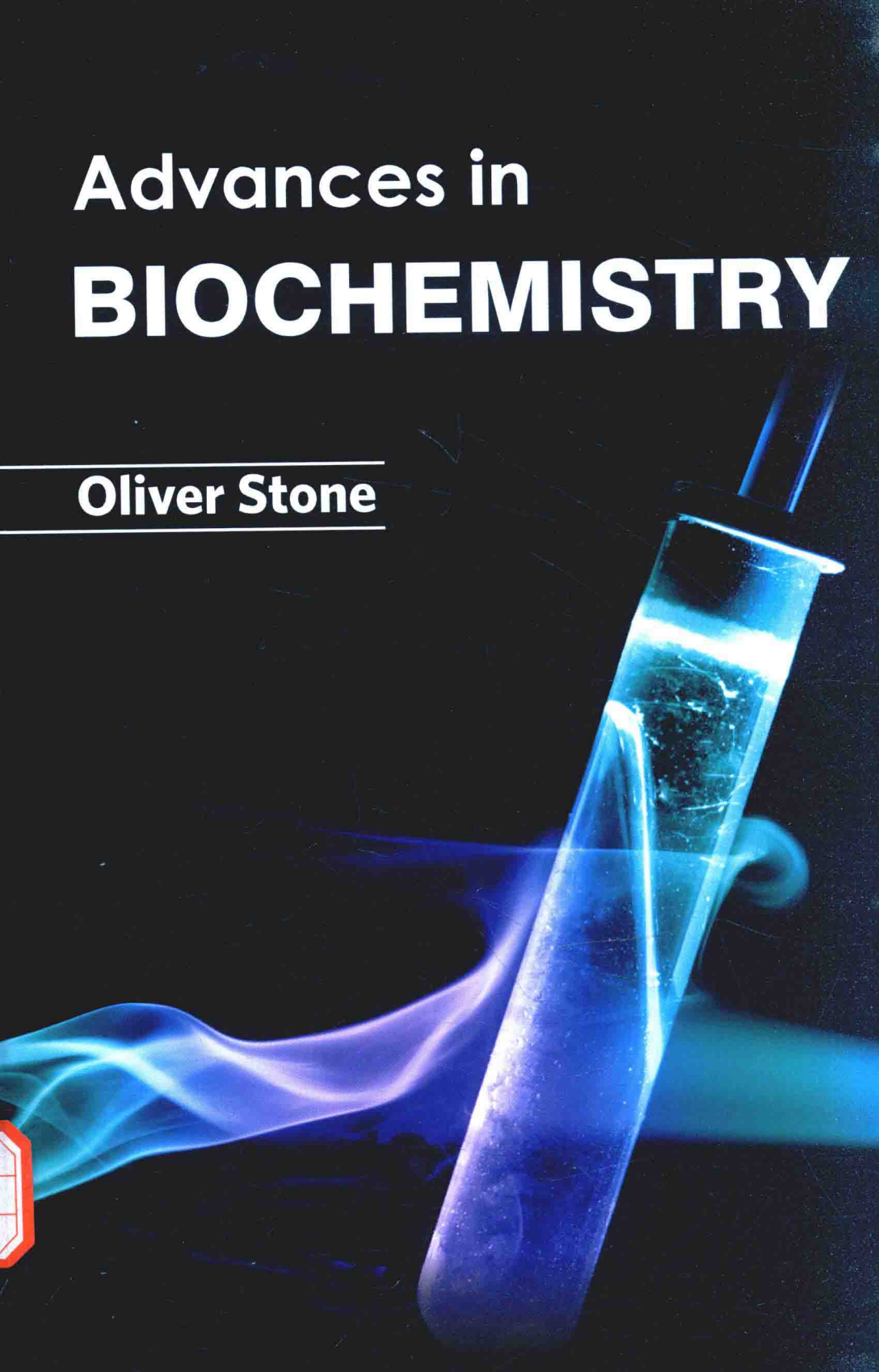
Advances in BIOCHEMISTRY

Oliver Stone



Advances in Biochemistry

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Preface

This book was inspired by the evolution of our times; to answer the curiosity of inquisitive minds. Many developments have occurred across the globe in the recent past which has transformed the progress in the field.

