25.8	
$\overline{K2}$	29.1	25.7	26.3	25.6	
$\overline{K3}$	25.2	25.9	26.6	25.7	
R	6.2	0.3	2.3	0.2	

DH: degree of hydrolysis
ED: enzyme dosage
HTi: hydrolysis time
KWR: frozen krill and water ratio

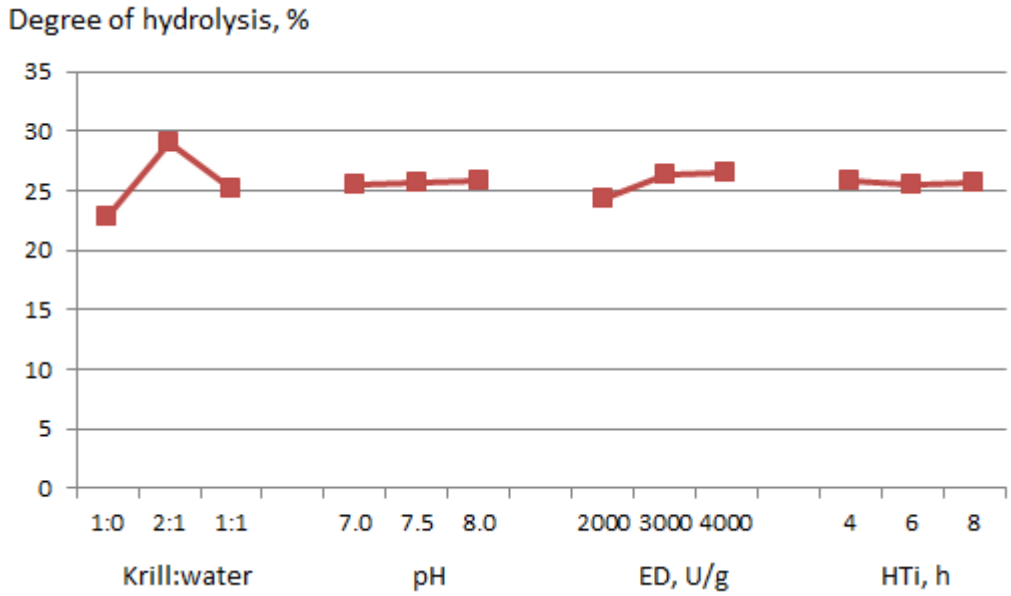


Figure 5. Degree of hydrolysis as dependent on krill:water ratio, pH, alcalase concentration (ED), and hydrolysis time (HTi), analyzed by orthogonal experiment analysis

Range analysis in orthogonal experiments (Table 15, Fig. 5) gave the following ranking of influencing factors: KWR (R = 6.2) > ED (R = 2.3) > pH (R = 0.3) > HTi (R = 0.2). Time was the least influencing factor, since DH was almost the same at 4h as at 6 and 8h. Therefore, 4h was considered as optimal hydrolysis time.

In conditions of KWR = 2:1, HTe = 55ºC, pH = 8.00, alcalase 3000U/g, HTi = 4h, DH = 31.1 % was found. Peptides with an average length of 3.2 and a molecular mass of 352 were obtained.

3.3.8 Effect of end pH

Table 16. Acid value and krill oil appearance obtained by different end pH.

	pH=6.6	**pH=7.0**	**pH=7.4**
Acid value	29.9	29.9	29.2
Krill oil appearance	Little opaque High fluidity	Opaque Medium fluidity	Opaque Low fluidity

Neutral $pH_{50ºC}$ is 6.5. This means that both $pH_{55ºC}$=6.6, 7.0 and 7.4 were alkaline (Table 16). As pH increased, krill oil turned opaque and the fluidity decreased. This can partly be rationalized by saponification of the lipids. Higher fatty acid salts (e.g. sodium stearate) are opaque and will cause decreasing fluidity. However, the acid value did not vary much. Thus, adjusting acid value through titration was not considered practical.

On the other hand, as can be seen from Picture 4 in tAppendix III, lipid and water phase of end pH=6.6 separated more completely compared pH=7.0 and 7.4. Better separation allows the centrifuge to be run at lower speed and for a shorter time, both resulting in increased capacity and reduced energy consumption.

It is necessary to adjust end pH to 6.5~6.7 for alcalase hydrolysis. When neutral protease was applied, incubation was simplified, without any need for pH adjustment. However, neutral protease was more expensive than alcalase and resulted in lower DH.

3.4 Centrifugation speed

Table 17. Separation effect obtained by different centrifugation speeds.

(5min)	266xG	600xG	1066xG
separation effect	Soft solidity PEF No oil float	Medium solidity PEF No oil float	Medium solidity PEF Little oil float

After adjusting end pH = 6.5~6.7, T = 75~80ºC, time = 5 min, heating to inactivate protease, separation occurred into a lipid phase and a water phases in the same time. Centrifugation was applied immediately for further separation, when the hydrolysate was still warm.

The effect of speed was investigated with time fixed at 5min (Table 17). The speed resulting in 600xG gave medium solidity and no oil float. At lower speed (266xG), more water remained in PEF. At higher speed (1066xG), oil was floating on the top (three layers). Based on this, 600xG gave optimal separation.

3.5 Drying

3.5.1 Comparison of air drying and freeze drying

Table 18. Dried state and acid value obtained by air drying and freeze drying.

