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MINI-REVIEW

Functions, applications and production of 2-O-D-glucopyranosyl-L-ascorbic acid

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Abstract Vitamin C (VC) is an essential nutrient that cannot be synthesized by the human body. Due to its extreme instability, various VC derivatives have been developed in an attempt to improve stability while retaining the same biological activity. One of the most important VC derivatives, 2-O-D-glucopyranosyl-L-ascorbic acid (AA-2G), has attracted increasing attention in recent years with a wide range of applications in cosmetics, food, and medicine. In this mini-review, we first introduce the types and properties of different VC glycosyl derivatives. Next, we provide an overview of the functions and applications of AA-2G. Finally, we discuss in-depth the current status and future prospects of AA-2G production by biotransformation.

Keywords L-ascorbic acid · 2-O-D-glucopyranosyl-L-ascorbic acid · Applications · Biotransformation

Introduction

L-Ascorbic acid (L-AA) is a water-soluble vitamin commonly known as vitamin C (VC). VC is an essential nutrient, which means our body cannot synthesize it and must be absorbed from foods and supplements (Rumsey and Levine 1998). If our bodies lack VC, scurvy as well as many chronic diseases can occur, including heart disease, cancer, eye diseases, and neurodegenerative conditions (Jacob and Sotoudeh 2002), which scavenge reactive oxygen species in vivo as a potent water-soluble antioxidant (Fujinami et al. 2001). In addition, VC is widely used in food, cosmetic, and pharmaceutical industries by virtue of its functions in collage formation, iron absorption, and carnitine synthesis (Englard and Seifter 1986; Naidu 2003). However, VC is extremely instable in aqueous solution especially under particular oxidative conditions, such as heat, light, metal ions, and ascorbate oxidase (Yamamoto et al. 1990a). Exposure to these oxidative conditions results in the irreversible degradation of VC (Fig. 1). The instability of VC directly reduces its biological activity and greatly limits its applications. Therefore, preparation of a new VC substitute with increased stability that retains the biological activities of L-AA has been a research hotspot in both academics and industry (Yamamoto and Tai 1999).

Various VC derivatives such as ascorbyl (acyl-, -phosphate, and -sulfate) (Mead and Finamore 1969; Mima et al. 1970; Watanabe et al. 2002), ascorbyl palmitate (Dresser et al. 2002; Hsieh et al. 2006), ascorbyl methyl ether (Lu et al. 1984), and ascorbyl glucoside (Yamamoto et al. 1990a) have been chemically or biologically synthesized. However, most of these are not satisfactorily improved in terms of anti-oxidation and releasing L-AA in vivo. L-AA glucoside is an important VC derivative and various ascorbyl glucosides have been reported (Li and Shi 2007). The 2-, 3-, 5-, and 6-hydroxyl groups of L-AA were modified by different chemical and biological approaches to synthesize different L-AA glucosides. Figure 2 shows the chemical structure of 5-O-D-glucopyranosyl-L-ascorbic acid (AA-5G), 6-O-D-glucopyranosyl-L-ascorbic acid (AA-6G), 3-O-glycosyl-L-ascorbic acid (AA-3G), 2-O-D-

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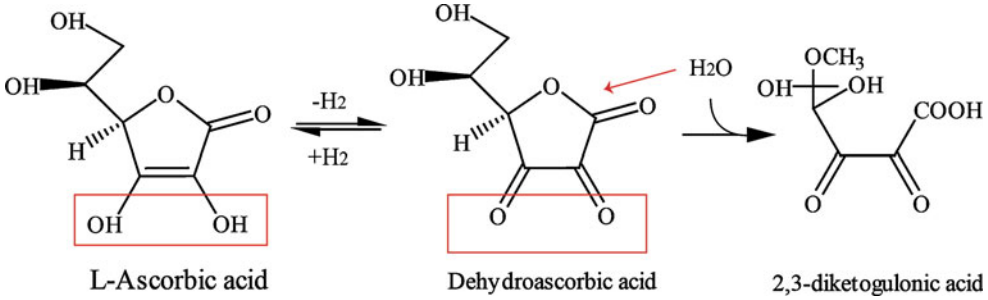

