A Dissertation

for the Degree of Master of Science

Development of functional feed additives by encapsulation of α-tocopherol in β-cyclodextrin and co-treatment of α-linolenic acid / betaine

알파-토코페롤의 베타-사이클로덱스트린 내 캡슐화 및 알파-리놀렌산과 베테인 동시 처리를 통한 기능성 사료첨가제의 개발

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Summary

Functional materials are agents that have biological function with small amount, such as vitamins, hormones and neurotransmitters. There are various types of functional materials, and their biological functions can be classified into anti-inflammation effect, anti-oxidation effect and methyl-group donation etc. Functional materials are on the border between food and medicine because of its high efficiency of biological functions. Especially, many types of functional materials are contained in livestock products, and by strengthening the amount of those materials, functional livestock products can be produced.

Functional bioactive materials have some obstacles until entering into the body and functioning. Because of low stability in high temperature or UV light, they can be degraded during storage, delivery and processing. After intake, the low pH in stomach and enzymatic reaction can degrade the functional molecules. Furthermore, many of the functional materials such as unsaturated fatty acids or lipophilic vitamins are hardly dissolved in water, resulting in low absorption rate by intestinal epithelial barrier. The low stability and low bioavailability of the material are the key obstacles of their usage.

To overcome this obstacles, we tried two strategies. The first study is encapsulating α-tocopherol in β-CD with surfactant mixture system. β-CD is popular encapsulation material which can include other hydrophobic material inside of its cyclic structure, forming inclusion complex. But β-CD encapsulation still has limited solubility, and has no way of optimization for

each guest molecule. So, we used surfactant mixture system together with β-CD encapsulation to improve the solubility and optimize encapsulation efficiency and release efficiency. The second study is about determining synergistic effect of ALA and betaine. By finding out synergistic composition of ALA and betaine, we can develop feed additives composed of two synergistic materials together.

In chapter 1, α-tocopherol was encapsulated in β-CD with various ratio of tween 80 and span 80. The encapsulated α-tocopherol showed better encapsulation efficiency, better release rate, better stability, and finally enhanced accumulation of α-tocopherol in yolk when supplemented to hens. In chapter 2, the synergistic effect of ALA and betaine on myoblast proliferation and differentiation was analyzed. Single treatment of ALA and betaine had a little effect on myoblast proliferation or differentiation, but co-treatment of ALA and betaine enhanced both proliferation and differentiation of myoblasts.

These results indicated that α-tocopherol encapsulation can enhance better stability and bioavailability, which can be applied to developing efficient functional feed additives. Moreover, ALA and betaine were found out to be have synergistic effect, so supplement of ALA and betaine together to animals probably have better effects than single supplement of ALA or betaine.

Key words: α-tocopherol, β-cyclodextrin, surfactants, spray drying, α-linolenic acid, ω-6/ω-3 balance, betaine, muscle growth, feed additives

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List of Abbreviations

β-CD: β-cyclodextrin

ALA: α-linolenic acid

DHA: docosahexaenoic acid

EPA : eicosapentaenoic acid

Tw : tween 80

Sp : span 80

SGF : simulated gastric fluid

SIF : simulated intestinal fluid

FE-SEM : field-emission Scanning Elctron Microscope

DLS : dynamic light scattering

Dex : dextrose

Mal : maltose

Suc : sucrose

Sta : starch

Fla : flaxseed

HPLC : high-performance liquid chromatography

GC : gas chromatography

BCA assay : bicinchoninic acid assay

TEER : transepithelial electrical resistance

DPPH : di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium

GOT : glutamic-oxaloacetic transaminase

PCNA : proliferating cell nuclear antigen

FGFR : fibroblast growth factor receptor

CDK : cyclin-dependent kinase

IGF-1R : insulin-like growth factor -1 receptor

PBS : phosphate buffered saline

DMEM : Dulbecco's modified Eagle's medium

FBS : fetal bovine serum

HS : horse serum

DMSO : dimethylsulfoxide

Introduction

The three main functions of foods are supply of the energy source for maintenance of life, suggestion of happiness derived from sense such as flavor and taste, and lastly, bioactive function which can affect our health in a positive way. The foods used in terms of the last function are called functional bioactive materials. These functional materials are widely being used in the food industry, cosmetics and pharmaceutical industries. In livestock industry, the functional materials are usually used for providing positive effect on the growth performance or for producing functional livestock products by accumulating the functional materials in the product (Lowry et al., 2005; Smith, Tokach, Goodband, Nelssen, & Richert, 1997; Surai et al., 1998; Yang, Beauchemin, & Rode, 1999).

There are various types of functional materials used as feed additives. But these materials lose their functions during processing, storage and delivery, and even after being fed, because the low pH in the stomach and various digestive enzymes in the small intestine can affect the stability of the materials. Also, some hydrophobic materials have low absorptive rate in the epithelial layer. These barriers lower the effectiveness of the functional materials and also can lead to lower productivity and higher cost. So strategies to overcome these problems are needed.

In this study, two strategies were tried to overcome these limitations and enhance the effectiveness of the functional materials as feed additives. The first

strategy is β-cyclodextrin (β-CD) encapsulation. β-CD as a cyclic oligosaccharide composed of seven glucose can load hydrophobic materials into the inner side of the cyclic structure (Challa, Ahuja, Ali, & Khar, 2005). It can protect the loaded material from the surrounding environment and can enhance the solubility of the loaded material at the same time. Encasulation of α-tocopherol as a strong antioxidant in β-CD were prepared by spray-drying method with mixed surfactants at the same time to optimize the encapsulation efficiency, release efficiency and solubility. The second strategy is to study the effect of α-linolenic acid (ALA) and betaine on myoblast proliferation and differentiation *in vitro*. By confirming the synergistic effect of ALA and betaine on muscle growth, the growth enhancement effect of animals by the two materials when co-delivered as feed additives can be expected.

Review of Literature

1. Functional feed additives

1) Purpose of supplementation of functional feed additives

In livestock industry, it is common to supplement feed additives to basic feed for several purposes. Feed additives generally include every additional feed used for prevention of disease, supplementing deficient nutrients, improving feed conversion rate or improving growth performances of the animals(Cromwell, 2002; Windisch, Schedle, Plitzner, & Kroismayr, 2008). Also, there is another purpose of supplementing feed additives for producing functional livestock products, which can be achieved by accumulation of the supplemented functional materials into the final livestock product such as meats, eggs or milk.

2) Obstacles in using functional materials

Functional materials used as feed additives are also widely used in pharmaceuticals, cosmetics, foods and so on. These materials should be cost-effective, stable and highly absorptive and efficiently accumulated. However, most of these materials have their own obstacles to be used efficiently. For example, poly unsaturated fatty acids such as conjugated linoleic acid(CLA) or α-linolenic acid(ALA) have variety of positive effects on heath but they are very unstable under high temperature during processing or long-time storage. Furthermore, as many of the materials having hydrophobic property have some

difficulties on mixing or processing with other soluble materials, they are hard to be absorbed at epithelial barriers in small intestine because of its low dispersion rate. Especially in livestock industry, these obstacles leads to low cost efficiency, and they limit the usage of more variety of functional materials as feed additives.

3) α-Tocopherol

α-Tocopherol is a subtype of vitamin E, which is a most preferentially used type in the body (Rigotti, 2007). As shown in Figure 1, α-tocopherol has long hydrophobic carbon chain and benzene ring derivative which has the antioxidant function by stabilizing free radicals.

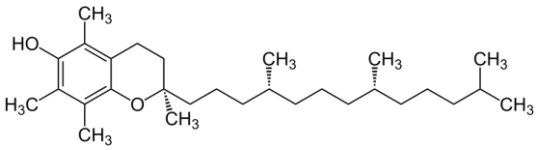


Figure 1. Structure of α-tocopherol

In developed mammalians, vitamin E is the most abundant lipophilic antioxidant in serum or tissue (Meydani, 2002). Vitamin E is usually stored in the lipid bilayer of the cell membranes, and acts as antioxidant by removing free radicals generated inside of the cells immediately and protecting the cells from oxidative damage. The importance of antioxidants as food supplements has also been emphasized for many years, because oxidative stress is strongly related to aging, Alzheimer disease, diabetes, coronary heart diseases, cancer, inflammational diseases (Butterfield, Boyd-Kimball, & Castegna, 2003; Griendling & FitzGerald, 2003; Han & Meydani, 2000).

In livestock industry, vitamin E is usually supplemented as feed additives as a form of total vitamin mixture with other various types of vitamins. This kinds of vitamin E supplementation generally affect positively to the growth performance of the livestock and resistance against diseases.

4) α-Linolenic acid (ALA)

ALA is one of essential ω-3 fatty acid composed of C18 carbon chain and 3 double bonds in the structure (Figure 2). Supplementation of ω-3 fatty acids is very important in terms of positively affecting to the ratio of ω-3 and ω-6 fatty acids in daily diets. As a ω-3 fatty acid, ALA has anti-inflammation effects, anti-cancer effects, prevention of cardiovascular diseases, development of central nerve system and prevention of some kinds of cancers (Simopoulos, 2002).

In livestock industry, one of the defects of the livestock product is high ratio of ω-3 to ω-6 fatty acids, because of selecting corn as the basic source of the feeds. Various attempts have been tried to modulate and improve the ω-3:ω-6 balance in the final livestock products. Therefore, flaxseed oil containing a lot of ALA content was supplemented to affect the ratio of ω-3 to ω-6 in the meats and eggs.

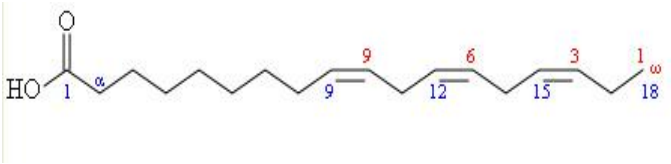


Figure 2. Structure of ALA

5) Betaine

Betaine, a trimethyl-glycine, contains both cationic groups and anionic groups, thus it's so-called zwitterion compound (Figure 3). Betaine has its biological function in the body by two different ways (Lever & Slow, 2010). The first function derives from its osmoprotective effects. In the body, cells have to maintain the cellular membrane potential and this is usually achieved by continuous movement of ions by energy-expending pumps. By providing enough both positive charge and negative charge at the same time, the cells can save its energy by easily regulating membrane potential, and this saved energy can be used for growth.

The second function of betaine is methyl group donation. Providing methyl group is a significant step during formation of amino acids (Park & Garrow, 1999).

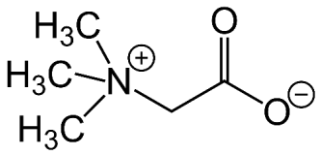


Figure 3. Structure of betaine

For producing methionine, other essential aminoacids and other metabolic derivatives such as creatine, epinephrine and carnitine, there exists a methyl group transfer mechanism. Sufficient amount of methyl group has to be supplied to make amino acids and sustain active metabolisms essential for being alive such as DNA methylation. The core of this methyl transfer-mediated mechanisms is that homocysteine receives methyl group from other molecules

for forming methionine, and methionine acts as methyl donor to other derivatives through various metabolism. Among this process, homocysteine has to get methyl group from methyl donor such as choline or its derivative, betaine from diet. Choline becomes betaine by enzymatic interaction in the body, and betaine becomes dimethyl glycine through donating its methyl group by an enzyme called betaine-homocysteine methyl transferase (BHMF). Some of dimethyl glycine is discharged as urine, some becomes glycine and they are recycled in the body. Therefore, supplementation of betaine promotes general metabolism and helps formation of amino acids, hormones and other essential metabolic derivatives essential for normal growth.

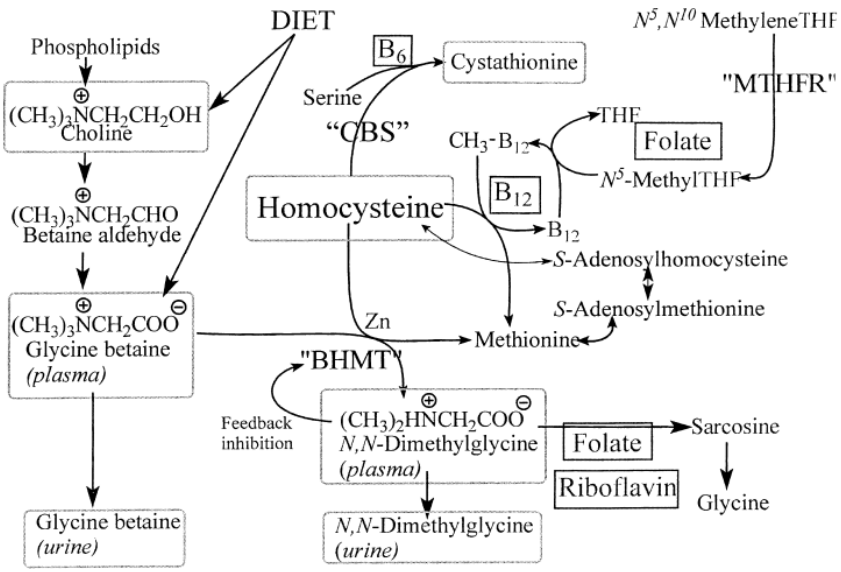


Figure 4. Scheme of trans-methylation mechanism by BHMT (McGregor *et al.*, 2002)

In livestock industry, betaine has been used as feed additives to substitute choline, because it's cheaper than choline, and usage of betaine directly saves

one step of enzymatic reaction, the conversion of choline to betaine. Furthermore, supplementation of betaine has been known to improve general growth performance of animals. Although the specific mechanism of betaine is not clear, the osmoprotective effect and methyl group donation were reported. Betaine also accelerates functioning of other functional materials because betaine activates general metabolism. For this reason, usage of betaine as a synergistic reagent with other functional materials is also actively being investigated.

2. Nutrient delivery system

1) Background of nutrient delivery system

In the past, ‘nutrient’ is generally indicated as a basic source of energy and body composition for maintaining of normal metabolism and normal health. During last decades, strong novel therapeutic methods and agents have been developed along with the overwhelming pharmaceutical and medical developments. However, as these therapies are specific and strong they also have potential dangers of side effects as well, although most of them acts after the diseases occurs. As preventive medicine has been developed due to aging, scientific effects of active molecules in daily supplemented diets have been revealed. Especially, some active antioxidant molecules such as phytochemicals, ascorbic acid and vitamin E have been used as preventive agent and therapeutic agent, developing the novel word ‘nutraceutical’ which is combination form of nutrient and pharmaceutical.

Bioactive functional materials widely exist in meats, eggs, milk, fish, vegetables, but these materials in foods are very small amount and modern people are hard to maintain and control balanced diet all the time. Thus, modern people tend to purchase the foods intensified with functionality or intake nutraceuticals as food supplements separately.

Therefore, for making functional livestock products by supplementing functional feed additives or for supplementing these materials directly as food supplements, these functional materials should be processed properly, transported and absorbed into intestine efficiently and finally function properly. However, these materials are unstable under light and high temperature, and hardly absorbed in intestinal epithelial barrier because of low solubility. To overcome these limitations, recent studies graft drug delivery system (DDS) technology onto nutrient delivery system.

Representative methods for delivery of functional materials can be divided into three categories as shown in Table 1. First method is chemical processing which includes covalently attaching other molecules for enhancing stability and solubility. PEGylation is the most common method of chemical processing and there also are many methods to form pro-forms of materials which can be converted into mature form in the body by enzymatic reactions. Chemical processing method can dramatically change the physicochemical property of the functional material, but it has to newly synthesize a novel chemical compound. Therefore, they have some difficulties on global permission of their usage and industrialization. The second method is physical encapsulation which

physically coats the functional material into the coating material for enhancing physical stability and solubility. This method is relatively easier than chemically modifying methods, although selection of proper coating materials and control of releasing efficiency are obstacles to their general usage. The last method is emulsifying method, which uses surfactants or liposomes as an emulsifying agent and emulsifying the lipophilic functional molecules forming micelles to enhance the solubility and improve physicochemical properties into more efficient way.

Table 1. Strategies to enhance stability and bioavailability of functional materials

Types	Chemical processing	Physical encapsulation	Emulsifying with surfactant
Characteristics	• Chemical covalent bond • Need for enzyme for the reaction • Forming totally new material	• Hydrophobic, or hydrogen interaction • Forming physical inclusion complex • Controlled release	• Emulsifying by forming micelle • Easily affected by environment • High enhanced solubility
Examples	PEGylation Enzymatic modification	chitosan, dextran, modified starch, **cyclodextrins**, CMC(carboxymethylcellulose), ethylcellulose	**span**, **tween**, wax, gum arabic, sodium alginate, diacylglycerols

2) Cyclodextrin encapsulation

Cyclodextrin (CD) is a family of compounds made up of sugar molecules bound together in a ring, forming cyclic oligosaccharides. It divides into 3 subtype depending on the number of glucose composing of the cyclic structure (Figure 5); α-CD, β-CD, γ-CD. Each type of CD has different pore size and solubility, but β-CD is most abundantly used type because it's easier to be synthesized than other types.

