Effect of Calcium Compounds Obtained from Fish Processing By-Product on Calcium Metabolism in Rats

Phatchareerat Tongchan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Technology

Prince of Songkla University

2011

Thesis Title	Effect of Calcium Compounds Obtained from Fish Processing By-Product on Calcium Metabolism in Rats
Author	Mrs. Phatchareerat Tongchan
Major Program	Food Science and Technology

Major Advisor:

……..
(Assist. Prof. Dr. Chakree Tongreung)

Co-advisor:

……
(Dr. Sunisa Siripongvutikorn)

……
(Dr. Sathaporn Prutipanlai)

Examining Committee:

…….Chairperson
(Assist. Prof. Dr. Suphitchaya Chanthachum)

.....
(Prof. Dr. Nateetip Krishnamra)

…….
(Assist. Prof. Dr. Chakree Tongreung)

…….
(Dr. Sunisa Siripongvutikorn)

…….
(Dr. Sathaporn Prutipanlai)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Food Science and Technology

…….
(Prof. Dr. Amornrat Phongdara)
Dean of Graduate School

ชื่อวิทยานิพนธ์	ผลของสารประกอบแคลเซียมที่ผลิตจากเศษเหลือใช้ของกระบวนการแปรรูปปลาต่อกระบวนการแคลเซียมเมทาบอลิซึมในหนูขาวใหญ่
ผู้เขียน	นางสาวพัชรีรัตน์ ทองจันทร์
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา	2553

บทคัดย่อ

โครงกระดูกปลาเป็นของเหลือที่เป็นของแข็งหลักที่เกิดขึ้นในอุตสาหกรรมการแปรรูปปลาแช่เยือกแข็ง โดยทั่วไปเศษเหลือดังกล่าวถูกนำไปผลิตเป็นปลาป่นที่มีมูลค่าต่ำ การวิเคราะห์องค์ประกอบเคมีของโครงปลาตาโตพบว่าประกอบด้วยโปรตีนและเถ้าร้อยละ 54.95 และ 31.41 (น้ำหนักแห้ง) ตามลำดับ โดยมีกล้ามเนื้อที่ติดอยู่ที่กับกระดูกปลาประมาณร้อยละ 41.5 ของน้ำหนักโครงปลา การกำจัดกล้ามเนื้อดังกล่าวทำได้โดยต้มในกรดแลคติคเข้มข้นร้อยละ 5 ที่อุณหภูมิ 85 องศาเซลเซียสเป็นเวลา 5 นาที การผลิตสารประกอบจากกระดูกที่สะอาดกระทำโดยการเผา โดยทำให้มีอุณหภูมิเพิ่มขึ้น 10 องศาเซลเซียสต่อหนึ่งนาที และคงไว้ที่อุณหภูมิ 1300 องศาเซลเซียสเป็นเวลา 0, 1, 2 และ 3 ชั่วโมง ซึ่งทำให้น้ำหนักตัวอย่างลดลงร้อยละ 30 เมื่อตรวจสอบด้วย XRD พบวัฏภาคของแคลเซียมไฮดรอกซีแอปาไทต์และแคลเซียมไตรฟอสเฟสในวัสดุหลังการเผา

การใช้แคลเซียมที่ผ่านการเผาที่อุณหภูมิ 1300 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ไปเสริมให้แก่หนู 4 กลุ่ม เพื่อให้แต่ละกลุ่มได้รับแคลเซียม 0, 11, 22 และ 44 มิลลิกรัม แคลเซียมต่อวัน เป็นเวลา 7 สัปดาห์ พบว่าการเสริมแคลเซียมทำให้น้ำหนักตัวเฉลี่ยของหนูเพิ่มขึ้น ในขณะที่หนูกลุ่มที่ไม่เสริมแคลเซียมมีน้ำหนักตัวเฉลี่ยลดลงร้อยละ 9 ของน้ำหนักเริ่มต้น การเสริมแคลเซียมทำให้การดูดซึมและการกักเก็บแคลเซียมไว้ในร่างกายหนูเพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) การเสริมแคลเซียมทำให้น้ำหนักกระดูกโคนขาของหนูเพิ่มขึ้นเมื่อเปรียบเทียบกับกลุ่มควบคุม หนูกลุ่มควบคุมมีช่องว่างปรากฏขึ้นในกระดูกโปร่ง (trabecular bone) ในขณะที่กลุ่มที่เสริมแคลเซียมพบว่ากระดูกโปร่ง (trabecular bone) มีช่องว่างที่แคบและต่อเนื่องกว่ากลุ่มควบคุม ดังนั้นหนูจึงสามารถนำสารประกอบแคลเซียมที่เตรียมได้จากเผากระดูกปลาตาโตไปใช้ประโยชน์ทางชีวภาพได้

Thesis Title	Effect of Calcium Compounds Obtained from Fish Processing By-Product on Calcium Metabolism in Rats
Author	Mrs. Phatchareerat Tongchan
Major Program	Food Science and Technology
Academic Year	2010

ABSTRACT

Fish frame is a main solid by-product of the frozen fish processing. It is normally used for fish meal production because low value. Proximate analysis of the bigeye snapper frame revealed content of 54.95% protein and 31.41% ash (dry basis). The frame was attached with muscle tissue for 41.5% of total weight. It was removed by boiling the frame in lactic acid solution (5% w/w) at 85 ^{o}C for 15 min. The muscle free-bone was burned with the heating rate of 10 ^{o}C/min and sintered at 1300 ^{o}C for 0, 1, 2 or 3 hours. This heating processing reduced about 30% of the frame weigth. Evaluation of morphologic of calcium compound by using X-ray diffractometer (XRD) revealed the existence of calcium hydroxyapatite and whitlockite phase. The sintering at 1300 ^{o}C did not cause significant effect on the XRD patterns.

Calcium compound derived from sintering the frame at 1300^{0}C for 1 hour was supplement to 4 groups of male Wistar rats receive 0, 11, 22, or 44 mg Ca/day for 7 weeks. Supplementation of calcium increased mean body weight of rats whereas a 9% loss of mean body weight of control group was observed. Absorption and retention rate of the supplemented calcium were increased significantly ($p<0.05$) with increasing of the calcium intake. Femur weight of rats was increased by increasing amount of calcium supplement with respect to that of the control rats. Abnormal trabecular conformation was noticed in the control rats whereas thicken and narrowed inter-trabecular spaces were observed in the calcium supplement groups. The results thus revealed that Wistar rat could be use the sintered calcium compound for bone normal development.

ACKNOWLEDGEMENT

I would like to express my deep appreciation and sincere gratitude to my advisor, Assist. Prof. Dr. Chakree Tongreung for his guidance during study. His encouragement will be always appreciated. I also would to thank him for knowledge and personal life.

My great thankfulness goes to my co-advisor, Dr. Sunisa Siripongvutikorn for her question, valuable suggestion and recommendations. Her advice can help me stop and thinking carefully about my work. I am also extremely thankful to Dr. Sathaporn Prutipunlai of Faculty of Science, Prince of Songkla University who another one of my co-advisor for her guidance and helpful throughout practice work with animal laboratory.

I would like to thankful the examining committees, Assist. Prof. Dr. Suphitchaya Chanthachum and Prof. Dr. Nateetip Krishnamra for their valuable suggestions and recommendations.

I wish to thank the staff of animal laboratory, Science faculty, Prince of Songkla University.

My deep acknowledgement is due to all of my friends and staffs who gave me their helps and shared a hard time with me during study.

My deepest gratitude to my father and mother for their presence in my life, they always support and try to understand me towards finishing my thesis. I also thank my brother and sister for their encouragement for me throughout my life.

This study could not be succeeded without the financial support from Graduate School Prince of Songkla University and the Agro-Industry Practice in Agro-Industry Scholarship. I gratefully acknowledge these financial supports.

Phatchareerat Tongchan

CONTENTS

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Osteoporosis is the most common metabolic bone disease in human that often found in postmenopausal woman. It is characterized by low bone mass, micro architectural deterioration, compromised bone strength, and an increase in the risk fracture (Pongchaiyakul *et al.*, 2008). WHO reported that this disease has effect more than 75 million people in the United States, Europe and Japan. Osteoporosis causes more than 8.9 million fractures annually worldwide, of which more than 4.5 million occur in the America and Europe. The lifetime risk for a wrist, hip or vertebral fracture has been estimated to be in the order of 30 % to 40 % in developed countries. With rapid ageing of Asian population, osteoporosis has become one of the prevalent and costly health problems (Lau, 2002). In other words, very close to that for coronary heart disease. In a nation-wide survey during 2000-2001, the age adjusted prevalence of osteoporosis in Thai woman ranging from 40-80 years was 13.6% and 19.8 % for femoral neck and lumbar spine, respectively (Limpaphayom *et al.*, 2001). The age-specific prevalence of osteoporosis among Thai woman below 50 years of age was less than 5% and the prevalence increase with advancing age, i.e., more than 50% found after age of 70. Comparatively, a study from Khon Kaen province, a rural area of Thailand reported that the prevalence of osteoporosis showing a bit higher than the aforementioned study. The prevalence of osteoporosis in the latter report was found to be 19.3 and 24.7% at the femoral neck and lumbar spine, respectively (Pongchaiyakul *et al.*, 2002). Differences in the disease prevalence are probably due to the dissimilarity of the reference database of the mean peak bone mass used for the WHO measurable criteria. The study in Khon Kaen province used the mean peak bone mass developed from rural women that was higher than the one developed mainly from an urban area. For men, the age-adjusted prevalence of osteoporosis was 12.6, 4.6 and 3.9% at the femoral neck, lumbar spine and both sites, respectively (Pongchaiyakul *et*

al., 2006). These figures of prevalence in both men and women are comparable with previous studies in Western countries and in some other Asian countries (Melton, 2003). Over the past decade, osteoporosis has emerged as one of the most common diseases in the elderly population and has represented as one of the most significant public health problems due to its morbidity, mortality, and financial cost related to fractures, particularly hip fracture. From the studies in a Thai population, osteoporosis may be preventable, as a number of environmental factors are open to intervention by effective pharmacological agents in with appropriate non-medical modality (Pongchaiyakul *et al.*, 2008).

Annually, more than 50% of total fishery products are discarded as inedible byproduct, such as bone, fin, internal organ and head. Praseadsun (1992) reported that fishery processing in Thailand spend 30-85 % by-product including of 25-30% solid and 30-35 % liquid. Thus, many studies have been performed to utilize the large amounts of protein, oil, mineral, carbohydrate and nucleic acid originating from fishery byproducts, and to improve their functional properties (Nagai and Suzuki, 2000).

Many different by-products are generated by marine products processing plants each year. Fish frame is a by-product from frozen seafood processing. As such, fish frame is rich in useful inorganic substances that containing a proper balance of calcium and phosphorus that can be used as a calcium food supplement (Kim *et al.*, 2000; Tsutagawa *et al.*, 1994) However, it has been used chiefly for applications in animal feed or disposed of, and the latter contributing to environmental pollution. A few studies on the extract of calcium from tuna bone, a by-product of canned tuna, have been conducted (Kim *et al.*, 1999; Lee *et al.*, 1997).

Utilization the fish frame as calcium sources is possible because it could be formed microcrystalline hydroxyapatite after sinter treatments (Jung *et al.*, 2006). However, studies on the utilization of organic components or mineral in the fish bone are scarce. Additionally, the use of seafood by-product in term of calcium source would be a possible means to calcium supplement. The outcome of this study will be of great benefit for usable of by-product to produce a new source of calcium.

1.2 Review of literature

1.2.1 Bone

Bones are rigid organs that form part of the endoskeleton of vertebrate with a specialized connective tissue composed of both mineral and organic phases that exquisitely designed for its role as the load re-bearing structure of the body. To accomplish this task, it is formed from a combination of dense compact bone and cancellous (trabecular) that is re-unforced at point of stress. Cortical bone is dense and compact. It forms the outer layer of the bone. Trabecular bone makes up the inner layer of the bone and has a spongy, honeycomb-like structure. The mineral phase of the skeleton contributes about two-thirds of its weight; the remaining one-thirds is organic matrix, consisting primarily of type I collagen and small among of non-collagenous protein (Figure 1) (Hill, 1998). Collagen is a protein that provides a soft framework, and calcium phosphate is a mineral that adds strength and hardens the framework. This combination of collagen and calcium makes bone strong and flexible enough to withstand stress. More than 99 percent of the body's calcium is contained in the bones and teeth. The remaining 1 percent is found in the blood. Living bone is not dry, brittle, or dead. It is a moist, changing, productive tissue that is continually resorbed (dissolved and assimilated), re-formed, and remodeled (replaced and renewed) (Wynsberghe *et al.*, 1995). The bones in adult human body are 206 pieces (Katja *et al*., 2007).

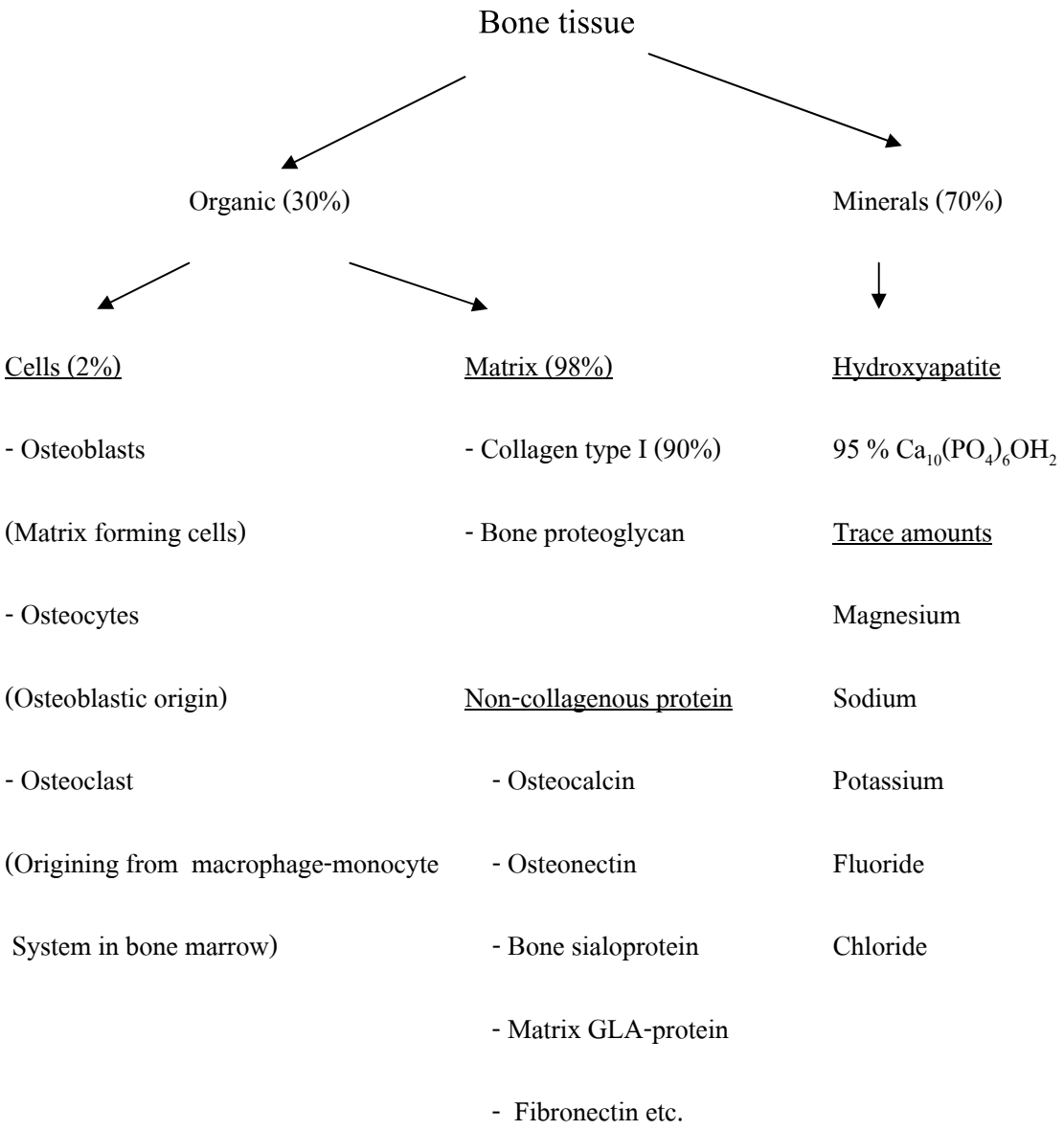

Figure 1. Bone compositions

Source: Dogan and Posaci (2002)

