Determination of the Nutritional Value, Protein Quality and Safety of Krill Protein Concentrate Isolated Using an Isolelectric Solubilization/Precipitation Technique

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ABSTRACT

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Despite its abundance and nutritional value, krill has not been widely utilized for human consumption due to the lack of proper technology for protein recovery. The study objectives were to isolate krill protein concentrate (KPC) and to determine the nutritional value, health benefits, and safety of KPC for human consumption. Proximate analysis indicated KPC on a dry basis is composed of ~ 78% protein and ~ 8% fat of which ~27% are omega-3 polyunsaturated fatty acids (ω-3 PUFAs). The amino acid composition of KPC indicated that all nine essential amino acids were present in amounts that met the requirements for adult humans assuming sufficient protein was consumed. Protein quality measurements were determined by feeding KPC or casein diets to growing female rats. In regards to bioavailability, KPC was equal to that of casein in digestibility, protein digestibility corrected for amino acid score (PDCAAS), and protein efficiency ratio (PER). In terms of safety, there were no differences in the absolute weights of the major organs except for the kidneys. Kidney weights and total mineral content were higher *(P*<0.001*)* in rats fed the casein compared to the KPC diet. Based on the nutritional and safety analysis, KPC appears to be a promising high quality protein source for human consumption with the advantage of being a rich source of ω-3 PUFAs.

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1.0. Literature Review

1.1 Krill Abundance

The term krill refers to ~80 species of small shrimp-like crustaceans belonging to the order *Euphausiacea*. Despite growing only to a full length of ~2 inches, krill has been estimated to be one of most abundant multicellular organisms on the planet (Nicol, James & Pitcher, 1987). Interest in utilizing krill as a food source for humans is due to its biomass, which has been cited as high as ~500 million tons (Nicol & Endo, 1997). Of the different species of krill, only two species of krill are harvested annually; the Antarctic krill (*Euphausia superba*), and North Pacific krill (*Euphausia pacifica*). The focus of our research is on the Antarctic krill because the market for Antarctic krill spans the globe.

Antarctic krill are schooling animals that are capable of forming swarms that may contain up to a ton of krill with over 20,000 krill within a cubic meter. These large swarms are generally found within the top 200 meters of the water column. The large biomass and formation of surface swarms contributes to the ease and accessibility of krill to harvesting. However, only ~100,000 tons of krill are currently harvested each year, with a majority of the harvest being directed to aquacultural feeds (Nicol & Endo, 1997). The low harvest of Antarctic krill is primarily due to lack of a world-wide demand. Demand would presumably increase if krill were used for human consumption. An area where krill might gain interest as a food for humans is as an economical replacement as a high quality protein source. Products where krill protein may substitute for other

proteins include but not limited to, nutritional supplements, sports drinks, infant formulas, and milk replacers. For these products, protein concentrates rather than whole krill or krill meat could/would be used. However, lack of proper technology for protein recovery from krill has hindered progress in the commercial development of protein products from krill.

1.2 Protein Recovery Techniques

Protein concentrates are produced by technologies that concentrate the proteins in food so that protein levels are higher than that in the original food. A common method for isolation of proteins from food products involves protein hydrolysis. This process isolates protein components by hydrolyzing proteins, and allowing the products of hydrolysis to solubilize in solution. Following solubilization different methods are used to recover these products from the solution, such as drying. Problems associated with the hydroysis technique are proteins are isolated as a powder resulting in loss of functional properties which decreases the range of uses for the recovered protein.

A technique for extracting proteins has recently been developed and applied to isolate protein from krill (Chen & Jaczynski, 2007). The advantage of the method of isoelectric solubilization/precipitation technique is that it allows for the recovery of krill protein with various functional characteristics that pertain to the food industry

The methodology of the isoelectric solubilization/precipitation (Chen et al., 2007) is centered upon the isoelectric point of the protein of interest. The

isoelectric point (pI) is the pH at which the net charge of the protein is zero. By manipulating the pH of the protein containing solution, it is possible to change the solubility of the protein in that solution. The pH adjustment accompanied by centrifugation allows other components in the solution to be removed, leaving the protein solubilized in the supernatant. A pH shift into the range of the pI would allow for the proteins to precipitate ut of solution, and be collecdted using centrifugation. However before the protein recovered from krill can be used as a protein supplement, the quality of the protein needs to be determined.

1.3 Protein Quality Measurements

Humans must consume enough protein in the diet to meet the requirements for the essential amino acids (EAA). Adult humans require 9 EAAs and infants require 10 EAA because these amino acids are not produced in sufficient quantities by the body to meet the body's needs for that amino acid. The body's use for amino acids spans from catabolism for energy to synthesis of crucial enzymes and other compounds critical for survival. The current recommendation for protein intake for adults is 0.8g protein per kg of body weight (Whitney & Rolfes, 2005). Sources of protein in typical human diets vary from meat and plant sources to dietary supplements. Protein supplements can vary from individual amino acid supplements to isolated protein powders. Commonly used whole protein supplements are the milk proteins whey and casein, soy concentrate, egg albumin, or a combination of these proteins.

Protein quality is a measure of the EAAs present in the protein as well as the digestibility of that protein. Digestibility is important because even if a protein has all EAA in sufficient quantities needed by the body, it may not be accessible to the body if the protein is poorly digested. Therefore, a high-quality protein must have all the EAA in relative amounts needed by the body as well as a high bioavailability.

Various methods exist to estimate protein quality. Net Protein Utilization (NPU) is a measure used to estimate protein retention and is calculated as:

$$NPU = (I – (FN – EFN) - (UN – EUN))/ (I).$$

Another protein quality measurement, the true biological value (BV), evaluates how efficiently food protein can be turned into body tissue. Similar to NPU, BV estimates the retention of protein which is then corrected for digestibility. BV is calculated as:

$$BV = (I – (FN – EFN) – (UN – EUN))/ (I – FN – EFN).$$

I is nitrogen intake, FN is fecal nitrogen excreted, EFN is endogenous fecal nitrogen excreted measured from the protein free group, UN is urinary nitrogen excreted, EUN is endogenous urinary nitrogen excreted measured from the protein free group. Nitrogen rather than protein is measured due to ease of measurement and because all amino acids contain nitrogen.

True digestibility is another measurement that accounts for protein bioavailability using the formula:

$$D = (I – (FN – EFN)) / I).$$

There are two main protein quality measurements used for humans: the Protein Digestibility-Corrected for Amino Acid Score (PDCAAS) and protein efficiency ratio (PER). In both calculations of protein quality, the test protein is compared to reference proteins, in order to scale their nutritional value. The reference proteins is usually either egg albumin or the milk protein casein because these proteins support the growth and development of growing animals (Whitney & Rolfes, 2005).

The PDCAAS is currently used by the Committee on Dietary Reference Intakes to assess protein quality for individuals over one years of age (Whitney & Rolfes, 2005). This measurement directly compares the amino acid composition of the test protein to the amino acid score of a reference protein, usually casein or egg albumin. The resultant ratio is then multiplied by the true digestibility of the test protein to yield a PDCAAS score. Egg, casein, and whey proteins are examples of proteins that score a 1.0 in PDCAAS, the highest value possible.

