Full Length Research Paper

A comparison of acidic and enzymatic hydrolysis of rutin

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Rutin and its hydrolysis products (isoquercitrin and quercetin) are widely used as important materials in food and pharmaceutical industry. In this study, the effects of various acids and enzymes as catalysts on the hydrolysis reaction of rutin were studied. In comparison with acidic and enzymatic catalysis of rutin, the research results indicated that there was a sharp difference in the selectivity of hydrolysis product between the methods. When 2.5% H_3PO_4, 1% HCl and 0.5% H_2SO_4 were used as catalysts, transformation yields of isoquercitin hydrolyzed from rutin were 9.60, 0.69 and 1.25%, but those of quercetin were 11.13, 100 and 2.57%, respectively. When hesperidinase, snailase and cellulase-T2440 were used as catalysts, transformation yields of isoquercitin hydrolyzed from rutin were 43.21, 3.07 and 0.00%, but those of quercetin were 58.10, 96.39 and 30.89%, respectively. In conclusion, the aglycon of rutin was deglycosolated easily under mild acidic hydrolysis conditions at appropriate temperatures, but its secondary glucoside was difficult to be obtained. Contrarily, the prepared isoquercitrin by enzymatic hydrolysis of rutin was preferable to the acidic hydrolysis, especially for hesperidinase.

Key words: Rutin, isoquercitrin, quercetin, hydrolysis, acid, enzyme.

INTRODUCTION

Flavonoids are important dietary constituents owing to their health-promoting properties (Wang et al., 2008). For example, rutin which is widely distributed in plant-derived food items is believed to protect against many diseases (Mauludin et al., 2009). Rutin (quercetin-3-O-rutinoside) is composed of one molecule of quercetin as aglucon and rutinose, or isoquercitrin (quercetin-3-O-glucoside) as secondary glycoside and rhamnose. As shown in Figure 1, there is a structural similarity because the three active components have identical molecule of quercetin, so they

have been found to have various similar biological activities including antiproliferative effects on several cancer cells, anti-inflammatory and anti-allergic effects, anti-oxidative activity, and atherosclerosis-preventing effect (Wach et al., 2007; Silva et al., 2009; Motoyama et al., 2009; Fernandez et al., 2005; Erlund, 2004; Seyoum et al., 2006). This chemical family is an abundantly found and widely distributed secondary metabolite in plants. Human beings regularly consume the three components in various fruits, vegetables or herbal medicines such as apple, onion and sophora flower.

Although there is a structural similarity in rutin, iso-quercitrin and quercetin, there are some noticeable diffe-rences in physical, chemical and biological properties (Seyoum et al., 2006). Quercetin is generally accumulated

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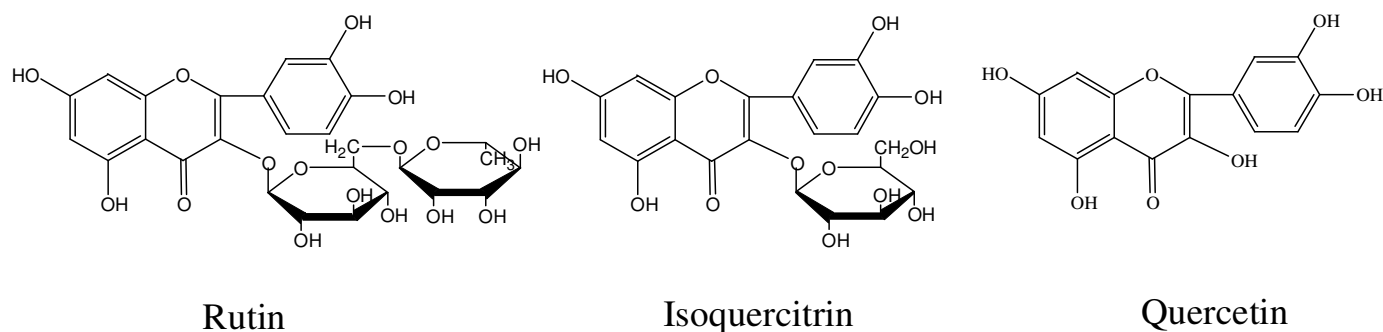*Corresponding author. E-mail: fuword@163.com. Tel: 86-511-8561-6571.

