CHAPTER I

INTRODUCTION

1.1 L-Ascorbic acid

1.1.1 Chemistry

L-ascorbic acid (AA) is a simple sugar, well known as vitamin C. The structure of L-ascorbic acid is given in Figure 1. The molecule has an almost planar five-membered ring. The two chiral centers at positions 4 and 5 determine the four stereoisomers; L-ascorbic acid, D-ascorbic acid, D-araboascorbic acid (erythorbic acid) and L-araboascorbic acid (Figure 1). Besides L-ascorbic acid, only erythorbic acid shows a limited level of vitamin C activity (about 1/20 L-ascorbic acid activity). L-ascorbic acid is a white to yellow-tinged crystalline powder. It crystallizes out of water solution as square or oblong crystals and slightly solubles in acetone and lower alcohols. The physical properties of ascorbic acid are listed in Table 1.

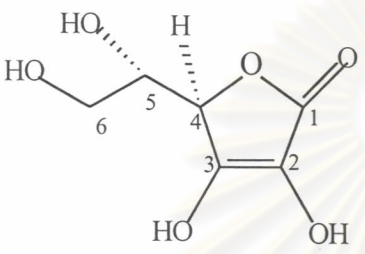
As evident from the structure, AA contains two enolic groups at 3- and 2-hydroxyls. Due to the enediol structure, the proton at 3-hydroxyl is highly acidic ($pKa_1 = 4.17$). The reversible oxidation to semidehydro-L-ascorbic acid and further to dehydro-L-ascorbic acid is the most important chemical property of ascorbic acid and is the basis for its known physiological activities.

1.1.2 Synthesis of L-ascorbic acid

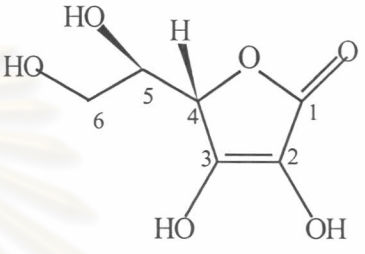
Most plants and higher animal species have ability to synthesize AA from D-glucose or D-galactose via the glucuronic acid pathway (Figure 2). In the first phase of its synthesis, glucose is converted through several stages to D-glucuronic acid which is then reduced to L-gulonate. Subsequently, L-gulonate lactonizes to form

Table 1. Physical Properties of L-ascorbic acid (Moser and Bendich, 1998)

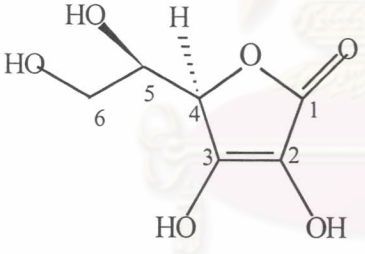
Property	Characteristics
Appearance	White, odorless, crystalline solid
Formula	$C_6H_8O_6$; MW 176.13
Crystal form	Monoclinic; usually plates, sometimes needles
Melting point	190-192°C (decomposition)
Density	1.65
Optical rotation	$[\alpha]^{23}_D$ = +20.5° to +21.5° (C = 1 in water)
	$[\alpha]^{23}_D$ = +48° (C = 1 in methanol)
pK_1	4.17
pK_2	11.57
Redox potential	First stage: E_0 + 0.66V (pH 4)
Solubilities	1 g dissolves in 3 ml water, 30 ml 95% ethanol, 50 ml absolute ethanol, 100 ml glycerol USP, or 20 ml propylene glycol. Insoluble in ether, chloroform, benzene, petroleum ether, oils, fats, and other fat solvents



L-ascorbic acid

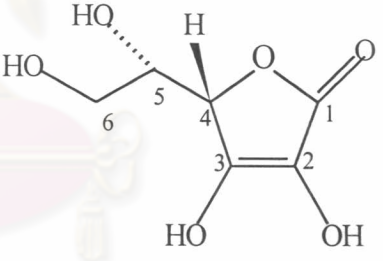


D-ascorbic acid



D-araboascorbic acid

Erythorbic acid



L-araboascorbic acid

Figure 1. Isomers of ascorbic acid (Moser and Bendich, 1998)

L-gulono-γ-lactone, which is oxidized to 2-keto-L-gulonolactone. This reaction is catalyzed by microsomal flavoenzyme, L-gulono-γ-lactone oxidase. The oxidized product in its subsequent step is spontaneously isomerized to form L-ascorbic acid.

However, there are some species such as humans, other primates, guinea pigs, ODS-od/od (ODS) rat, red-vented bulbul, indian fruit eating bat, and some other birds lack the ability to synthesize AA due to lack of hepatic L-gulono-γ-lactone oxidase. Thus, vitamin C must be obtained through the diet in the form of food or supplement (Chatterjee, 1978).

Industrial production of ascorbic acid is based on the classical method developed by Reichstein and Grussner (1934) with D-glucose as starting material (Moser and Bendich, 1998). In a first step, D-glucose is reduced to D-sorbitol, which is then fermentatively oxidized to L-sorbose. The hydroxyl groups are protected by treatment with acetone and acid, and the formed diacetone-L-sorbose can then be oxidized to the corresponding acid, diacetone-2-keto-L-gulonic acid, which is easily converted to L-ascorbic acid by hydrolysis, lactonization, and enolization (Figure 3).

Much time and effort has been devoted to finding other methods of vitamin C synthesis by fermentation (Kulhanek, 1970). An important breakthrough was recently achieved using the recombinant DNA technology. It is possible to introduce the 2,5-diketo-D-gluconic acid reductase gene from *Corynebacterium* into *Erwinia herbicola*, which gives it the ability to produce 2-keto-L-gulonic acid directly from D-glucose (Anderson *et al.*, 1985). The use of this technology for the cost-effective production of L-ascorbic acid, however, requires further improvements of the process.

