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Research Article

Facile Immobilization of Soybean Lipoxygenase on Nanoporous Rice Husk Silica by Adsorption

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2. Keywords

Soybean lipoxygenase; Rice Husk Silica (RHS); Immobilization; Adsorption

1. Abstract

Soybean lipoxygenase (LOX, EC. 1.13.11.12) catalyzes the generation of hydroperoxy fatty acids, which occurs in the first committed step of oxylipin biosynthesis in plants. As industrial interest in producing hydroperoxide derivatives of poly unsaturated fatty acids has increased, numerous attempts have been made to immobilize oxylipin biosynthesis enzymes including LOX. However, there have been difficulties in immobilization efficiency and enzyme stability. In this study, we tried to immobilize soybean LOX on nanoporous Rice Husk Silica (RHS) via an adsorption method and investigated the characteristics of adsorbed LOX in comparison with its free form. The amount of adsorption on RHS was increased in proportion to the amount of soybean LOX, and at least 30-35% was adsorbed. The specific activity of RHS-adsorbed soybean LOX was 73.17% of the free form. The dissociation kinetics of the free form from RHS-adsorbed soybean LOX indicated that the adsorbed form is maintained for twelve weeks and is more stable in sodium phosphate buffer than in Tris-HCl buffer. The optimal pH for the adsorption of soybean LOX on RHS was 7.02. The adsorbed soybean LOX showed an initial 60% activity after five re-use cycles. The results indicate that RHS is a suitable matrix for soybean LOX immobilization and that adsorption is a direct, simple, rapid, and less expensive method for its immobilization.

3. Introduction

Jasmonic Acid (JA), one of plant stress hormones triggered by various local and systemic signals, has been known as an anticancer agent [1]. In plants, JA synthesis is initiatedby the release of α-Linolenic Acid (LnA) via a lipase [2, 3, 4, 5]. The released LnA is oxygenated to 13-Hydroperoxy Octadecatrienoic acid (13-HPOT) by the action of 13-Lipoxygenase (13-LOX). Then, Allene Oxide Synthase (AOS) converts the 13-HPOT produced by 13-LOX to an unstable 12, 13-Epoxy Octadecatrienoic acid (12,13-EOT), which is then metabolized to 12-Oxo Phytodienoic Acid (12-OPDA) by Allene Oxide Cyclase (AOC). Finally, the 12-OPDA is reduced to 3-oxo-2-(2'-pentenyl) cyclopentane-1-Octanoic acid (OPC 8:0) by 12-oxo Phytodienoate Reductase (OPR) and then converted to JA by three subsequent cycles of β-oxidation.

The conversion of LnAto 12-OPDA by sequential three enzymatic reactions including LOX, AOS, and AOC occurs in chloroplasts [6, 7, 8], where as the reduction of 12-OPDA to OPC by OPR and the three steps of β-oxidation occur in peroxisomes [9, 10, 11, 12]. JA biosynthesis, which is involved in the production of unstable intermediates by participating enzymes located in other cellular organelles, has encountered obstacles with in vivo production and regulation. To overcome this inconvenience, enzyme immobilization can effectively mimic the multistep JA biosynthesis by co-immobilization regardless of cellular location [13].

Recently, soluble enzymes as catalytic molecules have been frequently used to synthesize useful valuable compounds for commercial and academic purposes. However, the use of soluble forms has several disadvantages in producing these compounds, including difficulty in enzyme recovery and reuse, contamination of products by the used enzymes, an additional purification process, and high costs. To overcome these hurdles and improve productivity, enzyme immobilization has been used to separate the

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enzymes from the reaction product. A variety of methods are used to immobilize enzymes including adsorption, entrapment, and cross-linking or covalent binding as the most common methods [14]. Adsorption and entrapment are physical methods based on molecular interactions between the enzyme and matrix, whereas cross-linking or covalent binding is covalent bond formation between them [15]. Compared to others, the adsorption technique is relatively simpler and less expensive and retains higher catalytic activity because enzyme immobilization occurs in the enzyme solution [16, 17], suggesting that the adsorption technology could offer better commercial potential [18, 19].