Fig. 1 Oxidation and degradation of L-AA

glucopyranosyl-L-ascorbic acid (AA-2G), and 6-O-acyl-2-O-D-glucopyranosyl-L-ascorbic acid (6-acyl-AA-2G). Compared with other VC derivatives, AA-2G offers several advantages. First, in humans, AA-2G is more readily absorbed. For instance, AA-2G is superior to ascorbyl ester derivatives in cosmetic applications because it can be easily absorbed by the skin and easily degraded in vivo to release L-AA and D-glucose, both of which are readily and safely absorbed (Kouki et al. 1992; Jun et al. 2001). In addition, because of the simple reaction steps, high regiospecificity, and low production cost, enzymatic production of AA-2G is more attractive than chemical synthesis of the other VC derivatives. Therefore, AA-2G is considered the best VC derivative and has recently received increasing attention.

In this mini-review, we first introduce the types and properties of L-AA glucosides. Next, we discuss the functions and applications of AA-2G, and finally we describe the current status and prospects of AA-2G preparation using the biotransformation approach.

Types and properties of L-AA glycosides

The L-AA glucosides mainly include AA-5G, AA-6G, AA-3G, AA-2G, and 6-acyl-AA-2G (Li and Shi 2007; Muto et al. 1991; Yamamoto et al. 1992). AA-5G is the derivative that has a glycosyl modification at the C-5 position of VC (Fig. 2a). It easily dissolves in aqueous solution, 0.1 M