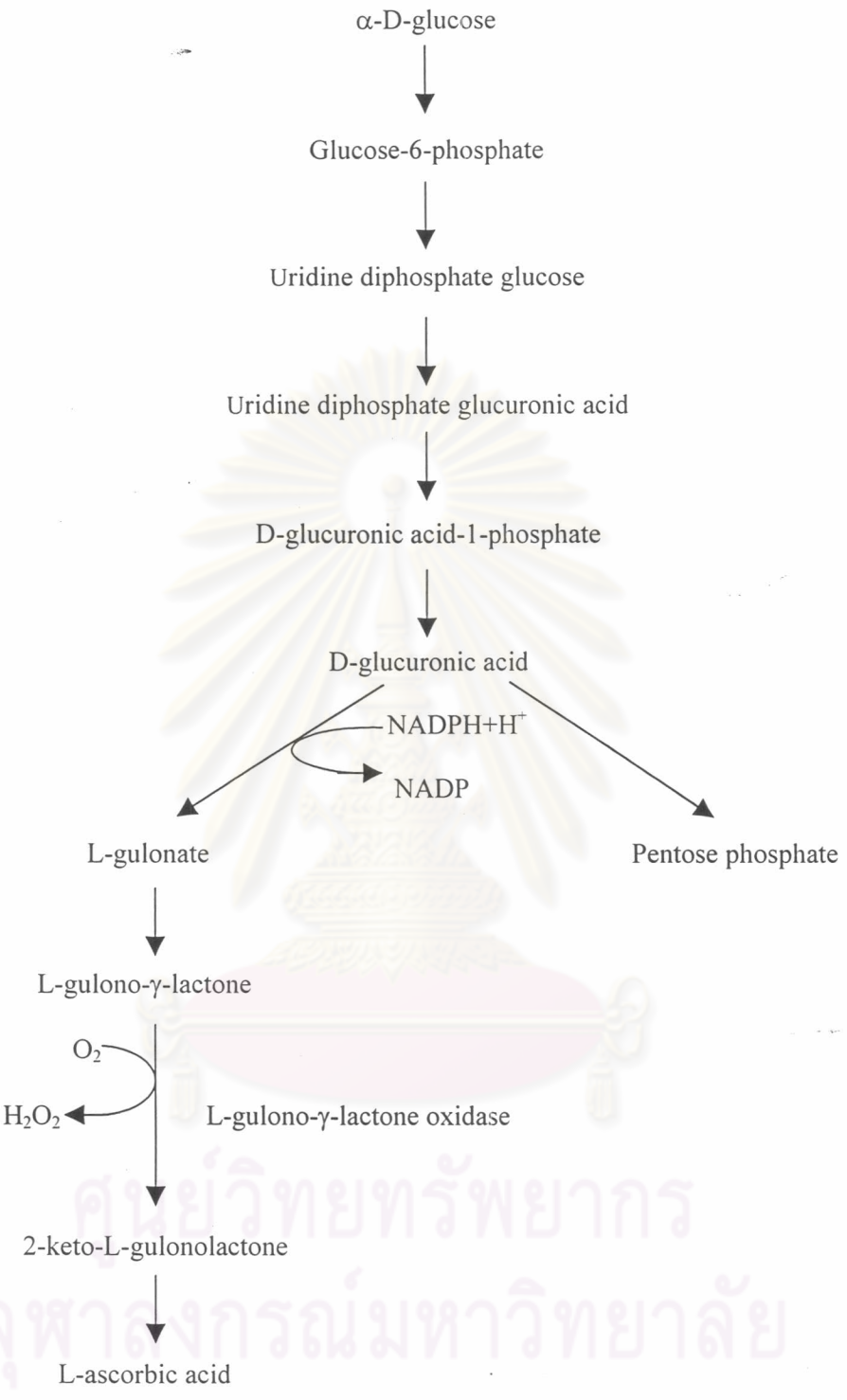


Figure 2. The biosynthesis of L-ascorbic acid (Basu and Dickerson, 1996)

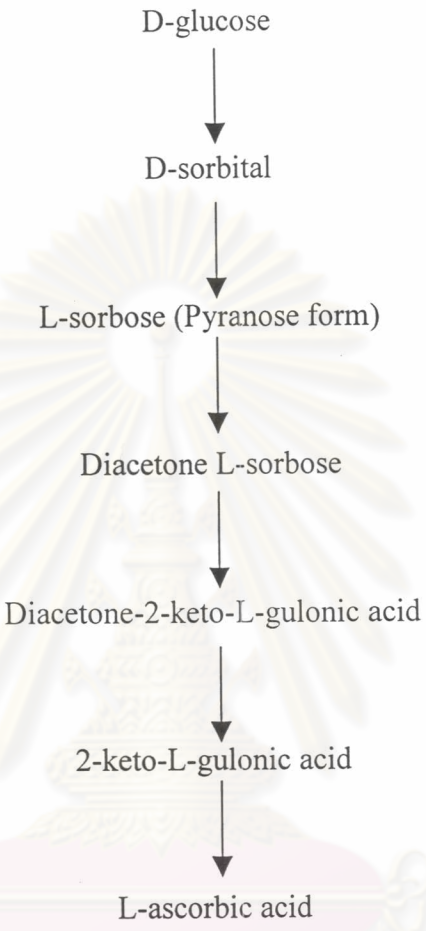


Figure 3. Chemical synthesis of L-ascorbic acid (Moser and Bendich, 1998)

1.1.3 Dietary sources of ascorbic acid

L-ascorbic acid is widely distributed in both plants and animals, especially plentiful in fresh fruits and vegetables (Bendich, 1997). The amounts of AA vary greatly from species to species and in different samples of the same species. Only small amounts are found in animal organs such as liver, kidney and muscle meats. Some berries, guava and rose hips are accumulators of AA and consequently are rich sources. While oranges, lemons, strawberries and most green leafy vegetables are moderately rich sources of ascorbic acid. Potatoes and cabbage are probably important on account of the large amount generally eaten. For practical reasons, citrus and other fruits are good daily sources of ascorbic acid, as they are generally eaten raw and are, therefore, not subjected to cooking procedures that can destroy ascorbic acid. The vitamin C content of some representative foods is listed in Table 2.

