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Impact of Solvent pH on Direct Immobilization of Lysosome-Related Cell Organelle Extracts on TiO_2 for Melanin Treatment

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Techniques for immobilizing effective enzymes on nanoparticles for stabilization of the activity of free enzymes have been developing as a pharmaceutical field. In this study, we examined the effect of three different pH conditions of phosphate buffer, as a dissolving solvent for lysosomal enzymes, on the direct immobilization of lysosomal enzymes extracted from Hen's egg white and *Saccharomyces cerevisiae*. Titanium(IV) oxide (TiO_2) nanoparticles, which are extensively used in many research fields, were used in this study. The lysosomal enzymes immobilized on TiO_2 under each pH condition were evaluated to maintain the specific activity of lysosomal enzymes, so that we can determine the degree of melanin treatment in lysosomal enzymes immobilized on TiO_2. We found that the immobilization efficiency and melanin treatment activity in both lysosomal enzymes extracted from Hen's egg white and *S. cerevisiae* were the highest in an acidic condition of phosphate buffer (pH 4). However, the immobilization efficiency and melanin treatment activity were inversely proportional to the increase in pH under alkaline conditions. In addition, enhanced immobilization efficiency was shown in TiO_2 pretreated with a divalent, positively charged ion, Ca^{2+}, and the melanin treatment activity of immobilized lysosomal enzymes on TiO_2 pretreated with Ca^{2+} was also increased. Therefore, this result suggests that the immobilization efficiency and melanin treatment activity of lysosomal enzymes can be enhanced according to the pH conditions of the dissolving solvent.

Keywords: Lysosomal enzymes, solvent pH, TiO_2, melanin treatment, divalent positively charged ion

Introduction

Lysosomes contain an assortment of soluble, acid-dependent hydrolases and a set of highly glycosylated integral membrane proteins [5]. Lysosomes extracted from cells are morphologically heterogeneous, often resembling other organelles of the endocytic and secretory pathways. Presently, lysosomal enzymes are believed to participate in several functions: a cysteine protease, cathepsin B, or an aspartic protease, cathepsin D, are released from lysosomes into the cytosol or the nucleus [3, 4, 12] and act as proteolytic enzymes. Furthermore, the lysosomes isolated from egg white and *Saccharomyces cerevisiae* have useful functions, like antimicrobial activity to kill bacteria such as *Escherichia coli*, *Deinococcus radiophilus*, *Shigella flexneri*, *etc.* [19, 20]. However, there are numerous enzymes in lysosomes, and the functional properties of each lysosomal enzyme fraction have been neither identified nor characterized [6].

Titanium (IV) oxide (TiO_2) has been widely studied for a variety of applications, such as sensory devices [1, 16], heterogeneous catalyst materials [18], hydrogen fuel storage materials [13], and photocatalytic devices [2]. Interest in interaction with bioavailable and nonmetal materials like TiO_2 and silicon dioxide led to a range of techniques

related to immobilization and adsorption using several effective enzymes [15]. Immobilization by adsorption has been used to increase the interaction force with enzymes by modifying the surface of the material [8, 14] or by changing the pH [11].

Melanin pigments consist of the colors of the skin, hair, and eye. Melanin may have the biological function of protecting the underlying tissues from harmful ultraviolet radiation [10]. These pigments are typically divided into two types: the black eumelanins and the reddish-brown pheomelanins [7]. Large amounts of melanin in light skinned/freckled individuals appear to make them more susceptible to skin cancer [9].

In this study, to decrease the absorbance of melanin through treatment with lysosomal enzymes, we examined the effect of the pH condition of the dissolving solvent for lysosomal enzymes immobilized on TiO_2. Here, lysosomal enzymes isolated from Hen's egg white and *S. cerevisiae* were used to evaluate the activity of melanin treatment of immobilized lysosomal enzymes on TiO_2. In addition, TiO_2 pretreated with divalent positive ions was compared with untreated TiO_2 to assess the immobilization efficiency and melanin treatment activity.

Materials and Methods

Isolation of Lysosomal Enzymes from Hen's Egg White and *S. cerevisiae*

The lysosomal enzymes were extracted by removing the membranes of lysosomes, which were isolated from egg white using the cell fraction method [3]. The yolk was separated from the white component of the eggs and discarded. The white component of the Hen eggs was homogenized until the viscosity of the egg white decreased significantly. Two particulate fractions were obtained by centrifuging the homogenate successively for 5 min at 500 ×*g* (unbroken cells and nuclei) and 30 min at 20,000 ×*g* (lysosomal fraction). The remaining pellet was shaken 10 times for 10 min with lysis buffer (1% NP-40, 1 mM DTT, and 1 mM PMSF) at a ratio of 1:1 and was allowed to react on ice. The solution was centrifuged for 10 min at 15,000 rpm, and the remaining pellet was used to determine the immobilization efficiency and melanin treatment activity of the fraction.