Figure 1. Chemical structures of rutin, isoquercitrin and quercetin.

in plants as glycosides such as glucosides, rutinosides and xylosides (Mauludin et al., 2009). These quercetin glycosides show higher solubility in water than quercetin due to the hydrophilicity of the sugar moieties. Indeed, quercetin-4-O-β-D-glucopyranoside has higher bioavailability than its aglycone in human beings, suggesting that conjugation with glucose would enhance quercetin absorption in the small intestine (Cornard et al., 1999; Wach et al., 2007; Chang et al., 2005). Moreover, orally administered quercetin is poorly absorbed, and the bioavailability of quercetin administered in capsule form to human beings was reported to be less than 1% (Erlund, 2004). Based on this, several trials have been conducted to increase the bioavailability of quercetin by optimizing its formulation. Nowadays, pharmacological congresses show that isoquercitrin possess a wide range of biological activities (allelopatic, antioxidant, atheroprotective, plaque-stabilizing, and anti-inflammatory activities) than rutin (Motoyama et al., 2009; Salim et al., 2004; Fernandez et al., 2005). Especially in Japan, enzymatically modified isoquercitrin (EMIQ) has been approved as a food additive (Shimada et al., 2010), and in USA, FDA declared EMIQ generally regarded as safe (GRAS) for use in multiple food categories (Salem et al., 2010). Therefore, in comparison with rutin and quercetin, isoquercitrin is better absorbed than other quercetin forms, and is expected to be a relative good material for application in medicinal and food application.

Isoquercitrin is a special natural product distributed in few plant sources, and its concentration is 0.01%, so isolating isoquercitrin from plants could be time consuming due to wide variety of impurities present (Williams et al., 1953). However, rutin is widely distributed in many plants in the world (Wang et al., 2010), such as *Alabastra sophorae japonicae* with concentration range of 10- 28% in China (Mauludin et al., 2009). Therefore, synthesizing isoquercitrin from rutin by hydrolysis method seems to be a good alternative.

Nowadays, there are two basic synthesis method of isoquercitrin: chemical hydrolysis and enzymatic hydrolysis (Bojarov and Kren, 2009). With the cleavage of one

rutinose by hydrolysis, rutin can be transformed into quercetin; by cleaving one terminal rhamnose by hydrolysis, rutin can be transformed into isoquercitrin which has several advantages over rutin (Takahama et al., 2009). In order to obtain highly purified isoquercitrin, a suitable hydrolysis method needs to be chosen to obtain isoquercitrin from hydrolysates of rutin for further pharmacological investigation and application research as natural anti- oxidant. Unfortunately, less attention has been paid to the hydrolysis of rutin and its selectivity of reaction product. Therefore, in this study, a comparison of acids and enzymes used as catalysts on the hydrolysis product selectivity of rutin was studied for the selective hydrolysis of rutin for the production of isoquercitrin.

MATERIALS AND METHODS

Chemicals and reagents

Standard isoquercitrin was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and standards of rutin and quercetin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China).

Hesperidinase was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and snailase and cellulase-T2440 were purchased from Sanland chemical Co., LTD (Xiamen, China). All reagents used were of analytical grade except methanol and acetonitrile, which were of HPLC grade purchased from TEDIA Co. (Fairfield, OH, USA). All other solvents and reagents were of analytical grade. Water was purified using an Elga Purelab Option-Q purification system (Elga Labwater, High Wycombe, Bucks, UK) and had a resistivity of not less than 18.0 MΩ cm. This water was used for cleaning procedures and in the preparation of all buffer solutions. All water solutions were prepared with ultrapur water filtered through a 0.45 μm membrane filter.

Analytical methods

HPLC-UV was performed using HITACHI Pump L-7100 with a UV-VIS Detector L-7420 (Techcomp Ltd., Shanghai, China) and N-2000 workstation (Hangzhou Mingtong S&T Ltd., Hangzhou, China).