1.1.4 Biochemical functions of L-ascorbic acid

AA is an essential micronutrient involves in a wide variety of biochemical processes in human body. The molecular mechanisms of the antiscorbutic effect of AA are largely, although not completely, understood. AA is a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters (Burri and Jacob, 1997; Tsao, 1997). AA takes part in the hydroxylation of proline and lysine which are necessary to synthesize collagen. A deficiency of vitamin C results in a weakening of collageneous structures, causing tooth loss. Joint pains, bone and connective tissue disorders, and poor wound healing, all of which are characteristics of scurvy (Basu and Dickerson, 1996). The biosynthesis of carnitine required AA as an enzyme cofactor (Burri and Jacob, 1997). Carnitine is essential for the transport of activated long chain fatty acids into the mitochondria. In addition, AA is used as a cofactor for catecholamine biosynthesis, in particular the conversion of

dopamine to form the neurotransmitter norepineprine (Combs, 1998). AA has also been implicated in the metabolism of cholesterol to bile acids (Ginter, 1973) and in steroid metabolism in adrenal (Burri and Jacob, 1997). Hydroxylation of aromatic drugs and carcinogens by hepatic cytochrome P450 is also enhanced by AA (Burri and Jacob, 1997). AA acts as an antioxidant by donating electrons and hydrogen ions and reacting with reactive oxygen species or free radical. AA helps to facilitate the adsorption of iron especially non-heme iron. AA reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{3+}) (Basu and Dickerson, 1996). Other proposed activities of AA include maintenance of enzyme thiols in a reduced state and sparing of glutathione, an important intracellular antioxidant and enzyme cofactor (Meister, 1994). AA participates in immune system via the increase of leukocyte and effects relating to cancer (Combs, 1998). Moreover, many biochemical, clinical, and epidemiologic studies have indicated that AA may be of benefit in chronic diseases such as cardiovascular disease, cancer and cataract (Combs, 1998).

Table 2. Ascorbic acid contents of some uncooked foods (Combs, 1998)

Food	Vitamin C (mg/100g)	Food	Vitamin C (mg/100g)
Fruits		**Vegetables**	
Apple	10-30	Asparagus	15-30
Banana	10	Bean	10-30
Blackcurrants	200	Broccoli	90-150
Cherry	10	Cabbage	30-60
Grapefruit	40	Carrot	5-10
Guava	300	Cauliflower	60-80
Hawthorn	160-800	Celery	10
Melons	13-33	Collard greens	100-150
Orange, lemon	50	Corn	12
Peach	7-14	Kale	120-180
Raspberry	18-25	Leek	15-30
Rose hips	1000	Oat, wheat	0
Strawberry	40-90	Onion	10-30
Tangerine	30	Pea	10-30
Animal products		Parsley	170
Meats	0-2	Pepper	125-200
Liver	10-40	Potato	10-30
Kidney	10-40	Rhubarb	10
Milk		Rice	0
Cow	1-2	Spinach	50-90
Human	3-6		

1.1.5 Application of L-ascorbic acid

Nowadays the use of AA is not limited to agents which enrich vitamin C as an essential nutritive element, but is extending in various applications. More particularly, because of the chemical structure and physiological activities, AA is useful as a souring agent, reductant, antioxidant, bleaching agent and stabilizer in various chemical reagents, foods and beverages. AA is also utilized as a cosmetic ingredient for skin care and extramedicine in commercial cosmetics because of its beneficial role against skin aging, such as promotion of collagen synthesis and inhibition of melagenesis (Murad et *al.*, 1981). The application of L-ascorbic acid in various industries was summarized in Table 3.

1.1.6 Degradation of L-ascorbic acid

The major drawback of AA is that it readily loses the physiological activities because of its high susceptibility to oxidation. AA is easily oxidized when in aqueous solution, especially in alkaline medium and on exposure to heat, light, traces metal ion likes copper and ascorbate oxidase (Tolbert *et al.*, 1975). The fist stage of oxidation, AA is oxidized reversibly to dehydroascorbic acid which is irreversibly degraded further by hydrolytic opening of the lactone ring to yield 2,3-diketogulonic acid (a biologically inactive compound). The intermediate 2,3-diketogulonic acid undergoes either further degradation to the various five carbon compounds (xylose, xylonic acid, lyxonic acid), or oxidation to oxalic acid and L-threonic acid (Figure 4). In acidic solutions, the degradation proceeds in forming L-(+)-tartaric acid, furfural, and other furan derivatives, as well as some condensation product (Yuan and Chen, 1998). Alkaline-catalyzed degradation results in over 50 compounds, mainly mono-, di-, and tricarboxylic acids (Moser and Bendich, 1998). The degradation of ascorbic acid is not only important in nutrition, but is also related to flavor and color changes of foods

Table 3. Industrial applications of L-ascorbic acid

(Sakai, *et al.*, 1998)

Functions	Products
Foods and beverages	
1) Color and flavor stabilizer	Fruit juice, canned fruit
2) Preventing of taste deterioration	Pet foods, animal feeds
3) Antioxidant	Carbonated beverage, beer, pet foods
4) Sough conditioner	Breads, cereals, confectionery
5) Speed curing process	Hams, bacon, sausage
6) Vitamin C enrichment	Animal feeds, pet foods, fruit juice
7) Souring agent	Confectionery, beverage
Cosmetics	
1) UV absorbent	Skin lotion, sunblock cream
2) Melanin formation inhibitor	Sunblock cream, foundation
3) Skin refining agent	Skin moisturizing lotion, lipstick
4) Skin whitening agent	Milky lotion, foundation,
Pharmaceuticals	
1) prevention and remedy for viral and bacterial disease	
2) astringent	
Plastics	
1) UV absorbent	
2) Deterioration-preventing agent	