Bone remodeling

Bone is subject to a continuous process of breakdown and renewal, called remodeling, by which bone mass is adjusted throughout adult life. This remodeling the process of resorption followed by replacement of bone with little change in shape. Bone ossification are continually remodelled from the time that initial calcification occurs until the final structure appears. Remodelling is the replacements of old bone tissue by new bone tissue (Gerard and Nicholas, 1990). After the intramembranous and endochondral bones form, the actions of osteoclasts and osteoblasts continually remodel them. Thus, throughout life, osteoclasts resorp bone tissue and osteoblasts replace the bone. These opposing processes of resorption and deposition are well regulated so that the total mass of bone tissue within an adult skeleton normally remains nearly constant, even though 3% to 5% of bone calcium is exchanged each year (David *et al.*, 1999). Bone is constantly undergoing bone remodeling which is a complex process involving the resorption of bone on a particular surface, followed by a phase of bone formation. In normal adults, there is a balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteobasts (Frost, 1991). Bone remodeling occur in small packets of cells called basic multicellular units (BMUs), which turn bone over in multiple bone surfaces at any one time, about 20% of the cancellous bone surface is undergoing remodeling. Calcium plays a very important role in bone remodeling. In this process bone is deposited by osteoblast and resorption is carried out by osteoclasts. In the bone deposition process bone is created by calcium and phosphate ions binding to create hydroxyapatite crystals. In the bone resorption process, the hematopoietic stem cell derived osteoclasts release enzymes that digest the bone and in doing so release the calcium back into the body circulation. Winzenberg *et al.* (2006) reported that calcium supplementation has little effect on bone remoding in children. Calcium supplementation may reduce bone remodelling rather than or as well as increasing bone modelling, accounting for the transient benefit of supplementation seen in some studies (Heaney, 2001). The purpose of remodeling is to regulate calcium homeostasis, repair micro-damaged bones (from everyday stress) but also to shape and sculpture the skeleton during growth. Bone remodeling can be divided into the following phases (Figureure 2) (Isabel *et al.*, 2006)

1. Quiescent phase: The bones are still rest. The factors that initiate the remodeling process remain unknown.

2. Activation phase: the first phenomena that occurs is the activation of the bone surface prior to resorption, through the retraction of the bone lining cells (elongated mature osteoblasts existing on the endosteal surface) and the digestion of the endosteal membrane by collagenase action. Once exposed, the mineralized surface attracts the circulating osteoclasts coming from the nearby vessels.

3. Resorption phase: the osteoclasts then begin to dissolve the mineral matrix and decompose the osteoid matrix. This process is completed by the macrophages and permits the release of the growth factors contained within the matrix, fundamentally transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF), insulin-like growth factor I and II (IGF-I and II).

4. Formation phase: simultaneously in the resorbed areas the preosteoblast grouping phenomena is produced, attracted by the growth factors liberated from the matrix which act as chemotactics and in addition stimulate their proliferation (Lind *et al.*, 1995). The preosteoblasts synthesize a cementing substance upon which the new tissue is attached, and express bone morphogenic proteins (BMP) responsible for differentiation. A few days later, the already differentiated osteoblasts synthesize the osteoid material which fills the perforated areas.

5. Mineralization phase: mineralization begins thirty days after deposition of the osteoid, ending at 90 days in the trabecular and at 130 days in the cortical bone. The quiescent or ‘at rest’ phase then begins again platelet derived growth factor (PDGF), insulin-like growth factor I and II (IGF-I and II).

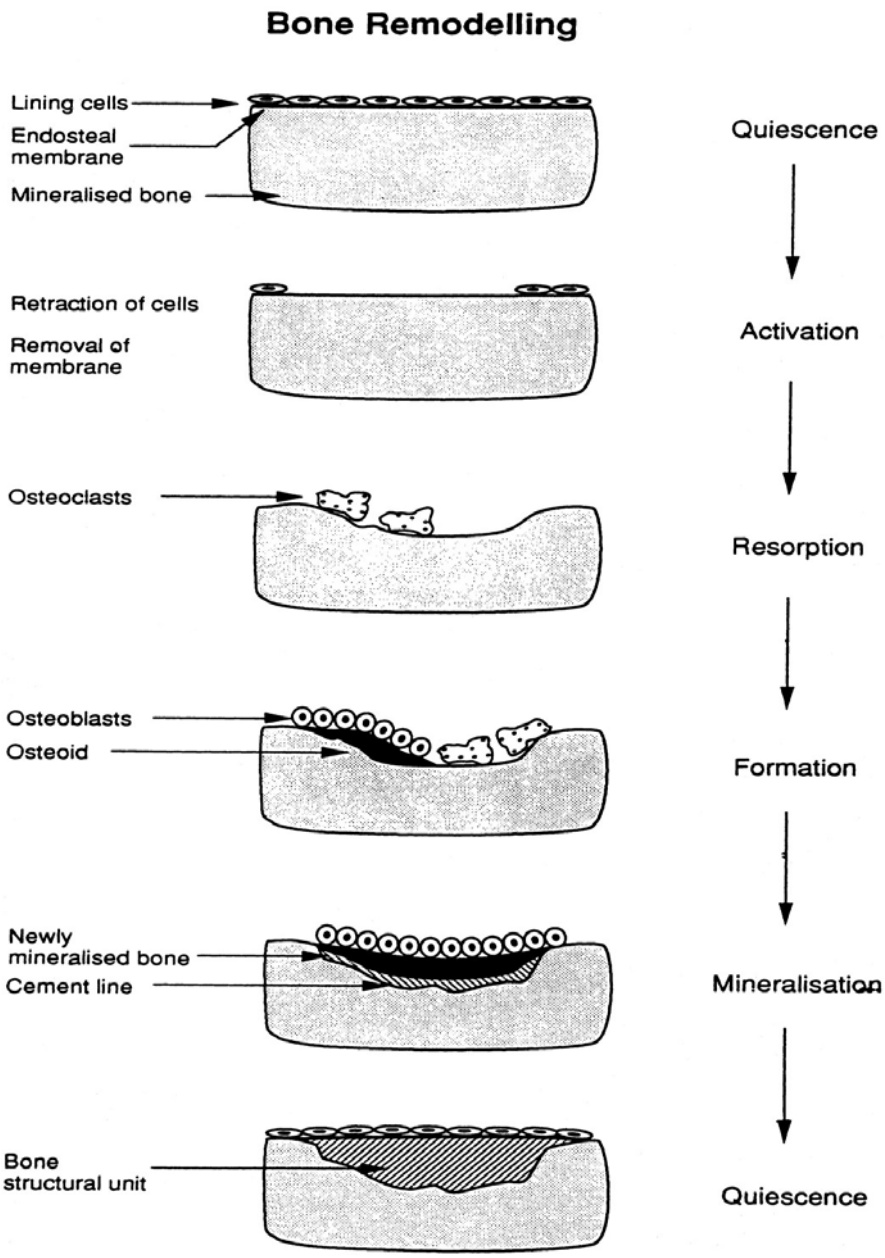

Figure 2. Bone remodeling phases.

Sources: Modified from Compston *et al*. (2007)

Bone is constantly renewed but the rate of the remodeling process is not evenly distributed throughout the skeleton. It is higher in cancellous bone which is more abundant in the vertebrae and in the metaphyseal regions of the long bones (i.e., the osteoporotic fracture-relevant sites) than in cortical bone. The increased of calcium intake was suppresses the number of bone-remodeling sites results in an apparent increase in bone density. (Dawson *et al.*, 1997.; Karl, 2009; Mackerras and Lumley,

1997; Nielsen *et al.*, 1998) Nielsen *et al*., (1998) has shown that increasing calcium intake in young horses results in a higher calcium retention. This rise in available calcium may be beneficial altering bone remodeling and promoting a greater mineral density, which is associated with increased bone strength. Maehira *et al*. (2009) reported that the calcium source had effect on biomechanical properties of bone with bone remodeling. Solubility of calcium contributes to bone quality though the dual action of stimulating bone formation and inhibiting bone resorption.

a. Mineral deposition

Mineral deposition (mineralization) is crystallization processes in which calcium, phosphate, and other ions are taken from the blood plasma and deposited in bone tissue. It begin in fetal ossification and continuous throughout life. Osteoblasts begin the process by laying down collagen fibers in a helical pattern among the length of the osteon. These fibers then become encrusted with minerals especially calcium phosphate that harden the matrix. Calcium phosphate crystals do not form unless the products of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) concentration in the tissue fluids reaches a critical value called the solubility product. Most tissues have inhibitors to prevent this, so they do not become calcified. Osteobalst, however, apparently neutralize these inhibitors and thus allow the salts to precipitate in the bone matrix. The first few hydroxyapatite crystals to form act as “seed crystals” that attract more calcium and phosphate from solution. The more hydroxyapatite that forms, the more it attracts additional minerals from the tissue fluid, until the matrix is thoroughly calcified (Kenneth and Saladin, 2007; Rhoades and Pflanzer, 1992)

b. Mineral resorption

Mineral resorption is the process by which osteoclasts break down bone and release the minerals, resulting in a transfer of calcium from bone fluid to the blood. High levels of calcium, magnesium, phosphate and products of collagen will be released into the extracellular fluid as the osteoclasts tunnel into the mineralized bone. The resorption involves the preparation of bone surface by removal of the unmineralized osteoid layer by the linning osteoblasts which produce a variety

of enzyme, (metalloproteinase, collagenase and gelatinase). This facilitated access of the osteoclasts to the underlying mineralized bone (Hill, 1998).

1.2.2 Calcium

Calcium is a divalent cation. The most obvious function of calcium is as a component of the skeleton, where it occurs as a salt with phosphate. More than 99% of total body calcium is stored in the bones and teeth where it functions to support their structure (Shils *et al*., 1999). Although the absolute amount of calcium in the extra cellular fluid is small, this fraction is stringently regulated. Calcium plays important roles in several cellular processes, including cell division, growth, bone formation, blood coagulation, hormone signaling, and neuromuscular functions. A 70-kg man contains about 1.3 kg of calcium. The remaining 1% is found throughout the body in blood, muscle, and the fluid between cells. This calcium is not metabolically inert. About 0.5 g of calcium leaves the bone and is deposited back into the bone each day. A constant level of calcium is maintained in body fluid and tissues so that these vital body processes function efficiently. Calcium is lost from the body in feces, urine, and sweat. The fecal calcium consists of unabsorbed dietary calcium, the amount of which depends on dietary intake and other factors, and a small portion of the endogenously secreted calcium (about 100 to 150 mg/day), which escapes reabsorption. Urinary calcium excretion of adults is about 100 to 250 mg/day, but varies widely among persons consuming self-selected diets (Nordin *et al*., 1967). As in infancy and during the pubertal growth spurt in adolescence. Calcium supplementation in children and adults has been shown to have beneficial effects on bone mineral density (BMD) (Matkovic *et al.*, 2005; Vatanparast and Whiting, 2006)

Calcium stores

Skeletal calcium largely is in a crystalline form that resembles the mineral hydroxapatite with other ions in the crystal lattice. The steady state content of calcium in bone reflects the net effect of bone resorption and formation. Calcium is an essential mineral in the body. More than 99% of calcium in the body is in bone and teeth and the small fraction is in the extracellular fluid which fraction is stringently regulated. The total serum calcium concentration is 10 mg/dl (2.5 mM) divided into three pools as follow.

1. Protein-bound (40%)
2. Complexes with small anion (10%) such as phosphate and citrate
3. Ionized calcium (50%)

The complexes and ionized pools represent the differentiable form of calcium.

1.2.2.1 Calcium metabolism

a. Calcium absorption

Calcium absorption mostly occurs in the small intestine and the rate of calcium absorption varies among different segments. Duodemun is most effective part for calcium absorption because it has capability to extract and absorb calcium from low-calcium diet by the active transport process (Charoenphandhu *et al.*, 2006). Transepithelial absorption of calcium, like that of other solutes, can be conceptually described by the relation calcium absorption that is summation of passive transport and active transport. Passive absorption is the sum of diffusion and solvent drag. Active transport is an energetically dependent transcellular process. Cellular calcium absorption across polarized epithelial cells is a two-step process where entry across apical (mucosal, luminal) membranes is followed by extrusion across basolateral (serosal, contraluminal) membranes into interstitial fluid and thence into the circulation. Calcium influx down its electrochemical gradient across apical membranes is generally thought to be mediated by calcium channels. Basolateral efflux, in contrast, involves energy-dependent extrusion that is accomplished by the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na+/Ca^{2+} exchanger. Calcium absorption by proximal intestine is mediated by a combination of passive and active transport mechanisms. Most evidence suggests that small intestine calcium transport is dominated by passive diffusion with a small component of solvent drag mechanisms. Together, these two mechanisms account for about 90% of proximal tubule calcium absorption. Furthermore, these passive transport mechanisms would dictate a paracellular route for absorption. The ability of the body to absorb calcium from food is greatly variably affected by the presence of other nutrients and substances in the diet, calcium bioavailability from food, as well as by individual physiological factor (Drueke., 1990). The central feature of

transepithelial calcium movement in the small intestine is that it occurs by two independent processes. The first is a saturable, transcellular route that is subject to physiological and nutritional regulation via vitamin D and takes place largely in the proximal intestine, i.e., the duodenum and the upper jejunum. The second process is nonsaturable, essentially independent of nutritional and physiological regulation, and concentration-dependent, and exists all along the small intestine (Pansu *et al*., 1981; Pansu *et al.*, 1999).

b. Factors affecting calcium absorption

(1). Calcium intake

The amount of calcium absorbed in the intestine depends on habitual calcium intake. The low calcium intake, active transcellular calcium transport in the duodenum is unregulated and a larger proportion of calcium is absorbed. In rats on low calcium intakes, active transport in the duodenum can account for about 50% of the total absorbed but the fraction accounted for by active transport diminishes rapidly as calcium intake increases (Bronner and Pansu, 1999). Yin *et al.* (2010) reported that calcium absorption efficiency in Chinese boys increased with calcium intake up to 665 mg/day with calcium intake ranged from 352 to 1323 mg/day.

(2). Hormones

Calcium absorption correlated with two major hormones that composed of parathyroid hormone and 1,25-dihydroxyvitamin D. A third hormone, calcitonin plays a limited role. Gastrointestinal tract are subject indirectly to control by parathyroid hormone (PTH). Widmaier *et al*. (2004) reported that PTH production is controlled by the extracellular calcium concentration, acting directly stimulates the formation of 1,25-dihydroxyvitamin D, which then increases intestinal absorption of calcium. Decreased plasma calcium concentration stimulates parathyroid hormone secretion and an increased plasma calcium concentration does just the opposite. Facilitating calcium absorption from the small intestine would clearly serve to elevate blood levels of calcium.

(3). Age

Net calcium absorption can be as high as 60% in infants and young children, when the body needs calcium to build strong bones. Absorption slowly decreases to 15-20% in adulthood and even more as one age. Because calcium absorption declines with age, recommendations for dietary intake of calcium are higher for adults ages 51 and over. Yin *et al*. (2009) also reported that are important factors for calcium retention and absorption during growth.

