

ABSTRACT

REYNOLDS, RONG. Spray Drying of β -Lactoglobulin-Vitamin A and β -Lactoglobulin-Vitamin D Complexes. (Under the direction of Jonathan C. Allen)

β -Lactoglobulin (β -LG) is a major whey protein of ruminant species. Its amino-acid sequence and 3-dimensional structure show that it is a lipocalin, a serum retinol binding protein. β -LG has been reported to be able to bind a variety of ligands, many of which are hydrophobic compounds. These compounds include retinol (vitamin A), fatty acids, vitamin D, cholesterol, etc. The importance of the binding property is that it can be implemented to deliver these nutrients using β -LG as a carrier without the presence of the fat in which they normally associate.

In the study, two complexes (β -Lactoglobulin-Vitamin A and β -Lactoglobulin-Vitamin D) were produced through spray drying. During the drying process, heat tends to denature the protein and causes the dissociation of the complex, which can result in low retention of vitamins. Certain sugars, such as lactose, were found to stabilize whey protein during spray drying. The study tested the hypothesis that the addition of lactose into the complexes would yield higher recovery of vitamins from spray drying.

The β -Lactoglobulin-Vitamin A complex was formed by mixing retinyl palmitate with 2% β -LG solution in DI water. Cholecalciferol was mixed with 2% β -LG solution in DI water to

form β -Lactoglobulin-Vitamin D complex. Both complexes were incubated at 40 °C for 2 hr. Binding of vitamin to β -LG was confirmed by fluorescence quenching of the protein at wavelength 332 nm. Lactose was then added into the above complex solutions at 5:1 weight ratio to the protein. Each mixed complex was then pumped into the spray dryer and powder was collected. The content of vitamins in the powder complexes was determined by HPLC analyses.

The results showed that in the presence of lactose, the spray-dried powder had 4-5 times more retention of vitamin A than without lactose and that the content of vitamin D in the powder complex was 1-2 times greater than without lactose.

The study also used the β -lactoglobulin-vitamin A complex to fortify a lemon lime soda and studied the stability of the vitamin A in the complex under different types of light for a period of seven (7) days. The results demonstrated that both lights and acidity lead to the quick degradation of vitamin A in the beverage.

Spray Drying of β -Lactoglobulin-Vitamin A and β -Lactoglobulin-Vitamin D Complexes

By

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BIOGRAPHY

Rong Reynolds was born in December 1969 to father, Fu Jing Cai and mother, Wu Guo Xiu. It was a time of man's landing on the moon. Although she didn't know much about astronomy, she always admired people with great contribution to society. Rong grew up in China and had a great childhood. The love and support from her parents surrounded her all the way through college before she left them for the United States in 1995 to establish her own family with her husband James Reynolds. The following few years of motherhood added new dimension to her life, although the love for learning was never diminished. She studied computer programming at a community college while being home with her two children Raymond and Renee. It wasn't until both children started elementary school when she decided to continue her study in the field of Food Science. She was accepted into the graduate program in the Department of Food Science at North Carolina State University under the advisory of Dr. Jonathan C. Allen in August 2003. She graduated with a Master of Science degree in Food Science.

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TABLE OF CONTENTS

LIST OF FIGURES.....	VI
LIST OF TABLES.....	VII
CHAPTER 1 INTRODUCTION.....	1
1.1 β-LACTOGLOBULIN	1
1.1.1 History	1
1.1.2 Structure	1
1.1.3 Conformation.....	3
1.1.4 Function (ligand-binding)	4
1.1.5 Physiological Functions	7
1.1.6 Binding Affinity.....	7
1.2 VITAMIN A.....	7
1.2.1 General properties.....	7
1.2.2 Biological Function	10
1.2.3 Metabolism	10
1.2.4 Deficiency and Diseases.....	10
1.2.5 Sources and Requirement	12
1.3 VITAMIN D.....	12
1.3.1 General properties.....	12
1.3.2 Biological function	13
1.3.3 Metabolism	13
1.3.4 Deficiency and Disease.....	15
1.3.5 Sources and Requirement	16
1.4 SPRAY DRYING.....	17
1.4.1 Overview.....	17
1.4.2 General process.....	18
1.5 OBJECTIVE OF THE STUDY	21
REFERENCES:.....	25
CHAPTER 2 SPRAY DRYING OF THE β-LACTOGLOBULIN-VITAMIN A AND β-LACTOGLOBULIN-VITAMIN D COMPLEXES.....	29
2.1 INTRODUCTION.....	29
2.2 MATERIALS AND METHODS	30
2.2.1 Preparation of the Complexes	30
I. β -lactoglobulin and Vitamin A complex	30
II. β -lactoglobulin and Vitamin D complex	31
2.2.2 β -Lactoglobulin –Vitamin A Binding Study	31
2.2.3 Spray Drying of the Complexes	32
2.2.4 Powder Recovery.....	32
2.2.5 Vitamin Analyses	33
I. Vitamin A analysis	33
Standards	33
Sample preparation and extraction.....	34
Apparatus.....	34
II. Vitamin D analysis	35
Standards	35
Sample preparation and saponification	36
Extraction.....	36
Apparatus.....	37
2.3 RESULTS	38

I. <i>β</i> -lactoglobulin and Vitamin A complex	40
II. <i>β</i> -lactoglobulin and Vitamin D complex.....	44
2.4 DISCUSSION.....	50
REFERENCES:.....	54
CHAPTER 3 FORTIFICATION OF A LEMON-LIME SODA WITH THE β-LACTOGLOBULIN-VITAMIN A COMPLEX.....	55
3.1 INTRODUCTION.....	55
3.2 MATERIALS AND METHODS	56
3.2.1 Preparation of <i>β</i> -lactoglobulin-vitamin A complex solution	56
3.2.2 Analyzing vitamin A in the complex solution.....	57
3.2.3 Fortifying process.....	57
3.2.4 Stability study	58
I. Seven day under fluorescent and UV light.....	58
II. One day under UV light.....	59
3.2.5 Vitamin A analysis	59
3.3 RESULTS AND DISCUSSION	59
I. Seven day under fluorescent and UV light.....	59
II. One day under UV light.....	62
CONCLUSION OF THE STUDY	63
REFERENCES:.....	65

LIST OF FIGURES

Figure 1. 1 An illustration of (a) β -lactoglobulin and its super family (b) lipocalin	2
Figure 1. 2 An illustration showing the (a) binding of cholesterol to the central cavity of β -lactoglobulin (b) binding of vitamin D ₂ to the central cavity of β -lactoglobulin	5
Figure 1. 3 Structures of vitamin A metabolites and esters	8
Figure 1. 4 Various functions of vitamin A	9
Figure 1. 5 A general view of the metabolism of natural retinoids and of β -carotene	11
Figure 1. 6 Common forms of vitamin D	13
Figure 1. 7 Metabolism of vitamin D.....	14
Figure 1. 8 Schematic representation of a typical industrial spray drying process.....	21
Figure 2.1 Fluorescence change upon binding of retinyl palmitate to β -lactoglobulin	39
Figure 2. 2 Standard curve of Retinyl Palmitate with Retinyl Acetate.....	41
Figure 2. 3 HPLC chromatogram of vitamin A palmitate in 50 mg β -LG-vitamin A-lactose powder.....	42
Figure 2. 4 HPLC chromatogram of vitamin A palmitate in 50 mg β -LG-vitamin A powder.....	43
Figure 2. 5 Standard curve of vitamin D ₃ with vitamin D ₂ as internal standard	45
Figure 2. 6 HPLC chromatogram of vitamin D ₃ in 50 mg β -LG-vitamin D-lactose powder	46
Figure 2. 7 HPLC chromatogram of vitamin D ₃ in 50 mg β -LG -vitamin D powder	47
Figure 2. 8 Comparison of vitamins A and vitamin D content in complexes with and without lactose	49
Figure 3. 1 Vitamin A degradation in lemon-lime soda under dark, fluorescence and UV light	61
Figure 3. 2 Vitamin A degradation under UV in lemon-lime soda fortified with β -lactoglobulin-vitamin A complex	63

LIST OF TABLES

Table 1. 1 Some basic molecular properties of bovine β -Lactoglobulin.....	3
Table 1. 2 Some of the ligands reported to bind to β -Lactoglobulin.....	6
Table 1. 3 Recommended dietary allowance of vitamin D, USA, 1989.....	17
Table 1. 4 Vitamin D requirements, 1997.....	17
Table 2. 1 Vitamin A (Retinyl Palmitate) standards.....	34
Table 2. 2 Vitamin D ₃ (Cholecalciferol) standards.....	36
Table 2. 3 Powder recovery after spray drying.....	40
Table 2. 4 Vitamin content before and after spray drying.....	48

Chapter 1 Introduction

1.1 β -Lactoglobulin

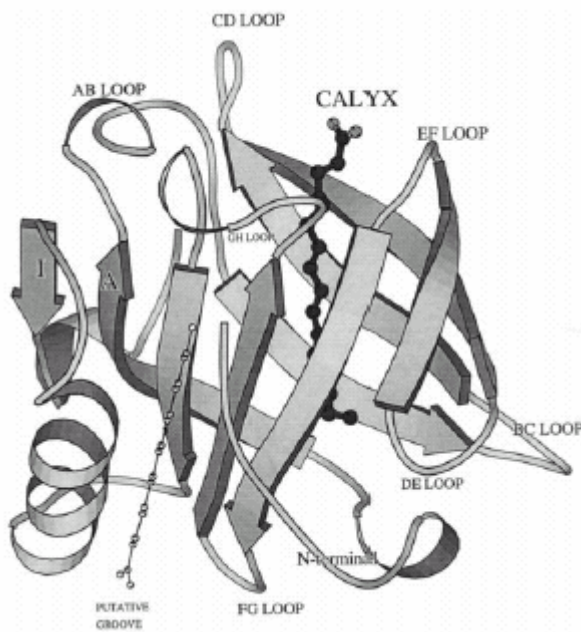
1.1.1 History

β -lactoglobulin (β -LG) is the major milk protein in the whey fraction (3 g/l, 50% of whey protein). It was discovered by Palmer (1934) during the salt fractionation of fresh skim milk and a sample of commercial acid whey. In 1942, Cannan et al. proposed the name β -lactoglobulin, by which this protein is known today. McKenzie (1971) discussed numerous studies that have been done to obtain pure β -LG in the earlier stage of the research of this protein. The general methods used include salt fractionation (Palmer, 1934), acid precipitation of whey protein (McKenzie, 1971 and Aschaffenburg, 1957) and chromatography (Armstrong et al., 1970). With the use of Zone Electrophoresis, the genetic variants of β -LG were discovered and scientists were able to make more discoveries and detailed analysis of these genetic variants (Bell, 1962). McKenzie also mentioned the differences of β -LG among species (1971). For the research described in this thesis, only bovine β -LG was used and genetic variants were ignored.

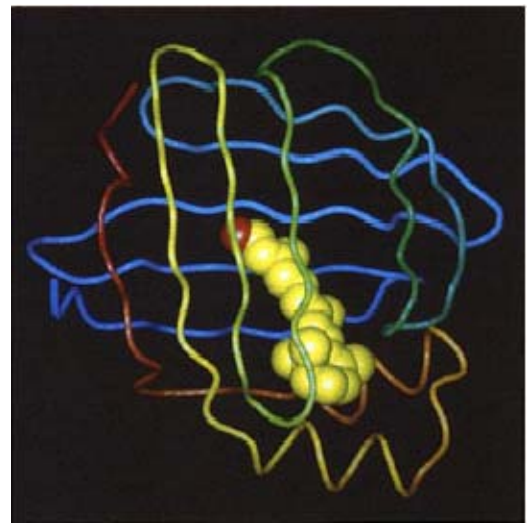
1.1.2 Structure

Since its discovery, many studies have been done to investigate the structure and function of β -LG. In the review written by Kontopidis et al. (2004), β -LG is described as a small globular protein with 162 amino acid residues (MW $\sim$ 18,400). It folded up into an 8-stranded, anti-parallel β -barrel with a 3-turn α -helix on the outer surface and a ninth β -strand (I) flanking the first strand (A) (Figure 1.1). Judged by its amino acid sequences and 3-D

structure, β -LG is considered to be in the lipocalin family of proteins, which are generally transporters of small hydrophobic molecules (Papiz et al., 1986; Brownlow et al., 1997). It exists as a dimer made up of two identical subunits under pH 5.3 and reaches dimer-monomer equilibrium under physiological pH (Sawyer, 2000). It dissociates into monomers at very low pH and is very acid stable. Table 1.1 lists some basic molecular properties of bovine β -lactoglobulin.



a (Wu et al., 1999)



b (Papiz et al., 1986)

Figure 1. 1 An illustration of (a) β -lactoglobulin and its super family (b) lipocalin

Table 1. 1 Some basic molecular properties of bovine β -Lactoglobulin

Number of amino acids		162
Monomeric Mr (B genetic variant)		18 277
Isoelectric point		
B genetic variant, native		5.407
reduced and denaturing conditions		4.968
Extinction coefficient: 1 mg/ml at 280 nm		0.96
Monomer radius, Rg		1.75 nm
Axial ratio (dimer)		2:1
Dimer Kd (B genetic variant)	pH 2.7	5.08×10^{-5} M
	pH 6	7.04×10^{-6} M
	pH 7.5	7.94×10^{-6} M
Octamer dissociation constant	pH 4.65	1.58×10^{-12} M ³

(Sawyer, 2000)

1.1.3 Conformation

Though β -LG is a small protein, its secondary and tertiary structures determine the conformation that is very important to ligand binding. Circular dichroism spectral analysis indicated that β -LG contains 16% α -helix, 54% β -sheet and 30% aperiodic amino acid sequences (Mattarella et al., 1983). The less of the β -sheet and more of the α -helix, the less likely is the binding to hydrophobic molecules. The change of the conformation could be influenced by dielectric constant lowering solvents such as alcohol, pH, ionic strength, protein concentration and thermal stress. These factors can cause protein denaturation or aggregation and affect its function.