Over the years, biochemistry has become significant in classifying living processes so much so that many scientists in the field of life sciences are involved in biochemical research. This book presents an analysis of the research area of proteins, enzymes, cellular mechanisms and chemical compounds that are used in relevant methods. It includes the basic issues and some of the current advancements in biochemistry. This book caters to students, researchers, biologists, chemists, chemical engineers and professionals who are keen to know more about biochemistry, molecular biology and other related fields. The chapters within the book have been contributed by renowned international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics; many of whom are active in biochemical and biomedical research. It will provide information for scientists about the complexities of some biochemical procedures; and will stimulate both professionals and students to devote a part of their future research in understanding related mechanisms and methods of biochemistry.

This book was developed from a mere concept to drafts to chapters and finally compiled together as a complete text to benefit the readers across all nations. To ensure the quality of the content we instilled two significant steps in our procedure. The first was to appoint an editorial team that would verify the data and statistics provided in the book and also select the most appropriate and valuable contributions from the plentiful contributions we received from authors worldwide. The next step was to appoint an expert of the topic as the Editor-in-Chief, who would head the project and finally make the necessary amendments and modifications to make the text reader-friendly. I was then commissioned to examine all the material to present the topics in the most comprehensible and productive format.

I would like to take this opportunity to thank all the contributing authors who were supportive enough to contribute their time and knowledge to this project. I also wish to convey my regards to my family who have been extremely supportive during the entire project.

Editor

Contents

Part 1

Metabolism and Mechanism

1

Enzyme-Mediated Preparation of Flavonoid Esters and Their Applications

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1. Introduction

Flavonoids comprise a group of plant polyphenols with a broad spectrum of biological activities. They have been shown to exert beneficial effects on human health and play an important role in prevention and/or treatment of several serious diseases, such as cancer, inflammation and cardiovascular disease (Middleton et al., 2000; Rice-Evans, 2001). Flavonoids are important beneficial components of food, pharmaceuticals, cosmetics and various commodity preparations due to their antimutagenic, hepatoprotective (Stefani et al., 1999), antiallergic (Berg & Daniel, 1988), antiviral (Middleton & Chithan, 1993) and antibacterial activity (Tarle & Dvorzak, 1990; Tereschuk et al., 1997; Singh & Nath, 1999; Quarenghi et al., 2000; Rauha et al., 2000). They are known to inhibit nucleic acid synthesis (Plaper et al., 2003; Cushnie & Lamb, 2006), cause disturbance in membranes (Stepanovic et al., 2003; Stapleton et al., 2004; Cushnie & Lamb, 2005) and affect energy metabolism (Haraguchi et al., 1998). But the most studied activity is their antioxidant action since they can readily eliminate reactive oxygen and nitrogen species or degradation products of lipid peroxidation and are thus effective inhibitors of oxidation (Ross & Kasum, 2002).

However, their commercial applications are limited due to low solubility in lipophilic environment and low availability for a living organism. Although aglycons, prenylated and methoxylated flavonoid derivatives may be implemented into such systems, they are rarely found in nature and are often unstable. In some plant species, the last step in the flavonoid biosynthesis is terminated by acylation which is known to increase solubility and stability of glycosylated flavonoids in lipophilic systems. Selectively acylated flavonoids with different aliphatic or aromatic acids may not only improve physicochemical properties of these molecules (Ishihara & Nakajima, 2003) but also introduce various beneficial properqties to the maternal compound. These include penetration through the cell membrane (Suda et al., 2002; Kodelia et al., 1994) enhanced antioxidant activity (Viskupicova et al., 2010; Katsoura et al., 2006; Mellou et al., 2005), antimicrobial (Mellou et al., 2005), anti-proliferative (Mellou et al., 2006) and cytogenic (Kodelia et al., 1994) effect and improvement of thermostability and light-resistivity of certain flavonoids.

