Chapter I

Introduction

Recently, there has been considerable interest worldwide in the development of food colorants from natural sources. One indication of this is the number and distribution of food colorant patents issued in the years 1969 through 1984. The number of patents issued in the last five years from 1987 frame is approximately equal to that of the preceding ten-year span, 1969-1978. In addition, natural colorants are attracting more interest than synthetic colorants, since there were 356 patents on natural sources compared to 71 on synthetics (Francis,1987).

The algae biliproteins, sometimes called phycobiliproteins, are a group of pigments which occur in organisms of the division Rhodophyta (red algae) and cyanophyta (blue-green algae) where they function as light absorbers. The absorption spectra of the major biliproteins lead to the classification of these proteins into three groups: phycoerythrins, red biliproteins with

the phycoerythrobilin chromophores having absorption maxima at 490-570 nm ; phycocyanin, blue biliproteins with the phycocyanobilin chromophores having a major absorption band at approximately 620 nm ; and allophycocyanin, the blue biliprotein with a sharp absorption maximum at 650 nm. In primary structure all of the major biliproteins are oligomers of an $\alpha\beta$ monomer, where α and β are dissimilar polypeptide chains of approximately 160-180 residues (Glazer,1984). Phycobiliproteins are intensely colored. Thus, phycocyanin and phycoerythrin could be utilized as natural pigments for food, drug and cosmetics industries to replace the currently used synthetic pigments that are suspected of being carcinogens. Pigment preparations soluble in either water or alcohol can be prepared and are suggested for use in chewing gum, frozen confections,sherbets confectioneries, candied ices (Francis, 1987) and mix in liquor (Dainippon Ink and Chemicals Inc, 1980) .Ten patents were applied in the year 1979 through 1987 , of three deal with Spirulina (Francis,1987) . Phycocyanin from Spirulina has already been commercialized by Dainippon Ink & Chemicals of Japan under the name of "Linablue". The product is an odorless nontoxic blue powder with a slight sweetness and has brilliant blue with a faint reddish fluorescence in water. In another patent, Dainippon Ink describes the

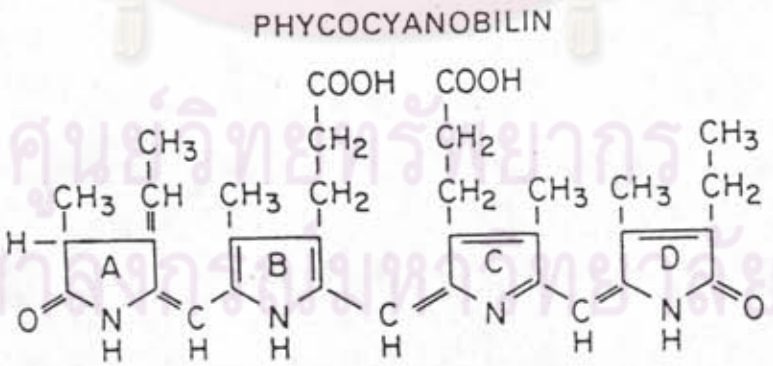
buffer extract of Spirulina, the blue pigment obtained being used for eye shadow, eye liner and lipstick. Since it is not water-soluble, it does not run when it is wet by water or sweat and does not irritate the skin (Richmond, 1986). For other applications, the phycobiliproteins have been widely applied as fluorescent tags in a variety of analytical and diagnostic procedures particularly in multiparameter fluorescence activated cell analyses (Glazer, 1988).