1.1.4 Function (ligand-binding)

Like its super family, β -LG is also capable of binding retinol (Table 1.2). Evidence showed (Wu et al., 1999) that the major binding site is inside the hydrophobic pocket with its carboxyl group of amino acid residues such as lysine. It is this central cavity, the calyx, that provides the ligand-binding site. The so-called calyx, or β -barrel, is conical and is made of β -strands A-D forming one sheet, and strands E-H forming a second (Fig. 1.2). Strand A bends through a right angle such that the C-terminal end forms an anti-parallel strand with H; strands D and E also form a less significant interaction completely closing the calyx. On the outer surface of the β -barrel, between strands G and H, is the 3-turn α -helix. The loops that connect the β -strands at the closed end of the calyx, BC, DE, and FG are generally quite short, whereas those at the open end are significantly longer and more flexible. In particular, the EF loop acts as a gate over the binding site. At low pH, it is in the “closed” position, and binding is inhibited or impossible, whereas at high pH it is open, allowing ligands to penetrate into the hydrophobic binding site (Kontopidis et al., 2004).

Several studies have challenged the notion of interior cavity as the major binding site for the ligands. Evidence provided by Lange et al. (1998) using the method of fluorescence resonance energy transfer favor the surface cleft of β -LG as the binding site rather than the internal cavity and the same conclusion was reached by Monaco et al. (1987) using crystallographic analysis of a β -lactoglobulin-retinol complex.

This discrepancy, as pointed out by Kontopidis, could be due to the difference in methods.

The binding of cholesterol and vitamin D₂ to β -LG (Fig 1.2) was characterized by Kontopidis et al (2004) based on crystallographic analysis and the result was consistent with the analysis of fluorescence quenching of the Trp upon binding by Wang et al. (1997).

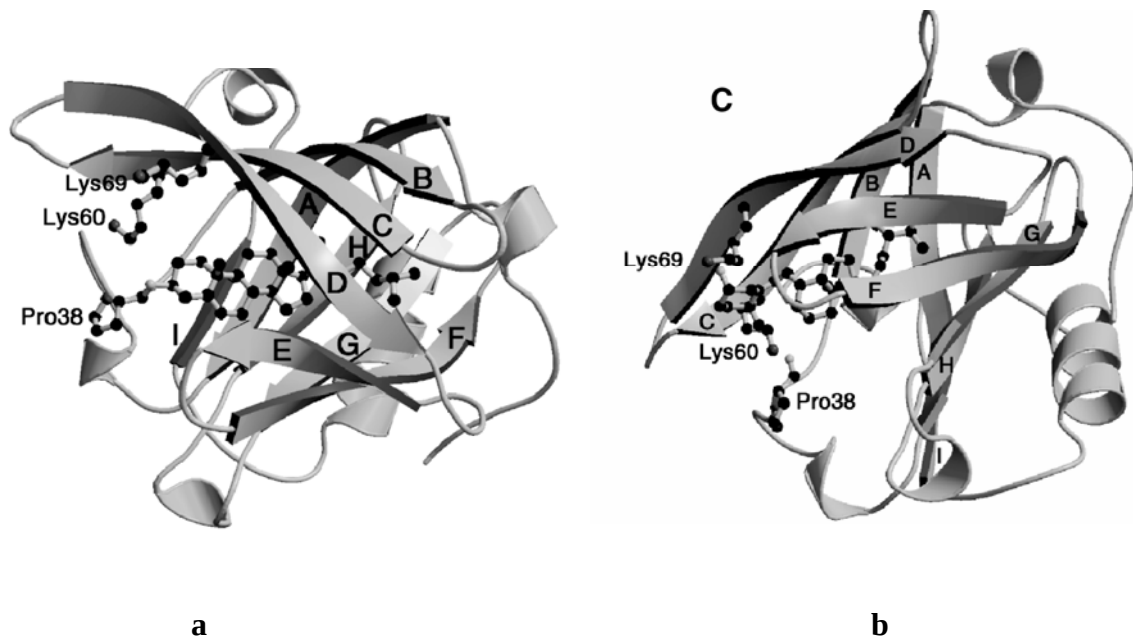


Figure 1. 2 An illustration showing the (a) binding of cholesterol to the central cavity of β -lactoglobulin (b) binding of vitamin D₂ to the central cavity of β -lactoglobulin (Kontopidis et al., 2004)

Table 1. 2 Some of the ligands reported to bind to β -Lactoglobulin

Ligand	K_d (M)	n^a	Interactions	pH	Method	β -Lg ^b	Reference
Palmitate	1.0×10^{-7}	0.9		7.0	Fluorescence	B, home	Frapin <i>et al.</i> (1993)
	1.5×10^{-6}	1		7.4	Equilibrium dialysis	AB, Pentex	Fletcher and Spector (1970)
	0.6×10^{-6}	1	Not ANS	7.0	Fluorescence	AB, Sigma	Narayan and Berliner (1998)
Stearate	1.2×10^{-7}	1		7.0	Fluorescence	B, home	Frapin <i>et al.</i> (1992)
Nonanone	4.1×10^{-4}	1		6.7	Equilibrium dialysis	AB, Sigma	O'Neill and Kinsella (1993)
Iodobutane	3.6×10^{-4}	1			Equilibrium dialysis		Wishnia and Pinder (1966)
SDS	3.2×10^{-6}	1					Ray and Chatterjee (1967)
<i>n</i> -OBS ^c	1.6×10^{-5}	1		6.8	Equilibrium dialysis	AB, home	Hill and Briggs (1956)
5-DSA ^d	8.0×10^{-7}	1	Not retinol,	7.0	EPR	AB, Sigma	Narayan and Berliner (1997)
	4.0×10^{-7}	1	Palmitate	7.0	Fluorescence	AB, Sigma	Narayan and Berliner (1997)
Retinol	1.5×10^{-7}	0.85		7.5	Fluorescence	AB, Sigma	Laligent <i>et al.</i> (1991)
	5.8×10^{-7}	0.63		7.4	Fluorescence	AB, Mann	Futerman and Heller (1974)
	2.0×10^{-8}	1		7.5	Fluorescence	A,B Sigma	Fugate and Song (1980)
	3.6×10^{-8}	1		7.5	Affinity chromatography		
	6.7×10^{-5}	1		7.2	Equilibrium dialysis	AB, Sigma	Jang and Swaisgood (1990)
	3.8×10^{-8}	1		8.0	Equilibrium dialysis	AB, home	Puyol <i>et al.</i> (1991)
	6.5×10^{-8}	1		7.0	Equilibrium dialysis	Recombinant	Cho <i>et al.</i> (1994)
	6.5×10^{-8}	1		7.0	Fluorescence	AB, Sigma	Narayan and Berliner (1998)
Retinoic acid	2.0×10^{-7}	1	1.0×10^{-8} *	7.0	Fluorescence	A, Sigma	Macleod <i>et al.</i> (1996)
	1.8×10^{-7}	1	2.7×10^{-10} *	7.0	Fluorescence	B, Sigma	Macleod <i>et al.</i> (1996)
Retinoic acid	3.9×10^{-8}	0.8		8.0	Fluorescence	A, recombinant	Cho <i>et al.</i> (1994)
<i>cis</i> -Parinaric acid	2.8×10^{-8}	0.8		7.0	Fluorescence	B, home	Dufour <i>et al.</i> (1992)
β -Ionone	6.0×10^{-7}	1		3.0	Fluorescence	B, home	Dufour <i>et al.</i> (1994)
DPH ^f	9.3×10^{-8}	0.8		7.0	Fluorescence	B, home	Dufour <i>et al.</i> (1994)
Toluene	2.2×10^{-3}	2	1.7×10^{-2} *	5.8	Equilibrium dialysis	A	Robillard and Wishnia (1972)
Ph.CF ₃	2.4×10^{-3}	2	4.3×10^{-2} *	5.8	Equilibrium dialysis	A	Robillard and Wishnia (1972)
PNP _h	3.1×10^{-5}	1		6.0	Fluorescence	A, home	Farrell <i>et al.</i> (1987)
	6.3×10^{-5}	1		6.0	Fluorescence	B, home	Farrell <i>et al.</i> (1987)
ANS	4.0×10^{-5}	1		7.5	Fluorescence	AB, Sigma	Laligent <i>et al.</i> (1991)
Bis-ANS	1.2×10^{-5}	0.9		7.0	Fluorescence	AB, Sigma	Narayan and Berliner (1998)
2Me-ANS	3.0×10^{-6}	1		8.0	Fluorescence		Lovrien and Anderson (1969)
Ellipticene	9.0×10^{-8}	0.9		8.5	Fluorescence	B, home	Dufour <i>et al.</i> (1992)
	1.4×10^{-6}	0.5	Not ret/pal	8.0	Fluorescence	AB, Sigma	Dodin <i>et al.</i> (1990)
Bromophenol Blue	5.0×10^{-4}			7.0	Spectroscopy	B, Miles	Waissbluth and Grieger (1974)
Protoporphyrin	4.0×10^{-7}	0.8	Not retinol	7.0	Fluorescence	B, home	Dufour <i>et al.</i> (1990)

^a Figures other than 1 are derived from analysis of the binding curves.

^b Home—prepared in the laboratory; AB—assumed mixed where not specified.

^c *n*-OBS—*n*-Octylbenzene-*p*-sulphonic acid.

^d 5-DSA—5[4,4-Dimethylloxazolidine-*N*-oxyl]-stearic acid.

^e These K_d arise from a different method of analysis.

^f DPH—1,6-Diphenylhexa-1,3,5-triene.

^g These figures refer to the second binding site.

^h PNP—*p*-Nitrophenylphosphate.

Except from: β -Lactoglobulin: Structural Studies, Biological Clues (Sawyer *et al.*, 1998)

1.1.5 Physiological Functions

The physiological functions of β -LG are still uncertain. However, it has been suggested that β -LG may play a role in the transporting of retinol and /or fatty acids (Perez and Calvo, 1995). Perez et al. (1992) suggested that the interaction of the protein with fatty acids increased the activity of pregastric lipase indicating that ruminant β -LG could participate in fat digestion during the neonatal period. There is evidence that β -LG in milk contains endogenously bound fatty acids (Perez et al., 1989).

1.1.6 Binding Affinity

The studies of the interaction of β -LG to many hydrophobic molecules were not only focused on the binding site, but also the comparison of the affinity with the various ligands based on several techniques, such as fluorescence spectra change and stoichiometry. Studies have shown that one mole of ligand is capable of binding at lease one mole of per dimer protein (Wang et al., 1997) and vitamin D may bind more to β -LG than does retinol.

1.2 Vitamin A

1.2.1 General properties

Vitamin A refers to all isoprenoid compounds from animal products that posses the biological activity of all-*trans* retinol. The structures of all-*trans* retinol and its important metabolites are shown in Fig 1.3. Oxidation of the alcohol moiety of all-*trans* retinol yields all-*trans* retinal. Further oxidation produces all-*trans* retinoic acid. Acetate and palmitate esters of all-*trans* retinol are the primary commercial forms of vitamin A available to the

pharmaceutical and food industry (Eitenmiller and Landen, 1999). Retinols are very sensitive to oxidation and peroxidation. Esterification greatly stabilizes the vitamin toward oxidation.

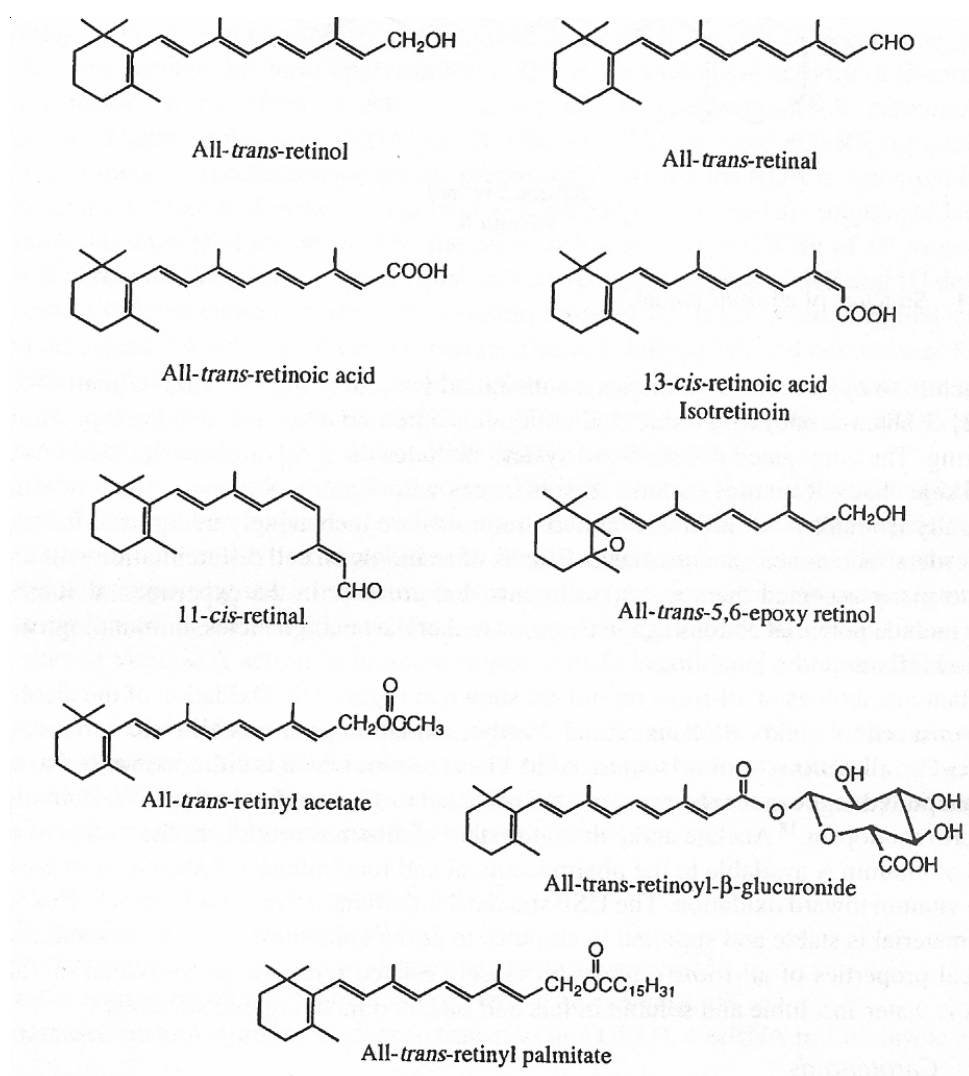


Figure 1. 3 Structures of vitamin A metabolites and esters
(Eitenmiller and Landen, 1999)

In the plant kingdom, especially in colorful fruit and vegetables, certain carotenoids, especially beta-carotene, possess provitamin A activity, meaning they are metabolic

precursors of vitamin A. Carotenoids are converted into active vitamin A after hydrolysis by acid. Retinol contains five conjugated double bonds with UV absorption maximum at 325 nm in hexane or ethanol. Vitamin A is water-insoluble and soluble in fats, oils, and most organic solvents.

