ALGAE AND THEIR BIOTECHNOLOGICAL POTENTIAL

Proceedings of the 4th Asia-Pacific Conference on Algal Biotechnology, 3-6 July 2000 in Hong Kong

Edited by

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Preface

The term 'algae' is a very difficult one to define. It may appear in textbooks of Botany, Zoology and Microbiology. In general, algae are organisms that include seaweeds and a number of single-celled and multicellular microscopic forms. Algae are ubiquitous; they inhabit oceans, freshwater bodies, rocks, soils and trees. There may be over 50,000 algal species on the earth.

Man's uses of algae have a long history. In China, marine algae were used as food as far back as 600-800 BC. In recent decades, there has been renewed interest in the utilization of algae as sources of health food and high-value chemicals and pharmaceuticals, and for aquaculture, agriculture and wastewater treatment. Even so, the biotechnological potential of algae is still far from fully exploited. With the aim of promoting algal biotechnology particularly in the Asia-Pacific region, the Fourth Asia-Pacific Conference on Algal Biotechnology was planned and successfully held in Hong Kong during 3-6 July 2000. The Conference attracted more than 250 participants from 30 countries and regions. Some 230 papers were presented at the Conference. Among them, 40 papers were selected after peer review. A part of the selected papers have been published in Journal of Applied Phycology (Vol. 13 No. 4). This book consists of another part of the selected papers, which deal with the various aspects of algal biotechnology with emphasis on the biotechnological potential of algae.

This book cannot be made possible without the help and effort of many. First of all we are indebted to the authors of the various chapters for their excellent contributions. Second, we would like to thank the reviewers whose critical comments and constructive suggestions have helped to improve the quality of this book greatly. Third, we would like to thank the generous support of the Innovation and Technology Commission of the Government of the Hong Kong Special Administrative Region. Finally, we would like to acknowledge the assistance of Martine van Bezooijen and the other staff at Kluwer in producing the book.

Feng (Steven) Chen
Yue Jiang

Hong Kong, September 2001

Contents

POLYUNSATURATED FATTY ACIDS: BIOLOGICAL SIGNIFICANCE, BIOSYNTHESIS, AND PRODUCTION BY MICROALGAE AND MICROALGAE-LIKE ORGANISMS

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1. Abstract

Growing interest in the nutritional and pharmaceutical importance of polyunsaturated fatty acids (PUFAs) has created an increasing demand for purified PUFAs. As the traditional sources are insufficient for satisfying this demand, alternative sources are being sought. Microalgae are a great source of many highly valuable products and they are considered a potential alternative for the large-scale production of PUFAs. Investigations have been actively carried out for screening of potential microalgal strains and development of feasible culture techniques for the commercial production of these vital compounds.

2. Introduction

Polyunsaturated fatty acids (PUFAs) (Fig. 1), especially those long-chain fatty acids in the n-3 (originally named ω-3) family, have entered the biomedical and nutraceutical arenas as they perform many vital functions in biological membranes and as precursors of a variety of lipid regulators of cellular metabolism (Berdanier, 2000; Hwang, 2000). The beneficial effect of n-3 PUFAs was first noticed by Dyerberg and Bang in the early 1970s. They reported that the Inuit (Greenland Eskimos) population had a lower incidence of heart disease than that of an equivalent population of Danes. They also found that these Greenland Eskimos had a favourable plasma lipid profile: with low levels of triglycerides, plasma cholesterol and very low-density lipoproteins (VLDL) and high levels of high-density lipoproteins (HDL) (Dyerberg *et al.*, 1975). As the Eskimos consume a large amount of marine mammals and arctic fish in their diet that are rich in n-3 fatty acids, n-3 PUFAs are presumed to be a major factor responsible for the healthy effect of fish oil. The report by Dyerberg *et al.* (1975) had spurred many investigators to perform epidemiological studies in other countries to investigate the beneficial effects of n-3 PUFAs on humans. It has become clear that dysfunctions of PUFA-derived eicosanoids may lead to illnesses and disorders including cardiovascular, respiratory, gastrointestinal, renal, dermal and immune diseases, cerebral and ocular underdevelopment, as well as carcinogenesis (Horrocks and Yeo, 1999). Other studies relating to PUFAs such as the biosynthesis mechanisms of PUFAs, exploitation of sources of PUFAs, and production of PUFAs have also been carried out extensively. This review