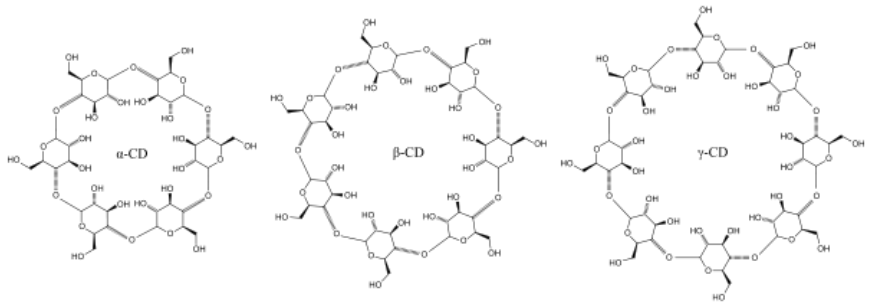


Figure 5. The types of cylclodextrins

As the glucose molecules composing of CD is layered with polarity, hydrophobic residues of the glucoses heads to inner side and hydrophilic residues heads to outer surface (Figure 6). This conformational features determine the potential of CD as a coating agent by positioning hydrophobic guest molecules inside of the CD for improving their stability and solubility. The form of CD including guest molecule inside its pore is called 'inclusion complex'. CD is approved as GRAS (generally regarded as safe), so broadly being used in pharmaceutical, food and cosmetic industries.

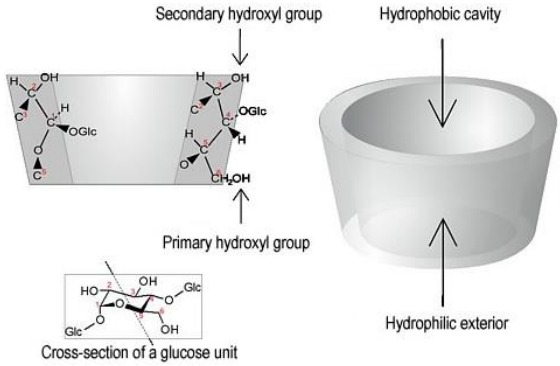


Figure 6. Three-dimensional structure of cyclodextrins (Szejtli *et al*., 1996)

There are several methods of forming inclusion complex using CD, and depending on the type of guest molecules or purpose of the usage, proper method should be selected (Table 2).

Table 2. Methods of CD encapsulation

Method	Reference
Kneading	(Patel, Suhagia, Shah, Rathod, & Parmar, 2007)
Co-precipitation	(Park et al., 2002)
Dry mixing	(Ozdemir & Ordu, 1998)
Slurry complexation	(Wong & Yuen, 2001)
Neutralization	(Koester et al., 2001)
Spray drying	(Sinha, Anitha, Ghosh, Nanda, & Kumria, 2005)
Freeze drying	(Cao, Guo, & Ping, 2005)
Solvent evaporation	(Dua et al., 2007)

Although CD encapsulation is efficient way of improving stability and solubility of included functional molecules, it still has some limitations of its usage. CD is quite soluble in water, but when it's in the form of inclusion

complex with other lipophilic compound, its solubility decreases. Moreover, CD encapsulation is hard to control its release and has quite different inclusion efficiency rate depending on the type of guest molecules. So, strategies to overcome these limitations has been investigated such as chemical modification of CD.

3) Surfactant mixture system and hydrophile-lipophile balance (HLB)

Surfactants, also called as detergents, emulsifiers and dispersants, are any compounds that lower the surface tension between two molecules which have distinct physicochemical properties. This can be achieved by the molecular property of surfactants that have both hydrophilic and lipophilic part in one molecule and can form polarized layer such as micelles.

Surfactants are used as emulsifier to enhancing solubility of hydrophobic molecules in the water. There are a plenty of types of surfactants, but their property as an emulsifier can be represented as HLB (hydrophile-lipophile balance) value, which indicates the degree of how much it is hydrophilic or lipophilic, and this can act as the standard of selecting surfactant (Griffin, 1949). The more the number decreases, the more lipophilic the surfactant is. For example, HLB value of span 80 is 4.3 and that of tween 80 is 15. When two or more surfactants are blended, HLB value can be recalculated considering the ratio of the blended surfactants.

3. Muscle growth

1) Mechanism of muscle growth

During fetal development, myogenic progenitor cells derived from somites go through skeletal myogenesis differentiating to form primary muscle fibers (Buckingham *et al*., 2003). After fetal development, the resident progenitor cells are re-positioned around primary myofibers (Kassar-Duchossoy *et al*., 2005; Manceau, Marcelle, & Gros, 2005; Relaix, Rocancourt, Mansouri, & Buckingham, 2005). In adults, the satellite cells usually remain to be mitotically quiescent and are located in the space between sarcolemma and basal lamina in muscle fiber. In this condition, very limited genes are expressed and the quiescent state is maintained. Then, these quiescent cells are activated to proliferate in response to stress or microenvironment change (Charge & Rudnicki, 2004). There is still no clear explanation about the factors which activate quiescent satellite cells. However, those activation-inducing factors are classified into several groups. The increase of intrinsic factors such as sphingosine-q-phosphate (Nagata, Partridge, Matsuda, & Zammit, 2006) are one of them, or change of signals induced by external physical stretching such as hepatocyte growth factors (HGF) or nitric oxide (Pisconti *et al*., 2006; Wozniak & Anderson, 2007), and micro-environmental growth factors such as fibroblasts growth factors (FGF) (Jones *et al*., 2005). After activation starts by these kinds of factors, satellite cells move to the outside of basal lamina and proliferate with expressing Pax7 and myoD. After several division, the expression level of Pax7 decreases and that of myoD increases, accompanied

by cell fusions to differentiate into myofiber. Among these satellite cells experience cell division, some parts don't participate in differentiation characterized by no expression of myoD. These divided cells without becoming myotubes are called 'self-renewing myoblasts'. The myogenesis after birth is distinguished from fetal myogenesis, and are called 'regeneration' (Le Grand & Rudnicki, 2007).

In livestock industry, regulation of muscle mass to produce 'meat' as a livestock product is very important economically. In terms of muscle growth, increase of accumulated protein in muscle fiber called as 'muscle hypertrophy' is basic concept. However, growth of muscle size by increasing size of myotubes has limitation because of the limited number of nuclear. So, for efficiently inducing more muscle hypertrophy, muscle regeneration for providing more nuclei should be accompanied.

2) Effect of PUFA and betaine on muscle growth

Tachibana *et al*. investigated the effect of various fatty acids on modulation of proliferation and differentiation of myoblast *in vivo*. In that study, a certain type of conjugated linoleic acid (CLA), c9, t11 CLA and n-6 poly unsaturated fatty acids have effects on muscle cell proliferation, and oleic acid, linoleic acid and c9, t11 CLA also modulate differentiation of myoblasts (Lee, Tachibana, Morinaga, Fujimura, & Yamada, 2009).

Terruzzi *et al*. determined the effect of betaine on differentiation of murine myoblast cell line and found out that the insulin-like growth factor-1 (IGF-1) signal plays an important role in this process. In that study, they performed

dose-dependent betaine treatment on differentiating myoblasts, and 10mM of betaine significantly increased IGF-1 signaling, synthesis of Myosin heavy chain (MyHC) and neomyotube length (Senesi, Luzi, Montesano, Mazzocchi, & Terruzzi, 2013).

Fernandez-Fıigares *et al*. conducted an experiment to determine the effect of supplementary betaine, CLA or both betaine and CLA on growth performance and carcass composition in Iberian pigs. As a result, betaine or CLA were not shown to have any effect on growth performance and carcass composition individually, while supplement of both betaine and CLA enhanced growth performance and imporved carcass composition in which the portion of protein increases and that of lipid decreases (Fernandez-Figares, Conde-Aguilera, Nieto, Lachica, & Aguilera, 2008).

Chapter 1. Encapsulation of α-tocopherol in β-CD with surfactant mixture

1. Introduction

There are many types of functional materials which have excellent effects, but they have some obstacles of usage. The materials such as α-tocopherol and α-linolenic acid (ALA), are unstable at UV or heat, and has low absorption rate because of their hydrophobicity. To improve the stability and solubility, dual strategy having β-cyclodextrin (β-CD) encapsulation and surfactant mixture system were tried. Although β-CD itself is a widely used encapsulation material, it still has some limitations such as deficient solubility, no optimization, and no release control. Therefore, surfactant system together with β-CD was used to enhance solubility and control the release rate (Figure 7). In this study, two distinct types of surfactants, tween 80 and span 80 were mixed by various ratios to optimize the encapsulation level, protection efficiency, release rate and intestinal permeability.

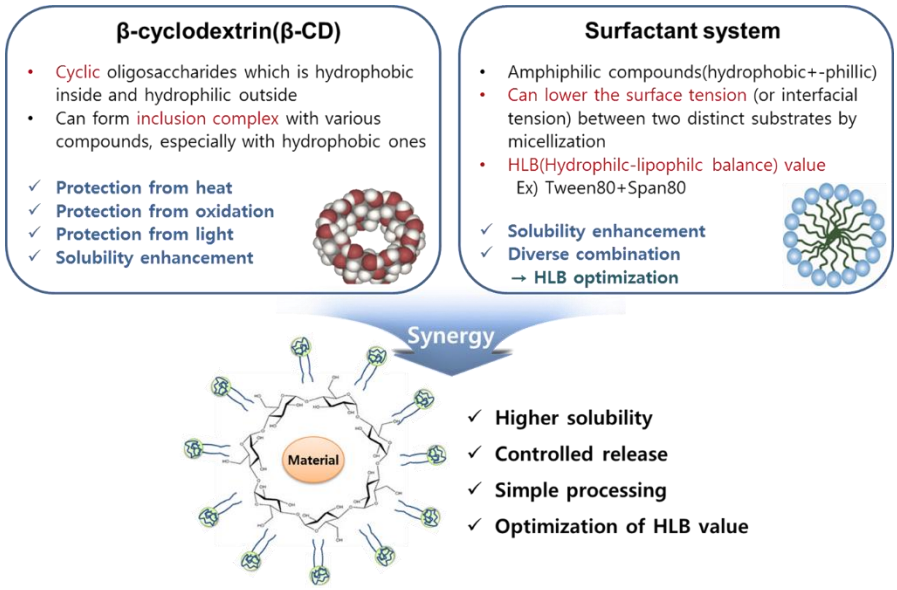


Figure 7. Scheme of the strategy of α-tocopherol encapsulation

2. Materials and methods

1) Materials

α-Tocopherol, β-cyclodextrin (β-CD), chloroform, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Triton X-100 and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Mo. USA). Ethanol, methanol and hexane were from Merck (Darmstadt, Germany). Bicinchoninic acid (BCA) reagents were purchased from THERMO Scientific (PA, USA), and Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone (Utah, USA). The surfactants, tween 80 and span 80 were purchased from DAEJUNG (Siheung, Korea). Cell culture insert (6-well, 3.0 um pore size) was purchased from BD bioscience (CA, USA). Rabbit-anti-ZO-1 antibody and Alexa 488-conjugated anti-rabbit Fc-antibody were purchased from Santa Cruz Biotechnology (CA, USA). Poultry feed, flaxseed oil, and processed flaxseed used in vivo assay were purchased from Seoul Feed (Incheon, Korea), and Amplex Red cholesterol assay kit was purchased from Invitrogen (CA, USA).

2) Encapsulation of α-tocopherol in β-CD

5.44g of β-CD was dissolved in 320ml of DW by stirring. Then, 2.06g of α-tocopherol was dissolved in 80ml of ethanol, and dissolved α-tocopherol was added to β-CD solution. Tween 80 and span 80 with various mixing ratios were added to the solution with the surfactant concentration 0.1%. Then the solution was stirred for 24h at room temperature. After stirring, samples were dried using spray-dryer (Mini Spray Dryer B-290, Buchi). The conditions for spray-

drying is as below : aspirator : 90 ; inlet temperature : 170℃ ; outlet temperature : 90℃ ; PUMP : 15.

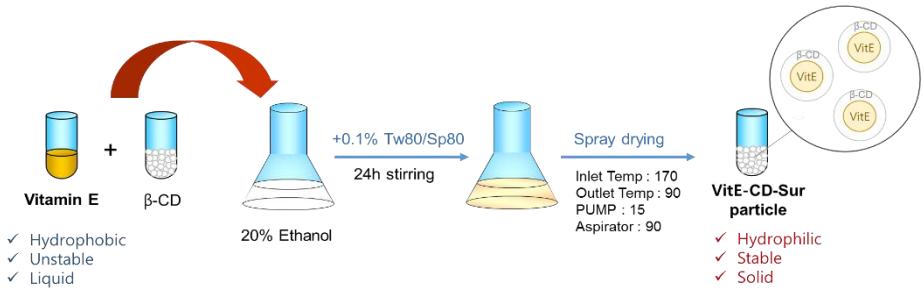


Figure 8. Scheme of the encapsulation step of α-tocopherol in β-CD

3) Measurement of size and observation of morphology

The size and morphology of the solid powder prepared by spray-drying was determined by Field-Emission Scattered Electron Microscopy (FE-SEM, AURIGA). The morphology of the particles dispersed in water was measured by dynamic light scattering spectrophotometer (DLS-7000).

4) Determination of encapsulation efficiency

α-Tocopheorol was released from the samples (10mg/ml) by adding hexane with vortexing for 1 min. Then, samples were centrifuged at 14000 rpm for 5 min, and the supernatant was collected to be analyzed by HPLC. The encapsulation efficiency was calculated as below : (α-tocopherol content in the final sample) / (α-tocopherol content before spary-drying) x 100.

5) *In vitro* release assay

Simulated gastric fluid (SGF) was prepared by adding HCl to PBS until the pH reaches 1.2 and simulated intestinal fluid (SIF) was prepared by adding NaOH to PBS until the pH reaches 7.4. 0.1% Tween 80 was added to both SGF and SIF. 60mg of each sample was weighed and loaded into 14ml round tube, and SGF or SIF was added into each tube. After brief vortexing (1-2sec), the solution was incubated in 37℃ at shaking incubator for 8 h. At 0, 0.5, 1, 1.5, 2, 4, 8 h, 50ul of each sample was collected and additional 50ul of free SGF or SIF was added to maintain the total volume of the solution. After the releasing step, solutions were centrifuged at 1400 rpm for 3 min, and the clear supernatant was collected into the new tubes. The amount of α-tocopherol was analyzed by BCA assay.

6) Heat stability assay

For preparing control samples, α-tocopherol and one of various solid powders (CD, glucose, maltose, sucrose, starch and flaxseed) were mixed evenly. Small amount of each sample or prepared controls were loaded into 1.5ml tube, and incubated at 40, 60 or 80 ℃ for 10, 20 or 30 min by heat-block, with total 9 conditions. After heating, samples were released by adding 10mg/ml ethanol, vortexing for 1min, and centrifuging for 5 min, finally the supernatant was collected. The amount of α-tocopherol in released sample was analyzed by BCA assay, and the protection efficiency was calculated as below : (The amount of α-tocohperol per sample 1 g after heating) / (The amount of α-tocopherol per sample 1 g before heating) x 100.

7) UV stability assay

Control samples were prepared by the same method as heat stability assay. Each sample and controls were loaded onto 6-well plate, and incubated under the germicidal UV lamp (SANKYO DENKI G40T10) located in the clean bench (CLEAIR, CHC Lab) by 68.3cm distance for 24h, 36h and 72h. After incubation, α-tocopherol was released from the samples by adding ethanol, vortexing and centrifugation, and analyzed by BCA assay. The protection efficiency was calculated as the same method as heat stability.

8) BCA assay for α-tocopherol quantification

For preparing standard curve, 2 mg of α-tocopherol was dissolved in 1ml of ethanol and diluted 7 times as the BSA standard curve preparation protocol indicated. 20ul of α-tocopherol released in ethanol or ethanol/PBS (1:1) or 20ul of standard curve samples were loaded into 96-well plate, and 100 ul of BCA reagents prepared by adding reagent B into A was added to each well by multi-pippette. After short incubation, the amount of α-tocopherol in each well was analyzed by detecting absorption rate at 562 nm by microplate reader.

9) Caco-2 cell culture

Caco-2 cells (human colorectal adenocarcinoma cell line; ATCC number HTB-37) were seeded and grown in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. When cells were grown to be 80% confluent, cells were sub-cultured by trypsinization method. For preparing Caoc-2 permeability assay, detached cells were re-seeded onto trans-well insert for 6-well plate by

adding 2 ml of media under the insert and 1ml of media into the insert. Media was changed every other day during trans-well cell culture. After 10 days, cells were fully grown and cells were used in permeability assay when TEER value reaches 500Ω·cm^2.

