From the Kitchen to the Lab: Discovery and Application of Food Catalysts to Promote the Coupling of Quinone with Amines

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ABSTRACT: Biocatalysts are used to catalyze specific reactions in various industries, and they often provide environmentally friendly and sustainable alternatives to chemical catalysts. Enzymes are well-known examples of biocatalysts; however, their catalytic activities are lost upon denaturation. We report the discovery of novel protein-based biocatalysts from processed foods, including skimmed milk, soy milk, cheese, and tofu (dried). Considering the reaction of the physiologically interesting pyrroloquinoline quinone (PQQ) dehydrogenase coenzyme with amino acids, it was found that the reaction with glycine to form imidazolopyrroquinoline (IPQ) did not proceed when PQQ was present at very low concentrations. In contrast, in the presence of protein-based foods, this reaction was accelerated, and it even proceeded at significantly lower PQQ concentrations (i.e., 600-times lower). It was also deduced that milk can accelerate the reaction between PQQ and various amino acids, primary amines, and secondary amines. Investigations into the reaction rate revealed that food catalysis is a non-enzymatic reaction. Furthermore, nuclear magnetic resonance spectroscopy was used to demonstrate that the components of milk interacted with the amino substrates owing to the ability of amines to react with quinones on colloidal surfaces. Finally, the application of skimmed milk enhanced the PQQ detection limit during HPLC following IPQ derivatization. Such food catalysts therefore show promise in the fields of biocatalysis and food technology.

Introduction

Biocatalysis is a rapidly evolving research field. As a result, biocatalysts such as enzymes can now be efficiently designed using genetic engineering and applied to a variety of highly selective reactions in aqueous solutions.[1,2] The use of enzymes for high-performance liquid chromatography (HPLC) derivatization reactions has also been reported.[3] However, enzymes suffer from two main drawbacks, namely, their high cost and the facile denaturation of their protein structures by heat, acids, bases, or other factors, which ultimately renders them inactive.[4,5]

Proteins are an important source of nutrients, and many foods contain proteins as a main component. Many of these foods are processed by heating, freezing, drying, salting, fermentation, seasoning, or the addition of additives. Importantly, these processes can also lead to enzyme deactivation. However, processed foods have a number of benefits, including increased shelf lives, reduced cooking times, and improved nutritional value, taste, and texture. Even though

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processed foods are inexpensive compared to purified enzymes and reagents, it is generally impossible to accelerate organic reactions in processed food media. The use of processed foods to promote reactions would therefore represent a significant breakthrough in the field of biocatalysis.

Pyrroloquinoline quinone (PQQ) is a dehydrogenase coenzyme whose structure contains quinone, pyridine, pyrrole, and tricarboxylic acid moieties (Figure 1). This enzyme binds to calcium, magnesium, and lanthanoids to perform redox reactions.[6] In addition, its quinone structure acts as the active site in the reduction of PQQ in the presence of a reducing reagent.[7] In terms of its derivatives, the quinone and acetal forms of PQQ exist in equilibrium in an aqueous environment. In addition, PQQ disodium trihydrate, which is produced by fermentation,[8,9] is known to promote longevity,[10] improve brain function,[11] and suppress fat accumulation.[12] To date, a range of analytical techniques have been employed for the detection of PQQ, including HPLC–ultraviolet (UV) detection,[13] HPLC–mass spectrometry (MS), enzyme assays,[14] and redox-based HPLC approaches.[15] Despite its high stability in air and water, PQQ easily reacts with organic materials (such as under extraction conditions), which renders its quantitative analysis particularly challenging.

To address the above issues, our group previously developed a strategy to convert and analyze stable PQQ derivatives. More specifically, PQQ was reacted with amino acids to form imidazolopyrroquinoline (IPQ)[16] or imidazolopyrroquinoline with amino acid residues (R-IPQ) bearing an amino acid substituent.[17] An analytical quantification method was developed based on the reaction between PQQ and glycine to generate the less reactive IPQ. The developed method was designed to proceed smoothly under neutral to alkaline pH conditions.[18] However, when the concentration of PQQ was low, the reaction was hindered, thereby complicating the analysis. To date, no catalyst has been reported that promotes this reaction.

