An effective biphase system accelerates hesperidinase-catalyzed conversion of rutin to isoquercitrin

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Isoquercitrin is a rare, natural ingredient with several biological activities that is a key precursor for the synthesis of enzymatically modified isoquercitrin (EMIQ). The enzymatic production of isoquercitrin from rutin catalyzed by hesperidinase is feasible; however, the bioprocess is hindered by low substrate concentration and a long reaction time. Thus, a novel biphase system consisting of [Bmim][BF4]:glycine-sodium hydroxide (pH 9) (10590, v/v) and glyceryl triacetate (151, v/v) was initially established for isoquercitrin production. The biotransformation product was identified using liquid chromatography-mass spectrometry, and the bonding mechanism of the enzyme and substrate was inferred using circular dichroism spectra and kinetic parameters. The highest rutin conversion of 99.5% and isoquercitrin yield of 93.9% were obtained after 3 h. The reaction route is environmentally benign and mild, and the biphase system could be reused. The substrate concentration was increased 2.6-fold, the reaction time was reduced to three tenths the original time. The three-dimensional structure of hesperidinase was changed in the biphase system, which α-helix and random content were reduced and β-sheet content was increased. Thus, the developed biphase system can effectively strengthen the hesperidinase-catalyzed synthesis of isoquercitrin with high yield.

Isoquercitrin (quercetin-3-O-β-D-glucoside), a natural, rare ingredient with several biological activities, has attracted increasing attention. Isoquercitrin is generally used as an antithrombotic drug to treat myocardial ischemia, cerebral hypoxia and ischemic disease because of its non-oxidizable1, anti-inflammatory2, anti-depressant, hypotensive, hypolipidemic3, anti-mutagenesis4, and anti-virus properties as well as other pharmacological effects. In addition, isoquercitrin is a key precursor for the biosynthesis of enzymatically modified isoquercitrin (EMIQ), which was recently approved as a multiple food additive5,6. However, isoquercitrin rarely exists in nature7 and the extraction yield of isoquercitrin is extremely low by using traditional extraction methods, which results in high production costs8. Isoquercitrin is a derivative of rutin that lacks a rhamnose moiety in its structure. Recently, several methods for the transformation of rutin to isoquercitrin have been investigated, including acid hydrolysis9, heating10, microbial transformation11, and enzymatic transformation techniques12. Due to its significant economic benefits and ecological acceptability compared to solvent extraction from natural sources and chemical synthesis13, there are increasing reports indicating that isoquercitrin can be efficiently synthesized from rutin using enzymatic hydrolysis process under suitable reaction conditions13.

In recent years, the enzymatic production of isoquercitrin by selective transformation of rutin in buffer systems has been successively established. Hesperidinase (hesperidin-α-1,6-rhamnosidase, EC 3.2.1.40) is an excellent commercially available biocatalyst for the biotransformation of rutin to isoquercitrin with high selectivity14. Compared with crude and recombinant α-L-rhamnosidases, hesperidinase was proposed as a more technically feasible catalyst through the control of pH instead of temperature14,15. Subsequently, a buffer medium containing a specific proportion of the ionic liquid (IL) [Bmim][BF4] was developed as a co-solvent system9. [Bmim][BF4]:glycine-sodium hydroxide buffer (pH 9) (10:90, v/v) was determined to be the optimal medium, which effectively improved rutin solubility and hesperidinase catalytic efficiency. These results indicated that the IL can effectively enhance the selective synthesis of isoquercitrin and the reaction process is simple and eco-friendly14. When the substrate concentration of rutin was increased by 9.8-fold, the reaction time was reduced from 30 h to 10 h, the conversion of rutin was improved to 93.4% (1.7-fold), and the isoquercitrin yield was enhanced to 91.4% (2.3-
fold). However, further industrial application of the hesperidinase-catalyzed transformation in the co-solvent system remains limited by low substrate solubility, slow catalytic efficiency and long reaction time. Therefore, it is imperative to explore more efficient processes for the hesperidinase-catalyzed synthesis of isoquercitrin from rutin via rhamnose hydrolysis.