The lysosomal enzyme from *S. cerevisiae* was provided by the Korea Research Institute of Bioscience and Biotechnology and was grown in YPD medium (1% yeast extract, 2% peptone, 4% glucose) at 30°C in a shaking incubator at 200 rpm for 12 h. The yeast grown until the log-phase was harvested through centrifugation at 3,000 rpm for 10 min, and the supernatant was discarded. The collected pellet was mixed with Tris-SO_4 buffer (containing 1 ml of 1 M DTT solution per 100 ml). After suspending the mixture, it was centrifuged at 3,000 rpm for 10 min, and the supernatant was discarded. The pellet was resuspended and recollected in 25 ml of sorbitol K^+ buffer. To remove the unnecessary remainders of the cells, the samples were ultrasonicated at 40 W for 5 min (10:0 sec on/off pulses) and centrifuged at 3,000 rpm for 10 min. After collecting the cells, the supernatant was thoroughly mixed with the breaking buffer (containing 50 ml of 1 M PMSF solution per 50 ml), ultrasonicated at 30 W for 5 min (10:10 sec on/off pulses), and centrifuged at 500 rpm for 5 min. We could get lysosomes after centrifuging at 20,000 ×*g* for 30 min and discarding the supernatant. Finally, the extraction of lysosomal enzymes from lysosome followed the same method as the enzymes extracted from Hen`s egg white.

Preparation of Lysosomal Enzymes Immobilized on TiO_2

The 8 mg of anatase (~200 nm) TiO_2 particles used in this experiment was purchased from Sigma-Aldrich, and the reactions were conducted in a sodium phosphate buffer (0.1 M, pH 4, 6, and 10) for the designated reaction time. This dosage of TiO_2 (8 mg) was used for the immobilization of lysosomal enzymes in this study because lower amounts of TiO_2 do not have any melanin treatment activity. The TiO_2 particles were treated with 0.8 mg of lysosomal enzyme and respectively, 0.1 M sodium phosphate buffer at each pH condition for 10 min. The mixture (total of 10 ml) of extracted lysosomal enzyme and TiO_2 was continuously shaken on an orbital shaker (150 rpm) at room temperature; the lysosomal enzymes immobilized on TiO_2 were separated by centrifugation and they were washed three times with 0.1 M sodium phosphate buffer (pH 4, 6, and 10, respectively) and maintained at 4°C until used. The amount of lysosomal enzyme immobilized on TiO_2 was determined by measuring the initial and final concentrations of enzymes in the mixing solution using the Bradford protein assay method (Bio-Rad, Richmond, CA, USA). In addition, to evaluate the effect of TiO_2 pretreatment with monovalent and divalent positive ions, 8 mg of TiO_2 was mixed at room temperature in 1 ml of 0.1 M $CaCl_2$ and 0.1 M KCl. The TiO_2 pretreated by Ca^{2+}, K^+, respectively, was mixed with the buffer before starting the immobilization test.

Melanin Treatment

Melanin (100 ppm) purchased from Sigma-Aldrich (USA) was dissolved in 1 M sodium phosphate buffer, pH 7.0. Then 100 mg amounts of immobilized lysosomal enzymes under each pH condition were used to treat the 100 ppm melanin. The residues of melanin treated by immobilized lysosomal enzymes were measured as absorbance at 450 nm, using the Multiskan Ascent (Thermo Electron Corporation, USA).

Data Analysis

All the data were obtained from three independent samples measured simultaneously for error analysis, and the results are shown with the standard deviation and the correlation with cell mortality under several experimental conditions. The data were analyzed using Sigma Plot (SPS Chicago, IL. USA).