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first introduces the biological significance and beneficial effects of polyunsaturated fatty acids, then briefly describes the biosynthesis of PUFAs and finally discusses the various sources of PUFAs and the techniques involved in the microbial production of PUFAs, with emphasis on the biotechnological potential of microalgae.

all-cis-5,8,11,14-arachidonic acid (AA, 20:4n-6)

all-cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, 22:6n-3)

Figure 1. Chemical structure of two biologically important PUFAs: AA and DHA. Trivial nomenclature of PUFA: the first number denotes the number of carbon atoms. The number after the colon denotes the number of double bonds, and the number after n- denotes the position of the last double bond from the methyl end of fatty acids.

3. Significance of polyunsaturated fatty acids

3.1. BIOLOGICAL FUNCTIONS

Polyunsaturated fatty acids (PUFAs) are classified mainly into four families designated n-3, n-6, n-7 and n-9. The n-3 and n-6 families of fatty acids predominate in plants and animals. The parent fatty acid of the n-3 family is α-linolenic acid and of the n-6 family, linoleic acid. These precursor acids are elaborated into longer chains and more highly unsaturated fatty acids, for examples, arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) by a series of desaturations and elongations.

PUFAs are structural components of cell and organelle membranes (mainly as *sn*-2 phospholipids). They are crucial for regulating the membrane structure, fluidity, phase transitions and permeability as well as for the control of membrane-associated processes (Berdanier, 2000; Gill and Valivety, 1997). PUFAs also act as the precursors of many metabolites that regulate vital biological functions. In plants, PUFAs are converted by a variety of enzymes to various oxygenated compounds, acting as anti-infectives, wound-response mediators, chemotactic agents, aroma and flavour compounds (Gill and Valivety, 1997). In lower animals, such as insects and marine invertebrates, PUFA-derived metabolites mediate cellular processes and ecological responses including metamorphosis, reproduction, chemotaxis and immune function (Gill and Valivety, 1997).

In higher animals, long-chain PUFAs are precursors of a diverse series of oxygenated fatty acids termed 'eicosanoids' that are crucial to the development and the proper maintenance of homeostasis (Fig. 2) (Hwang, 2000). PUFA-derived eicosanoids in humans including prostaglandins, prostacyclins, thromboxanes and leukotrienes are produced through two main pathways: the cyclo-oxygenase and lipoxygenase pathways, each is catalyzed by a distinct group of enzymes (Fig. 3). These compounds have a short lifespan. They exert potent biological activities even at very low concentrations and they are linked to many physiological and pathophysiological syndromes (Gurr, 1999; Gurr and Harwood, 1991).

3.2. ESSENTIAL FATTY ACIDS: LINOLEIC ACID AND α-LINOLENIC ACID

Higher animals including humans are unable to produce fatty acids over C18 as they only possess Δ4, Δ5, Δ6 and Δ9 desaturases (lacking Δ12 and Δ15); they cannot form linoleic acid (LA, 18:2n-6) and α-linolenic (ALA, 18:3n-3) from oleic acid *de novo* (Fig. 4). However, they can further elaborate LA and ALA to longer PUFAs (Gurr, 1999). LA and ALA are hence considered to be essential fatty acids (EFA) and must be obtained from the diet, as they are the parent acids of the n-3 and n-6 families of PUFAs.

Linoleic acid is abundant in several seed oils and its major sources come from the seeds of sunflower, corn and soybean (White, 2000). Diets deficient in LA or having unusual ratios of LA to ALA induce changes in the PUFA composition of neuronal and glial membranes. Such changes have been linked to alterations in retina and brain functions (Fernstrom, 1999). Linoleic acid deficiency may also lead to skin lesions (Gurr, 1999).