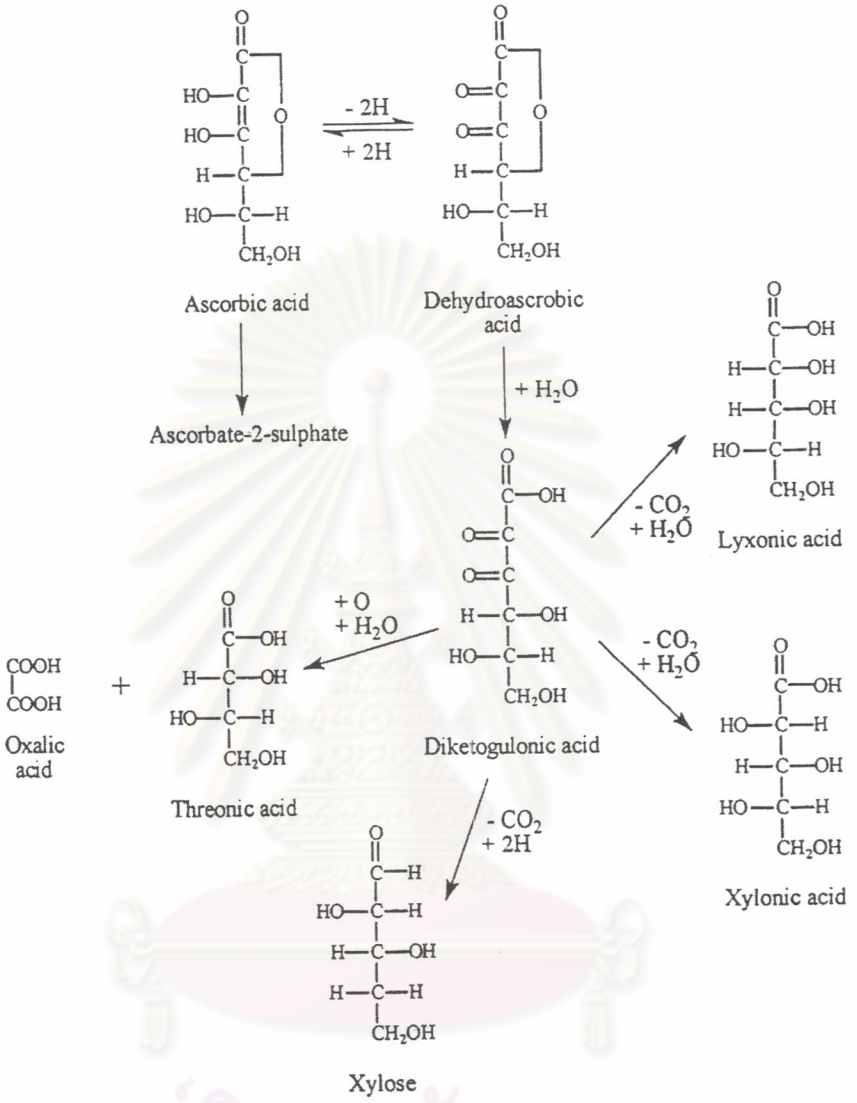


Figure 4. Degradation of ascorbic acid (Basu and Schorah, 1982)

especially juices. The browning in processed fruit juices is mainly caused by the degradation of ascorbic acid (Rodriguez *et al.*, 1991; Solomon *et al.*, 1995). Therefore, users must use AA in a higher amount that far exceeds a theoretical level, and must pay close attention to the storing and handling of the compound. The instability against such oxidative environments hampers its application in food and various industries.

1.1.7 Ascorbic acid glucoside

To overcome the instability of AA, many efforts have been proposed to generate more stabilized AA derivatives with maintaining the inherent *in vivo* biological activity. The numerous stable derivatives of AA have been synthesized by chemical methods (Tolbert *et al.,* 1975). Some examples of 2-O-substitued L-ascorbic acid such as L-ascorbic acid 2-O-sulfate (AA-2S) (Figure 5A) (Mead and Finamore, 1969), L-ascorbic acid 2-O-phosphate (AA-2P) (Figure 5B) (Mima *et al.*, 1970) and L-ascorbic acid 2-O-methylester (AA-2M) (Figure 5C) (Lu *et al.,* 1984) have been demonstrated to be stable *in vitro* and have shown nonreducing activity. Among them, AA-2S and AA-2M were found as naturally occurring metabolites of AA. However, they were deficient of a substantial vitamin C activity in monkeys and guinea pigs (Kuenzing *et al.,* 1974; Machlin *et al.*, 1976). On the other hand, AA-2P exerted an antiscorbutic activity in monkeys and guinea pigs although it had not been unequivocally identified as a metabolite of AA in these animals. AA-2P can also enhance collagen synthesis in cultured human skin fibroblasts (Machlin *et al.*, 1979). However, AA-2P was not persistent because AA was released very rapidly from AA-2P by hydrolysis and the hydrolyzed AA was unstable in the culture medium (Kumano *et al.*, 1998). Derivatives which are substituted at not only the C2 or C3

positions but also at the C6 position by a fatty acyl group, alkyl group or phospholipid were endowed with hydrophobic properties (Nishiyama *et al.*, 1993).