	Air Drying	Freeze Drying
Drying temperature	50°C	-50°C
Dried state	Paste	Sticky powder
Acid Value (mg KOH/g)	29.6	27.2

Two different drying methods were compared (Table 18). Air drying has higher processing capacity and is cheaper than freeze drying. However, freeze drying resulted in better lipid quality (acid value 27.2 mg KOH/g), compared to air drying (29.6 mg KOH/g). Freeze drying also produced material with a better dried state:

sticky powder, easy to disperse in solvent. Thus, freeze drying was preferred compared to air drying.

3.5.2 Comparison between PEF and Olymeg

Table 19. Chemical composition obtained by PEF and Olymeg.

	Moisture content (%)	Crude protein (%)	Total fat （%）	PL in total fat (%)	Ash (%)	Acid value (mg KOH/g)
Olymeg	6.4	44.9	43.2	44.3	5.0	11.4
PEF	1.2	23.0	74.4	31.0	7.4	27.2

PL: phospholipid

Table 19 shows that after 4h alcalase hydrolysis, PEF contained more fat (74.4% > 43.2%) and less protein (23.0% <44.9%) than Olymeg. Most krill protein hydrolyzed to small peptide and was recovered from the water phase. The rest protein or non-water soluble peptide remained in PEF. Therefore, protein concentration decreased and fat concentration increased by adequate hydrolysis. Furthermore, the acid value increased from the value in frozen krill at 18.1 mg KOH/g to 27.2 mg KOH/g in PEF.

The acid value from frozen krill was higher than Olymeg (11.4 mg KOH/g). The most probable reason was that the krill was captured in May, analyzed in November. Although the frozen krill was stored in -20ºCduring 6 months, low temperature still cannot stop hydrolyzing. In another experiment, I handled krill meat stored in -20ºC for 2 years. After thawing, the texture of this krill meat was just like gravy. The acid value was up to 100 mg KOH/g, and the PL concentration in KO was as low as 5~6%. However, the AX concentration did not change much.

Thus, the PEF manufacturing process should be moved to the ship, immediately after catching the krill, in order to avoid storage-induced hydrolysis and deterioration of the krill.

3.6 Extraction

3.6.1 Solvent selection

Table 20. Krill oil extractability, phospholipid (PL) concentration, astaxanthin (AX) concentration and acid value obtained by different solvents used for extraction.

	99.5% Ethanol	**95.5% Ethanol**	**Acetone**	**Ethyl acetate**	**Chloroform methanol 2:1**
Extractability (%)	70	54	50	62	79
PL (%)	33.0	40.4	9.4	30.5	30.3
AX（ppm）	166.5	113.8	223.8	181.1	151.1
Acid value (mg KOH/g)	28.0	46.2	30.6	27.8	30.6

PL: phospholipid
KO: krill oil
AX: astaxanthin

Table 20 shows that the use of different solvents highly differed in extraction efficiency in conditions of PEF: solvent = 1:10, ETe = 25ºC, ETi = 2h.

Acetone is generally applied for industrial PL purification (removal of non-polar lipid), as illustrated by the low proportion of PL in the lipid extract. However, krill oil extracted from acetone still contained 9.4% PL. This demonstrated that krill PL has special features. The most striking feature is the high concentration of *n-3* PUFA in PL, leading to special physicochemical characteristics.

Ethyl acetate also can be used as a solvent for PL rich KO extraction (PL=30.5%), but it is expansive and less commonly used than ethanol.

99.5% ethanol had higher extractability (70% > 54%), gave higher AX concentration (166.5ppm>113.8ppm), but lower PL concentration (33.0% < 40.4%) compared with 95.5% ethanol. This indicates that 99.5% ethanol was an efficient solvent to extract both triglyceride, PL and AX.

The residue after extraction with 99.5% ethanol was a powder, while 95.5% ethanol residue was more like an oily paste.

The extract obtained with 95.5% ethanol extract had the higher acid value (46.2 mg KOH/g) than that obtained with 99.5% ethanol (28.0 mg KOH/g) and other solvents. One main reason for the differences in extractability is that 95.5% ethanol contains more water than 99.5% ethanol. This may facilitate extraction of proteins and other amphoteric substances that may increase acid value during KOH titration. However,

protein concentrations did not differed much (KO from 99.5% ethanol: protein concentration 9.7%; KO from 95.5% ethanol: protein concentration 10.1%). A more probable reason may be that 95.5% ethanol had higher free fatty acid extractability than 99.5% ethanol and the other solvents.

As impurity extractability and PL concentration increased, KO from 95.5% ethanol had bad fluidity and opaque appearance. Chloroform methanol 2:1 is a standard method to analyze total lipids concentration (Folch et al., 1957). The use of the same solvent system resulted in the highest extractability (79%). However, chloroform and methanol are toxic and their use is not allowed in food or pharmaceutical industry.

Both considering total extractability of total lipid, PL and AX, and the acid value of the extract, 99.5% ethanol seemed optimal to extract KO.

3.6.2 Orthogonal experiment（$L_9(3^3)$ PER, ETe, ETi）

Table 21. Krill oil extractability, phospholipid (PL) concentration, astaxanthin (AX) concentration and acid value obtained by orthogonal experiment (L_9 (3^3) PER, ETe, ETi).