A group of isomers of linoleic acid collectively termed conjugated linoleic acid (CLA) has received considerable attention in recent years. They are oxidatively unstable compounds that may form in small amounts during partial hydrogenation, during oxidation, and during normal or abusive heating. Food lipids originating from ruminant animals (beef, dairy and lamb) also contain CLA. CLA appears to exhibit anticarcinogenic and antiatherogenic properties (Ha *et al.*, 1987, 1989; Hunter, 2000). Effort to confirm and extend the potential benefits of CLA is expected to be an area of continuing research interest.

The main dietary sources of α-linolenic acid are canola oil, rapeseed and soybean oil (White, 2000). A study of a rural population in France and the UK confirmed that ALA could lower the clotting activity of platelets and the response of platelets to aggregation by thrombin (Renaud, 1995; Renaud *et al.*, 1986). There are researches indicating that ALA rich diets may improve some aspects of cardiovascular functions and protect against heart attacks but further confirmation is needed (Gurr, 1999). Nevertheless, ALA is essential as it is the parent acid of the physiologically important long-chain PUFAs of the n-3 family. ALA is converted to longer-chain n-3 PUFAs by the same desaturases used for the n-6 or n-9 families, but the extent to which ALA is converted to long-chain PUFAs in humans is not known (Hwang, 2000).

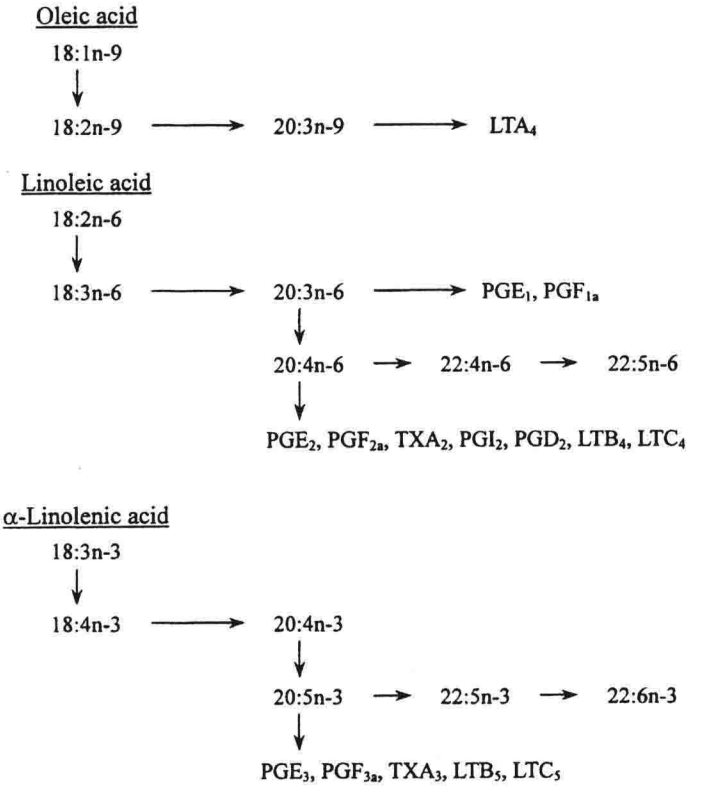

Figure 2. Conversion of dietary fatty acids to long-chain PUFAs and precursor acids for eicosanoids. LTA_4, leukotriene A_4; PGE_1, prostaglandin E_1; TXA_2, thromboxane A_2 (Hwang, 2000).

3.3. N-6 PUFAS: γ-LINOLENIC ACID, ARACHIDONIC ACID AND DOCOSAPENTAENOIC ACID

Certain plant oils such as evening primrose seed and borage seed oil contain considerable amounts of γ-linolenic (GLA, 18:3n-6) (López-Alonso and García-Maroto, 2000). GLA is converted from its parent acid LA by Δ6 desaturase and provides a substrate for further desaturation and elongation reactions, yielding the precursors of eicosanoids, dihomo-γ-linolenic acid (DGLA, 20:3n-6) and arachidonic acid (AA, 20:4n-6). DGLA and AA give rise to series-1 and series-2 prostaglandins, and series-3 and series-4 leukotrienes, respectively. There have been claims for therapeutic potency of GLA; GLA is beneficial to patients suffering from diseases related to inflammation. The anti-inflammatory effect of GLA may be due to accumulation of dihomo-γ-linolenic acid (20:3n-6) derived from GLA, and DGLA competes with AA for the same enzyme system, resulting in suppression of formation of prostaglandins derived from AA, which are highly potent eicosanoids (Gurr, 1999; Hwang, 2000).