It has been reported that certain vitamins are found naturally in glucose-bound form with a speculation that it may be a means by which the organisms stored native vitamin in a stabilized form. Hence, some attempts to obtain a stable glucosylated ascorbic acid by chemical procedures have been made (Ishido *et al.,* 1983; Masamoto *et al.,* 1983). In these methods, glucose has been conjugated to AA in the β-configuration. Studies on this β-D-glucopyranosyl type derivative confirmed that they may not be a promising precursor of AA, because the physiological release of an active AA should be dependent on β-glucosidase which is distributed in a trace amount in a limited number of tissue. This means that they hardly exhibit desired physiological activities in living body. Furthermore, conventional organic chemical process have the drawbacks that they are inferior in economical efficiency because the reaction is very complicated, time-consuming and low yield, as well as that the establishment of non-toxicity and safeness for the resultant derivatives is very difficult.

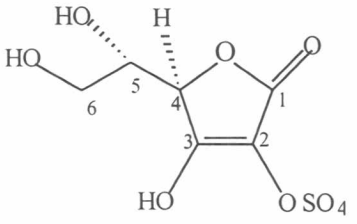
AA has also been described to be conjugated with glucose by the enzymatic method utilizing a saccharide-transfer reaction. The glucosylated AA has been synthesized from AA and maltose or other α-glucans by regioselective transglucosylation with various enzymes from different sources. Two types of glucosylated AA, namely 6-O-α-D-glucopyranosyl-L-ascorbic acid (AA-6G) (Figure 5D) and 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) (Figure 5E) have been reported to be synthesized enzymatically. AA-6G was synthesized in the presence of maltose and AA with α-glucosidase from *Aspergillus niger* by Suzuki *et al.* (1973). However, AA-6G is not satisfactory improved in chemical stability over AA in

aqueous solutions (Yamamoto, Muto, Nagata *et al.*, 1990). The enzymatic transglycosylation to synthesize AA-2G has been studied using various enzymes from mammalian tissues (Muto *et al.*, 1990), microorganisms (Aga *et al.*, 1991) and plants. Among various enzymes, rice seed α-glucosidase (EC 3.2.1.20, α-D-glucohydrolase) (Muto *et al.*, 1990) and cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) (Yamamoto, Muto, Murakami *et al.*, 1990; Aga *et al.*, 1991; Tanaka *et al.*, 1991 and Jun *et al.*, 2001) are able to form AA-2G efficiently. Both enzymes showed the same regioselectivity in this transglucosylation as mammalian α-glucosidase.

The conventional methods using rice seed α-glucosidase, however, continue to have some problems in the purification of enzymes from these sources and a low yield of AA-2G. It is easier to purify CGTase from bacteria than α-glucosidase from rice (Shin *et al.*, 2000; Nam *et al.*, 2001). Furthermore, CGTase is more stable at high temperature and brings a good productive efficacy. Therefore, the enzymatic transglucosylation using CGTase is suitable for the mass production of AA-2G.

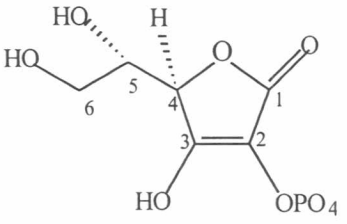
AA-2G is the most promising AA derivative that overcomes the drawbacks of AA. This AA derivative has no reducing group within its molecule so that they have an extremely high tolerance to thermal and oxidative degradations in an aqueous solution (Yamamoto, Muto, Nagata *et al.*, 1990). AA-2G is bioavailable as an ascorbate source *in vivo* and *in vitro* after its enzymatic degradation to free AA by α-glucosidase (Yamamoto, Suga, e*t al.*, 1990, 1992 and Kumano *et al.*, 1998). In addition, AA-2G acts as an effective antiscorbutic vitamin in guinea pig (Yamamoto, Suga *et al.*, 1990; Wakamiya *et al.*, 1992) and stimulates collagen synthesis in human skin of fibroblasts *in vitro* (Yamamoto, Suga *et al.*, 1990). The enzymatic production of AA-2G is more applicable than chemical synthesis of other AA derivative such as AA-2P, AA-2S or AA-2M in terms of reaction steps,

A)



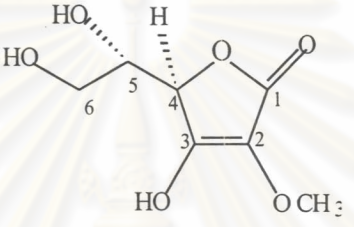
L-ascorbic acid-2-O-sulfate

B)



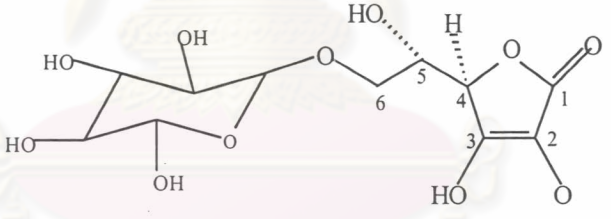
L-ascorbic acid-2-O-phosphate

C)



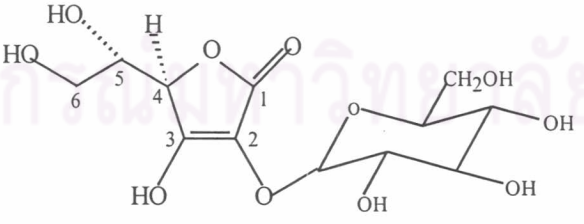
L-ascorbic acid-2-O-methylester

D)



6-O-α-D-glucopyranosyl-L-ascorbic acid

E)



2-O-α-D-glucopyranosyl-L-ascorbic acid

Figure 5. Derivatives of L-ascorbic acid (Sakai *et al.*, 1998)

regiospecificity and production costs. These aspects of AA-2G provide a further basis for its use replace L-ascorbic acid as an effective pharmacological agent, experimental reagents, a promising food additive and cosmetics.