In nature, flavonoid acylation is catalyzed by various acyltransferases which are responsible for the transfer of aromatic or aliphatic acyl groups from a CoA-donor molecule to hydroxyl residues of flavonoid sugar moieties (Davies & Schwinn, 2006). Acylation is widespread especially among anthocyanins; more than 65% are reported to be acylated (Andersen & Jordheim, 2006). While the exact role of plant acylation is not yet fully understood, it is known that these modifications modulate the physiological activity of the resulting flavonoid ester by altering solubility, stability, reactivity and interaction with cellular targets (Ferrer et al., 2008). Acylation might be a prerequisite molecular tag for efficient vacuolar uptake of flavonoids (Kitamura, 2006; Nakayama et al., 2003). Some acylated flavonoids have been found to be involved in plant-insect interactions; they act as phytoalexins, oviposition stimulants, pollinator attractants (Iwashina, 2003), and insect antifeedants (Harborne & Williams, 1998). With respect to novel biological activities, acylation of flavonoids can result in changes in pigmentation (Bloor, 2001), insect antifeedant activity (Harborne & Williams, 1998) and antioxidant properties (Alluis & Dangles, 1999).

Over the past 15 years, there has been a substantial effort to take advantage of this naturally occurring phenomenon and to implement acylation methods into laboratories. However, the use of acyltransferases as modifying agents is rather inconvenient, as they require corresponding acylcoenzyme A, which must be either in stoichiometric amounts or regenerated *in situ*. Natural acyltransferases and cell extracts from *Ipomoea batatas* and *Perilla frutescens* containing acyltransferases were applied for selective flavonoid modification with aromatic acids (Tab.1) (Nakajima et al., 2000; Fujiwara et al., 1998).

Acyltransferase	Plant source	References
hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucosid-6''-*O*-acyltransferase	*Perilla frutescens*	Yonekura-Sakakibara et al., 2000
malonyl-CoA:anthocyanin 3-*O*-glucosid-6''-*O*-malonyltransferase	*Dahlia variabilis*	Wimmer et al., 1998
hydroxycinnamoyl-CoA:anthocyanin 5-*O*-glucosid-6''-*O*-acyltransferase	*Gentiana triflora*	Tanaka et al., 1996
hydroxycinnamoyl-CoA:anthocyanidin 3-rutinosid acyltransferase	*Petunia hybrida*	Brugliera & Koes, 2003
malonyl-CoA:anthocyanidin 5-*O*-glucosid-6''-*O*-malonyltransferase	*Salvia splendens*	Suzuki et al., 2001

Table 1. Acyltransferase catalysis of flavonoid acylation and their nature sources.

To solve this problem, the chemical approach was first investigated. It possessed a low degree of regioselectivity of esterification and drastic reaction conditions had to be applied (Patti et al., 2000). Later on, hydrolytic enzymes (lipases, esterases and proteases) have been recognized as useful agents due to their large availability, low cost, chemo-, regio- and enantioselectivity, mild condition processing and no need of cofactors (Collins & Kennedy, 1999; Nagasawa & Yamada, 1995).

Since the enzymatic preparation of flavonoid derivatives is a matter of several years, commercial applications have just been emerging. There are several patented inventions available to date, oriented on the flavonoid ester production and their use for the manufacture of pharmaceutical, dermopharmaceutical, cosmetic, nutritional or agri-foodstuff compositions

(Fukami et al., 2007; Moussou et al., 2004, 2007; Ghoul et al., 2006; Bok et al., 2001; Perrier et al., 2001; Otto et al., 2001; Nicolosi et al., 1999; Sakai et al., 1994).

This review presents available information on enzyme-mediated flavonoid acylation *in vitro*, emphasizing reaction parameters which influence performance and regioselectivity of the enzymatic reaction. In the second part, the paper focuses on biological effects of synthesized flavonoid esters as well as of those isolated from nature. Finally, the paper ends with application prospects of acylated flavonoids in the food, pharmaceutical and cosmetic industry.

2. Flavonoid esterification

Presently, the enzyme-catalyzed flavonoid esterification in organic media is a well-mastered technique for synthesis of selectively modified flavonoids. Results in this field suggest that a high degree of conversion to desired esters can be achieved when optimal reaction conditions are applied. The key factors, which influence regioselectivity and the performance of the enzymatic acylation of flavonoids, include type and concentration of enzyme, structure and concentration of the substrates (acyl donor, acyl acceptor and their ratio), nature of the reaction media, water content in the media, reaction temperature and nature of the reaction as reviewed in Chebil et al., 2006, 2007.