We herein report our discovery that certain foods are able to promote this reaction. In addition, an analytical method is developed to monitor the reaction, and the discovery and properties of various food catalysts are discussed, along with their potential applications in analytical chemistry.

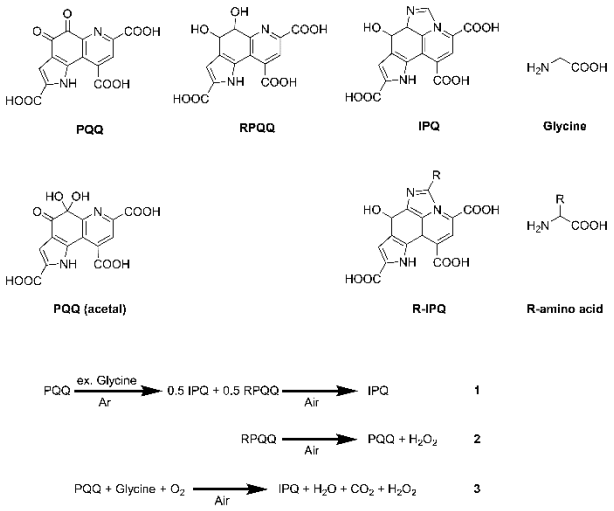

FIGURE 1 Chemical structures of the compounds of interest and the reaction process discussed herein.

Results

Conversion of PQQ into IPQ

The reaction of PQQ with glycine forms IPQ, wherein the amino group reacts with the C=O bond of the quinone moiety to promote cyclodecarboxylation. IPQ is less reactive and more stable than PQQ, even in the presence of excess amino acids. Thus, the reaction of PQQ with excess glycine in an inert atmosphere was monitored using NMR spectroscopy (Supporting Information, Figure S1). It was found that IPQ and reduced PQQ (RPQQ) were produced. The subsequent bubbling of air through this reaction solution yielded only IPQ (Figure 1, Equation 1). This was accounted for by considering that RPQQ was reoxygenated to form PQQ (Figure 1, Equation 2).[19] With these considerations in mind, Equation 3 was derived, indicating that the complete conversion of PQQ to IPQ requires oxygen.

Screening of the food catalysts

During the development of an analytical approach for PQQ derivatization based on the above reaction, we found PQQ in some foods was converted to IPQ more easily than that in a pure form. In particular, the reaction was accelerated in the presence of whole milk powder (Figure 2a). Thus, the components responsible for promoting this reaction

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were initially investigated. The amino acid concentration was kept low during the reaction to observe the effects of the additives more easily. As outlined in Figure 2b and Table S1, starch was also investigated as a carbohydrate, lactoglobulin and lactalbumin were examined as pure proteins, and soymilk and tofu (dried) powder were employed as soybean-derived foods. Various food products promoted the PQQ derivatization reaction, with significantly higher yields (2×) being obtained in the presence of whole milk powder, skimmed milk, cheese, whey protein, soymilk, and tofu (dried) powder. Improved yields were also obtained using casein and lactalbumin (1.4–1.5×), whereas no improvements were observed using butter, starch, or lactoglobulin.