10) Immunofluorescence analysis

The apical and basal side of caco-2 trans-well insert was washed by PBS. The cells were fixed by 4% PFA, and washed with PBS again. Blocking solution (PBS with 3% BSA and 0.2% Triton X-100) was added to permeabilize the cell, and rabbit-anti-ZO-1antibody as primary antibody was treated with blocking solution. Cells were washed with PBS with 0.2 % Trion X-100 and Alexa488-conjugated secondary antibody in blocking solution was added to cells. Cells were washed by PBS with 0.2% Triton X-100. 1ug/ml DAPI was added and cells were washed again by PBS. Finally, the membrane of trans-well insert was scalped and analyzed by Super-resolution Confocal Microscope (SP 8 X STED, Leica) microscopy after adding mounting solution, air-drying and sealing.

11) Caco-2 epithelial permeability assay

Caco-2 cells were prepared by washing the insert with PBS. Sample solution was prepared by adding each sample to PBS and was normalized by 5mM α-tocopherol. Free α-tocopherol with 0.1% DMSO and physical mixture of α-tocopherol and β-CD were also prepared as solution to be used for controls. Caco-2 inserts were moved to new 6-well plate filled with 1.6ml PBS / well. 0.8ml of sample solutions or control solutions were added into each Caco-2

insert. After incubation at 37℃ for 30 min, the inserts were removed and basal solutions were collected to each new 1.5ml tube. Collected solutions were stored at -20℃ until BCA analysis.

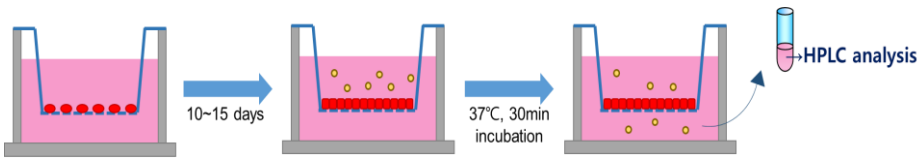


Figure 9. Schematic illustration of Caco-2 permeability assay

12) *In vivo* assay for ALA supplementation (*in vivo* assay - 1)

315 56-week old laying hens were divided into 7 groups randomly, using 45 hens per group and housed as 5 hens per cage. For 8 weeks, each group of hens were fed with feed supplemented with various compositions of ALA. On day 0, 7, 14, 28, 42 and 56, eggs were collected and used for GC analysis. Feed and water were provided ad libitum.

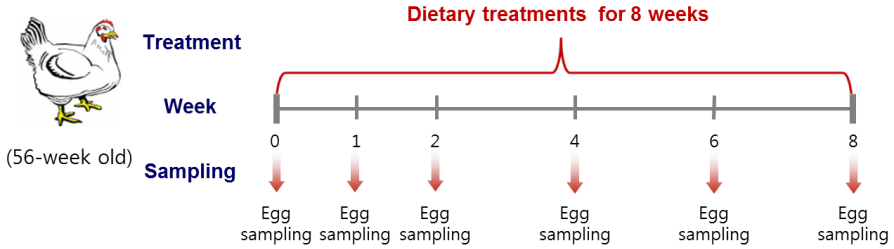


Figure 10. Summary of the experimental plan (*in vivo* assay - 1)

13) *In vivo* assay for ALA and encapsulated α-tocopherol supplementation (*in vivo* assay - 2)

30-week old laying hens (70 hens) were divided into 7 groups randomly, becoming 10 hens per group and housed as 5 hens per cage. For 4 weeks of experimental duration, each group of hens was supplemented with basic feed including ALA supplemented with α-tocopherol. Basic feed contains 3.25g/kg ALA and 20mg/kg of α-tocopherol. Free vitamin E, physical mixture of vitamin E and β-CD, or vitE-CD-Tw/Sp(1:2) was fed to the hens of each group respectively. On day 0, 7, 14, 21 and 24, eggs were collected. 15 eggs were collected from each group, and 5 yolks were pooled together by making 3 poolings per group. Then pooled yolks were analyzed by HPLC, GC, cholesterol assay and DPPH assay, etc. On day 0, 14 and 28, blood sampling was conducted. The blood corpuscle analysis and serum analysis was conducted separately.

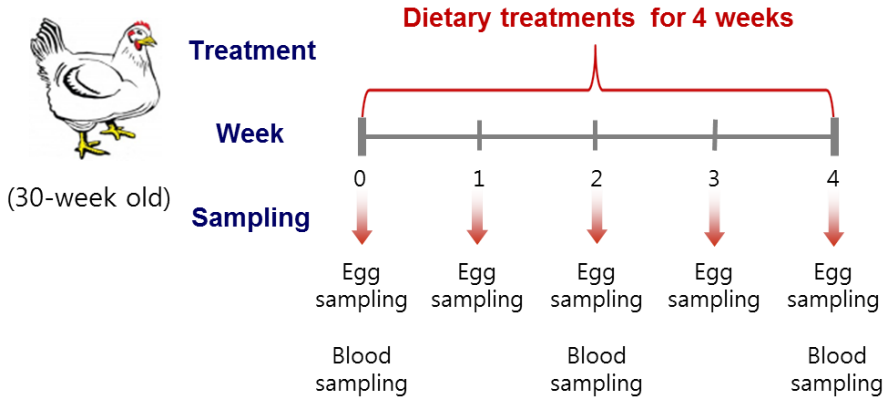


Figure 11. Summary of the experimental plan (*in vivo* assay - 2)

14) Yolk cholesterol analysis

Yolk total lipids were extracted by the method of Folch *et al*.(Folch & Lebaron, 1956). After adding Folch solution (chloroform : methanol = 2:1) at 67mg/1ml, yolk fragments were homogenized by homogenizer in 1.5ml tubes, centrifuged and supernatant was collected. The lipid extract was diluted with the working buffer and analyzed by cholesterol assay kit (Red cholesterol assay kit, Amplex) according to the suggested protocol in the kit.

15) Yolk total antioxidant activity analysis

The total antioxidant activity of yolk was analyzed by DPPH scavenging assay. Lipid of each yolk sample was extracted by Folch method as the same as cholesterol assay. The lipid extract diluted with methanol to be 1/5. 50ul of diluted lipid extract sample were loaded into 96-well plate, and 100ul of 0.2mM DPPH in methanol was added to each well. Free methanol with DPPH solution was used as a positive control, and free methanol without DPPH solution was used as a negative control. After 37 ℃ incubation for 30 min, the absorbance at 400nm was detected by microplate reader.

16) Statistical analysis

Every data was expressed with the mean ± standard deviation. Statistical significance calculated using unpaired t-tests were presented when p value is under 0.05.

3. Results and discussion

1) Particle size and morphology

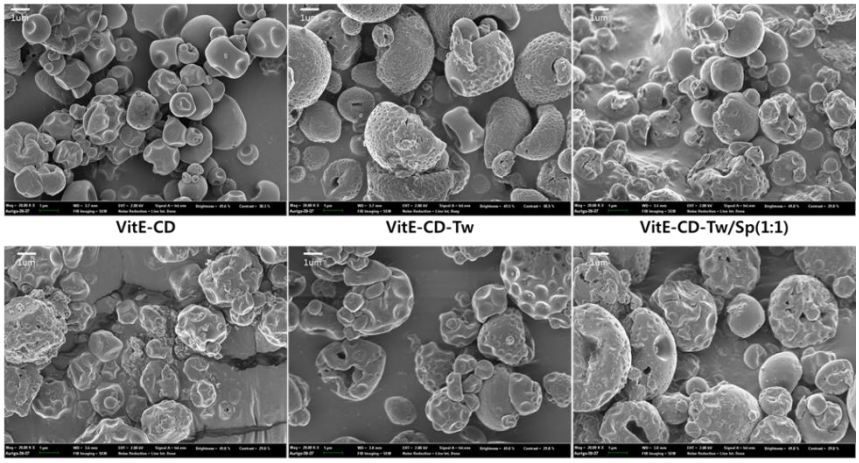
The α-tocopherol-β-CD inclusion complexes made by spray drying were prepared as 6 different types depending on the ratios of surfactants (table 3).

Table 3. The types of α-tocopherol-β-CD inclusion complexes depending on surfactant ratios

No.	Name(VitE-CD-Tw:Sp(ratio))	HLB value
1	VitE-CD	
2	VitE-CD-Tw	15
3	VitE-CD-Tw/Sp(1:1)	9.65
4	VitE-CD-Tw/Sp(1:2)	7.8
5	VitE-CD-Tw/Sp(1:4)	6.44
6	VitE-CD-Sp	4.3

After sample preparation, morphology and size of the particles were observed by FE-SEM. The surface morphologies of the particles were irregular. The sizes of the particles ranged from 1 to 10 um, whereas the sizes of the samples using surfactants increased. Particle size of samples dispersed in water was also measured by DLS. The resultd of DLS analysis showed that the sizes of the samples ranged from 90 to 250nm, whereas the sizes of the samples with mixed surfactants incresed. The difference of sizes between SEM and DLS comes from aggregation of samples during sample preparation of SEM observation..

(a)



(b)

No.	Formulation	Average Diameter (nm)
1	VitE-CD	91.6
2	VitE-CD-Tw	92.83
3	VitE-CD-Tw/Sp(1:1)	114.07
4	VitE-CD-Tw/Sp(1:2)	250.97
5	VitE-CD-Tw/Sp(1:4)	135.67
6	VitE-CD-Sp	99.1

Figure 12. Size and morphology of the spray-dried samples. (a) FE-SEM images of the samples. (b) Particle sizes of samples measured by DLS.

2) Encapsulation efficiency

To analyze encapsulation efficiency, α-tocopherol was released from each samples and analyzed by HPLC. Encapsulation efficiency was calculated as below : EE(Encapsulation efficiency) = (α-tocopherol content in the final sample) / (α-tocopherol content before spary-drying) x 100. The encapsulation efficiency was strongly affected by existence of surfactants. While the encapsulation efficiency of VitE-CD was about 18%, VitE-CD-Tw and VitE-CD-Tw/Sp (1:1) showed about 60% encapsulation efficiency, and VitT-CD-

Tw/Sp (1:2) and VitE-CD-Tw/Sp (1:4) showed about 70% encapsulation efficiency, which is more than 3 times higher than VitE-CD. It may be regarded that addition of surfactants during stirring-step improved the total dispersion rate of α-tocopherol and β-CD and resulted in enhanced encapsulation efficiency of samples with surfactants.

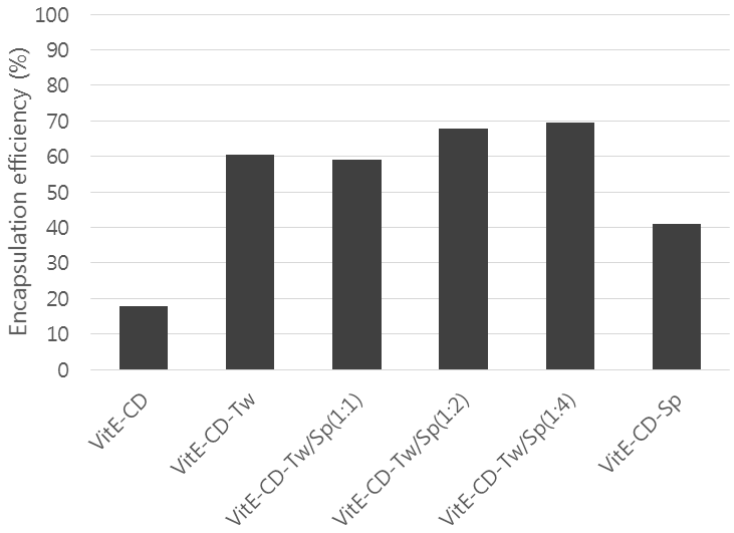


Figure 13. α-Tocopherol encapsulation efficiency. α-Tocopherol was released from each sample and quantified by HPLC. Encapsulation efficiency was calculated as the percent of the level of released α-tocopherol to the level of formulated α-tocopherol before drying.

3) *In vitro* release assay

Release assay was conducted using SGF (PBS, pH 1.2) and SIF (PBS, pH 7.4) to imitate the gastric environment and small intestinal one, respectively. AtSGF release assay, VitE-CD showed the lowest release rate which is under 10% for 4 h. VitE-CD-Tw/Sp (1:1) and VitE-CD-Tw/Sp (1:2) showed similar

release rate in SGF with about 20%. VitE-CD-Tw/Sp (1:4) showed the highest release rate in SGF with about 40%. At SIF release assay, VitE-CD and VitE-CD-Tw/Sp (1:1) showed relatively low release rate, while VitE-CD-Tw/Sp (1:2) and VitE-CD-Tw/Sp (1:4) showed relatively high release rate. As results, VitE-CD-Tw/Sp (1:2) which showed low release rate at SGF and high release rate at SIF with a controlled release.

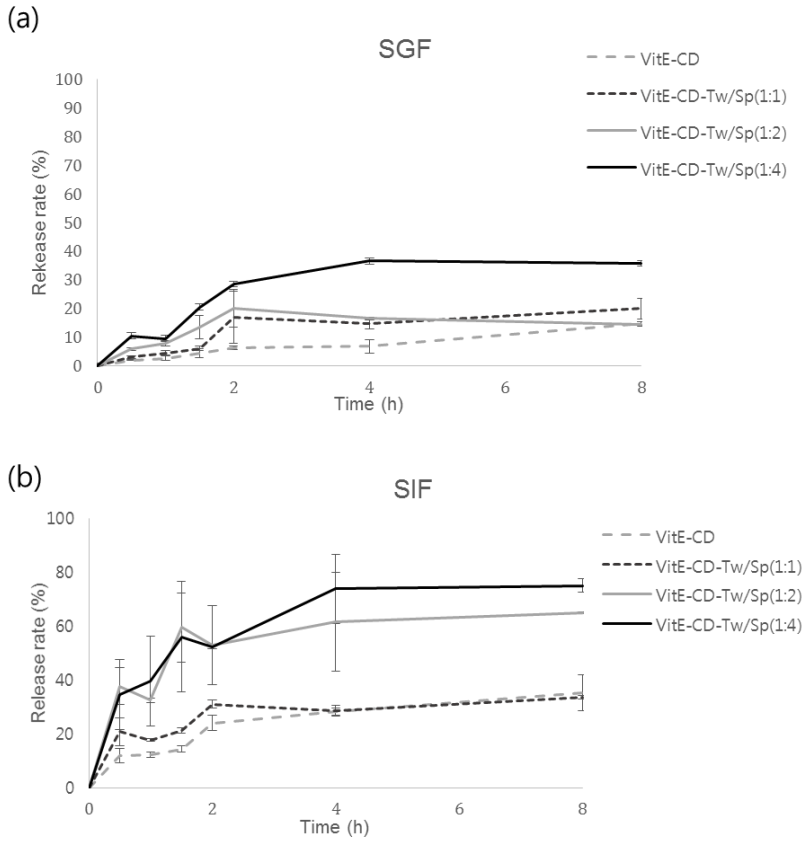


Figure 14. *In vitro* release assay. Each sample was loaded in SGF (pH1.2 PBS buffer) (a) or SIF (pH7.4 PBS buffer)(b) and incubated in 37℃ shaking incubator. At 0.5, 1, 1.5, 2, 4 and 8h time points, an aliquot of sample was removed and the amount of α-tocopherol in the supernatant was analyzed by HPLC.

4) Heat stability assay

Heat stability assay was conducted to confirm whether the encapsulated α-tocopherol is stable under high temperature which imitated the processing step of pellet feed or long-term storage high temperature in the summer. Physical mixtures of α-tocopherol with various types of powders (β-CD, dextrose, sucrose, maltose, starch and flaxseed) are used as control. Controls showed different heat stability between the type of powders, but are generally shown to stable until 80 ℃ incubation for 30 min except the mixture of α-tocopherol and sucrose. Every VitE-CD sample showed at least 80% of heat stability.