Lipoxygenase (LOX) (EC.1.13.11.12) selectively oxidizes Linoleic Acid (LA) and Linolenic Acid (LnA) to 9-/13-Hydroperoxyoctadecadienoic acid(9-/13-HPOD) and 9-/13-hydroperoxyoctadecatrienoic acid (9-/13-HPOT), respectively. Hydroperoxide derivatives of polyunsaturated fatty acids (PUFA) have attracted increased industrial interest [20, 21]. To enhance the commercial potential of this reaction, numerous methods for LOX immobilization have been reported in literature [22, 23, 24], including the adsorption of LOX on glutenin and gliadin [1] and on glass and glass wool [25], and the covalent immobilization of LOX on Cyanogen Bromide (CNBr), Glutardialdehyde (GDA) [26], and oxirane acrylic beads [27]. Most of these methods were shown to fail at enzyme stabilization.

The supporting matrix is an essential factor in enzyme immobilization. Several properties such as form, shape, density, porosity, pore size distribution, operational stability, and particle size distribution of the matrix influence the catalytic reactions of enzymes. The ideal supports are believed to be cheap, inert, and physically strong and stable [28]. Nanoporous Rice Husk silica (RHS) prepared by acid treatment and carbonization-oxidation has available functional groups on the silica surface, about 4-5 nm pore size and about 50 nm average particle size [29, 30]. In particular, rice husk, the material of RHS production, is a safe, cheap, environmentally friendly raw material and is formed as a by-product of rice production. Previously, we performed covalent immobilization of soybean LOX, rice AOS, and rice AOC on RHS with two linkers, glutardialdehyde and polyethylene glycol, showing low specific activities (1.3-2.5% and 18.5-23.0%, respectively) and a wide range of immobilization efficiencies (50-92% and 24-51%, respectively) [13]. In this study, soybean LOX was non-covalently immobilized on nanoporous Rice Husk Silica (RHS) by an adsorption method. The features of RHS-adsorbed soybean LOX including immobilization efficiency, specific activity, dissociation kinetics, pH dependence on enzyme adsorption, and reusability were studied.

4. Materials and Methods

4.1. Materials

Linoleic Acid (LA) (99%), soybean lipoxygenase (soybean LOX type 1-B, EC 1.13.11.12), and Xylenol Orange sodium salt were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and were commercially available.

4.2. Immobilization of Soybean LOX on Nanoporous Rice Husk Silica (RHS) by Adsorption

Nanoporous Rice Husk Silica (RHS) was obtained from Dr. Chong Soo Han (Nano Bio Research Center, Jeollanamdo, South Korea) and was prepared as described previously [13, 31]. To immobilize soybean LOX on nanoporous RHS by adsorption, soybean LOX was mixed with 200 μL of 50 mM sodium phosphate buffer (pH 7.5) containing 20 mg RHS. After rocking at 100 cycles/min for 30 min at room temperature, the supernatant and RHS-adsorbed soybean LOX were respectively recovered by centrifugation at 12000 × g for 1 min at room temperature. The amount of soybean LOX adsorbed on RHS was calculated by subtracting the amount of protein in the supernatant from the initial amount of protein. The protein contents were analyzed using the Bicinchoninic Acid (BCA) method. Bovine Serum Albumin (BSA) was used as the standard [32].