Separation and determination of rutin and isoquercitrin by using HPLC-UV method was on an Alltima C$_{18}$ column   (250mm× 4.6mm;
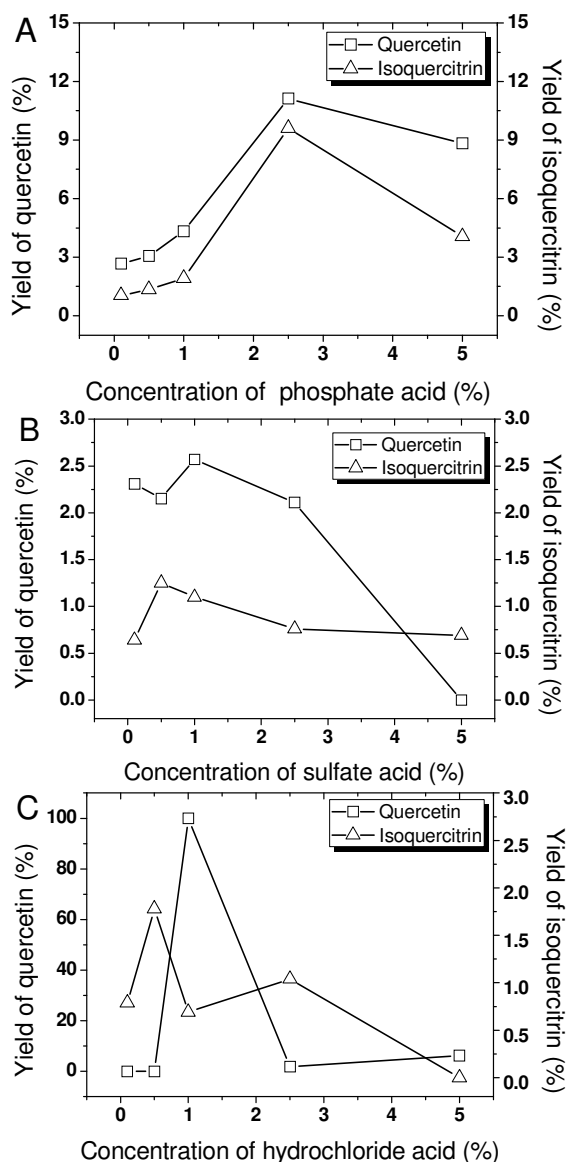
Figure 2. Acidic hydrolysis of rutin by phosphate acid, sulfate acid and hydrochloride acid.

i.d. 5μm) with a mobile phase consisting of acetonitrile: 0.02% phosphoric acid solution (20:80, v/v), and the determination of quercetin was with another mobile phase which consisted of acetonitrile: pH 4 ammonium acetate buffer (27:73, v/v) at a flow rate of 1.0 mL·min^{-1}, detected at 360 nm (Wang et al., 2009). All solutions were filtered through a 0.45 μm filter before injection. All samples were determined in triplicate.

Acidic and enzymatic hydrolysis of rutin

Rutin was weighed exactly 100 mg and dissolved into 100 ml MeOH solution, and then was stored at 4°C. H_3PO_4, H_2SO_4 and HCl were chosen as chemical catalysts, and different amount of the test acids with concentration of 10% were placed in 10 ml air-tight tube. 1 ml of the stock solution of rutin was added into each tube, then,

was diluted with water to a final volume of 5 ml. The tubes were then shaken in a water bath shaker at 70°C for 20 h with an electromagnetic stirring (120 rpm), respectively.

Hesperidinase, snailase and cellulase-T2440 were chosen as enzymatic catalysts, and different amount of the test enzymes with enzymatic concentration of 5 mg were placed into 10 ml air-tight tube and 1 ml of the stock solution of rutin was added to each tube, then, was diluted with water to a final volume of 5 ml. The tubes were then shaken in a water bath shaker at 40°C for 12, 24, 36 and 48 h with an electromagnetic stirring (120 rpm).

When the reaction was finished, the crude hydrolysis products of rutin were centrifuged at 10000 rpm for 10 min and the supernatants were filtered through a 0.45 μm filter before injection. All samples were determined in triplicate by HPLC-UV.