(4). Vitamin D

Vitamin D is mobilized by the addition of hydroxyl groups, first in the liver by the enzyme 25-hydroxylase and then in certain kidney tubular cells by 1-hydroxylase. The end result of these changes is 1, 25-dihydroxyvitamin D (calcitriol) the active form of vitamin D. The major action of 1,25-dihydroxyvitamin D is to stimulate the absorption of calcium by intestine. Thus, the major event in vitamin D deficiency is decrease intestinal calcium absorption, resulting in decreased plasma calcium (Winzenberg *et al.*, 2006). Calcium absorption process needs vitamin D for help. Active vitamin D plays with calcitriol hormone forming. Vitamin D supplementation stimulates intestinal calcium absorption and prevents the reduction in maturation-related periosteal bone gain by inducing accumulation of calcium from cancellous and endocortical bone (Iwamoto *et al.*, 2003).

(5). Plant

Some plant in natural containing phytic acid and oxalic acid. It may bind to calcium and prevent it from being absorbed optimally. These substances affect the absorption of calcium from the plant itself not the calcium contained in foods eaten at the same time. Weaver *et al*. (1987) found that oxalic acid in spinach was reduced absorption of calcium in rat model. Kennefick and Cashman (2000) reported that fiber extracts from wheat bran and barley hull were reduced calcium absorption associated with phytate content in these fiber extract. Dietary fiber is known exhibit various health-promoting biological activities. Harrington *et al.* (2001) also reported that fractional absorption of calcium was significantly reduced by phytate from wheat when compare with fiber prepare from apple or orange. This may

have been due to the phytate in the wheat fiber extract rather than the fiber components in orange and apple.

(6). Lactose

Lactose is disaccharide that is resistant to metabolism in the small intestine. Animal studies produced strong evidence that the disaccharide lactose has beneficial effects on intestinal calcium absorption. Lactose enhanced calcium ion uptake in intestine of rats. Lactose is interacting with the tissue rather than forming a complex with calcium ion to either maintain the calcium ion in solution or to aid its movement into the tissue (Armbrecht and Wasserman, 1976). In adults with lactose-tolerant, the effect of lactose on calcium absorption are inconclusive (Zittermann *et al.*, 2000). In rats, lactose has been shown to enhance calcium absorption. Addition of 5–15% lactose to the diet results in an increase in fractional calcium absorption of 5–10% (Brommage *et al.*, 1993; Lengemann *et al.*, 1959) Nishimukai *et al*. (2008) also reported that the absorption of calcium could be increase by epilactose from cow milk or addition of phosvitin peptides from egg york (Choi *et al.*, 2005). Epilactose increased calcium absorption in the jejunal and ileal sacs of rats when epilactose was added to the mucosal fluid in rat.

(7). Others

Erba *et al*. (2002) found that calcium absorption increases independently of the calcium concentration when the casein phosphopeptide/calcium ratios ranged from 5 to15. The more the casein phophopeptide amounts increased, the more the phosphate groups linked to the serine residues increased, and the serine residues then competed with inorganic phosphate to bind calcium. In previous study reported that calcium seems to have consistently greater affinity than to organic phosphate (Erba *et al.*, 2001). Calcium could be bound to the peptides and easily released in the intestinal lumen for absorption. Krall and Hughes (1999) found that cigarette use appeared to be lowered the efficiency of intestinal calcium absorption and be a risk factor accelerated bone loss in elderly men and women. Jung *et al*. (2006) reported that fish-bone peptide from hoki-bone-protein hydrolysate can be absorbed as same as casein phosphopeptide and suggests that preparation of fish-bone peptide by use enzyme could increase calcium solubility under natural pH. Kawase *et al*. (2007) studied the useful of additive gum arabic in rats and found that

administration of gum arabic with calcium could be increase the efficiency of oral calcium absorption. Some studies have reported the effects of citrate on minerals. An in vitro experiment suggested that dietary citric acid enhances the absorbability of Ca, Mg and Zn (Walter *et al.*, 1998).

1.2.2.3 Calcium balance

Figure 3 shows the main pathways of calcium in adult humans. Human adults lose approximately 0.3% of their bone mass each year; this means that their calcium balance is negative and they lose about 10 mg of calcium each day. This loss of bone mass may be ten times greater in post-menopausal woman. The ultimate goal of all hormone regulation of intestinal absorption, bone resorption and renal tubular reabsorption of calcium is keep the plasma calcium concentration, particularly the 50% of calcium in the ionic form. PTH and calcitriol are the most important hormones on calcium homeostasis. This complex control mechanism also regulates extracellular calcium of which there are about 900 mg in the human body. Extracellular fluid (ECF) contains about 10^{-3} M calcium; the concentration of calcium ions in the cytosol is more than a 100 mg (Gueguen *et al.*, 2000). Excess absorbed calcium that cannot be store in bone is excreted in urine, feces and sweat. The calcium balance in adult humans is zero, so all absorbed calcium is excreted by these routes, possibly after being incorporated into and then released from bone (Gueguen *et al.*, 2002). Almost all the calcium reabsorbed by the intestinal tract comes from secretions like the bile, and the endogenous calcium excreted in feces is the fraction that is not reabsorbed. The urinary loss results from glomerular filtration (about 10 g calcium per day) and tubular reabsorption which retrieves over 98% of the filtered load (Broadus, 1993). The changes in the amount of calcium excreted in the urine may therefore have a major impact on calcium balance (Heaney, 1996). Urinary excretion is influenced by hormonal and dietary factors. Among the latter are protein, sodium, and some carbohydrates, which increase calcium excretion, and phosphorus, which decreases it. Except under conditions of extreme sweating, loss of calcium from the skin is small (about 15 mg/ day) (Greenwood and Maria, 2008).

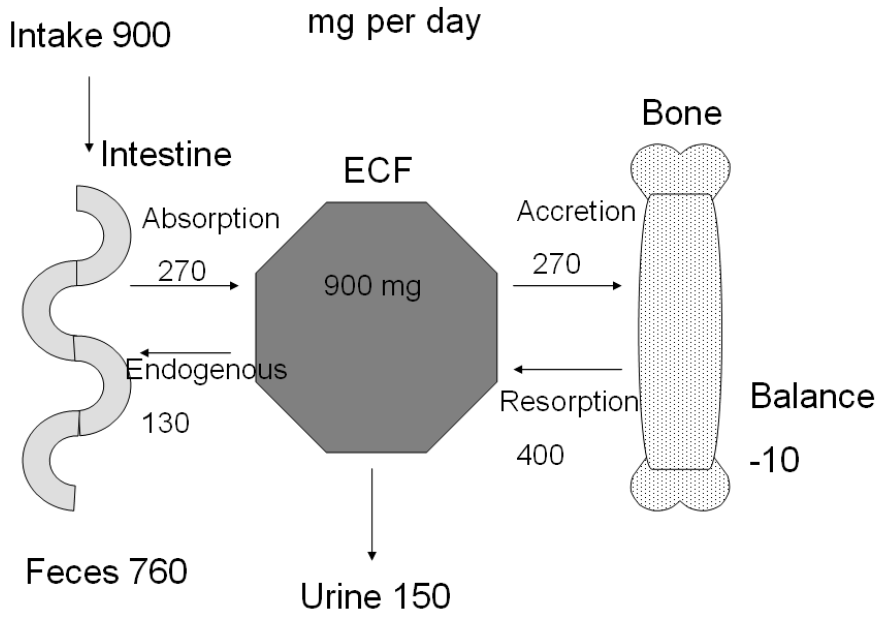

Figure 3. The main pathways of calcium in adult human

Source: Gueguen *et al*. (2000)

1.2.3 Calcium use as calcium supplementation

1.2.3.1 Calcium carbonate

The most common forms of calcium available to the consumer are calcium carbonate. Studies on the bioavailability of calcium carbonate have yielded varying results. Smith *et al*. (1989) studied 169 woman aged from 35 to 65 years who were given calcium carbonate supplements for four year. The calcium carbonate supplements could reduced bone loss in menopause woman. Domrongkitchaiporn *et al.* (2002) found that calcium carbonate supplementation with a meal or combined calcium carbonate with estrogen therapy did not increase urinary calcium oxalate excretion in 56 postmenopausal women. Straub (2007) reported that the bioavailability of calcium carbonate was found to be equivalent to skim milk and orange juice fortified with calcium-citrate malate in 12 elderly subjects. Changes in the level of serum, urinary calcium, and PTH were not significantly different between sources including skim milk, calcium carbonate, or orange juice fortified with calcium

citrate malate. Calcium carbonate is well-absorbed when taken with a meal. Calcium carbonate supplements provide greater amounts of elemental calcium and consequently require fewer tablets than other forms of calcium (Deborah and Straub, 2007). However, calcium carbonate may cause side affected such as upset stomach vomiting, stomach pain, belching, constipation, loss of appetite metallic taste. Henzlik *et al*. (2005) reported that the serum calcium of rats fed with calcium carbonate were changes followed closely the group of rat fed with placebo. The results suggested that the calcium carbonate and placebo groups there were no significant differennces in any of the phamacokinetic parameters for calcium.

1.2.3.2 Calcium citrate

calcium citrate was found to have better bioavailability in 25 postmenopausal women than calcium carbonate when given with a meal (Heller *et al.*, 2000). Kenny *et al.* (2004) found that calcium citrate decreased the markers of bone resorption significantly more than calcium carbonate in postmenopausal women, although no difference in their effect on calcium excretion or PTH were detected. Huang *et al.* (2009) reported that nano calcium citrate can enhance the serum calcium concentration and maintain the whole-body bone mineral density in ovariectomized mice than micro calcium citrate.

1.2.3.3 Calcium hydroxyapatite (HA)

Calcium hydroxyapatite is a calcium compound in form of $(Ca_{10}\ (PO_4)_6(OH)_2)$ and widely used as bioactive ceramics or in particulate forms in various bone repairs. Recently, HA has been used for a variety of biomedical applications, including matrices for drug release control. Due to the chemical similarity between HA and mineralized bone of human tissues (Murugan and Ramakrishna, 2007). Zhang and Gonsalves (1997) reported that HA has an exact stoichiometric Ca/P ratio of 1.67 and is chemically very similar to the mineralized human bone. Research on hydroxyapatite as a source of calcium is limited. Kiely (1987) reported that microcrystalline calcium hydroxyapatite compound has a significant effect in preventing the development of osteoporosis in corticosteroid-treated rheumatoid patients. Ruegsegger *et al.* (1995) found that hydroxyapatite was more effective than calcium carbonate in slowing peripheral trabecular bone loss from the distal tibia and the distal radius in 40 osteoporotic patients. Patients were followed

up to 20 months, and bone densities were evaluated every 4 months using high-precision peripheral quantitative computed tomography. At the end of the study, the loss of trabecular bone was 0.8% in the hydroxyapatite group compared with 1.8% in the calcium carbonate group. Gao *et al.* (2008) reported that nanometer pearl powder could be produced calcium hydroxyapatite supplementation. The bone, serum calcium content, femur weight and length of rats fed with nanometer pearl powder were significant higher than rats fed with laboratory diet. However, very little information is available on a beneficial effects of calcium hydroxyapatite from fish processing byproducts is a useful calcium source and few attempts have been made to test their usage for benefits of human health.

1.2.3.4 Tricalcium phosphate

Tricalcium phosphate is a compound with formula $Ca_3(PO_4)_2$. It is also known as bone ash (calcium phosphate being one of the main combustion products of bone. Calcium phosphate is used as a nutrition supplement and occurs naturally in cow milk. Although the most common and economical forms for supplementation are calcium carbonate and calcium citrate. There is some debate about the different bioavailability of the different calcium salts. Lupton *et al*. (1995) reported that tricalcium phosphate may inhibit colon tumor. These incidences more effectively than calcium lactate. Because the calcium phosphate group had lower colonic proliferative status than calcium lactate group.

1.2.4 Utilization of byproduct from fish processing

Marine capture fisheries contribute over 50% of total world fish production and more than 70% of this production has been utilized for processing (FAOSTAT, 2001). As a result, fish processing is a large quantity of processing byproducts as fin, frame, heads, skin and viscera. Recent estimates revealed that current discards from the world's fisheries exceed 20 million tons equivalent to 25% of the total production of marine capture fisheries (FAOSTAT, 2001). However, these byproducts had potential to convert to valuable products. Majority of fisheries byproducts are presently employed to produce fish oil, fishmeal, fertilizer, pet food and fish silage (Choudhury and Bublitz, 1996). Most of these products possess low economic value. Therefore, development of new technologies in search of novel

products from fish processing byproduct will bring more value and opportunities for the seafood industry.

1.2.4.1 Byproduct (fish bone) as a potential calcium sources

Fish bone is considered as a potential source of calcium. Fish bone material derived from processing of large fish is useful calcium source. The study in bioavailability and clinical trial of calcium were prepare from bone suggested that calcium prepare from bone is good much better than common calcium powder (Venugopal, 2009). Calcium fish powder was prepared from lizard fish used as a source of calcium for northeastern pregnant women in Thailand. The results shown that most of pregnant woman were accepted calcium from Lizard fish. Calcium fish powder was low cost and high potential to be used as an alternative choice to promote calcium intake in various groups whose calcium consumption is limited (Lorkerpon *et al*., 2005). Larsen *et al*. (2000) reported that calcium in fish could be absorbed to the body as tested in vivo. In order to incorporate fish processing byproduct into calcium-fortified food it should be converted into an easy edible form. This can be achieved utilizing different methods including hot water treatment, acetic acid solutions and sintered. In addition, Ishikawa *et al*. (1990) used superheated steam to reduce the loss of soluble components from fish tissue and that enabled better recovery of bone within a shorter period. Two studies from Denmark have shown that absorption of calcium from small soft bones fish was comparable to that from skimmed milk both in rats (Larsen *et al*., 2000) and in humans (Hansen *et al.*, 1998). There are few reports on the availability of calcium from fish bones, and due to the potential nutritional value of this material. Malde *et al*. (2009) also reported that calcium from fish bones may be a useful and well absorbed calcium source. Due to the high mineral content of the bone fraction, salmon bones can be well suitable as a natural calcium and phosphorus source in, for example, food or as supplement. However, very little information is available on the use of fish processing byproduct as calcium sources for benefits of human health.

1.2.4.2 Byproduct (fish bone) in medicinal science

Recently, hydroxyapatite has been introduced as a bone graft material in a rang of medical and dental applications because of their similar chemical composition with in human bone and teeth. Fish bone material becomes an important source for biomedical applications due to the presence of hydroxyapatite as the major inorganic constituent.

1.2.4.3 Byproduct (fish bone) in others useful

Admassu and Breese (1999) using fishbone as a natural apatite for removal of various aqueous metal ions and compare with synthetic hydroxyapatite. Fishbone apatite is cheaper than synthetic hydroxyapatite and also compares favorably to apatite ores because of its purity and lower substitution of undesirable metals. Based on this investigation, fishbone apatite would be an effective alternative to synthetic hydroxyapatite for removal of divalent heavy metal ions from aqueous solutions.

1.3 Objectives of study

1. To produce calcium compound from fish processing by-product.
2. To study bioavailability of calcium compound from fish processing by-product in rats.
3. To study approximate dose of calcium compound for daily use with base on daily calcium requirement in rat.