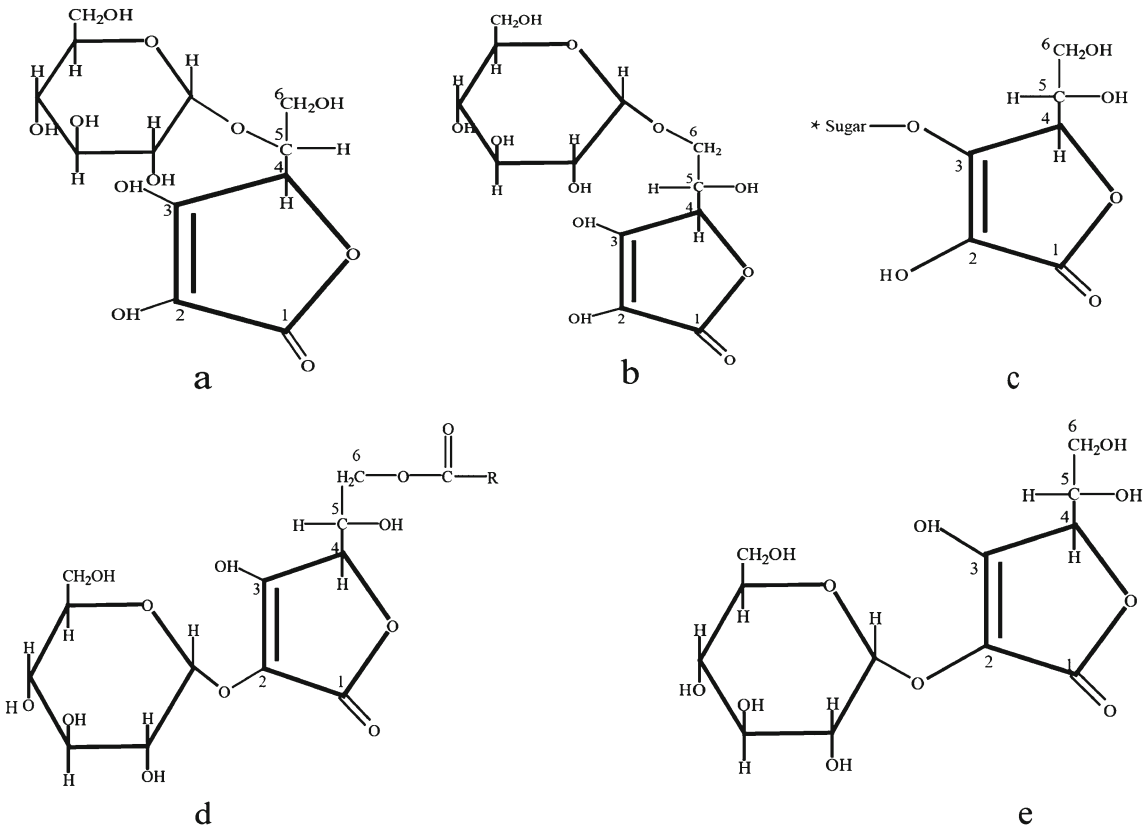

Fig. 2 The chemical structures of L-AA glucosides. (**a** AA-5G; **b** AA-6G; **c** AA-3G; **d** 6-acyl-AA-2G; **e** AA-2G. *Asterisk* sugars include: lactose, isomaltose, gentiobiose, melibiose, cellubiose, chitobiose, maltose, and *N*-acetamido-lactose)

NaOH, or 0.1 M acetic acid and slightly dissolves in methanol or ethanol. However, it is insoluble in diethyl ether and benzene. AA-5G also can be easily reduced and has a color reaction. Additionally, it has been demonstrated that AA-5G is superior to L-AA in terms of increased stability in water solutions (Mandai et al. 1993).

Suzuki et al. (1973) first produced AA-6G by transglucosylation from maltose to the C-6 position of L-AA with *Aspergillus niger* α-glucosidase (Fig. 2b). AA-6G is more stable and reducing than L-AA, yet is still not satisfactorily improved with respect to stability in an aqueous solution, and the glucoside is not absorbed absolutely in vivo (Yamamoto and Muto 1992).

A new L-AA glycosyl derivative, AA-3G, has recently been prepared chemically by Li and Shi (2007). The preparation method involved protecting the 5,6-dihydroxyl of L-AA, then coupling with 1-haloacylglycosyl, obtaining the intermediate 3-O-(acylglycosyl)-(5,6-O-isopropylidine)-L-ascorbic acid, followed by removing the isopropylidine and acyl from the intermediates, thereby obtaining the target compound AA-3G (Fig. 2c). Although it may have better physiological function than other glycosyl derivatives, including AA-2G, it is still in the research stage and much work must be done to realize the industrial production of AA-3G.

The derivative 6-acyl-AA-2G is produced by molecular modifications of AA-2G (Fig. 2d) that include: 6-butyryl-AA-2G, 6-caproyl-AA-2G, 6-capryloyl-AA-2G, and 6-octadecanoyl-AA-2G. It has been demonstrated that these VC derivatives can effectively transform to L-AA and AA-2G with enzymatic activity (Tai et al. 2001; Yamamoto et al. 2002). In addition, with an increase in acetyl groups, the stability and radical scavenging activity of 6-acyl-AA-2G are accordingly enhanced (Fujinami et al. 2001; Takebayashi et al. 2002; Tai et al. 2002). Therefore, 6-acyl-AA-2G is superior to other VC derivatives in terms of radical scavenging activity.

AA-2G is prepared by transferring a glycosyl residue from α-1, 4-glucan to the C-2 position of VC and bound with α-1, 2-linkage (Fig. 2e). The physicochemical properties of AA-2G are as follows: formula, $C_{12}H_{18}O_{11}$; molecular weight, 338.26; melting point, 158.5–159.5 °C; ultraviolet absorption wavelength, λ_{max} 238 nm at pH 2.0 and 260 nm at pH 7.0; colorless; and pillared crystal structure. Due to its high thermostability and inability to be oxidized, AA-2G has become one of the most important VC derivatives and has attracted much attention.