Biphasic systems have long attracted attention. Biphasic extraction has been used to extract isoquercitrin from a mixture during the reaction process. In this type of system, the catalyst is located in the mobile phase. It is generally believed that the reaction occurs in the aqueous phase or at the phase interface. After the completion of the reaction, a simple phase-separating operation could allow the separation of the catalyst and product. For the hesperidinase-catalyzed synthesis of isoquercitrin from rutin, there is no doubt that this type of extraction is one of the best and simple methods to prepare isoquercitrin in an IL-buffer co-solvent system. In our follow-up study, we determined that glycercyl triacetate is a more suitable extracting agent and the solvent can be recycled and reused. According to dissolve balance principle, isoquercitrin molecules in the enzymatic reactants could be quickly enter the extraction phase, reducing the target product, which shifts the equilibrium reaction in the desired direction. Therefore, if a biphasic system was applied in the hesperidinase-catalyzed synthesis of isoquercitrin from rutin, it would accelerate the biocatalytic progress and also significantly improve isoquercitrin yield. Based on the above, the investigated enzymatic reaction of isoquercitrin biosynthesis using selective conversion of rutin catalyzed by hesperidinase was shown in Fig. 1. However, to the best of our knowledge, no report has been published concerning the application of a biphasic system for the hesperidinase-catalyzed synthesis of isoquercitrin from rutin.

In general, enzymes are the globular proteins whose catalytic activity depends on native configuration of their active sites. Different systems may cause various alterations to enzyme secondary structure, leading to better exposure of active sites of enzyme and possibly altering the equilibrium reaction in the desired direction. Therefore, if a biphasic system was applied in the hesperidinase-catalyzed synthesis of isoquercitrin from rutin, there is no doubt that this type of extraction is one of the best and simple methods to prepare isoquercitrin in an IL-buffer co-solvent system. In our follow-up study, we determined that glycercyl triacetate is a more suitable extracting agent and the solvent can be recycled and reused. According to dissolve balance principle, isoquercitrin molecules in the enzymatic reactants could be quickly enter the extraction phase, reducing the target product, which shifts the equilibrium reaction in the desired direction. Therefore, if a biphasic system was applied in the hesperidinase-catalyzed synthesis of isoquercitrin from rutin, it would accelerate the biocatalytic progress and also significantly improve isoquercitrin yield. Based on the above, the investigated enzymatic reaction of isoquercitrin biosynthesis using selective conversion of rutin catalyzed by hesperidinase was shown in Fig. 1. However, to the best of our knowledge, no report has been published concerning the application of a biphasic system for the hesperidinase-catalyzed synthesis of isoquercitrin from rutin.

Figure 1 | Biosynthesis of isoquercitrin using selective conversion of rutin catalyzed by hesperidinase.
Figure 2 | The effect of reaction conditions on enzymatic synthesis of isoquercitrin from rutin during the enzymatic transformation of rutin using a biphasic system. Reaction conditions: the effect of pH on rutin conversion (A) and isoquercitrin yield (B) using the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system in the presence of hesperidinase (10 U/L) at 40 °C and 180 rpm for 6 h; the rutin concentration was 0.33 mmol/L, and the volume ratio of substrate and glyceryl triacetate was 1:1. The effect of temperature on rutin conversion (C) and isoquercitrin yield (D) using the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system at 180 rpm for 6 h; the rutin concentration was 0.33 mmol/L, and the volume ratio of substrate and glyceryl triacetate was 1:1. The effect of substrate concentration on rutin conversion (E) and isoquercitrin yield (F) using the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system in the presence of hesperidinase (10 U/L) at 180 rpm for 6 h; and the volume ratio of substrate and glyceryl triacetate was 1:1. The effect of enzyme concentration on rutin conversion (G) and isoquercitrin yield (H) using the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system in the presence of hesperidinase (10 U/L) at 40 °C and 180 rpm for 6 h; the volume ratio of substrate and glyceryl triacetate was 1:1. The effect of the volume ratio of the two phases on rutin conversion (I) and isoquercitrin yield (J) using the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system in the presence of hesperidinase (10 U/L) at 45 °C 180 rpm for 6 h; the substrate concentration was 3.3 mmol/L.
hesperidinase-catalyzed transformation of rutin in a biphase system were investigated at 30–55 °C. The concentration of aqueous rutin solution was 3.3 mmol/L and pH was 9.0. The results indicated that the optimal temperature range for the selective biotransformation of rutin to isoquercitrin was 40 to 50 °C. The highest isoquercitrin yield occurred at 40 °C. Additionally, the hesperidinase z-L-rhamnosidase activity was slightly reduced above 50 °C.