The protein efficiency ratio (PER) is a ratio of grams of protein ingested per g of body weight gained. Ability of a protein to support growth is specifically important in regards to growing children, when energy and protein requirements are high. The ability of a protein source to support weight gain during growth in humans and animals is an indication of high quality. Due to ethical issue in humans, growing rats are routinely used to determine PER. Rats are fed the test protein, and the protein quality determined by measuring nitrogen balance. This data can then be applied to determine how well the protein was utilized in the body. In addition to protein, lipid is another nutritional component of interest. To

determine lipid nutritive value, it is often of interest to determine the fatty acid composition of foods containing lipid.

1.4 Fatty Acid Composition and Oxidation

Generally, fatty acids can be classified into one of three different categories. The saturated fatty acids (SFAs) are those in which the carbon chain is fully saturated with hydrogen. The monounsaturated fatty acids (MUFAs) contain a single double bond within the carbon chain, whereas the polyunsaturated fatty acids (PUFAs) contain more than one double bond. Each type of fatty acid has different effects on health. The ingestion of large amounts of SFAs is positively correlated with the occurrence of CVD (Artaud-Wild, Connor, Sexton, & Connor, 1993), whereas consumption of PUFAs in place of SFAs can decrease the risk for CVD (Kriketos et al., 2001). The omega-3 PUFAs are not only required, but may have added health benefits when consumed. Of the omega-3 PUFAs, eicosapentanoic acids (EPA, 20:5ω-3) and decosahexanoic acid (DHA, 22:6ω-3) has commonly been linked to reducing the risk of CVD.

Seafood not only contains less total lipid when compared to other protein sources, but it also contains the essential fatty acids required by humans, linolenic acid (18:2n-6) and linolenic acid (18:3n-3). Fats derived from seafood are termed healthy, and it is recommended that 2 servings of fish should be consumed weekly. As is with most seafoods, krill is low in total lipids (Suzuki & Shibata, 1990). Kolakowska et al. (1994) reported that omega-3 PUFAs accounted for ~19% of total fatty acids in Antarctic krill. Of the omega-3 PUFAs, EPA and

DHA were particularly abundant. Overall, the fatty acid profile of krill resembles that of shrimp as well as fish. However, most of the fatty acids in fish are incorporated into triglycerides, whereas 65% of the fatty acids in crustaceans are incorporated into phospholipids (Weihrauch et al., 1977). This value is slightly lower according to Bottino (1975), who reported that ~58% of the total lipid in krill may be in the form of phospholipids. Evidence exists that consuming PUFAs in the form of phospholipids may allow more efficient absorption of the incorporated PUFAs (Werner, Havinga, Kuipersm, & Verkade, 2003). These subtle differences in the lipid composition may explain some of the benefits found in consuming krill oil (Bunea, 2004).

Despite the similar fatty acid compositions different results were found in trials comparing krill oil to fish oil. Bunea (2004) compared lipid and lipoprotein profiles of subjects administered varying amounts of either krill oil or fish oil. The authors found that daily consumption of of 1-1.5g/d krill oil resulted in a greater reduction in serum cholesterol and triglycerides and increased HDL concentration compared to daily consumption of 3 g/d of fish oil. The authors attributed these findings to the high phospholipid and omega-3 fatty acid content.

In regards to safety, PUFAs are susceptible to oxidation and the ingestion of oxidized fats has been shown to have negative effects on health. Ingestion of oxidized fatty acids has been shown to increase the risk of CVD in mice (Khan-Merchant, Penumetcha, Meilhac, & Parthasarthy, 2002). Due to susceptibility to oxidation, foods high in PUFAs may have a short shelf life, and may be a source of oxidized fatty acids. Krill is high in PUFAs but has also been shown to be high

in various antioxidants, such as vitamin E and a carotenoid, astaxanthin (Suzuki & Shibata, 1990). In addition, the omega-3 PUFAs in krill are in the form of phospholipids whereas in fish omega-3 are in the form of triglycerides. In vitro studies have suggested that DHA incorporated into phospholipids may be less likely to undergo autoxidation than DHA in the form of triglycerides (Song and Miyazawa, 1997). In vivo, feeding rats DHA as phospholipids resulted in lower plasma and liver peroxidation compared to feeding DHA as triglycerides (Song and Miyazawa, 2001). These combined features may limit the rate of PUFA oxidation in krill oil, and thus increase stability compared to fish oil.

1.5 Safety of KPC

KPC is a rich source of protein and therefore, the physiological impacts of KPC intake needs to be considered. Different protein sources have been shown to cause fluctuations in mineral balance. Thus, protein may have adverse effects on kidney function.

Nephrocalcinosis is a condition in which there is an accumulation of calcium in the kidneys. There have been great advances in understanding the cause of this condition. Meyer et al. (1982) discovered that altering the source of dietary protein has greater effects on nephrocalcinosis than by altering the mineral content of the diet. Whiting & Draper (1980) showed that hypercalciuria was proportional to the concentration of sulfur containing amino acids in the diet. The authors suggested that the results were due to generation of acidic sulfur byproducts of methionine and cysteine degradation. It was noted that acidic

plasma filtrate inhibits various components in reabsorption of calcium in the kidney (Lemann, Litzow, & Lennon, 1967). Therefore, widespread acceptance of krill as a food source for human consumption will require the determination of the nutritional value, health benefits and safety.

2.0 Study Objectives

Objective 1. To isolate and determine nutrient analysis of krill protein concentrate (KPC)

Objective 2. To determinate KPC protein quality *in vivo*

Objective 3. To assess the potential health benefits and safety issues associated with consuming KPC

Chapter 1:

Determination of the Nutritional Value, Protein Quality and Safety of Krill Protein Concentrate Isolated Using an Isoelectric Solubilization/Precipitation Technique

3.0 Introduction

The term "krill" refers to ~85 different species of pelagic crustaceans belonging to the order *Euphausiacea*. Krill are estimated to have the largest multi-cellular biomass on the planet, with estimates as high as ~500 million tons (Nicol & Endo, 1997). Despite this large biomass, only ~12% of the total krill catch is consumed by humans (Ichii, 2000). Due to its abundance and under-utilization, krill offers a relatively untapped potential food source for human consumption.

Krill is composed of 60-80% protein, 7-26% lipid and 12-17% ash on a dry weight basis (Grantham, 1977). The protein derived from krill is considered high quality based on chemical analysis showing krill protein contains all nine essential amino acids (EAAs) in sufficient quantities to meet the FAO/WHO/UNU requirements for human adults (Chen, Tou, & Jaczynski, 2007). However, in addition to the amino acid content, bioavailability must also be assessed to determine protein quality. In an animal feeding study comparing whole krill to egg protein, Iwantani et al. (1977) reported rats fed whole krill gained less weight and whole krill resulted in reduced protein quality measurements of protein efficiency ratio, biological value, and net protein utilization. The authors suggested that the decreased digestibility of krill protein may have been due to the presence of the exoskeleton. A challenge to protein recovery from krill has been the difficulty in removing the chitinous exoskeleton of krill. Removal of the exoskeleton is important because indigestible

polysaccharides such as chitin can impede digestion and absorption (Ikegamie, Tsuchihashi, Harada, Nishide & Innami, 1990).