CHAPTER 2

CHARACTERISTICS OF CALCIUM COMPOUND FROM BIGEYE SNAPPER FRAME (*Priacanthus tayenus*)

2.1 Abstract

Calcium compound was produced from frame of bigeye snapper (*Priacanthus tayenus*) by thermal sintering process. The principal frame compositions included protein (54.95%), lipid (12.48%) and ash (31.41%). The muscle tissue attached to the frame accounted for 41.5% of total weight. The muscle tissue was effectively removed by heating in citric acid 5% (w/w) at 85 ^{o}C for 15 min. The muscle free-bone was heated with the heating rate of 10 ^{o}C/min and sintered at 1300 ^{o}C for 0, 1, 2 or 3 hrs. The sample weight was reduced by about 30% by those heating conditions. The morphologic of calcium compound was evaluated by using X-ray diffractometer (XRD). And, the XRD patterns revealed the mixture of calcium hydroxyapatite and calciumtriphosphate phase. All holding times at 1300 ^{o}C did not cause significant effect on the XRD patterns. The results thus revealed that bigeye snapper frame can be a potent source for production of calcium compound by using thermal sintering process.

2.2 Introduction

The fishery industry generates by-products such as bone, fin, internal organ and head accounted more than 50% of initial raw material weight. According to estimation of the Food and Agricultural Organization of the United Nations, over 100 million metric tons of fish waste or discard is annually generated worldwide. And, only a small portion is used in the production of fishmeal and fish oil (Kilpatrick, 2003). Many studies have been performed to utilize these discarded materials (Kim *et al*., 2000; Nagai and Suzuki, 2000).

In general, by-products from fish were used to produce fish meal, a principal mixture of feed. Fish meal containing about 10% mineral is accounted for a major

mineral source for feed formulation (Nordum *et al.*, 1997). Addition of dried fish bone in diet for cod showed a positive effect on growth and feed efficiency compared to traditional diet (Toppe *et al*., 2006). Apart from utilization as the mineral source for feed production, it is possible to transform fish bone calcium to human mineral supplement. Calcium in fish bone was formed into microcrystalline hydroxyapatite by sinter treatment (Jung *et al*., 2006). Since, it is the calcium phase existed in human bone thus it is likely to possess high bioavailability. However, studies intended to utilize minerals of fish bone are scarce (Kim *et al*., 2000; Larsen, *et al*., 2000).

Bigeye snapper is one of prominent raw materials of frozen fish fillet in Thailand. Its by-products; head, viscera, and frame, generated by the processing are exclusively for feed production. This study thus aimed to apply a sintering process at high temperature in order to produce calcium compound from bigeye snapper frame. The characterization of the obtained material would be carried out for its further application.

2.3. Materials

2.3.1 Materials

By-product from frozen bigeye snapper (*Priacanthus tayenus*) processing consisting of fish frame and head was used for this study. The sample was collected from the SS Frozen Food Co., Ltd. (Songkhla Thailand). The sample was placed on ice with a sample/ice ratio of 1:1 (w/w) and transported to the Department of Food Science and Technology, Prince of Songkla University within 2 hours. The sample was kept at 4°C until analysis within 7 days.

2.3.2 Reagent

Nitric acid, citric acid and ethylene diamine tetraacetic acid (EDTA) were obtained from Sigma (St. Louis, MO, USA).

2.4. Methods

2.4.1 Sample preparation

2.4.1.1 Proximate analysis

The fish frame was manually cut into small pieces before subjected to analyze for moisture, ash, fat and protein contents according to the method of AOAC (1999). Total calcium and phosphorus were measured by using the inductively coupled plasma optical emission spectrometry (Optical Emission Spectrometer, Optima 4300 DV, Perkin Elmer Corp., London, U.K.)

2.4.1.2 Removal of fish muscle residue

The fish muscle residue attached to fish bone was removed by following the modified procedure described by Sathivel (2003). Briefly, the sample was soaked in a 3 % (v/w) citric acid solution with solution/the frame ratio of 1:1 and heated at 85 ^{o}C for 45 min (Tongchan *et al*., 2009) then blended for 30 second by using a food processor. The treated frame was manually cleaned to remove coagulated muscle under running tap water. The muscle-free bone was then dried at 110ºC for 12 hours in hot air oven before bought to determine for moisture, ash, fat, protein calcium and phosphorus content.

2.4.2 Preparation of Calcium Compound

2.4.2.1 Bone sintering process

Calcium compound powder was generated by the following the method of Ozawa and Suzuki (2002). The muscle-free bone was sintered in an electrically heated box furnace by using the CARBOLITE STF (Model CARBOLITE STF 15/75/450 England). Temperature was increased from 30 ºC to 1300ºC with at heating rate of 10 ºC/min. The holding times of sample at 1300 ºC were 0, 1, 2 or 3 hours.

2.4.1.3 Characterizations of the sintered powder

2.4.1.3.1 Weight change of the sample during heating to 1300 ºC was measured by using a thermogravimetric analyzer (Perkin Elmer, tga7, USA).

2.4.1.3.2 The phase of calcium compound powder was evaluated by using a x-ray diffraction (XRD). The sintered powder was crushed to fine powders and examined by using Philips diffractometer (XRD x'pert, Netherland). A time constant of 1 s and a scanning rate of 2 min were used. The XRD patterns were recorded with a chart drive speed of 2 cm min^{-1} and the position values were varied from 10 to 80 ºC.

2.4.1.3.3 Total Ca and P of the powder were performed by using the inductively coupled plasma optical emission spectrometry (Optical Emission Spectrometer, Optima 4300 DV, Perkin Elmer Corp, London. U.K.). The Ca/P ratio of the material was then calculated.

2.5 Results and Discussion

2.5.1 Chemical composition of bigeye snapper frame and bone

The chemical compositions of bigeye snapper frame and muscle-free bone were analyzed as shown in Table 1. Approximately 44.82 % of total protein content of the bigeye snapper frame was decreased by using acidic pre-treatment. This is likely due to removal of the attached muscle residue facilitated by thermal and acidic coagulation of fish muscle protein. The result is in an agreement with the crude protein of salmon frames, 48%, removed by an enzymatic hydrolysis (Liaset *et al*., 2003). Prabakaran and Rajeswari (2006) showed that using a strong water jet after boiling of fish frame for 1 hour could decrease protein content by 30.23%. Lipid content of the frame agreed with the value (1-27%) reported by John (1997). Pretreatment before bone sintering thus also aided in lipid removal. Calcium and phosphorus were principal components in organic part of the frame and bone. The Ca to P ratios in the frame and bone were 1.70 and 1.15, respectively. It is possible that application of heat and acid caused leaching out of calcium from fish frame. The calcium content of the bone was in accordance with that of salmon bones (135-147

g/kg) reported by Toppe *et al*. (2007). However, its calcium content was lower than the figure of salmon bone (185±15 g/kg) reported by Liaset *et al*. (2003).

Table 1. Compositions of fish frame and fish bone of bigeye snapper.

Compositions	Fish frame	Fish bone
Protein (% dry weight)	54.95 ± 0.2	30.38 ± 0.54
Lipid (% dry weight)	12.48 ± 0.06	0.87 ± 0.38
Ash (% dry weight)	31.41 ± 1.2	68.75 ± 0.45
Ca (% dry weight)	6.48 ± 0.32	14.70 ± 0.1
P (% dry weight)	3.81 ± 0.38	12.83 ± 0.26
Moisture	68.99 ± 0.26	8.19 ± 0.34

Values are given as mean ± SD from triplicate determinations.

Mineral composition in the bone powder is shown in Table 2. The ratio of Ca/P was 1.35. However, the value of Ca content of this study was higher than that of sea bream (220–230 mg Ca/kg ash) reported by Orban *et al*. (2000). Similarly, Tahvonen *et al*. (2000) found that Ca content of whole Baltic herring was 44–1158 mg Ca/kg ash. Helland *et al*. (2005) found that calcium content in salmon bone were 272 mg Ca/kg ash. It was reported that phosphorus content in fish was independent to fish specie (Toppe *et al.*, 2007). Phosphorus content of this result was, however, higher than the value (121 mg P/kg ash) reported by Shearer *et al*. (1992). It is known that a variation in the mineral composition of marine foods is closely related to seasonal and biological differences, area of catch, processing method, food source, and environmental conditions (Rodrigo *et al*., 1998).

Table 2. Percentage of mineral composition in bone powder (*Priacanthus tayenus*)

Mineral	mg Ca/kg ash
Ca	370.98 ± 0.09
P	280.33 ± 0.30
Al	7.4 ± 0.02
Mg	2.8 ± 0.19
S	2.2 ± 0.08
Sr	2.9 ± 0.20
Si	0.7 ± 0.06
Zn	0.4 ± 0.09
Other	320.5 ± 0.12

Mean ± SD from triplicate determinations

2.5.2 Effect of sintering processing on bone weight loss

Figure 4 shows the weight loss of the fish bone during heating from 30°C to 1300°C. The sintering process caused totally reduction in the sample weight by 30%. Reduction of the fish bone weight of this study was in good correspondence as the value (35%) of Japanese sea bream bone reported by Ozawa and Suzuki (2002). The sintering process from 30°C - 250 °C caused a 12.21 % reduction of initial bone weight. This is likely due to evaporation of moisture and thermal decomposition of protein. The further significant weight loss (19.58%) was occurred at temperatures between 380-600°C. It was corresponded to pyrolysis of organic components such as protein and lipid in the bone.

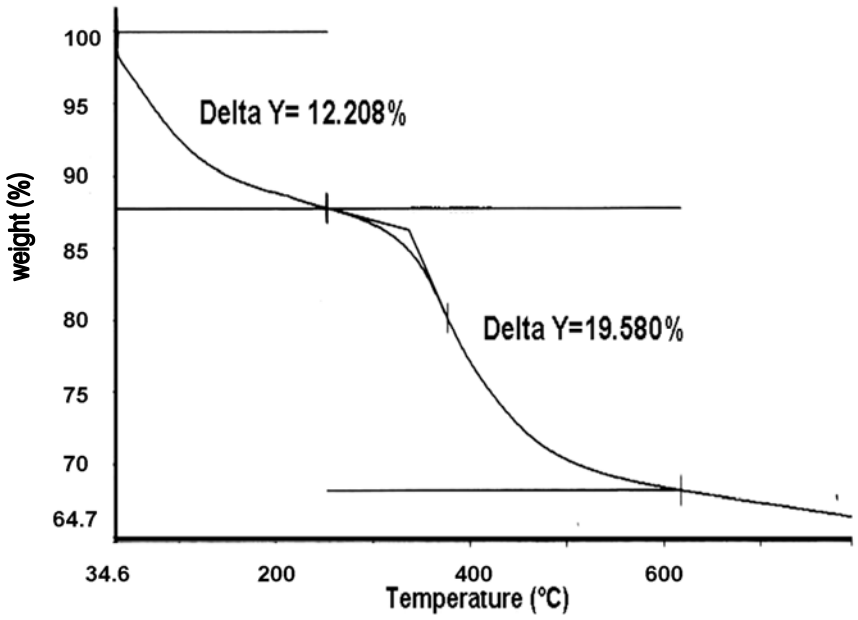

Figure 4. Typical weight change of fish bone after heating from 30° C to 1300°C.

2.5.3 Effect of holding times at 1300°C on characteristic of calcium compound

XRD patterns of the calcium compound obtained by using the sintering process with different holding times at 1300°C are shown in Figure 5. The results revealed non significant effect of holding time on the XRD patterns. There was no effect of holding times on synthesization of hydroxyapatite crystallites from mixture of chemical compounds according to phase and particle morphology (Earl *et al*., 2006). In general, the XRD patterns exhibited broad and merged peaks indicating the hydroxyapatite phase ($Ca_5(PO_4)_3(OH)$ and tricalciumphosphate or whitelockite phase ($Ca_3(PO_4)_2$. Existence of the tricalciumphate phase might be generated from deformation of natural hydroxyapatite from bone after sintering at 1200°C or higher (Joschek *et al.*, 2000). A well-crystallized hydroxyapatite crystal was produced from calcium matrix of bone of Japanese sea bream after sintering at temperature between 400-900°C for 24 hours in air (Ozawa *et al*., 2002). Moreover, Prabakaran and Rajeswari (2006) reported that pure hydroxyapatite phase was existed by heating at

900°C. The result thus suggested necessity to lower the sintering temperature if the hydroxyapatite phase is primary phase.

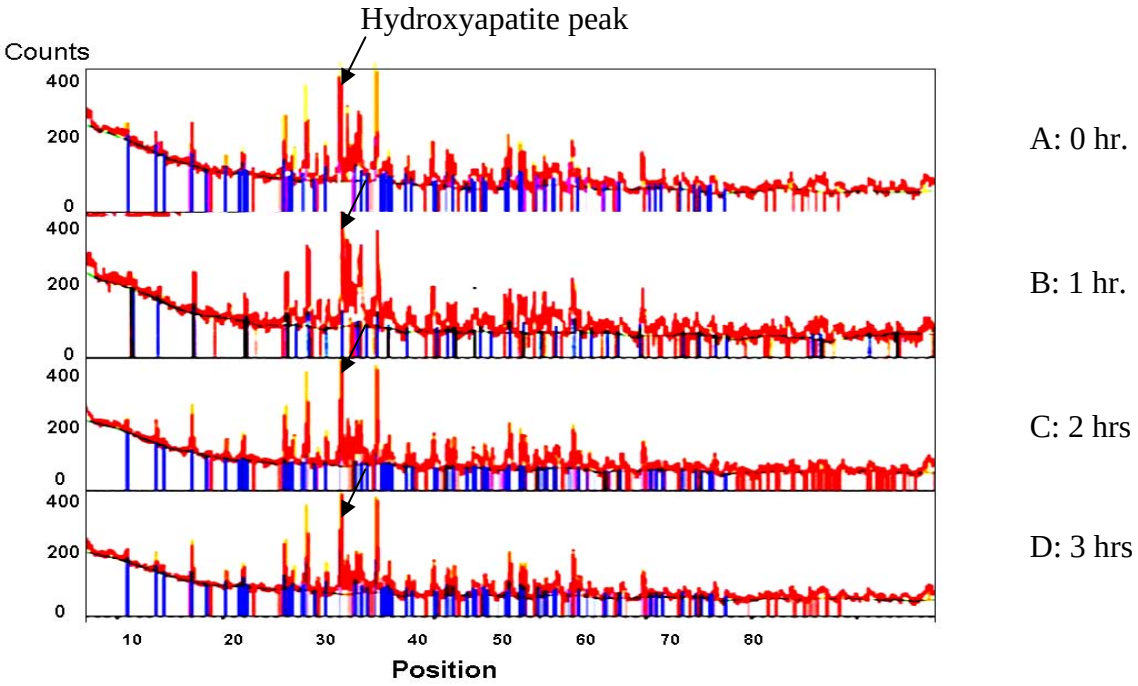

Figure 5. XRD patterns for fish bone heated at 30°C to 1300 °C and holding times A = 0 hr, B = 1 hr, C = 2 hrs and D = 3 hrs.

2.6 Conclusions

The result revealed that calcium compounds could be produced from bigeye snapper bone by the sintering process. The results showed that the use of hot citric acid was effective in removal of muscle residue. The sintering process caused bone weight reduction by 30%. Non significant effect of holding time at 1300 °C on phase of calcium compound was found. The sintered calcium compounds composed of hydroxyapatite and tricalciumphosphate phases.

CHAPTER 3

EFFECT OF SINTERED CALCIUM COMPOUND FROM *Priacanthu tayenus* BONE ON CALCIUM METABOLISM IN RAT

3.1 Abstract

Calcium compound was prepared by sintering muscle-free fish bone of *Priacanthu tayenus* at 1300 ^{0}C for 1 hour. Four groups of 6 male Wistar rats were fed for seven weeks with basic diet with marginal calcium content. And those three groups were fed with the sintered calcium compound as a supplement to gain 11, 22, or 44 mg Ca/d. Effects of the sintered calcium compound on metabolism of calcium in rats were monitored. Supplementation of calcium increased body weight of rats whereas a 9% loss of mean body weight of control group was observed by the seventh week. Absorption and retention rate of the supplemented calcium were increased significantly ($P<0.05$) with increasing of the calcium intake. Femur weight of rats was increased by increasing amount of calcium supplement with respect to that of the control rats. Abnormal trabecular conformation was noticed in the control rats whereas thicken and narrowed inter-trabecular spaces were observed in the calcium supplement groups. The results thus revealed that calcium compound prepared by sintering process from fish frame has high bioavailability.