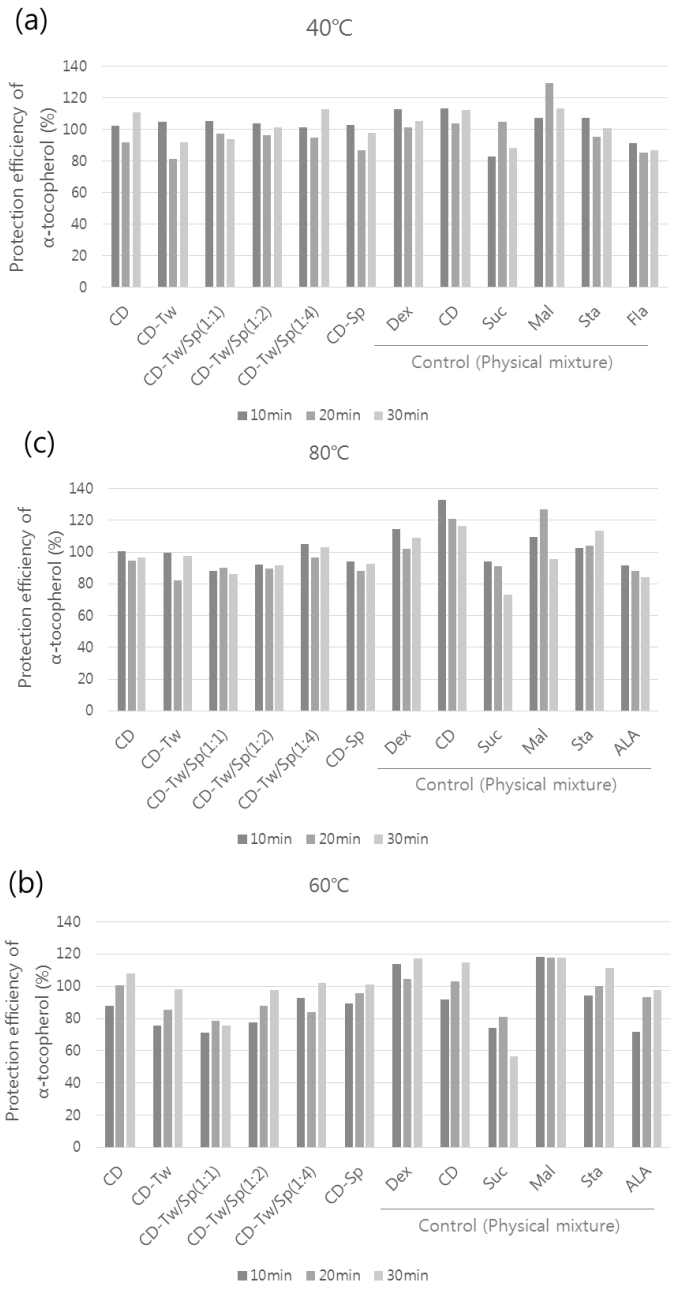


Figure 15. Heat stability assay. Each sample was incubated under 40℃ (a), 60℃ (b) and 80℃ (c) for 10, 20 and 30 min. Physical mixtures of α-tocopherol and other powder-type materials were used as controls. The remaining percentage of α-tocopherol after incubation was calculated after HPLC analysis.

5) UV stability assay

As α-tocopherol is most unstable under UV light, UV stability assay was conducted to determine the protection efficiency of β-CD encapsulation. Samples were incubated under germicidal UV lamp in clean bench for 24, 48 and 72 h. Physical mixtures of α-tocopherol and various powders were used as controls. Except VitE-CD samples, all VitE-CD with surfactant samples showed ability to protect the α-tocopherol included in the particles compared to controls made as physical mixture. Among controls, the protection efficiency was dependent on the type of powder mixed with α-tocopherol.

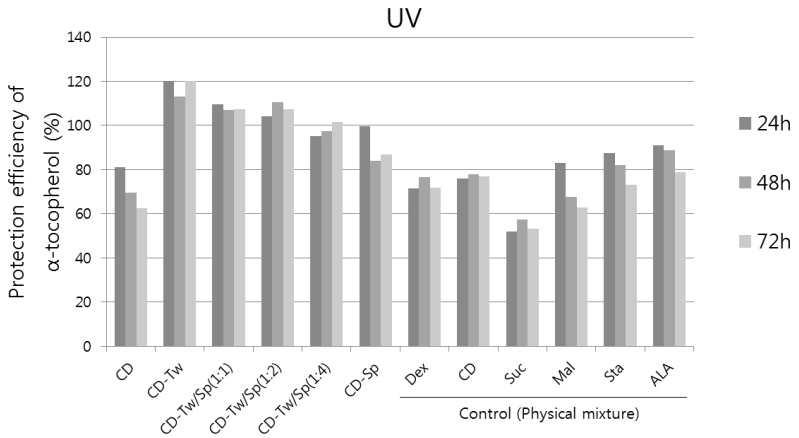


Figure 16. UV stability assay. Each sample was incubated under UV light from germicidal lamp located 68.3cm above the samples for 10, 20 and 30min. Physical mixtures of α-tocopherol and other powder-type materials were used as controls. The remaining percentage of α-tocopherol after incubation was calculated by HPLC analysis.

6) Caco-2 permeability assay

Caco-2 permeability assay was conducted to predict whether encapsulated α-tocopherol had improved epithelial absorption rate. First, formation of tight junction was confirmed by TEER index and then, FITC-dextran was treated to confirm the permeability between cells. Finally, tight junction specific protein ZO-1 was stained by immunostaining. The results indicated that Caco-2 seeded and grown in insert had enough tight junction formation in starting Caco-2 permeability assay.

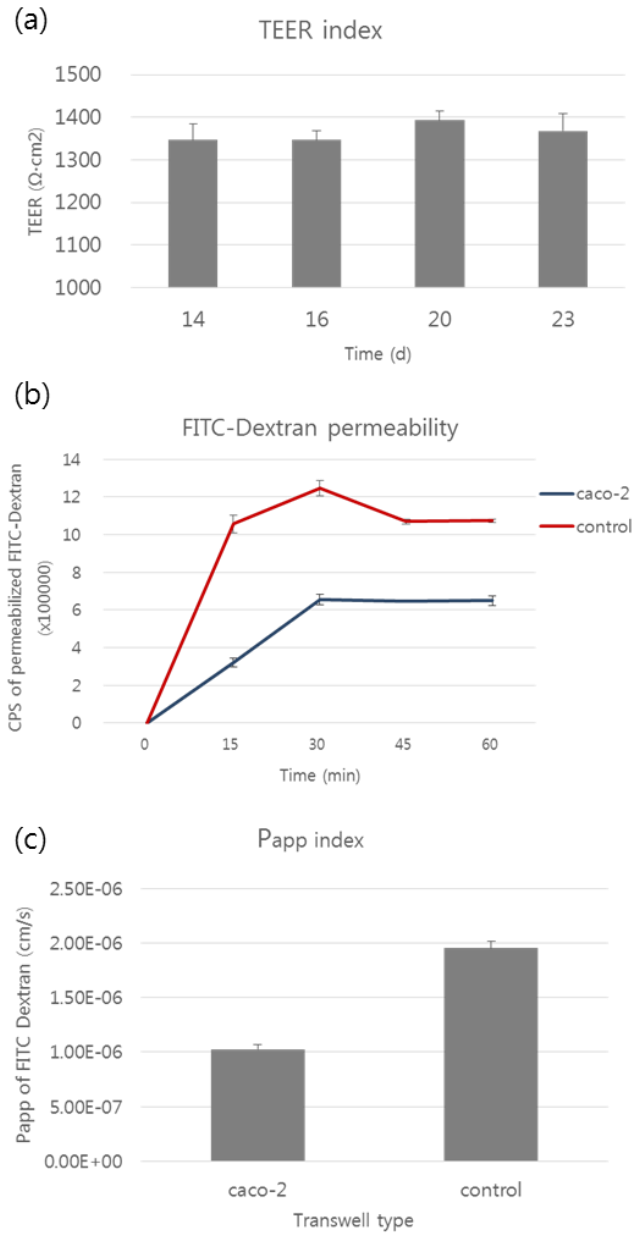


Figure 17. Confirmation of tight junction. Tight junction formation was confirmed by measuring TEER index (a), FITC-dextran permeability (b), and Papp index (C).

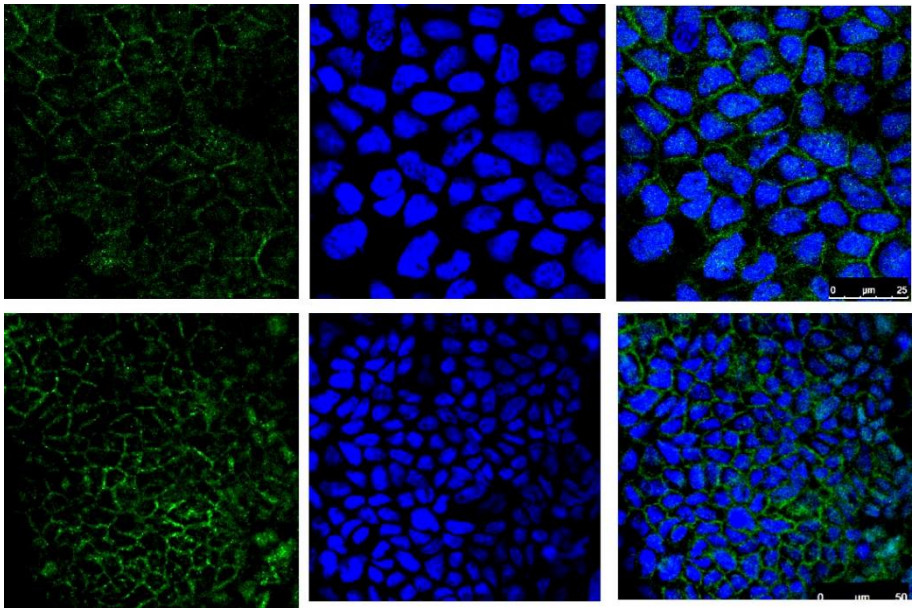


Figure 18. Immunofluorescence assay for ZO-1. Tight junctions were stained by anti-ZO-1 antibody and Alexa488-conjugated secondary antibody (green signal), and nuclei were stained by DAPI (blue signal).

Free VitE and VitE-CD mixture showed similar permeability whereas β-CD itself did not affect permeability of the Caco-2 layer. Among encapsulated α-tocopherol samples, VitE-CD-Tw/Sp (1:2) and VitE-CD-Tw/Sp (1:4) showed 2-fold higher permeability compared to free VitE treated group, while VitE-CD-Tw/Sp (1:1) showed no significant improvement in permeability.

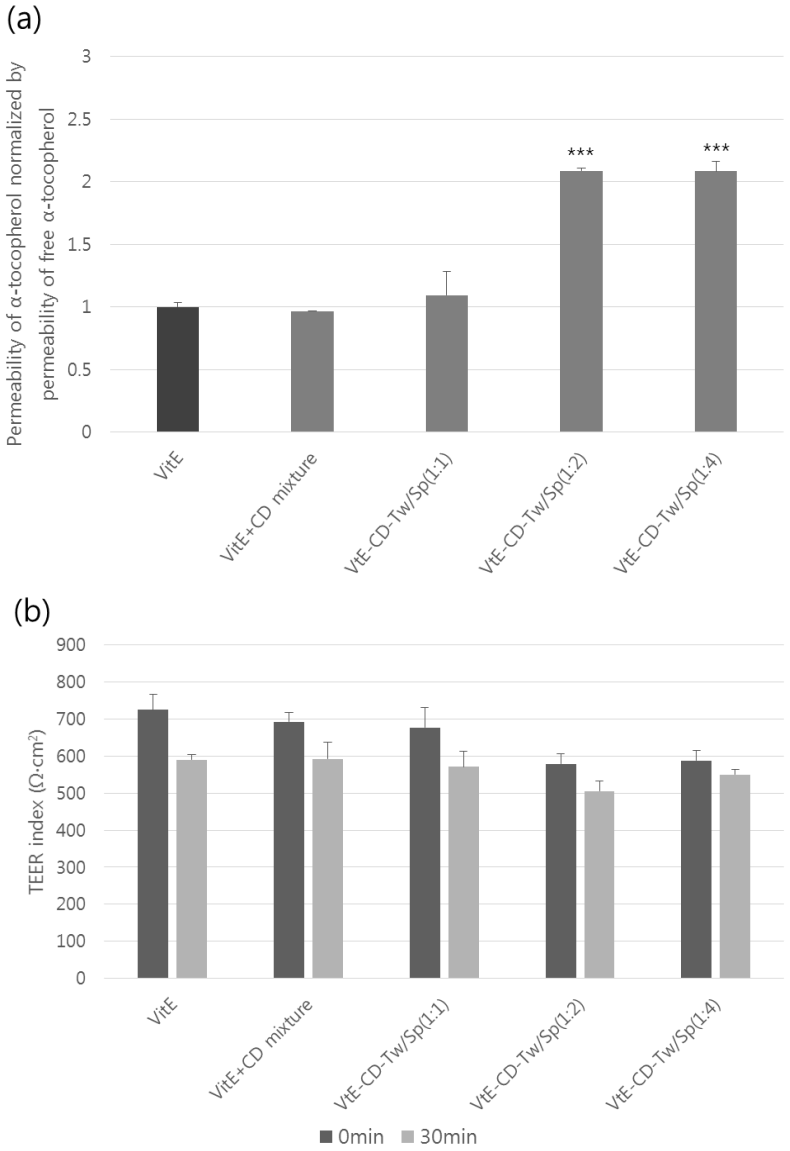


Figure 19. Caco-2 permeability assay. 5mM of free vitamin E or various VitE-CD samples were loaded on the Caco-2 trans-well inserts for 30 min and the amount of permeabilized α-tocopherol was analyzed (a). TEER index before and after the incubation was calculated to confirm maintenance of tight junction during incubation (b). (Error bar represents standard deviation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, one-way ANOVA).

7) *In vivo* assay of ALA-supplement (*in vivo* assay – 1)

In vivo assay was conducted with 315 hens for 8 weeks to determine the effect of supplementing flaxseed oil and processed flaxseed which is the major natural source of ALA on fatty acid composition on egg yolks. Flaxseed oil is 55% ALA source and processed flaxseed contains 17.4% ALA. However, the price of processed flaxseed oil is much cheaper than the price of flaxseed oil. So through this *in vivo* assay, the possibility of replacing flaxseed oil by processed flaxseed and the ω-6/ω-3 fatty acid ratio under 4 can be achieved by and minimum ALA content in feed.

Table 4. Experimental test groups for *in vivo* assay -1

(g/kg)

Test groups	ALA content	Flaxseed oil/ /Processed flaxseed	Surfactant content	Basic oil content	Coloring Agent content	chickens
NC	-	-	-	10	-	45
PC	2.75	5/0	-	5	-	45
T1	2.75	0/15.8	-	5	-	45
T2	2.75	2.5/7.9	-	5	-	45
T3	1.38	2.5/0	-	7.5	0.4	45
T4	1.38	2.5/0	1.25	7.5	0.4	45
T5	1.38	2.5/0	2.5	7.5	0.4	45

As results, all groups supplemented with ALA showed increased ALA, EPA and DHA contents and improved ω-6/ω-3 ratio. Compared with PC, T1 and T2, processed flaxseed can replace flaxseed oil in terms of essential ω-3 fatty acid content and ω-6/ω-3 ratio. However, groups supplemented with lower amount of ALA (T3, T4 and T5) showed lower ω-3 fatty acid content and more ω-6/ω-3 ratio than 4. Conclusively, to produce eggs in which ALA, DHA and EPA is enhanced and ω-6/ω-3 ratio is under 4, at least 2.75g/kg ALA has to be

supplemented in the poultry feed.

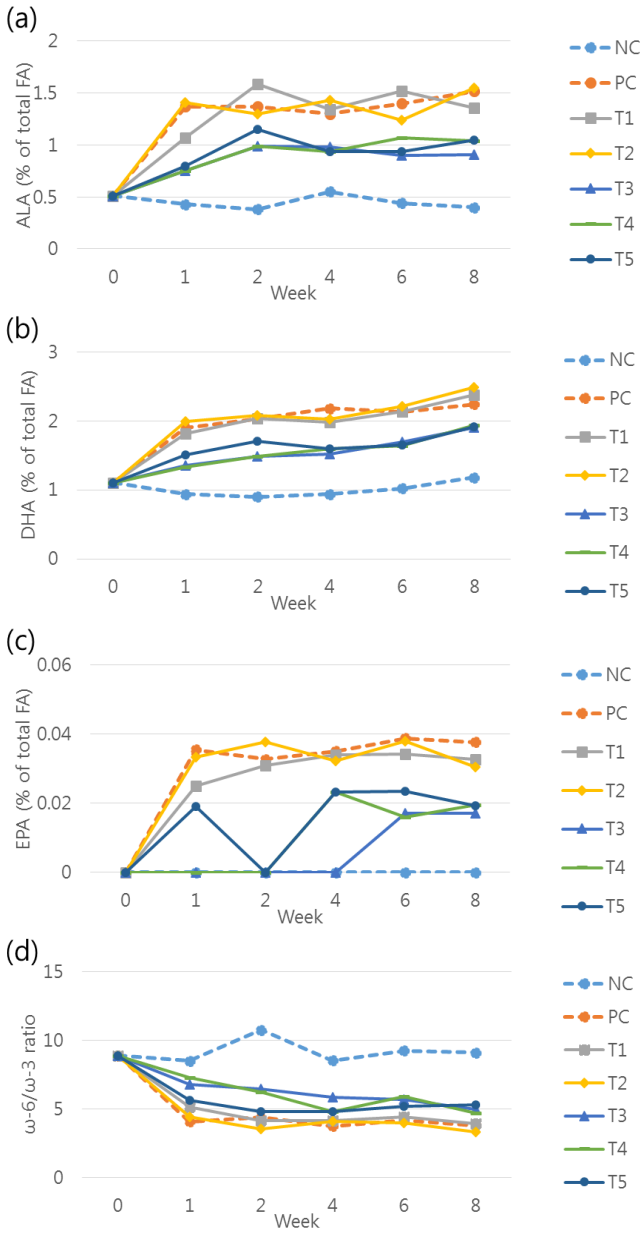


Figure 20. Fatty acid composition of the yolks from each experimental group for 8 weeks. During 8 weeks of experiment, yolks from each test group were collected and the ALA content (a), DHA content (b), EPA content (c) and ω-6/ω-3 fatty acids ratio were analyzed by GC.