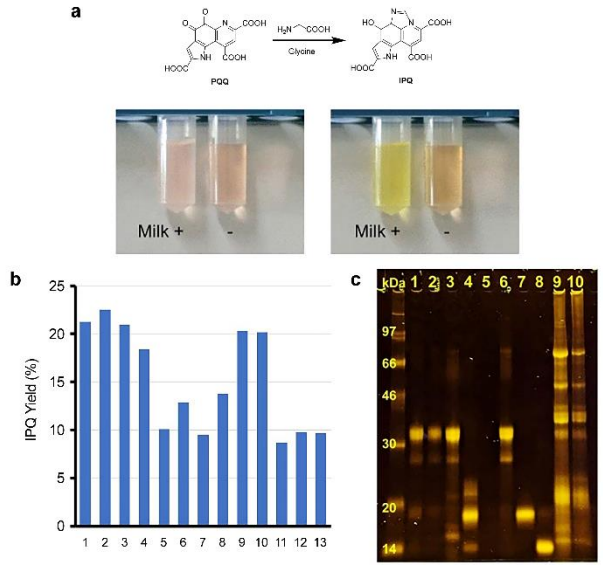

FIGURE 2 Food catalyst discovery, screening, and SDS–PAGE results. a) Discovery of the food catalysts: Left, after 0 h, and right, after 0.5 h at 37 °C. Reaction conditions: PQQ, 117 μM; glycine, 0.133 M; ± whole milk powder, 1 g L^{-1}. b) IPQ yield using different food samples as catalysts. 1 = whole milk powder, 2 = skimmed milk, 3 = cheese, 4 = whey protein, 5 = butter, 6 = casein, 7 = lactoglobulin, 8 = lactalbumin, 9 = soy milk, 10 = tofu (dried), 11 = starch, 12 = silica gel, and 13 = control (buffer). Reaction conditions: 37 °C, 2 h; food sample, 1.0 g L^{-1} (for soy milk, the concentration was adjusted based on the solids content); PQQ, 117 μM; glycine, 3.3 mM; sodium phosphate buffer, 50 mM, pH 7.0. c) Protein analysis (SDS–PAGE) of different food samples.

Protein analysis (SDS–PAGE)

Since protein-rich foods were found to effectively promote the reaction, all food specimens of interest were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) for protein analysis (Figure 2c). The whole milk powder contained casein and lactoglobulin, the skimmed milk and cheese were primarily composed of casein, and the whey protein mainly comprised lactoglobulin with a minor amount of lactalbumin. Butter contained very few proteins, while the soymilk and tofu (dried) specimens comprised mixed proteins with various molecular weights.

Effect of reaction conditions

After heating the skimmed milk and tofu (dried) powder specimens in the buffer solution at 80 °C for 30 min, comparable IPQ yields were obtained. The reaction was also repeated under an Ar atmosphere, giving IPQ yields of 22.5% (skimmed milk) and 20.2% (tofu, dried), which were significantly higher than the 9.3% yield obtained for the control (buffer). Comparable yields were obtained under both sets of conditions (i.e., air and Ar gas). Thus, as a model experiment, a primary amine (aminoethanol, 81.9 mM (5 g L^{-1})) and secondary amine (*N*-methylamino ethanol, 66.6 mM (5 g L^{-1})) were reacted with PQQ (2.34 mM) in the presence of skimmed milk (2 g L^{-1}) under an Ar atmosphere at 37 °C. In the case of ethanolamine, the PQQ conversion reached 61.0% after 1 h for the reaction containing skimmed milk (c.f., 44.5% for the control), while a conversion of 43.8% (21.9% control) was obtained for the *N*-methylamino ethanol reaction after 3 h. These results clearly indicate skimmed milk accelerated the addition of amines to the quinone carbonyl moiety.

Investigation of the amino acid scope

As previously reported, substituted IPQ can be obtained by reacting various amino acids with PQQ.[17] Thus, the corresponding reactions with leucine, isoleucine, valine, asparagine, and arginine were investigated, both in the presence and absence of skimmed milk (60 °C for 3 h). The HPLC results are summarized in Figure S2 and Table 1.

Table 1 Reaction of various amino acids with PQQ at 60 °C for 3 h*

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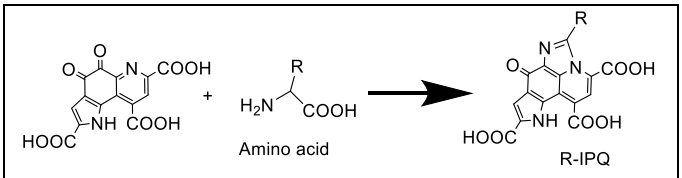

Amino acid	R-IPQ yield (Milk)	R-IPQ yield (control)	Ratio (Milk +/−)
Leu	54.0	21.3	2.5
Ile	86.5	37.1	2.3
Val	59.3	25.5	2.3
Asn	13.2	4.8	2.7
Arg	36.7	12.3	3.0

*Reaction conditions (2 mL reaction tube, 1 mL total volume): PQQ, 2.34 mM; amino acid, 5 g L^{-1} (Leu 38.1, Ile 38.1, Val 42.7, Asn 37.8, Arg 28.7 mM); and skimmed milk, 2 g L^{-1} in water. The arginine solution was adjusted to pH 5 using phosphoric acid.