3.2 Introduction

Considerable amount of total catch fishes is discarded as processing leftovers including fin, frame, head, skin, viscera, and trimming by-product. There is an estimate that current discards from world's fisheries exceed 20 million tons equaled to 25% of the total production of marine capture fisheries (FAO, 2003). Fish frame is the main solid by-product of the frozen fish processing. It accounts for 25-30% of fish weight. Thus there is increasing interest to utilize these by-products especially for human consumption.

Chronic inadequate intake of calcium from the diet is one factor in the etiology of several disorders (Weaver and Heaney, 1999). Adequate calcium intake during growth is critical to the achievement of peak bone mass that may reduce the risk of osteoporosis (Heaney *et al*., 2001). Osteoporosis has become an important degenerative disease in the world especially in Asia. Bone fractures associated with osteoporosis can occur in any of the bone with the prevalence in the hip. In Thailand, an epidemiological survey has indicated that the incidence of hip fracture is 162/100000 population over the age of 50 (Lau *et al*., 2002). Nutrition interventions to increase calcium intake are consumption of high calcium containing foods and use of calcium supplements.

In Thailand, most of fish frame generated from processing of frozen fish fillet is used for animal feed production. Although, with regard to its chemical composition, fish frame could be transformed to high value product. For instance fish-bone phosphopeptide with the high affinity to calcium had been isolated from hoki (*Johnius belengerii*) skeletons (Jung *et al*., 2006). In addition microcrystalline hydroxyapatite was successfully prepared from fish bone by sinter treatment (Ozawa and Zuzuki, 2002; Jung *et al*., 2006). Even though, hydroxyapatie and calciumtriphosphate are principle calcium compound existed in bone. The bioavailability of the those calcium compound was less proposed (Kim *et al*., 1997; Larsen *et al*., 2000). There is no study aimed to clarify metabolism of hydroxyapatite derived from fish bone. Thus, the objective of this study was to evaluate the bioavailability of calcium compound sintered from fish frame.

3.3 Material and Methods

3.3.1 Material

By-product from frozen fish processing consisted of fish frame and head of *Bigeye snapper* was used for this study. The sample was collected from the SS Frozen Food company in Songkhla, Thailand. The sample was placed on ice with a sample/ice ratio of 1:1 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. The sample was kept at 4°C and analyed within 7 days.

3.3.2 Calcium compound processing

The fish frame was manually and boiled in hot citric acid (3%) to remove muscle residue then sintered at 1300ºC for 1 hour. The obtained sintered compounds were subjected to confirm calcium triphosphate and calciumhydroxyapatite form by using an X-ray diffractometer (XRD).

3.3.3 Animal and diet

Male Wistar rats weighing about 200-230 g obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Thailand were used. The animals were housed under control conditions with an ambient temperature of the Primate Research Unit of the university in a room at 23 $\pm$ 2 °C and a 12-h light/dark cycle. They were fed with a standard rat diet (C. P. 082, Lot No. 17, S. W. T. Co., Ltd, Thailand) for 2 weeks. During inhabitant and experimental period, rats were fed with the basic diet, AIN-93M, advised by NRC (NRC, 1995). Water was supplied *ad libitum*. The experimental designs were approved by the Ethics Committee for experimental animals No. Ref 09/50 Prince of Songkla University.

3.3.4 Experiment protocol

Five-week-old male rats were randomly divided into 4 groups of 6 animals/group and fed with the basic low calcium containing diet (Table 3). The calcium compounds were mixed with arabic gum with a calcium/gum ratio of 1:1

(w/w) and dissolved in distill water 10 ml. The mixture was prepared freshly and administered oral by syringe to the rat to receive calcium supplement of 0, 11, 22 or 44 mg Ca/d for 7 weeks. Animals were allowed tap water *ad libitum*. Body weights of rats were monitored everyday at room temperature.

Table 3. Formulation of basic low calcium containing diet.

Composition	Total (g)
Corn starch	448
Casein	200
DL-Methionine	3
Sucrose	200
Corn oil	50
Cellulose	52
Mineral mix	35
Vitamin mix	10
Choline bitartrate	2

3.3.5 Sample collection

a. Urine and fecal sample collections

On the last week of experiment, each group of rats was placed individually in metabolic cage to collect urine and fecal for a 24-hour period. Fecal weight and urine volume were measured and stored at 4 ºC until use for analysis.

b. Serum sample collection

After 7-week feeding periods, the rats were fasted overnight. It was become unconscious by using diethyl ether as an anesthesia on the morning. Whole blood was drawn from the Ophthalmic Venous (Orbital Sinus) 2.0 ml per rat (≤ 10 % of total blood volume) into the capillary tube preloaded with heparin solution for prevent blood clotting and immediately transfer into 15.0 ml tubes. The obtained bloods were centrifuged to separate serum before storage at -80 ºC until use.

c. Femur sample collection

At the end of experiment, rats were sacrificed by cervical dislocation according to the method of IACUC. (2008). Its right femur of each rat was defleshed from adjacent tissues, wrapped in saline-soaked gauze bandages to prevent

dehydration, and stored frozen at -20 °C in small Ziploc freezer bags until the histopathology evaluation.

3.3.6 Analysis

a. Total calcium in urine and faces

Calcium in urine and fecal were determined by using an inductive coupled plasma atomic emission spectrometer (PERKIN'ELMER: ICP-OES) according to the method described by Yoon *et al.* (2005). Calcium concentrations were presented as mg/kg/d of fecal and urine on a wet weight basis. Analytical limits of detection were determined as 0.01 μg/L wet weight.

b. Calcium absorption and calcium absorption rate

Calcium absorption and calcium absorption rate were determinate. Calcium adsorption was calculated by subtraction total calcium in feces from total calcium intake according the method of Choi (2005).

Calcium absorption = Calcium intake – calcium in fecal

Absorption of calcium can be calculated according to the method described by Cui *et al.* (2005).

$$\text{Absorption of calcium (\%)} = \frac{\text{Total calcium intake} - \text{calcium content in feces} \times 100}{\text{Total calcium intake}}$$

c. Calcium retention

Retention rate of calcium can be calculated according to the method described by Alam *et al.* (2005) as followed.

$$\text{Retention of } Ca^{2+} \text{ (\%)} = \frac{\text{Total } Ca^{2+} \text{ intake} - Ca^{2+} \text{ in feces} - Ca^{2+} \text{ in urine} \times 100}{\text{Total absorbed calcium}}$$

d. Femur length and weight

Femoral length was measured with a caliper (IS 11205-150-2 England) and its weight was obtained by using a precision electrical balance (China)

e. Bone mineral density

After sacrificed, the right femur of rat was selected for

histopathological study. Bones were defleshed and placed in 10% phosphate-formalin buffer at least 72 hours. Bones were cut to a small size and then decalcified in EDTA-G solution (EDTA disodium salt 14.50 g, NaOH 1.25 g, glycerol 15 ml and distilled water 100 ml) for 3 weeks by changing EDTA-G solution every week. After three weeks, the decalcified bones were dehydrated in series of ethanol gradient and clearing in xylene. They were then embedded in paraffin, cut into section of 5 μm thicknesses, and stained with Hematoxylin and Eosin. The slides were analyzed under the light microscope (ZEISS: Axiostar plus) and photographed using a digital camera (SONY: DSC-S85). The method of bone histopathological study was slightly modified from Miao and Scutt (2002).

3.3.7 Statistic analysis

Experimental values are presented as the mean ± SD of the number of experiments indicated in the legends. Significance was assessed by using Oneway – ANOVA ($P<0.05$ as significant) SPSS version 14.

3.4 Results and discussion

3.4.1 Effect of sintered calcium compound on body weight of rats

Figure 6 shows the percentage weekly body weight changes of rats in each feeding regime over the seven weeks of feeding period. In first four weeks, the percentage body changes in groups of rat received the basic low calcium containing diet (group A) was presented the lowest body weight changes significantly ($P<0.05$). Since calcium content of basic low calcium diet was 2.64 mg/kg thus habitual feed intake (22 mg/d) would provide calcium equivalent to 12% of the required Ca/d (NRC, 1995). The insufficient intake of calcium might cause abnormal growth as observed by low percentage body weight changes. The result is in accordance with the study of Paradis and Cabanac (2005) reported that the mean body weight of rat fed with low-calcium was lower weight than control group after 6 weeks. The percentage body weight changes in group of rat fed with 11 mg Ca/d (group B) was higher than the rat in group A but lower than the rat in group C and D significantly ($P<0.05$). However the percentage body weights changes of rats received 22 mg Ca/d (group C) and 44 mg Ca/d (group D) were not significant difference during first four weeks. The results suggested that differentiations level of calcium supplement for rat was affected on the percentage of body weight changes in first four weeks. Thereafter, decreasing in the percentage body weights was observed in all groups. This may due to lose of electricity at 4^{th} week. The temperature increased was possible affected to low consume of rats and resulted in percentage of body weight changes was low in this phenomenon.

Thus, the results confirmed the consumption of low calcium containing food affected the growth rate, while enough calcium concentration produced a normal growth rate. The deficiency of calcium caused abnormal physical development. Nevertheless, this study found that the rat received basic low calcium diet showed inactive and hair lacked luster. The results also agreed with the study of Jiancong *et al*. (2010) reported that the rats had luster less fur and were sluggish when fed with low calcium diet. The cause of hair lacked luster, abnormal growth, inactive, and coarse in rat resulted from the deficient of calcium (Cui, 2005).

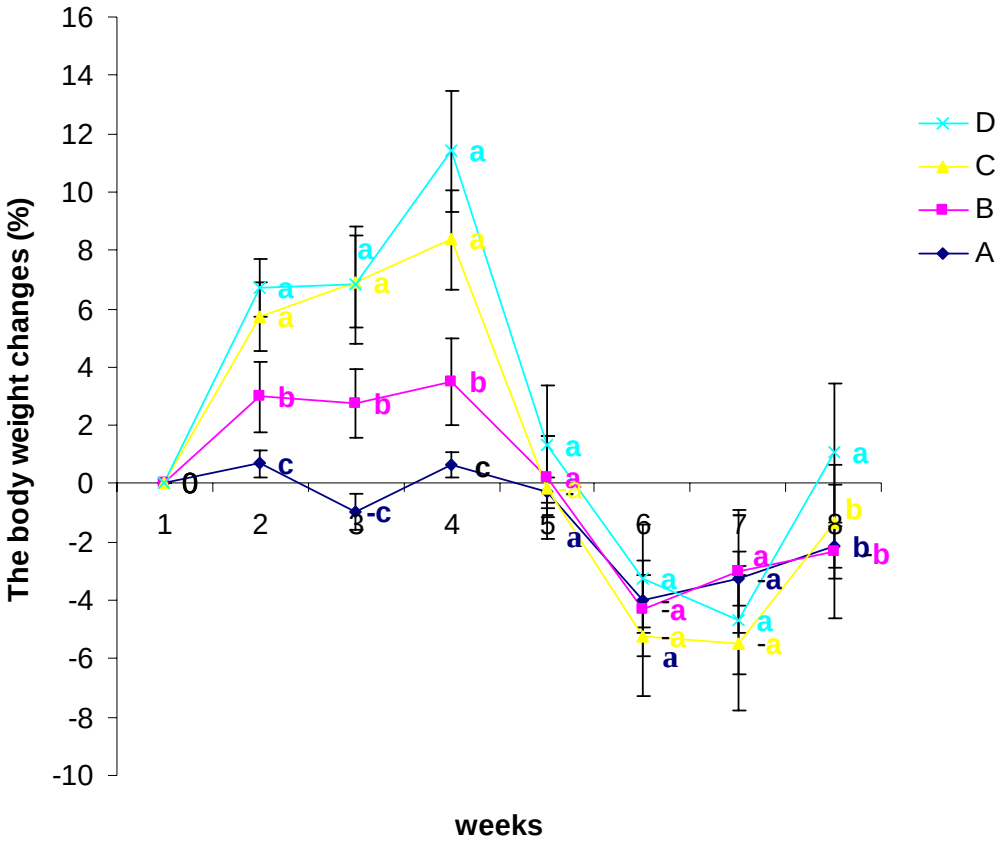

Figure 6. The body weights changes (%) of male Wistar rats fed with different levels of calcium supplement.

(a-c) Mean average body weight changes within the week with difference letters are significantly difference at $P < 0.05$.

Remark:

A : Rat fed with low calcium containing diet

B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)

C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)

D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

3.4.2 The effect of sintered calcium compound on absorption and retention rate in rats

3.4.2.1 Calcium in feces

Calcium absorption determined by calcium intake and calcium in feces. The effect of supplement with sintered calcium compound on feces calcium content is depicted in Figure 7. Throughout the 7 weeks duration, calcium content in feces was recorded. It has been shown that calcium supplementation of 11, 22 and 44 mg/d of calcium exhibited significant increase when compared with non supplement group *($P<0.05$)*. Predominately unabsorbed calcium from diet or supplement and endogenously secreted moderate calcium in faces were reported by Nordin *et al*. (1967). By this study found that the lowest calcium content was recorded for feces of the rats received insufficient calcium intake (Group A). The result highlighted adaptation of animal to obtain the required calcium by increasing its absorption. The results indicated this group of rat was show calcium deficiency symptom accordant with average weight (Fig 6). Accordant with Boelter and Greenberg (1994) who reported that the calcium deficient in animal usually show outstanding characteristic different from the normal in 7-10 weeks. It was found that calcium content in feces was increased with increase of calcium supplement in feed. However, this study found that calcium residue in feces of the groups supplemented with 11 mg/d was not significant difference with the group received sufficient calcium intake, 22 mg Ca/d. The highest calcium content in feces of the group fed with 44 mg Ca/d suggested that it was high calcium intake or over absorption limited. It was found that rat fed with high calcium intake showed a larger proportion of unabsorbed calcium with relative to that of the rat with low calcium intake Bronner (2003). As well as, similar conclusion was made in human study (Jaconsen *et al.*, 2005).

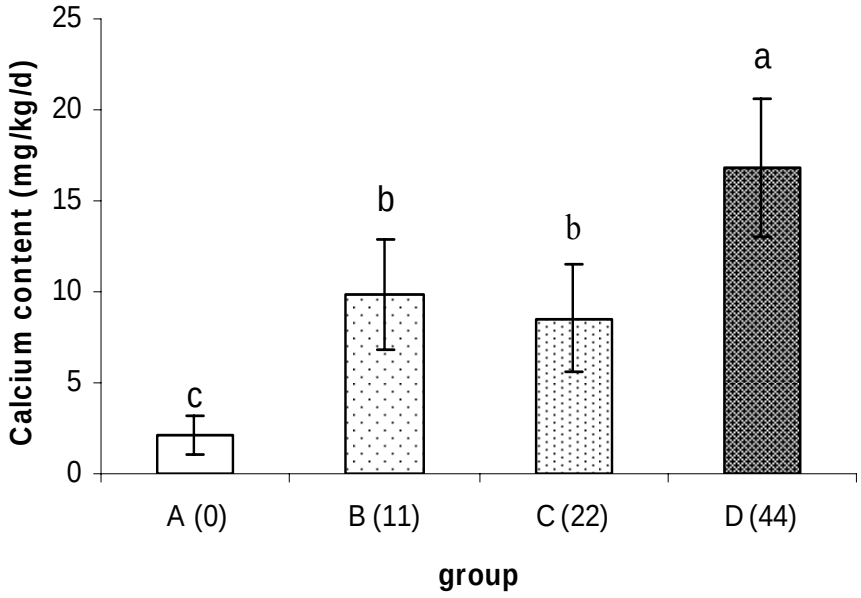

Figure 7. Calcium content in feces per day of rat fed with sintered calcium compound.
Different letters on the bars indicated the significant differences ($P < 0.05$).