The thylakoid membranes of phycobilisome, uncommon with those of higher plants, contain Photosystem I (PS I) and Photosystem II (PS II). There are, however, some noteworthy differences in the composition and organization of photosynthetic structure in these organisms and in green plants. Higher plants contain both chlorophyll a and chlorophyll b. The latter is a prominent component of the antenna of PS II (Glazer,1983). Cyanobacteria and red algae contain only chlorophyll a. However, additional light harvesting capacity is provided by a family of brightly colored proteins (biliprotein) present in large amounts. The grana regions , formed of appressed thylakoids, so characteristic of higher plant chloroplasts, are absent in cyanobacteria and red algae. Instead, the outer surface of their thylakoid membranes is studded with

regularly spaced phycobilisomes multiprotein complexes of which biliproteins make up over 85 % (Tandeau de Marsac and Cohen-Bazire,1977 ;Yamanaka, Glazer and Williams, 1978). The strong absorption bands of the major biliproteins lie in the region of 470-650 nm, whereas those of the chlorophyll a complexes are at approximately 430-440 nm and 670 nm. This separation of the major absorption bands permits analysis of the relative contributions of the biliproteins and of chlorophyll a to the action spectra of PS I and PS II (Glazer,1984). It is found that in cyanobacteria, P-700, the reaction center of PS I, is associated with an antenna of some 140 chlorophyll a molecules. The reaction center of PS II is associated with only approximately 20-50 chlorophyll a molecules, but receives most, if not all, of the energy harvested by phycobilisomes (Glazer,1984). Membrane-bound pigment-protein complexes that function in harvesting or absorbing radiant energy and transferring it to the photosynthetic reaction centers are found in all photosynthetic organisms. In prokaryotic cyanobacteria and eukaryotic red algae, the major light-harvesting complex is the phycobilisome, a water-soluble, supermolecular structure attached to the stromal surfaces of the thylakoid membranes (Conley, Lemaux and Grossman, 1988). The phycobilisome is far more efficient

than any man-made device for the transduction of solar energy. Depending on their organismal origin, phycobilisomes contain between 300-800 tetrapyrrole chromophores which absorb light over much of the visible spectrum. The design of the phycobilisome is such that excitation energy is delivered from any one of these many chromophores to a reaction center in the photosynthetic membrane with an efficiency approaching 100 % (Glazer, 1984). The characteristic of phycobiliprotein is conferred by covalently linked linear tetrapyrrole chromophores (bilins).

Figure 1 Chemical structure of phycocyanobilin



Crespi and Smith (1970) proposed that phycocyanobilin was doubly linked to the polypeptide chain, one bond being an ester involving the β-carboxyl group of an aspartyl residue and the hydroxyl group of the enol form of ring A of the bilin (see Figure 1) and the other bond a thioether derived from a cysteine side-chain to the methine carbon of the ethylidene group at position 2 of ring A of phycocyanobilin (Glazer,1976). The intensely colored phycobiliproteins allophycocyanin, phycocyanin and phycoerythrin have two dissimilar polypeptide chains (α and β) that vary in molecular weight from 17 to 22 kDa. The number of chromophores varies for the different phycobiliprotein subunits (Conley et.al., 1988). As shown in Figure 2, for example , R-phycocyanin , $(\alpha\beta)_3$, contains six phycocyanobilin (PCB) and three phycoerythrobilin (PEB) groups per trimer; each α subunit carries one PCB, whereas each β subunit carries one PCB and one PEB. The absorption and fluorescence emission maxima are given for the higher aggregate of each of the biliproteins, i.e., in the case of R-phycocyanin , the values refer to the $(\alpha\beta)_3$ aggregate.The precise values differ slightly with the organismal source of the protein (Glazer, 1984).