1.2 Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (1,4-α-D-glucan: 1,4-α-D-glucopyranosyl-transferase, EC 2.4.1.19, CGTase) is a unique enzyme capable of producing cyclodextrins (CDs) from starch. CDs are cyclic oligosaccharides in which six or more glucose units are joined by means of α-1,4-glucosidic bonds. Depending to the number of glucose unit (six, seven or eight), they are named α-, β-, or γ-CDs, respectively. CDs are able to form inclusion complexes with various organic and inorganic compounds and improve their physiological and chemical properties (Saenger, 1982). This property made CDs having numerous applications in food, pharmaceutical, chemical and cosmetic industries (Schmid *et al.*, 1989).

CGTase is a multifunctional enzyme that catalyzes several transferase reactions in which a newly made reducing end of an oligosaccharide is transferred to an acceptor molecule. Depending on the nature of the acceptor molecule, four transferase reactions can be distinguished: cyclization, coupling, disproportionation, and hydrolysis as the scheme shown in Figure 6 (Bart *et al.*, 2000).

The CGTase produces CDs from starch and related α-1,4-glucans through an intramolecular transglycosylation reaction known as cyclization. Cyclization is a single-substrate reaction in which an α-glycosidic bond in amylose or starch was cleaved and transferred to its own nonreducing end, resulting in formation of CD. In the presence of substrates and suitable acceptors such as glucose, xylose or sucrose, the enzyme also catalyzes coupling and disproportionation reaction through

intermolecular transglycosylation reactions. Coupling is the reverse reaction of cyclization in which a cyclodextrin ring is cleaved and the resulting linear maltooligosaccharide formed is transferred to an acceptor. This reaction proceeds according to a random ternary complex mechanism. Disproportionation is the major transferase reaction involving cleavage of a linear maltooligosaccharide and transferring of one part to another linear acceptor molecule. It proceeds according to a Ping-Pong mechanism. Furthermore, CGTase has a weak hydrolytic action on α-1,4-glucans and CDs. In hydrolysis the newly made reducing end is transferred to water. The summary of enzyme mechanism was shown in Table 4.

The cyclization in thought to be a special type of disproportionation, the nonreducing end of one chain itself serving as an acceptor, whereas the helical conformation of substrate is thought to be a prerequisite for cyclization. It should be mentioned that the acceptor binding sties of enzyme are not absolutely specific for glucose or maltooligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrates containing 16-80 glucopyranosyl residues. If chain length is greater than 100 units, disproportionation reaction dominates. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 5. High concentration of maltooligosaccharides or glucose favors the reversed coupling reaction resulting in linear and products with negligible amount of CDs (Kitahara *et al.*, 1978). The action of CGTase is different from that other starch degrading enzymes in that the products are cyclic and non-reducing.

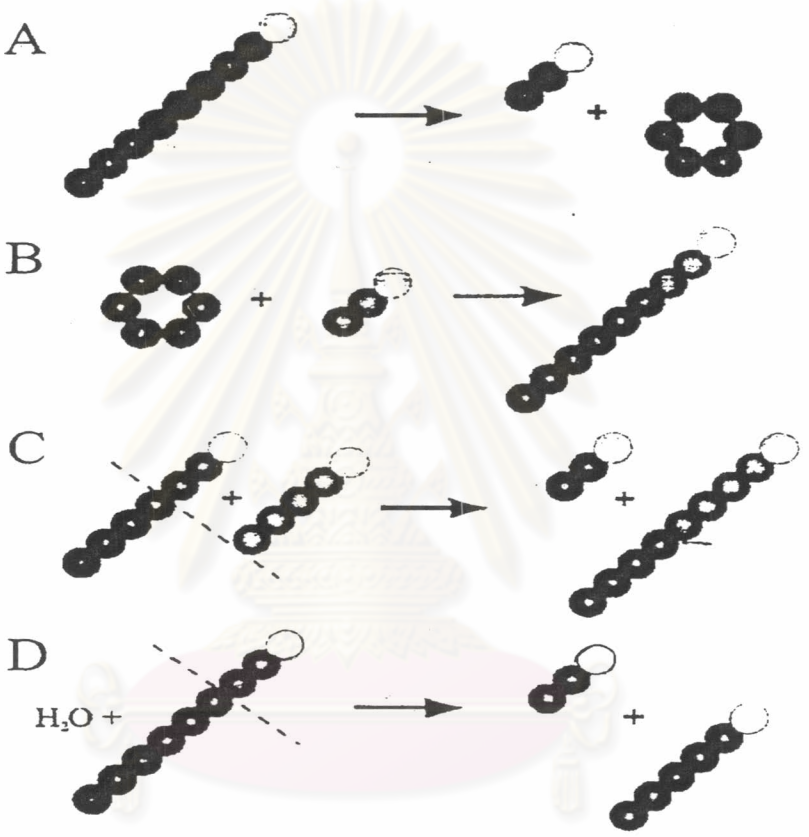


Figure 6. Schematic representation of the CGTase-catalyzed transglycosylation reactions (Bart *et al.*, 2000)

(A) Cyclization; (B) Coupling; (C) Disproportionation; and (D) Hydrolysis.

● = glucose residues; ○ = the reducing end glucoses.

Table 4. Summary of CGTase mechanisms. (Okada and Kitahata, 1975)

Reaction	Action
Cyclization	Starch ⟶ Cyclodextrin
Coupling	Cyclodextrin + $(glucose)_n$ ⟶ Oligosaccharide
Disproportionation	$(oligosaccharide)_m$ + $(oligosaccharide)_n$ ⟶ $(oligosaccharide)_{m-x}$ + $(oligosaccharide)_{n+x}$

Table 5. Relationship between length of substrate and mechanisms of CGTase (Szejtli, 1988).