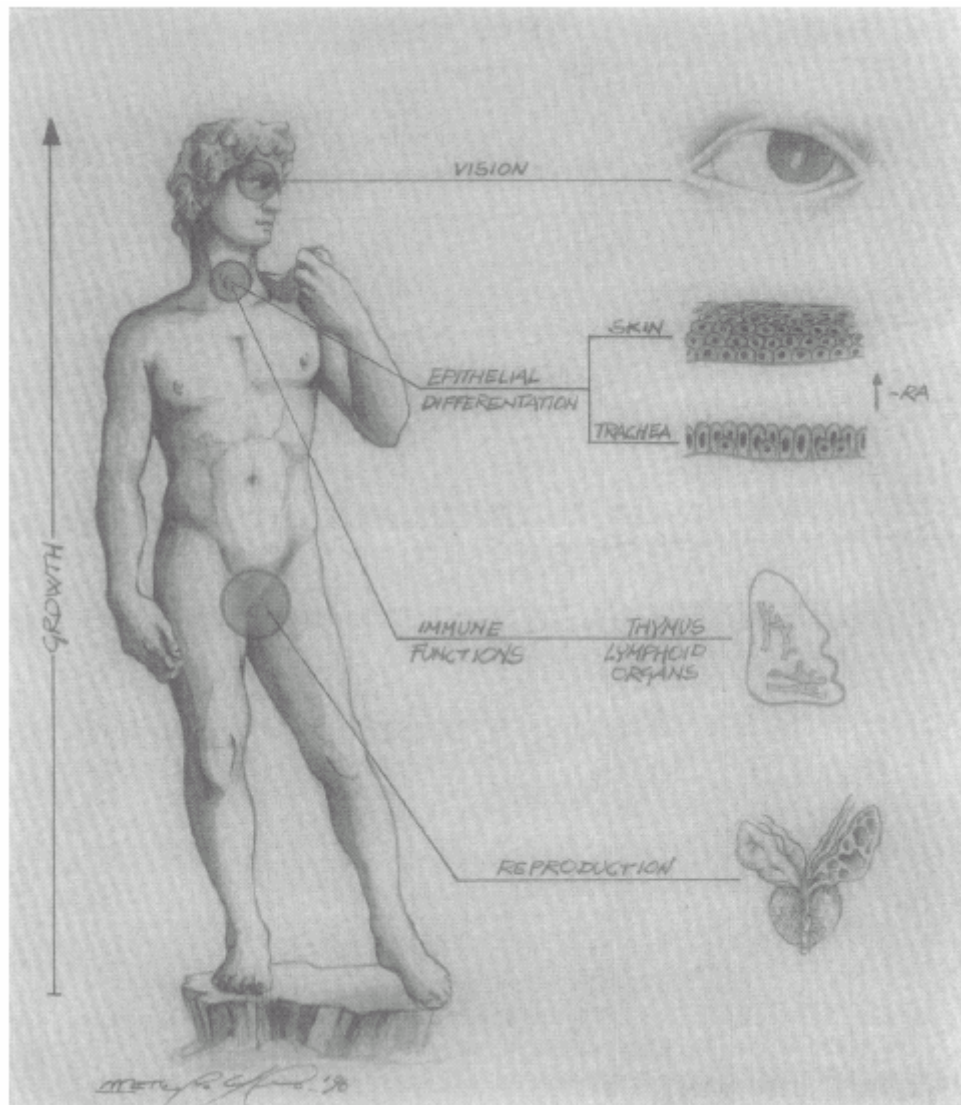


Figure 1. 4 Various functions of vitamin A

(De Luca et al., 1997)

1.2.2 Biological Function

Vitamin A functions primarily in vision, cell differentiation, and embryonic development (Fig 1.4). It consequently influences many physiologic processes, including growth, reproduction, and the immune response (Barua et al., 2000). Within cells, vitamin A is largely bound to specific carrier proteins, termed cellular retinoid-binding proteins. At least two such proteins bind retinol; another two bind retinoic acid, and another, uniquely in the eye, binds retinal. An additional binding protein for retinol and retinal, termed interphotoreceptor (interstitial) retinoid binding protein (IRBP), is found in the interphotoreceptor matrix between the retinal pigment epithelium and the outer segments of rod cells. The binding proteins serve as transport and protective agents for retinoids and, in some cases enhance their enzymatic transformations (Barua et al., 2000).

1.2.3 Metabolism

Soon after ingestion, foods containing preformed vitamin A in the form of retinyl ester and the carotenoids undergo enzymatic hydrolysis. Afterwards, the subsequent release and aggregation to lipid globules takes place (Fig 1.5).

1.2.4 Deficiency and Diseases

Deficiency of vitamin A, such as night blindness has been recognized since 1500 B.C. It is estimated that as many as one-half million children every year become partially or completely blind as a result of vitamin A deficiency (Underwood, 1994). Other symptoms of

vitamin A deficiency include skin lesions, loss of appetite, epithelial keratinization, lack of growth and increased susceptibility to infections (Eitenmiller and Landen, 1999). Both clinical and experimental studies have shown that vitamin A deficiency is associated with decreased resistance to infection, leading to the breakdown of epithelial barriers or changes in immune system defenses that normally counteract environmental pathogens (Lu et al., 2000).

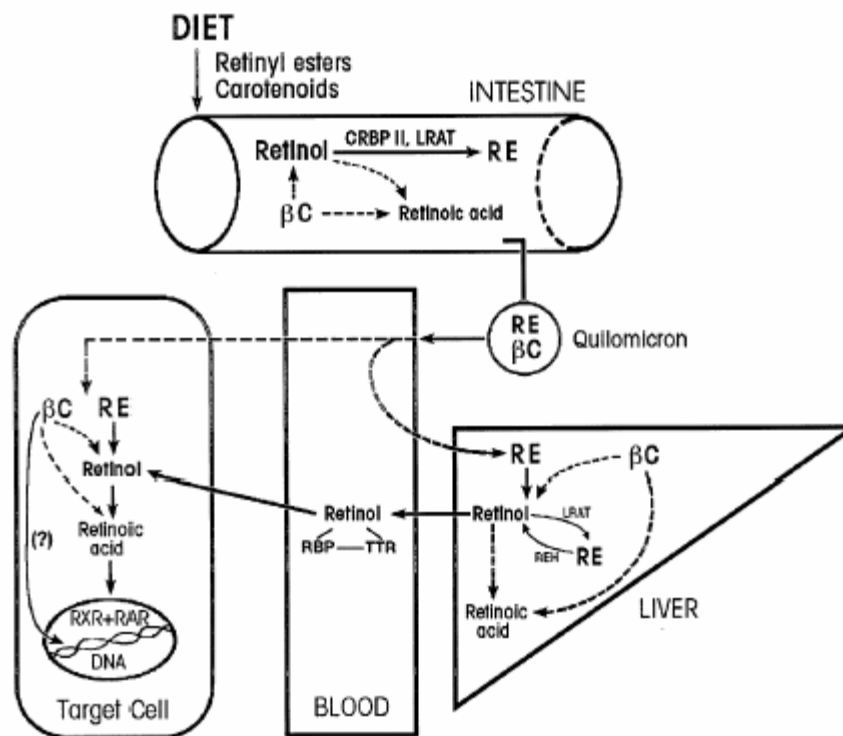


Figure 1. 5 A general view of the metabolism of natural retinoids and of β-carotene from their presence in the intestinal lumen to their actions at the target cell level. (β-Carotene, βC; retinyl ester, RE; lecithin retinol acyltransferase, LRAT; cellular retinol-binding protein type II, CRBP II; retinyl ester hydrolase, REH; retinol binding protein, RBP; transthyretin, TTR; retinoic acid receptor, RAR; retinoid X receptor, RXR) (Silveira and Moreno, 1998).

1.2.5 Sources and Requirement

Dietary sources of vitamin A include organ meats (the liver contains the highest amount), fish oils, butter, eggs, whole milk and fortified low fat milk, other dairy products, and fish (particularly fish with higher fat content like tuna and sardines). Margarine, fluid milk, and dry milk are typically fortified with retinyl palmitate in many countries. These products play a dramatic role in preventing vitamin A deficiency in countries where fortification is mandatory. In the United States, milk is fortified with not less than 2000 IU of vitamin A (retinyl palmitate). Margarine is fortified with not less than 15,000 IU per pound (retinyl palmitate and β -carotene) (Eitenmiller and Landen, 1999). The Recommended Dietary Allowance (RDA) for men and women is 900 and 700 μg retinol activity equivalents (RAE)/day respectively according to Food and Nutrition Board (2001).

1.3 Vitamin D

1.3.1 General properties

Vitamin D was discovered by Edward Mellanby in 1919 during his classic experiments with rickets. It is a family of compounds consisting of 9,10-secosteroids, which differ in their side-chain structures. They are classified into five forms: vitamin D₂, ergosterol; D₃, cholecalciferol (Fig 1.6); D₄, 22,23-dihydroergocalciferol; D₅, sitosterol (24-ethylcholecalciferol) and D₆, stigmasterol. Vitamin D is derived from a cholesterol-like precursor, 7-dehydrocholesterol (provitamin D₃). When human skin is exposed to sunlight, the UV-B photons (between 290–315 nm) interact with 7-dehydrocholesterol causing photolysis and cleavage of the B-ring of the steroid structure, and thermoisomerization

results in a secosteroid. In order to produce physiological activity, vitamin D has to be further metabolized (Mehta and Mehta, 2002).

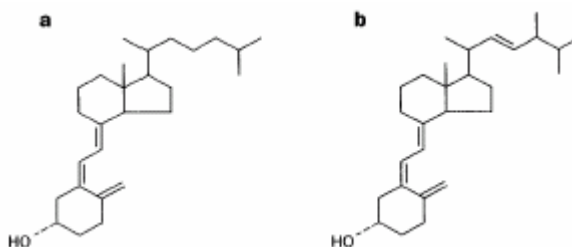


Figure 1. 6 Common forms of vitamin D

(a) vitamin D₃, cholecalciferol (b) vitamin D₂, ergosterol

1.3.2 Biological function

Vitamin D, derived from the diet or by bioactivation of 7-dehydrocholesterol, is inert and must be converted to the active hydroxylated forms to exert its biological activity. The metabolic functions of vitamin D are accepted as that of a steroid hormone and its overall biological system is described as the vitamin D endocrine system (Eitenmiller and Landern, 1999). Biological responses include mobilization and accretion of calcium and phosphorous (bone), calcium absorption (intestine), and reabsorption of calcium and phosphorous (kidney). Other accepted roles for vitamin D related to the vitamin D endocrine system include osteoblast formation, fetus development, pancreatic function, neural function, immunity, and $1\alpha,25(\text{OH})_2\text{D}_3$ mediated cellular growth and differentiation effects.

1.3.3 Metabolism

Vitamin D from skin or dietary sources does not circulate for long in the bloodstream, but instead is immediately taken up by adipose tissue or liver for storage or activation. In the

serum, bound to a vitamin D binding protein (DBP), vitamin D₃ is transported to the liver, where it is hydroxylated to 25(OH)D₃, the storage form of vitamin D (Fig 1.7). In the kidneys, 25(OH)D₃ is further metabolized to 1 α , 25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the biologically active form of vitamin D that functions as a hormone (Hyppönen, 2004).