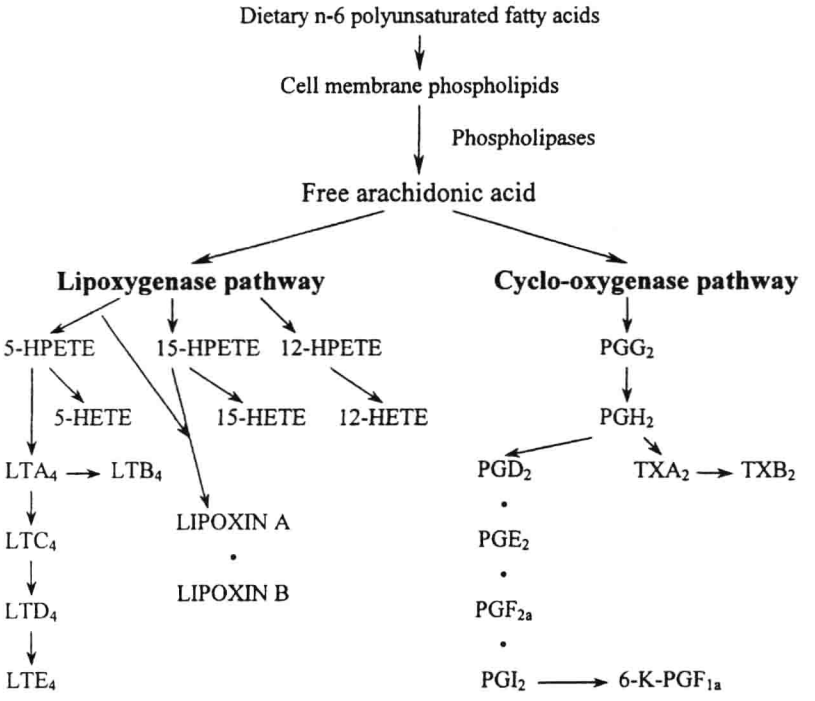

Figure 3. Eicosanoid formation from arachidonic acid via the cyclo-oxygenase and lipoxygenase pathways. LTA_4, leukotriene A_4; TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid (Hwang, 2000).

Arachidonic acid is exclusively found in animal products although it is also found in certain fungi. AA is important to infant development, as it is an essential component of the infant central nervous system. AA is normally supplied to the infant through breast-feeding. Its deficiency in pre-term infants may lead to impaired growth over the first year of life (Carlson *et al.*, 1993). Dietary arachidonic acid is incorporated into tissue phospholipids, which has to be released as the free fatty acid by the action of phospholipases before the initiation of the synthesis of eicosanoids (Berdanier, 2000) (Fig. 3). The kinds of eicosanoids synthesized vary with the type of tissue. AA derived eicosanoids are biologically active metabolites involved in wound healing and inflammatory response. They also exert diverse actions on the cardiovascular, reproductive, respiratory, renal, endocrine, skin, nervous, and immune systems. Excessive or imbalanced synthesis of these eicosanoids has been implicated in various pathological conditions, including thrombosis, inflammation, asthma, ulcers and kidney disease (Hwang, 2000).

Docosapentaenoic acid (DPA, 22:5n-6) is formed by further elongation and desaturation of AA. Its content in most organisms is low and the physiological function of n-6 DPA has not been fully clarified. However, it has been found that deficiency of n-

3 PUFAs, especially docosahexaenoic acid (DHA, 22:6n-3) in animals caused a compensatory rise in the n-6 DPA level in the brain and retina (Homayoun *et al.*, 1988). Retroconversion of n-6 DPA to AA by β-oxidation occurred in rats when the AA content was decreased by high DHA administration (Tam *et al.*, 2000).