4.3. Assay of LOX by Measurement of Conjugated Dienes of Lipid Hydroperoxide

Soybean LOX activity was determined as described previously [33]. The assay was carried out in a 2.5-mL reaction solution containing 50 mM Tris-HCl (pH 7.2), 2.5 mM LA, 0.2% Tween 20 (v/v), and 0~0.2 mg soybean LOX. LOX activity was determined at 25°C by monitoring absorbance at 234 nm indicating the formation of the conjugated diene of hydroperoxy dienoic acid (HPOD, ε= 25,000 $M^{-1}cm^{-1}$) from Linoleic Acid (LA) as a substrate, using a UV-visible spectrophotometer (Shimadzu UV-2550) [33]. To measure the activity of RHS-adsorbed soybean LOX, the adsorbed LOX was gently agitated in a 50-mL reaction mixture containing 50 mM Tris-HCl buffer (pH 7.2), 50 nM LA, and 0.004% Tween 20 (v/v) at 25°C. At intervals of 30 s, a constant volume (600 μL) was taken from the reaction mixture and LOX was inactivated by adjusting the pH with 3 M HCl. After recovering the RHS-adsorbed soybean LOXs by centrifugation at 12000 × g for 1 min at room temperature, the amount of HPOD in individual supernatants was measured at 234 nm.

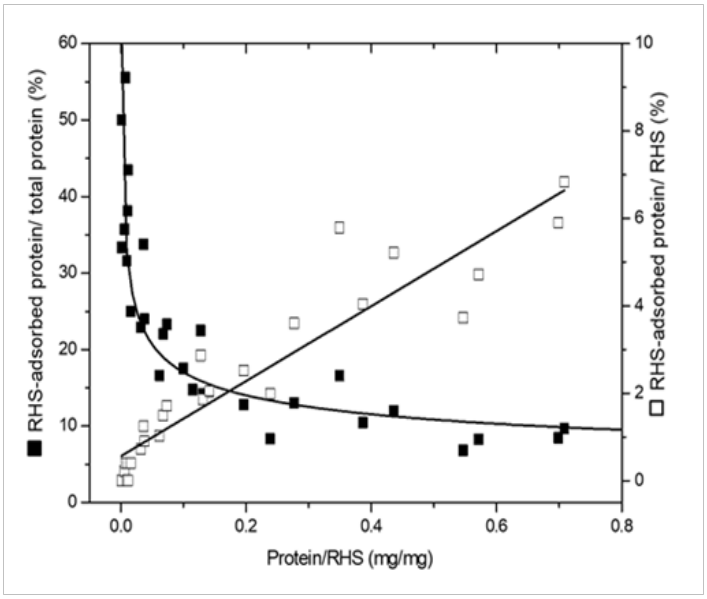

Figure 1a: Protein immobilization on nanoporous RHS by adsorption. Bovine serum albumin and soybean LOX

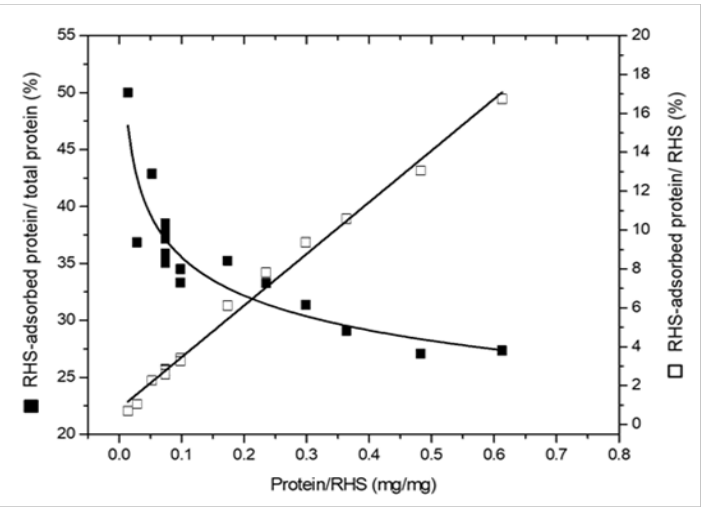

Figure 1b: ranging from 0.02 to 1.4 mg was mix with RHS (2mg) as described in the Materials and methods. The contents of RHS-adsorbed protein were calculated by subtracting the amount of unadsorbed protein from that of the used protein.