As the temperature further increased, the selectivity of isoquercitrin production was reduced via rutin hydrolysis in the [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system. This may be attributed to the hesperidinase, a complex enzyme containing z-rhamnosidase and /-glucosidase, however, z-rhamnosidase primarily catalyzes the hydrolysis reaction29. Additionally, above 50 °C there may be sufficient energy to disrupt some intra-molecular attractions between polar groups as well as disrupt the hydrophobic forces between non-polar groups within the protein structure. When those forces are altered by external factors, they alter the tertiary and secondary enzyme protein structures, which will alter the conformation of the enzyme active site30. Therefore, the optimal enzymatic reaction temperature should be 45 °C for industrial application. Compared with the [Bmim][BF4]-buffer system, the reaction temperature increased 5 °C in the biphase system, which is favorable for industrial production.

### Effect of substrate concentration

Figs. 2E and 2F show the effects of substrate concentration (0.16–4.9 mmol/L) on rutin conversion and isoquercitrin yield in the hesperidinase-catalyzed transformation of rutin in the [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system. The reactions were incubated in aqueous rutin solution with pH 9 at 45 °C. The reaction was sampled every hour for 6 h. As the volume ratio of glyceryl triacetate and substrate increased, the reaction temperature increased 5° C in the biphase system, which is favorable for industrial production.

| Medium | Temperature of reaction (°C) | Substrate concentration (mmol/L) | Enzyme concentration (U/L) | Reaction time (h) | Vm/Km (h⁻¹) | Rutin conversion (%) | Isoquercitrin yield (%) |
|--------|-----------------------------|-------------------------------|--------------------------|------------------|-------------|---------------------|------------------------|
| [Bmim][BF4]:glycine-sodium hydroxide [pH 9] (10:90, v/v) | 40 | 1.3 | 100 | 10 | 1531.4 | 93.4 | 91.4 |
| [Bmim][BF4]:glycine-sodium hydroxide [pH 9] (10:90, v/v) | 45 | 3.3 | 10 | 3 | 6.9 | 99.5 | 93.9 |

### Table 1: Comparative results of the enzymatic conversion of rutin to isoquercitrin in [Bmim][BF4]: glycine-sodium hydroxide (pH 9) (10:90, v/v)/glyceryl triacetate (1:1, v/v) and [Bmim][BF4]: glycine-sodium hydroxide (pH 9) (10:90, v/v) systems

The reaction was executed35. Therefore, isoquercitrin yield probably demonstrated a strange tendency, which shows a strongly different behavior. In general, when the rutin concentration was 1.6 mmol/L, a higher isoquercitrin yield should be obtained. However, if isoquercitrin is abundantly produced and the substrate concentration is appropriately increased, the yield and efficiency should be greatly improved. Therefore, the putative optimal substrate concentration for isoquercitrin production is 3.3 mmol/L.