2.1 Enzymes

To date, the use of proteases, esterases, acyltransferases and lipases has been investigated in order to find the most potent biocatalyst for selective flavonoid acylation. These enzymes are often in the immobilized form which improves enzyme stability, facilitates product isolation, and enables enzyme reuse (Adamczak & Krishna, 2004).

2.1.1 Proteases

Proteases represent a class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. They represent the first group of hydrolytic enzymes used for flavonoid modification. They perform both hydrolytic and synthetic functions. Since they are physiologically necessary for living organisms, proteases occur ubiquitously in diverse sources, such as plants, animals, and microorganisms. They are also classified as serine proteases, aspartic proteases, cysteine proteases, threonine proteases and metalloproteases, depending on the nature of the functional group at the active site.

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. Proteases are also extensively used in the pharmaceutical industry (Rao et al., 1998). Protease subtilisin was the first enzyme used for flavonoid ester synthesis conducted by Danieli et al. (1989, 1990). Later on, subtilisin was used for selective rutin acylation in organic solvents (Xiao et al., 2005; Kodelia et al., 1994). However, it has been reported that reactions catalyzed by subtilisin led to low conversion yields and a low degree of regioselectivity was observed (Danieli et al., 1990). These authors reported that the structure of the sugar moiety affected the regioselectivity. For flavonoid acylation, especially

serine proteases (subtilisin) have been used in ester synthesis (Danieli et al., 1989, 1990; Kodelia et al., 1994).

2.1.2 Esterases

Esterases (carboxyl esterases, EC 3.1.1.1) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds with wide distribution in animals, plants and microorganisms. A classification scheme for esterases is based on the specificity of the enzymes for the acid moiety of the substrate, such as the carboxyl esterases, aryl esterases, acetyl esterases, cholin esterases, cholesterol esterases, etc. (Jeager et al., 1999). Esterases show high regio- and stereospecificity, which makes them attractive biocatalysts for the production of optically pure compounds in fine-chemicals synthesis (reviewed in Bornscheuer, 2002).

They have the same reaction mechanism as lipases, but differ from them by their substrate specificity, since they prefer short-chain fatty acids, whereas lipases usually prefer long-chain fatty acids. Another difference lies in the interfacial activation (Hidalgo & Bornscheuer, 2006). In contrast to lipases, only a few esterases have commercial applications in organic synthesis because lipases are generally more entantioselective and resistant to organic solvents. The most widely used esterase is the preparation isolated from pig liver (Hidalgo & Bornscheuer, 2006). The practical applications of esterases in enzymatic transformation of flavonoids are not very attractive as it enables the implementation only of the molecule of a short aliphatic chain length, such as acetate, propionate and butyrate (Sakai et al., 1994).

2.1.3 Lipases

Today lipases stand amongst the most important biocatalysts in industry. Among them, microbial lipases find the biggest application use. They can be classified according to sequence alignment into three major groups: mammalian lipases (e.g. porcine pancreatic lipase), fungal lipases (*Candida rugosa* and *Rhizomucor* family) and bacterial lipases (*Staphylococcus* and *Pseudomonas* family) (Hidalgo & Bornscheuer, 2006). More than 50% of the reported lipases are produced by yeast in the forms of various isozymes (Vakhlu & Kour, 2006).

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) belong to the class of serine hydrolases. They catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis (Vakhlu & Kour, 2006). Under natural conditions, they catalyze the hydrolysis of ester bonds at the hydrophilic-hydrophobic interface. At this interface, lipases exhibit a phenomenon termed interfacial activation, which causes a remarkable increase in activity upon contact with a hydrophobic surface. The catalytic process involves a series of differentiated stages: contact with the interface, conformational change, penetration in the interface, and finally the catalysis itself (Hidalgo & Bornscheuer, 2006). Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol (Saxena et al., 1999). This synthetic activity of lipases is being successfully utilized also in flavonoid ester production.