	ETe（°C）	PER	ETi (h)	Extractability (%)	PL (%)	AX (ppm)	Acid value (mg KOH/g)
1	25	1:6	1	61	33.9	164.6	29.0
2	25	1:8	2	68	32.6	166.1	28.8
3	25	1:10	3	70	31.7	166.8	30.5
4	35	1:6	2	64	33.1	170.6	30.6
5	35	1:8	3	69	32.4	168.4	30.3
6	35	1:10	1	69	32.2	165.6	30.9
7	45	1:6	3	68	32.3	167.0	29.5
8	45	1:8	1	70	32.0	172.1	31.3
9	45	1:10	2	74	32.1	170.1	32.1

AX: astaxanthin
ETe: extraction temperature
ETi: extraction time
KO: krill oil
PER: PEF and 99.5% ethanol ratio
PL: phospholipid

Table 21 shows the values obtained by an orthogonal experiment (L9(3^3) PER, ETe, ETi). These data were used in the subsequent orthogonal analyses.

3.6.3 Extractability

Table 22. Krill oil extractability obtained by orthogonal experiment.

	ETe（°C）	PER	ETi (h)	Extractability (%)
1	25	1:6	1	61
2	25	1:8	2	68
3	25	1:10	3	70
4	35	1:6	2	64
5	35	1:8	3	69
6	35	1:10	1	69
7	45	1:6	3	68
8	45	1:8	1	70
9	45	1:10	2	74
$\overline{K1}$	66.3	64.3	66.7	
$\overline{K2}$	67.3	69.0	68.7	
$\overline{K3}$	70.7	71.0	69.0	
R	4.4	6.7	2.3	

ETe: extraction temperature
ETi: extraction time
PER: PEF and 99.5% ethanol ratio

Range analysis in orthogonal experiments (Table 22) gave the following ranking of influencing factors: PER (R = 6.7) > ETe (R = 4.4) > ETi (R = 2.3)

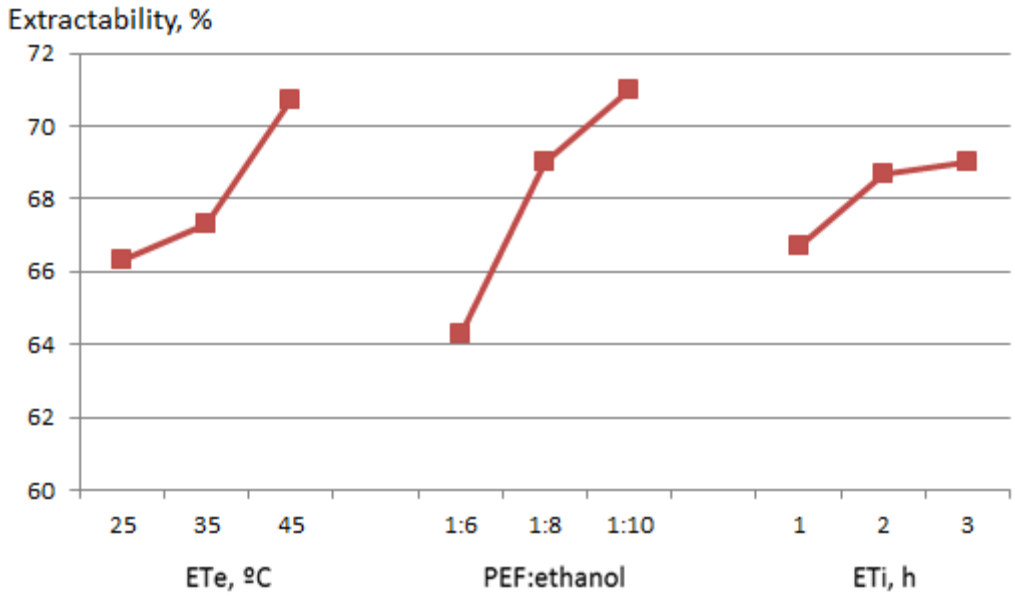


Figure 6. Extractability as dependent on extraction temperature (ETe), PEF: 99.5% ethanol ratio, extraction time (ETi), analyzed by orthogonal experiment analysis

The orthogonal experiment (Fig. 6) showed high ETe (25ºC < 35ºC < 45ºC), high PER (1:6 < 1:8 < 1:10) and long ETi (1h < 2h < 3h) resulting in high extractability.

In condition of 45ºC, 1:10, 3h, the highest extractability from PEF was 74%; PL concentration was 32.1%.

Total krill oil extractability was 74% / 74.4% = 99%
Total krill PL extractability was 74%×32.1% / (74.4%×31.0%) = 103%

3.6.4 Phospholipid concentration

Table 23. Phospholipid concentration obtained by orthogonal experiment.

	ETe（°C）	PER	ETi (h)	PL (%)
1	25	1:6	1	33.9
2	25	1:8	2	32.6
3	25	1:10	3	31.7
4	35	1:6	2	33.1
5	35	1:8	3	32.4
6	35	1:10	1	32.2
7	45	1:6	3	32.3
8	45	1:8	1	32.0
9	45	1:10	2	32.1
$\overline{K1}$	32.7	33.1	32.7	
$\overline{K2}$	32.6	32.3	32.6	
$\overline{K3}$	32.1	32.0	32.1	
R	0.6	1.1	0.6	

ETe: extraction temperature
ETi: extraction time
PER: PEF and 99.5% ethanol ratio
PL: phospholipid

Range analysis in orthogonal experiments (Table 23) gave the following ranking of influencing factors: PER (R = 1.1) > ETe (R = 0.6) = ETi (R = 0.6)