4.4. Assay of LOX by Ferrous Oxidation-Xylenol Orange Method

HPOD contents were measured spectrophotometrically using the xylenol orange method [34]. The xylenol orange reagent contained 125 μM xylenol orange, 100 mM sorbitol, 25 mM ferrous ammonium sulfate, and 25 mM sulfuric acid. The reagent (1 ml) was added to the LOX reaction mixture (100 μl) and then incubated at room temperature for 45 min. The amount of HPOD was calculated with the extinction coefficient (ε = 267,000 $M^{-1}cm^{-1}$) for hydrogen peroxide at 560 nm [35].

4.5. Stability of the RHS-Adsorbed Soybean LOX

To investigate the stability of the RHS-immobilized soybean LOX according to storage period, soybean LOX (~ 250 μg) was adsorbed on RHS (12 mg) in 50 mM Tris-HCl buffer (pH 7.2) and 50 mM sodium phosphate buffer (pH 7.2), respectively, and was then stored at 4°C for 6 months. At weekly intervals, the RHS-adsorbed soybean LOX was briefly vortexed for 3 s and then centrifuged at 12000 × g for 1 min at room temperature. Total protein contents with in the supernatant were measured by the BCA method to determine the amount of soybean LOX dissociated from the RHS.

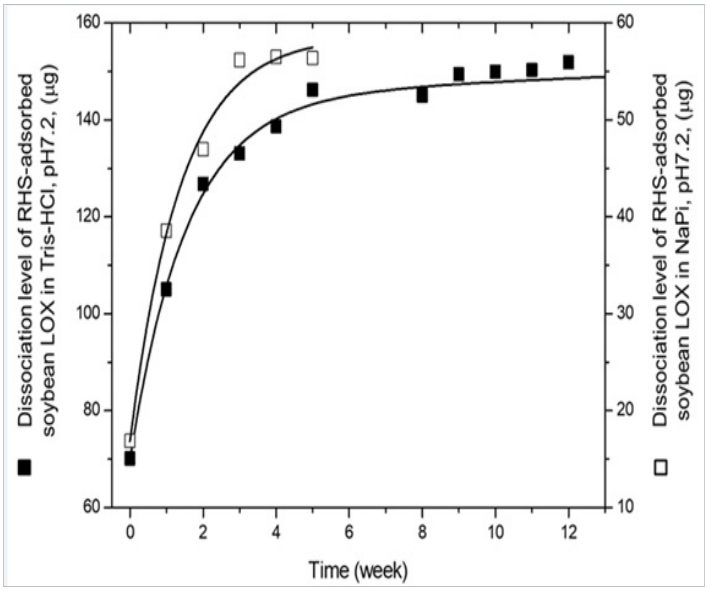

Figure 2: Dissociation kinetics of RHS-adsorbed soybean LOX in 50 mM Tris-HCl (▪) and 50 mM sodium phosphate (NaPi, ◦) buffers depending on storage period. The RHS-adsorbed soybean LOX (~250 μg) in RHS (12 mg) was suspended in 1.5 ml of 50 mM Tris-HCl buffer (pH 7.2) and 50 mM sodium phosphate (pH 7.2), respectively. Then, at every week, the leaked protein contents were determined using the BCA method. The detailed procedure used is described in the Materials and Methods section.