Candida antarctica lipase B (CALB) is one of the most widely used biocatalysts in organic synthesis on both the laboratory and the commercial scale (Anderson et al., 1998; Uppenberg et al., 1995) due to its ability to accept a wide range of substrates, its non-aqueous medium tolerance and thermal deactivation resistance (Degn et al., 1999; Anderson et al., 1998; Cordova et al., 1998; Drouin et al., 1997). CALB belongs to the α/β hydrolase-fold superfamily with a conserved catalytic triad consisting of Ser105-His224-Asp187 (Uppenberg et al., 1995). It comprises 317 amino acid residues. The active site contains an oxyanion hole which stabilizes the transition state and the oxyanion in the reaction intermediate (Haeffner et al., 1998). Reaction mechanism of CALB follows the bi-bi ping-pong mechanism, illustrated in Fig.1 (Kwon et al., 2007). The substrate molecule reacts with serine of the active site forming a tetrahedral intermediate which is stabilized by catalytic residues of His and Asp. In the next step alcohol is released and the acyl-enzyme complex is created. A nucleophilic attack (water in hydrolysis, alcohol in transesterification) causes another tetrahydral intermediate formation. In the last step, the intermediate is split into product and enzyme and is recovered for the next catalytic cycle (Patel, 2006).

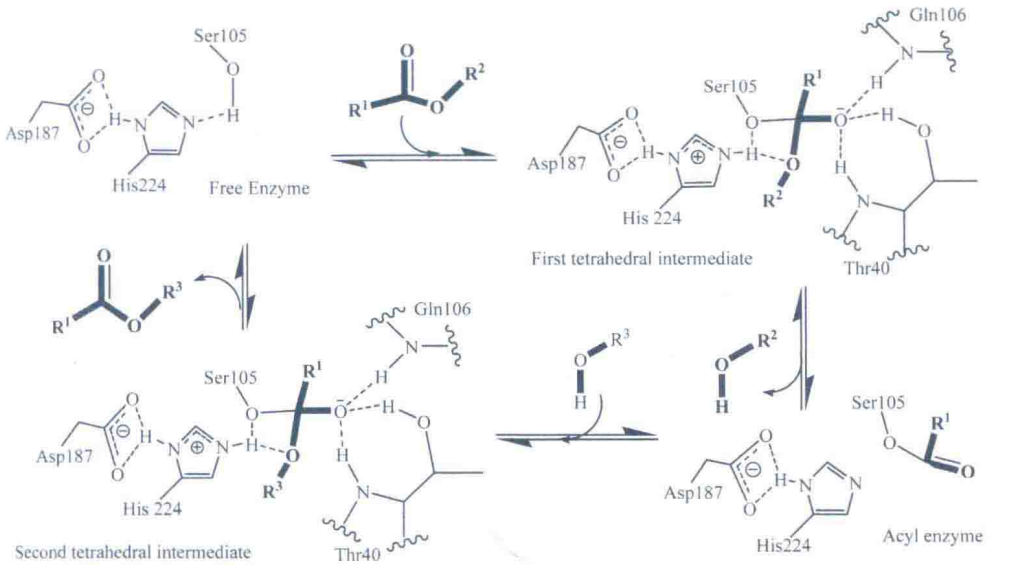

Fig. 1. Reaction mechanism catalyzed by *Candida antarctica* lipase (Kwon et al., 2007).

The active site of CALB consists of a substrate-nonspecific acyl-binding site and a substrate specific alcohol-binding site (Cygler & Schrag, 1997; Uppenberg et al., 1995). It is selective for secondary alcohols (Uppenberg et al., 1995), as reflected by the geometry of the alcohol-binding site (Lutz, 2004). In contrast to most lipases, CALB has no lid covering the entrance to the active site and shows no interfacial activation (Martinelle et al., 1995). CALB is being frequently used in acylation of various natural compounds such as saccharides, steroids and natural glycosides, including flavonoids (Riva, 2002; Davis & Boyer, 2001). The proper enzyme selection plays multiple roles in flavonoid acylation. The biocatalyst significantly influences the regioselectivity of the reaction. Information is available mainly on the use of lipases for flavonoid ester synthesis; especially the use of lipase B from *Candida antarctica*,

which is preferred due to its acceptance of a wide range of substrates, good catalytic activity and a high degree of regioselectivity (Viskupicova et al., 2010; Katsoura et al., 2006, 2007; Ghoul et al., 2006; Mellou et al., 2005, 2006; Stevenson et al., 2006; Ardhaoui et al., 2004a, 2004b, 2004c; Passicos et al., 2004; Moussou et al., 2004; Gayot et al., 2003; Ishihara & Nakajima, 2003; Ishihara et al., 2002; Kontogianni et al., 2001, 2003; Nakajima et al., 1999, 2003; Gao et al., 2001; Otto et al., 2001; Danieli et al., 1997).