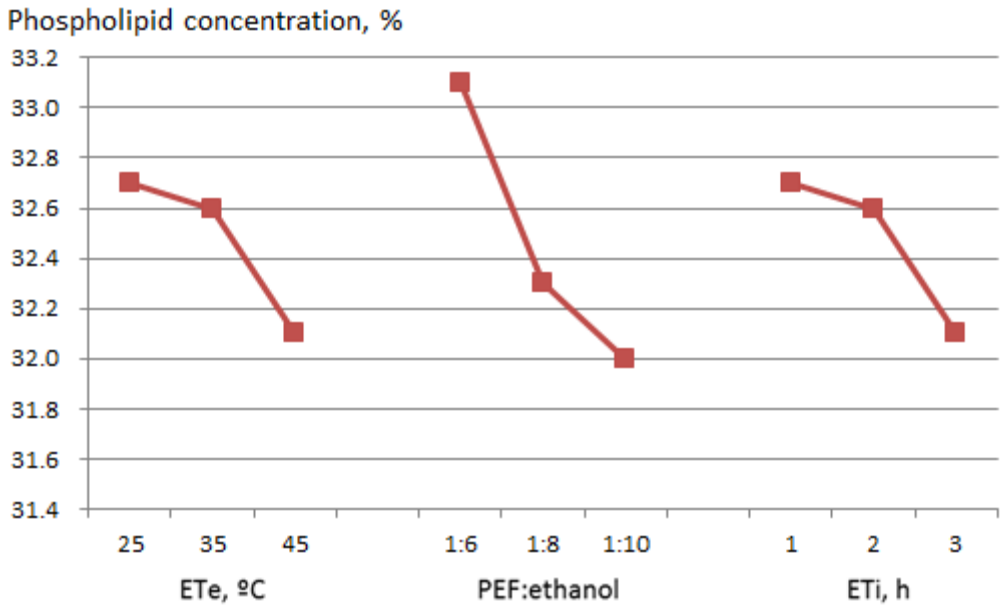


Figure 7. Phospholipid concentration in krill oil as dependent on extraction temperature (ETe), PEF: 99.5% ethanol ratio, extraction time (ETi), analyzed by orthogonal experiment analysis

PL concentration in KO is a major quality criterion, and PL in commercial KO normally is between 40%-45%.

The orthogonal experiment (Fig. 7) showed that low ETe (25ºC > 35ºC > 45ºC), low PER (1:6 > 1:8 > 1:10) and short ETi (1h > 2h > 3h) resulted in high PL concentration.

In condition of 25ºC, 1:6, 1h, the highest PL concentration was 33.89% in KO; extractability from PEF was 61%.

Total krill PL extractability was 61%×33.9% / (74.4%×31.0%) = 90%
Total krill oil extractability was 61% / 74.4% = 82%

3.6.5 Astaxanthin concentration

Table 24. Astaxanthin concentration obtained by orthogonal experiment.

	ETe（°C）	PER	ETi (h)	AX (ppm)
1	25	1:6	1	164.6
2	25	1:8	2	166.1
3	25	1:10	3	166.8
4	35	1:6	2	170.6
5	35	1:8	3	168.4
6	35	1:10	1	165.6
7	45	1:6	3	167.0
8	45	1:8	1	172.1
9	45	1:10	2	170.1
$\overline{K1}$	165.8	167.4	168.1	
$\overline{K2}$	168.9	168.9	168.9	
$\overline{K3}$	169.7	168.1	167.4	
R	3.9	1.5	1.5	

AX: astaxanthin
ETe: extraction temperature
ETi: extraction time
PER: PEF and 99.5% ethanol ratio

Range analysis in orthogonal experiments (Table 24) gave the following ranking of influencing factors: ETe (R = 3.9) > ETi (R = 1.5) = PER (R = 1.5)

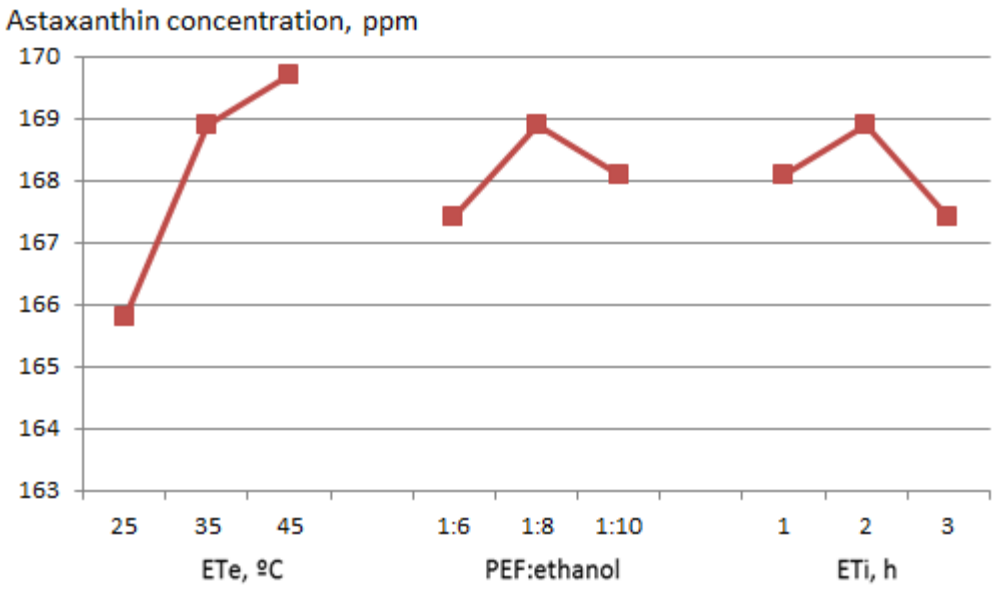


Figure 8. Astaxanthin (AX) concentration in krill oil as dependent on extraction temperature (ETe), PEF: 99.5% ethanol ratio, extraction time (ETi), analyzed by orthogonal experiment analysis.