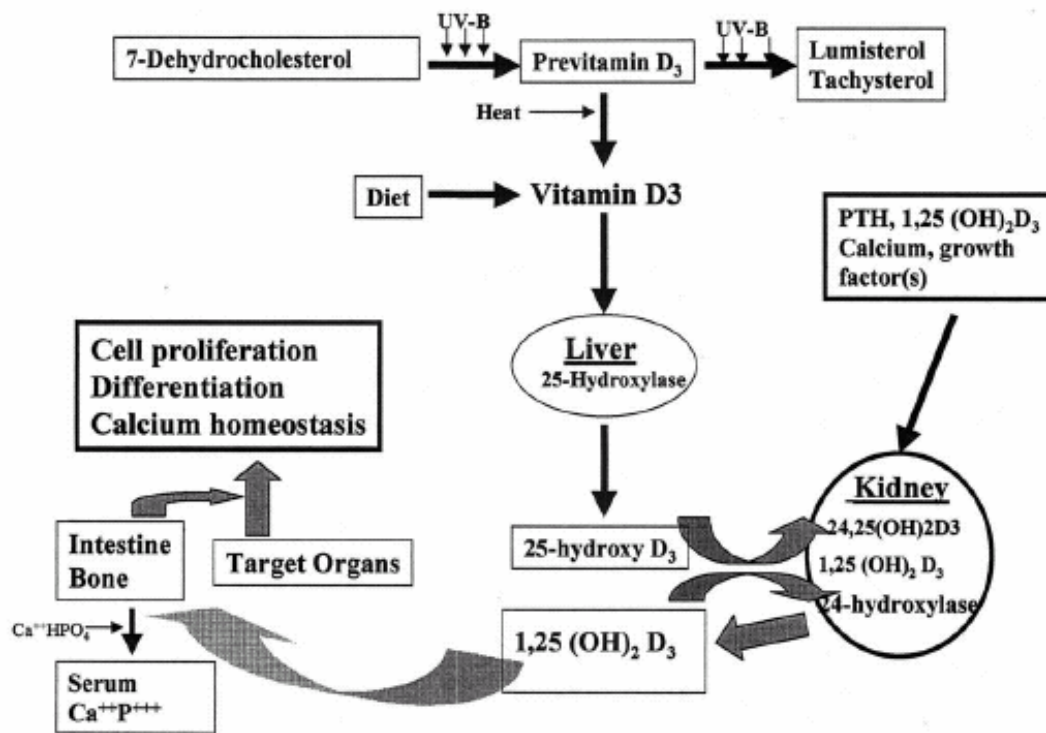


Figure 1. 7 Metabolism of vitamin D

Conversion of 7-dehydrocholesterol to previtamin D₃ by UV light and its subsequent processing to vitamin D₃ and active metabolite 1,25-dihydroxyvitamin D₃ is schematically shown. Vitamin D metabolism by liver and its processing by kidney is also shown in this diagram (Mehta and Mehta, 2002).

1.3.4 Deficiency and Disease

Vitamin D deficiency can cause rickets in infants and children, which is characterized by weakened, deformed bones, muscle spasms and seizures. Vitamin D deficiency can also produce osteomalacia in adults, a defect in bone mineralization with the accumulation of unmineralized osteoid on bone surfaces (Fraser, 1995). In elderly people, vitamin D deficiency is associated with muscle weakness and may lead to falls and subsequent fractures. Older people are prone to develop vitamin D deficiency because of various risk factors: decreased dietary intake, diminished sunlight exposure, reduced skin thickness, impaired intestinal absorption, and impaired hydroxylation in the liver and kidneys (Janssen et al., 2002). These symptoms associated with bone mineralization can be corrected and prevented by vitamin D supplementation. Besides its established role in maintaining the homeostasis of calcium and phosphate and its essentiality for the proper development and maintenance of bone, vitamin D is being investigated for possible clinical importance for protection from several cancers and diabetes. For the past 20 years it has been consistently reported and well established that the active metabolite of vitamin D, $1,25(\text{OH})_2\text{D}_3$ exhibits potent cell differentiating property in leukemia cells as well as cancer cells such as breast, colon and prostate cancer (Mehta and Mehta, 2002). It was also reported that low serum concentration of $25(\text{OH})\text{D}_3$ in hypovitaminosis D patients is associated with insulin resistance and β cell dysfunction which are manifestations of type 2 diabetes (Chiu et al., 2004).

1.3.5 Sources and Requirement

Sources of vitamin D include the vitamin D₃ synthesized in the body and food sources.

Formation through sunlight exposure provides humans with most of their vitamin D requirements. Food sources of vitamin D are limited. Provitamin D compounds, however, are abundant in plant and animal tissue that can be converted to provitamin D compounds by UV irradiation. 7-Dehydrocholesterol (provitamin D₃) in animals is converted to provitamin D₃ by exposure of the skin to sunlight. Provitamin D₃ then undergoes isomerization to vitamin D₃ (cholecalciferol). Provitamin D₂ (ergosterol) in plant tissue is converted to provitamin D₂ by irradiation. Provitamin D₂ isomerizes to vitamin D₂ (ergocalciferol). The biological activities of vitamin D₂ and D₃ are approximately equal for humans (Eitenmiller and Landern, 1999). The major dietary sources of vitamin D in the USA, Canada, and a few other technologically advanced nations are fortified dairy products, especially milks and infant formulas, eggs, fortified margarines, and fishes, including their oils. In less advanced nations where the consumption of dairy products, eggs, and fish is minimal or non-existent, vitamin D intakes must be extremely low. For example, foods like cassava, maize (corn), yam, plantain, rice, and other grains contain no vitamin D. Thus, subtropical and tropical peoples of the world must depend almost totally on skin biosynthesis to meet their tissue requirements of vitamin D (Anderson and Toverud, 1994).

The quantitative requirements for vitamin D are very small (Table 1.3). Even when supplied artificially in the diet, as little as 10 µg per day can maintain the vitamin status of adults (Fraser, 1995). In Canada and the United States, vitamin D is added to fluid milk at a level of 10 µg (400 IU) per quart or 9.6 µg (385 IU) per liter (Calvo and Whiting, 2003).

However, the Recommended Daily Allowance of vitamin D was redefined as Adequate Intake in 1997 by the Institute of Medicine due to the variability in consumption of vitamin D containing foods and levels of sun exposure (Holick, 1998; Weaver and Fleet, 2004).

Table 1. 3 Recommended dietary allowance of vitamin D, USA, 1989
(Anderson and Toverud, 1994)

Category	Age (years)	Vitamin D, μg^*
Infants	0.0–0.5	7.5
	0.5–1.0	10
Children	1–10	10
Males	11–24	10
	25–+	5
Females	11–24	10
	25–+	5
Pregnant/Lactating	—	10

*As cholecalciferol: 10 μg cholecalciferol = 400 IU of vitamin D.

Table 1. 4 Vitamin D requirements, 1997
(Weaver and Fleet, 2004)

1997 dietary reference intakes for vitamin D				
Population age	AI		Tolerable UL	
y	$\mu\text{g}/\text{d}$	IU/d	$\mu\text{g}/\text{d}$	IU/d
0–50	5	200	50	2000
51–70	10	400	50	2000
> 70	15	600	50	2000

1.4 Spray drying

1.4.1 Overview

Spray drying is a dehydration technology that converts a suspension or solution into dry powder. It is widely used in food and pharmaceutical industries. The major applications in the food industry include the drying of dairy products (milk, whey, cheese, buttermilk,

sodium caseinate, coffee whitener, butter, ice cream mixes), fruit and beverage, and other food items such as egg yolk, soy protein, etc. Spray-drying highly volatile flavors minimizes loss and maintains natural properties such as color and aroma (Deis, 1997). The spray-drying process is older than might commonly be imagined. Earliest descriptions date to the 1860s, and the first patent of note is dated 1872. Refining the process is ongoing. Spray drying involves transforming a fluid, pumpable medium into a dry-powdered or particle form. This is achieved by atomizing the fluid into a drying chamber, where the liquid droplets are passed through a hot-air stream. The objective is to produce a spray of high surface-to-mass ratio droplets (ideally of equal size), then to uniformly and quickly evaporate the water. Evaporation keeps product temperature to a minimum, so little high-temperature deterioration occurs.

1.4.2 General process

The general spray drying process involves: (Excerpt from the booklet on Anhydro spray dryer)

CENTRIFUGAL ATOMIZATION OF THE PRODUCT

The liquid or slurry to be dried is fed to the eye of the centrifugal atomizer disc, and as a result of the high speed at which this disc revolves, the feed is thrown out in the form of a fine mist. The liquid is not under pressure, nor has it to pass through narrow orifices, thus there is very little risk of clogging.

NOZZLE ATOMIZATION OF THE PRODUCT

When using the nozzle atomizer, the liquid is dispersed into a mist in a two-fluid nozzle by means of compressed air. The nozzle is situated in the middle of the drying chamber, spraying upwards, thus giving the particles the longest trajectory obtainable in the compact plant and permitting the production of coarser particles.

DRYING OF THE PARTICLES

Hot drying air is simultaneously introduced through an annular opening in the drying chamber ceiling. This hot air mixes continuously with the mist of atomized liquid and a practically instantaneous evaporation of the volatile liquid takes place. The non-volatile part is left in the form of small dry particles of powder.

On account of the rapid evaporation, the heat in the air is absorbed so quickly that the temperature in the drying zone is very low throughout the entire period of drying. It is only when the particles are dry that their temperature gradually rises towards the temperature of the outlet air.

POWDER RECOVERY

The powder falls down towards the outlet aperture in the conical bottom of the chamber and from there it goes with the outlet air to the dynamic cyclone separator. From there, the powder falls down into a container while the drying air is discharged from the top of the cyclone.

The atomizer is a critical component of the spray dryer. The degree of atomization influences the drying rate, the required particle residence time, particle size and particle size

distribution, which in turn relates to dispersibility of the product for rehydration (Mermelstein, 2001). More importantly, the drying chamber will affect the quality of the products. Many components of food are heat sensitive, so it is important to keep the residence time to a minimum, especially once the dry particle is formed and the desired amount of moisture has been removed. The exposure to high temperature should be kept to a minimum, and this relates to the uniformity of particle size distribution. If different size droplets are present, by the time the largest droplet is dried, the smallest droplet will have been exposed longer than necessary. Thus, a narrow particle size distribution would improve the quality of any heat-sensitive component of the product, such as vitamins, color, or flavor. The outlet temperature of the air leaving the tower is used for control and provides a measure of the severity and rate of drying. A high outlet temperature (e.g. 120°C) indicates fast, severe drying and a low outlet temperature (e.g. 80°C) indicates slow, mild drying. The drying rate may be altered by changing the air flow rate, the air temperature or the slurry rate (Meenan et al., 1997). The figure below (Fig 1.8) shows a typical industrial spray drying process.

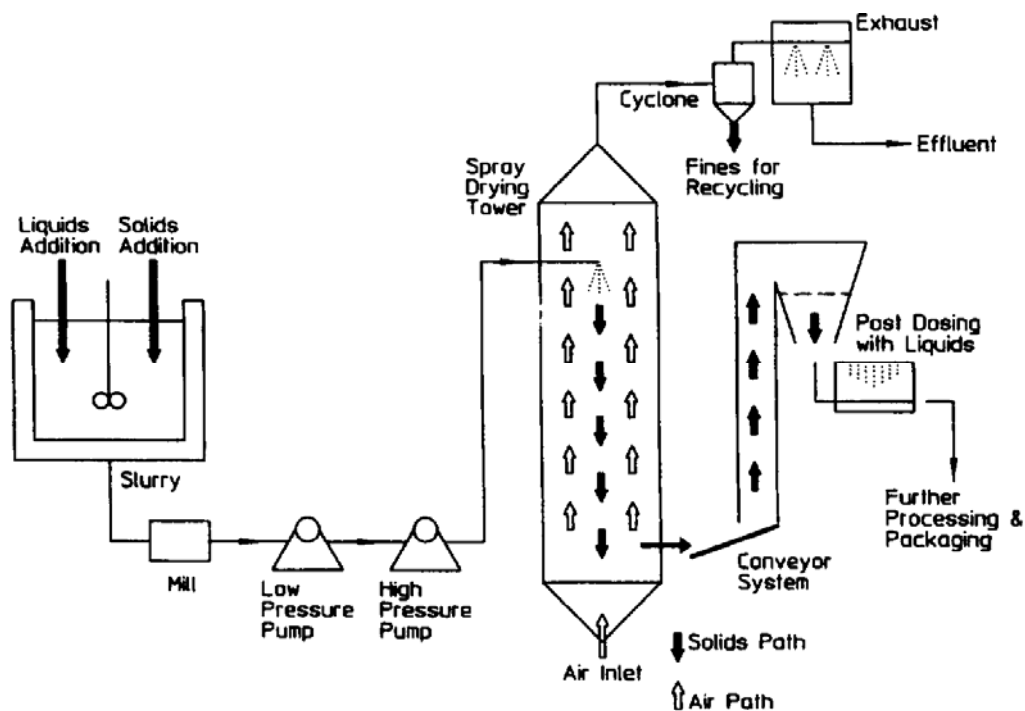


Figure 1. 8 Schematic representation of a typical industrial spray drying process
(Meenan et al., 1997)

1.5 Objective of the study

Since its discovery 60 years ago, the structure and functions of β -lactoglobulin have been studied extensively. Researchers at the Dept. of Food Science at North Carolina State University have been engaging in the study since 1992. Wang and Swaisgood (1993) developed a bioselective adsorption method to obtain purified β -LG from raw milk with almost 90% purity. More studies characterized the binding of β -LG using the purified form to various fat soluble molecules, such as vitamin A, D, E, K and fatty acids. The binding of β -LG to cholesterol was also characterized soon after. Its capability to bind retinol, fatty acid and other molecules makes β -LG a possible ingredient to deliver these nutrients in a fat free food system. β -LG apparently has nutritional value as well as functional characteristics. The fortifications of both fat soluble vitamins, vitamin A and vitamin D, are usually carried out

with some forms of fat as a carrier because of their solubility in fat. However, with the increased health consciousness of consumers, the choices of products are focused on less fat or fat free products. This trend parallels consumers' demands for more nutrient-dense foods. Such consumer demands create a need for food ingredients that can fulfill both requirements.

The general applications of β -LG as a food ingredient are for emulsification, gelation and nutrition. It is used by athletes as a good protein source to boost their muscle strength and sold in many health food stores such as General Nutrition Centers. Such an ingredient with both protein and vitamin function has not been introduced in the market and has obvious market value for the industry and consumers. Since β -LG can be produced as a spray-dried powder, it is logical to market vitamin-fortified β -LG as a powder form not only for its convenience and versatile use in different food applications but also for its shelf stability.