Substrate chain length (residues)	Effect on mechanism of CGTase
1 (D-glucose)	- no catalysis
2-4	- inhibit initial reaction of cyclization
5-14	- good substrate for coupling reaction
16-80	- good substrate for cyclization reaction
> 100	- good substrate for disproportionation reaction

CGTase is an extracellular enzyme produced by a variety of microorganisms, such as *Klebsiella pneumoniae* (Bender, 1977), *Brevibacterium* sp. (Mori, *et al.*, 1994), and mainly *Bacillus* sp. (Bender, 1986; Komitani, *et al.*, 1993), as listed in Table 6. The CGTase can be classified into three types: α-, β-, and γ- depending on the major kind of CD produced. The enzymes from different sources show slightly different characteristics such as working pH, temperature, and molecular weight. Each CGTase enzyme yields different ratio of CD-product, i.e. *Bacillus macerans* and alkalophilic *Bacillus* 38-2 CGTase produce α-, β- and γ-CDs in relative amounts of 2.7: 1.0: 1.0 (Depinto and Campbell, 1968) and 1: 11.5: 1.5 (Nakamura and Horikoshi, 1976), respectively, while the CGTase of *Bacilus fermus* 290-3 is known to produce γ-CD in the intestinal phase of the enzyme production (Englbrecht *et al.*, 1990).

Table 6. Properties of bacterial cyclodextrin glycosyltransferases

Organism	Main CD produced	Optimal pH	Optimal temperature (°C)	MW (Dalton)	pI	References
Bacillus macerans IFO 3490	α-CD	5.0-5.7	55	5,000	4.6	Kitahata *et al.*,1974
Bacillus stearothermophilus	α-CD	6.0	ND	68,000	4.5	Kitahata and Okada, 1982
Bacillus sp. AL-6 (alkalophilic strain)	γ-CD	7.0-10.0	60	74,000	ND	Fujita *et al.*, 1990
Bacillus cereus NCIMB 13123	α-CD	5.0	40	ND	ND	Jamuna *et al.*, 1993
Bacillus sp. IMMIA A. 7/1	β-CD	6.0	50	44,000	ND	Abelian *et al.*, 1994
Bacillus halophilus INMIA 3489	β-CD	7.0	60-62	71,000	ND	Abelian *et al.*, 1995
Klebsiella pneumonia M5 al	α-CD	6.0-7.2	ND	68,000	4.8	Bender, 1982
Thermoanaaerobacterium thermosulfu-rigenes EM 1	β-CD	4.5-7.0	80-85	68,000	ND	Abelian *et al.*, 1995
Bacillus circulans A11						
Isoform 1	β-CD	6.0-7.0	40	72,000	4.73	Kaskangam, 1998
Isoform 2	β-CD	6.0-7.0	40	72,000	4.49	
Isoform 3	β-CD	6.0	50	72,000	4.40	
Isoform 4	β-CD	7.0	50-60	72,000	4.31	

ND = no data

1.3 Enzyme immobilization

Conventional methods which directly utilize the soluble enzyme has been very limited their use in industrial applications. Because the soluble enzymes are generally unstable and readily deactivated, the cost of enzyme isolation and purification still high, and it is technically expensive to recover active enzyme from the reaction mixture after completion of the catalytic process. The aforementioned problems will, of course, lead to an increase cost in the use of enzymes for commercial purposes. One approach to solving these problems is the use of the active and stable water insoluble or immobilized enzymes.

The immobilization of enzyme can offer the possibility of wider and more economical exploitation of enzyme, including allowing reuse of expensive enzyme, simplifying the separation of product from the reaction mixture containing any residual reactant. Other favorable considerations in the use of immobilized enzymes include a greater stability of enzymic activity over broad ranges of pH and temperature, and the possibility for their use in continuous or semicontinuous process (Kenedy and Cabral, 1987).

1.3.1. Enzyme immobilization methods

Several methods are available for enzyme immobilization. The methods used for enzyme immobilization may be divided into four general methods (Chibata, 1978). The first method is to adsorb the enzyme on the support surface by physical forces (hydrogen bond, van der waals forces, hydrophobic interaction, etc.) or ionic interaction. The second method is entrapment involved the physical entrapping of enzyme molecules within a gel lattice. The third method is a crosslinking by means of bi- or multifunctional reagents which may induce intermolecular crosslinking between enzyme molecules. The fourth method is immobilization of enzyme by covalent

binding in which enzymes are immobilized on the support through covalent bonds (Kenedy and Cabral, 1987).

Although a number of immobilization techniques have been applied to many enzymes, there is no perfect technique applicable for enzyme immobilization. Each immobilization technique all possesses some limitations which detract from their uses in industrial process. Therefore, in practice, it is necessary to find a suitable method and conditions for the immobilization of a particular enzyme in the light of the final application designed for the enzyme. There are several factors that need to be considered in developing an immobilized enzyme system. These factors include activity retention, mechanical properties and operational stability of the enzyme preparation, cost and difficulty of the immobilization technique (Kenedy and Cabral, 1987). Some of the relative advantages and disadvantages of the different immobilization techniques are summarized in Table 7.

Immobilization by physical adsorption, ionic binding can be achieved simply under mild conditions. The adsorbed enzymes are easily desorbed from the carrier during utilization by temperature fluctuations and even more readily by changes in substrate and ionic concentrations, due to the relative weakness of adsorptive binding forces. Crosslinking is usually an unsuitable method. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity. In the entrapping method, no binding between enzyme and carrier should occur in theory so that the immobilized enzyme preparations with high retention activity are obtained. However the application of this technique is limited to small molecular substrate and product molecules. If considering the reaction characteristic of CGTase, the substrate is a large molecule-starch. Therefore, the large diffusional

barrier imposed by the membrane to transport the substrate and product will lead to reaction retardation. Covalent binding method has found wide applications and may be used either as the sole immobilization technique or as an integral part of many of the methods already described in which crosslinking reactions are employed. The operational stability of these immobilized enzyme preparations is high, due to the strong covalent bonds between enzyme and carrier. However, the destructive of enzyme caused by chemical modification may be occurred. In order to prepared highly active immobilized enzyme, the immobilization procedure should be carried out under very mild and extremely well controlled conditions.