### Effect of enzymatic activity

Figs. 2G and 2H show the effects of the enzymatic activity (0.1–50 U/L) on rutin conversion and isoquercitrin yield in the hesperidinase-catalyzed transformation of rutin in [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system. When the enzyme concentration was increased from 0.1 U/L to 50 U/L, the isoquercitrin yield and rutin conversion both generally increased. Isoquercitrin yield began to maintain a relatively stable value (80 ± 5%) at 3 h, and then decreased with increasing reaction time. The results indicated that under certain conditions of temperature and pH, the reaction rates increases with increasing amount of enzyme concentration. After 3 h, a longer reaction time caused such a loss of isoquercitrin. To reduce costs of enzymatic reaction in the biphase system, the optimal enzyme amounts was selected as 10 U/L. The maximum isoquercitrin yield, 85.0%, was obtained in the biphase system after 3 h, which was 3.3 times faster than the [Bmim][BF4]-buffer (10:90, v/v) system (10 h).

### Effect of the volume ratio of two phases

In the previous experiment, the reaction was executed35. Therefore, isoquercitrin yield probably demonstrated a strange tendency, which shows a strongly different behavior. In general, when the rutin concentration was 1.6 mmol/L, a higher isoquercitrin yield should be obtained. However, if isoquercitrin is abundantly produced and the substrate concentration is appropriately increased, the yield and efficiency should be greatly improved. Therefore, the putative optimal substrate concentration for isoquercitrin production is 3.3 mmol/L.

Because hesperidinase in a biphase system is a free enzyme, its secondary structure is exposed, which is favorable for protein and rutin interaction during enzymatic hydrolysis. When the hesperidinase active site is obscured by adjacent sites36–38, it cannot sufficiently interact with substrates, preventing the catalytic activity of the enzyme molecules and reducing the catalytic efficiency of hesperidinase.

### Effect of the volume ratio of two phases

Figs. 2I and 2J show the effects of the volume ratio of the two phases on rutin conversion and isoquercitrin yield in the hesperidinase-catalyzed transformation of rutin in the [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system. Five different phase proportions were selected: 1:0.5, 1:1, 1:2, 1:3 and 1:4. As the reaction progressed, the rutin conversion rate gradually increased and isoquercitrin yield gradually improved to 85.2% after 6 h. As the volume ratio of glyceryl triacetate and substrate increased, the time required for the reaction decreased. However, when the volume ratio of the two phases was 1:1, the final isoquercitrin yield was higher than a volume ratio of 1:4. In the previous experiment, this extraction method in the reaction rarely occurred. During the enzymatic reaction, isoquercitrin was extracted by glyceryl triacetate, shifting the chemical equilibrium to the right and improving the reaction rate.

The reaction catalyzed by hesperidinase occurs in the aqueous bulk phase, not at the organic interface. Thus, the mass-transfer rate of the extraction agent from the organic phase to the aqueous phase may also influence the reaction rate39. No significant difference was
observed as the volume ratios were examined, suggesting that the phase volume ratio may not be a sensitive parameter for affecting enzyme stability in a biphase system. If excess organic solvent is added, the product generates some amount of pollution. In addition, excess organic solvent causes waste according to the principles of green chemistry.

The reaction time and isoquercitrin extracted repeatedly. The effect of reaction time on enzymatic synthesis of isoquercitrin was investigated over a range of 0.25–5 h. Fig. 3A shows the rutin conversion during the enzymatic reaction in the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system. The rutin conversion was 96.9% and 99.5% after 1 h and 3.5 h, respectively. Fig. 3B shows the isoquercitrin yield during the enzymatic reaction in a biphase system. With an increased reaction time, the isoquercitrin yield increased linearly during the early stage of enzymatic hydrolysis. However, the isoquercitrin yield was only 75.6% and 83.1% after 1 h and 3.5 h, respectively.

An additional experiment was conducted with a 0–4 h reaction time to inspect the isoquercitrin yield. After terminating the reaction, the organic phase was removed. Fresh organic phase (glyceryl triacetate) was added and extracted for 5 min ultrasonically; then, the organic phase was removed. After extracting 5 times using this method, the rutin conversion and isoquercitrin yield increased to 99.5% and 93.9% after 3 h, respectively. Therefore, 3 h was determined to be a suitable reaction time.

Table 1 also shows the optimized time and temperature using a [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system in comparison with the previous experiments. Using the biphase system, the reaction time was reduced from 10 h to 3 h.