Functions and applications of AA-2G

As a VC derivative, AA-2G exhibits almost all of the in vivo bioactivity of VC. When products containing AA-2G are used on the skin, the action of α-glucosidase gradually releases VC, providing the benefits of VC over a prolonged period of time. Moreover, AA-2G can enhance antibody production and collagen synthesis due to its structural integrity, so it plays an important role in maintaining skin elasticity and repairing damaged skin (Yamamoto et al. 1993). Most importantly, because of the protection of the C-2 glycosyl, AA-2G is extremely stable in response to prolonged oxidation and heating in aqueous solutions and is non-reductive in its intact form (Yamamoto and Muto 1992).

The main application of AA-2G is in cosmetic fields. Due to its non-reducing and anti-oxidizing properties, AA-2G avoids the problem L-AA has in that it cannot be directly incorporated into aqueous solution cosmetics. In addition, AA-2G shows more salutary physiological action than L-AA in terms of collagen synthesis and cytotoxicity inhibition because it had no inhibitory effects on cells and was much more stable (Kumano et al. 1998b; Yamamoto et al. 1992, 1993). Compared with conventional VC derivatives, ascorbic acid 2-phosphate, the conversion of AA-2G to L-AA was sustained for a longer time and AA-2G showed better inhibition ability in melanin synthesis in B16 melanoma cells (Kumano et al. 1998a). Therefore, AA-2G has been used as a primary skin care ingredient and a medical additive in commercial cosmetics (Takebayashi et al. 2002).

AA-2G is easily processed by enzymes such as α-glucosidase to release active L-AA in vivo and in vitro. Thus, it often mimics the physiological effects of L-AA in medical and health care. For example, Yamamoto et al. found that AA-2G had anti-scorbutic activity in guinea pigs (Yamamoto et al. 1990c) and stimulated collagen synthesis in cultured human skin fibroblasts (Yamamoto et al. 1992). Miyai et al. found that AA-2G was the most effective compound among several L-AA derivatives in preventing UV-induced erythema in human and guinea pig skin (Miyai et al. 1996a). AA-2G was also essential for the reduction of actinic injury and can significantly suppress the cytotoxicities of hydrogen peroxide and superoxide anion produced by xanthine and xanthine oxidase (Miyai et al. 1996b). AA-2G also prevents epidermal cell injury by AA-induced positive regulation of cellular antioxidative ability (Miyai et al. 1996b). In addition, AA-2G potentiates B cell function in the humoral immune system because it enhances antibody production in cultured splenocytes via a continuous supplementation of L-AA (Yamamoto et al. 1993). Recently, Taniguchi et al. found that AA-2G could protect dermal fibroblasts from oxidative stress and cellular senescence (Taniguchi et al. 2012). AA-2G was also found to effectively scavenge radiation-induced radicals and protect against the enhanced effects of radiation in an abscopal region after local gamma ray irradiation in the head (Kinashi et al. 2010). Therefore, AA-2G has great potential in the health care and medical industries.

AA-2G also has potential in the animal husbandry and aquaculture fields (Yamamoto et al. 1993; Muto et al. 1991). VC is indispensable for the growth of young animals and can prevent or treat many diseases, including skin diseases and scurvy. Because of its instability, VC is rarely used in forage directly. Instead, AA-2G is widely added to forage as an important component due to its stability and steady L-AA bioactivity. Moreover, as an L-AA derivative, AA-2G retains most of the biological and physiological functions of VC so it can be used in almost every field where VC can be used.

Production of AA-2G by biotransformation

At present, biotransformation is the main method for AA-2G production. Although Toyoda-Ono et al. tried to synthesize AA-2G by a chemical approach, they found that the procedure was too complicated and was thus unsuitable for large-scale production (Toyoda-Ono et al. 2004).