Remark :
A : Rat fed with basic low calcium containing diet
B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)
C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)
D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

3.4.2.2 Calcium content in urine

Calcium content in urine per day of rats fed with different calcium supplement is presented in Figure 8. Urinary calcium content per day in rat fed with basic diet without calcium supplement was less than animal that received calcium supplement at a dose 22 and 44 mg/d. Two times of urinary calcium content per day from group C (22 mg/d) was higher when compared with group of rat fed with calcium 11 mg/d and control group significantly ($P<0.05$) while the rat fed with calcium content 44 mg/d was also increased higher than the rat in group A and B ($P<0.05$). The low calcium content in urine of those groups was affected by receive insufficient calcium and calcium reabsorped. The calcium concentration in blood of rat in both groups (group A and B) may was changes into lower than its normal level. In generally, when calcium deficiency state, parathyroid hormone (PTH) was released (Persson *et al*., 1993). This hormone secreted into the circulation and then acts primarily on kidney and bone, where it activated PTH receptor. This receptor directly enhances the tubular calcium reabsorption and it stimulates increases calcium absorption from the intestine (Joost *et al*., 2005). Accordant with Gueguen *et al*. (2002) who reported that the calcium balance in adult human is zero. All absorbed calcium is excreted by urine routes. It may possibility after being incorporated and then released from bone. Thus, the results indicated calcium content occur in urine of both group was released from bone (Figure 13A, 13B) and reabsorbed in tubular yet. The urinary calcium content in rat fed with 22 mg Ca/d had non significant different with rat fed with 44 mg Ca. It was found that blood calcium content of rat fed with basic feed was about 0.7 mg Ca/kg/d.

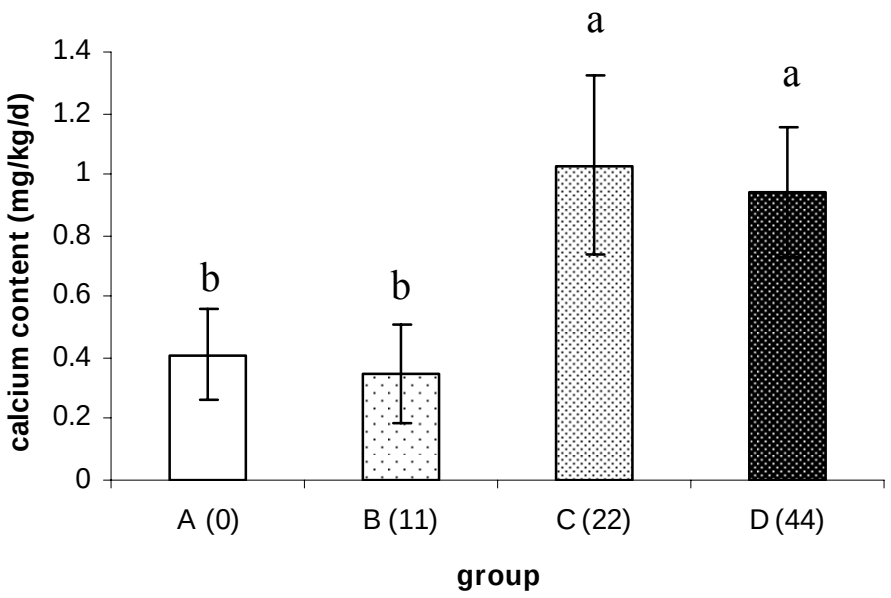

Figure 8. Calcium content in urine per day of rat fed with sintered calcium compound.

Different letters on the bars indicated the significant differences ($P < 0.05$).

Remark : A : Rat fed with low calcium containing diet

B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)

C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)

D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

The effect of supplement level of sintered calcium compound on calcium absorption per day in rats is shown in Figure 9 and Table 4. Increasing of calcium absorption was observed with an increase of calcium intake from 0 to 44 mg/d ($P<0.05$). The result corresponded with decrease of calcium content in feces (Figure 7). The highest calcium absorption of the group with the highest supplement dose suggested at least partially that the sintered calcium compound composed of the high absorbable calcium. Absorption of calcium carbonates increased linearly with increasing of its supplement dose up to 450 mg/d (Pansu *et al*., 1993). On another hand, the result highlighted the possibility that the sintered calcium compound could be absorbed even the intake concentration was two times higher than the recommended amount (NRC, 1995). It is important to note that calcium found in feces of the control group was mainly endogenous and the digestive juice calcium which has not been reabsorbed (Figure 6).

The effect of supplement level of sintered calcium compound on calcium absorption rate is show in Figure 10 and Table 4. The results show that absorption rate has no significant different in calcium absorption rate but the group of rat fed with calcium 22 and 44 mg/d slightly higher than the rat in group 11 mg/d, while control group was not show absorption rate because 0 mg calcium intake (data not show). The result corresponded with Jiancong *et al.* (2010) reported that the calcium absorption rate of rat fed with the high-dose haddock bone calcium tablet was higher than that of the low-dose supplement. However, the results confirm with the finding that calcium absorption is upregulated by low calcium intake and downregulated by high calcium intake (Bronner, 2003).

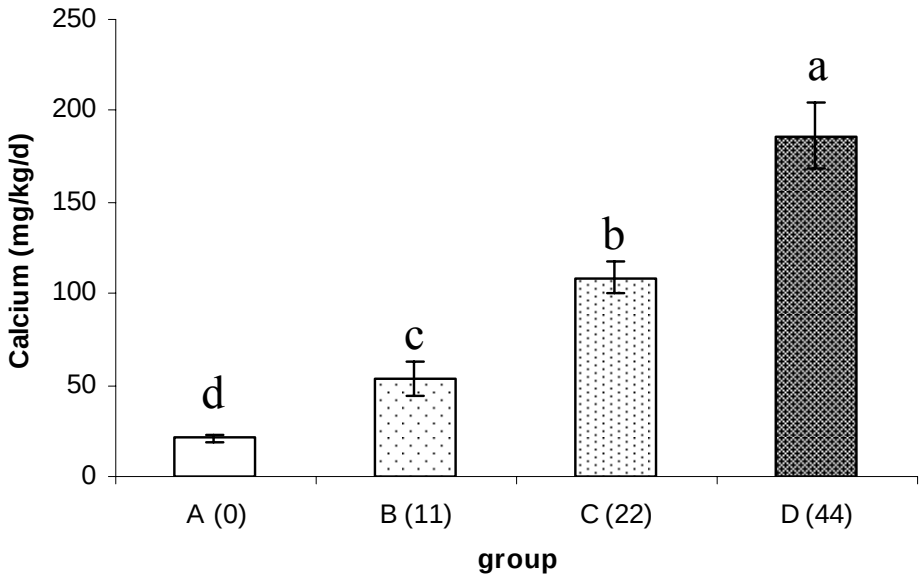

Figure 9. Calcium absorption per day of rat fed with sintered calcium compound. Different letters on the bars indicated the significant differences ($P < 0.05$).

Remark : A : Rat fed with low calcium containing diet

B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)

C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)

D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

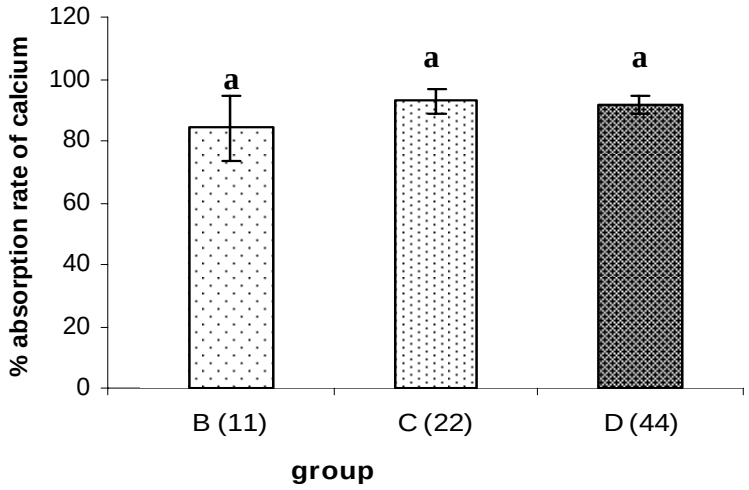

Figure 10. Calcium absorption of rat fed with sintered calcium compound. Different letters on the bars indicated the significant differences ($P < 0.05$).

Remark : B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)

C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)

D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

The retention rate of calcium in rat fed with differ level calcium compound are present in Figure 11. There has no significant difference in retention rate of calcium among different supplement doses with average calcium retention rate in range of 80-90%. This is accounted by high absorption and low urinary losses of calcium. The result is correspond with the finding that calcium retention in rats fed with haddock bone tablets increased with increasing of supplement dose from 14 mg/d to 50 mg/d (Jiancong *et al.*, 2010). The result suggested that sintered calcium compound has high bioavailability when compared with amount of calcium intake. However it is worth to mention that the calcium absorption rate was higher than the general value (30%)

reported by several research groups (Bronner *et al*., 1999). This difference may originate from collecting of rat feces and urine of this study was performed just 24 hours thus it is possible that significant amount of feed or feces may remain in the rat intestine.

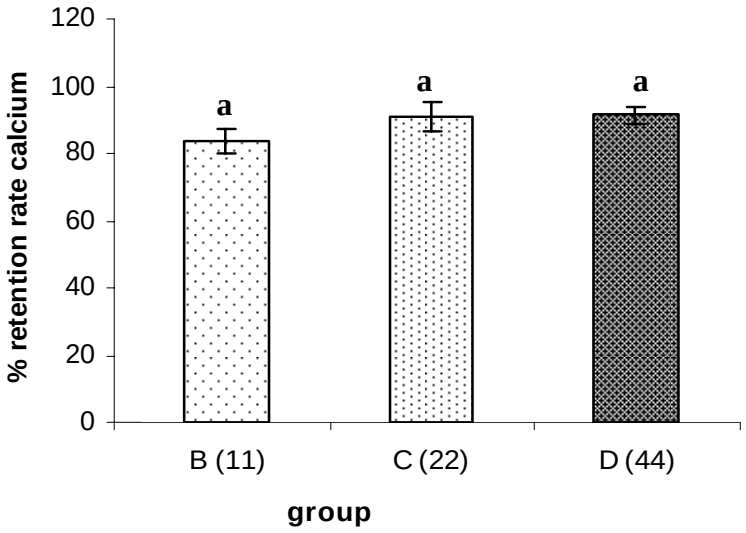

Figure 11. Retention rate of rat fed with sintered calcium compound. Different letters on the bars indicated the significant differences ($P < 0.05$).

Remark : B is a rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)

C is a rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)

D is a rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

Table 4. Effect of sintered calcium compound on absorption and retention rate in Wistar rats

Treatment (mg Ca/d)	*n*	Calcium content in feces (mg/kg/d)	total calcium absorption (mg/kg/d)	Absorption rate of Calcium (%)	Calcium content in urine (mg/kg/d)	Retention rate of calcium (%)
0	6	2.15± 1.02^{c}	21.17± 2^{d}	-	0.41±0.15^{b}	-
11	6	9.87±3.01^{b}	53.48± 8.77^{c}	84.19±10.6^{b}	0.35±0.16^{b}	83.63±3.6^{a}
22	6	8.56±2.97^{b}	108.64± 8.56^{b}	92.76±4.32^{a}	1.03±0.29^{a}	90.96±4.50^{a}
44	6	16.82±3.83^{a}	186.17±18.10^{a}	91.78±2.91^{ab}	0.94±0.21^{a}	91.3±2.87^{a}

Values in the same column followed by different superscript letters are significantly different *($P < 0.05$).*

3.4.3 Effect of sintered calcium compound on rat femur length and weight

Effects of calcium supplement levels on femur weight and length of rats are presented in Figure 12. There was non significant difference in rat femur bone length among the supplement regimes (Figure 12A). This is possibly due to adult rat with fully bone development was used for this experiment. Thus, bone growth and elongation did not change by amount of absorbed calcium. In case of bone weight, there was a statistically significant effect of sintered calcium compound. The rat in control group and fed with low calcium supplement was expressed the lowest bone weight (Figure 12B). This abnormal development of bone correlates very well with their insufficient calcium intake. The result is supported by the finding that calcium deficiency reduced maturation of femoral bone (Iwamoto *et al.*, 2003). In addition, the results shown the femur weight increased with an increasing of calcium supplement. The heaviest bones were observed in the rats received calcium supplementation of 44 mg Ca/d. Since average initial femur weight is not measured in this study thus the recorded weight would be considered as degree of bone healthiness. It is clearly seen that level of calcium supplement affected bone development and consequently altered bone morphological.

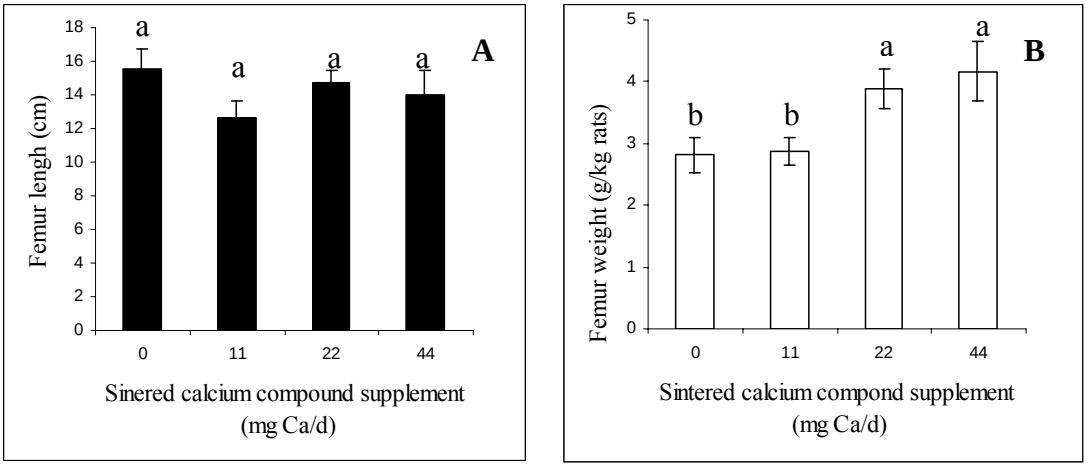

Figure 12. Effect of sintered calcium compound supplements levels on femurs length (A) and weight (B).
Different letters on the bars indicated the significant differences ($P < 0.05$).

Remark :
A : Rat fed with low calcium containing diet
B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)
C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)
D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

3.4.4 Effect of amount of sintered calcium compound on bone density

Histological sections of rats fed with different contents of calcium supplement are shown in Figure 13. Histological sections in Figure 13A and 13B revealed sparse and thinner trabeculae resulting in bone with great inter-trabeculae spaces. These abnormal trabeculae conformations suggested an insufficient calcium intake to reach calcium homeostasis for the groups with non Ca and 11 mg Ca/d supplement. It was found that calcium deficiency reduced maturation-related cortical bone gain as a result of decreased periosteal bone gain and enlarged marrow cavity (Miao and Scutt,

2002). The thicker trabeculae with high connectivity and narrowed inter-trabeculae spaces were observed in rats received 22 and 44 mg Ca/d (Figure 13C and 13D). The results thus confirmed that the adsorbed calcium is utilizable by rat. Together with the calcium absorption and retention studies, the results thus supported high bioavailability of the sintered calcium compounds. This is the key characteristics of a calcium source with high potential food supplement. Calcium carbonate, for instance, although it has high solubility and absorption rate but its utilization is very much lower than that of ossein-hydroxyapatite, the lower soluble and absorbed calcium compound (Ruegsegger *et al*., 1995).