The orthogonal experiment (Fig. 8) showed that high ETe (25ºC < 35ºC < 45ºC), PER = 1:8 and ETi = 2h resulted in high AX concentration.

In condition of 45ºC, 1:8, 2h, the highest AX concentration was 172.2ppm; extractability from PEF was 72%.

Total krill AX extractability was 72%×172.2ppm / (79% × 151.1ppm) = 104%
Total krill oil extractability was 72% / 79% = 91%

3.6.6 Acid value

Table 25. Acid value obtained by orthogonal experiment.

	ETe (°C)	PER	ETi (h)	Acid value (mg KOH/g)
1	25	1:6	1	29.0
2	25	1:8	2	28.8
3	25	1:10	3	30.5
4	35	1:6	2	30.6
5	35	1:8	3	30.3
6	35	1:10	1	30.9
7	45	1:6	3	29.5
8	45	1:8	1	31.3
9	45	1:10	2	32.1
$\overline{K1}$	29.4	29.7	30.4	
$\overline{K2}$	30.6	30.1	30.5	
$\overline{K3}$	31.0	31.2	30.1	
R	1.6	1.5	0.4	

ETe: extraction temperature
ETi: extraction time
PER: PEF and 99.5% ethanol ratio

Range analysis (Table 25) in orthogonal experiments gave the following ranking of influencing factors: ETe (R = 1.6) > PER (R = 1.5) > ETi (R = 0.4)

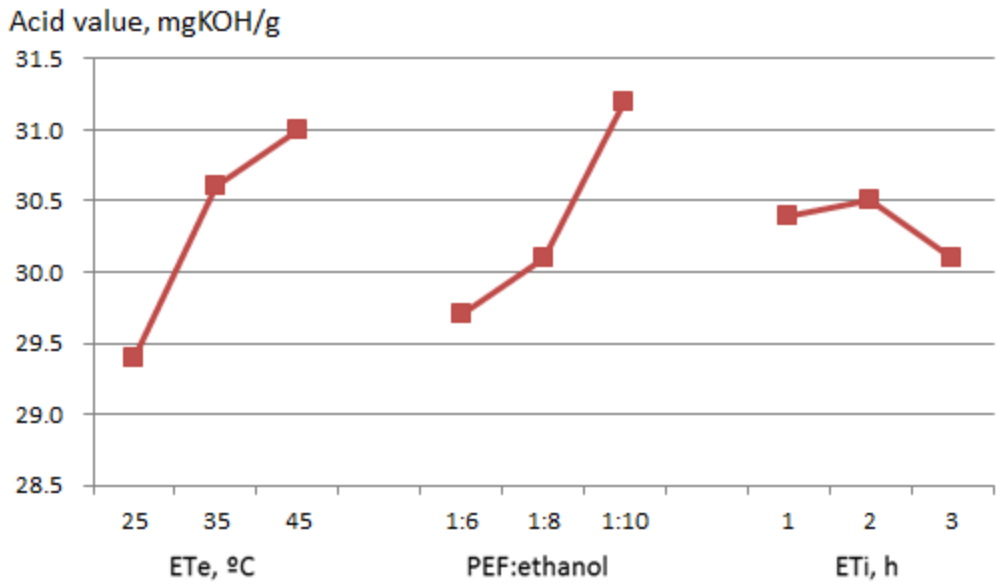


Figure 9. Acid value in krill oil as dependent on extraction temperature (ETe), PEF: 99.5% ethanol ratio, extraction time (ETi), analyzed by orthogonal experiment analysis

The orthogonal experiment (Fig. 9) showed that low ETe (25ºC < 35ºC < 45ºC), low PER (1:6 < 1:8 < 1:10) and ETi = 3h resulted in low acid value.

In condition of 25ºC, 1:6, 3h, the lowest acid value was 28.6 mg KOH/g.

Based on these results, it is recommended that fresh krill first processed to PEF and stored carefully in ship, then krill oil may be extracted from the PEF on land. PEF concentrated most of krill lipids without water and lipase activity, therefore, the acid value will be much less than starting material as krill that has been stored frozen for a long period of time.

3.7 The deep enzymolysis process

Table 26. Advantages and disadvantages compared by Olympic process and improved process.

Process name	Advantages	Disadvantages
Olympic	SFE (no solvent remaining) Low enzyme cost	Expansive Three layers (part of AX and n-3 triglyceride loss)
Improved process	Cheap (99.5% Ethanol extraction) High extractability	High enzyme cost Long incubation time

AX: astaxanthin
SFE: supercritical fluid extraction

The improved process, suggested by this thesis, has similarities to the Olympic process by using enzymolysis and solvent extraction (Table 26).

However, applied deep enzymolysis achieved higher degree of hydrolysis and formed two layers: lipid phase on the top and water phase on the bottom. According to Olympic patent CN102170795A, three layers formed after centrifugation: The first layer consists of neutral oil. The second layer is peptide enriched layer. The bottom layer consists of a complex of protein and lipid.