3.4. SIGNIFICANCE OF LONG-CHAIN N-3 PUFAS

Long-chain polyunsaturated fatty acids of the n-3 family include eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The major sources of these fatty acids are usually the oils of marine fatty fish (Ackman, 2000). EPA is the precursor of another family of eicosanoids that are widely involved in metabolic regulation (Fig. 2). There have been evidences suggesting that EPA is a potential anticachexia and anti-inflammatory agent (Babcock *et al.*, 2000; Calder, 1997). DHA is essential for the growth and functional development of the brain and retina in infants (Agostoni *et al.*, 2000; Hamosh, 2000; Hoffman, 2000). Deficiency of DHA in infants is related to deficits in learning ability (Horrocks and Yeo, 1999). DHA is also required for maintenance of normal brain functions in adults (Horrocks and Yeo, 1999).

DPA of the n-3 family is a potent stimulator of endothelia cell (EC) migration, but it has no effect on smooth muscle cell (SMC) migration (Kanayasu-Toyoda *et al.*, 1996). EC migration is an important process in the control of wound-healing responses of blood vessels. In contrast, SMC migration plays a central role in the genesis of atherosclerosis. It is also found that EPA, DPA and DHA can be actively inter-converted in endothelial cell lipids. EPA can be formed by retroconversion of DPA in endothelial cells and inhibit the production of prostacyclins in endothelial cells when stimulated with endogenous arachidonic acid-mobilizing agents (Benistant *et al.*, 1996). DPA is shown to be the most potent inhibitor of collagen or AA induced platelet aggregation among the long-chain n-3 PUFAs (Akiba *et al.*, 2000). It is suggested that DPA might interfere with the cyclo-oxygenase pathway and accelerate the lipoxygenase pathway.

Long-chain n-3 PUFAs may possess therapeutic potency to many cardiovascular and inflammatory diseases. Fish oil supplements, which are rich in EPA and DHA, have been found to improve triglyceride and lipoprotein cholesterol profile in patients with several forms of hyperlipoproteinemia (Clarke, 2001; Durrington *et al.*, 2001; Horrocks and Yeo, 1999; Nestel, 1990; Nettleton, 1995a). Experimental studies suggest that EPA and DHA can reduce the risk of atherosclerosis by inhibiting the proliferation of vascular smooth muscle cells (Shiina *et al.*, 1993; Terano *et al.*, 1997). EPA and DHA are also capable of reducing the tendency toward thrombosis together with an increase in plasminogen activator activity and thus increase in fibrinolysis (Freese and Mutanen, 1997; Nettleton, 1995a). Many epidemiological studies and clinical trials have demonstrated that dietary supplementation of patients suffering from chronic inflammatory diseases including rheumatoid arthritis, asthma, psoriasis and inflammatory bowel disease (IBD) with high dose of fish oil is accompanied by mild to moderate improvement in these diseases (Boissonneault, 2000; Broughton *et al.*, 1997; Cleland *et al.*, 1988; Endres *et al.*, 1995; Kremer *et al.*, 1990). Evidences from animal studies also indicate that EPA and DHA exert suppressive effects on tumour formation and metastasis,

including breast cancer and colon cancer (Glauert, 2000; Nettleton, 1995b; Palakurthi *et al.*, 2000; Rose *et al.*, 1995).

One of the mechanisms for the therapeutic potency of n-3 PUFAs is that eicosanoids produced from n-6 precursors exert strong effects on body tissues, whereas n-3 precursor-derived eicosanoids possess a different or weaker potency with respect to various cellular responses (Gurr, 1999). EPA and DHA competitively inhibit cyclo-oxygenase from working on AA by binding to the enzyme, occupying the sites that will otherwise be available to AA (Corey *et al.*, 1983; Gurr, 1999). Competition may also exist between the two PUFA classes for incorporation into membrane phospholipids (Hwang, 2000).

It has become clear that imbalanced consumption of PUFAs can cause disturbances of homeostasis and lead to the development of certain chronic diseases. To date, balanced PUFA intake has been recommended by many health organizations throughout the world (FAO/WHO, 1993). PUFAs are now generally accepted as 'functional foods' for the prevention of diseases and a range of PUFA-fortified foods (mainly with the addition of EPA and/or DHA) including bakery goods, butter, milk, meat products, snacks and infant formulas are now widely available in many countries.