Isolation of protein from krill is also challenging due to the presence of hydrolytic enzymes. These enzymes are released into the surrounding tissue and result in rapid tissue liquefication and spoilage upon the demise of the krill. Various protein isolation methods have been used to isolate krill protein (Rys & Koreleski 1979, Heinz, Henk & Kesting, 1981). Many techniques used to isolate proteins employ protein hydrolysis; however, this decreases the functional characteristics of proteins. Chen and Jaczynski (2007), using an isoelectric solubilization/precipitation technique isolated protein from trout byproducts with functional characteristics that allow for a broad range of use in the food industry. We used the isoelectric solubilization/precipitation technique to recover protein from Antarctic krill (*Euphausia superba*) in order to assess the nutritional value of krill protein *in vivo*.

In addition to being a promising source of high quality protein, krill also offers the advantage of being high in the omega-3 polyunsaturated fatty acids (ω-3 PUFAs). The ω-3 PUFAs have been reported to have various beneficial health effects including decreasing the risk of cardiovascular disease (Simopoulos, 2002). On the other hand, the safety of krill protein for human consumption also needs to be considered. Previous research has found that changes in dietary protein alone may have negative effects on the kidney (Zhang & Beynen, 1992). Meyer et al. (1982) found that changes in the source of dietary proteins fed to rats had greater effects on kidney calcification than fluxuations in the mineral

composition. Therefore, the effect of KPC on kidney function needs to be assessed.

Widespread acceptance of krill as part of the human diet will depend on the consumer's perception of krill as a nutritious, healthy and safe food. Therefore, the objectives of this study were to evaluate the nutritional value, potential health benefits, and safety of krill protein concentrate (KPC) isolated using an isoelectric solubilization/precipitation technique.

4.0 Materials and Methods

4.1. Krill protein isolation

Whole, frozen Antarctic krill (*Euphausia superba*) were purchased from Krill Canada (Langley, BC, Canada). The krill blocks were transported overnight to our laboratory in heavily insulated industrial strength boxes filled with dry ice. Upon arrival, Antarctic krill was immediately stored at -80ºC.

Protein was isolated from whole krill using an isoelectric point solubilization / precipitation method according to Chen & Jaczynski (2007). Briefly, 428 g frozen Antarctic krill was blended in a 1:6 w/v krill: deionized distilled water (ddH_2O) mixture. The krill ddH_2O mixture was homogenized at a temperature of 4^{o}C (PowerGen 700 Homogenizer, Fairlawn, NJ), and the pH adjusted to 11.5 using NaOH. The krill homogenate was centrifuged at 10,000 *g* for 10 min and the supernatant collected. The pH of the supernatant was adjusted to 5.5 using HCl than centrifuged at 10,000 *g* for another 10 min. The resultant KPC was collected, freeze-dried, and stored at -80^{o}C.

4.2. Proximate analysis

The proximate composition of frozen, whole Antarctic krill and freeze-dried KPC was determined in triplicates according to standard AOAC methods (Association of Official Analytical Chemists, 1995). Moisture content of Antarctic krill and KPC was determined by placing samples (2 g) in an aluminum dish and oven-drying at 100ºC. Samples were weighed at regular intervals until the weight was constant (18 h). Ash content was determined by ashing the KPC

and whole Antarctic krill at 550ºC for 24 h in a muffle furnace (Lindberg 515A2, Watertown, WI). Total crude protein of whole Antarctic krill and KPC was determined by the Kjeldahl method (Kjeltec Auto 1030 Analyzer, Foss North America Inc., MN). Total lipid of the KPC and whole Antarctic krill was determined by soxhlet extraction**.**

4.3. Amino acid profile

The analysis of essential and non-essential amino acids was conducted according to the AOAC method 982.30 E (a, b, c) by the Agriculture Experiment Station Chemical Laboratories University of Missouri-Columbia, Columbia, MO. The freeze-dried whole Antarctic krill and KPC were hydrolyzed with 6N HCl for 24 h. Amino acids were quantified using the Beckman Amino Acid Analyzer (Model 6300, Beckman Coulter, Inc., CA) employing sodium citrate buffers as step gradients with the cation exchange post-column ninhydrin derivatization method.

To determine whether the amino acid content of KPC met the human amino acid requirements, whole Antarctic krill and KPC was compared to FAO/WHO/UNU amino acid requirements for human adult and infants.

4.4. Animal feeding study

All animal procedures were conducted in accordance with the guidelines set forth by the National Research Council for the Care and Use of Laboratory

Animals (1996) and approved by the Animal Care and Use Committee at West Virginia University.

Immature (age 28 d), female Sprague-Dawley rats were individually housed in a metabolic cage to determine food intake and to collect urine and feces throughout the experiment. Rats were kept in rooms maintained at 21^{o}C with a 12 h light/dark cycle. During a 14 d acclimation period, animals were given *ad libitum* access to deionized distilled water (ddH_2O) and AIN-93G diet (Harklan Teklad; Indianapolis, IN). The AIN-93G meets all the nutrient requirements for growing rats as defined by the Nutritional Research Council (1995).

Following the 14 d acclimation period, rats were randomly assigned (n=10 rats/group) to be fed for 4 weeks, *ad libitum,* one of three isocaloric diets consisting of: 1) 10% protein supplied as casein, 2) 10% protein supplied as KPC or 3) a protein-free diet. To determine endogenous nitrogen losses, animals were fed a protein-free diet for 2 weeks after an initial 2 weeks of feeding a 10% casein diet. Replacement of the protein as either KPC or casein at a level of 10% in AIN-93G diet was corrected for protein and lipids so that the diets were isocaloric. Calcium and phosphorus contents of the diets were also matched (Table 1). Diets containing KPC were prepared weekly and kept stored at 4^{o} C. The assigned diets and ddH_2O were measured and replaced with fresh diet every 2 days. Body weights of all animals were measured weekly.

Urine and fecal samples were collected and measured weekly. Ascorbic acid (0.1%) was added to the urine collection tube as a preservative along with 1 ml of mineral oil to prevent evaporation. Collected urine samples were

centrifuged at 1,500 *g* for 10 min at 4°C. Urine samples were then aliquoted into fresh tubes and stored at -20°C until assayed for nitrogen content. Fecal samples were freeze-dried for 48 h then stored at -20°C until assayed for nitrogen content.