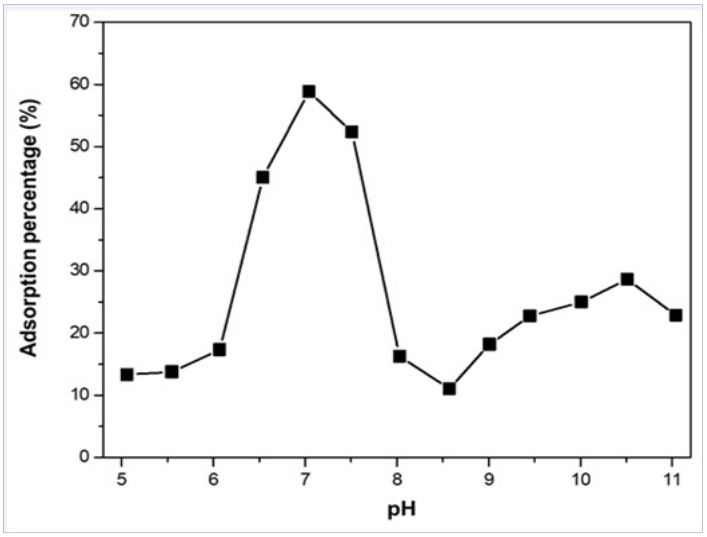

Figure 3: pH dependence of soybean LOX adsorption onto RHS. The binding experiment was conducted as described in the Materials and Methods section. The buffers used include 50 mM citric acid buffer (pH 5.06 and 5.55), 50 mM sodium phosphate buffer (pH 6.07, 6.54, 7.04, and 7.51), 50 mM Tris-HCl buffer (pH 8.03 and 8.57), and 50 mM boric acid buffer (pH 9.01, 9.45, 10.01, 10.51, and 11.04).

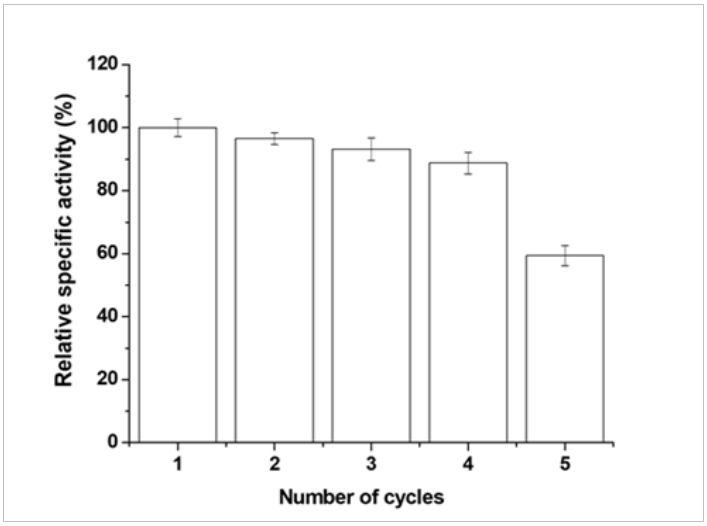

Figure 4: Reusability of RHS-adsorbed soybean LOX. The assays were performed at room temperature in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 100 μM LA and RHS (2 mg) with RHS-adsorbed soybean LOX (~30 μg). Specific activity was measured by the ferrous oxidation-xylenol orange method as described in Materials and Methods.

Table 1. Comparison of free, unadsorbed, and RHS-immobilized soybean LOX activity

Table 1: Comparison of free, unadsorbed, and RHS-immobilized soybean LOX activity			
Soybean LOX type	Specific activity		Total activity
	(μM/min·μg)	(%)	(μM/min)
Free	0.984 ± 0.02	100.00 ± 2.1	196.80 ± 4.20
Unadsorbed	0.816 ± 0.01	82.93 ± 0.9	105.26 ± 1.16
RHS-immobilized	0.720 ± 0.02	73.17 ± 2.4	48.96 ± 1.63

Note: Continuous assay (A_{234}) was used for free and unadsorbed soybean LOX used, and noncontinuous assay (A_{560})was employed for RHS-immobilized used as described in materials and methods.

The precipitated RHS was resuspended with the same buffer and again stored at 4°C.

4.6. Effect of pH on RHS Adsorption of Soybean LOX

RHS (2 mg) and soybean LOX (~ 150 μg) were mixed in pH-specific buffers by rocking at 100 cycles/min for 30 min at room temperature. The mixtures were centrifuged at 12000 × g for 1 min at room temperature. Soybean LOX contents within the supernatant were measured by the BCA method. The amount of RHS-immobilized LOX was determined by subtracting the measured amount in the supernatant from the initial used amount. The used buffers depending on pH include 50 mM citric acid buffer (pH 5.06/5.55), 50 mM sodium phosphate buffer (pH 6.07/6.54/7.04/7.51), 50 mM Tris-HCl buffer (pH 8.03/8.57), and 50 mM boric acid buffer (pH 9.01/9.45/10.01 /10.51/11.04).