The production of AA-2G by biotransformation was jointly discovered by Hayashibara Biochemical Laboratories of Japan and the Department of Pharmacology of Okayama University (Aga et al. 1991; Yamamoto et al. 1998). The transformation was catalyzed by cyclomaltodextrin glucanotransferase (CGTase) with α-cyclodextrin and L-AA as substrates, and the purity of AA-2G can achieve 97 % (Aga et al. 1991). So far, five enzymes have been used for AA-2G production, including α-glucosidase (EC 3.2.1.20) (Muto et al. 1990), CGTase (EC 2.4.1.19) (Aga et al. 1991), amylase (EC 3.2.1.1) (Lee et al. 2004), sucrose phosphorylase (EC 2.4.1.7) (Kwon et al. 2007), and α-isomaltosyl glucosaccharide-forming enzyme (Mukai et al. 2005). Table 1 shows the source, glycosyl donor, and disadvantages of these enzymes.

The α-glucosidase derived from mouse intestine and corn seed were first found to catalyze AA-2G synthesis (Muto et al. 1990; Yamamoto et al. 1990b). In the biosynthesis process, the glycosyl from maltose was transformed to L-AA by α-glucosidase. This enzyme was obtained from many sources, including animal (mouse kidney and intestine, dog and pig intestine), plants (seed of corn), and microorganisms (*Mucor*, *Penicillum*, *Aspergillus*, and *Saccharomyces*) (Yamamoto et al. 1998). Compared to the enzymes extracted from animals or plants, the enzymes produced by microbial fermentation have much superiority such as the simple productive process, low production cost, short production cycle, and potentiality for large-scale production. At present, *A. niger* is the commonly used wild-type microbe for the production of α-glucosidase. To improve the enzyme yield, many recombinant α-glucosidase has been investigated. For example, *A. niger* α-glucosidase was expressed in *Aspergillus nidulans* (Nakamura et al. 1997) and *Emericella nidulans* (Ogawa et al. 2006), and the expression level was reported to be 0.04 and 0.96 U/mg, respectively. Chen et al. expressed *A. niger* α-glucosidase gene in *Pichia pastroris* and the enzyme activity in the culture supernatant of a 3-L fermentor reached 2.07 U/mL (Chen et al. 2010). For AA-2G biosynthesis with α-glucosidase as the catalyst, few intermediate products were produced, but the synthesis efficiency of AA-2G was low because α-glucosidase could catalyze AA-2G hydrolysis at the same time as it was synthesizing it (Yamamoto et al. 1998).

CGTase is considered to be the best enzyme for large-scale production of AA-2G due to its high substrate specificity (Tanaka et al. 1991). Microbial fermentation is the main source for CGTase production, and the main microbes include *Paenibacillus macerans*, *Bacillus* sp., and *Bacillus circulans*. Usually the activity of CGTase from the wild-type strains is very low. For instance, the activity of CGTase from *Bacillus firmus* was only 7.05 U/mL (Gawande et al. 1998), and that from *Bacillus cereus* was 106 U/ml under the optimal conditions (Jamuna et al. 1993). In order to improve the enzyme yield, the

Table 1 Comparison of the enzymes for AA-2G synthesis from different sources

Enzyme	Source of enzyme	Glycosyl donor	Advantages/disadvantages	References
α-Glucosidase	Animal intestinum tenue, rice seeds and microorganisms	Oligosaccharide	Few mid-products and vice-products, but low synthesis efficiency and limited source	Muto et al. 1990; Yamamoto et al. 1990a, b
Cyclomaltodextrin glucanotransferase	*Bacillus stearothermophilu*, *Paenibacillus* sp., *Paenibacillus macerans*	Oligosaccharide and cyclodextrin	High transformation efficiency but need glucoamylase	Aga et al. 1991; Jun et al. 2001; Zhang et al. 2011b
Amylase	*Bacillus*	Starch	Few mid-products but low substrate specificity	Lee et al. 2004
Sucrose phosphorylase	*Bifidobaterium longum*	Sucrose	A novel enzyme	Kwon et al. 2007
α-Isomaltosyl glucosaccharide-forming enzyme	*Arthobacter golbiuformis*	Oligosaccharide	Few or no side-products, it is still being studied in the laboratory	Kubota et al. 2007; Mukai et al. 2005