The reaction rate and the calculation of apparent Michaelis constant in a biphase system. Fig. 4A shows the effect of substrate concentrations on the initial reaction rate during the hesperidinase-catalyzed conversion of rutin for the production of isoquercitrin in a biphase system. The rutin reaction rate was rapid during the initial reaction in the biphase system. As the reaction continued, the rutin reaction rate gradually decreased until 1.5 h. After a 2 h reaction, less rutin remained in the system and the reaction rate was relatively slower.

Fig. 4B illustrates the apparent $K_m$ value and $V_m$ by using non-linear regression methods in a biphase system. The apparent Michaelis constant and the maximum velocity of the reaction ($V_m$)
Enzyme and the biphasic system contain [Bmim][BF₄]:glycine-sodium hydroxide (pH 9) (10:90, v/v) system. A decrease in the apparent Michaelis constant indicates the higher affinity. In the developed biphasic system, the initial rate of isoquercitrin production was 0.017 mol/L·h, the apparent kinetic parameter Vₐ/Kₐ (Vₐ = 9.6 mmol/L·h, Kₐ = 1.4 mmol/L) was 6.9 h⁻¹, which was much less than that in a monophase system (1531.4 h⁻¹). But in this experiment, the affinity of hesperidinase toward rutin in a biphasic system was far lower than that in a monophase system. This maybe because of the existing of the reaction interface in the biphasic system, most of the substrates transferred to the interface and the enzyme molecular could not contact rutin.

Table 1 shows the comparative results of the enzymatic transformation of rutin to isoquercitrin in a biphasic [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system and [Bmim][BF₄]-buffer (10:90, v/v) system. Compared with the co-solvent system, the biphasic system had significant advantages in both reaction parameters and catalytic results with high yield and selectivity. According to the enzymatic reaction parameters, the substrate concentration of rutin in the biphasic system was 2.6-fold higher than the [Bmim][BF₄]-buffer (10:90, v/v) system and the apparent kinetic parameter was reduced to 5% the original value in [Bmim][BF₄]-buffer (10:90, v/v) system. Regarding the catalytic results, the rutin conversion and isoquercitrin yield in the biphasic system were 1.07-fold and 1.03-fold higher, respectively, than in the [Bmim][BF₄]-buffer (10:90, v/v) system.

This reaction route is environmentally benign and mild, the enzyme and the biphasic system contain [Bmim][BF₄]:glycine-sodium hydroxide (pH 9) and glyceryl triacetate could be reused. Importantly, glyceryl triacetate is nontoxic and clean. Isoquercitrin extracted by glyceryl triacetate also could be used as a safe and harmless food additive, or a precursor for the synthesis of EMIQ. Thus, the biphasic system can effectively strengthen the hesperidinase-catalyzed synthesis of isoquercitrin from rutin with high yield.

**Effect of different systems on the structure of hesperidinase.** The analysis of the hesperidinase CD spectra in three systems (including aqueous, co-solvent, and biphasic systems) are shown in Fig. 5 and are summarized in Table 2. The content of α-helix, β-sheet, turn and random of hesperidinase was calculated to understand the association between enzyme activity and secondary structure. As shown in Table 2, the fraction of α-helix decreased in the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system. A decrease in the α-helical fractions in hesperidinase, resulting from accelerated extracted mass transfer, may be attributed to turbulence and can induce structural transformations that may affect the enzyme active site. Table 2 shows that in the biphasic system, the contents of α-helix and random in the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system were decreased by 9.6% and 30.1%, and those in the [Bmim][BF₄]-buffer (10:90, v/v) system were decreased by 6.5% and 26.1%, respectively, compared with untreated hesperidinase. The β-sheet content in the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system was increased by 35.1%. The contents of α-helix and random in the [Bmim][BF₄]-buffer/glyceryl triacetate (1:1, v/v) system were decreased by 3.1% and 3.3% compared with the [Bmim][BF₄]-buffer (10:90, v/v) system.