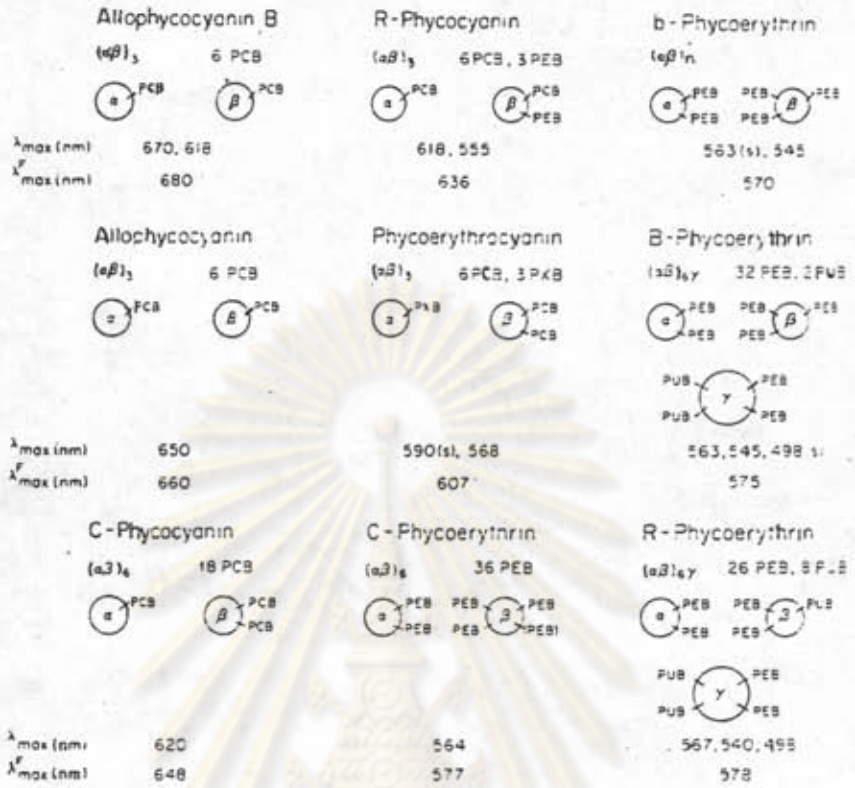


Figure 2 The nature and distribution of bilin prosthetic groups among the subunits of the various biliproteins and the numbers present in higher aggregates of each protein

Phycobilisomes have unusual stability properties. They are stable in concentrated solutions of certain salts (for example, 0.65-1.0 M sodium potassium phosphate or 0.8 M Na_2SO_4 at pH 7-8, but dissociate into a mixture of their constituent complexes

upon dilution of the salt. Either partial or complete dissociation into water-soluble subcomplexes can be achieved by appropriate manipulation of conditions. The final and perhaps most important feature is that the open-chain tetrapyrrole chromophores (bilins) of the biliproteins are all covalently linked to the polypeptide chains through stable thioether bonds (Glazer,1984).

The basic building block of all of the biliproteins is a monomer , $\alpha\beta$ made up of two different polypeptide chains. As documented later, in phycobilisomes the biliproteins of the rod (phycoerythrins, phycoerythrocyanins,phycocyanins) are present as hexameric, $(\alpha\beta)_6$, complexes with linker polypeptides, and the complicated allophycocyanin-containing complexes of the core can be broadly described as trimeric (Glazer, 1984).

Depending on the source of the protein, the pH ,ionic strength and protein concentration, the biliproteins are isolated in any one of a number of aggregation states ,which include $\alpha\beta$, $(\alpha\beta)_2$, $(\alpha\beta)_3$, $(\alpha\beta)_4$, and $(\alpha\beta)_6$ (Glazer, 1984). Native phycocyanin is unstable at pH values below 4.0 and higher than 9.0 (Glazer, 1976).

There has been increasing interest for the study of Spirulina which is commonly known in Thailand as Saa Rai Kliew Thong (its meaning is golden spiral algae which may be so called because of its appearance as spiral shape and the high value as gold). Spirulina is a member of the prokaryotic algae and belongs to genus Spirulina of the Oscilatoriaceae family. Spirulina is a ubiquitous organism. After first isolation by Turpin in 1827 from a fresh water stream, species of Spirulina have been found in a variety of environments : soil, sand, marshes, brackish water,sea water and fresh water (Ciferri,1983). In general, the higher the pH, the conductivity and predominantly sodium carbonate, the more marked was the predominance of Spirulina (Ciferri, 1983). Spirulina is a multicellular, filamentous cyanobacterium. Under the microscope, Spirulina appears as blue green filaments composed of cylindrical cells arranged in unbranched, helicoidal trichomes. The filaments are motile,gliding along their axis, without heterocysts. The helical shape of the trichome is characteristic of the genus but the helical parameters (i.e., pitch length and helix dimensions) vary with the species and even within the same species, differences have been observed in these parameters or may be induced by changing the environmental conditions such as growth temperature (Ciferri,1983). Spirulina has granular cytoplasm usually