As for flavonoid aglycons, only two enzymes have been reported to be capable of acylating this skeleton - lipase from *Pseudomonas cepacia* and carboxyl esterase. Lambusta et al. (1993) investigated the use of *P. cepacia* lipase for catechin modification. They discovered that the acylation took place on the C5 and C7 hydroxyls. Sakai et al. (1994) observed that carboxyl esterase showed regioselectivity towards C3-OH of catechin. Sakai et al. (1994) explored the use of carboxyl esterase from *Streptomyces rochei* and *Aspergillus niger* for the 3-*O*-acylated catechin production.

2.2 Reaction conditions

The performance and regioselectivity of the enzyme-catalyzed flavonoid transformation is affected by several factors, including the type of enzyme, the nature of medium, reaction conditions, water content in the media, structure and concentration of substrates and their molar ratio. By varying these factors, significant changes in ester production and regioselectivity can be achieved.

2.2.1 Reaction media

Reaction media play an important role in enzymatic transformations. Methodologies for enzymatic flavonoid acylation have focused on searching a reaction medium which allows appropriate solubility of polar acyl acceptor (flavonoid glycoside) and nonpolar acyl donor as well as the highest possible enzymatic activity. Moreover, the medium has often been required to be nontoxic and harmless to biocatalyst. In order to meet the above-mentioned requirements, several scientific teams have dealt with proper medium selection (Viskupicova et al., 2006; Mellou et al., 2005; Kontogianni et al., 2001, 2003; Gao et al., 2001; Nakajima et al., 1999; Danieli et al., 1997).

Non-aqueous biocatalysis has several advantages over conventional aqueous catalysis: the suppression of hydrolytic activity of the biocatalyst which is carried out in water (Fossati & Riva, 2006), the enhanced solubility of hydrophobic substrates, the improvement of enzyme enantioselectivity, the exclusion of unwanted side reactions, the easy removal of some products, the enhanced enzyme thermostability and the elimination of microbial contamination (Rubin-Pitel & Zhao, 2006; Torres & Castro, 2004). Laane (1987) pointed out that log P, as a solvent parameter, correlated best with enzyme activity. Zaks & Klibanov (1988) reported that the activity of lipases was higher in hydrophobic solvents than in hydrophilic ones. Narayan & Klibanov (1993) claimed that it was hydrophobicity and not polarity or water miscibility which was important, whereas the log P parameter could be called a measure of solvent hydrophobicity. Trodler & Pleiss (2008), using multiple molecular dynamics simulations, showed that the structure of CALB possessed a high stability in solvents. In contrast to structure, flexibility is solvent-dependent; a lower dielectric constant led to decreased protein flexibility. This reduced flexibility of CALB in

non-polar solvents is not only a consequence of the interaction between organic solvent molecules and the protein, but it is also due to the interaction with the enzyme-bound water and its exchange on the surface (Trodler & Pleiss, 2008). In organic solvents, the surface area has been suggested to be reduced, leading to improved packing and increased stability of the enzyme (Toba & Merz, 1997).

Polar aprotic solvents such as dimethyl sulfoxid (DMSO), dimethylformamide (DMF), tetrahydrofuran (THF) and pyridine were first investigated (Nakajima et al., 1999; Danieli et al., 1997). However, it was observed that enzyme activity was readily deactivated in these solvents. To date enzymatic acylation of flavonoids has been successfully carried out in various organic solvents (Tab.2), while the most frequently used are 2-methylbutan-2-ol and acetone because of their low toxicity, their polarity allowing proper solubilization of substrates and high conversion yields.