4.5. Protein quality measurements

Nitrogen measurements were obtained using the Kjeldahl method, and the following protein quality measurements were determined: true digestibility (D), true biological value (BV), net protein utilization (NPU), protein digestion corrected for amino acid score (PDCAAS) and protein efficiency ratio (PER). Protein quality measurements were calculated according to Pellet & Young (1980). BW is body weight gain, I is nitrogen intake, I is ingested nitrogen, FN is fecal nitrogen excreted, EFN is endogenous fecal nitrogen excreted measured from the protein free group, UN is urinary nitrogen excreted, EUN is endogenous urinary nitrogen excreted measured from the protein free group.

True Digestibility (D) $D = (I - (FN - EFN)) / I)$

True Biological Value (BV) $BV = (I - (FN - EFN) - (UN - EUN))/ (I - (FN - EFN)$

Net Protein Utilization (NPU) $NPU = (I - (FN - EFN) - (UN - EUN))/ (I)$

Protein Digestion Corrected for Amino Acid Score (PDCAAS)

PDCAAS = (the amount of limiting amino acid in the test protein /the measured amount of limiting amino acid in reference protein) x True Digestibility

Protein Efficiency Ratio (PER) $PER = BW / (I*6.25)$

4.6. KPC Fatty acid analysis

Extraction of lipids from KPC was performed according to Bligh and Dyer (1959). To quantify fatty acids 48 µL of heptadecenoic acid (17:1) was added as a standard during the initial weighing of the samples. Conducted in duplicates, powdered freeze-dried KPC was mixed in a (2:1:0.015 v/v/v) chloroform:methanol:acetic acid solution. Following centrifugation at 900 *g* for 10 min at 10^{o}C, the chloroform layer was collected. The collected chloroform was then filtered through 1-phase separation filters to remove any remaining water and precipitated material. The extracted lipid was then transmethylated following the procedure described by Fritsche and Johnston (1990). Briefly, extracted fatty acids were methylated by adding 4% H_2SO_4 in anhydrous methanol to the collected chloroform layer followed by incubation in 90^{o}C water bath for 60 min. Samples were dried under nitrogen gas, and iso-octane was used as a diluent. The fatty acid profile was analyzed by gas chromatography (CP-3800, Varian, CA) with an initial temperature of 140^{o}C held for 5 minutes and then increased 1^{o}C per min to a final temperature of 220^{o}C. Nitrogen was used as the carrier gas, and total separation time was 110 minutes. Peak area and amount of each fatty acid was calculated by Star GC Workstation computer software (Varian Inc., CA).

4.7. Measurement of Lipid Oxidation

Fatty acid oxidation of KPC was determined by measuring thiobarbituric acid reactive substances (TBARs) in duplicates to oxidation at 0, 1, 3, and 7 days

according to Yu & Sinnhuber (1957). TBARs were determined by mixing powdered, freeze-dried KPC with an antioxidant solution (tertiary butyl hydroxy quinone in propylene glycol), to prevent further oxidation during the process. Thiobabituric acid was added to the solution with trichloroacetic acid (TCA-HCl) while incubated in 100^{O}C water bath for 30 minutes to permit the formation of malondialdehyde. The chloroform layer was then collected and absorbance measured at 535 nm by spectrophotometry (Du Series 500, Beckman, CA). Concentration of malondialdehyde was calculated using Beer's law with a extinction coefficient of 156,000 M^{-1} cm^{-1}.

4.8. Serum lipid and lipoprotein measurements

At the end of the 4 week feeding study, rats were euthanized by CO_2 inhalation. Trunk blood was collected and centrifuging at 1,500 *g* for 10 minutes at 4^{o}C. Serum was collected and stored at -80^{O}C until analyzed. Serum triglyceride, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were determined by Hemagen serum lipid rotors using the Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics Inc., Columbia, MD).

4.9. Organ weights and clinical blood measurements

Following euthanasia, the major organs i.e. brain, liver and kidneys were excised, trimmed, blotted, and weighed as indicators of toxicity. The adrenals

were weighed as an indicator of chronic stress. The retroperioteneal and gonad fat pads were also excised and weighed.

Clinical blood measurements were determined by the Vet-16 rotor using the Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics Inc., Columbia, MD). Liver function was assessed by measuring serum aspartate amino transferase activity (AST), and alanine transferase activity (ALT). Kidney function was assessed by measuring albumin, blood urea nitrogen (BUN), calcium, creatinine, total protein, and urinary output.

4.10. Statistical Analysis

The animal feeding study was a completely randomized design, with n=10 rats per diet treatment. The t-test was used to determine differences between casein and KPC diets, and one-way ANOVA was used to determine differences among the three treatment groups. Post hoc multiple comparison tests were performed using Tukey's test. t-test and one-way ANOVA were performed using Sigma Stat 3.1 (2004). To compare recorded body weights, repeated measures was performed using mixed model procedure in SAS 9.1 (2003). SAS 9.1 was also used to analyze organ weights with final body weight used as the covariate. All differences were considered significant at $P < 0.05$.

5.0 Results and Discussion

5.1. Krill protein isolation and proximate analysis

Figure 1 shows the proximate analysis of whole Antarctic krill and KPC. The composition of whole Antarctic krill was 81.2% moisture and on a dry basis 76.5% crude protein, 12.1% total lipid and 17.4% total ash. Values for whole krill are in agreement with other studies reporting proximate analysis of krill on a dry basis composing of 45-80% crude protein, 7-30% total lipid, and 10-20% total ash (Savage & Foulds 1987, Sidhu, Montgomery, Holloway, Johnson & Walker 1970, Grantham 1977).

Protein recovered from krill by the isoelectric solubilization/precipitation technique resulted in KPC with a protein recovery yield of ~46% (dry basis). The proximate composition of KPC recovered by the isoelectric solubilization/precipitation technique was 3.3% moisture, and on a dry basis 77.7% crude protein, 8.1% total lipid and 4.4% total ash.

The isoelectric solubilization/precipitation technique resulted in a 33% reduction in total lipid content while the protein content remained relatively constant compared to whole krill. Total ash was reduced ~75%, due to removal of the exoskeleton which is the predominate source of minerals in crustaceans. Removal of the exoskeleton is important because indigestible polysaccharides such as chitin, impedes digestion (Ikegamie et al., 1990).

Based on results of the proximate analysis, the isoelectric solubilization/precipitation technique successfully isolated krill protein and removed the exoskeleton. The protein quality of the KPC recovered by the

isoelectric solubilization/precipitation procedure was assessed to determine its suitability as a protein source for human consumption.

5.2. Amino acid profile

The amino acid content of KPC was compared to whole Antarctic krill, and the WHO/FAO/UNU amino acid requirements for human adults and infants (Table 2). The isoelectric solubilization/precipitation method effectively concentrated the essential and non-essential amino acid content of KPC compared to whole krill (Table 2). The total essential amino acids for KPC were 531.5 mg/g protein compared to 212.1 mg/g protein for whole krill. The presence of all nine essential amino acids in sufficient amounts to meet FAO/WHO/UNU requirements for human adults indicates that KPC is a high quality protein for human consumption.