Liu, (2003) investigated the possibility of developing a powder form of the complexes of β -LG binding with vitamin A or vitamin D and using it to fortify fat-soluble vitamins in skim milk. Two drying methods (freeze-drying and spray-drying) were employed to compare the effectiveness of obtaining these complexes. Each of these two drying processes has its advantages and disadvantages. Freeze drying, or lyophilization, is sometimes preferred for unstable or heat labile foods or when product quality and structural integrity are critical. However, freeze-drying requires a lengthy processing time and has capital and energy costs three times those of other drying methods. Spray drying is the dehydration process best suited for liquid food products with high initial moisture contents and has been used to dry

dairy products since the early 1900s. The quality of spray-dried products is considered excellent due to the protection of the product solids by evaporative cooling (Resch, 2004).

The study (Liu, 2003) showed that these two methods didn't dry products with the same efficiency. In particular, freeze-drying yielded relatively better vitamin content in the powder than did spray drying. This was possibly due to protein denaturation and dissociation of the complex during heating in the spray-dryer and exposure of vitamins to heat and air.

The stability and bioavailability of β -LG-vitamin complex were also studied using a rat model (Liu, 2003). The powder produced by freeze drying was used to perform a fortification study in rats and the study concluded that the complex was effective at delivering the vitamins in rats and had the potential to be developed as a fortifying food ingredient.

However, spray drying is used more prevalently than freeze drying in the food industry for heat sensitive foods due to the encapsulation of the ingredients. Since higher energy cost is required for freeze-drying to yield better vitamin recovery in the powder than did spray-drying, further investigation as to whether spray drying can still be utilized to produce a complex powder with satisfying vitamin recovery was needed. The present study was to further investigate the spray drying process and look into ways to eliminate heat destruction of protein and vitamins in order to produce a powder form of the β -LG-vitamin complex with higher yield of vitamins. The study also looked at adding the protein-vitamin powder complex to aqueous beverages to test the stability of the vitamins under different

environmental conditions, such as the type of food system and the effects of lighting on the vitamins.

The outcome of producing a powdered form of the complex will not only expand the functionality of whey protein as a food ingredient, but also enable the fortification of fat-soluble vitamins without the consumption of excess fat.

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Chapter 2 Spray Drying of the β -Lactoglobulin-Vitamin A and β -Lactoglobulin-Vitamin D Complexes

2.1 Introduction

Spray drying has become the most important method for dehydration of fluid foods in the Western world. Spray-dried dairy products-- milk, whey, cheese, buttermilk, sodium caseinate, coffee whitener, butter, ice cream mixes-- comprise a large industry. Many protein sources (soy powders, isolated soy protein, whey proteins and various vegetable sources) also come in spray-dried form. Spray drying can be used to create nonfood products. Vitamins or medications might be made of compressed, spray-dried powders (Deis, 1997). However, the spray drying of the β -lactoglobulin and vitamin complex encounters a few problems. The heat applied during the spray drying could lead to denaturation of β -lactoglobulin and cause the complex to dissociate. The vitamin added into the complex was not in its native matrix and has no oil to encapsulate during the drying process and is very susceptible to degradation from the exposure to hot air. In order to produce such complex with high vitamin yield, it is critical in protecting β -lactoglobulin from denaturation during the drying process so the binding of vitamins in the complex will remain stable.

Sugars, such as lactose and trehalose, were found to stabilize the conformation of β -lactoglobulin when drying whey protein (De Wit, 1981; Murray and Liang, 2000). The present experiment tested the hypothesis that sugars will protect the complex from destruction by stabilizing protein and the vitamin will be retained more in the dried powder.

2.2 Materials and Methods

BioPURE β -lactoglobulin was provided as powder by Davisco Foods International, Inc. (Le Sueur, MN) containing 95% β -lactoglobulin. The powder was analyzed by HPLC and found to contain no vitamin D; a trace amount of retinyl palmitate that was detected was not quantifiable. Pure all-trans-retinyl palmitate, retinyl acetate, cholecalciferol, ergosterol and α -lactose monohydrate were purchased from Sigma Chemical Co (St. Louis, MO). All other chemical reagents were purchased from Sigma or Fisher (Pittsburgh, PA) and were of the highest analytical quality.

2.2.1 Preparation of the Complexes

I. β -lactoglobulin and Vitamin A complex

Four hundred mL of 2% w/v of BioPURE β -lactoglobulin (~8g) in DI water was prepared by slowly adding deionized water into the protein while stirring gently to avoid heavy foaming. The mixture was allowed to sit at room temperature until a homogenous clear solution was formed. All-trans-retinyl palmitate 0.2565g (MW~ 524.5) dissolved in 400 μ L absolute ethanol were pipetted into the solution to reach 1:1 molar ratio of vitamin to protein. The protein solution containing the vitamin was incubated at 40°C for 2 hrs according to the method used by Kontopidis et al. (2004) when studying the ligand binding of β -LG. For the complex with lactose, 5:1 weight ratio of lactose (~40 g) to protein was added to the solution after the incubation and allowed to sit until the lactose was dissolved before the spray drying.

II. β -lactoglobulin and Vitamin D complex

Four hundred mL of 2% w/v of β -lactoglobulin solution were prepared the same way as for the β -lactoglobulin-vitamin A complex. 0.18 g cholecalciferol (vitamin D₃) (MW~ 384.65) dissolved in 400 μ L absolute ethanol were pipetted into the solution to reach 1:1 molar ratio of vitamin to monomer of protein. The protein solution containing the vitamin was incubated at 40°C for 2 hrs following the same method described above. For the complex with lactose, 5:1 weight ratio of lactose (~40 g) to protein was then added to the solution after the incubation and allowed to sit until the lactose was dissolved before the spray drying.

2.2.2 β -Lactoglobulin –Vitamin A Binding Study

The binding of retinol, fatty acids and vitamin D to β -LG was characterized by Wang et al. (1997) using the method of fluorescence spectroscopy. In a previous study, Liu (2003) also validated the binding of vitamin A and vitamin D to β -LG. In the current study, fluorescence spectroscopy was used to verify the binding of vitamin A to the protein after the complex solution was formed and before spray drying. The solution tested did not contain a pH buffer.

The experiment was performed on a System 3 Scanning Spectrofluorometer (Optical Technology Devices, Inc., Elmsford, NY). The fluorescence emission spectrum between 220-500 nm of 2% β -lactoglobulin in DI water (pH ~ 7.0) was measured in the absence and in the presence of retinyl palmitate (1:1 molar ratio) dissolved in ethanol. The emission spectrum of the complex solution with lactose was also measured. Water served as blank.

The measurement was performed at room temperature using an excitation wavelength of 280 nm and a scan speed of 50 nm/min.

2.2.3 Spray Drying of the Complexes

The spray drying was performed on a pilot scale spray dryer (Anhydro, Denmark). The spray dryer was warmed up until the outlet air temperature reached 80°C with air pressure at 15 psi. DI water was then pumped into the spray dryer using a MasterFlex peristaltic pump (Model 7518-10, Cole-Parmer Instrument Co., Vernon Hills, IL) to rinse and stabilize the system. The samples were pumped when the inlet air temperature was at 120-130 °C and the outlet air temperature was at 72-74°C. The protein-vitamin solution was pumped at a constant flow rate of ~ 5 mL/min (0.5 on the speed scale). The speed of the pump is very important to keep the outlet temperature at a constant to avoid heat destruction to the protein and vitamins. If the outlet temperature is too low, the powder won't be dry. Meanwhile, both inlet and outlet air temperatures were under close monitoring to maintain at a constant level.

2.2.4 Powder Recovery

When the complex solution was pumped out, the DI water was fed into the system again to rinse the system and power was turned off to cool down the spray dryer and stop the air from blowing while the powder was being collected from the collecting bucket. The powder was weighed and flushed with nitrogen before it was stored in dark bottles in a freezer (-20 °C) for further analysis.

2.2.5 Vitamin Analyses

I. Vitamin A analysis

Vitamin A levels based on retinyl palmitate concentrations in the β -lactoglobulin-vitamin A complex were determined in duplicate by HPLC according to the procedure described by Liu (2003). All-trans retinyl acetate was used as the internal standard.

Standards

Stock solutions were prepared by weighing 0.02 g of all-trans-retinyl palmitate or all-trans retinyl acetate into a 100-mL volumetric flask and diluting to volume with absolute ethanol. Concentrations were determined on a 1:100 dilution (working solution) by measuring the absorbances at 325 nm on a Genesys 2 Spectrophotometer (Spectronic, Thermo Electron Scientific Instruments Co. Madison, WI) and calculated based on $C=A/E$. The absorption coefficients E (1%/1cm) are 940 and 1560 for retinyl palmitate and retinyl acetate respectively.

Standard solutions were prepared in 4-mL vials according to Table 2.1. The solvents were evaporated under nitrogen until dry. Two mL of HPLC grade hexane was then added to each tube afterwards and vortexed on a Genie Vortex mixer (Fisher Scientific, Raleigh, NC) before twenty five μ L was injected for HPLC analysis.

Table 2. 1 Vitamin A (Retinyl Palmitate) standards

vial #	Retinyl Palmitate working solution	Retinyl Acetate Working solution
	(4 µg/mL, 250 µL = 1 µg)	(4.5 µg /mL, 220 µL = 1 µg)
#1	250 µL	220 µL
#2	500 µL	220 µL
#3	750 µL	220 µL
#4	1000 µL	220 µL
#5	1250 µL	220 µL
#6	1500 µL	220 µL

Sample preparation and extraction

For each sample, 50 mg of the powder complex was added into 15-mL screw cap test tubes and dissolved with 2 mL DI water. One µg of internal standard retinyl acetate was added to each tube. Then 5 mL of absolute ethanol was added, vortex mixed, and allowed to stand for 5 minutes. To each tube, 5 mL of hexane was added. Tubes were vortex mixed and allowed to stand for 5 minutes. The vortex step was repeated two more times with 5-min standing times between treatments. To each tube 3 mL of distilled water was added and inverted three times. Tubes were centrifuged 1500 rpm (100 to 200x *g*) with a bench top centrifuge. The hexane layer was transferred to 4-mL vials and stored in a freezer until HPLC analyses.

Apparatus

HPLC analyses were performed using a Linear UVIS 203 detector (Scientific Instrument, Inc) with manual injection. The injector was from Waters Associate (Model U6K) and the

syringe (microliter #805, 0.05 ml) was purchased from Hamilton Co. (Reno, Nevada). The column was a 4.6 x 250-mm Prodigy 5 μ Silica Column (Phenomenex, Inc., Torrance, CA) maintained at 20°C with hexane/isopropanol (99.6: 0.4, v/v) as the mobile phase at a flow rate of 1 mL/min. The wavelength of 325 nm was used to quantitate the results and peak area of retinyl palmitate were integrated by using the Dynamax Software Package (Waters Associates).

II. Vitamin D analysis

Vitamin D levels in β -lactoglobulin-vitamin D complex were determined in duplicates by HPLC based on vitamin D₃ concentration with vitamin D₂ as an internal standard (Liu, 2003).

Standards

Vitamin D₂ and vitamin D₃ stock solutions were made 200 μ g/mL in methanol by weighing 0.02 g vitamins and dissolving into methanol in a 100 mL volumetric flask. Concentrations were determined on a 1:100 dilution (working solution) by measuring the absorbance at 264 nm on a spectrophotometer and calculating based on $C=A/E$. The absorption coefficients E (1%/1cm) are 460 and 485 for vitamin D₂ and vitamin D₃ respectively.

Standard solutions were prepared in 4 mL vials according to Table 2.2. Each vial was filled up to 0.5 mL with methanol. Fifty μ L of each standard solution was injected by syringe (Hamilton CO. Reno, Nevada. microliter #805) for HPLC analysis.

Table 2. 2 Vitamin D₃ (Cholecalciferol) standards

vial #	Vitamin D₃ working solution (2.05 µg/mL, 20 µL = 0.05 µg)	Vitamin D₂ working solution (2.5 µg /mL, 60 µL = 0.15µg)
#1	20 µL	60 µL
#2	40 µL	60 µL
#3	80 µL	60 µL
#4	120 µL	60 µL
#5	160 µL	60 µL

Sample preparation and saponification

For each sample, 50 mg powder was weighed into 45-mL tubes with screw-on tops and dissolved in 15 mL DI water. One hundred µL of the working solution (~0.2 µg vitamin D₂) and 15 mL of 1% ethanolic pyrogallol were successively added and mixed. The tubes were cooled in an ice bath and flushed with N₂ while about 6.0 g of KOH pellets were added. Tubes were immediately closed and mixed while still on ice until the KOH pellets were dissolved. The tubes were then wrapped in aluminum foil and shaken (shaker, New Brunswick Scientific Co. Inc, Edison, NJ; Speed =100) in the dark at room temperature for at least 18 hours.

Extraction

The saponified samples were transferred to 125-mL separatory funnels with 15 mL of DI water used to rinse the saponification tubes, 5 mL of ethanol, and 45 mL of hexane. The

extraction mixture in each tube was shaken for 1 min and allowed to stand for 4 min. The hexane layer was transferred to a 250-mL actinic separatory funnel. The aqueous layer was extracted two more times with 45 mL of hexane as described above, then discarded. All hexane layers were added to the 250-mL separatory funnel. The pooled hexane was then successively washed with 50 mL of 5% KOH, 50 mL of dH₂O, and 50 mL of 55% ethanol in water. The mixtures were allowed to stand for 5 min, except the last wash, which was allowed to stand 10 min before the aqueous layer was discarded. The hexane layer was evaporated to dryness at 40 to 50 °C in an evaporator (Rotarvapor, BÜCHI, SWITZERLAND) in a water bath to control the temperature. The residue was suspended in absolute ethanol and then evaporated to dryness. Immediately after drying, the residue was recovered by rinsing with small amounts of hexane (~5 mL first time, 3 mL second time and 1 mL last time). The hexane was evaporated under a stream of N₂ and 0.5 mL methanol was added to dissolve the residue. The methanol was then filtered (Acrodisc, LC PVDF, 0.45 µm, Fisher Scientific Co.) into a 4-mL vial with a 1-mL syringe (Hamilton Co., Reno Nevada. Model: Gastight #1001) and stored in a 4 °C refrigerator for HPLC analysis.