Solvent	Reference
2-Methylbutan-2-ol	Ghoul et al., 2006; Ardhaoui et al., 2004a, 2004b, 2004c; Passicos et al., 2004; Gayot et al., 2003
Acetone	Ghoul et al., 2006; Mellou et al., 2005, 2006; Kontogianni et al., 2001, 2003; Ishihara et al., 2002, Ishihara & Nakajima, 2003; Nakajima et al., 1999, 2003; Danieli et al., 1997
Acetonitrile	Ghoul et al., 2006; Ishihara & Nakajima, 2003; Nakajima et al., 1997, 1999
2-Methylpropan-2-ol	Ghoul et al., 2006; Stevenson et al., 2006; Mellou et al., 2005; Moussou et al., 2004; Kontogianni et al., 2001, 2003; Otto et al., 2001
Dioxane	Ghoul et al., 2006; Danieli et al., 1997
Pyridine	Danieli et al., 1990, 1997
THF, DMSO, DMF	Kontogianni et al., 2001, 2003; Danieli et al., 1997
Binaric mixtures of solvents	Ghoul et al., 2006; Gao et al., 2001; Nakajima et al., 1999; Danieli et al., 1997

Table 2. Organic solvents used in flavonoid acylation.

The effect of the solvent on conversion yield depends on the nature of both the acyl donor and the flavonoid (Chebil et al., 2006). Although much has been done in this area, it is quite difficult to deduce any general conclusion on solvent choice because the available data are controversial and sometimes even contrary.

Recently, ionic liquids have received growing attention as an alternative to organic solvents used for the enzymatic transformation of various compounds (Katsoura et al., 2006; Kragl et al., 2006; Jain et al., 2005; Lozano et al., 2004; Reetz et al., 2003; Van Rantwick et al., 2003). The potential of these "green solvents" lies in their unique physicochemical properties, such as non-volatility, nonflammability, thermal stability and good solubility for many polar and less polar organic compounds (Jain et al., 2005; Wilkes, 2004; Itoh et al., 2003; Van Rantwick et al., 2003). Probably the most promising advantage of the use of ionic liquids is their potential application in food, pharmaceutical and cosmetic preparations due to their reduced toxicity (Jarstoff et al., 2003). Due to the many above-mentioned advantages of ionic liquids for enzyme-mediated transformations, several flavonoid esters have been recently

prepared in such media (Katsoura et al., 2006, 2007; Kragl et al., 2006). The biocatalytic process showed significantly higher reaction rates, regioselectivity and yield conversions compared to those achieved in organic solvents. Thus ionic liquid use seems to be a challenging approach to conventional solvent catalysis.

The solvent-free approach for elimination of the co-solvent of the reaction has been recently introduced as an alternative for conventional solvents (Enaud et al., 2004; Kontogianni et al., 2001, 2003). It is based on the use of one reactant in the role of the solvent. The authors reported rapid reaction rates; however, the conversion yields were slightly decreased. In spite of the attractiveness, the use of solvent-free systems is characterized by a serious drawback due to the necessity to eliminate the excess of the acyl donor for the recovery of the synthesized products (Chebil et al., 2006).

2.2.2 Water content

Water content in reaction media is a crucial parameter in lipase-catalyzed synthesis as it alters the thermodynamic equilibrium of the reaction towards hydrolysis or synthesis. Moreover, it is involved in noncovalent interactions which keep the right conformation of an enzyme catalytic site (Foresti et al., 2007). The amount of water required for the catalytic process depends on the enzyme, its form (native or immobilized), the enzyme support, and on the solvent nature (Arroyo et al., 1999; Zaks & Klibanov, 1988). The influence of water content in the reaction system on enzyme activity is variable with various enzymes (lipase from *Rhizomucor miehei, Rhizomucor niveus, Humicola lanuginosa, Candida rugosa, Pseudomonas cepacia*).

In general, the water amount which is considered to be optimal for esterifications in organic solvents is 0.2 - 3% (Rocha et al., 1999; Yadav & Piyush, 2003; Iso et al., 2001). The enzymatic esterification of flavonoids in non-aqueous media is greatly influenced by the water content of the reaction system (Ardhaoui et al., 2004b; Gayot et al., 2003; Kontogianni et al., 2003). Ardhaoui et al. (2004b) observed the best enzyme activity when water content was maintained at 200 ppm. Gayot et al. (2003) found that the optimal value of water in an organic reaction medium equaled 0.05% (v/v). Kontogianni et al. (2003) reported that highest flavonoid conversion was reached when initial water activity was 0.11 or less.

2.2.3 Temperature

Temperature represents a significant physical factor in enzyme-catalyzed reactions. It affects viscosity of the reaction medium, enzyme stability, and substrate and product solubility.