Products where krill protein may substitute for other proteins are: sports drinks, infant formulas, and milk substitutes. For use as a protein source in foods for infants, KPC was lower in leucine and tryptophan concentration than FAO/WHO/UNU requirements for human infants. However, protein quality is determined not only by the essential amino composition but also by its digestibility. Therefore, an animal feeding study was performed to determine the bioavailability of KPC.

5.3. Protein quality measurements

Figure 2 displays the body weights of rats fed KPC and casein. Growth of rats fed KPC was equal to rats fed casein, suggesting KPC protein is high quality. Table 3 shows protein quality measurements of digestibility, BV, NPU, PDCAAS and PER. Lower (P<0.001) BV and NPU for KPC than casein indicated that there was less nitrogen retention by the rats fed the KPC compared to casein diet. However, the casein diet was supplemented with DL-Methionine whereas the diet with KPC was not (Table 1). The addition of limiting amino acids such as DL-Methionine is known to increase protein quality values (Sarwar, 1997). This in turn, may explain the lower scores in NPU and BV in rats fed the KPC compared to the casein diet. The NPU and BV measurements also have limitations. Primarily, they do not account for the amount of essential amino acids present in the test protein.

Currently, the PDCAAS is the preferred measurement for protein quality (Schaafsma, 2000) and the protein information on food labels are assessed using the PDCAAS. The PDCAAS takes into consideration the amino acid content as well as the digestibility of the protein of interest. The results of our study showed that PDCAAS scores were equal for KPC and casein (Table 3).

The PER, a ratio of body weight gain per g protein ingested, is used to determine the quality of protein sources to be added to foods for infants. The Food and Drug Administration specifies the use of casein as the reference protein for the PER and rats are the species that are routinely used to determine the quality of proteins to be used in infant formulas (Gropper, Smith & Groff, 2005).

In the current study, there was no difference in PER between rats fed the KPC and compared to the casein diet (Table 3). Furthermore, Figure 2 shows that KPC supported the growth of young female rats equal to casein.

Table 3 shows that there were no differences in total weight gain between rats fed casein or KPC. However, rats consuming KPC had greater total (P = 0.01), retroperitoneal (P = 0.02), and gonadal (P = 0.03) fat pad weights than rats fed casein. Fat mass was greater in rats consuming the KPC diet despite diets being isocaloric and no significant differences in food intake and feed efficiency displayed between rats fed KPC and casein (Table 3). A greater excretion ($P < 0.001$) of total nitrogen in rats fed KPC (1.8ml ± 0.07) than rats fed the casein diet (1.4ml ± 0.07) suggests that more amino acid deamination may have occurred in rats fed KPC. It is possible that excess amino acids not available for protein synthesis were being converted for energy storage. Further studies will be needed to address the mechanism responsible for the greater fat mass.

Based on the current study KPC appears to be a high quality protein that is comparable to casein for digestibility, PER and PDCAAS scores and supports growth. In addition, the ~8% lipid content of KPC may provide the advantage of having a healthier lipid composition than other animal proteins. Therefore, we evaluated the fatty acid profile of the lipid component of KPC.

5.4. KPC Fatty Acid Composition

The fatty acid composition of KPC (Table 4) was ~37% saturated fatty acids, ~21% monounsaturated fatty acids and ~27% ω-3 PUFAs. Of the ω-3

PUFAs, eicosapentanoic acid (EPA, 20:5, ω-3) accounted for 12.7 % and decosahexanoic acid (DHA, 20:6, ω-3) for 12.3%. KPC appears to contain higher amounts of EPA and DHA (~27%) compared to Coho Salmon (~21%), a fatty fish considered to be a rich source of ω-3 PUFAs. The lipid content of KPC appears to be rich in the biologically active ω-3 PUFAs, EPA and DHA. The ingestion of EPA and DHA has been linked to various health benefits (Ruxton, Reed, Simpson & Millington, 2004). However, ω-3 PUFAs are susceptibility to oxidation. Oxidized oils are associated with a short shelf-life and have negative health effects such as increased risk of CVD when ingested (Khan-Merchant, Penumetcha, Meilhac, & Parthasarathy, 2002). Therefore, we determined the oxidative stability of KPC.

5.5. Measurement of Lipid Oxidation

Figure 3 shows the results of the lipid oxidation measurement. TBAR values measured were lower than values obtained in "fresh" lipid fed to rats in other literature, with values recorded as being fresh ranging from 0.01-2.3 mmol MDA/kg (Eder, Keller, Hirche, & Brandsch, 2003). This would suggest that the KPC used in the current study was not oxidized, even after seven days at room temperature (Figure 3). Given that PUFAs are extremely susceptible to oxidation, the results are contrary to the expected results. Krill oil contains a high percentage of PUFAs making it susceptible to oxidation. However, krill oil has been shown to be high in various antioxidants such as vitamin E and the carotenoid, astaxanthin. Vitamin E is a well-known lipid soluble vitamin with

potent antioxidant properties and astaxanthin has been cited as being 10 times more potent antioxidant than β-carotene (Miki, 1991). The polyunsaturated fats found in fish are commonly associated with triglycerides, whereas krill has been shown to contain higher percentage of phospholipids. Bottino (1975) reported that ~58% of the total lipid in krill may be in the form of phospholipids. In vitro studies showed that DHA in phospholipid form are more stable against autoxidation than DHA incorporated into triglycerides (Song and Miyazawa, 1997). The presence of these components may explain the oxidative stability of krill oil.

5.6. Serum Lipid and Lipoprotein Profile

Total cholesterol (P=0.04) and HDL (P=0.003) was lower in rats fed KPC compared to the rats fed the casein diet (Table 5). Previous research has found that concentration of EPA may be responsible for decrease in HDL cholesterol. Using human males, Childs et al. (1990) found that fish oils rich in EPA lad to a similar decrease in HDL cholesterol. However, Bunea et al. (2004) found that krill oil was more effective at improving blood lipids and lipoproteins than fish oil. Individuals diagnosed with hyperlipidemia showed an increase in HDL and a decrease in both LDL and total cholesterol with krill oil consumption. There was no effect on total cholesterol or LDL in individuals provided equal amounts of fish oil. Bunea suggested that the greater lipogenic effects of krill oil was due to the ω-3 PUFAs in krill being associated with phospholipids; whereas, the ω-PUFAs in fish are mainly associated with triglycerides. In addition, the sterols

from shellfish, like krill, have been shown to interfere with cholesterol absorption (Vahouny, Connor, Roy, Lin & Gallo, 1981) and this in turn may improve the blood lipid and lipoprotein profile.