HPLC analysis of PQQ derivatization reaction

The derivatization of PQQ using glycine to form stable IPQ facilitates the analysis of reactive PQQ (Figure S3). A two-step reaction was conducted (pH 6: PQQ ⟶ IPQ + RPQQ; pH 10: reoxygenation of RPQQ) (Figure S4). Notably, large excesses of glycine and catalyst were used to ensure that the reaction reached completion. In the absence of a catalyst, PQQ concentrations of 10,000, 500, and 50 μM gave IPQ yields of 95%, 90%, and 50%, respectively, whereas in the presence of 1 wt% skimmed milk as the catalyst, IPQ yields of 99% and 96% were obtained at PQQ concentrations of 50 and 15 μM PQQ, respectively (Figure 3a). These results demonstrate the significant catalytic action of skimmed milk proteins. Chromatographic analysis confirmed that IPQ was the main product, with no other interfering peaks observed (Figure 3b). This suggests that this method is suitable for the quantitative analysis of PQQ.

Three chocolate samples, each containing 0.1 wt% PQQ (PQQ in extract solution: 58 μM), were analyzed using this approach. Importantly, the calibration curve constructed for the developed method was linear in the concentration range of 25–110 μM with an R^2 value of 0.999 (Figure 3c). All three analyzed samples were found to contain 0.1 wt% PQQ (Table S2), which corresponded to 100% recovery.

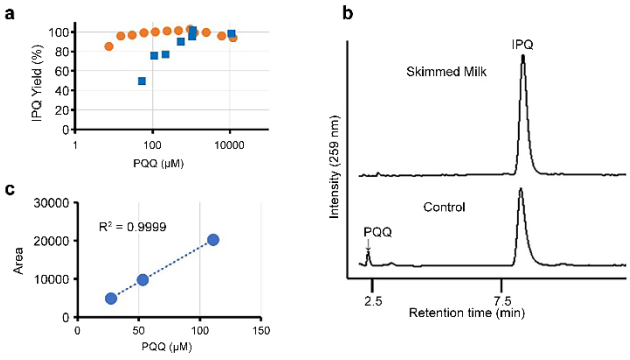

Figure 3 HPLC analysis of the PQQ derivatization reaction carried out in the presence of skimmed milk. a) Effect of the PQQ concentration on the IPQ yield in 1.33 M (10 wt%) glycine (blue: no catalyst; orange: 1 wt% skimmed milk catalyst). b) Chromatographs for reaction solutions with and without skimmed milk using an initial PQQ concentration of 117 μM. c) Influence of PQQ concentration on the HPLC peak area in the presence of skimmed milk.

Investigation of reaction mechanism

The effects of different reactant/reagent concentrations on the reaction were subsequently studied. For this purpose, the glycine concentration was reduced to 0.133 M (10 g L^{-1}), and the effects of milk supplementation were examined over a reaction time of 1 h (Figure 4a). In the presence of 117 μM PQQ, the IPQ yield was 19% in the absence of a catalyst. However, the addition of 0.01 wt% skimmed milk doubled the yield to 39%, while the addition of 0.2 wt% skimmed milk gave a yield of 71%. Increasing the skimmed milk loading further had little effect on the IPQ yield. Next, the effect of the initial concentration of PQQ was studied using a 0.133 M aqueous glycine solution, 0.01 wt% skimmed milk, and reaction time of 1 h (Figures 4b and 4c). In the absence of a catalyst, the relationship between the PQQ concentration and IPQ yield was almost linear. In contrast, in the presence of skimmed milk, the IPQ yield increased rapidly as the PQQ concentration increased and then plateaued at higher PQQ concentrations. This corresponds to a linear increase in IPQ concentration against initial PQQ concentration, as plotted in Figure 4c.

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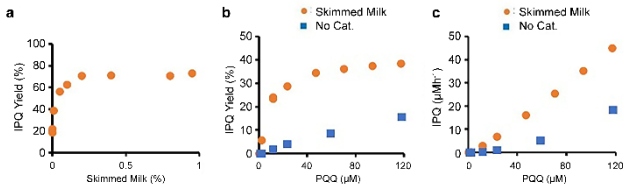

FIGURE 4 Effects of PQQ and skimmed milk concentrations on derivatization reaction. a) Effect of skimmed milk concentration (PQQ, 117 μM; glycine 0.133 M). b) Effect of PQQ concentration. c) Relationship between PQQ and IPQ concentrations. Note that (b) and (c) use the same data but with different vertical axes (IPQ yield (%) and IPQ concentration (μM/h), respectively).