8) *In vivo* assay of ALA and encapsulated vitamin E-supplement (*in vivo* assay – 2)

In vivo assay – 2 was conducted to determine whether encapsulated α-tocopherol can enhance accumulation of α-tocopherol in egg yolks and whether supplementation of ALA/α-tocopherol mixture can affect the accumulation of ALA and α-tocopherol together. Experimental test groups are shown in Table 5. ALA content in feed was composed of processed flaxseed 20g and flaxseed oil 2g, and the ALA-supplemented feed was used as basic feed. The α-tocopherol content in basic feed was 20mg/kg, and additional α-tocopherol was added according to the test group composition.

Table 5. Experimental test groups for *in vivo* assay – 2

Test groups	ALA content (g/kg)	α-tocopherol (mg/kg)	β-CD (mg/kg)	Surfactant content (g/kg)	chickens
Control	3.25	20	-	-	10
+VitE(1X)	3.25	120	-	-	10
+VitE(2X)	3.25	220	-	-	10
+VitE+CD mix(1X)	3.25	120	439	-	10
+VitE+CD mix(2X)	3.25	220	878	-	10
+VitE-CD-Sur(1X)	3.25	120	439	32	10
+VitE-CD-Sur(2X)	3.25	220	878	64	10
Total					70

The amount of α-tocopherol in egg yolks after 4 weeks of experimental period was firstly measured. From the first week, each group showed different α-tocopherol content with maintaining by week 2. Among VitE-1X groups, VitE-CD-Sur group showed about 1.2-fold higher α-tocopherol content on

week 2. Among VitE-2X groups, VitE-CD-Sur group showed about 1.5-fold higher α-tocopherol content on week 2. Therefore, it was confirmed that encapsulation in α-tocopherol with β-CD and surfactant enhanced accumulation of α-tocopherol in the yolks although VitE+CD mixture group showed little differences compared to free VitE-group indicating that physical mixture of α-tocopherol and β-CD cannot enhance accumulation of α-tocopherol in yolks. VitE (2X) group showed 1.6-fold higher accumulation of α-tocopherol than VitE (1X) group, while VitE-CD-Sur (2X) group showed more than 2-fold higher accumulation than VitE-CD-Sur (1X) group. Thus, it is clear that the effect of encapsulation becomes strong as α-tocopherol concentration increases (Figure 21).

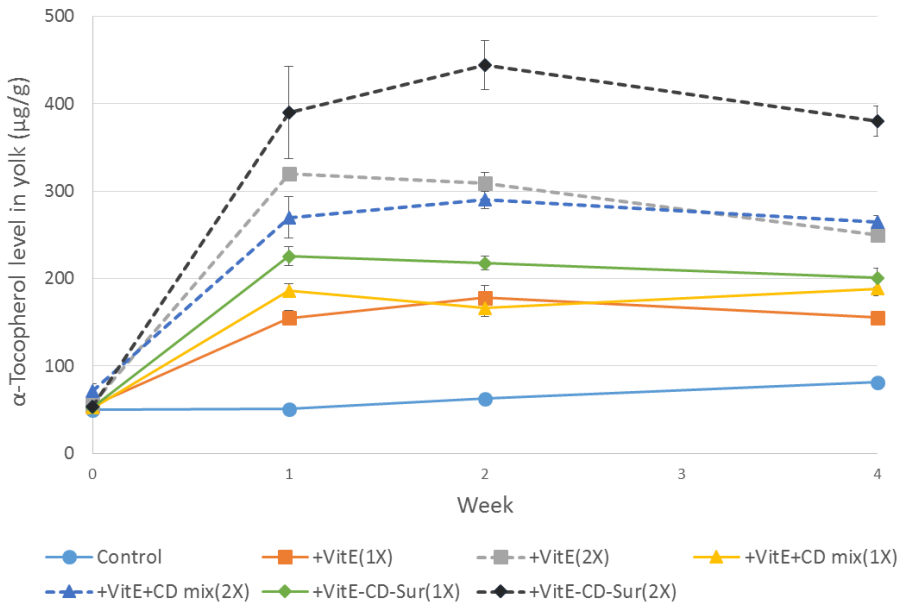


Figure 21. The amount of α-tocopherol in egg yolk

To determine the total antioxidant activity of the yolks supplemented with experimental feed, DPPH free radical scavenging assay was conducted.

Interestingly, groups supplemented with VitE-CD-Sur only showed significant enhancement of total antioxidant activity. As VitE-CD-Sur (2X) showed higher radical scavenging activity than VitE-CD-Sur (1X), this effect is strongly related to the amount of α-tocopherol in the yolk although its tendency was not exactly matched to the result of α-tocopherol accumulation in yolk. Therefore, there is probably some interaction between α-tocopherol and other antioxidants in the body, and between free α-tocopherol and encapsulated α-tocopherol had some differences in terms of this interactions in the body.

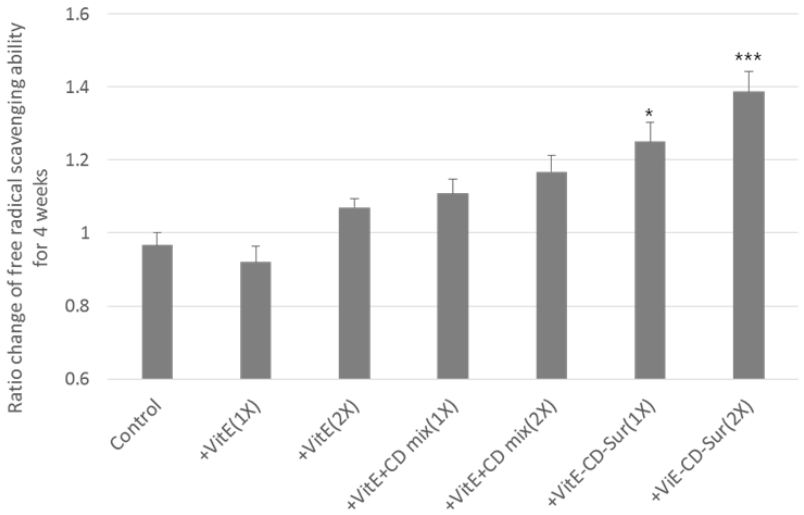


Figure 22. DPPH free radical scavenging assay. 0.2mM of DPPH free radical was added to the diluted yolk extracts and the scavenging activity was analyzed. (Error bar represents standard deviation, *p < 0.05, **p < 0.01, ***p < 0.005, one-way ANOVA).

Next, the cholesterol level of yolk was analyzed. As expected, supplementation of α-tocopherol or β-CD did not show any significant differences compared with control group.

Serum cholesterol level and serum glutamic-oxaloacetic transaminase (GOT) index were analyzed. There was little difference of serum cholesterol level among the test groups. GOT level was analyzed because excessive supplementation of lipophilic vitamins damaged the liver (Hathcock *et al*., 1990; Rothman *et al*., 1995). During the experimental period, there was little difference in serum GOT level in between test groups, indicating that supplemented α-tocopherol or β-CD did not induce liver damage or liver burdening at least for 4 weeks.

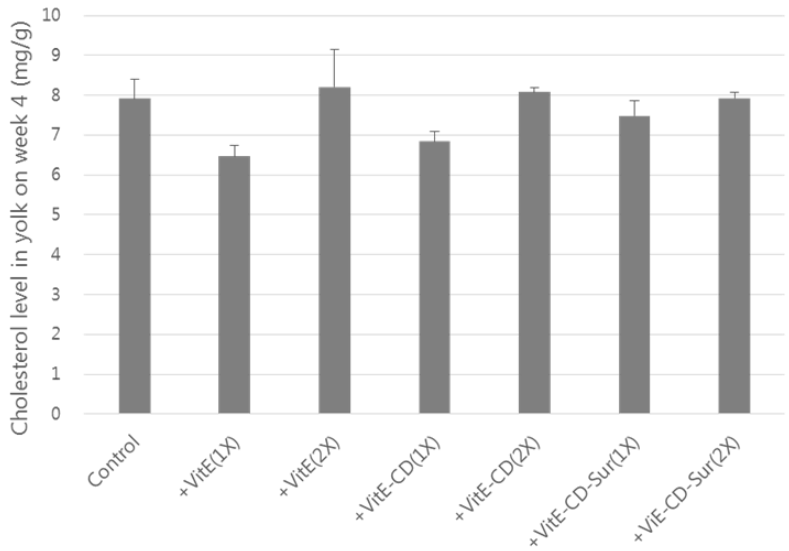


Figure 23. The amount of cholesterol in egg yolks (Error bar represents standard deviation, *p < 0.05, **p < 0.01, ***p < 0.005, one-way ANOVA).

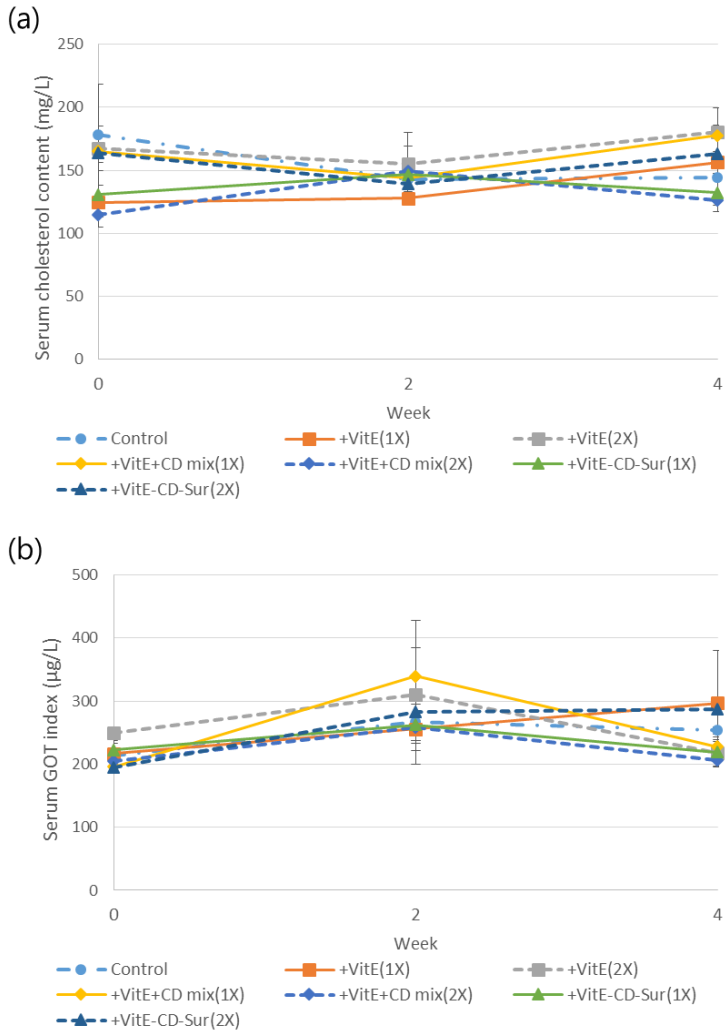


Figure 24. Serum cholesterol level and GOT index. During 4 weeks of *in vivo* assay, serum was extracted on week 0, week 2 and week 4. (a) Serum cholesterol level were investigated by serum biochemical analysis and (b) serum GOT index. (Error bar represents standard deviation).

During the 4 weeks of experimental period, laying performance was recorded to confirm whether the experimental feed had any effects on laying performance of the hens. Among all the test groups, egg production rate was

from 70 % to 100 %, without not much change of egg production among test groups. Another parameter of laying performance, the average weight of eggs, was also quite similar among the test groups. Thus, supplementing of the experimental feed did not affect general lying performances of the hens during 4 weeks.

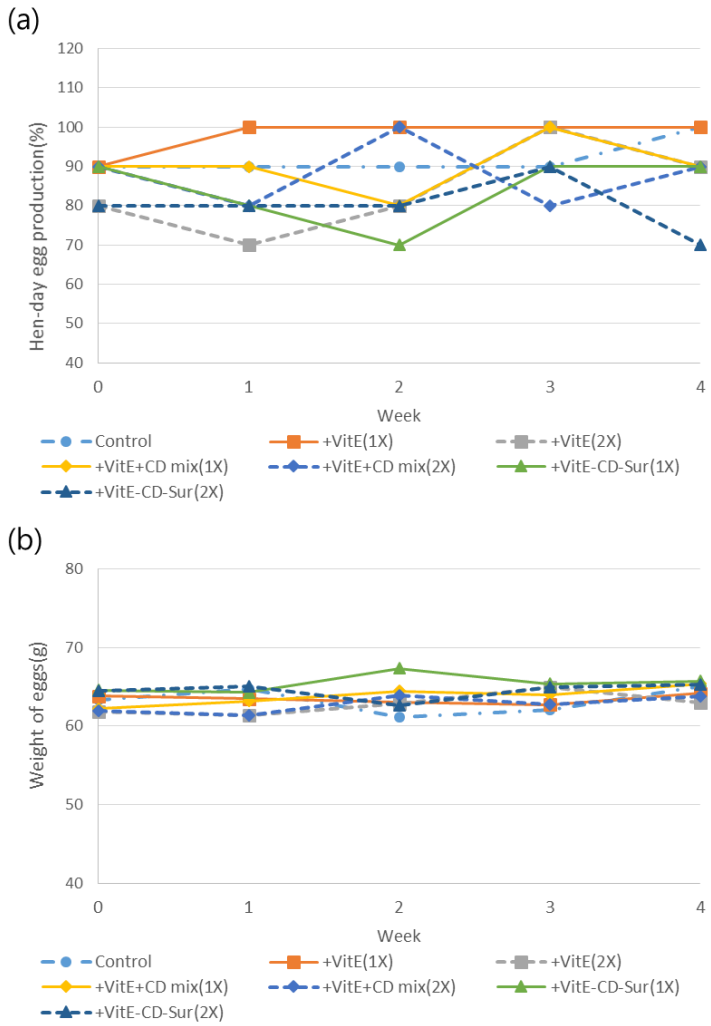


Figure 25. Laying performance of the hens supplemented with various types of α-tocopherol. (a) The number of eggs layed per group (n=10) per day. (b) The average weight of eggs of each group.

4. Summary

As α-tocopherol is unstable and hydrophobic functional material, efficiency of its supplementation as a feed supplement can be improved by overcoming stability and solubility problem. In this study, α-tocopherol as the most bioactive form of vitamin E in the body were encapsulated using β-CD and surfactants. The encapsulated α-tocopherol showed improved encapsulation efficiency, controlled release, stability and epithelial permeability. By using various ratios of two surfactants, HLB value of surfactants can be regulated, and this regulation made it possible to optimize the best condition for final selection. The final purpose of this study was to produce functional eggs which has improved ω-6/ ω-3 ratio of fatty acids and enhanced content of α-tocopherol in the yolks. Through *in vivo* assay, it was suggested that supplementing encapsulated α-tocopherol enhanced α-tocopherol content in yolk dramatically from the first week of experiment, and the amount was maintained after third week. During supplementing the experimental feeds, there were no severe influence such as liver toxicity, yolk and serum cholesterol level and laying performances. In conclusion, encapsulation of α-tocopherol in β-CD enhanced stability *in vitro* and bioavailability of α-tocopherol *in vivo*.

Chapter 2. Determination of synergistic effect of ALA and betaine on myoblast proliferation and differentiation

1. Introduction

Muscle development is one of the main interests of livestock industry because it is directly related to the productivity and economic efficiency. In adults, muscle cells called satellite cells are in quiescent state, and starts to proliferate and differentiate upon external or internal signaling. Muscle development can be achieved by both hypertrophy which is the result of enhanced protein amount in muscle fiber and hyperplasia which is induced by adding more nuclei to muscle fiber. If both hypertrophy and hyperplasia of muscle fiber can be induced, muscle growth will be achieved more effectively.

