Aqueous two-phase extraction and enzymatic conversion of mulberry anthocyanins

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Over the past 100 years, the use of artificial colors accounted for a large part of the ratio. However, toxicology studies have found that some synthetic pigments are harmful to the human body, and even cause teratogenic cancer. So, in recent years, interest in natural pigments has increased considerably (Agcam et al., 2017), mainly because of their apparent non-toxicity and eco-friendliness. As natural pigments, anthocyanins have been reported to possess antineoplastic, and neuro-protective effects (El et al., 2016). Mulberry, the edible fruit of Morus of the family Moraceae. Mulberry anthocyanins (MAY), commonly called mulberry red pigments, is a very good natural pigment. It is high coloring, safety, water-soluble and has numerous applications in the food industry, such as additives in desserts, baked foods and so on (CastañedaOvando et al., 2009). Anthocyanins in mulberry including cyanidin 3-*O*-rutinoside (C_3G), cyanidin 3-glucoside (C_3G), cyanidin 3-*O*-galactoside glucoside (C_7G) and other elements. Among them, C_3G is the main coloring component, accounting for about 60 % of the total. Thus, the directional hydrolysis of C_3R (about 30 % of the total) with glycosidase can make the content of C_3G close to or even exceed 90 %.

Mulberry is a berry, water content, soft and juicy, thin and easy to break. Due to the weather and storage conditions, the fresh mulberries are very easy to rot or mold. Also, during picking or transportation, broken mulberries often discarded by people. Moreover, if natural or pest disasters occur, large mulberries will deteriorate and cause huge economic losses to the farmers. According to the statistics, the annual output of mulberry is about 6.5 million tons. The picking period is less than one month. It is extremely perishable under natural conditions and seriously affects the economic value of mulberry. For a variety of reasons, the anthocyanins in mulberries are not well utilized, which is a great waste of resources. In order to take advantages of these active substances, anthocyanin extraction and catalytic conversion have been achieved in mulberry waste (Chen et al., 2006).

Fig. 1 Rotten mulberries

It is well known that anthocyanins are soluble in polar solvents and commonly extracted by aqueous mixtures of organic solvent such as ethanol, methanol or acetone which contain a small amount of hydrochloric acid or formic acid to avoid the degradation of acylated anthocyanins (Byamukama et al., 2005). The most common extraction method for natural pigments is traditional solvent extraction with a long time (Pan et al., 2002). Aqueous Two-Phase Extraction (ATPE) is recognized as an effective, versatile and important emerging technique for the downstream processing of biomolecules (Zou et al., 2012). The aqueous two-phase system (ATPS) not only can avoids the anthocyanin acylation (Patil et al., 2009), but also can be very effective to extract MAY (Liu et al., 2008). In this study, using the ATPS consisted of ethanol and ammonium sulfate buffer. In order to obtain the optimum conditions, the effects of several factors on the distribution behaviors of MAY in the developed ATPS was investigated, respectively.

Anthocyanins in mulberry are mainly composed of C_3R and C_3G (Wu et al., 2011). C_3G can significantly inhibit the growth of lung cancer *in vitro* and *in vivo* (Gutiérrezquequezana et al., 2017). In this study, the crude enzyme solution of *E. coli* was added to catalyze the conversion of C_3R and C_3G. The enzyme solution was expressed from unpurified RhaB1 by BL21-pET21a-rhaB1 to form recombinant RhaB1. Through calculating the yield, the process conditions including reaction time, reaction temperature, material ratio were optimized. The standard curves were plotted by HPLC to calculate the linear regression equation for C_3R and C_3G, respectively.

The mobile phase of the HPLC consisted of 0.5 % formic acid in water (solvent A) and 100 % methanol (solvent B) at a flow rate of 1.0 mL/min. The column temperature was maintained at 30 °C and the detection wavelength was 513 nm. Sample solution was filtrated through a syringe filter (0.45 μm) and the injection volume was 10 μL. The fitting formula of C_3R and C_3G were $Y=2.74354\times10^7X-162908.29$ ($R^2=0.9947$) and $Y=1.74063\times10^7X-386867.05$ ($R^2=0.9979$), respectively.

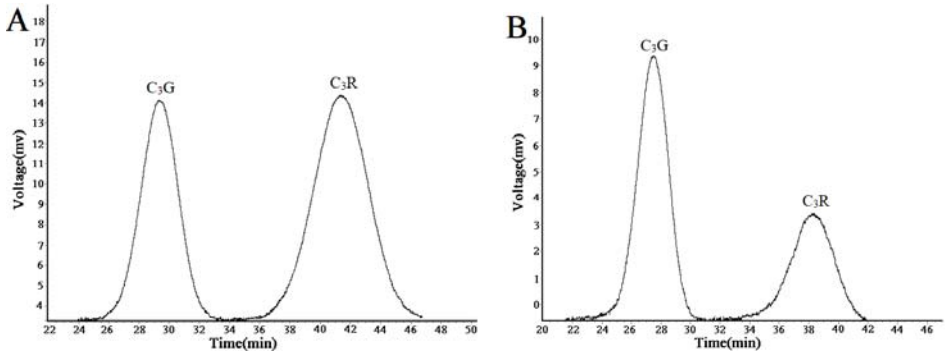

Fig.2.The HPLC chromatogram of C_3G and C_3R in the mulberry juice before (A) and after (B) reaction.

Fig.2A shows the contents of the original C_3G and C_3R in the mulberry juice before the addition of the crude enzyme solution for enzymatic conversion. Fig.2B shows the contents of C_3G and C_3R after the addition of the crude enzyme solution for enzymatic conversion. The experimental conditions of Fig.2B are the crude enzyme solution and mulberry juice mixing by 1: 1 ratio and going catalytic reaction for 1 hour at 40 ° C water shaking bath. By comparing two chromate graphs, it is very clear that the content of the C_3R is significantly reduced and the content of C_3G is increased, indicating that the catalytic reaction has begun. Thus, the developed ATPS could be used for the extraction and enzymatic conversion of mulberry anthocyanins.

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