4.7. Recycling of RHS-Adsorbed Soybean LOX

Supplementary Table 1.Dissociation kinetics of soybean LOX adsorbed on RHS

Supplementary Table 1. Dissociation kinetics of adsorbed soybean LOX on RHS				
Time (weeks)	Dissociated amount in 50mM Tris.HCl (pH 7.2)		Dissociated amount in 50mM NaPi (pH 7.2)	
	(μg)[a]	(%)	(μg)[b]	(%)
0	70.12	27.61	16.88	6.78
1	105.02	41.35	38.57	15.49
2	126.78	49.91	46.96	18.86
3	133.12	52.41	56.17	22.56
4	138.63	54.58	56.5	22.69
5	146.22	57.57	56.39	22.65
8	145.05	57.1		
9	149.39	58.81		
10	149.96	59.03		
11	150.32	59.18		
12	151.87	59.79		
[a] RHS (12 mg) contained 254 μg of adsorbed soybean LOX				
[b] RHS (12 mg) contained 249 μg of adsorbed soybean LOX				

To investigate its reusability, the RHS-adsorbed soybean LOX was recovered using centrifugation at 12000 × g for 1 min at room temperature after rocking at 100 cycles/min for 30 min at room temperature. The recovered LOX (~ 30 μg proteins in 2 mg RHS) was mixed with 200 μl of 50 mM sodium phosphate buffer (pH 7.2) and then assayed in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 100 μM LA and 0.008% Tween 20 (v/v) at room temperature. At 5, 10, 20, 40, 60 and 80-s intervals, 50 μl of the assay mixture was mixed with 1 ml of xylenol orange reagent to stop the enzyme reaction and was incubated at room temperature for 45 min. HPOD levels were measured by absorbance using spectrophotometric detection at 560 nm. The remaining solution after the first cycle assay was washed with 1 ml of 50 mM sodium phosphate buffer (pH 7.2) and recovered by the centrifugation at 12000 × g for 1 min at room temperature. The recovered RHS-adsorbed LOX was reused for assaying the enzyme reaction solution. Protein contents in the supernatant of each washing step were also measured using the BCA method to calculate the amount of RHS-adsorbed soybean LOX in each assay cycle.

5. Results

5.1. Soybean Lipoxygenase Immobilization on Nanoporous Rice Husk Silica (RHS) by Adsorption

In this study, to observe the capacity of RHS in protein immobilization by adsorption, proteins such as Bovine Serum Albumin (BSA) and soybean LOX were mixed with RHS (2 mg) according to the amount. The amount of RHS-adsorbed proteins

was measured by subtracting the amount of unadsorbed protein from the amount of used protein. The adsorption of BSA (Figure 1a) and soybean LOX (Figure 1b) to RHS showed two features. One is that at the amount ratio of the used protein to RHS greater than 0.2, a certain percentile of protein content used is adsorbed to RHS. 10-15% of total BSA (Figure 1a) and 25-30% of total soybean LOX (Figure 1b) were only adsorbed on RHS. The other is that depending on the quantitative ratio of protein to RHS, the amount of RHS-adsorbed protein increases. At 0.7 ratio of BSA to RHS, 7% of total RHS is involved in adsorbing BSA (Figure 1a). At 0.6 ratio of soybean LOX to RHS, 17% of total RHS is participated in adsorbing soybean LOX (Figure 1b).