These alterations increased the uniformity and flexibility of hesperidinase, which enhances its activity and improves catalytic efficiency. The decreased proportion of α-helix and random and the increased proportion of β-sheet in hesperidinase is more conducive for isoquercitrin production. Wang et al. reported similar results for changes in the composition of cellulase structure with an 8.85% decrease in α-helix content. Combined with the results of the previous experiments, the small proportion of α-helix in the reaction system was more favorable for the reaction.
In conclusion, the extraction agent glyceryl triacetate was successfully applied in a biphasic system for the selective biotransformation of rutin for isorquercitrin production. The rutin conversion and isoquercitrin yield in the [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system were 99.5% and 93.9%, respectively. Compared with the co-solvent system, the developed biphasic system increased the substrate concentration 2.6-fold, decreased the reaction time to three tenths the original time, increased the reaction temperature from 40°C to 45°C, and hesperidinase had a higher substrate affinity to rutin in a biphasic system. The results suggest that the developed biphasic system could enhance the hesperidinase-catalyzed synthesis of isorquercitrin from rutin with high yield and selectivity.

**Methods**

**Synthesis of isoquercitrin in a biphasic system.** In this study, disodium hydrogen phosphate-citrate buffer (pH 4–8) and glycitein-sodium hydrogen dioxide buffer (pH 9–10) were used in a biphasic system. The system contains: 720 μL rutin solution, 100 μL IL, 180 μL hesperidinase solution, and 1 mL glyceryl triacetate. The hesperidinase (contains α-L-rhamnosidase and β-D-glucosidase activities, ≥10 units/g solid) produced by Penicillium was purchased from Sigma Co. (St. Louis, MO, USA). All enzymatic reactions were performed in a temperature-controlled incubator shaker. In a typical experiment, rutin solution was added with the IL to a 10 mL centrifuge tube. The reaction was initiated by adding hesperidinase and glyceryl triacetate buffered solution and the mixtures were incubated for different amounts of time at various pH values. IL concentrations, substrate concentrations, enzyme concentrations, reaction times, and the volume ratio of two phases with different pH values were used in a biphase system. Different substrate concentrations of rutin solution (0.82, 1.6, 2.4, 3.3 and 4.0 mmol/L) were prepared to obtain the initial rate of isoquercitrin production in a [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system with hesperidinase activity of 10 U/L at 45°C and 180 rpm in a shaker. All samples were assayed in triplicate and were obtained at 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 210 and 240 min. To attain equilibrium, the samples were placed in a refrigerator at −20°C before HPLC detection. The reaction rate of different time points were calculated using the HPLC data.

**Determination of kinetic parameters and apparent michaels constant.** Kinetic parameters were conventionally determined from the initial rate data and non-linear regression to the corresponding rate equations. The concentrations of rutin and hesperidinase solution were 3.3 mmol/L and 10 U/L, respectively. All experiments were performed in a [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system at 45°C and 180 rpm in a shaker. All samples were assayed in triplicate and were obtained at 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 210 and 240 min. To attain equilibrium, the samples were placed in a refrigerator at −20°C before HPLC detection. The reaction rate of different time points were calculated using the HPLC data.

**Multiple interfacial isoquercitrin extraction methods.** Because an interface exists in a biphasic system, there may be an amount of isoquercitrin adsorbing at the interface. Isoquercitrin can be easily extracted using organic phase glyceryl triacetate; therefore, repeated extractions can accelerate the reaction time. The reaction system was a [Bmim][BF4]-buffer/glyceryl triacetate (1:1, v/v) system with hesperidinase activity of 10 U/L at 45°C, 180 rpm/min in a shaker. Every sample was obtained at 15 min to determine the initial conversion rate. Apparent K_m, ρ and V_max were calculated by using non-linear regression methods.