Apparatus

HPLC analyses were performed on a Waters Millipore Automated Gradient Controller with Linear UVIS 203 detector and manual injection. A reversed-phase 4.6 × 250-mm Vydac TP201 C18, 5 µ micron column with a guard column (Vydac, Hesperia, CA) was used at room temperature with the mobile phase acetonitrile/ethyl acetate/chloroform (88:8:4, v/v/v) at a flow rate of 1 mL/min. The column was washed and equilibrated with methanol after each sample injection. The wavelength of 264 nm was used to quantitate the results and a

Dynamax Software Package (Waters Associates) was used to integrate the peak areas of vitamin D.

2.3 Results

The association of vitamin A with 2% β -LG solution in DI water can be seen in Fig 2.1. The binding of retinyl palmitate to β -LG will result in the fluorescence quenching of the Trp in β -LG at 332 nm (Wang et al., 1999). The fluorescence emission spectrum of β -LG-vitamin A complex solution both with and without lactose shifted to longer emission wavelengths relative to the solution of β -LG alone, indicating that binding of vitamin A to β -LG did occur after the addition of vitamin A into the protein solution. It also showed that lactose did not affect the binding because the fluorescence emission spectrum of the β -LG-vitamin A solution with lactose was close to that of β -LG-vitamin A without the lactose. This suggests that the vitamin A associates with β -lactoglobulin but not lactose in terms of encapsulation during the spray drying.

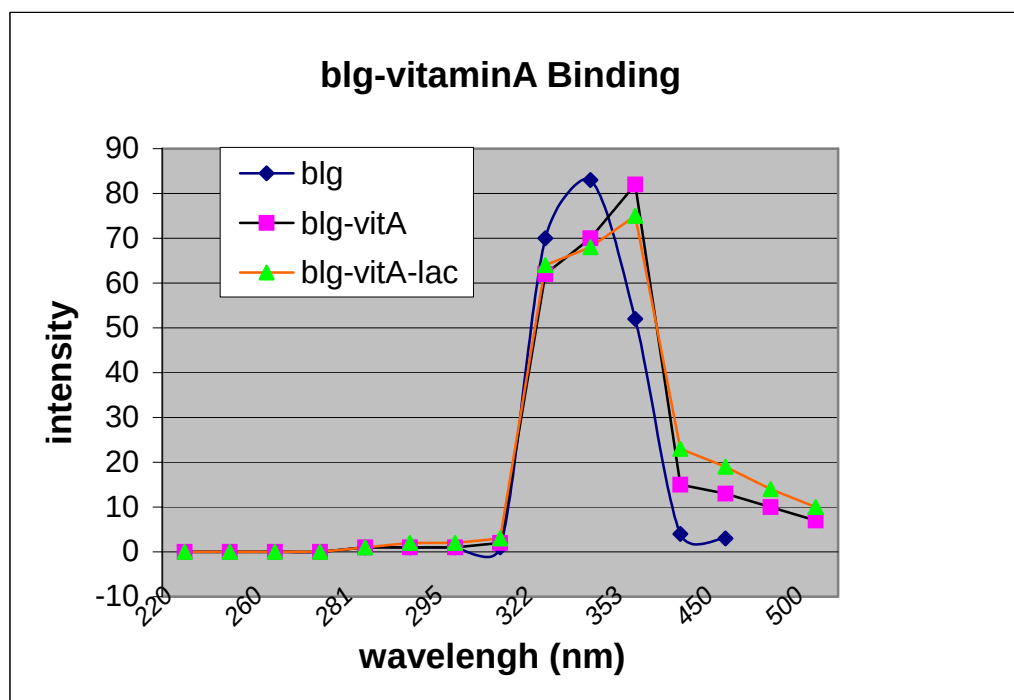


Figure 2.1 Fluorescence change upon binding of retinyl palmitate to β -lactoglobulin

The powder recovered from the spray drying was about 12-70% of the original solid content (Table 2.3). The loss of solids was due to the powder sticking on the wall of the spray dryer and the leakage of air carrying fine particles from the collecting bucket. The rate of operation was about 200 mL/per hour.

Table 2. 3 Powder recovery after spray drying

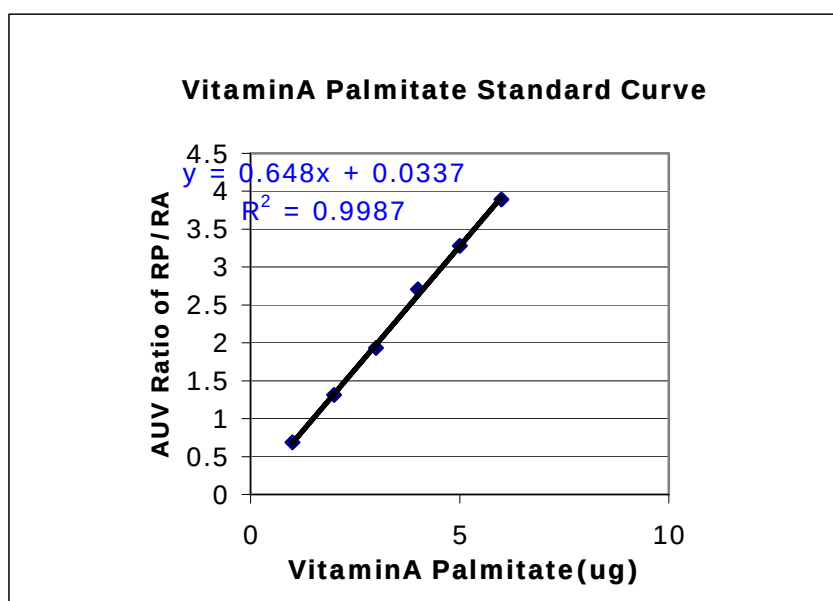
Powder	Temperature(°C)	Powder collected(g)¹
vitD-BLG	Inlet:130-150 / Outlet:78-80	20/30 $\checkmark$ 67%
vitD-BLG	Inlet: 120-130 / Outlet:70-72	10/30 $\checkmark$ 33%
vitD-BLG	Inlet: 120 / Outlet:72-74-76	11/30 $\checkmark$ 37%
vitA-BLG	Inlet: 120 / Outlet:72-74	5/30 $\checkmark$ 16.7%
vitA-BLG	Inlet: 120 / Outlet:76-78,occasional 80	14.5/30 $\checkmark$ 48.3%
vitD-BLG	Inlet: 120-130 / Outlet:72-74	2.1/8 $\checkmark$ 26%
vitD-BLG-lac	Inlet: 120-130 / Outlet:70-72	44.35/120 $\checkmark$ 37%
vitA-BLG	Inlet: 120-130 /Outlet:72-74	1/8 $\checkmark$ 12.5%
vitA-BLG-lac	Inlet: 120-130 /Outlet:72-74	14.9/48 $\checkmark$ 31%
Mean recovery ($\pm$ SD)		34 $\pm$ 16 %

¹(g recovered/g injected) $\checkmark$ percent recovery

I. β -lactoglobulin and Vitamin A complex

The quantity of vitamin A palmitate in the powder was calculated based on the standard curve shown in Fig 2.2 using the ratio of peak area of retinyl palmitate to retinyl acetate in the HPLC chromatograph. The chromatograms showed that the powder complex with lactose (Fig 2.3) had a larger retinyl palmitate peak area than the one without lactose of the same amount (Fig 2.4), indicating higher vitamin A content. Furthermore, the same amount of powder with lactose contains less quantity of β -LG than without lactose, suggesting more association of vitamin to the protein in the presence of lactose. It demonstrated an effect lactose had in stabilizing the protein during the heating process. Further investigation on how sugar encapsulated the complex and whether sugar may facilitate the binding of vitamin to the protein would be worthwhile.

Figure 2. 2 Standard curve of Retinyl Palmitate with Retinyl Acetate as internal standard



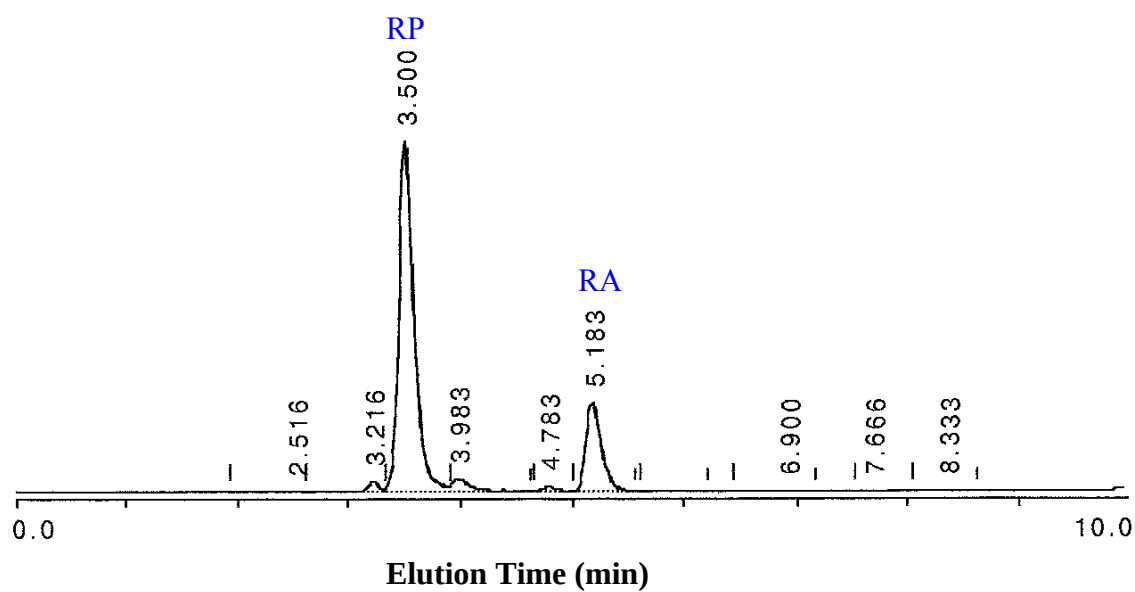


Figure 2. 3 HPLC chromatogram of vitamin A palmitate in 50 mg β -LG-vitamin A-lactose powder
(with retinyl acetate as internal standard. **RP**:retinyl palmitate; **RA**:retinyl acetate)

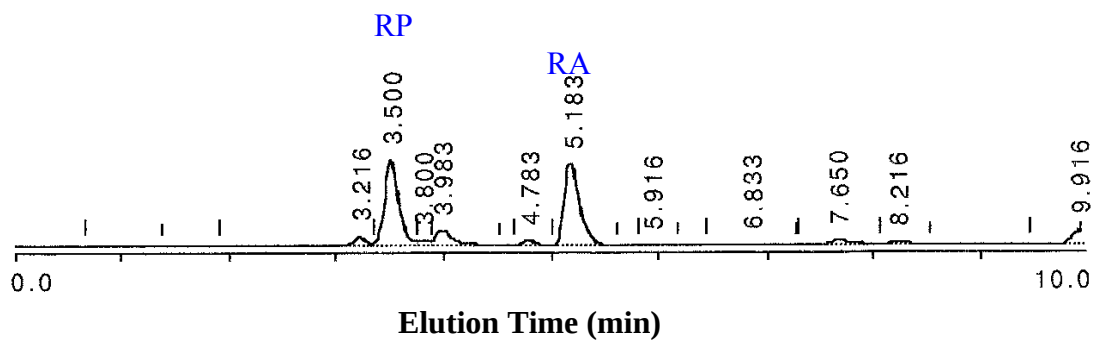
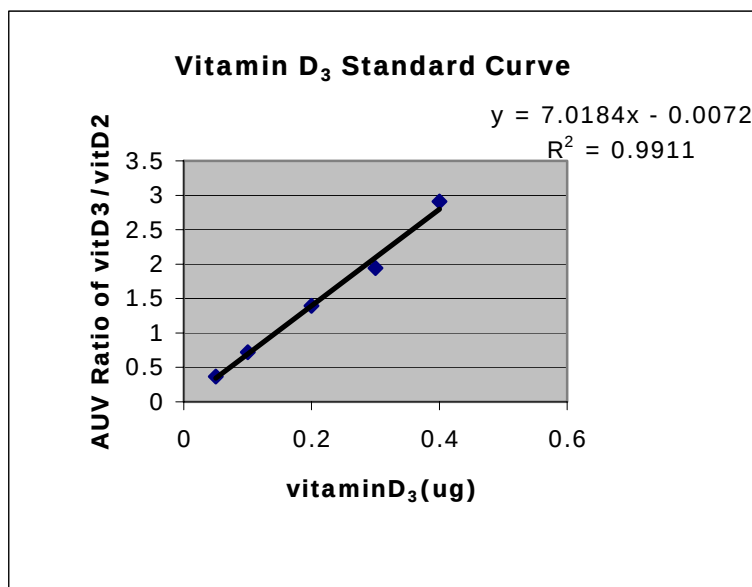


Figure 2. 4 HPLC chromatogram of vitamin A palmitate in 50 mg β -LG-vitamin A powder
(with retinyl acetate as internal standard. RP:retinyl palmitate; RA:retinyl acetate)

II. β -lactoglobulin and Vitamin D complex

The quantity of vitamin D in the powder was calculated based on the standard curve shown in Fig. 2.5 using the ratio of peak area of vitamin D₃ to vitamin D₂ in the HPLC chromatographs. The chromatograms showed that the powder complex with lactose (Fig. 2.6) had a slightly higher amount of vitamin D content than did the one without lactose (Fig. 2.7), comparing same amount of powders. But the difference was not as great as it was for the β -lactoglobulin-vitamin A complex in terms of vitamin content between the powders with and without lactose.