RESULTS AND DISCUSSION

Acidic hydrolysis of rutin by phosphate acid, sulfate acid and hydrochloride acid

Traditionally, flavonoid aglycons and secondary glucosides have been formed by acidic hydrolysis (Takahama et al., 2009). Some glucosidic bonds of flavonoids are easily broken by acidic hydrolysis method; however, some bonds are slowly degraded (Garegg, 2004). In this study, the basic acidic reactions of rutin degradation were the break- age of α-L-rhamnoside bond and β-D-glucoside bond. So, the hydrolytic degradation behaviors of rutin catalyzed by different acid was observed, and the relationship between the yields of each hydrolytic products and the concen- trations of test acids were analyzed systematically. Figure 2 shows the acidic hydrolysis product selectivity of rutin catalyzed by phosphate acid, sulfate acid and hydro- chloride acid, respectively.

Figure 2 is a comparison of the yields of quercetin and isoquercitrin at 70°C for 20 h produced due to the hydrolysis of rutin by phosphate acid, sulfate acid and hydrochloride acid with five different concentrations, respectively. As can be seen in Figure 2A, the results indicated that phosphate acid possessed mild catalysis properties for the breakage of α-L-rhamnoside bond and β-D-glucoside bond of rutin in the same time. The production of quercetin and isoquercitrin was almost instantaneous for phosphate acid-catalyzed hydrolysis reaction when phosphate acid was added to the rutin solution, while it started off from a low value. The yields (and rates of hydrolysis) of quercetin and isoquercitrin that were transformed from rutin were both increased when the concentration range of phosphate acid was 0.1 - 2.5%. The maximal yields of quercetin and isoquercitrin were 11.1 and 9.6% when the concentration of phosphate acid was 2.5%, respectively. However, with the increase of phosphate acid concentration, the yields of quercetin and isoquercitrin both began to decrease. A possible reason for this effect might be due to the instability of quercetin and isoquercitrin with time in the presence of phosphate

acid. This percentage conversion of quercetin and isoquercitrin was improved by catalyzing with 2.5% phosphate acid and separation of the hydrolysis products *in situ*, thus improving both their rates of hydrolysis and yields. Therefore, selecting optimum phosphate acid concentration and other hydrolysis conditions is essential to obtain maximum conversion rate of quercetin and isoquercitrin.

As can be seen in Figure 2B, although sulfate acid possessed more weak catalysis properties for the breakage of α-L-rhamnoside bond and β-D-glucoside bond of rutin than phosphate acid, there was impalpable difference in the production process of quercetin and isoquercitrin. When the concentration of sulfate acid increased from 0.25 to 0.5%, the yield of isoquercitrin increased from 0.64 to 1.25%, however, the yield of quercetrin decreased from 2.31 to 2.15%. On the contrary, when the concentration of sulfate acid increased from 0.5 to 1%, the yield of isoquercitrin decreased from 1.25 to 1.10%, however, the yield of quercetrin increased from 2.15 to 2.57%. Nevertheless, a further degradation of the product isoquercitrin and quercetin at the high pH condition with the concentration of sulfate acid increase from 1 to 5% was also observed. According to the data obtained for this study, the production of isoquercitrin and quercetin mainly depended on the pH value of the hydrolysis system of rutin. Moreover, a possible reason for this effect might be due to the oxidization of sulfuric acid by the view of ionic polarization, which is closely related to the hydrolysis product selectivity of rutin because of the different oxidization between concentrated and dilute sulfuric acid. Therefore, it was difficult to prepare isoquercitrin and quercetin by sulfate acid-catalyzed hydrolysis of rutin with the presence of sulfate acid.