**CD spectroscopic assay.** CD measurement was carried out with Jasco-810 spectropolarimeter (Japan) in Yangzhou University. The CD spectra were measured in 190–300 nm at room temperature using a 0.1 cm quartz cuvette. The scanning speed was 100 nm/min; bandwidth was 1 nm; spectral resolution was 0.1 nm. The data points were taken on an average of four times. The α-helix and β-sheet structure contents in the protein were calculated using the software provided by the manufacturer (Jasco-810 Analytical Manager System) corresponding to standard substances. All the experiments were carried out at 25°C. To attain equilibrium, the reaction samples were placed in a water thermostat at 25°C before the experiments.

**Statistics analysis.** Triplicate experiments were performed for each parameter investigated. Standard deviations were calculated to verify the reliability of the results. The differences in mean values were evaluated using the analysis of variance (ANOVA) method. Significance was determined at a 95% level of probability.

| Medium | α-helix | β-sheet | Turn | Random | Total |
|--------|---------|---------|------|--------|-------|
| Pure water | 22.2 | 12.0 | 23.8 | 42.0 | 100 |
| [Bmim][BF4]-glyceronic sodium hydroxide [pH 9] (10:90, v/v) | 15.7 | 0 | 69.1 | 15.2 | 100 |
| [Bmim][BF4]-glyceronic sodium hydroxide [pH 9] (10:90, v/v)/glyceroly triacetate (1:1, v/v) | 12.6 | 47.1 | 28.4 | 11.9 | 100 |

*All samples were assayed in triplicate.

1. Li, R., Yuan, C., Dong, C., Shuang, S. & Choi, M. F. In vivo antioxidative effect of isoquercitrin on cadmium-induced oxidative damage to mouse liver and kidney. Naunyn-Schmiedeberg's Arch. Microbiol. 383, 437–445 (2011).
2. Kim, Y., Narayanan, S. & Chang, K. Inhibition of influenza virus replication by plant-derived isoquercetin. Antivir. Res. 88, 227–235 (2010).
3. Gasparotto Junior, A., et al. Anti-inflammatory effects of isoquercitrin and extracts from Tropaeolum majus L.: Evidence for the inhibition of angiotensin converting enzyme. J. Ethnopharmacol. 134, 363–372 (2011).
4. Amado, N. G. et al. Isoquercitrin isolated from Hyptis fasciculata reduces gliblastoma cell proliferation and changes β-catenin cellular localization. Anti-Cancer Drug Des. 20, 543–552 (2009).
5. Massouka, N., Matsuda, M. & Kubo, I. Characterisation of the antioxidant activity of flavonoids. Food Chem. 131, 541–545 (2012).
6. Wang, J. et al. Selective hydrolysis by commercially available hesperidinase for isoquercitrin production. Mol. Catal. B: Enzym. 81, 37–42 (2012).
24. Vertegel, A. A., Siegel, R. W. & Dordick, J. S. Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme. *Sci. Rep.* **2**, 2876–2890 (2012).

25. Moore, F. L. Separation of zirconium from other elements by liquid-liquid extraction. *Bioresource Technol.* **153**, 278–283 (2014).

26. Wang, Z. et al. Effects of low intensity ultrasound on cellulase pretreatment. *Biotechnol. Biofuels* **7**, 1–9 (2014).

27. Chaudhari, R. V., Bhanage, B. M., Deshpande, R. M. & Delmas, H. Enhancement of interfacial catalysis in a biphasic system using catalyst-binding ligands. *Nature* **373**, 501–503 (1995).

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Author contributions

J.W. conceived the idea and designed the experiments. A.G. prepared the samples and performed characterization with the assistance from C.Y., Q.B., X.S. and B.H. J.W. discussed with A.G., X.W. and F.W. for the analysis and discussion of results. A.G. and J.W. were mainly responsible for preparing the manuscript with further inputs from other authors. All the authors discussed the results and commented on the manuscript.

Additional information

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Brown, S. & Griffiths, L. A. New metabolites of the naturally-occurring mutagen, quercetin, the pro-mutagen, rutin and of taxifolin. *Experientia* **39**, 198–209 (1983).

Szczezinski, E., Hawryl, M. A. & Hawryl, A. Retention behavior of some flavonoids in 2D-TLC systems on cyano bonded polar phases. *Chromatographia* **54**, 789–794 (2001).