4. Biosynthesis of PUFAs

The biosynthesis of polyunsaturated fatty acids comprises two processes. One is the *de novo* synthesis of saturated or monounsaturated fatty acids from acetate and the other is the conversion of these fatty acids to polyunsaturated fatty acids through a series of desaturation and elongation processes.

4.1. *DE NOVO* SYNTHESIS OF SATURATED FATTY ACIDS

In most organisms, *de novo* synthesis of fatty acids from acetate is catalyzed by two enzyme systems: acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Schweizer, 1989). In plants, fatty acids synthesis takes place in the plastid and the process starts with the repeated incorporation of two-carbon units derived from acetate. Acetate must first be activated to form acetyl-CoA in order to serve as a primer for the condensation reaction (Ohlrogge *et al.*, 1993). There is a hypothesis that acetyl-CoA may also be produced from pyruvate and CoA by the pyruvate dehydrogenase reaction (Randall *et al.*, 1989). Acetyl-CoA is then converted to malonyl-CoA, which is an ATP- and Mn^{2+}- dependent carboxylation reaction catalyzed by ACC. Malonyl-CoA supplies all the carbon atoms of the fatty acid chain, with the exception of the two methyl terminal carbons, which are supplied by acetyl-CoA (Gurr and Harwood, 1991).

The conversion of acetyl-CoA and malonyl-CoA to fatty acids is catalyzed by FAS. There are two types of FAS. Type I, which consists of a multifunctional protein complex catalyzing the individual partial reactions, is found in animals and yeasts. Type II is found in plants and most bacteria, which is a collection of separable enzymes (Gurr and Harwood, 1991). The reactions of FAS are essentially the same for all organisms. In FAS (type II), the process involves a series of condensation reactions driven by the decarboxylation of malonyl-CoA, resulting in the extension of the fatty acyl chain to 16

(C16:0) or 18 (C18:0) carbons in length, producing palmitic or stearic acid (Ohlrogge *et al.*, 1993). The reactions require the involvement of acyl carrier protein (ACP). The enzyme acetyl-CoA:ACP transacylase catalyzes the transfer of acetyl-CoA to acetyl-ACP, which is regarded as the primer for fatty acid synthesis. Another similar enzyme, malonyl-CoA:ACP transacylase, catalyzes the transfer of malonyl-CoA to malonyl-ACP (Fig. 5) (Ohlrogge *et al.*, 1993).

3-Ketoacyl-ACP synthase (KAS) is a group of condensing enzymes containing three isozymes: KAS I, II, and III. They catalyze the condensation of acyl-ACP and malonyl-ACP to produce 3-ketoacyl-ACP. Biosynthesis of fatty acid is initiated by KAS III using acetyl-CoA as the primer. KAS I extends the acyl chain up to 16 carbons while KAS II elongates C14:0-ACP and C16:0-ACP to C16:0-ACP and C18:0-ACP, respectively (Gurr and Harwood, 1991). 3-Ketoacyl-ACP is converted to 3-hydroxyacyl-ACP through a reductive step by the action of 3-ketoacyl-ACP reductase. Then the enzyme 3-hydroxyacyl-ACP dehydrase removes water molecule from 3-hydroxyacyl-ACP to form enoyl-ACP, which is finally reduced to the corresponding saturated acyl-ACP by enoyl-ACP reductase (Ohlrogge *et al.*, 1993).

The release of the acyl chains from the ACP requires another enzyme system: either acyl-ACP hydrolase (thioesterase) or acyltransferase. Inside the plastid, the acyl chain will be first desaturated by an enzyme, stearoyl-ACP desatruase, which is highly specific for 18-carbon chains. The product, oleoyl-ACP, will then provide a substrate for the acyl-ACP thioesterase. This enzyme has the highest affinity for oleoyl-ACP. Therefore the 18-carbon oleic acid becomes the major product of fatty acid synthesis in plastid. Acyltransferase, an enzyme specific for palmitoyl-ACP, is localized on the inner membrane of the chloroplast envelope. This enzyme ensures that the 16-carbon fatty acids are the major components of membranes (Gurr and Harwood, 1991; Ohlrogge *et al.*, 1993; Schweizer, 1989).