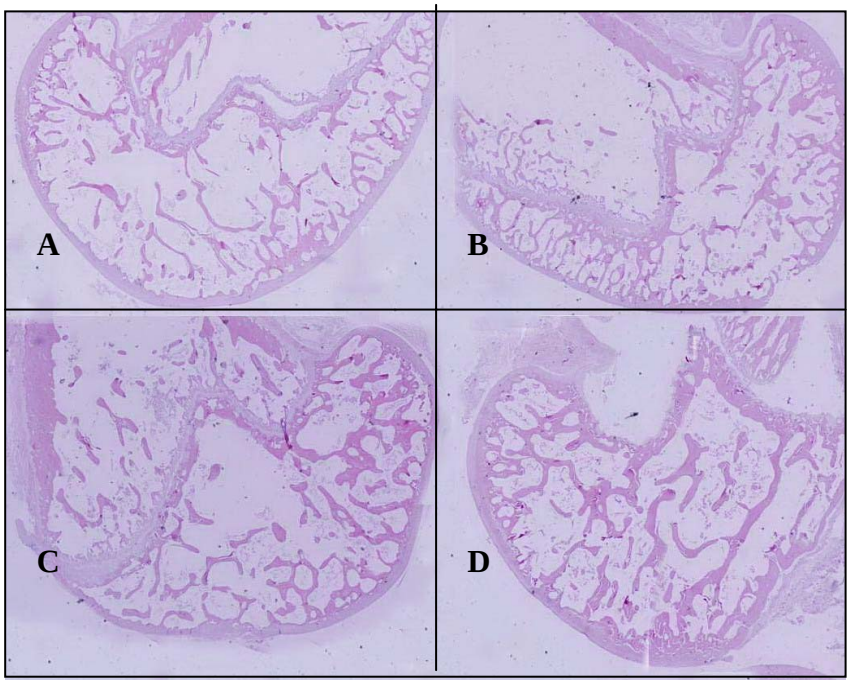

Figure 13. Histological sections of rat's bone fed with different sintered calcium compound.

Remark : A : Rat fed with low calcium containing diet

B : Rat fed with low calcium containing diet + sintered calcium compound under requirement (11 mg/d)

C : Rat fed with low calcium containing diet + sintered calcium compound followed requirement (22 mg/d)

D : Rat fed with low calcium containing diet + sintered calcium compound over requirement (44 mg/d)

3.5 Conclusions

The study revealed that high calcium bioavailability material could be prepared from fish bone by sintering process. Calcium supplement levels of this study supported normal growth and bone development of rats. Absorption and retention rate of calcium were non significant difference among rats fed with different calcium supplement levels. Since sintering a 90 mg of the fish bone produced a 10 mg calcium compound thus the solid by-product could be considered as a potent material for production of a calcium supplement.

CHAPTER 4

SUMMARY AND FUTURE WORKS

4.1 Summary

1. Chemical composition of fish frame by-product was included 54.95 ± 0.2 % protein and 31.41 ± 1.2% ash. Used of citric acid was 70% protein removed.

2. By-product from frozen fish processing could be produced calcium compound in two phases. Calcium hydroxyapatite and tricalciumphosphate were found after sintered at 1300^{o}C. The holding time of sintered had no affected on calcium compound phase.

3. Calcium compound from fish processing by-product could be used as source of calcium supplement in rats. The bioavailability of calcium compound was present in rats.

4. The approximately dose of calcium compound from fish processing by-product is between 22-44 mg per day.

4.2 Future works

1. Calcium compound enhancing bioavailability in rat should be further evaluated.

2. Use of calcium compound from fish processing by-product as source of calcium and human and its toxicity.

REFERENCES

Admassu, W. and Breese, T. 1999. Feasibility of using natural fishbone apatite as a substitute for hydroxyapatite in remediating aqueous heavy metals. J. Hazard. Mater. 69: 187-1271.

Alam, M.R., Kabir, A.K.M.A., Amin, M.R., and McNeill, D.M. 2005. The effect of calcium hydroxide treatment on the nutritive and feeding value of *Albizia procera* for growth goats. Anim. Feed. Sci. Tech. 122: 135-148.

Armbrecht, H.J. and Wasserman, R.H. 1976. Enhancement of Ca^{++} uptake by lactose in the rat small intestine. J. Nutr. 106: 1265-1271.

Belitz, H.D. and Grosch, W. 2001. Schieberle, P. Lehrbuch der Lebensmittelchemie, ISBN 3-540-41096-15. Aufl. Springer Verlag, Berlin Heidelberg New York.

Bregestovski, P. and Spitzer, N. 2005. Calcium in the function of the nervous system: New implications. Cell Calcium. 37: 371-374.

Boelter, M.D. and Greenberg, D.M. 1994. Severe calcium deficiency in growing rats. J. Nutr. 2: 67-74.

Broadus, A. E. 1993. Physiological functions of calcium, magnesium and phosphorus and mineral ion balance. In: Favus, M. J. (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. pp. 44-46. New York: Rven press.

Brommage, R., Binacua, C., Antille, S. and Carrie, A. L. 1993. Intestinal calcium absorption in rats is stimulated by dietary lactulose and other resistant sugars. J. Nutr. 123: 2186-2194.

Bronner, F. and Pansu, D. 1999. Nutrition aspects of calcium absorption. J. Nutr. 129: 9-12.

Bronner, F. 2003. Mechanisms of international calcium absorption. J. Cell. Biochem. 88: 387-393.

Charoenphandhu, N., Tudpor, K., Pulsook, N. and Krishnamra, N. 2006. Chronic metabolic acidosis stimulated transcellular and solvent drag-induced calcium transport in the duodenum of female rats. Am. J. Physiol. Gastrointest. Liver. Physiol. 291: 446-455.

Choi, I., Jung, C., Choi, H., Kim, C. and Ha, H. 2005. Effectiveness of phosvitin peptides on enhancing bioavailability of calcium and its accumulation in bones. Food Chem. 93: 577-583.

Chompston, E.J., Veli, S., Kaptoge, S. and Seeman, E. 2007. Bone remodeeling rate and remodeling balance are not co-regulated in adulthood: Implication for the use of activation frequency as an index of remodeling rate. J. Bone. Miner. 5: 1031-1036.

Choudhury, G.S. and Bublitz, C.G. 1996. Computer-based controls in fish processing industry. In: Mittal, G.S. (ed) Computerized Control Systems in the Food Industry. New York: Marcel Dekker Inc. pp. 513–538.

Coelho, T.M., Nogueira, E.S., Weinand, W.R., Lima, W.M., Steimacher, A., Medina, A.N., Baesso, M.L. and Bento, A.C. 2007. Thermal properties of natural nanostructured hydroxyapatite extracted from fish bone waste. J. Appl. Phys. 101: 74-81.

Cui, S.F., Yong, Z., Sun, W., Cao, P. and Tang, Q. 2005. Effect of nano pearl powder on the calcium absorption and utilization in rats. Acta. Lab. Anim. Sci. Sin. 13: 204-207 (Chinese).

David, S., Jackie, B. and Ricki, L. 1999. Hole's Human Anatomy & Physiology. United stage of America: McGraw-Hill Companies, Inc.

Dawson, B., Harris, S.S., Krall, E.A. and Dallal, G.E. 1997. Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. N. Engl. J. Med. 337: 670-676.

Dogan, E. and Posaci, E. 2002. Monitoring hormone replacement therapy by biochemical markers of bone metabolism in menopausal women. J. Postgrad. Med. 78: 727-731.

Domrongkitchaiporn, S., Ongphiphadhanakul, B., Stitchantrakul, W., Chansirikarn, S., Puavilai, G. and Rajatanavin, R. 2002. Oxalate nephrolithiasis in postmenopausal women supplemented with calcium or combined calcium and estrogen. Maturitas. 41: 149-156.

Earl, J.S., Wood, D.J. and Milne, S.J. 2006. Hydrothermal synthesis of hydroxyapatites. J. Phys. Conf. Ser. 26: 268-272.

Erba, D., Ciappellano, C. and Testolin, G. 2001. Effect of caseinphosphopeptides on inhibition of calcium intestinal absorption due to phosphate. Nutr. Res. 21: 649-653.

Erba, D., Ciappellano, C. and Testolin, G. 2002. Effect of the ratio of casein phosphopeptides to calcium (w/w) on passive calcium transport in the distal small intestine of rats. Nutr. Res. 18: 143-146.

FAO 2002. Human vitamin and mineral requirement. Rome: FAO.

FAOSTAT, FAO statistical databases, fisheries data 2001. Food and Agriculture Organization of the United Nation, Rome, Italy (online). Available from http://www.fao.orgurlhttp://www.fao.org (20 September 2010)

Frost, H. 1991. A new direction for osteoporosis research. Bone. 12: 429-437.

Gao, H., Chen, H., Chen, W., Tao, F., Zheng, Y., Jiang, Y. and Ruan, H. 2008. Effect of nanometer pearl powder on calcium absorption and utilization in rats. Food Chem. 109: 493-498.

Gerard, J.T. and Nicholas, P.A. 1990. Principles of Anatomy and Physiology. United Stage of America: Biological Sciences Textsbook.

Greenwood, M.R.C. and Maria, M. 2008. Use of dietary supplements by military personel. Washington D.C: The National Academy of Sciences.

Gueguen, L., MsSaAgr, A. and Pointillart, A. 2002. The bioavailability of dietary calcium. J. Am. Coll. Nutr. 19: 119-136.

Hansen, M., Thilsted, S.H., Sandstrom, B., Kongsbak, K.T.L., Jensen, M. and Sorensen, S.S. 1998. Calcium absorption from small soft-boned fish. J. Trace. Elem. Med. Biol. 12: 148-154.

Hanzlik, P.R., Fowler, S.C. and Fisher, D.H. 2005. Relative bioavailability of calcium from calcium formate, calcium citrate and carbonate carbonate. J. Phamacol. 313: 1217-1222.

Harrington, M.E., Flynn, A. and Cashman, K.D. 2001. Effects of dietary fibre extracts on calcium absorption in the rat. Food Chem. 73: 263-269.

Heaney, R.P., Saville, P.D. and Recker, R.R. 1995. Calcium absorption as a function of calcium intake. J. Lab. Clin. Med. 85: 881-890.

Heaney, R. P. 1996. Principle of Bone Biology. New York: Academic Press.

Heaney, R. P. 2001. The bone remodeling transient: interpreting interventions involving bone-related nutrients. Nutr. Rev. 59: 327-334.

Helland, S., Refstie, S., Espmark, A., Hjelde, K. and Baeverfjord, G. 2005. Mineral balance and bone formation in fast-growing Atlantic salmon parr (*Salmo salar*) in response to dissolved metabolic carbon dioxide and restricted dietary phosphorus supply. J. Aquac. Nutr. 250: 364–376.

Heller, H.J., Greer, L.G., Haynes, S.D., Poindexter, J.R. and Pak, C.Y. 2000. Pharmacokinetic and pharmacodynamic comparison of two calcium supplements in postmenopausal women. J. Clin. Pharmacol. 40: 1237-1244.

Hill, P.A. 1998. Bone remodeling. Bri. J. Ort. 25: 101-107.

Hiller, J.C., Thompson, T.J.U., Evison, M.P., Chamberlain, A.T. and Wess, T.J. 2002. Bone mineral change during experimental heating : an X-ray scattering investigation. Biomaterials. 24: 5091-5097.

Huang, S., Chen, J.C., Hsu, C. and Chang, W.H. 2009. Effects of nano calcium carbonate and nano calcium citrate on toxicity in ICR mice and on bone mineral density in an ovariectomized mice model. Nanotech. 20: 375-382.

IACUC. 2008. Guidelines for the use of cervical dislocation for rodent euthanasia. The university of Texus. Institional Animal Care and Use Committee (Online). Available http://www.avma.org./issues/animalwelfare/ euthanasia.pdf (20 September 2010).

Isabel, F.T.H., Miguel, A.A.G., Mariano, C.P. and Luis, B.J. 2006. Physiological bases of bone regeneration II. The remodeling process. Med. Oral. Patol. Oral. Cir. Bucal. 11: 151-157.

Ishikawa, M., Kato, M., Mihori, T., Watanabe, H. and Sakai, Y. 1990. Effect of vapor pressure on the rate of softening of fish bone by super-heated steam cooking. Nippon. Suisan. Gakk. 56:1687-1691.

Iwamoto, J., Yeh, J.K., Takeda, T., Ichimura, S. and Sato, Y. 2003. Comparative effects of vitamin K and vitamin D supplementation on prevention of osteopenia in calcium-deficient young rats. Bone. 33: 557-566.

Jaconsen, R., Lorensen, J.K., Toubro, S., Mikkelsen, I.K. and Astrup, A. 2005. Effect of short-term high dietary calcium intake on 24-h energy expenditure, fat oxidation, and fecal fat excretion. Int. J. Obes. 29: 292-301.

Jiancong, H., Shanggui, D., Chao, X. and Guozhong, T. 2010. Preparation and biological efficacy of haddock bone calcium tablets. Chin. J. Oceanol. Linmol. 28: 371-378.

Johns, P. 1997. The structure and components of collagen containing tissues. In: Ward, A.G. and Cours, A. (eds) Science and Technology of Gelatin. Academic Press, London, pp. 31-72.

Joost, G.J.H., Nilius, B. and Bindels, J.M. 2005. Calcium absorption across epithelia. Physiol. Rev. 85: 373-422.

Joschek, S., Nies, B., Krotz, R. and Gopferich, A. 2000. Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. Biomaterials. 21: 1645-1658.

Jung, W.K., Lee, B.J. and Kim, S.K. 2006. Fish-bone peptides increases calcium solubility and bioavailability in ovariectomised rats. Bri. J. Nutr. 95: 124-128.

Karl, M. 2009. The calcium quandary. J. Nutr. 25: 655-656.

Katja, H., Marieb, A. and Elaine, N. 2007. Human Anatomy & Physiology. San Francisco: Benjamin Cummings.

Kawase, A., Hirata, N., Tokunaga, M., Matsuda, H. and Iwaki, M. 2007. Gum arabic enhances intestinal calcium absorption in rats. J. Health Sci. 53: 622-624.

Kennefick, S. and Cashman, K.D. 2000. Inhibitory effect of wheat fibre extract on calcium absorption in Caco-2 cells : evidence for a role of associated phytate rather than fibre. Eu. J. Nutri. 39: 12-17.

Kenneth, S. and Saladin, A. 2007. Anatomy & Physiology. Georgia College and Stage University: McGRAW- hill international edition.

Kenny, A.M., Prestwood, K.M., Biskup, B., Robbins, B., Zayas, E., Kleppinger, A., Burleson, J.A. and Raisz, L.G. 2004. Comparison of the effects of calcium loading with calcium citrate or calcium carbonate on bone turnover in postmenopausal women. Osteoporos. Int. 15: 290-294.

Kiely, E.M. 1987. Microcrystalline calcium hydroxyapatite compound in corticosteroid-treated rheumatoid patients: a controlled study. Bri. Med. J. 68: 1124.

Kilpatrick, J.S. 2003. Fish processing waste: Opportunity or liability In: P.J. Bechtel (ed) Advances in Seafood Byproducts, 2002 Conference Proceedings. University of Alaska Sea Grant College Program (publisher), Alaska, pp. 1-10.

Kim, J.S., Yang, S.K. and Heu, M.S. 1999. Component characteristics of cooking tuna bone as a food resource. J. Fish Soc. 33: 38-42.

Kim, J.S., Cho, M.L. and Heu, M.S. 2000. Preparation of calcium powder from cooking skipjack tuna bone and its characteristics. J. Kor. Fish Soc. 33: 158-173.

Krall, E.A. and hughes, B.D. 1999. Smoking increases bone loss and decreases intestinal calcium absorption. J. Bone. Miner. Res. 14: 215-220.

Larsen, T., Thilsted, S.H., Konsbak, K. and Hansen, M. 2000. Whole small fish as a rich calcium source. Bri. J. Nutr. 83:191-196.