There are many growth-enhancing agents used as feed additives. But most of them are not exactly confirmed about its growth promoting efficiency, or are not clear how those materials influence growth performance of animals. In this study, we tried to figure out whether ALA, one of essential ω-3 poly unsaturated fatty acid, and betaine, methyl-donating agent have synergistic effect and to suggest clear evidences of the mechanism regarding the effects *in vitro*. As there have been some studies revealed the effect of poly unsaturated fatty acids (PUFA) and betaine on muscle development *in vitro* and *in vivo*, we hypothesized that ALA and PUFA might have synergistic effect on muscle development. The study was proceeded to two separate assay, myoblast proliferation assay and myoblast differentiation assay. By confirming the effect

of ALA and betaine on each step of muscle development, we can suggest some evidences of effectiveness of supplementing ALA and betaine together.

2. Materials and methods

1) Materials

α-Linolenic acid (ALA), betaine and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, Mo, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS) and penicillin/streptomycin were purchased from Hyclone (Utah, USA). Rabbit-anti-IGF-1R-antibody and goat-anti-rabbit-FC regions-antibody conjugated with Alexa-488 were purchased from Santa Cruz Biotechnology (CA, USA). Primers were synthesized from Bioneer (Daejeon, Korea). Trizol was from Invitrogen (CA, USA). SyBR green was from Enzynomics(Daejeon, Korea) and Quantitect Reverse Transcription kit was from QIAGEN (Hilden, Germany).

2) C2C12 proliferation assay

C2C12 cells (mouse myoblast cell line; ATCC number CRL-1772) were seeded into 6-well plate with very low density, which was 0.03×10^6 cells/well in growth medium made of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were grown for 48h to be about 30~40% confluent. Then, the medium of each well was changed to DMEM with 5% FBS and 1% penicillin/streptomycin containing different concentration of ALA or

betaine. After further incubation for 24h in 37℃, cell counting and quantitative RT-PCR were conducted.

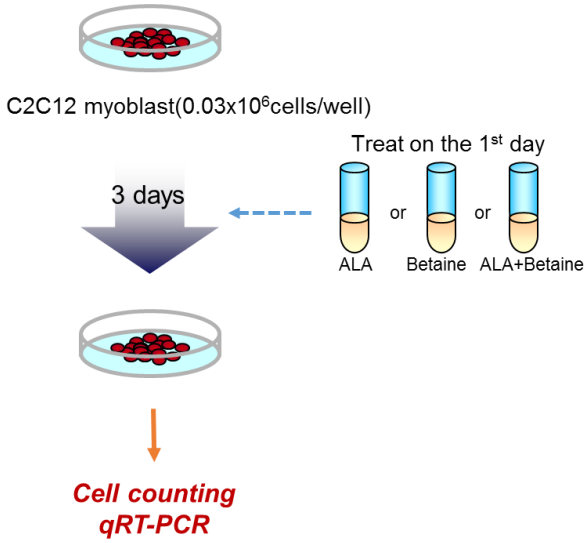


Fig 26. Schematic illustration of C2C12 proliferation assay. C2C12 myoblasts were seeded with very low density (0.03x106cells/well) in 6-well plate and grown in 10% FBS DMEM. On the 2nd day, ALA, betaine, or both ALA and betaine was added with 5% FBS DMEM and incubated for further 2 days.

3) C2C12 differentiation assay

C2C12 cells were seeded into 6-well plate with very high density, which was 0.8 x 106 cells/well in growth medium and grown for 24h, reaching at 90% confluency. Then differentiation was induced by replacing medium by differentiation medium made of DMEM with 2% HS and 1% penicillin/streptomycin. The media was changed every day for 72h, and then the media was replaced to DM containing different concentration of ALA or betaine. After further incubation for 24h in 37℃, quantitative RT-PCR and

immunofluorescence assay were conducted.

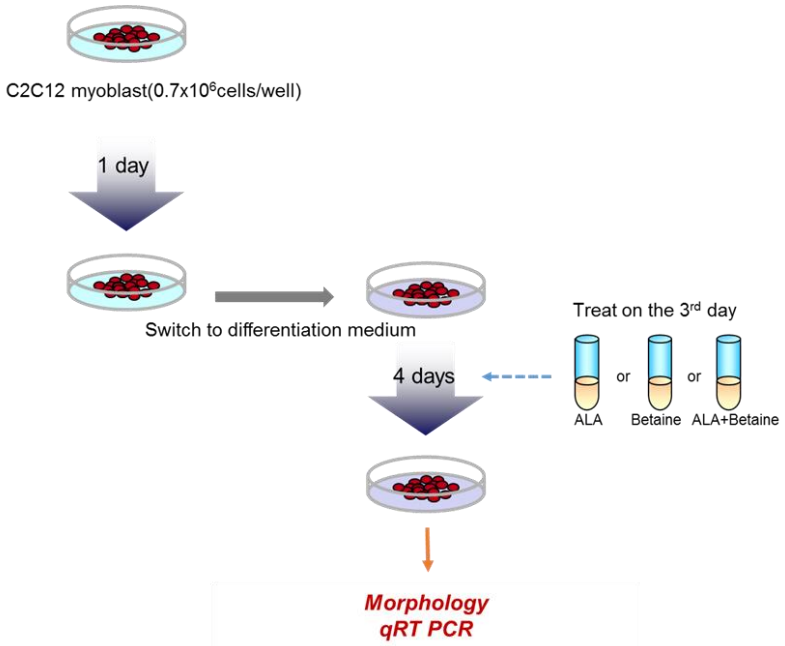


Fig 27. Schematic illustration of C2C12 differentiation assay. C2C12 myoblasts were seeded with very high density (0.7x106cells/well) in 6-well plate and grown in 10% FBS DMEM. From the 2nd day to 4th day, the media was changed to 2% HS DMEM every day. On the 5th day, ALA, betaine, or both ALA and betaine was added with 2% HS DMEM and incubated for further 1 day.

4) Cell counting

Cells grown in 6-well plate was detached and dissociated into single cells by trypsin treatment. The detached cells from each well were collected by centrifugation and loaded into 1.5ml tube by resuspending with 500ul of 10%FBS-containing DMEM. Then, the number of cells per volume was calculated by hemocytometer.

5) Quantitative Real Time-PCR

Total RNA was extracted using TriZol solution following manufacture's protocol and cDNA was synthesized using Quantitect Reverse Transcription kit (QIAGEN). Quantitative real-time PCR was conducted by using TOPrea qPCR 2X PreMIX (SYBR Green) and iCycler Real-Time Detection System (BioRad). Primers used for proliferation assay : PCNA-sense : GGAGACAGTGGAGTGGCTTT, PCNA-antisense : TGGATAAAGAAGA GGAGGCG, FGFR1-sense : GTGGACCAGGAGAGACTCCA, FGFR1-antisense : TCACAGCCACTCTCTGCACT, Myod1-sense : GTCGTAGCCA TTCTGCCG, Myod1-antisense : AGCACTACAGTGGCGACTCA, Ckdn1a-sense : ATCACCAGGATTGGACATGG, Ckdn1a-antisense : CGGTGTCAGA GTCTAGGGGA. Primers used for differentiation assay : Actn2-sense : AGGT GTGAGTTGCACCAGG, Actn2-antisense : ATGATCCAGGAGGAGGAGT G, Cdh2-sense : CGTCCACCTTGAAATCTGCT, Cdh2-antisense : AAGGA CAGCCCCTTCTCAAT, Myf6-sense : TGTCCACGQTGGQQGQQQGG, Myf6-antisense : GTGTTTCGGATCATTCCAGG, Myog-sense : GTGGGAG TTGCATTCACTGG, Myog-antisense : CTACAGGCCTTGCTCAGCTC. The mRNA expression level of each gene was normalized by GAPDH mRNA expression level.

6) Immunofluorescence

Rabbit-anti-IGF-1R antibody was used as primary antibody and Goat-anti-rabbit-Fc region antibody conjugated with Alexa-488 was used as secondary antibody. The protocol was the same as described above in chapter 1.

7) Statistical analysis

Every data is appeared with the mean ± standard deviation. Statistical significance calculated using unpaired t-tests were presented, when p value is under 0.05.

3. Results and discussion

1) Cell counting for proliferation assay

The number of cells after treatment of each concentration of ALA, betaine or both ALA and betaine was shown in Figure 28. When 20uM of ALA was treated, the number of cells increased compared to control and other concentrations, indicating that certain concentration of ALA can enhance myoblast proliferation. However, any concentration of betaine treatment did not have any effects on myoblast proliferation. When ALA and betaine were treated together, the number of the cells increased even more than ALA-treated cells, although betaine itself did not show any effect on myoblast proliferation. Therefore, it can be possible to predict that co-treatment of ALA and betaine has some synergistic effects on myoblast proliferation.

2) Expression level of genes related to myoblast proliferation

The mRNA expression level of genes related to myoblast proliferation was analyzed (Figure 29). PCNA, which function as major scaffold protein during DNA replication used as first marker of myoblast proliferation. The mRNA expression level of PCAN had no significant change among groups, in which

only 20uM ALA treated group showed increasing tendency. Fgfr1 is a gene for fibroblasts growth factor receptor (FGFR) and known to increase during proliferation of fibroblast or fibroblast-like cells such as myoblasts. Fgfr1 increased only in 20uM ALA and 20mM betaine-treated group. Cdkn1a which codes cell cycle arresting protein, p21, had no change. Myod1 which is a factor for stop of proliferation and induction of differentiation also had no change but 20mM betaine-treated group. Therefore, Co-treatment of 20uM ALA and 20mM betaine have synergistically increase mRNA expression levels related to myoblast proliferation, and had no change in genes for induction of differentiation.

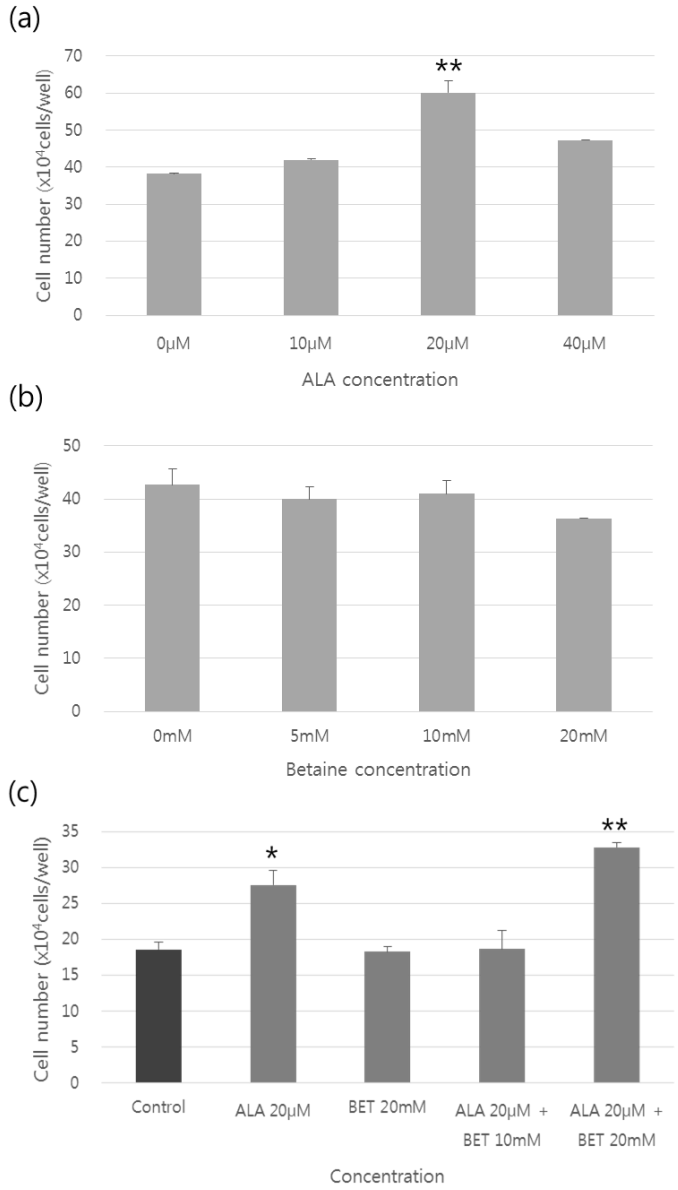


Figure 28. C2C12 cell counting assay. Cells treated with indicated concentrations of ALA (a), betaine (b) or both ALA and betaine (c) were detached by using trypsin-EDTA and resuspended into new media containing FBS. Then, the number of cells was counted by hemocytometer. (Error bar represents standard deviation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, one-way ANOVA)

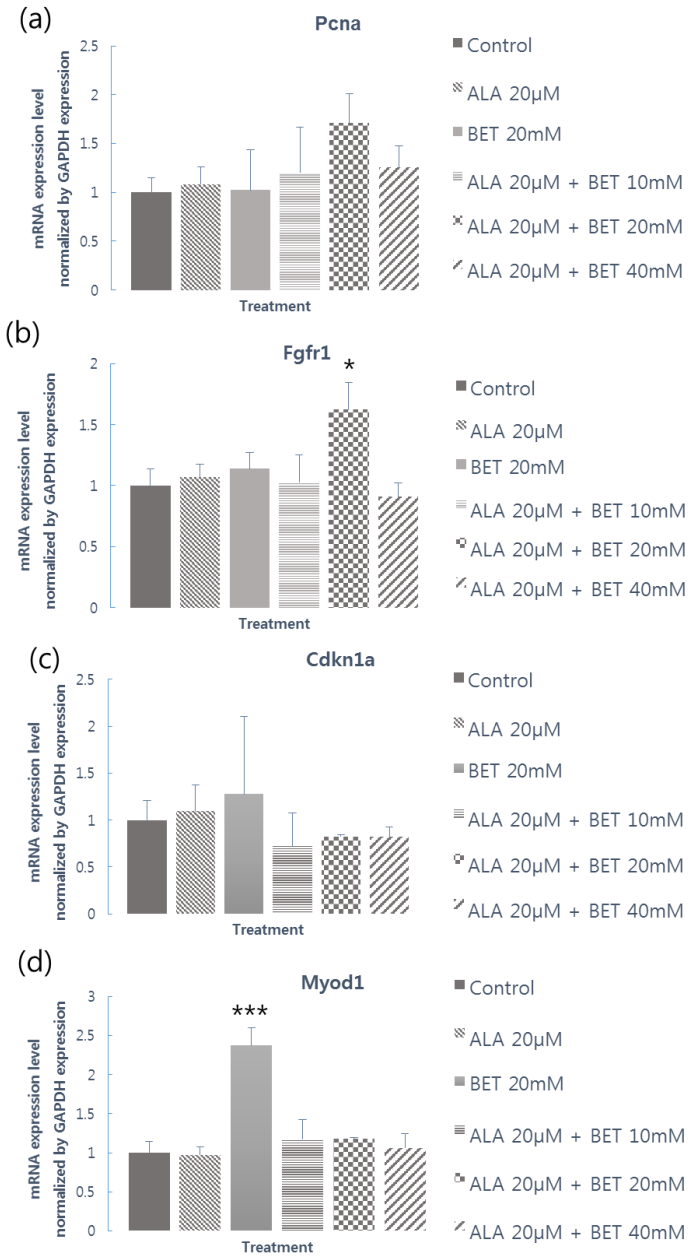


Figure 29. mRNA expression level of genes related to myoblast proliferation. mRNA of C2C12 cells treated with each indicated concentration of ALA, betaine or both ALA and betaine was extracted and the mRNA expression level of PCNA (a), Fgfr1 (b), Cdkn1a (c) and Myod1 (d) was analyzed by quantitative real-time PCR. (Error bar represents standard deviation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, one-way ANOVA)

3) Expression level of genes related to myoblast differentiation

To determine whether treatment of ALA or betaine can affect myoblast differentiation, C2C12 cells which had been induced to differentiate were treated with ALA or betaine. The mRNA expression level of five genes related to myoblast differentiation were analyzed. Cdh2 is a gene coding cadherin, which is Ca2+ dependent adhesion protein between cells. In case of muscle, as myoblasts fusion and elongate to form myotubes, this cadherin also increases. Actn is a gene coding actinin which is a protein located in z-disc in muscle fiber and mainly functions as linker between actin filament and z-disc in the muscle structure. So increase of Actn is related to formation of myotube during differentiation. Myf6 and Myog is coding transcription factors which activate the expression of myogenic inducers or factors, so usually used as markers of myoblast differentiation. The final one, Igf1r is a gene coding insulin-like growth factor receptor (IGF-1R) which is a transmembrane receptor transducing various signals for cell growth and anabolic metabolism. As IGF-1R signaling is the major signaling for muscle growth which has opposite effect from myostatin signaling, mRNA level of Igf1r was also analyzed.

First, the effect of single treatment of ALA or betaine on mRNA expression level was investigated (Figure 30). Expression level of Cdh2 did not changed after treatment of ALA or betaine, but expression level of Actn2 increased after 5mM or more concentration of betaine was treated. Expression level of Myf6, one of the transcription factors, increased after treatment of 20mM and 40mM of betaine, while Myog did not show any change between the groups.

Expression level of Igf1r was increased in the group treated 40mM of betaine. From these results, it became clear that certain concentration of betaine can enhance myoblast differentiation, while ALA does not have any effect on myoblast differentiation when treated alone.