4.2. BIOSYNTHESIS OF UNSATURATED FATTY ACIDS

Unsaturated fatty acids are synthesized by two basically different mechanisms; one is anaerobic and the other is aerobic. Anaerobic desaturation is only limited to certain bacteria, whereas aerobic pathway involves oxygen-dependent desaturation and is the most commonly found mechanism in non-parasitic organisms (Gurr and Harwood, 1991; Schweizer, 1989).

In the aerobic desaturation, oleic acid (18:1n-9) is desaturated by a Δ12 desaturase to form linoleic acid (LA, 18:2n-6) and further by a Δ15 desaturase to form α-linolenic acid (ALA, 18:3n-3), which are the precursors of the n-6 and n-3 fatty acid families, respectively. Longer-chain polyunsaturated fatty acids are produced by a series of desaturation and elongation reactions from these precursors. The biosynthesis of the n-9, n-6 and n-3 families of fatty acids is shown in Fig. 4. Some primitive organisms such as algae, fungi and bacteria have the ability to desaturate the fatty acid chain in either direction. They possess the array of desaturase and elongase activities required for the *de novo* production of various PUFAs. Therefore, these microorganisms are actually the primary synthesizers of PUFAs in nature (Gill and Valivety, 1997). Plants are able to insert new double bonds on the methyl side. However, they normally lack the requisite

enzymes to produce PUFAs above C18. Animals normally contain enzymes capable of introducing new double bonds on the carboxyl side only (Gurr and Harwood, 1991). Generally, Δ6 desaturase has a low activity in human tissues and is the rate-limiting step in the biosynthesis of long-chain PUFAs (Gurr, 1999). The parent acids of the three fatty acid families, oleic acid, LA and ALA also compete with one another for the enzyme Δ6 desaturase and each of them has a different affinity for the enzyme.

The double bonds of PUFAs occur in two isometric forms: *cis* and *trans*. Most plants and mammals contain fatty acids of the *cis* configuration, which is more flexible and has a greater fluidity than the *trans* isomeric configuration formed in ruminants' stomach by bacteria or by the technical hydrogenation of fat (Hunter, 2000). Fatty acids typically exist in storage oils and membrane lipids as glycerides, glycolipids, phospholipids, lipoproteins etc. (Gurr and Harwood, 1991).

5. Sources of PUFAs

5.1. CONVENTIONAL SOURCES

PUFAs are currently obtained from the oils of selected plant seeds (LA, ALA and GLA) and oils from certain marine fish (AA, EPA and DHA). Fishes such as salmon, sardine, mackerel, menhaden, anchovy and tuna are preferably used for fish oil production as their flesh usually contains a high proportion of fat tissues (up to 20 – 30% of fatty acids). Most fish oils are concentrated by the 'wet reduction process' under inert gas or in closed containers to reduce chances of deterioration in quality due to oxidation. Refined fish oils with increased levels of n-3 fatty acids are being produced by a process known as winterization, i.e., chilling and filtration. Saturated and monounsaturated fatty acids will be solidified and removed during the process, while the PUFAs remain in a liquid form. The oil rich in n-3 PUFAs is then subject to alkali treatment to remove non-saponifiable matters and eventually clay-bleached to remove colour. High-grade fish oils will be further deodorized to minimize fishy flavour and antioxidants will be added to prolong the shelf life (Yongmanitchai and Ward, 1989). However, the composition and content of fatty acids in fish oils are subject to seasonal and climatic variations, and also depend upon species of fish and geographical locations of catching sites. Other limitations of using fish oil for PUFA production include the undesirable fishy flavour of such products, the oxidative instability of fish oil and the difficulties of producing concentrates of the individual fatty acid from raw material (Ward, 1995). In addition, fish oil may be contaminated by pesticides and heavy metals due to environmental pollution. It also contains substantial amounts of undesirable fatty acids and cholesterol. It is expected that the supply of PUFAs from the fish source will be inadequate to meet the future demand. Therefore, alternative sources of PUFAs are now being exploited. Table 1 lists examples of commercial sources of PUFAs.