Two layers are easier to handle compared with three layers.

As deep enzymolysis decreased soluble protein concentration, hydrolysate viscosity decreased. At the same time, the lipid phase contained more lipids as water phase extracts more peptide. The division into an aqueous, peptide rich phase, and a lipid phase is also characterized by a high difference in density. This is evident based on Stokes' Law,

$$V_s = \frac{2}{9}\frac{r^2 g(\rho_p - \rho_f)}{\eta}$$

Vs: Particle free settlement velocity, m/s
r: Lipid particle radius, m
ρ_p: Water phase density, kg/m^3
ρ_f: Lipid phase density, kg/m^3
g: Acceleration of gravity, m/s^2
η : Kinetic viscosity of system, Pa·s

In condition of fixed lipid particle diameter and acceleration of gravity, both

increasing density difference (ρ_p-ρ_f) increasing, and kinetic viscosity of the system decreasing can lead to increased particle free settlement velocity. System viscosity also depends on temperature and soluble protein concentration.

In other words, as temperature and DH increase, lipid and water phase are prone to separate spontaneously. This is illustrated by applying 6000 U alcalase/g, heating to 55^{o}C, and incubating for 12 h. Under these conditions, lipid and water phases separated spontaneously at room temperature, and small lipid particles accumulated to big particles automatically.

According to krill oil analysis presented in Table 27, phospholipid concentration was only 32.1g/100g, far below the 40 g/100g limits normally set for high-quality commercial products. However, the EPA concentration was 11.9 g/100g and DHA concentration was 7.6 g/100g, which are in normal level. *n-3* fatty acids are mainly combined with phospholipid in krill oil, therefore, low phospholipid concentration will result in low EPA and DHA concentration.

Table 27. Krill oil composition analysis (1:10, 45^{o}C, 2h, krill oil extractability is 74%).

Item	Content	Assay method
Total lipids (g/100g)	93.4	GB/T 14772-2008*
Protein (g/100g)	8.7	GB 5009.5-2010*
Phospholipid (g/100g)	32.1	GB/T 5537-2008)
EPA (g/100g)	11.9	GB/T 5009.168-2003
DHA (g/100g)	7.6	GB/T 5009.168-2003
Ethanol and water (g/100g)	1.9	GB 5009.3-2010
Astaxanthin (ppm)	168.0	SN/T 2327
Fluoride (ppm)	2.8	GB/T 5009.18
Acid value	30.6	GB/T 5530-2005
Peroxide value (mEq peroxide/kg)	2.5	GB/T 5009.37-2003

* GB/T 14772-2008 is Soxhlet extraction

* GB 5009.5-2010 is Kjeldahl method

Table 28. Freeze dried extraction residue composition analysis.

Item	Content	Assay method
Total lipids (g/100g)	12.2	GB/T 14772-2008
Protein (g/100g)	50.5	GB 5009.5-2010
Phospholipid from total lipids (%)	3.0	GB/T 5537-2008)

The lack of decrease in EPA and DHA with low PL concentration represents an apparent controversy. However, Kolakowska (1986) pointed that stored in -22ºC for 6 months, up to70% of the phospholipid was decomposed, and free fatty acids increased by 6-20 fold.

In this study, phospholipid was measured by the molybdenum blue colorimetric method that meant low phosphorus concentration will lead to low phospholipid concentration.

Figure 10. PLase subgroups and site of action.

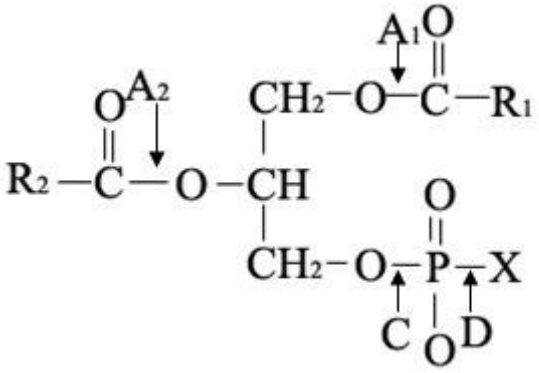


Fig.10 shows that PLase has four subgroups: PLaseA1, PLaseA2, PLaseC and PLaseD, acting on different part of the PL molecule. Only PLaseC had dephosphorilation function and keeps *n-3* fatty acids remaining in the basic structure. In other words, the current result indicates high activity of PLaseC during autolysis of krill PL.

The Kjeldahl method is used for the quantitative determination of nitrogen in chemical substances. In krill oil, not only protein contained nitrogen, but also phospholipid, including PC, PE and PS. The main PL of krill are PC and PE. PC and PE concentration of krill was 80.4% and14.9% of total PL, respectively. PI was also present but with very low percentage (0.7%) of total PL (Abdelkader 2012).

The MW of PC can be calculated as MW(PC) = (2EPA-2) + C_3H_5 + PO_3 + (Choline-1) = 842. This means that N (%) = 14 / 842=1.66%. Simultaneously, MW(PE) = (2EPA-2) + C_3H_5 + PO_3 + (Ethanolamine -1) = 782; N (%) = 14 / 782 = 1.79%: MW(PI) = (2EPA-2) + C_3H_5 + PO_3 + (Inositol-1) = 901; N (%) = 14 / 901= 1.55%.