Next, the effect of co-treatment of ALA and betaine on mRNA expression level of differentiation-related genes were confirmed (Figure 31). Expression level of Cdh2 which was not changed after single treatment of ALA or betaine increased significantly after treatment of 20mM betaine and 40uM ALA. Expression level of Actn2 in the group treated with both ALA and betaine was almost similar to the result of single treatments. Expression level of the transcription factors, Myf6 and Myog, were also increased in the groups of co-treatment linearly as the concentration of ALA increased. Expression of Igf1r also increased after co-treatment of ALA and betaine, and this effect became stronger as the concentration of ALA increased. Through these results, it was determined that co-treatment of ALA and betaine has synergistic effect on myoblast differentiation as well.

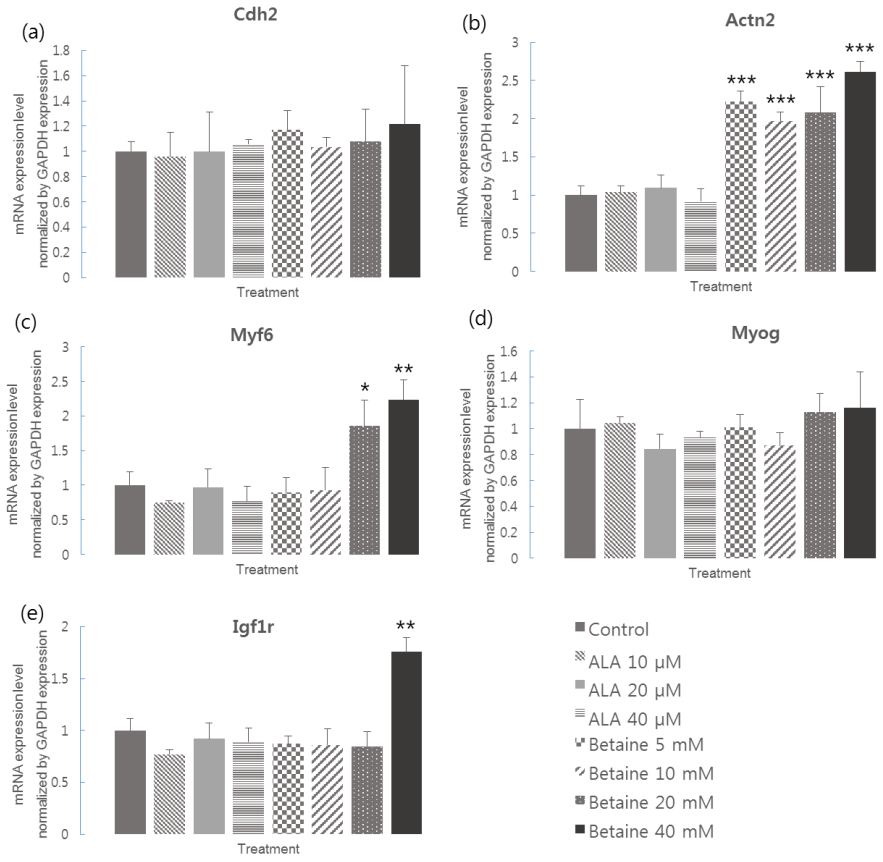


Figure 30. mRNA expression level of genes related to myoblast differentiation. mRNA of C2C12 cells treated with each indicated concentration of ALA or betaine was extracted and the mRNA expression level of Cdh2 (a), Actn2 (b), Myf6 (c), Myog (d) and Igf1r (e) was analyzed by quantitative real-time PCR. (Error bar represents standard deviation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, one-way ANOVA)

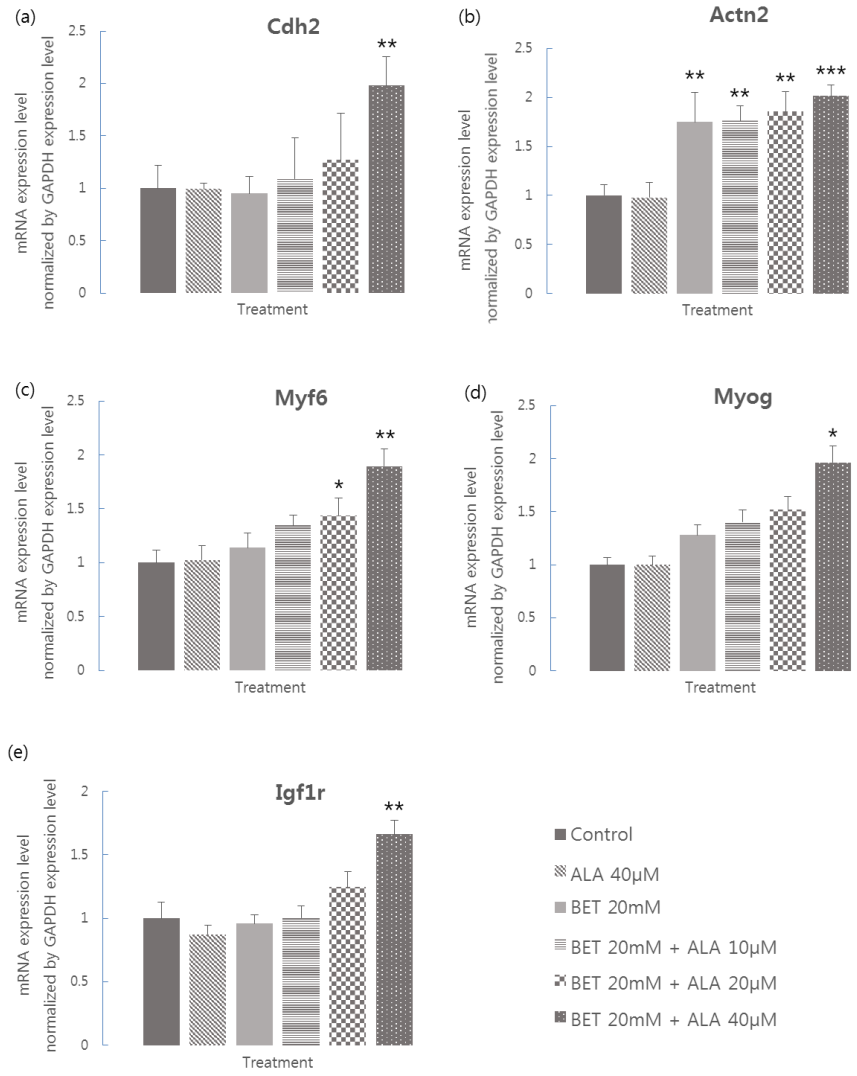


Figure 31. mRNA expression level of genes related to myoblast differentiation(co-treatment). mRNA of C2C12 cells treated with each indicated concentration of ALA, betaine or both ALA and betaine was extracted and the mRNA expression level of Cdh2 (a), Actn2 (b), Myf6 (c), Myog (d) and Igf1r (e) was analyzed by quantitative real-time PCR. (Error bar represents standard deviation, *p < 0.05, **p < 0.01, ***p < 0.005, one-way ANOVA)

Finally, we investigated immunofluorescence assay to clarify and confirm the effect of co-treatment of ALA and betaine on muscle differentiation and myogenesis. As shown in Figure 32, treatment of 40uM ALA had little effect on myogenesis. Treatment of 20mM betaine seemed to increase IGF-1R expression, but treatment of both 40uM ALA and 20mM betaine enhanced the expression of IGF-1R much more. Furthermore, The size, length and number of myotubes were also shown to be increased in co-treatment group. In conclusion, co-treatment of ALA and betaine can enhance myogenesis, especially enhancing the expression of Igf1r and and morphological change. The expression pattern of myogenesis-regulating genes were visualized by heat-map analysis and it became clear that co-treatment of ALA and betaine induced increase of all the genes regarding myogenesis (Figure 33).

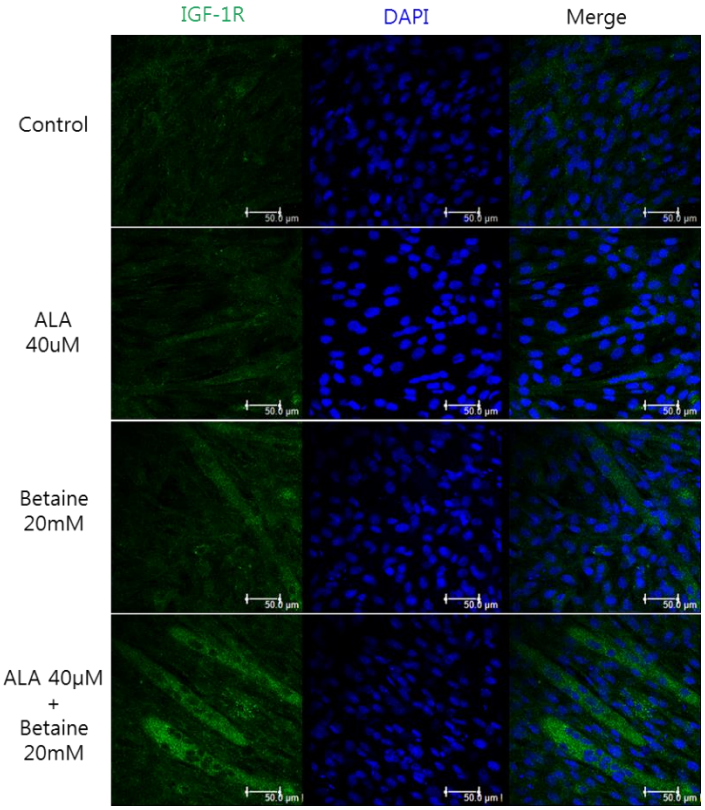


Figure 32. Immunofluorescence image of differentiated C2C12 cells. cells treated with 40uM of ALA, 20mM of betaine, or both 40uM of ALA and 20mM of betaine were stained with Alexa-488(primary Ab: anti-IGF-1R Ab, secondary Ab: anti-rabbit Ab, green signal) and DAPI(nuclei, blue signal). Scale bar: 50 μm.

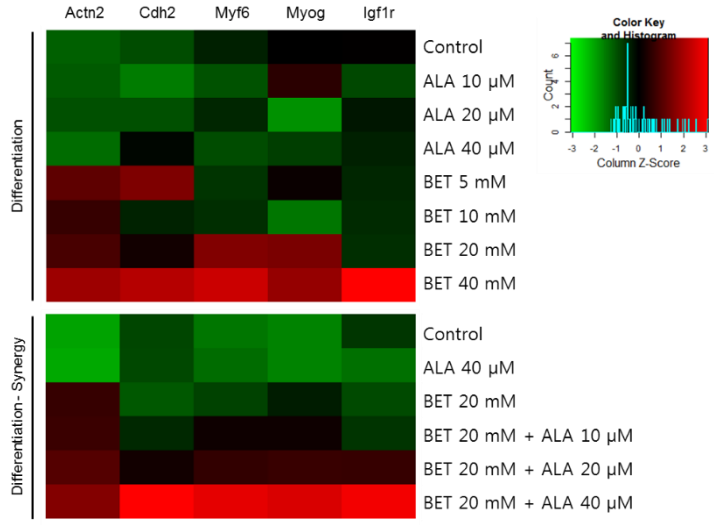


Figure 33. Heat-map analysis using genes related to myogenesis

4. Summary

Finding out muscle growth enhancer is very important in livestock industry. Here, we tried to revealed the synergistic effect of ALA and betaine on muscle development *in vitro*. Although there are some reports that some types of PUFAs such as c9, t11 CLA, LA and AA have positive effect on myoblast proliferation, there has been no evidence that ALA has effect on muscle development. Betaine has been known to induce myoblast differentiation, but there were no evidence that betaine also had effect on myoblast proliferation when treated together with other molecules. In this study, we found out that ALA alone have ability to enhance myoblast proliferation, and this effect get stronger if betaine is supplemented together. Also, in differentiation phase, supplementing both ALA and betaine together can enhance myoblast differentiation even more than sole treatment of betaine. We also found out that this effect is mainly related to IGF-1R signaling. This result is more meaningful because it became clear that ALA can act not only as essential fatty acid but also as muscle development enhancer. Furthermore, as betaine is metabolism enhancer by donating methyl-group in the body, it can enhance the effect of ALA even more in myoblast proliferation phase. In conclusion, supplementing ALA and betaine as muscle growth enhancer has possibility to have synergistic effect on muscle growth of animals, and this is concerned with IGF-1R signaling.

Conclusion and Future Prospect

In livestock industry, there are many functional feed additives which can enhance growth performance, protect animals from diseases, improve health condition of animals and accumulate functional materials in livestock products. There are many functional feed additives developed and used already, but many of them have low efficiency because of the physicochemical properties of the functional materials such as stability and hydrophobicity. Moreover, most of them are unclear about their effect and have no scientific evidences of the effect. To overcome the barriers and achieve efficient processing and delivery of functional materials to animals, we suggested β-CD encapsulation with surfactant mixture system. Moreover, to clarify the effect of ALA and betaine on growth performance and confirmation of synergistic effect between two materials, chapter 2 was planned and investigated.

There has been many studies to achieve accumulation of α-tocopherol in egg yolks by supplementing α-tocopherol alone or together with other substances (Bollengier-Lee et al. 1998; Meluzzi et al. 2000; Surai 2000). There also have been some studies of encapsulation of vitamin E in other coating materials such as liposome, proteins, nano-emulsions or chitosan to enhance stability or bioavailability, although these studies were for clinical applications or food supplements only and relatively expensive materials to be used in animal industry (Luo et al. 2011; Suntres and Shek 1994; Trombino et al. 2009; Pierucci et al. 2007; Liu and Park 2009). The encapsulation technology using β-cd in this study enhanced stability, solubility and bioavailability of a-

tocopherol and this effect was confirmed by both in vitro assay and in vivo assay. This is quite meaningful because previous studies have not tried supplementing encapsulated α-tocopherol because of cost issues but the encapsulation using β-CD and surfactants do not affect price too much and have enough effects on enhancing stability and bioavailability, suggesting possible application of encapsulation strategy to animal industry. Furthermore, this technology can be applying to other unstable and hydrophobic materials such as phytosterols.

The synergistic effect of ALA and betaine on muscle development is the first discovery, and there might be other combinations which have synergistic effect when treated together. It is not clear how ALA and betaine regulate muscle development specifically. However, ALA is a type of PUFAs which can affect plasma membrane fluidity and general signal transduction between cells, and betaine upegulates general metabolism such as synthesis of amino acids and DNA methylation through methyl donation effect. Therefore, anabolic metabolism essential for muscle development and upregulated signal transduction which affects expression of myogenesis-related genes can be suggested as the reason of their synergistic effect. Finding out synergistic effect between two distinct materials can be very effective way of improving the effect of functional feed additives, especially one of the synergistic material is relatively very expensive.

Through this two studies, we suggest more efficient and scientific way of developing functional feed additives or producing functional livestock products

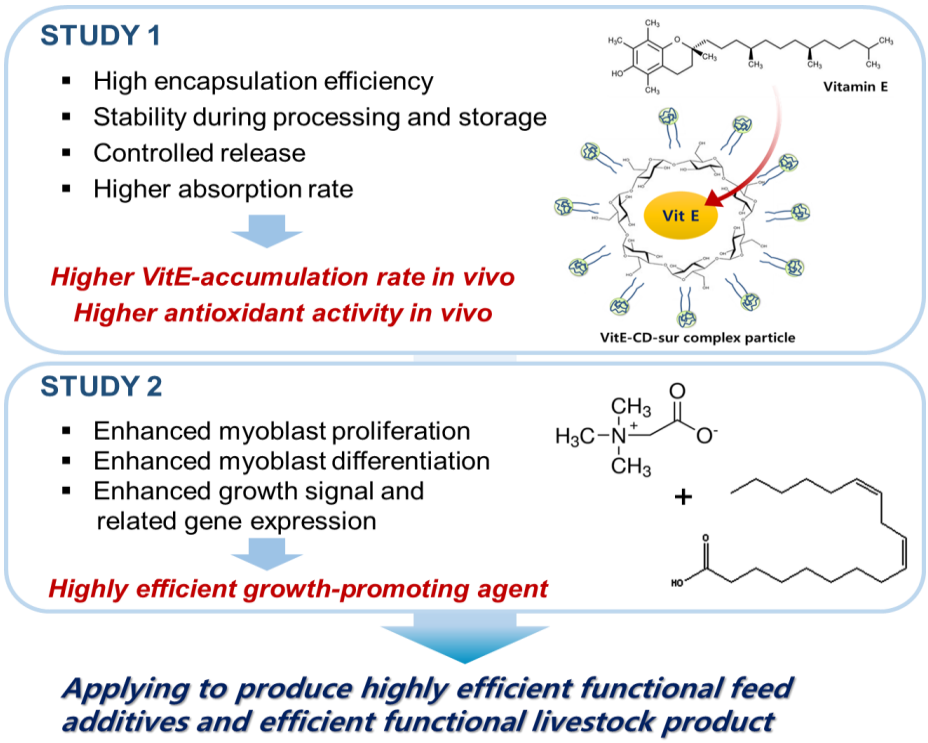


Figure 34. Scheme of the conclusion

Literature Cited

Bollengier-Lee, S., M. A. Mitchell, D. B. Utomo, P. E. Williams, and C. C. Whitehead. 1998. Influence of high dietary vitamin E supplementation on egg production and plasma characteristics in hens subjected to heat stress. Br Poult Sci 39 (1):106-112.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., & Relaix, F. 2003. The formation of skeletal muscle: from somite to limb. J Anat, 202(1): 59-68.