^{1}H NMR analysis

The intermolecular interactions between large skimmed milk molecules and small molecules can be observed using NMR based on peak broadening and changes to the relaxation time. Skimmed milk is a colloidal solution with a particle size of approximately 1200 nm, while the particle size of casein is 520 nm (Figure S5). Upon the addition of skimmed milk to the glycine solution, a broadening of the NMR peaks was observed. Since PQQ exists in an equilibrium between its PQQ (acetal) and quinone forms in water, four peaks were observed in the aromatic region when the solution was prepared in D_2O. Notably, the peaks corresponding to PQQ broadened upon the addition of skimmed milk (Figure 5b). Relaxation time measurements were performed for the two low-field aromatic peaks of PQQ, which corresponded to the pyridine ring protons of the quinone and acetal forms. The T_1 relaxation times of these peaks significantly reduced in the presence of skimmed milk (Figure 5c), demonstrating that both PQQ species interacted with skimmed milk.

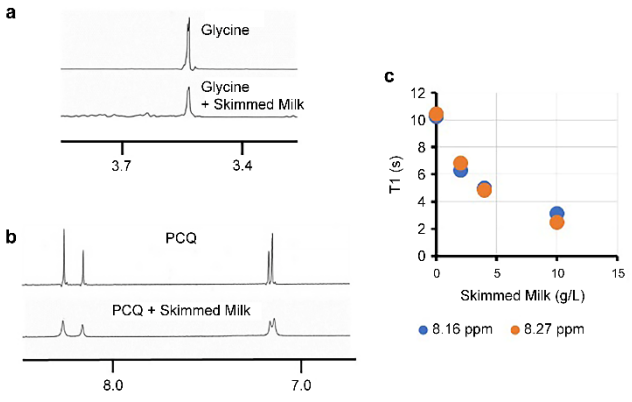

FIGURE 5 Effects of skimmed milk addition on the glycine and PQQ ^{1}H NMR spectra. a) ^{1}H NMR peak of glycine in the presence and absence of skimmed milk. b) ^{1}H NMR peaks of PQQ in the presence and absence of skimmed milk. c) T_1 relaxation times for the aromatic PQQ peaks.

Discussion

High-protein foods, such as whole milk powder, skimmed milk, cheese, and whey protein, were the most effective catalysts for the derivatization reaction. Soymilk and tofu (dried) exhibited slight reaction-promoting effects, while butter, starch, and silica gel (an inorganic substance) had no effect on the reaction. Based on the compositions of these foods, it was considered that proteins were responsible for catalyzing the derivatization reaction. The main protein identified in the whole milk powder (essentially milk with the water removed), skimmed milk (whole milk with the fat removed),[20] and cheese (denatured and coagulated milk proteins) samples was casein, whereas that in the whey protein sample (an isolated product with high protein purity) was lactoglobulin, which cannot be coagulated. In contrast, the butter specimen contained very little protein (Table S1). With these considerations in mind, it was clear that high-protein foods were the most active in promoting the reaction. However, when the reaction was repeated using purified casein and lactalbumin, the conversions were negligible, and no response enhancement was observed upon treatment with lactoglobulin.

Interestingly, soymilk and tofu (dried) both accelerated the reaction. These foods contained mixtures of proteins, including some denatured ones. They are prepared by adding water to soybeans and boiling (soymilk) or by coagulating soymilk using Ca and Mg, repeatedly freezing and thawing, and removing the water (tofu, dried).[21] Proteins that undergo coagulation due to heating, freezing, and thawing typically lose their original tertiary structures, so the fact that these foods exerted a catalytic effect is particularly interesting.