Thus, N(from PL) = N(from PC) + N(from PE) + N(from PI)= 40%×80.4%×1.66% + 40%×14.9%×1.79% + 40%%×0.7%×1.55%= 0.6448%.

By the Kjeldahl method, 40% PL of krill oil equaled to background protein values: N(from PL)×6.25 = 0.6448%×6.25 = 4.03%.

According to Table 26, extra% = Total lipids% + Protein% + Ethanol and water% - 100% = 93.38%+ 8.74% + 1.86% - 100% = 3.98%. Theoretically, 33.11% PL equaled to 3.34% background protein values, closed to 3.98% extra value. The true protein value was 8.74% – 3.34% = 5.40%.

4. Conclusions

Antarctic krill is an abundant source of *n-3* PUFA phospholipids, with considerable application potential to secure good human health. However, extraction of phospholipids from intact krill is complicated. Thus, enzymolysis should be employed to facilitate efficient extraction of phospholipid-rich krill oil.

The current research shows that most efficient extraction is achieved by two step proteolysis. For the first step, aimed at liquefying the krill and separating shells to reduce the content of fluoride, a combination of endogenous proteases from the krill and added protease should be employed. Alcalase was identified as the most suitable enzyme for enzymolysis of among 5 commonly used enzymes based on degree of hydrolysis. This first step hydrolysis should be terminated by heating, especially in order to deactivate endogenous phospholipase activity, since high concentration of free fatty acids reduces the quality of the krill oil.

The aim of the second step is to decompose lipid and protein complexes, thereby facilitating high krill oil extractability. The optimal hydrolysis conditions for alcalase were found to be:

First step hydrolysis: Frozen krill and water ratio = 2:1, hydrolysis temperature = 60~65ºC, hydrolysis time = 15min, alcalase dosage = 3000U/g. Centrifugation: 400xG, filter cloth 50~200 mesh.

Second step hydrolysis: Hydrolysis temperature = 55 ºC, pH = 8.0, hydrolysis time = 4 h, end pH = 6.5~6.7. Five min heating at 75~80℃ is needed for precipitation. Centrifugation: 600xG, 5min.

The phospholipid enriched fraction (PEF) was defined as freeze dried lipid phase after centrifugation. Extraction from PEF aimed at high krill oil extractability, high PL concentration, high astaxanthin (AX) concentration and low acid value. Comparing 5 commonly used solvents (99.5% ethanol, 95.5%ethanol, acetone, ethyl acetate and chloroform methanol 2:1), concluded that 99.5% ethanol was most suitable for extraction, based on a combined evaluation of extractability and consumer safety.

Maximum krill oil extractability was obtained at extraction temperature of 45 ºC, a PEF and 99.5% ethanol ratio at 1:10, and extraction time at 3h. Under these conditions, krill oil extractability was 74%.

Maximum phospholipid concentration was obtained at extraction temperature of 25 ºC, PEF and 99.5% ethanol ratio at 1:6, and extraction time at 1h. Under these conditions, the PL concentration in krill oil was 33.9%.

Maximum astaxanthin concentration was obtained at extraction temperature of 45 ºC, PEF and 99.5% ethanol ratio at 1:8, and extraction time at 2h. Under these conditions, astaxanthin concentration in krill oil was 172 ppm.

High acid value (AV) and low PL concentration due to storage was seen in frozen krill. This was probably due to hydrolysis during frozen storage for 6 months, and lipase and phospholipase (PLase) during the time from catch until the material was frozen, thawed and incubated. It is recommended that fresh krill is processed to PEF immediately after capture, rapidly frozen, and stored at stable, low temperatures in the ship. Extraction on land should occur rapidly after landing. If frozen krill is the raw material for krill oil extraction, high concentration of free fatty acids represents a potential risk of inferior quality.