containing gas vacuoles and easily visible septa (Glazer, 1984). Sexual reproduction being absent, Spirulina reproduces by binary fission. (Rippka, Deruelles, Waterbury, Herdman and Stanier, 1979). Spirulina platensis is characterized by a diameter of the helix of >35 to 50 mm and pitch of 20 mm (Ciferri, 1983). Electron microscope of ultrathin sections of Spirulina platensis revealed that the cell wall is composed of possibly four layers . The most external or outer membrane layer (L-IV) is composed of material arranged linearly in parallel with the trichome axis and is considered analogous to that present in the cell wall of gram-negative bacteria. Layer III is possibly composed of protein fibrils wound helically around the trichomes, whereas the peptidoglycan-containing layer (L-II) folds towards the inside of the filament,giving rise, together with a putative fibrill as inner L-I, to the septum separating the cells. The septum separating the cells would be composed of the peptidoglycan layer only. The septum appears as a thin disk,folded in part. This fold covers a portion of the septum surface, and its extent seems to be related to the pitch of the trichome; the larger the pitch, the smaller the folded area and vice versa (Drews and Weckesser, 1982).

Spirulina has high nutritional value, high

protein content of from 55 to 77 % (Zafaralla, Barril, Vidal, Serrana, Aguila and Tansinsin, 1985), recommended as health food and it is to serve as a vitamin food such as vitamin B1,B2 and B12 contents being relatively high. Today, it is better known as a high protein food supplement for human consumption. Spirulina contains all of the essential amino acids(Ciferri, 1983). The amino acid pattern of the alga compares favorably with the FAO standard (Zafaralla et.al., 1985). Compared with other algal SCPs like Chlorella and Scenedesmus, Spirulina is richer in crude protein as shown by its maximum reported crude protein content of 77 % as against those of the other algae which range from 40-55%. It compares favorably with soya bean, an animal protein feed with 35-40% protein. Spirulina has an advantage because of its high digestibility. This characteristic is a plus factor as it does away with the necessity of treating algal cells in order to counteract the digestibility problems presented by the tough cell wall as is the case in Chlorella SCP. Among the three most popular algae SCPs Spirulina contains the least amount of lipids while Chlorella has the most. Nevertheless, the former contains all of the essential fatty acids . Spirulina has phycobiliprotein about 60 % of total soluble cellular protein and low nucleic acid content about 4.2-4.4 % (Ciferri, 1983). Spirulina consists of 0.85 %

chlorophyll, 0.23 % beta-carotene, 0.12-0.15 % xanthophylls and phycobilin 12-15 % (Santillan, 1982). As a health food Spirulina SCP eliminates hunger pangs, relieves the stress of light dieting, satisfies the needs of weight watchers and cleanses the body of excess fat (Zafaralla, 1985). Furthermore, due to its high B12 content , a vitamin with a naturally chelated iron compound, Spirulina curbs stress among vegetarians. Spirulina mixed in regulated amounts with the normal diet of athletes and body builders improves their strength and stamina. This benefit which is derivable from the alga has been well exploited by advanced countries like the United States, Great Britain and Japan where Spirulina is sold as protein food (Zafaralla et.al., 1985). In Thailand the weather is very suitable for cultivation of Spirulina. The Siam Algae Co.Ltd, has been commercially producing Spirulina as a health food and also as a fish feed for fancy carps since 1978 (Richmond, 1986). Mass culture of Spirulina yields appreciably higher amounts of protein per unit area per unit of water than conventional crop plants (Richmond, 1986).