Figure 2. 5 Standard curve of vitamin D₃ with vitamin D₂ as internal standard



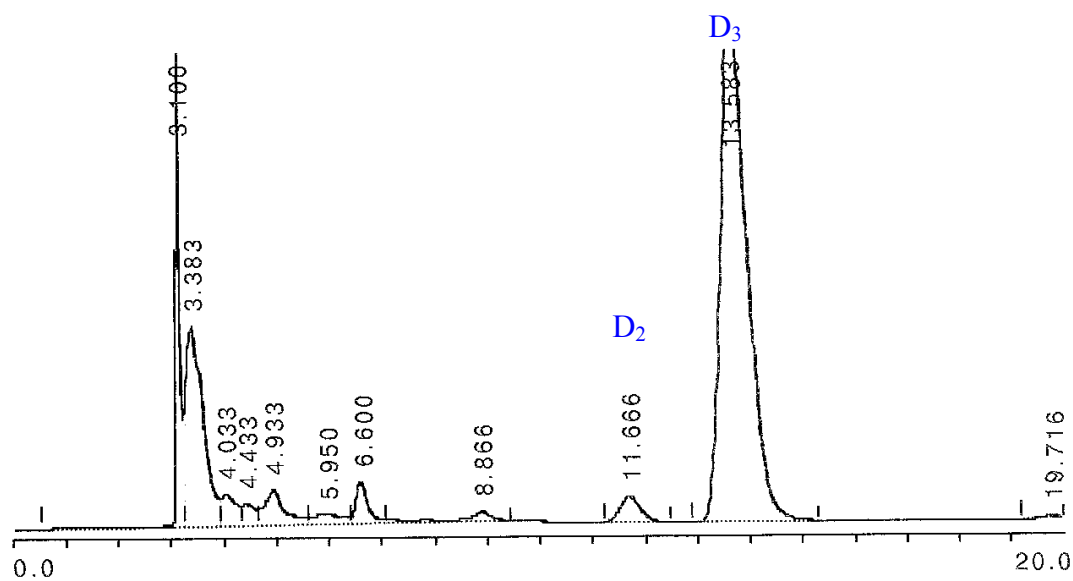


Figure 2. 6 HPLC chromatogram of vitamin D₃ in 50 mg β -LG-vitamin D-lactose powder
(with vitamin D₂ as internal standard)

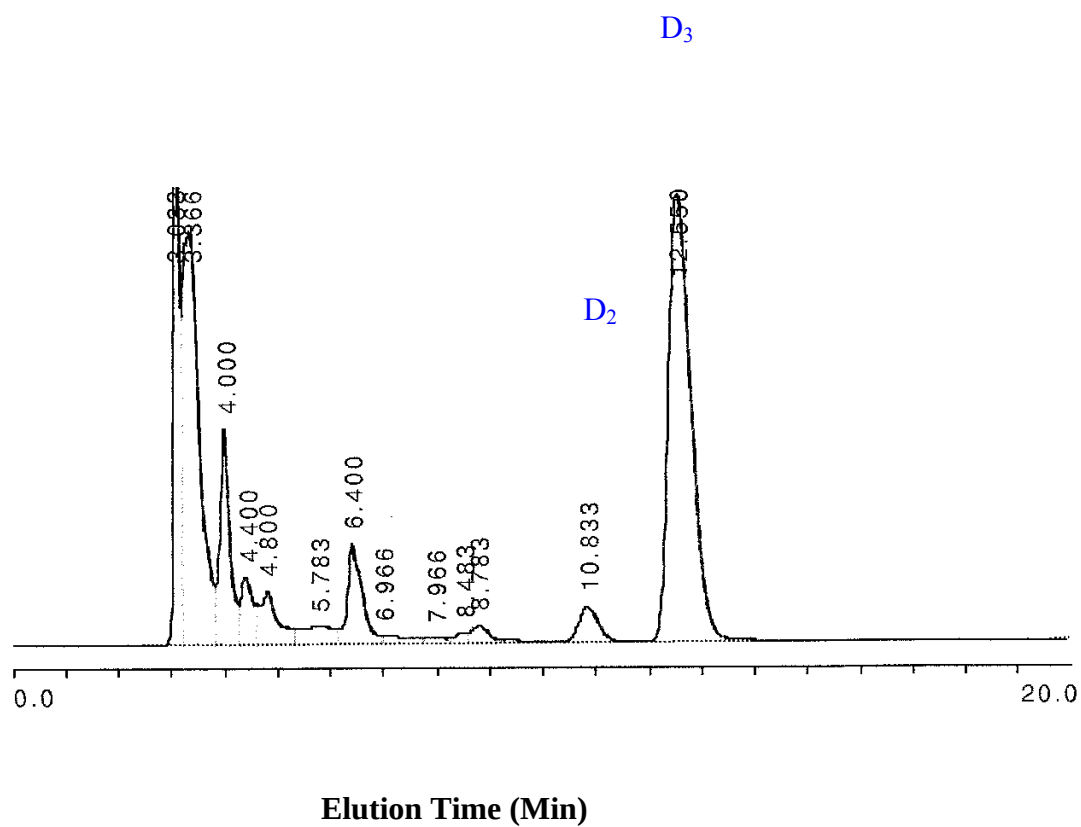


Figure 2. 7 HPLC chromatogram of vitamin D₃ in 50 mg β -LG -vitamin D powder with vitamin D₂ as internal standard

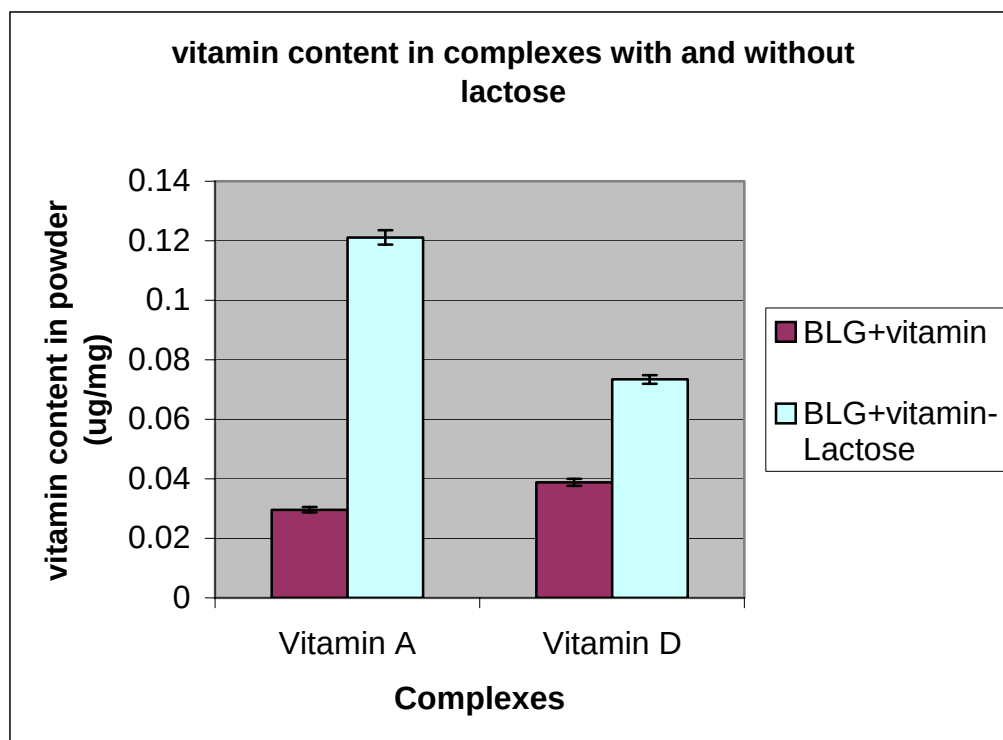
The spray dried powders were also analyzed for the protein content using the BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL). The results showed that the ratio of protein to lactose remained the same as the original amount before the spray drying (1:5, 17%).

As the chromatograms (Fig. 2.3, Fig. 2.4, Fig. 2.6, Fig. 2.7) from both complexes show, there were differences in vitamin contents. Table 2.4 lists the quantity of vitamin in each powder calculated from the chromatograms based on the standard curve (Fig. 2.2, Fig. 2.5). Vitamin A content in the β -LG-vitamin A-lactose complex is 4-5 time higher than the one without lactose, and the vitamin D content in the β -LG-vitamin D-lactose complex is 1-2 times higher than the complex without lactose. Fig. 2.8 clearly reflects the differences in vitamin quantities.

Table 2. 4. Vitamin content before and after spray drying (mean $\pm$ SD of duplicate samples)

Complexes	Vitamin A Original (ug/mg)	VitaminA in powder (ug/mg)	Vitamin D Original(ug/mg)	VitaminD in powder (ug/mg)
β-LG+vitamin	32.06	0.0294 $\pm$ 0.0002	22.5	0.0377 $\pm$ 0.0011
β-LG+vitamin- Lactose	5.34	0.1211 $\pm$ 0.00005	3.75	0.0611 $\pm$ 0.0123

Figure 2. 8 Comparison of vitamins A and vitamin D content in complexes with and without lactose



2.4 Discussion

High temperatures are considered to be detrimental to protein structure since they can lead to denaturation and coagulation. However, if the protein solution is not heated beyond a certain temperature and its pH is relatively low, denaturation will be probably reversible (Lapanje, 1978). The thermal stability of β -lactoglobulin under the spray drying condition will certainly affect the structure and conformation of the molecules and therefore affect the binding of vitamins. Fessas et al. (2001) investigated the thermal unfolding of monomeric and dimeric β -lactoglobulins at neutral pH. Spectroscopic studies demonstrated that irreversible modifications of the bovine β -LG structure occurred upon thermal treatment above 65-70 °C. As the temperature increases, the dissociation of dimer into monomer increases, which is reflected in the changes of circular dichroism (CD) spectra. Fessas et al. (2001) also observed decreased binding of hydrophobic probes at the protein surface as temperature rose in concurrence with dimer dissociation. These results indicated that the drying temperature used during the spray drying of the β -lactoglobulin-vitamin complex should remain below the denaturation temperature of β -LG.

However, the binding of retinol and fatty acid to β -LG may increase the heat stability of the protein and its resistance to denaturation (Puyol et al., 1994). The evidence from differential scanning calorimetry (DSC) indicated that the binding of palmitic acid and retinol increased the denaturation temperature by 8 and 4 °C respectively from 70°C.

De Wit (1981) showed that the addition of lactose to whey protein shifts the transition temperature of β -LG from $\sim 73^{\circ}\text{C}$ to several degrees higher. Furthermore, trehalose, a natural sugar capable of withstanding extreme conditions of low water activity and/or thermal stress, can stabilize the conformation of β -LG during drying (Murray and Liang, 2000). The results from the spray drying confirmed the importance of sugar in protecting the degradation of vitamin A and vitamin D during the spray drying.

Organic solvents such as alcohol that have lower dielectric constants than water can lead to protein denaturation, aggregation, or even precipitation (Lapanje, 1978). The use of ethanol to dissolve the retinyl palmitate and vitamin D precipitated a small amount of β -lactoglobulin from the water upon mixing the vitamins into the protein solution. Ethanol could cause slight alteration of the conformation of β -lactoglobulin and affect the binding. Thus, the concentration of the ethanol used to dissolve vitamins in the complex solution must be kept at a minimum level.

Vitamin A is subject to isomerization and nutritional loss during the processing of foods. Conversion of all-trans isomers to cis-isomers occurs easily. Light, acids, metals, lipooxygenase, and heat processing, acting independently or sometimes synergistically, can produce rapid isomerization (Ball, 1988). When spray dried, vitamin A is subject to heat destruction as well as oxidization because of the use of hot air in drying the products. So, the spray drying of vitamins is usually encapsulated in some forms of oil. With the protection of β -lactoglobulin when bonded as a complex, vitamin A could be encapsulated and exposed less to high temperature and air. Vitamin D, however, is considered to be relatively stable

during the spray drying of fluid milk into powder (Indyk et al., 1996) and the processing factors are less detrimental to vitamin retention. Fig. 2.8 indicated that the lactose had a greater protective effect on β -LG –vitamin A complexes than the vitamin D complex.

However, the spray-dried powder contained vitamins at only 0.1-3% of the original amount added to the protein (Table 2.4). In other words, the recovery rate of the vitamins from both products was very low. Several factors may contribute to the poor recovery of vitamin. Because vitamin was pre-dissolved in limited quantity of ethanol before it was transferred into the protein solution with the pipette, there could have been some loss of the vitamin during the transfer. It could also be poor binding of vitamin to β -LG in a solution containing no salt and buffer (amount bound to β -LG was not determined from the binding study, Fig. 2.1) with different concentration from the reported study (Wang et al., 1999). The unbound vitamin could adhere to the vessel or be pumped into the spray dryer with the solution but degraded by heat. Loss and isomerization during the extraction is another possibility.

During the spray drying of the complexes with lactose, one might suspect a browning reaction between the protein and sugar under heating conditions to occur, with the powder turning a brown color. However, the powder did not show any discoloration and its appearance was a fine, white free flowing powder. The Maillard reaction is complicated and can be influenced by several factors, such as water activity, temperature, pH and concentration of reactants. The result might be due to the fact that the major reactive amino acid lysine in β -LG was involved in the binding of vitamin and no free amino acids were available to react with sugar. The atomization and quick evaporation of the water may also

reduce the reaction. However, this may not rule out a possibility of a browning reaction occurring during long time storage if the powder has moisture and is stored at room temperature.