Lau, E.M. 2002. Osteoporosis-A Worldwide Problem and the Implications in Asia. Osteoporosis. 31: 67-68.

Lee, C.K., Choi, J.S., Jeon, Y.J., Byun, H.G. and Kim, S.K. 1997. The properties of natural hydroxyapatite isolated from tuna bone. Bull. Kor. Fish. Soc. 26: 553-560.

Lengemann, F.W., Wassermann, R.H. and Comar, C. 1959. Studies on the enhancement of radiocalcium and radiostrontium absorption by lactose in the rat. J. Nutr. 68: 443-456.

Liaset, B., Julshamn, K. and Espe, M.. 2003. Chemical composition and theoreticalnutritional evaluation of the produced fractions from enzyme hydrolysis of salmon frames with Protamex. Proc. Biochem. 38: 1747-1759.

Limpaphayom, K.K., Taechakraichana, N., Jaisamrarn, U., Bunyavejchevin, S., Chaikittisilpa, S. and Poshyachinda, M. 2001. Prevalence of osteopenia and osteoporosis in Thai women. Menopause. 8: 65-69.

Lind, M., Deleuran, B., Thestrup-Pedersen, K., Soballe, K., Eriksen, E.F. and C, B. 1995. Effects of osteotropic growth factors. APMIS. 103: 140-146.

Lupton, J.R., Chen, X.Q. and Frolich, W. 1995. Calcium phosphate supplementation results in lower rat fecal bile acid concentratios and a more quiescent colonic cell proliferation pattern than does calcium lactate. Nutr. Cancer. 23: 221-231.

Mackerras, D. and Lumley, T. 1997. First- and second-year effects in trials of calcium supplementation on the loss of bone density in postmenopausal women. Bone. 21: 527-533.

Maehira, F., Miyagi, I.B.S. and Eguchi, Y. 2009. Effect of calcium source and soluble silicate on bone metabolism and the related gene expression in mice. J. Nutr. 25: 581-589.

Malde, M.K., Graff, I.E., Siljander-Rasi, H., Venalainen, E. Julshamn, K., Pedersen, J.I. and Valaja, J. 2009. Fish bones-a hight available calcium siurce for growing pigs. J. Anim. Physiol. Anim. Nutr. 19: 1396-1439.

Marenzana, M., Shipley, A.M., Squitiero, P., Kunkel, J.G. and Rubinacci, A. 2005. Bone as an ion exchange organ: Evidence for instantaneous cell-dependent calcium efflux from bone not due to resorption. Bone. 37: 545-554.

Matkovic, V., Goel, P.K., Badenhop-Stevens, N.E., Landoll, J.D., Li, B. and Ilich, J.Z. 2005. Calcium supplementation and bone mineral density in females from childhood to young adulthood: a randomized controlled trial. Am. J. Clin. Nutr. 81: 175-188.

McDowell, L.R. 1992. Minerals in Animal and Human Nutrition. New York. Boston: Academic press, Inc.

Melton, L.J. 2003. Epidemiology worldwide. Endocrinol. Metab. Clin. N. Am. 32: 1-13.

Miao, D. and Scutt, A. 2002. Histochemical localization of alkaline phosphates activity in decalcified bone and cartilage. J. Histochem. Cytochem. 50: 33-40.

Miller, G.D. and Anderson, J.B. 1999. The role of calcium in prevention of chronic diseases. J. Am. Coll. Nutr. 18: 371-372.

Murugan, M. and Ramakrishna, S. 2007. Development of cell-responsive nanophase hydroxyapatite for tissue engineering. Am. J. Biochem. Biotechnol. 3: 118-124.

Nagai, T. and Suzuki, N. 2000. Isolation of collagen from fish waste material skin, bone and fins. Food Chem. 68: 277-281.

Nather, A., Ong, H.J.C. and Zameer, A. 2009. Structure of Bone (Online). Available http://www.worldscibooks.com/medsci/5695.html (16 September 2010)

Neuman, W.F., Neuman, M. W., Diamond, A.F., J, M. and Gibbons, W.S. 1982. Studies of the solubility characteristics of brushite:apatite mixtures and their stabilization by non-collagenous proteins of bone. Calcif. Tissue. Int. 34: 149-157.

Nielsen, B., Potter, G., Green, L., Morris, E., Murray, M., Smith, W. and Matin, M. 1998. Response of young horses in training to varying concentrations of dietary calcium and phosphorus. J. Equine. Vet. Sci. 18: 897-404.

Nishimukai, M., Watanabe, J., Taguchi, H., Senoura, T., Hamada, S., Matsui, H., Yamamoto, T., Wasaki, J., Hara, H. and Ito, S. 2008. Effects of epilactose on calcium absorption and serum lipid metabolism in rats. J. Agric. Food. Chem. 56: 10340-10345.

Nordin, B., Peacock, M. and Knowles, F. 1967. Effect of calcium administration and deprivation on serum and urine calcium in stone-forming and control subjects. J. Bri. Med. 2: 729-731.

Nordum, S., Asgard, T., Shearer, K.D. and Arnessen, P. 1997. Availability of phosphorus in fish bone meal and inorganic salts of Atlantic salmon (*Salmo salar*) as determined by retention. Aquaculture.157: 51-61.

NRC. 1995. Nutrient requirements of laboratory animal. National research council (Online). Available http://www. onlinebooks.library.uoenn.edu. (20 November 2010)

Orban, E., Di Lena, G., Ricelli, A., Paoletti, F., Casini, I., Gambelli, L. 2000. Quality characteristics of sharp snout sea bream (*Diplodus puntazzo*) from different intensive rearing systems. Food Chem. 70: 27–32.

Ozawa, M. and Suzuki, S. 2002. Microstructural Development of Natural Hydroxyapatite Originated from Fish-Bone Waste through Heat Treatment. J. Amer. Cer. Socie. 85:1315-1317.

Pansu, D., Duflos, C., Bellaton, C. and Bronner, F. 1993. Solubility and intestinal transit time limit calcium absorbtion in rats. J. Nutr. 123: 1396-1404.

Paradis, S. and Cabanac, M. 2005. Calcium deficiency cannot induce obesity in rats. Physiol. Behav. 85: 259-264.

Persson, P., Persson, R.G. and Hakanson, R. 1993. The effect of high or low dietary calcium on bone and calcium homeostasis in young male rats. Calcif. Tissue. Int. 52: 460-464.

Petenuci, M.E., Stevanato, F.B., Visentainer, J.E.L., Matsushita, M., Garcia, E.E., Souza, N.E. and Visentainer, J.V. 2008. Fatty acid concentration, proximate composition, and mineral composition in fishbone flour of Nile tilapia. Archivos latin de Nutri. 58: 87-90.

Pongchaiyakul, C., Rojroongwasinkul, N., Chotmongkol, R., Kosulwat, V., Charoenkiatkul, S. and Rajatanavin, R. J. 2002. Bone mineral density in rural Thai adults living in Khon Kaen province. J. Med. Assoc. Thai. 85: 235-244.

Pongchaiyakul, C., Apinyanurag, C. and Soontrapa, S. 2006. Prevalence of osteoporosis in Thai men. J. Med. Assoc. Thai. 89: 160-169.

Pongchaiyakul, C., Songpattanasilp, T. and Taechakraichana, N.J. 2008. Burden of Osteoporosis in Thailand. J. Med. Assoc. Thai. 91: 261-267.

Prabakaran, K. and Rajeswari, S. 2006. Development of Hydroxyapatite from natural fish bone through heat treatment. Trends Biomater. Artif. Organs. 20: 20-23.

Praseadsun, P. 1992. Usage of by-product. Department of Biotechnology Faculty of Agro-industry, Prince of Songkla University.

Rhoades, R. and Pflanzer, R. 1992. Human Physiology. United Stages of America: Saunders Collage Publishing.

Ritchie, L.D., Fung, E.B., Halloran, B.P., Turnlund, J.R., Loan, M.D., Cann, C.E. and King, J.C. 1998. A longitudinal study of calcium homeostasis during human pregnancy and lactation and after resumption of menses. Amer. J. Clinical. Nutri. 67: 693-701.

Rodrigo, J., Ros, G., Priago, J., Lopez, C., and Ortuno, J. 1998. Proximate and mineral composition of dried salted roes of hake (*Merluccius merluccius L.*) and ling (*Molva molva L.*). Food Chem. 63: 221-225.

Ruegsegger, P., Keller, A. and Dambacher, M.A. 1995. Comparison of the treatment effects of ossein-hydroxyapatite compound and calcium carbonate in osteoporotic females. Osteoporos. Int. 5: 30-34.

Samad, H.E., Goff, J.P. and Khammash, M. 2002. Calcium homeostasis and parturient hypocalcemia. J. Theor. Biol. 214: 17-29.

Shearer, K.D., Maage, A., Opstvedt, J., Mundheim, H., 1992. Effects of high-ash diets on growth, feed efficiency, and zinc status of juvenile Atlantic salmon (*Salmo salar*). Aquaculture. 106: 345-355.

Shils, M.E., Olsen, J.A, Shike, M. and Ross, A.C. 1999. Modern Nutrition in Health and Disease. Baltimore. Willams and Wilkins. pp.331-334.

Smith, E.L., Giligan, C., Smith, P.E. and Sempos, C.T. 1989. Calcium supplementation and bone loss in middle-aged woman. Am. J. Clin. Nutr. 50: 833-842.

Straub, A.D. 2007. Calcium supplementation in clinical practice: A review of forms, doses, and indications. Nutr. Clin. Pract. 22: 286-296.

Tahvonen, R., Aro, T., Nurmi, J., and Kallio, H. 2000. Mineral content in Baltic Herring and Baltic Herring products. J. Food. Comp. Anal. 13: 893-903.

Talmage, R.V., Matthews, J.L., Mobley, H.T. and Lester, G.E. 2003. Calcium homeostasis and bone surface proteins, a postulated vital process for plasma calcium control. J. Musculoskel. Neuro. Interact. 3: 194-200.

Tongchan, P., Thongreung, C., Prutipanlai, S. and Niyomwas, S. 2009. Effect of calcium compound obtained from fish by-product on calcium metabolism in rat. As. J. Food Ag-Ind. 2: 669-676.

Toppe, J., Aksnes, A., Hope, B. and Albrektsen, S. 2006. Inclusion of fish bone and crab by-products in diets for Atlantic cod, Gadus morhua. Aquaculture. 253: 636-645.

Toppe, J., Akbrektsen, S., Hope, B. and Aksnes, A. 2007. Chemical composition, mineral content and amino acid and lipid profile in bones from various fish species. Comp. Biochem. Physiol., Part B. 146: 395-401.

Tsutagawa, Y., Hosogai, Y. and Kawai, H. 1994. Comparison of mineral and phosphorus contents of muscle and bone in the wild and cutured horse mackerel. J. Food Hyg. Soc. Japan. 34: 315-318.

Vatanparast, H. and Whiting, S. 2006. Calcium supplementation trials and bone mass development in children, adolescents, and young adults. J. Nutr. Rev. 64: 204-209.

Venugopal, V. 2009. Marine Products for Healthcare : Functional and bioactive nutraceutical. Boca Raton: CRC Press.

Walter, A., Rimbach, G., Most, E. and Pallauf, J. 1998. Effects of citric acid supplements to a maize-soya diet on the in vitro availability of minerals, trace elements, and heavy metals. Zentralblatt. Veterinarmedizin. 45: 517-524.

Weaver, C., Martin, B.R., Ebner, J.S. and Krueger, C.A. 1987. Oxalic acid decreases calcium absorption in rats. J. Nutr. 117: 1903-1906.

Weaver, C.M. and Heaney, P.P. 1999. Calcium. In: Shils, M.E., Olsen, J.A, Shike, M. and Ross, A.C. (eds) Modern Nutrition in Health and Disease. Baltimore. pp.141-156.

Widmaier, E.P., Raff, H. and Strang, K.T. 2004. Human Physiology : The machnisms of body function. Boston: McGraw-Hill.

Winzenberg, T.M., Shaw, K.A., Fryer, J. and Jones, G. 2006. Calcium supplementation for improving bone mineral density in children. Cochrane Database of Systematic ReviewsArt. No.: CD005119. DOI: 005110.001002/14651858. CD14005119. pub14651852.

World Health Organization. 1994. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. No. 843 of Technical Reports Series Geneva.

Wynsberghe, D., V, Noback, C.R. and R, C. 1995. Human Anatomy and Physiology McGraw-Hill, Inc.

Yoon, G.A., Man, Kim, Y.M., Chi, G.Y. and Hwang, C.H. 2005. Effects of tuna bone and herbal extract on bone metabolism in ovariectomized rats. Nutr. Res. 25: 1013-1019.

Yin, J., Zhang, Q., Liu, A., Du, W., Wang, X., Hu, X. and Ma, G. 2010. Factors affecting calcium balance in Chinese adolescents. Bone. 46: 162-166.

Zhang, S. and Gonsalves, K.E. 1997. Preparation and characterization of thermally stable nanohydroxyapatite. J. Mat. Sci. 8: 25-28.

Zhang, Q. and Tordoff, M.G. 2004. No effect of dietary calcium on body weight of lean and obese mice and rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286: R669-R677.

Zhang, X. and Vecchio, K.S. 2005. Creation of dense hydroxyapatite (synthetic bone) by hydrothermal conversion of seashells. J. Mat. Sci. 26: 1445-1450.

Zheng, Y., Zhou, H., Modzelewski, J.R., Kalak, R., Blair, J.M., Seibel, M.J. and Dunstan, C.R. 2007. Accelerated bone resorption, due to dietary calcium deficiency, promotes breast cancer tumor growth in bone. Cancer. Res. 19 : 542-548.

Zittermann, A., Bock, P., Drummer, C., Scheld, K., Heer, M. and Stehle, P. 2000. Lactose does not enhance calcium bioavailability in lactose-tolerant, healthy adults. Am. J. Clin. Nutr. 71: 931-936.

APPENDIX

1. Animal feed preparation (Formulation of basic low calcium containing diet)

Method

1. Weight all ingredients depicted in Table 3.1
2. Mixed corn starch, casein, DL-methionine, sucrose, corn oil, cellulose and choline bitartrate together.
3. Mixed mineral and vitamin with the mixer.
4. Add distill water for the mixer ratio of 0.5 : 1 (w/w)
5. The mixer was mold and baked at 60 ^{O}C 1 hours.
6. Keep in plastics box until use.
7. The feed was given every day around 8.00-9.00 a.m.

2. The method of feeding calcium animal
   1. The calcium compounds were mixed with arabic gum with a calcium/gum ratio of 1:1 (w/w) and dissolved in distill water 10 ml.
   2. The mixture was prepared freshly and administered oral by use syringe to the rat.
   3. Animals were allowed tap water *ad libitum*.

VITAE

Name Phatchareerat Tongchan

Student ID 4911020018

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Animal Science)	Prince of Songkla University	2548

Scholarship Awards during Enrolment

Master Student Research Scholarship by Graduate School and Agro-Industry Practice in Agro-Industry Scholarship, Prince of Songkla University, Hat Yai, Thailand

List of Publication and Proceeding

1. Tongchan, P., Thongraung, C., Siripongvutikorn, S. and Prutipanlai, S., 2007 "Characteristics of calcium hydroxyapatites prepared from fish frame (*Bigeye snapper*)" International conference on 10th Asean Food (AFC 2007) August 21-23, Kuala Lumpur, Malaysia. pp 105.
2. Tongchan, P., Thongraung, C., Prutipanlai, S. and Niyomwas, S. 2009. "Effect of calcium compound obtained from fish by-product on calcium metabolism in rat" International conference on Food Innovation Asia (FIAC 2009) June 18-19, Bangkok International Trade & Exhibition Centre (BITEC), Bangkok, Thailand. Volume 2, Issue 04, pp 669-676.