Subsequently, the structure–activity relationships were examined for the pure proteins. First, the structures of lactalbumin and lactoglobulin were compared, considering

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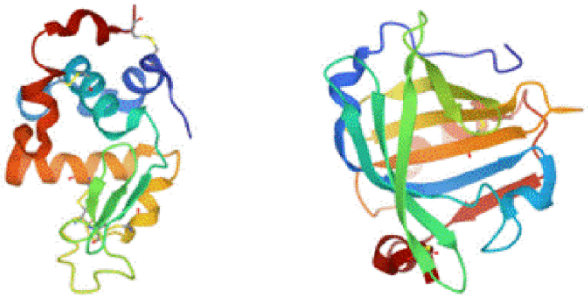
that lactalbumin was more active than lactoglobulin. The structures were modeled using data from the Protein Data Bank and the HOMCOS molecular modeling server. The results showed that the molecules in contact with lactoglobulin were fat-soluble compounds such as retinol, retinoic acid, and octanoic acid. Furthermore, lactalbumin forms an α-helical structure, whereas lactoglobulin forms a β-sheet structure (Figure 6). Because α-helices are more hydrophilic than β-sheets, these structural differences may be responsible for the observed differences in activity. However, although the active foods are hydrophilic, and the reactive surfaces of their proteins appear hydrophilic, these considerations are insufficient to explain the factors contributing to the detected high activities.



FIGURES 6 Three-dimensional structures of pure proteins: (left) lactalbumin and (right) lactoglobulin.

It was surprising that while whole milk powder, skimmed milk, cheese, and whey protein demonstrated high activities, purified casein, lactalbumin, and lactoglobulin did not. This result is of particular importance since it implies that these foods could be applied as active catalysts with fewer purification steps. The types of proteins and protein contents of the active foods varied from 23% for whole milk powder to 97% for whey powder. This indicates that the activity was not caused by a specific protein content or structure. Moreover, the difference in colloidal size between the food and protein molecules in water (e.g., skimmed milk: 1200 nm; casein: 520 nm) indicates that the surface properties are likely more important than the surface area in determining the activity. It was therefore assumed that food molecules can become colloidal during mixing or denaturation, and that the catalytic reactions occur on the surfaces of the colloidal particles.

Because the investigated food specimens contain other components that possess a range of functional groups, it is possible that these compounds played a role in determining the catalytic activity. This could be comparable to the effects of inorganic compounds with abundant active surface sites. Such an assumption could be supported by the fact that the catalytic activity remained stable at temperatures up to 80 °C, which is a significant advantage in terms of potential applications.

To demonstrate the potential applicability of these food-based catalysts, other amino acid reactions were investigated. The reaction was promoted when using these amino acids, including extremely bulky branched-chain amino acids and alkyl side chain-free amino acids such as arginine and asparagine. Importantly, the product yields were more than twice those recorded in the absence of any catalyst. Despite attempts to induce an enzyme-like reaction using skimmed milk, a linear relationship was observed between the PQQ concentration and IPQ molar yield, indicating the absence of Michaelis–-Menten kinetics.[22] These reactions are therefore non-enzymatic. Indeed, the catalytic activity of the skimmed milk system was particularly low compared to those of well-known enzymes. However, skimmed milk is significantly cheaper; therefore, it is still promising to replace enzymes in some situations.

The reaction between PQQ and IPQ involves several steps. To investigate the reaction in greater detail, a primary amine (ethanolamine) and secondary amine (methylethanolamine) were employed as model compounds for the reaction in an inert atmosphere. The PQQ conversion rates increased for both the primary and secondary amines in the presence of skimmed milk, further confirming that food-based catalysts promote the addition of amino groups to the quinone carbonyl groups of PQQ.

The NMR peaks of PQQ (in its equilibrium state) broadened in the presence of amino acids, indicating that interactions occur between skimmed milk, PQQ, and amino acids. The T_1 relaxation time is inversely proportional to molecular motion, wherein a slower molecular motion corresponds to a shorter T_1 relaxation time,[23] Skimmed milk therefore appears to reduce the relaxation times of both the

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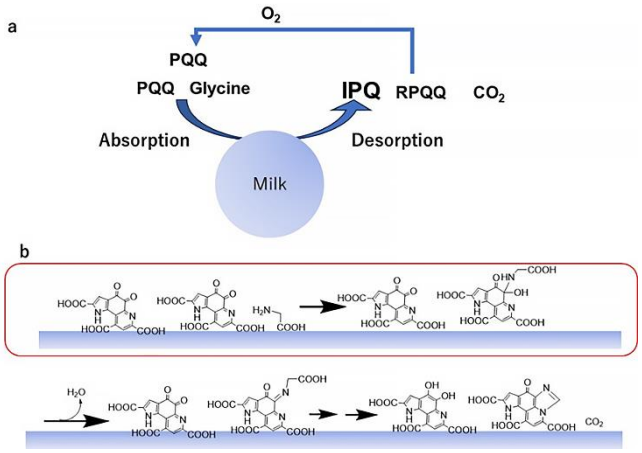
quinone- and acetal-type PQQ structures, likely because the movement of both chemical species is suppressed by the skimmed milk colloid. This result supports the formation of a PQQ–skimmed milk complex.