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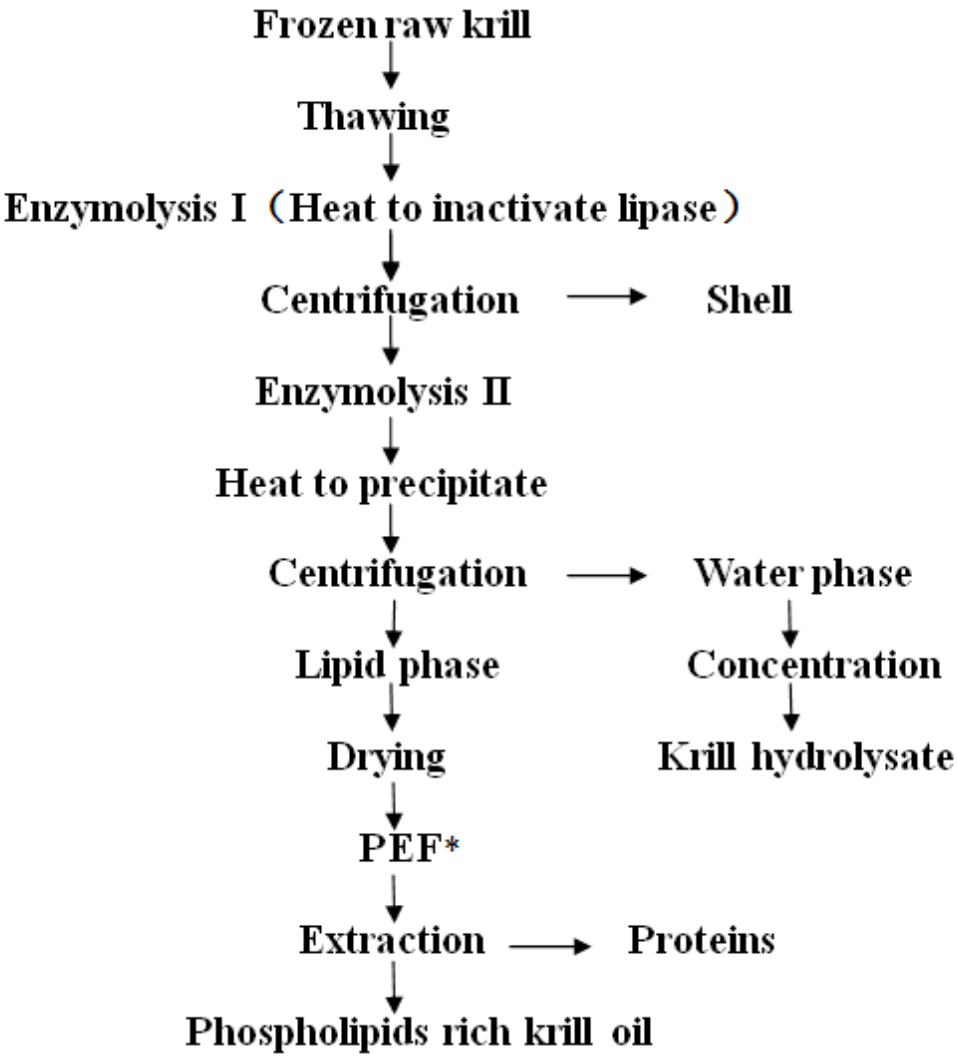
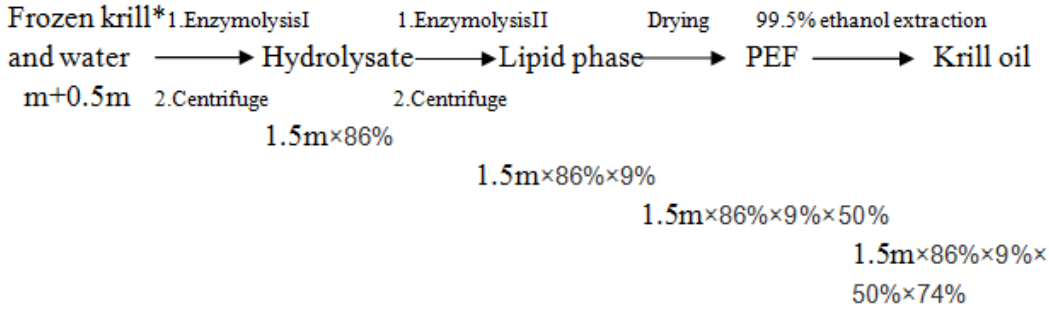
Appendix I Technical summary



*PEF was defined as freeze dried lipid phase in this study.

Thawing: KWR = 2:1, 55° C water bath (speed up thawing)
Enzymolysis I: Alcalase 3000U/g, 60~65°C, 15min
Centrifugation for shelling: link-suspended batch centrifugal, 400xG, filter cloth 50~200 mesh
Enzymolysis II: 55°C, start pH = 8.0, 4h, end pH = 6.5~6.7
Heat to precipitate: 75~80°C, 5min
Centrifugation for lipid and water phases separation: 600xG, 5min
Drying: Freeze drying or vacuum drying
Extraction: 99.5% Ethanol, PER=1:10, ETe=45° C, ETi=2h.

Appendix II Mass balance



	Moisture content	Crude protein	Total fat	PL in total fat (%)	Ash	NFE	Acid Value
Frozen krill*	79.6	12.1	4.2	31.1	2.7	1.4	18.1
PEF	1.2	23.0	74.4	31.0	7.4		27.2
Krill oil	1.9	8.7	93.4	32.1			30.6
Freeze dried residue		50.5	12.2	3.0			

*Frozen krill caught in May, analyzed in November, 2013

KWR = 2:1: m+0.5m
Yield of frozen krill and water to hydrolysate: 86%
Yield of hydrolysate to lipid phase: 9%
Lipid phase water content: 50%
Yield of PEF to KO in condition of 99.5% Ethanol, 1:10, 45^{o} C, 2 h: 74%
Total lipids conversion ratio from frozen krill to PEF: 1.5×86%×9%×50%×74.4% / 4.2% = 103%
Total PL conversion ratio from frozen krill to PEF: 1.5×86%×9%×50%×74.4%×31.0% / (4.2%×31.1%) = 103%
Total krill lipids extractability from PEF: 74%×93.4% / 74.4% = 93%
Total PL extractability from PEF: 74%×32.1% / (74.4%×31.0%) = 103%
Total KO yield from frozen krill: 1.5×86%×9%×50%×74% = 4.3%
Total KO extractability from frozen krill: 4.3%/4.2% = 102%
Total krill lipids extractability from frozen krill: 103%×93% = 96%
Total PL extractability from frozen krill: 103%×103% = 106%

Appendix III Photographs

Picture 1. Frozen raw Antarctic krill

Picture 2. Krill shell and hydrolysate after Enzymolysis I and centrifugation

Picture 3. Lipids and water phase separation effect of different KWR.

Picture 4. Lipid and water phase separation effect of different end pH

Picture 5. Lipid and water phase separation after 600G, 5min centrifugation

Picture 6. Freeze dried phospholipid enriched fraction (PEF)

Picture 7. Ethanol rotary evaporation