Traditionally, flavonoid aglycons have been formed by hydrochloride acidic hydrolysis. As can be seen in Figure 2C, quercetin was easily obtained by the hydrolysis of rutin catalyzed by hydrochloride acid when compared with isoquercitrin. The yield of quercetin that was transformed from rutin was increased when the concentration range of hydrochloride acid was 0.1 - 1%, and the maximal yield of quercetin was nearly 100% when the concentration of hydrochloride acid was 1%. However, the yield of isoquercitrin was less than 2% when the concentration range of hydrochloride acid was 0.1 - 5%. It was indicated that hydrochloride acid possessed more strong catalysis property for the breakage of β-D-glucoside bond of rutin than α-L-rhamnoside bond. Therefore, it was easy to prepare quercetin by hydrochloride acid-catalyzed hydrolysis of rutin with 1% sulfate acid solution.

Enzymatic hydrolysis of rutin by hesperidinase, snailase and cellulase-T2440

Flavonoid can also be hydrolysed enzymatically, and it is a more interesting way because it produces a more "natural" product in the mild reaction system, in which hydrolyzed products can be used as starting material for the synthesis of substances applied in pharmaceutics, cosmetics and food technology. Figure 3 shows the enzymatic hydrolysis of rutin catalyzed by hesperidinase, snailase and cellulase-T2440, respectively.

As can be seen in Figure 3, the transformation of rutin catalyzed by the varieties of enzymes showed that, rutin can be hydrolyzed by the three kinds of enzymes. Hesperidinase is a crude glycosidase containing α-L-rhamnosidase and β-D-glucosidase. α-L-Rhamnosidase (EC 3.2.1.40) catalyzes the cleavage of terminal rhamnoside groups from rutin to isoquercitrin and rhamnose (Suzuki, 1962). At the same time, β-D-glucosidase (EC 3.2.1.21) catalyzes the cleavage of terminal rutinoside groups from rutin to quercetin and rutinose (Yang et al., 2009). In theory, hesperidinase possess significant catalysis properties for the breaking of α-L-rhamnoside bond and β-D-glucoside bond of rutin at the same time. The experimental results were shown in Figure 3A; the maximal yields of quercetin and isoquercitrin were 74.5 and 43.2% when the enzymatic reaction time was 24 and 12 h, respectively. A possible reason for this effect might be due to the enzymatic activity of β-D-glucosidase which was stronger than that of α-L-rhamnosidase in the presence of hesperidinase. Therefore, selecting optimum enzymatic hydrolysis conditions is essential to obtain maximum conversion rate of quercetin and isoquercitrin.

Figure 3B shows the enzymatic hydrolysis result of rutin catalyzed by snailase, which indicated that snailase can catalyze the cleavage of terminal rutinoside groups from rutin to quercetin and rutinose. When the enzymatic reaction time was 12 h, the maximal yields of quercetin and isoquercitrin were 96.4 and 3.1%, respectively. However, after 12 h, with the increase of enzymatic reaction time, the yields of quercetin and isoquercitrin both began to decrease rapidly. It was indicated that quercetin and isoquercitrin as enzymatic hydrolysis products were unstable in this reaction system with the presenting snailase. The reason is that snailase is a crude glycosidase containing cellulase, pectase, amylase, protease, and so on (Han et al., 2007). Therefore, the enzymic hydrolysis of rutin was carried out with various enzymes which possibly acted sequentially according to two steps: firstly, α-L-rhamnosidase, made the cleavage of the terminal rhamnose, and the corresponding β-D-glucosides (isoquercitrin) was released; subsequently liberation of quercetin took place after the action of a β-D-glucosidase. Moreover, quercetin maybe continuously degraded to other products catalyzed by other enzymes at the same time.