5.2. Activity of RHS-Adsorbed Soybean LOX

To investigate the effect of RHS adsorption on the specific activity of soybean LOX, soybean LOX (200 μg) was mixed with RHS matrix (2 mg) and then RHS-unadsorbed and adsorbed LOX was separated by centrifugation. The average amount of RHS-adsorbed LOX was 68 μg, indicating that 34.5% of the used soybean LOX content is adsorbed on RHS at 0.1 quantitative ratio of soybean LOX to RHS as shown in (Figure 1a). Further more, the specific activities of the RHS-unadsorbed and adsorbed soybean LOX were measured using a continuous assay at 234 nm and the xylenol orange method (noncontinuous assay) at 560 nm, respectively. The specific activity of RHS-adsorbed soybean LOX was 73.17% of that of its free form (Table 1).

5.3. Dissociation Kinetics of RHS-Adsorbed Soybean LOX

To check the stability of RHS-adsorbed soybean LOX over the storage period, the RHS-adsorbed soybean LOX was stored in 50 mM Tris-HCl (pH 7.2) and 50 mM sodium phosphate buffer (pH 7.2) at 4◦, and the soybean LOX dissociated from RHS was quantified at weekly intervals. For this study, soybean LOX (~250 μg) was adsorbed onto RHS (12 mg), at which their quantitative ratio was about 0.02. (Figure 2) showed that 70.12 μg (27.61%) and 16.88 μg (6.78%) of soybean LOX were initially unadsorbed onto the RHS in 50 mM Tris-HCl (pH 7.2) and 50 mM sodium phosphate buffer (pH 7.2), respectively, indicating that 72.39% and 93.22% of the soybean LOX were adsorbed onto RHS in each buffer (Figure 2, Supplementary Table 1). The result suggests that sodium phosphate buffer is more effective than Tris-HCl buffer for RHS adsorption of soybean LOX.

Further more, depending on the storage period, leakage of soybean LOX from the RHS-adsorbed soybean LOX in 50 mM sodium phosphate (pH 7.2) buffer was saturated after three weeks. The leaked amount was ~56 μg, accounting for ~23% of the initial RHS-adsorbed soybean LOX content. However, the dissociated amount of soybean LOX in Tris-HCl (pH 7.2) buffer was found to be saturated after five weeks with ~145 μg (~55% of the initial RHS-adsorbed soybean LOX content) (Figure 2, Supplementary Table 1), indicating that dissociation of soybean LOX from RHS-adsorbed form in the Tris-HCl buffer occurs more easily than that in sodium phosphate buffer.

5.4. pH Dependence of Soybean LOX Adsorption on to RHS

pH is one of the most important factors to be considered for enzyme immobilization. Here, in order to investigate the effect of pH on soybean LOX adsorption onto RHS, four different buffer solutions were used according to pH, including 50 mM citric acid for pH 5.06 and 5.55, 50 mM sodium phosphate for pH 6.07, 6.54, 7.04, and 7.51, 50 mM Tris-HCl for pH 8.03 and 8.57, and 50 mM boric acid for pH 9.01, 9.45, 10.01, 10.51, and 11.04. Soybean LOX (~150 μg) was mixed with RHS (2 mg) in each buffer, and then RHS-adsorbed LOX was quantified by subtracting the amount of unadsorbed LOX from the initial total. The result showed that the adsorption of soybean LOX onto RHS is maximized at around pH 7.0 (Figure 3).

5.5. Recycling of RHS-Adsorbed Soybean LOX

To analyze the operational stability of immobilized soybean LOX, the adsorbed soybean LOX (~30 μg) on RHS (2 mg) was reused five times. As shown in (Figure 4), the activity of RHS-adsorbed soybean LOX showed gradual decreases according to the cycles of reuse. The specific activity after five cycles retained 59 ± 3.2% of the initial activity.