CGTase from *P. macerans* was expressed in *Escherichia coli* (Li et al. 2010), and the yield of extracellular recombinant CGTase reached 275.3 U/mL (Cheng et al. 2011), which was the highest ever reported. When CGTase is used for catalyzing the biosynthesis of AA-2G, α- and β-cyclodextrins are often used as glycosyl donors. Many intermediate products, such as AA-2G*n* ("*n*" means the number of glycosyls attached to the L-AA), are produced along with AA-2G, and thus, further treatment with glucoamylase is necessary. The transglycosylation to produce AA-2G using CGTase from *Paenibacillus* sp. was studied (Jun et al. 2001), and the amount of AA-2G produced was 2.987 g/L under optimal transformation conditions. Prousoontorn and Pantatan (2007) produced AA-2G using immobilized CGTase with alumina as the carrier. The yield of AA-2G was 2.92 % and the immobilized CGTase retained activity up to 74.4 % of its initial catalytic activity. Zhang et al. (2011b) first used CGTase from recombinant *E. coli* to produce AA-2G and studied the optimization process of enzymatic AA-2G production. In this system, maximal AA-2G production reached 13 g/L. After CGTase was immobilized by sodium alginate, the amount of AA-2G reached 21 g/L, the highest production ever reported (Zhang et al. 2011a).

The α-amylase can be produced from several bacteria (especially *Bacillus*), yeasts, and fungi, and bacterial amylase is generally preferred over fungal amylase (Pandey et al. 2000). The activity of α-amylase achieved 26,350 U/g dry bacterial bran by *Bacillus coagulans* in solid state fermentation (Babu and Satyanarayana 1995) and reached 16.42 UI by *Bacillus* sp. (Tanyildizi et al. 2005). Nowadays, the industrial production of α-amylase has been realized. However, for AA-2G synthesis, α-amylase is inferior to CGTase due to its poor substrate specificity.

The other two other enzymes [sucrose phosphorylase, (SPase) and α-isomaltosyl glucosaccharide-forming enzyme, (IMGase)] were also used to catalyze AA-2G synthesis. Transformation to AA-2G by SPase from *Bifidobacterium longum* was first reported by Kwon et al. (2007). Although SPase has not been used to produce AA-2G in large-scale production, it can use sucrose as a glycosyl donor, and this provides a new method for AA-2G preparation. The method using IMGase from *Arthrobacter globiformis* to produce AA-2G was also reported (Kubota et al. 2007; Mukai et al. 2005). The advantage of this enzyme is that the product has few (or no) by-products. However, this enzyme is still being studied in the laboratory, and no commercial IMGase is currently available.

At present, the most commonly used enzymes for AA-2G production are CGTase and α-glucosidase. Table 2 shows a comparison of AA-2G production by CGTase from *P. macerans* JFB05-01 and α-glucosidase from *A. niger* SG136 with different glycosyl donors. For CGTase, α-cyclodextrin was the best glycosyl donor for AA-2G synthesis. The conversion efficiency of β-cyclodextrin was 22 % lower than α-cyclodextrin, and that of maltose was even lower. Similar results were also reported in the literature (Aga et al. 1991; Jun et al. 2001; Zhang et al. 2011b). However, the cost of α-cyclodextrin is too high and β-cyclodextrin is insoluble in aqueous solution, so both are unsuitable for large-scale production of AA-2G. The substrate specificity of α-glucosidase was superior to CGTase because it can utilize maltose as a glycosyl donor, but there is increased difficulty of AA-2G purification due to the high concentrations of the by-product AA-6G. Therefore, obtaining a high AA-2G yield using maltose as a glycosyl donor is an attractive challenge because maltose is easily dissolved in water and inexpensive. To solve this problem, we can improve the substrate and product specificity of glycosyltransferases via rational and semi-rational design strategies. As shown in Fig. 3, we can analyze the molecular mechanisms (structures and functions of different domains) of CGTase and α-glucosidase by computer simulation and software analysis using proteomics and bioinformatics, and further construct recombinant enzymes via domain swapping and site-directed mutagenesis technologies, and finally employ kinetics reaction method to screen the target enzyme having high product (AA-2G) specificity with maltose as a glycosyl donor. This will be helpful for industrial production of AA-2G with a high yield and low cost.