5.7. Organ weights and Clinical blood measurements.

Major organ weights were measured as indicators of toxicity (Table 6). Of the major organs, only the kidney weights were lower (P=0.003) in the rats fed KPC compared to casein. When brain weights were analyzed, a difference was suggested in terms of the relative brain weight. When data was re-analyzed using body weight as a covariate however, no difference was observed. Studies have shown that feeding different types of protein can influence mineral deposition in the kidneys. Zhang & Beynen (1992) provided rats with casein, soybean, and cod meal and reported that female rats fed diets of ~18% casein had the highest kidney calcium (Ca) concentration among the three proteins. In our study, total kidney mineral content corrected for kidney weight was higher ($P < 0.001$) in rats fed the casein (0.3g/g ± 0.04) compared to the KPC (0.07g/g ± 0.01) diet. Similarly Ca ($P = 0.008$) and phosphorus (P) ($P < 0.001$) content was also higher in rats fed casein compared to the KPC fed rats. Although rats fed KPC excreted a greater (P=0.03) volume of urine on a daily basis compared to rats fed casein, there was no significant difference in kidney function as indicated by serum levels of creatinine, blood urea nitrogen, protein, Ca, and P in the rats fed KPC compared to the casein diet (Table 7).

5.8. Conclusion

In conclusion, the large biomass of krill makes it an economical and sustainable alternative to current commercially available protein sources. The isoelectric solubilzation/precipitation method enabled protein from Antarctic krill to be successfully recovered. Nutritional evaluation of the recovered KPC indicates it is a high quality protein that is suitable as a protein supplement in foods designated for adult and possibly infant consumption. The PDCAAS and PER score showed KPC to be equal to the milk protein, casein. KPC also had the added advantage of being high in ω-3 PUFAs and was shown to be safe to consume in regards to kidney function.

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Figure 1.

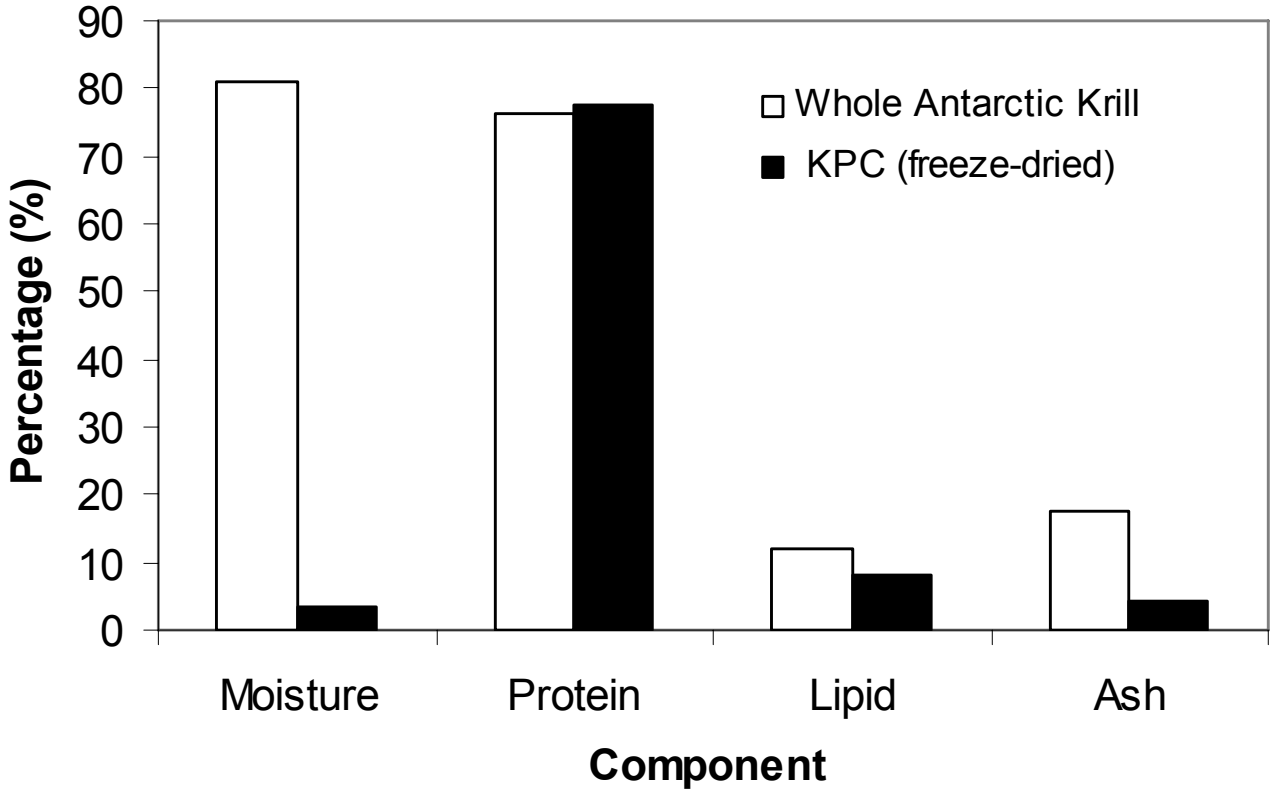
90
80
70
60
50
40
30
20
10
0
Percentage (%)
Whole Antarctic Krill
KPC (freeze-dried)
Moisture
Protein
Lipid
Ash
Component

Figure 2.