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Chapter 3 Fortification of a Lemon-Lime Soda with the β -Lactoglobulin-Vitamin A Complex

3.1 Introduction

Fortified dairy products are major sources of vitamin A. Current US regulations, as specified in the Pasteurized Milk Ordinance, require vitamin A fortification of all reduced fat fluid milk products (Murphy et al., 2001). Unfortified whole milk is considered to be a good source of vitamin A because vitamin A is primarily associated with the fat phase of the milk (Zahar et al., 1995). With the removal of fat in reduced and nonfat milk, the addition of vitamin A is essential to maintaining the daily requirements for the nutrient.

Vitamin A is susceptible to degradation, especially when milk is packaged in transparent containers that are exposed to light (De-Man, 1981; Gaylord et al., 1986; Senyk and Shipe, 1981). A study done by Whited et al. (2002) examined the detrimental effect of light exposure to the nutritional value and flavor quality of milk and showed that among whole, reduced and nonfat milk, more fat was associated with less loss of vitamin. The greater susceptibility of vitamin A in nonfat milks exposed to light may contribute to the higher prevalence of underfortification. In this study, we examined how the light exposure will affect the vitamin A content in the form of β -LG-vitamin A complex and whether the β -LG will shield vitamin A from the light effect in a fat-free environment or not. The study was to find out whether the complex might reduce the degradation of vitamin A by light.

To further assess the effect of UV light on the degradation of vitamin A in the β -lactoglobulin-vitamin A complex, a separate experiment was performed to investigate when the degradation started to occur.

3.2 Materials and Methods

One 2-liter bottle of lemon-lime soda was purchased from a local Food Lion store. Bio-Pure β -lactoglobulin was provided by Davisco Foods International Inc. (Le Sueur, MN). All-trans retinyl palmitate in fish gelatin matrix was purchased from Sigma Chemical Co. All other chemicals are the same as in the spray drying of β -lactoglobulin-vitamin A complex.

3.2.1 Preparation of β -lactoglobulin-vitamin A complex solution

Twenty g of Bio-Pure β -lactoglobulin was mixed in DI water to make a 10% solution in a volumetric flask. Two g all-trans retinyl palmitate in fish gelatin matrix containing 500,000 IU vitamin A per gram were added into the homogenized protein solution to reach molar ratio of 1:1 of protein to vitamin A palmitate. The beaker containing the solution was covered with aluminum foil and then placed on a stirring plate. The mixture had been stirred for ~6 hrs before it was refrigerated overnight. The complex solution stored overnight was separated into 15 mL centrifuge tubes and centrifuged for 25 minutes in a bench top centrifuge. However, the solution was too viscous and formed a suspension that was hard to purify from the gelatin matrix. Whatman filter papers (#4) were then used to filter the suspended particles out of the solution in the centrifuge tubes. The purified complex solution was pooled in a brown colored jar and stored in a refrigerator. The final concentration of vitamin A in the solution was determined with HPLC.

3.2.2 Analyzing vitamin A in the complex solution

Five hundred μL purified β -lactoglobulin-vitamin A complex solution was pipetted into a fifty mL volumetric flask and filled to volume with DI water to make a 1:100 dilution. Two mL of the dilution solution (20 μL of original purified complex solution) was used for extraction and HPLC analysis. The standard curve with the linear algorithm $y=0.648x + 0.0337$ ($R^2=0.9987$) was used to calculate the concentration of vitamin A. It was 0.19 mg/mL of the concentrated complex solution.

3.2.3 Fortifying process

The 2 liter soda was opened to test the pH (~ 3.04). Thirty-five mL β -lactoglobulin-vitamin A palmitate solution (pH ~ 7) was added into the bottle. The bottle was capped and rotated gently to avoid heavy foaming. The pH of the mixed soda was re-tested and it increased slightly. Because of the concerns that frequent opening of the bottle to sample during the one-week study period would cause the growth of bacteria in soda due to the high sugar content, a cold sterilization was performed that separated the beverage into aliquots in 50 mL sterilized cell culture flasks. Fifty mL plastic sterile syringes were used to pull a portion of the mixed soda in the 2 liter bottle and filtered through a syringe filter attached to a bell which sits on top of the opening to the cell culture flasks to remove possible bacteria. Each flask held less than the full volume to allow the headspace for carbon dioxide. The process was lengthy because the build-up of back pressure from the gas made the filtration difficult and the filter also need to be replaced often because the viscous solution caused the blockage.

3.2.4 Stability study

I. Seven days under fluorescent and UV light

All samples in the cell culture flasks were randomly divided into 3 groups and received different treatments in the same room.

1. Storage in dark, served as control group. Samples were placed in a cardboard box and wrapped in aluminum foils.
2. Fluorescence light. Samples were placed at a distance of 49 inches to the lights at room temperature. The intensity of the lights measured with a light meter (Sper Scientific, model 840006) at this distance was 383-396 lx.
3. UV light box Chromato-VUE, Model C-3 (Ultra-Violet Product Inc. San Gabriel, Calif.). Samples were placed inside at a distance of 11.5 inches to the long wave length light source. The intensity was measured at this distance with same meter used for the fluorescence lights. There were differences between the middle and two ends of the box. Facing the opening of the box, the intensity on the left was 28.0 lx, right was 44.7 lx and the middle was 83.4 lx. Samples were rotated frequently to avoid imbalance in light intensity.

Two samples were randomly taken from each group for vitamin A analysis every day for seven (7) consecutive days. Two random samples before the treatments were analyzed for vitamin A content to serve as the baseline.

II. One day under UV light

One 2-liter lemon-lime soda was purchased from a local Food Lion store. All other materials and equipment used are the same as in the previous experiment. The fortification process was performed the same way as in the previous experiment using the same complex solution. No cold sterilization procedure was performed. The 2 liter bottle was laid flat inside the UV box. Samples were taken at 0, 2, 4, 6, 8, 12 hr of UV exposure from the 2 liter bottle for vitamin A analysis.

3.2.5 Vitamin A analysis

Vitamin A was quantified based on the concentration of all-trans retinyl palmitate using the method described in Chapter 2.

3 .3 Results and Discussion

I. Seven day under fluorescent and UV light

The soft drink experienced a loss of carbonation during the experiment. It occurred during the opening of the soda bottle to test the pH and the major loss was during the lengthy cold sterilization procedure. The filtration step in the procedure was difficult to perform and may also have caused long exposure time of vitamin to air. There was also concern that some quantities of protein could be held back by the filters due to the clogging caused by the mixed beverage.

During the experimental period, a great amount of leakage was detected in most flasks, mostly after the first night. The leakage could be caused by pressure from carbon dioxide in

the flasks. The results indicated that the samples kept in the dark didn't show degradation until day 4 (96 hr) with a reduction of about 50% in vitamin A palmitate content, and stayed at a constant level for the remaining days (Fig. 3.1).

UV light had a detrimental effect on the vitamin A degradation in the samples at the wavelength used. The long wavelength is close to the maximum absorption (325 nm) in the spectra of all species of retinol, including retinyl palmitate. The samples had lost nearly all the vitamin A after one day exposure to the UV source. The one day under UV light experiment was conducted (Fig. 3.2) to further show the progress of the vitamin A degradation within one day.

The fluorescent light had less detrimental effect than UV light on the vitamin A degradation. The samples started showing degradation after 24 hr with a reduction of about 70% and remained at a constant level for the rest of experiment days.

The result is similar to several studies on light degradation of vitamin A. Whited et al. (2002) concluded that vitamin A loss was directly influenced by the length and intensity of light exposure and inversely influenced by the fat content of the milk. Study from Bartholomew and Ogden (1990) also presented some evidence that vitamin A added to milk was more light sensitive than indigenous vitamin A.

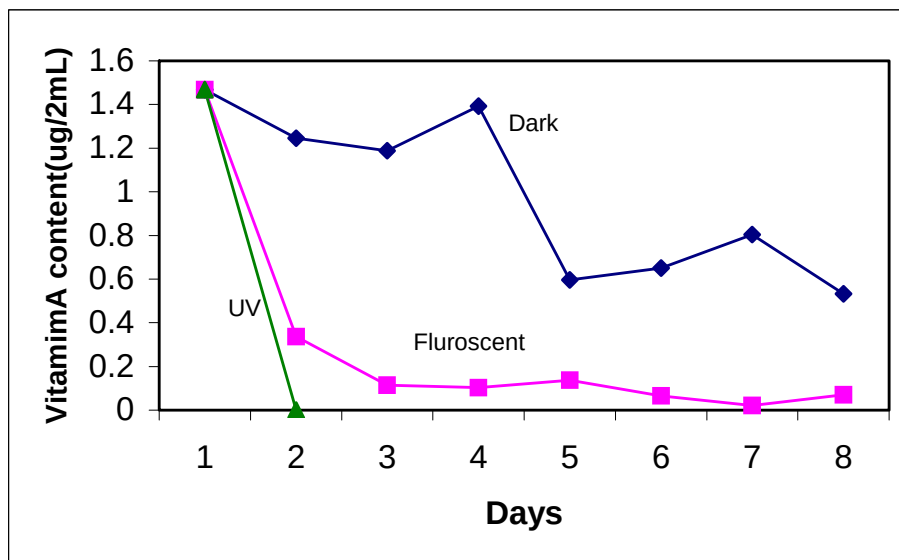


Figure 3. 1 Vitamin A degradation in lemon-lime soda under dark, fluorescence and UV light

From the results obtained during the 7-day study, there was no evidence concluding that beta-lactoglobulin had a protective effect for the vitamin A palmitate against the lighting sources used in this study. The degradation of vitamin A may not only be caused by light due to the fact that samples kept in the dark also experienced vitamin A reduction after 4 days. Many factors may contribute the loss of vitamin A: 1. β -lactoglobulin dissociates into monomers at low pH and the changed conformation affected the binding affinity;

2.Oxidation of vitamin A caused by leakage of flasks and exposure to air during the entire study time; 3. Vitamin A is very acid unstable and rapid isomerization occurred; 4. The unbound vitamin A dissociated from the complex degraded quickly under all the above conditions.

However, other aspects of study may be worth pursuing. One study performed by Carlotti et al. (2002) concluded that the use of butylated hydroxy toluene (BHT) protected retinyl palmitate from degradation induced by light and heat in the study of effects of sunscreen when exposed to UV radiation. The addition of the antioxidant BHT into the complex may protect retinyl palmitate from light degradation and contribute longer shelf stability to fat free foods fortified with such complex.

II. One day under UV light

Vitamin A can be quite unstable at pH values ≤ 4.5 . Low pH causes both isomerization of all-trans-retinol to cis form and de-esterification of vitamin A esters to the more labile free retinol (Erdman et al., 1988). Cis-trans isomerization can be directly promoted by light, the relative amount of different cis isomers depending on the wavelength and the light permeability of the containers (Murphy et al., 1988). To illustrate, Fig 3.2 shows that vitamin A content was reduced by 70% after 2 hr of UV exposure at the highest intensity of 83.4 lx. Progressive loss of vitamin A was observed for every 2 hr afterwards at a reduction rate of 5-30%. This study confirmed the previous result that the UV was detrimental to the vitamin A in the complex. The use of antioxidant such as BHT to protect the degradation of vitamin A can be further studied.

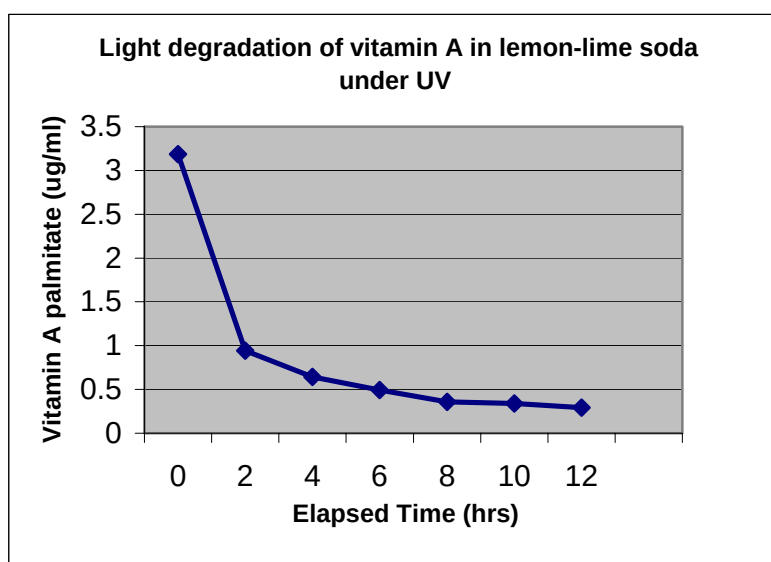


Figure 3. 2 Vitamin A degradation under UV in lemon-lime soda fortified with β -lactoglobulin-vitamin A complex

Conclusion of the study

The study investigated the possibility of developing a spray dried complex utilizing the low cost ingredient β -lactoglobulin as a carrier for fat soluble nutrients such as vitamin A and vitamin D. The processing parameters that influenced the stability of the complex were

studied. The addition of lactose into the complex helped yield higher amounts of vitamins and increased the solubility of the product. It is possible to use the method to further develop a spray-dried complex on a large scale after higher recovery rates of powder and vitamins can be achieved. The complex can be added as a dry ingredient into baking goods, nutrition bars and snacks. It can also be easily mixed into beverages such as sports drink to boost the nutritional contents.

The current study did not strongly support the conclusion that the complex can effectively carry the vitamins under heat processes, light exposure and low pH food systems.

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