Table 2 Comparison of cyclodextrin glycosyltransferase and α-glucosidase for catalyzing AA-2G synthesis

Specificity	Glycosyl donors	Cyclodextrin glycosyltransferase (from *Paenibacillus macerans*)	α-Glucosidase (from *A. niger*)
Substrate specificity (relative activity[a])	α-Cyclodextrin	100 %	11 %
	β-Cyclodextrin	78 %	13 %
	Maltose	36 %	100 %
Product specificity (proportion of production)	AA-2G	>90 %	15–50 %
	AA-5G	Little	Little
	AA-6G	Little	20–60 %
Number of glucosyls (*n*)		*n*=1, 2, 3, 4, 5, 6	*n*=1

All the data were from Lab of Bioprocess and Biosystems Engineering, Jiangnan University, China

[a] The relative activity was measured by the conversion efficiency of different glycosyl donors to AA-2G and by-products

After the reaction occurs, not only the product AA-2G was present in the solution but also L-AA and saccharides. To get purified AA-2G, further separation and purification are necessary. At present, ion exchange and electrodialysis are the two main methods for AA-2G purification. Ion exchange (Yamasaki et al. 2001) is one of the most commonly used methods for AA-2G purification (Fig. 4a). The demineralized reaction solution is first subjected to an anion ion-exchange resin column, which uses one in the form of a gel-, macroreticular-, or macroporous structure as a mother structure and uses strong- or weak-base styrene and acrylic