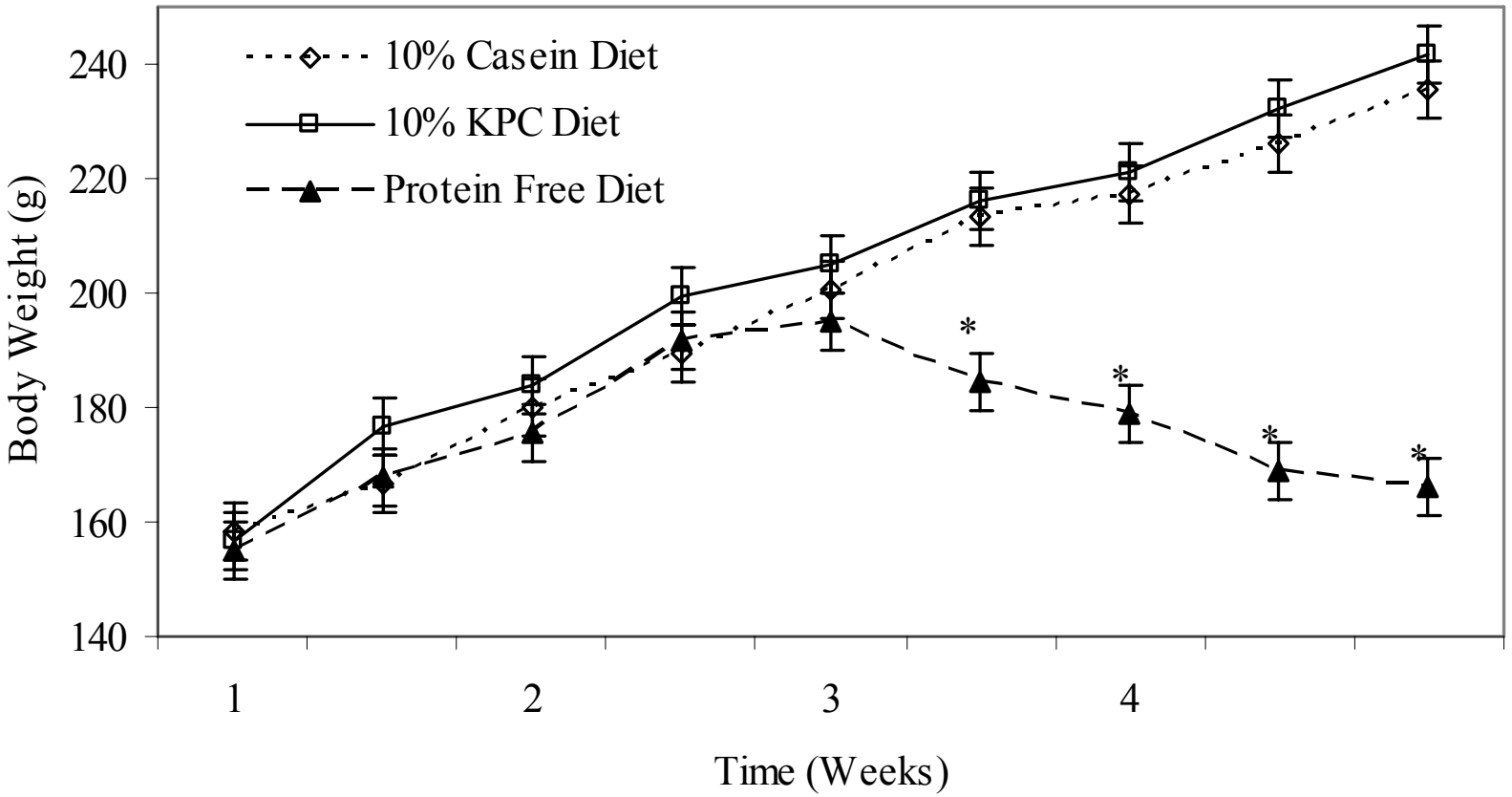
10% Casein Diet
10% KPC Diet
Protein Free Diet
Body Weight (g)
240
220
200
180
160
140
1
2
3
4
Time (Weeks)
*
*
*
*

Figure 3.

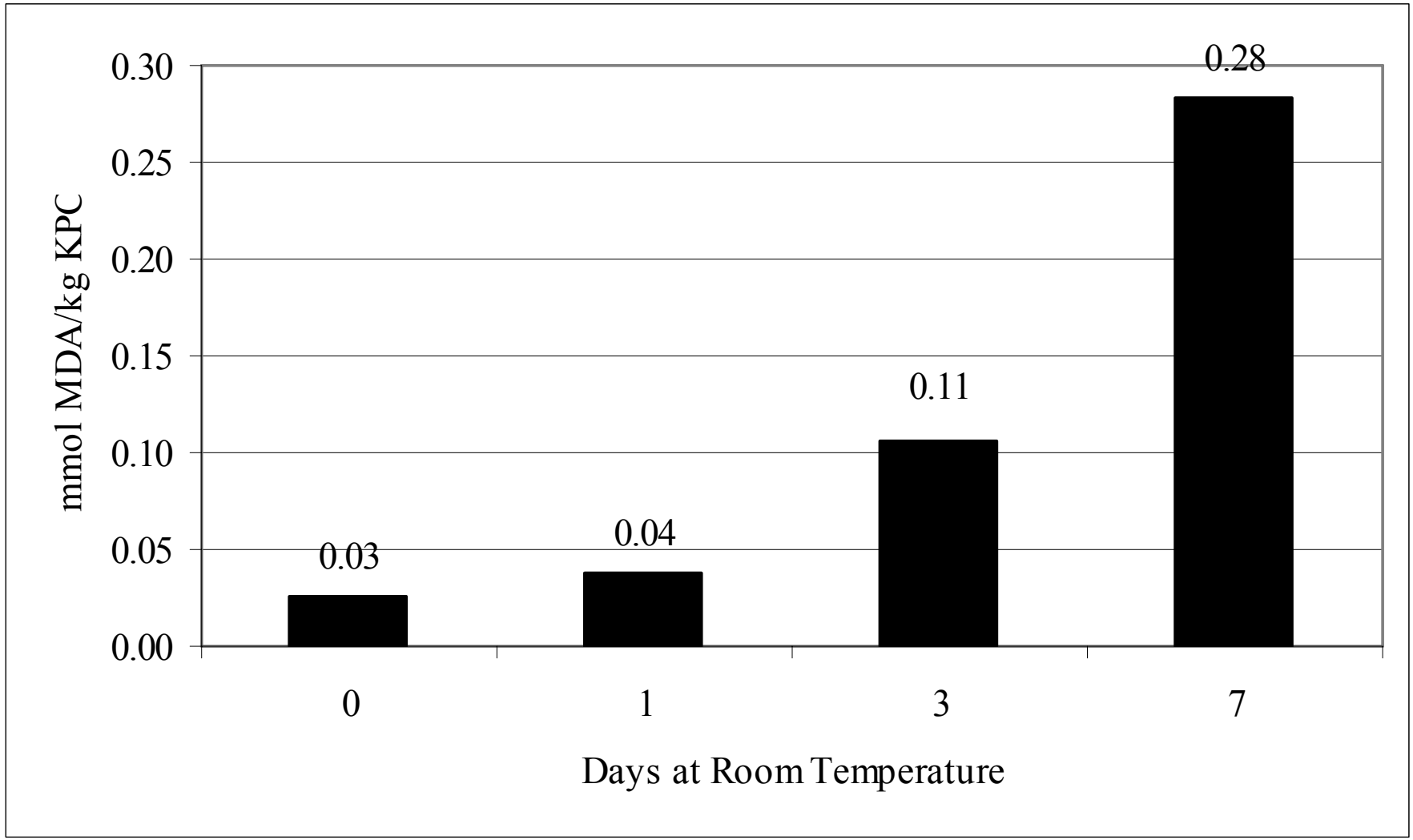
0.30
0.25
0.20
0.15
0.10
0.05
0.00
mmol MDA/kg KPC
0.03
0.04
0.11
0.28
0
1
3
7
Days at Room Temperature

Table 1. Diet Composition

Ingredients (g/kg diet)	10% Casein	10% KPC[b]	Protein Free
Casein	115	0	0
DL-Methionine	1.5	0	0
Sucrose	531.8	609.8	631.8
Corn Starch	200	229.4	200
Corn Oil	53.5	49.9	54.6
Cellulose	52	59.6	66.4
Vitamin Mix[a]	10	11.5	10
Ethoxyquin	0.01	0.01	0.01
Mineral Mix[a]	13.4	15.3	13.4
Calcium Phosphate	20.2	20.9	23.7
Calcium Carbonate	2.6	3.6	0.04

[a] Based on the AIN-93G diet vitamin and mineral mixes (Reeves et al., 1993).

[b] Diet formulated for 872g of diet + 128g addition of KPC

KPC = Krill Protein Concentrate

Table 2. Amino acid composition of whole krill, krill protein concentrate, and the FAO/WHO/UNU amino acids requirements for human adults and infants.