To summarize these results, a plausible reaction mechanism is illustrated in Scheme 1. Initially, glycine and PQQ are adsorbed onto the surface of the colloidal skimmed milk particles and react on the milk surface. This results in the generation of IPQ, RPQQ, and CO_2, wherein the produced RPQQ is reoxygenated to PQQ in the presence of oxygen. Subsequently, on the surfaces of the milk particles, the amine binds to the PQQ quinone moiety; this is the main reaction step promoted by the food catalyst. Intramolecular dehydration then proceeds, and subsequent Schiff base formation and cyclodecarboxylation generate IPQ and RPQQ.



SCHEME 1 Mechanism for the reaction between PQQ and glycine promoted by skimmed milk. a) Overall reaction mechanism. b) Reaction steps on the surfaces of the colloidal milk particles. Amine addition to the carbonyl group is accelerated by the food catalyst (red box).

The quinone group of PQQ is extremely reactive to amino acids, which can lead to inaccurate HPLC analysis when determining the PQQ content. The analysis of capsules containing both PQQ and various amino acids is therefore difficult. Derivatization is therefore required, and in the case of the current study, the low-cost and non-toxic glycine was selected for reaction under mild conditions. The results demonstrate that the developed method, based on derivatization and the addition of a skimmed milk catalyst, is suitable for combination with HPLC analysis. Importantly, this method is also suitable for systems containing low PQQ concentrations, with a linear calibration curve being obtained, even under such conditions. Although the standard PQQ intake by a single person is approximately 20 mg/d,[24] only trace amounts of PQQ are present in any specific food item. Thus, the development of analytical approaches for the determination of trace levels of PQQ, such as that reported herein, is essential in the context of commercial applications.

Although PQQ is abundant in breast milk,[25] its levels are likely lower than expected because milk boosts the reactivity of PQQ. This phenomenon is important for understanding the natural distribution of PQQ. More specifically, this phenomenon, in which highly denatured proteins catalyze reactions, suggests the presence of primitive biocatalysts. Food catalysts can therefore be considered a type of organic catalyst.

Conclusion

Denatured foods, such as skimmed milk, can promote catalytic reactions in a non-enzymatic manner. Using this biocatalyst, the derivatization of PQQ using amino acids proceeded efficiently even at low PQQ concentrations. This method was deemed suitable for the derivatization of PQQ prior to analysis by HPLC. The results of this research represent a new advance in the area of biocatalysis, namely food catalysts, which are advantageous in terms of their stability, price, and availability.

ASSOCIATED CONTENT

Supporting Information: Experimental methods, nutritional composition of food catalysts, chromatography data of reactions between amino acids and PQQ, additional HPLC data, and particle size distributions of skimmed milk and casein molecules (PDF).

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Author Contributions

K.I. designed the study and performed the experiments. N.S.M.I. investigated the protein structures. S.I. performed the HPLC analyses. K.I. wrote the manuscript. All authors discussed the results and reviewed the manuscript.

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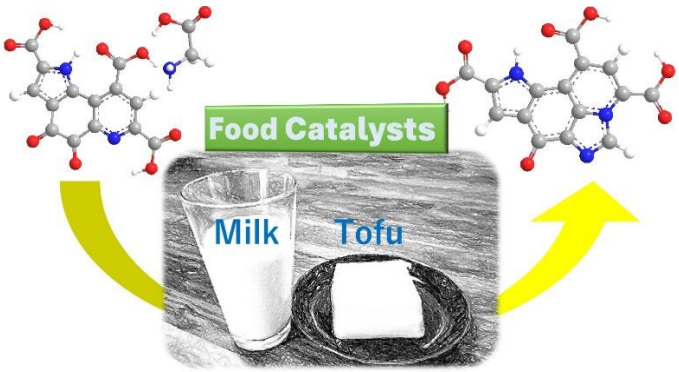

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