Since phycobiliproteins are the major proteins in cyanobacteria,a lot of work have been carried out to study these proteins. Allen and Smith (1969) were the first to point out the possibility that phycocyanin may

be a nitrogen storage compound in the cell and nitrogen deficiency reduce phycocyanin in Anacystis sp.. Later on Boussiba and Richmond (1980) showed that c-phycocyanin serves as a nitrogen source in Spirulina platensis during nitrogen starvation. De Loura (1987) reported that nitrogen deficiency during growth causes a change in pigment composition but no significant changes in whole cell lipid and fatty acid composition of the two cyanobacteria, Pseudanabaena sp. and Oscillatoria splendida. Nitrogen deficiency does not affect the cellular content in chlorophyll a , but it causes a selective loss in phycobiliproteins; carotenoid contents increase with phycocyanin depletion (De Loura ,Dubacq and Thomas, 1978).

Engelmann and Gaidukov (1902) reported that the pigmentation of certain cyanobacteria can be modified by light quality: the growth of such organisms behind a series of filters of different colors cause increased light absorption by the cells in the specific spectral region to which they have been exposed, a phenomenon which these authors termed " complementary chromatic adaptation". However, the existence of such a chromatic response was questioned by several investigators, who were unable to repeat the observations of Engelmann and Gaidukov on other strains of cyanobacteria.

The controversy was resolved by Boresch (1922) who was the first to confirm the results of Engelmann and Gaidukov and who showed that the chromatically induced change in the color of the cells is largely attributable to a change in phycoerythrin - phycocyanin ratio. Phycocyanin predominates after growth in red light and phycoerythrin predominates after growth in green light, the transition occurring at a wave length of approximately 590 nm. Bogorad (1975) also studied chromatic adaptation in two filamentous cyanobacteria, Tolypothrix tenuis and Fremyella diplosiphon. Two main conclusions have emerged from this work. The chromatically induced modification of the phycoerythrin-phycocyanin ratio involves de novo protein synthesis, not turnover (Emerson and Lewis, 1942). There is evidence, both indirect and direct, which suggests that the relative rate of phycoerythrin and phycocyanin synthesis are controlled by a regulatory pigment analogous to, but not identical with, phytochrome. This regulatory pigment appears to exist in two forms, interconvertible by irradiation with specific wavelengths of light (Tandeau De Marsac, 1977).

Tandeau De Marsac and Cohen - Bazire (1977) isolated phycobilisomes from eight different species of cyanobacteria and found that light quality affected

differentially the rate of phycoerythrin and phycocyanin synthesis ; phycobilisomes prepared from algae after growth in white, red and green light differed markedly in their phycobiliprotein composition. "Green-light " phycobilisomes had a high PE:PC ratio, "white-light" phycobilisomes had a somewhat lower PE:PC ratio, and "red-light" phycobilisomes were virtually devoid of phycoerythrin. These light-induced modifications of the major phycobilisomal light-harvesting protein were accompanied by marked changes in the relative concentrations of the colorless group II polypeptides (Bennett and Bogorad, 1971).

Reunjitchachawaly, Vetayasuporn, Chitnumsub and Tanticharoen (1988) studied optimal condition to produce pigment from Spirulina sp. and found that concentration of NaCl less than 3.0 % did not effect growth of cells. However at 3.0 % NaCl chlorophyll a and phycocyanin contents were decreased to 33 % and 13 % respectively.

In this study, the multicellular cyanobacterium, Spirulina spp. was chosen as a source of biliprotein because Spirulina spp. contains a single type of phycocyanin (Anusuya, 1982). Furthermore, Spirulina can be found predominant in various environments in Thailand.

The aims of this thesis are :

1.to isolate Spirulina spp. with high growth rate from natural water .

2. to find out the optimal conditions for Spirulina spp. to produce high yield of phycocyanin .

3. to partially purify phycocyanin.