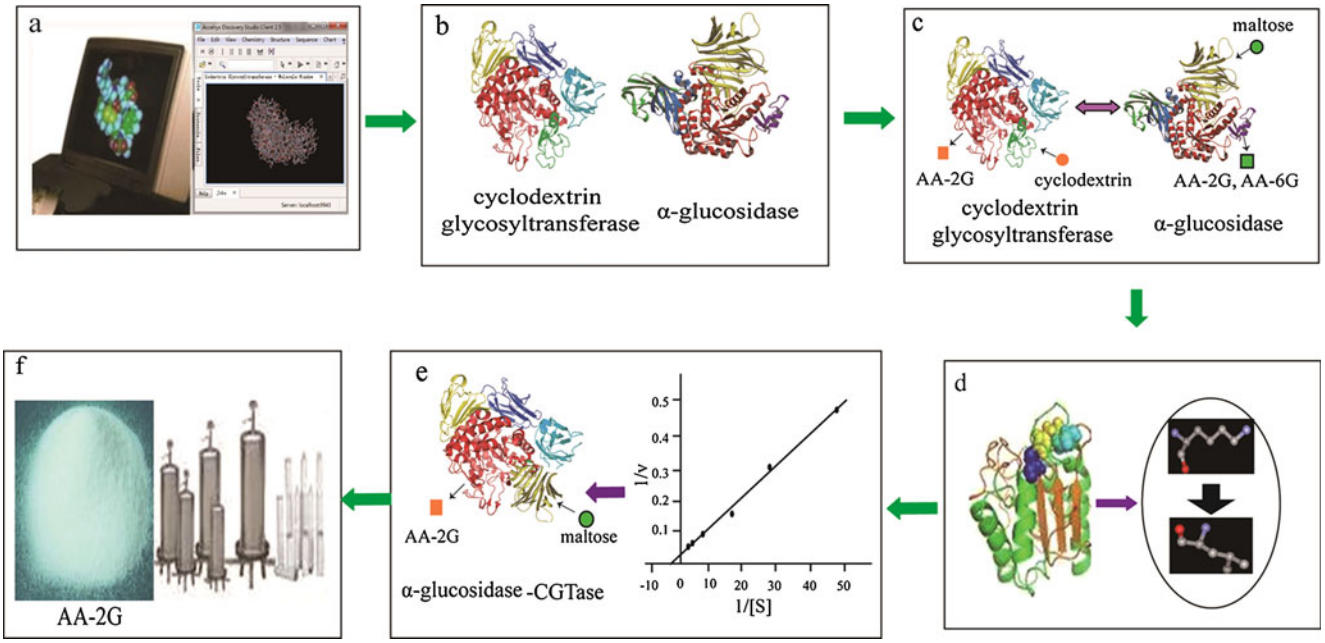

Fig. 3 Diagrammatic map of the molecular modifications of glucanotransferases (**a** computer simulation and software analysis; **b** analysis of structure and function of different glucanotransferases domains; **c** domain swapping with different glucanotransferases; **d** site-directed mutagenesis to construct different mutants; **e** target enzyme screening by the kinetics reaction; **f** high content AA-2G product)

anion-exchange resins as a basal material. Subsequently, AA-2G is adsorbed and L-AA and saccharides are removed by elution with solutions such as HCl, H_2SO_4, HNO_3, NaCl, KCl, Na_2SO_4, $NaNO_3$, citric acid, and citric acid sodium.

Electrodialysis (Aga et al. 1994) is the other method to separate and purify AA-2G (Fig. 4b). An electrodialysis is an anion-exchange membrane used to allow L-AA to predominantly permeate the membrane and to separate L-AA from AA-2G or to allow AA-2G to predominantly permeate the membrane and to separate AA-2G from the saccharide. Finally, a product with a high content of AA-2G is prepared that has satisfactory stability and physiological activity. The ion-exchange membranes utilized in this method are high molecular organic membranes that are mainly composed of stylene–divinyl copolymers and synthetic fibers as a reinforcing agent. The anion-exchange membranes are those that have a quaternary ammonium salt group, and the electrolytic solution is an approximately 0.1–2 N salt solution. If there is AA-2G*n* in the reaction solution before purification with the above two methods, glucoamylase should be used to hydrolyze AA-2G*n* to AA-2G and D-glucose. The resultant separated by the above two methods is then stored at room temperature, during which colorless crystals form. After a methanol wash and vacuum dry, purified AA-2G crystals are obtained (Mandai et al. 1992).

Conclusions

In this review, we introduced the properties of various VC glycosyl derivatives, provided an overview of the functions and applications of AA-2G, and finally discussed the current

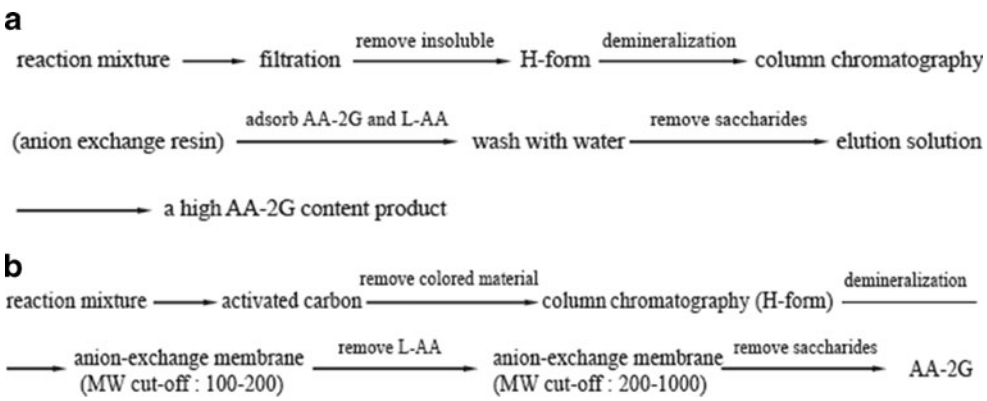

Fig. 4 Methods of separation and purification of AA-2G (**a** the process of ion-exchange method; **b** the process of electrodialysis method)

status of AA-2G preparation methods. The molecular modifications of glycosyltransferases via rational and semi-rational design strategies like domain swapping and site-directed mutagenesis should be an effective approach to improve substrate and product (AA-2G) specificity to achieve industrial AA-2G production.

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