Since lipase from *C. antarctica* belongs to thermostable enzymes, improved catalytic activity was observed at higher temperatures (Arroyo et al., 1999). To date, flavonoid transformation has been carried out in the temperature range 30 - 100°C (Ghoul et al., 2006; Katsoura et al., 2006; Stevenson et al., 2006; Mellou et al., 2005; Ardhaoui et al., 2004a, 2004b, 2004c; Moussou et al., 2004; Passicos et al., 2004; Enaud et al., 2004; Gayot et al., 2003; Kontogianni et al., 2003; Ishihara et al., 2002; Gao et al., 2001; Otto et al., 2001; Nakajima et al., 1999; Danieli et al., 1990). The choice of temperature depends on the enzyme and solvent used. The majority of authors performed flavonoid acylation at 60°C due to the best enzyme activity, good solubility of substrates and highest yields of resulting esters reached (Viskupicova et al., 2006, 2010; Ghoul et al., 2006; Katsoura et al., 2006; Stevenson et al., 2006;

Ardhaoui et al., 2004a, 2004b, 2004c; Moussou et al., 2004; Passicos et al., 2004; Enaud et al., 2004; Gayot et al., 2003; Otto et al., 2001). Our results on the effect of temperature on naringin conversion are presented in Fig.2 and are in accordance with other authors (Viskupicova et al., 2006).

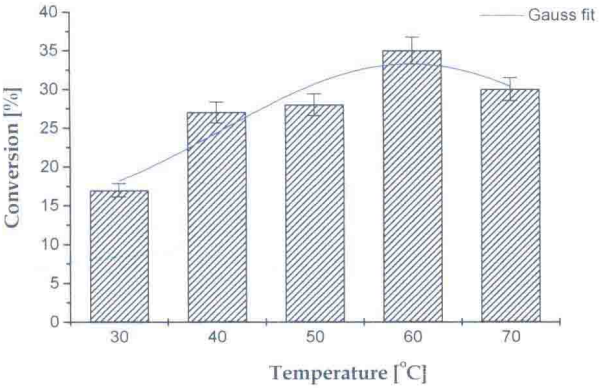

Fig. 2. Effect of temperature on naringin conversion to naringinpalmitate in 2-methylbutan-2-ol catalyzed by *C. antarctica* lipase after 24 h (Viskupicova et al., 2006).

2.3 Acyl donors and acceptors

2.3.1 Acyl donor

Since lipase-catalyzed acylation takes place through the formation of an acyl-enzyme intermediate, the nature of the acyl donor has a notable effect on reactivity. The ideal acyl donor should be inexpensive, fast acylating, and completely non-reactive in the absence of the enzyme (Ballesteros et al., 2006). Many acylating agents have been tested in flavonoid esterification, such as aromatic or aliphatic organic acids, substituted or not (Tab.3). Special attention was attributed to fatty acid ester production (Katsoura et al., 2006; Mellou et al., 2005, 2006; Ardhaoui et al., 2004a, 2004b, 2004c; Enaud et al., 2004; Gayot et al., 2003; Kontogianni et al., 2003). This approach enables to improve flavonoid solubility and stability in lipophilic systems. The proper acyl donor selection may significantly influence not only the physicochemical but also biological properties of the resulting esters.

A simple way to increase the reaction rate and conversion yield in acylation is to use an excess of acyl donor (Patti et al., 2000). Many authors have tried to determine the optimal molar ratio of flavonoid/acyl donor in order to achieve the highest possible yields. The molar ratios 1:1 to 1:15 (acyl acceptor/acyl donor) have been investigated, whereas the majority agreed on the ratio 1:5 to be the most suitable for the best reaction performance (Mellou et al., 2006; Gayot et al., 2003; Ishihara & Nakajima, 2003; Ishihara et al., 2002; Kontogianni et al., 2001). A better solution is offered by the use of special acyl donors which ensure a more or less irreversible reaction. This can be achieved by the introduction of electron-withdrawing substituents (esters), resulting in higher conversion yields and reaction rates. The use of vinyl esters allows a several times faster reaction progress than do other activated esters (Ballesteros et al., 2006). Enzymatic synthesis of flavonoid esters can be realized by two basic approaches, i.e. esterification and transesterification (Fig.3).