Cellulase is a complex of enzymes containing chiefly endo and exo β glucanases plus cellobiase (Xu et al., 2008). Since different cellulase preparations vary widely in the proportions of the different components, depending on source, growing conditions of the organism, harvesting
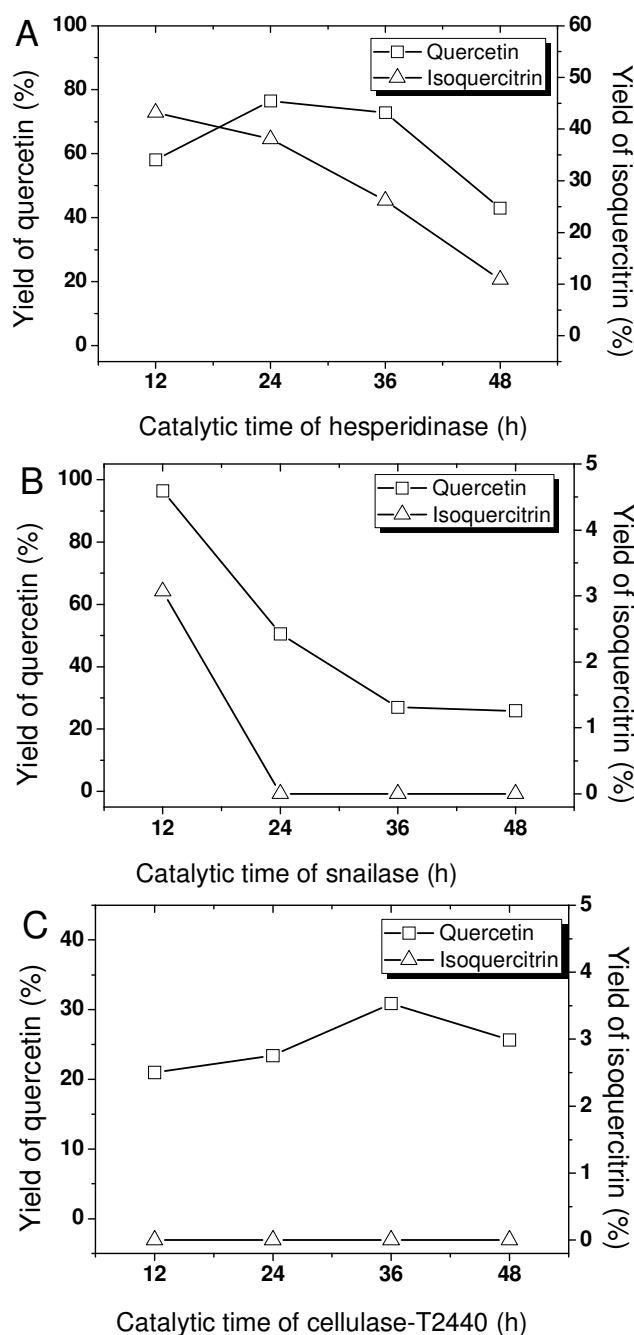
Figure 3. Enzymatic hydrolysis of rutin by hesperidinase, snailase and cellulase-T2440.

and handling procedures, and the rate and extent of the hydrolysis of cellulose substrates also varies widely (Singh et al., 2009). As can be seen in Figure 3C, cellulase-T2440 possessed strong catalysis properties for the breakage of β-D-glucoside bond of rutin; the obtained maximal yield of quercetin was 30.9% when the enzymatic reaction time was 36 h. However, cellulase-T2440 could not catalyze the cleavage of

terminal rhamnoside groups from rutin to isoquercitrin and rhamnose. The reason for this effect is due to the fact that cellulase-T2440 has mild enzymatic activity of β-D-glucosidase.

Comparison of acids and enzymes used as catalysts in the selectivity of hydrolysis product

Although acidic and enzymatic hydrolysis of rutin could obtain isoquercitrin and quercetin, there was a sharp difference in the selectivity of the reaction product. The comparison of acids and enzymes used as catalysts on the selectivity of the hydrolysis product is shown in Figure 4. As can be seen in Figure 4, when 2.5% H_3PO_4, 1% HCl and 0.5% H_2SO_4 were used as catalysts, transformation yields of isoquercitin hydrolyzed by rutin were 9.60, 0.69 and 1.25%, but those of quercetin were 11.13, 100 and 2.57%, respectively. Based on the selectivity of hydrolysis product, rutin could be hydrolyzed by all the hree kinds of acids. H_3PO_4 and HCl used as catalyst got better conversation yield when compared with H_2SO_4. It was indicated that aglycon of rutin was deglycosolated easily under mild conditions at appropriate temperature but its secondary glucoside was difficult to obtain in the acidic solutions. This is because the acid-catalyzed hydrolysis of glycosides is thought to proceed primarily through an A1 carbonium ion reaction (Laopaiboon et al., 2010).