6. Discussion

6.1. Nanoporous Rice Husk Silicaassupport Matrix for Adsorbing Soybean LOX

One property of mesoporous materials as the support matrix is that their large surface areas and numerous pores facilitate loading of higher enzyme contents per unit mass. Previously, we observed the covalent immobilization efficiencies of oxylipin biosynthesis enzymes including soybean LOX, rice AOS, and rice AOC to RHS with two linkers, Glutardialdehyde (GDA) and Epichlorohydrin and Polyethylene Glycol 8000 (ECH-PEG). The result showed that GDA-linked RHS and ECH-PEG-linked RHS exhibited protein immobilization efficiencies of 49.6-92.4% and

24.6-51.32%, respectively, depending on the protein samples, suggesting that the immobilization efficiency is affected by the molecular size of proteins and the hydrophobicity of the solvent used for dissolving the protein [27]. In this study, at the amount ratio of the used protein to RHS greater than 0.2, 10-15% and 25-30% of used BSA (Figure 1a) and soybean LOX (Figure 1b) were, respectively, adsorbed to RHS. At 0.7 quantitative ratio of BSA to RHS, 7% of total RHS was involved in adsorbing BSA (Figure 1a) and at 0.6 ratio of soybean LOX to RHS, 17% of total RHS was participated in adsorbing soybean LOX (Figure 1b), indicating that under constant RHS content, the amount of RHS-adsorbed protein is increased according to the amount of used protein and the adsorption efficiency to RHS is different depending on protein type.

Compared to our previous report [27], the efficiency of protein immobilization with RHS through adsorption showed to be lower than that with linkers on RHS by covalent binding. However, the specific activities of soybean LOX covalently immobilized with GDA-linked RHS and ECH-PEG-linked RHS showed 2.5 and 23.0% of that of free form, respectively, while the specific activity of RHS-adsorbed soybean LOX was 73.17% that of its free form (Table 1), indicating that the adsorption process is more useful to maintain LOX activity.

Further more, we observed that the leaked level of RHS-adsorbed soybean LOX in 50 mM sodium phosphate buffer is lower than that in 50 mM Tris-HCl buffer (Figure 2). The adsorption percentage of soybean LOX on RHS in 50 mM Tris-HCl buffer (pH 7.2) was approximately 35% at 0.075 quantitative ratio of soybean LOX to RHS (Figure 1), but that in 50 mM sodium phosphate buffer (7.2) was about 60% (Figure 3). These results indicate that the adsorption of soybean LOX onto the RHS is dependent on the pH and buffering agent, suggesting that the immobilization efficiency could be increased by optimizing the buffering reagent.

The main application of enzyme immobilization is the reuse of enzymes for multiple reaction cycles [36, 37, 38, 39, 40, 41]. Previously, the activities of three oxylipin synthesis enzymes namely, soybean LOX, rice AOS1, and rice AOC, covalently immobilized on RHS with a short arm linker (GDA-linked RHA) and a long arm linker (ECH-PEG-linked RHS) were shown to be maintained during ten cycles although there were differences depending on the linker and enzyme type [13]. In this study, the RHS-adsorbed soybean LOX was reused five times and its activity showed gradual decreases according to the cycles of reuse (Figure 4). However, considering that the specific activity of RHS-adsorbed soybean LOX is higher than that of the covalently immobilized soybean LOX, RHS could be efficiently used for immobilization purposes.

7. Conclusion

Nanoporous rice husk silica is generated from a by-product of rice production as a safe, cheap, environmental friendly raw material. We previously observed that RHS could be used as a suitable matrix for covalent immobilization of enzymes, although the activities of the immobilized enzymes were poor. In this study, to improve the activity of the immobilized enzymes for oxylipin synthesis in vitro, immobilization of soybean LOX on RHS was attempted by adsorption. The activity of RHS-adsorbed soybean LOX was about 73.17% compared to that of its free form and showed improved activity than that of soybean LOX covalently immobilizedon RHS. The adsorption efficiency of soybean LOX on RHS was optimized at pH 7.2. The dissociation kinetics of RHS-adsorbed soybean LOX according to the storage period indicated that the stability of the adsorbed soybean LOX is enhanced in sodium phosphate buffer (pH 7.2) than that in Tris-HCl (pH 7.2), indicating that it is possible to improve the efficiency of enzyme adsorption on RHS depending on buffering reagents. Further more, the operational stability allowed the RHS-adsorbed soybean LOX to be used repeatedly for at least five cycles.

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