Nam, H., Hong, S., Shin, K. & Oh, D. Quercetin production from rutin by a thermostable beta-rutinosidase from Pyrococcus furiosus. *Biotechnol. Lett.* **34**, 483–489 (2012).

Tian, G., Xiang, S., Noiva, R., Lennarz, W. J. & Schindelin, H. The crystal structure of yeast protein disulde isomerase suggests cooperation between its active sites. *Cell* **124**, 61–73 (2006).

Mimi Sakainah, A. M. et al. Effect of substrate and enzyme concentration on cyclodextrin production in a hollow fibre membrane reactor system. *Sep. Purif. Technol.* **124**, 61–67 (2014).

Xue, Y., Li, L., He, Y. & Guan, Z. Protease-catalysed direct asymmetric Mannich reaction in organic solvent. *Sci. Rep.** 2**, 761–767 (2012).

Buono, F. G., Chidambaram, R., Mueller, R. H. & Waltermire, R. E. Insights into palladium-catalyzed cyanation of bromobenzene: additive effects on the rate-limiting step. *Org. Lett.* **10**, 5325–5328 (2008).

Gong, P. & Xu, J. Bio-resolution of a chiral epoxide using whole cells of *Bacillus megaterium* ECU1001 in a biphasic system. *Enzym. Microb. Technol.* **36**, 252–257 (2005).

Dai, G., Li, J. & Jiang, L. Conformation change of glucose oxidase at the water–air interface. *Colloids Surf. B: Interfaces* **13**, 103–111 (1999).

Wang, M. et al. A green approach to the synthesis of novel "Desert rose stone"-like nanobiocatalytic system with excellent enzyme activity and stability. *Sci. Rep.* **4**, 6606 (2014).

Jiang, Y. et al. Pickering emulsion stabilized by lipase-containing periodic mesoporous organosilica particles: A robust biocatalyst system for biodiesel production. *Bioresource Technol.* **117**, 222–227 (2012).

36. Tian, G., Xiang, S., Noiva, R., Lennarz, W. J. & Schindelin, H. The crystal structure of yeast protein disulde isomerase suggests cooperation between its active sites. *Cell* **124**, 61–73 (2006).

Mimi Sakainah, A. M. et al. Effect of substrate and enzyme concentration on cyclodextrin production in a hollow fibre membrane reactor system. *Sep. Purif. Technol.* **124**, 61–67 (2014).

Xue, Y., Li, L., He, Y. & Guan, Z. Protease-catalysed direct asymmetric Mannich reaction in organic solvent. *Sci. Rep.** 2**, 761–767 (2012).

Buono, F. G., Chidambaram, R., Mueller, R. H. & Waltermire, R. E. Insights into palladium-catalyzed cyanation of bromobenzene: additive effects on the rate-limiting step. *Org. Lett.* **10**, 5325–5328 (2008).

Gong, P. & Xu, J. Bio-resolution of a chiral epoxide using whole cells of *Bacillus megaterium* ECU1001 in a biphasic system. *Enzym. Microb. Technol.* **36**, 252–257 (2005).

Dai, G., Li, J. & Jiang, L. Conformation change of glucose oxidase at the water–air interface. *Colloids Surf. B: Interfaces* **13**, 103–111 (1999).

Wang, M. et al. A green approach to the synthesis of novel "Desert rose stone"-like nanobiocatalytic system with excellent enzyme activity and stability. *Sci. Rep.* **4**, 6606 (2014).

Jiang, Y. et al. Pickering emulsion stabilized by lipase-containing periodic mesoporous organosilica particles: A robust biocatalyst system for biodiesel production. *Bioresource Technol.* **117**, 222–227 (2012).

Chaudhari, R. V., Bhanage, B. M., Deshpande, R. M. & Delmas, H. Enhancement of interfacial catalysis in a biphasic system using catalyst-binding ligands. *Nature* **373**, 501–503 (1995).