Amino Acid	KPC (mg/g protein)	Whole Antarctic Krill (mg/g protein)	FAO/WHO/ UNU 1985 Adult (mg/g protein)	FAO/WHO/UNU 1985 Infants (mg/g protein)
Essential Amino Acids				
Isoleucine	57.1	25.4	13	46
Leucine	88.4	39.9	19	93
Lysine	92	43.7	16	66
Cysteine + Methionine	48.5	24	17	42
Phenylalanine + Tyrosine	97.6	50	19	72
Threonine	46.4	21.5	9	43
Tryptophan	16.8	7.3	5	17
Valine	58.1	26	13	55
Histidine	26.6	11.4	16	26
Arginine	62.6	37.8		
Total EAA (mg/g protein)	531.5	212.1	127	460

Non-Essential Amino Acids		
Cysteine	14.9	8.5
Tyrosine	46.5	27.9
Alanine	54.4	29.4
Aspartate	106.3	53.4
Glutamate	116.7	66.9
Glycine	41.3	33.5
Proline	31.8	22.9
Serine	37.6	19.1
Taurine	4.2	15.5
Total NEAA (mg/g protein)	516.3	314.9

KPC = Krill Protein Concentrate, Total EAA = total essential amino acids, Total NEAA = total non-essential amino acids

Table 3. Protein quality measurements, body weight, fat pads and food intake measurements.[a]

Protein Quality Measurement	Casein	KPC
Digestibility (%)	93.3 ± 2.0	93.2 ± 1.0
Biological Value	0.69 ± 0.01	0.58 ± 0.02*
Net Protein Utilization (%)	64.7 ± 1.0	54.1 ± 2.0*
PDCAAS	1	1
Protein Efficiency Ratio (g BW/g protein)	1.57 ± 0.05	1.44 ± 0.15
Final Body Weight (g)	235.4 ± 5.8	241.8 ± 3.4
Total Body Weight Gain (BW) (g/4 wk)	73.3 ± 3.0	80.0 ± 3.5
Total Fat Pad Weights (g)	7.9 ± 0.4	9.6 ± 0.4*
Retroperitoneal Fat Pad (g)	2.5 ± 0.2	3.3 ± 0.3*
Gonadal Fat Pads (g)	5.3 ± 0.3	6.3 ± 0.2*
Total Food Intake (FI) (g)	451.1 ± 11.5	462.7 ± 11.6
Feed Efficiency (g BW/g FI)	0.16	0.17

[a] Values are mean ± SEM with n=10

* Significant difference ($P < 0.05$) by Student's *t*-test.

KPC = krill protein concentrate, PDCAAS = protein digestibility corrected for amino acid score

Table 4. Fatty acid composition of krill protein concentrate, whole Antarctic krill, and Coho salmon.

Fatty Acid (%)	KPC	Whole Antarctic Krill [a]	Coho Salmon [b]
SFAs			
14:0	10.0	4.9	4.5
16:0	25.2	18.8	12.7
18:0	2.1	1.0	3.5
MUFAs			
16:1	7.51	4.9	8.5
18:1	13.5	16.4	20.3
PUFAs			
ALA 18:3 (n-3)	1.5	1.1	2.6
EPA 20:5 (n-3)	12.7	17.4	7.2
DHA 22:6 (n-3)	12.3	12.4	11.1
18:2 (n-6)	3.1	3.3	6.5
20:4 (n-6)	1.2	0.5	2.2

[a] Values from Suzuki & Shibata, 1990

[b] Values from USDA, 2004

KPC = krill protein concentrate, SFAs = saturated fatty acids, MUFAs= monounsaturated fatty acids, PUFAs = polyunsaturated fatty acids, ALA = α-linolenic acid, EPA = eicosapentaenoic acid, DHA = Docosahexaenoic acid.

Table 5. Serum lipid and lipoprotein profile of rats fed 10% KPC or 10% casein diets.[a]

Clinical Measure	Casein	KPC
Triglycerides (mg/dl)	218.6 ± 24.6	235.1 ± 19.3
Total-CHOL (mg/dl)	97.9 ± 6.5	67.9 ± 11.0*
VLDL (mg/dl)	43.7 ± 4.9	47.1 ± 3.9
LDL (mg/dl)	38.1 ± 8.7	31.8 ± 5.2
HDL (mg/dl)	95.2 ± 7.3	66.3 ± 3.7*

[a] Values are given as mean ± SEM of n = 10

* Indicates significant difference at $P < 0.05$ by the Student's *t*-test.

KPC = krill protein concentrate, CHOL = cholesterol, VLDL = very low density lipoproteins,

LDL = low density lipoproteins, HDL = high density lipoproteins.

Table 6. Absolute and relative organ weights of rats fed either 10% Krill protein concentrate or casein.

Organ Weights	Casein	KPC
Absolute Brain Weight (mg)	1.7 ± 0.03	1.6 ± 0.03
Relative Brain Weight (mg/100g body weight)	720.1± 11.2	677.3 ± 14.8*
Absolute Heart Weight (mg)	0.8 ± 0.07	0.9 ± 0.06
Relative Heart Weight (mg/100g body weight)	357.2 ± 6.7	361.4 ± 7.4
Absolute Liver Weight (mg)	9.4 ± 0.3	10.1 ± 0.2
Relative Liver Weight (mg/100g body weight)	3973.9 ± 85.5	4190.7 ± 89.7
Absolute Kidneys Weight (mg)	2.1 ± 0.05	1.9 ± 0.04*
Relative Kidneys Weight (mg/100g body weight)	897.2 ± 18.2	780.7 ± 20.2*
Absolute Adrenals Weight (mg)	0.07 ± 0.003	0.08 ± 0.002
Relative Adrenals Weight (mg/100g body weight)	29.7 ± 0.7	31.2 ± 1.1

[a] Values are given as mean ± SEM of n = 10

* Indicates significant difference with $P < 0.05$ by the Student *t*-test.

KPC = krill protein concentrate.

Table 7. Clinical serum measurements of kidney and liver function.[a]

Clinical Measure	Casein	KPC
	Kidney Function	
Albumin (U/L)	4.2 ± 0.5	4.1 ± 0.9
BUN (U/L)	12.1 ± 1.3	10.1 ± 3.2
Calcium (U/L)	11.5 ± 1.3	11.4 ± 2.7
Creatinine (U/L)	0.5 ± 0.15	0.5 ± 0.1
Total Protein (U/L)	6.0 ± 0.7	6.3 ± 1.0
Phosphorus	11.3 ± 0.4	10.7 ± 0.5
Urinary output (ml/d)	6.0	14.5*

[a] Values are given as mean ± SEM

* Indicates significant difference at $P < 0.05$ using the Student's *t*-test

KPC = krill protein concentrate, BUN = blood urea nitrogen, AST = Aspartate amino transferase,

ALT = Alanine amino transferase.