Moreover, rutin could be transformed to quercetin and (or) isoquercitrin by the test enzymes. When hesperidinase, snailase and cellulase-T2440 were used as catalysts, transformation yields of isoquercitin hydrolyzed by rutin were 43.21, 3.07 and 0.00%, but those of quercetin were 58.10, 96.39 and 30.89%, respectively. These results again indicate that the enzymatic hydrolysis of rutin was preferable to the acidic hydrolysis, especially for rutin that occurred only in trace amounts. For the first time, isoquercitrin was identified as a second glucoside which can be prepared by enzymatic hydrolysis of rutin catalyzed by hesperidinase. Essentially, the cleavage of terminal rhamnoside group from rutin catalyzed by hesperidinase was attributed to the rhamnosidase. Rhamnosidase of gly- coside hydrolase (GH) family 78 are widely distributed in fungi, yeast and bacteria. Its industrial applications include the modification of the viscous property of gellan gum and its role in rhamnose production, or the biotransformation of antibiotics and steroids (Ribeiro and Ribeiro, 2008). Therefore, the preparation of isoquercitrin by enzymatic hydrolysis of rutin is a new application of hesperidinase.

Conclusion

In this study, the effects of various acids and enzymes as catalyst on the hydrolysis reaction of rutin were studied,
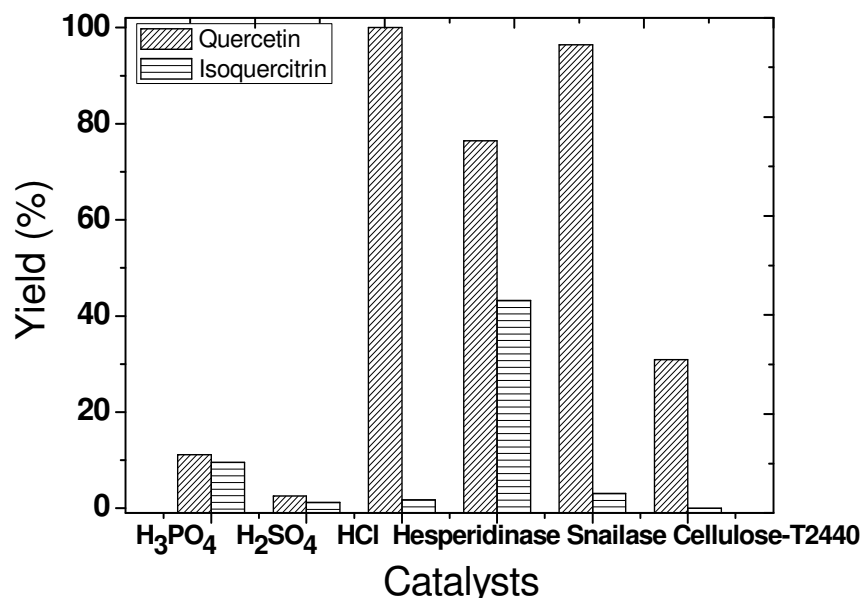
Figure 4. Comparison of the acids and enzymes used as catalysts in the selectivity of hydrolysis product.

respectively. Compared with acidic and enzymatic catalysis of rutin, the research results indicated that there was a sharp difference in the selectivity of hydrolysis product between the methods. When 2.5% H_3PO_4, 1% HCl and 0.5% H_2SO_4 were used as catalysts, the conversion yields of isoquercitin hydrolyzed from rutin were 9.60, 0.69 and 1.25%, but those of quercetin were 11.13, 100 and 2.57%, respectively. When hesperidinase, snailase and cellulase-T2440 were used as catalysts, the conversion yields of isoquercitin hydrolyzed from rutin were 43.21, 3.07 and 0.00%, but those of quercetin were 58.10, 96.39 and 30.89%, respectively. In conclusion, aglycon of rutin was deglycosolated easily under mild acidic hydrolysis conditions at appropriate temperatures, but its secondary glucoside was difficult to be obtained. Contrarily, the preparation of isoquercitrin by enzymatic hydrolysis of rutin was preferable to the acidic hydrolysis, especially when hesperidinase was used

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