1.3.2 CGTase Immobilization

The immobilizations of CGTase have been widely reported. CGTases from different sources have been successfully immobilized on various carriers by different methods. Nakamura and Horikishi (1977) studied on the adsorption of CGTase from an alkolophilic *Bacillus* sp. on a vinylpyridine copolymer, anion exchange resin. The enzyme was succinylated to increase the binding forces operating between the enzyme and the carrier. The immobilized enzyme has about 25% of the activity of soluble enzyme. Ivony *et al.* (1983) prepared immobilized *Bacillus macerans* CGTase by covalent binding on a polyacrylamide bead. The specific activity was 3.4-6.3% to that of soluble enzyme and the pH and thermal stability was markedly enhanced by immobilization. The CGTase from alkalophilic *Bacillus* sp. No 38-2 has also been immobilized onto synthetic adsorption resin, DIAION HP-20 (Kato and Horikoshi, 1984). No distinguish change in pH or thermal stabilities of immobilized enzyme were observed. Sakai *et al.* (1991) have immobilized CGTase from *Bacillus stearothermophilus* on FF-4611 (glycidyl methylcrylate polymer) for the continuous production of glucosyl-cyclodextrins (G_1-CDs) from branched dextrin.

Table 7. Comparison of the attributes of different classes of immobilization techniques (Kenedy and Cabral, 1987)

Characteristics	Crosslinking	Physical adsorption	Ionic binding	Covalent binding	Entrapping
Preparation	intermediate	simple	simple	difficult	difficult
Binding force	strong	weak	intermediate	strong	intermediate
Enzyme activity	low	intermediate	high	high	low
Regeneration of carrier	impossible	possible	possible	rare	impossible
Cost of immobilization	intermediate	low	low	high	intermediate
Stability	high	low	intermediate	high	high
General applicability	no	yes	yes	no	yes

Steighardt and Kleine (1993) have achieved covalent binding of *Bacillus macerans* CGTase on porous glass bead. About 25% of enzyme activity was bound. The batch reusability for CDs production of this immobilized enzyme proved to be at least 20 times with a residual CGTase activity of 65%. Okada and his collaborators (1994) used capillary membrane as carrier for CGTase immobilization. The enzyme was covalently bound on the porous sponge layer of this membrane using polyethyleneimine as a crosslinking agent. In 1999 Abdel-Naby established the immobilization of *Paenibacillus macerans* CGTase on aminated polyvinyl chloride by covalent bonding with glutaraldehyde. The immobilized enzyme retained about 85% of its initial catalytic activity after being used for 14 cycles of CD production. Recently, Martin *et al.* (2002) investigated the covalent immobilization of CGTase from *Thermoanaerobacter* sp. in activated silica and Sepharose 4B supports. The catalytic efficiencies of the immobilized CGTase in activated silica and Sepharose were 6% and 2.4%, respectively.

The cyclodextrin research group at the Department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on CGTase of *Paenibacillus* sp. A11, a strain isolated from Southeast Asian soil (Pongsawasdi and Yagisawa, 1987). The enzyme was purified and characterized for some properties such as molecular weight, working pH and temperature and the enzyme activity on various substrate (Techaiyakul, 1991). Effect of some carbohydrates on the induction of CGTase to produce higher CD-products mainly γ-CD was also studies (Rattapat, 1996). The enzyme was purified by chromatofocusing column and analysis on native-PAGE suggested that it may compose of 4 isozymes with different isoelectric points in the range of 4.40-4.90 (Rojtinnakorn, 1994) and was used in enzyme purification through immunoaffinity column chromatography (Kim, 1996). Optimization of

CGTase production in a 5 litre-fermenter and cyclodextrin production from rice starch by using immobilized CGTase in both batchwise and continuous processes and also free CGTase were studied (Rutchtorn, 1993; Kuttiarcheewa, 1994 and Malai, 1995). Siripornadulsil (1992), Vittayakitsirikul (1995) and Boonchai (1996) reported on molecular cloning techniques, gene expression, mapping and partial nucleotides sequence determination. Laloknam (1997) studied on the mutation of *Bacillus* sp. A11 for the production of higher CGTase activity. Kasgangam (1998) later isolated and characterized CGTase isozymes from this strain. Production of cyclodextrins form cassava starch was studied (Nilmanee, 2000) and Wongsangwattana (2000) reported on specificity of glycosyl acceptor in coupling and transglycosylation reactions of CGTase from *Bacillus circulans* A11. In addition reduction of naringin and limonin in tangerine *Citrus reticulata*, Blanco juice with β-cyclodextrin polymer was also studied (Rodart, 2001).

There is an increasing interest in developing efficient and scalable synthetic routes to modify various physiologically active substances for improving their properties and applications in various industries. Transglycosylase activity of CGTase, which catalyzes a coupling reaction, has been widely utilized for the purpose of developing many modified compounds. For these enzymatic reactions, the immobilization of enzyme constitutes an interesting strategy to achieve their stabilization and the separation of the biocatalyst from the reaction mixture. Hence, this research aims to study the immobilization of CGTase from *Paenibacillus* sp. A11 and evaluate the usefulness of the immobilized CGTase for use in the production of AA-2G. Characterization of the immobilized enzyme for catalytic properties and investigation of the optimum condition for AA-2G production will also be performed.

These experiments may establish the fundamentals for subsequent large-scale production of AA-2G in industrial application.

The objective of this research were:

i) To study the immobilization of CGTase by covalent coupling method

ii) To determine the optimum conditions for covalent attachment of CGTase

iii) To characterize catalytic properties of immobilized CGTase and compared them with the soluble enzyme.

iv) To evaluate the use of immobilized enzyme for the production of AA-2G