Butterfield, D. A., Boyd-Kimball, D., & Castegna, A. 2003. Proteomics in Alzheimer's disease: insights into potential mechanisms of neurodegeneration. J Neurochem, 86(6): 1313-1327.

Cao, F., Guo, J., & Ping, Q. 2005. The physicochemical characteristics of freeze-dried scutellarin-cyclodextrin tetracomponent complexes. Drug Dev Ind Pharm, 31(8): 747-756.

Challa, R., A. Ahuja, J. Ali, and R. K. Khar. 2005. Cyclodextrins in drug delivery: an updated review. AAPS PharmSciTech 6 (2):E329-357.

Charge, S. B., & Rudnicki, M. A. 2004. Cellular and molecular regulation of muscle regeneration. Physiol Rev, 84(1): 209-238.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production.

Anim Biotechnol, 13(1): 7-27.

Dua, K., Ramana, M. V., Sara, U. V., Himaja, M., Agrawal, A., Garg, V., & Pabreja, K. 2007. Investigation of enhancement of solubility of norfloxacin beta-cyclodextrin in presence of acidic solubilizing additives. Curr Drug Deliv, 4(1): 21-25.

Fernandez-Figares, I., Conde-Aguilera, J. A., Nieto, R., Lachica, M., & Aguilera, J. F. 2008. Synergistic effects of betaine and conjugated linoleic acid on the growth and carcass composition of growing Iberian pigs. J Anim Sci, 86(1): 102-111.

Folch, J., & Lebaron, F. N. 1956. The chemistry of the phosphoinositides. Can J Biochem Physiol, 34(2): 305-319.

Griendling, K. K., & FitzGerald, G. A. 2003. Oxidative stress and cardiovascular injury: Part II: animal and human studies. Circulation, 108(17): 2034-2040.

Griffin, W. C. 1949. Classification of surface active agents by HLB. J. Soc. Cosmet. Chem., 1: 311-326.

Han, S. N., & Meydani, S. N. 2000. Antioxidants, cytokines, and influenza infection in aged mice and elderly humans. J Infect Dis, 182 Suppl 1: S74-80.

Hathcock, J. N., Hattan, D. G., Jenkins, M. Y., McDonald, J. T., Sundaresan, P.

R., & Wilkening, V. L. 1990. Evaluation of vitamin A toxicity. Am J Clin Nutr, 52(2): 183-202.

Jones, N. C., Tyner, K. J., Nibarger, L., Stanley, H. M., Cornelison, D. D., Fedorov, Y. V., & Olwin, B. B. 2005. The p38alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. J Cell Biol, 169(1): 105-116.

Kassar-Duchossoy, L., Giacone, E., Gayraud-Morel, B., Jory, A., Gomes, D., & Tajbakhsh, S. 2005. Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev, 19(12): 1426-1431.

Koester, L. S., Guterres, S. S., Le Roch, M., Eifler-Lima, V. L., Zuanazzi, J. A., & Bassani, V. L. 2001. Ofloxacin/beta-cyclodextrin complexation. Drug Dev Ind Pharm, 27(6): 533-540.

Le Grand, F., & Rudnicki, M. A. 2007. Skeletal muscle satellite cells and adult myogenesis. Curr Opin Cell Biol, 19(6): 628-633.

Lee, J. H., Tachibana, H., Morinaga, Y., Fujimura, Y., & Yamada, K. 2009. Modulation of proliferation and differentiation of C2C12 skeletal muscle cells by fatty acids. Life Sci, 84(13-14): 415-420.

Lever, M., & Slow, S. 2010. The clinical significance of betaine, an osmolyte with a key role in methyl group metabolism. Clin Biochem, 43(9): 732-744.

Liu, N., and H. J. Park. 2009. Chitosan-coated nanoliposome as vitamin E carrier. J Microencapsul 26 (3):235-242.

Lowry, V. K., M. B. Farnell, P. J. Ferro, C. L. Swaggerty, A. Bahl, and M. H. Kogut. 2005. Purified beta-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against Salmonella enterica serovar Enteritidis. Int J Food Microbiol 98 (3):309-318.

Luo, Y., B. Zhang, M. Whent, L. L. Yu, and Q. Wang. 2011. Preparation and characterization of zein/chitosan complex for encapsulation of alpha-tocopherol, and its in vitro controlled release study. Colloids Surf B Biointerfaces 85 (2):145-152.

Manceau, M., Marcelle, C., & Gros, J. 2005. [A common somitic origin for embryonic muscle progenitors]. Med Sci (Paris), 21(11): 915-917.

McGregor, D. O., Dellow, W. J., Robson, R. A., Lever, M., George, P. M., & Chambers, S. T. 2002. Betaine supplementation decreases post-methionine hyperhomocysteinemia in chronic renal failure. Kidney Int, 61(3): 1040-1046.

Meluzzi, A., F. Sirri, G. Manfreda, N. Tallarico, and A. Franchini. 2000. Effects of dietary vitamin E on the quality of table eggs enriched with n-3 long-chain fatty acids. Poult Sci 79 (4):539-545.

Meydani, M. 2002. Antioxidants in the prevention of chronic diseases. Nutr Clin Care, 5(2): 47-49.

Nagata, Y., Partridge, T. A., Matsuda, R., & Zammit, P. S. 2006. Entry of muscle satellite cells into the cell cycle requires sphingolipid signaling. J Cell Biol, 174(2): 245-253.

Ozdemir, N., & Ordu, S. 1998. Improvement of dissolution properties of furosemide by complexation with beta-cyclodextrin. Drug Dev Ind Pharm, 24(1): 19-25.

Park, C. W., Kim, S. J., Park, S. J., Kim, J. H., Kim, J. K., Park, G. B., Kim, J. O., & Ha, Y. L. 2002. Inclusion complex of conjugated linoleic acid (CLA) with cyclodextrins. J Agric Food Chem, 50(10): 2977-2983.

Park, E. I., & Garrow, T. A. 1999. Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. J Biol Chem, 274(12): 7816-7824.

Patel, H. M., Suhagia, B. N., Shah, S. A., Rathod, I. S., & Parmar, V. K. 2007. Preparation and characterization of etoricoxib-beta-cyclodextrin complexes prepared by the kneading method. Acta Pharm, 57(3): 351-359.

Pierucci, A. P., L. R. Andrade, M. Farina, C. Pedrosa, and M. H. Rocha-Leao. 2007. Comparison of alpha-tocopherol microparticles produced with different wall materials: pea protein a new interesting alternative. J Microencapsul 24 (3):201-213.

Pisconti, A., Brunelli, S., Di Padova, M., De Palma, C., Deponti, D., Baesso, S., Sartorelli, V., Cossu, G., & Clementi, E. 2006. Follistatin induction by nitric oxide through cyclic GMP: a tightly regulated signaling pathway that controls myoblast fusion. J Cell Biol, 172(2): 233-244.

Relaix, F., Rocancourt, D., Mansouri, A., & Buckingham, M. 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature, 435(7044): 948-953.

Rigotti, A. 2007. Absorption, transport, and tissue delivery of vitamin E. Mol Aspects Med, 28(5-6): 423-436.

Rothman, K. J., Moore, L. L., Singer, M. R., Nguyen, U. S., Mannino, S., & Milunsky, A. 1995. Teratogenicity of high vitamin A intake. N Engl J Med, 333(21): 1369-1373.

Senesi, P., Luzi, L., Montesano, A., Mazzocchi, N., & Terruzzi, I. 2013. Betaine supplement enhances skeletal muscle differentiation in murine myoblasts via IGF-1 signaling activation. J Transl Med, 11: 174.

Simopoulos, A. P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother, 56(8): 365-379.

Sinha, V. R., Anitha, R., Ghosh, S., Nanda, A., & Kumria, R. 2005. Complexation of celecoxib with beta-cyclodextrin: characterization of the interaction in solution and in solid state. J Pharm Sci, 94(3): 676-687.

Smith, J. W., 2nd, M. D. Tokach, R. D. Goodband, J. L. Nelssen, and B. T. Richert. 1997. Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early-weaned pigs. J Anim Sci 75 (7):1861-1866.

Suntres, Z. E., and P. N. Shek. 1994. Incorporation of alpha-tocopherol in liposomes promotes the retention of liposome-encapsulated glutathione in the rat lung. J Pharm Pharmacol 46 (1):23-28.

Surai, P. F. 2000. Effect of selenium and vitamin E content of the maternal diet on the antioxidant system of the yolk and the developing chick. Br Poult Sci 41 (2):235-243.

Surai, P. F., I. A. Ionov, T. V. Kuklenko, I. A. Kostjuk, A. MacPherson, B. K. Speake, R. C. Noble, and N. H. Sparks. 1998. Effect of supplementing the hen's diet with vitamin A on the accumulation of vitamins A and E, ascorbic acid and carotenoids in the egg yolk and in the embryonic

Trombino, S., R. Cassano, R. Muzzalupo, A. Pingitore, E. Cione, and N. Picci. 2009. Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of beta-carotene and alpha-tocopherol. Colloids Surf B Biointerfaces 72 (2):181-187.

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. 2008. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci, 86(14 Suppl): E140-148.

Wong, J. W., & Yuen, K. H. 2001. Improved oral bioavailability of artemisinin through inclusion complexation with beta- and gamma-cyclodextrins. Int J Pharm, 227(1-2): 177-185.

Wozniak, A. C., & Anderson, J. E. 2007. Nitric oxide-dependence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. Dev Dyn, 236(1): 240-250.

Yang, W. Z., K. A. Beauchemin, and L. M. Rode. 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. J Dairy Sci 82 (2):391-403.

Summary in Korean

기능성 생리활성 물질이란 미량으로 생체의 기능에 영향을 미칠 수 있는 물질들을 총칭하며, 대표적으로 비타민, 호르몬, 신경전달 물질 등이 포함된다. 기능성생리활성물질의 종류는 매우 다양하지만, 이들이 체내에서 발휘하게 되는 긍정적 영향은 항염증반응, 항산화반응, 메틸기공여(methyl donation) 등의 몇몇 기작으로 분류될 수 있다. 이러한 기능성 생리활성물질들은 식품으로부터 일상적으로 섭취가 가능하면서도, 질병 예방 및 치료의 효과 또한 지니고 있다고 알려져 있어 최근에는 nutraceutical이라 불리우기도 한다. 특히 축산물에 기능성 생리활성물질들이 많이 함유되어있고, 이러한 물질들을 강화하여 고기능성 축산물을 개발하여 국내 축산물의 경쟁력 제고를 위한 한 전략으로서 이용하기 위한 연구들도 많이 진행되고 있다.

기능성 생리활성물질은 그 효능이 우수하고 자연계에 다양하게 분포하고 있지만, 생체내로 들어와 이용되기까지 다양한 한계점들이 존재한다. 우선, 보관, 유통 및 가공 과정에서 빛이나 열과 같은 외부 환경에 대해 안정성이 낮아 작용기가 파괴되는 문제점이 발생한다. 또한, 체내로 들어왔을 때 위에서의 낮은 pH로 인해, 혹은 소장에서의 소화효소들에 의해 작용기가 변성되거나 파괴되는 문제가 발생할 수 있다. 또한 기능성 불포화지방산이나 지용성 비타민과 같은 많은 종류의 기능성 생리활성물질들은 지용성 물질이기 때문에 소장상피세포층에서 물질이 잘 분산되지 않아 흡수효율이 매우 낮다는 한계점을 지니고 있다. 이러한 낮은 안정성과 생체이용률은 곧 낮은 효율성으로 이어지며, 이는 제품개발에 있어서의 비용문제로 연결될 수 밖에 없기에 이용효율성을 높이기 위해 이러한 한계점들을 극복할 수 있는 전략이 요구된다. 이러한 기능성 생리활성물질들의 이용에 있어서의 한계점을 극복하기 위해 이 연구에서는 베타-사이클로덱스트린을 이용한 포접체형성 및 두 가지 생리활성물질의 시너지효과 검증이라는 두 전략으로 고효율의 기능성 사료첨가제

개발을 시도하였다.

지용성물질의 포접체 형성을 위해서 모델 생리활성물질로서 대표적인 항산화제이자 식품업계, 화장품업계, 제약업계에서 널리 쓰이는 알파-토코페롤을 이용하였다. 알파-토코페롤은 10가지 비타민E의 형태 중 가장 생체이용성이 높은 물질로서 체내에서 ROS(reactive oxygen species)를 제거하고 lipid peroxidation을 막아주는 항산화작용을 수행하는 기능성 생리활성물질이다. 알파-토코페롤의 작용기인 페놀기는 외부환경, 특히 빛(UV)에 대해 낮은 안정성을 지니며 매우 낮은 수용성을 지니고 있어 체내에서의 흡수가 잘 이루어지지 않는 물질이다.

알파-토코페롤의 안정성과 생체이용률을 높이기 위해 베타-사이클로덱스트린이라는 캡슐화 제제를 이용한 코팅기술과 혼합유화제를 이용한 유화시스템을 동시에 이용하는 dual-strategy를 적용하였다. 베타-사이클로덱스트린은 고리형 다당체로서 포도당 7개가 고리형태로 연결되어 있으면서 고리 안쪽은 소수성 잔기들이 위치하고 고리 바깥쪽은 친수성 잔기들이 위치하고 있어 소수성 물질을 내부에 담지하면서 동시에 수용성을 높일 수 있다는 특징을 지니고 있다. 여기에 담지과정에서 혼합유화제를 함께 이용하여 수용성을 더욱 높일 수 있고 친유성 유화제와 친수성 유화제를 다양한 비율로 섞음으로써 혼합유화제의 HLB(Hydrophile-Lipophile Balance)값 조절을 통한 담지 효율 및 방출 효율의 최적화(optiization)를 수행하였다. 유화제 유무 및 HLB값에 따라 총 6가지 formula로 포접체를 제조하고 spray drying방법으로 고체형태의 최종 포접체 샘플을 제조하였다. 이 후에는 물리화학적 특성검정을 위해 particle의 모양 및 size를 SEM과 DLS로 확인하였고, SGF와 SIF에서 particle의 방출효율을 확인하였다. 또한 UV 및 고온으로부터 포접체의 알파-토코페롤 보호 효과를 확인하였고, 마지막으로 소장상피세포층에서의 흡수율 증진을 *in vitro*수준에서 확인하기 위해 대장상피세포주인 Caco-2를 이용한 epithelial

permeability assay를 진행하였다. 담지율, 방출효율, 보호효과 및 Caco-2 permeability를 고려하였을 때 최종적으로 유화제를 tween 80:span 80=1:2 비율로 이용하였을 때 가장 높은 효율을 보임을 확인하였다. 이를 최종 formula로 결정하여 마지막으로 산란계를 이용한 *in vivo* 실험을 진행하여 난황으로의 알파-토코페롤 전이율이 증가하였음을 확인하였다.

두 가지 생리활성물질간의 시너지효과 검정을 위해서 근육세포의 성장에 영향을 미치는 것으로 알려진 비테인과 기능성 오메가-3계 지방산인 알파리놀렌산이 근육아세포의 증식 및 분화에 미치는 효과를 *in vitro* 수준에서 검정하였다. 알파리놀렌산은 근육아세포의 증식에 영향을 미치며 비테인은 분화에 영향을 미치는 것이 일차적으로 확인이 되었으며, 이를 바탕으로 농도를 재설정하여 알파리놀렌산과 비테인을 동시에 처리해봄으로써 증식과 분화에 모두 시너지효과를 지님을 확인하였다. 또한 그 과정에서 IGF-1R 시그널이 매개됨을 확인하였다.

이러한 결과들을 종합적으로 고찰해보았을 때 기능성 생리활성물질을 이용한 사료첨가제를 개발하는 데 있어, 그 안정성과 생체이용률을 높이기 위해 베타-사이클로덱스트린과 유화제를 이용한 포접체 기술이 유용하게 이용될 수 있을 것임을 기대할 수 있다. 또한 알파-리놀렌산과 비테인을 함께 사료첨가제로 급여하였을 때, 각각 급여하였을 때 보다 더욱 증진 된 성장촉진효과가 있을 것임을 기대할 수 있는 분자생물학적 근거가 마련되었다는 데 이 연구의 의의가 있다.