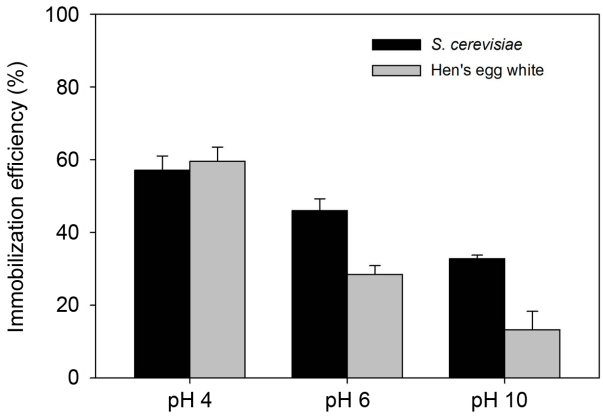

Fig. 1. Immobilization efficiency under different pH conditions on TiO_2 using lysosomal enzymes extracted from lysosomes isolated from Hen's egg white and *S. cerevisiae*.

Results and Discussion

Immobilization Efficiency of Lysosomal Enzymes on TiO_2 under Different pH Conditions of Dissolving Solvents

We measured the immobilization efficiency of lysosomal enzymes immobilized on TiO_2 at different pH conditions (pH 4, 6, and 10). To adapt the pH, we used sodium phosphate as a dissolving solvent. As shown in Fig. 1, the results indicated that the immobilization efficiency of immobilized lysosomal enzymes extracted from Hen`s egg white was the highest at pH 4 compared with the other pH conditions. In the same manner, it immobilized the most of those extracted from yeast at pH 4. From both results, we found that the higher the pH was increased, the more the immobilization efficiency decreased. The immobilization efficiency at different pH levels of lysosomal enzymes extracted from Hen`s egg white and *S. cerevisiae* showed very similar tendencies once immobilized.

Effect of Melanin Treatment Using Immobilized Lysosomal Enzymes Extracted from Two Sources at Various pH Conditions for Seven Days

We examined melanin treatment using immobilized lysosomal enzymes at different pH conditions and two independent sources, in triplicate. Samples (100 μg) of immobilized lysosomal enzymes extracted from both sources under all pH conditions were used in 500 μl of 100 ppm melanin, which melts in pH 6 PBS buffer. We confirmed that the residue of melanin had a measured absorbance at 450 nm. Through the immobilized lysosomal enzymes extracted from Hen's egg white, we found that the residues of melanin changed smoothly at all pH conditions and that

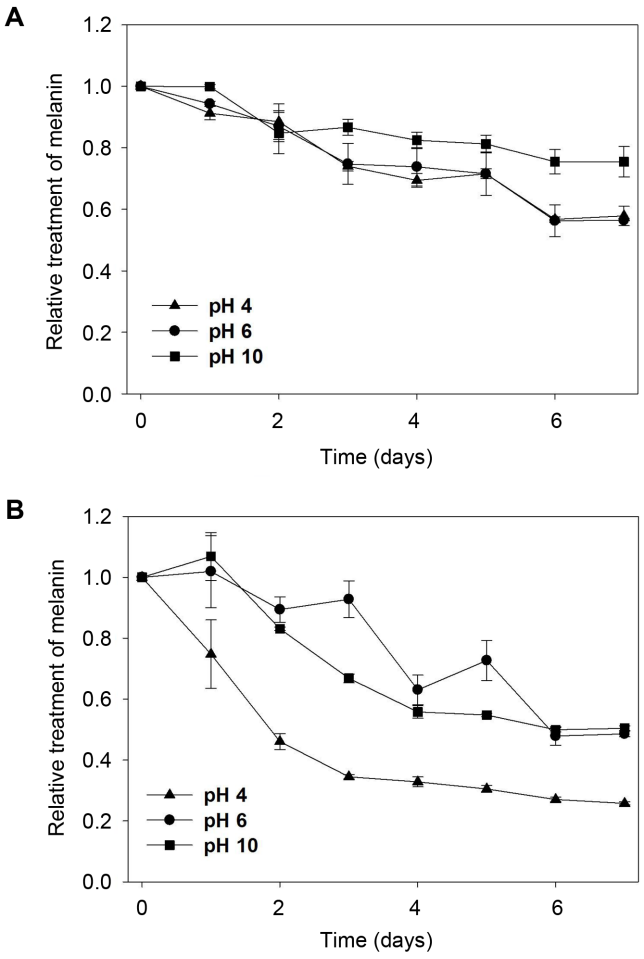

Fig. 2. Effect of melanin treatment using lysosomal enzymes, extracted from (**A**) Hen's egg white and (**B**) *S. cerevisiae*, immobilized on TiO_2 under different pH conditions.
These data correspond to the treatment efficiency relative to the melanin dissolved in phosphate buffer under each pH condition.

total residues fell similarly at pH 4 and 6. On the other hand, those of yeast indicated that the residues changed sharply at pH 4, and total residues showed a similar trend at pH 6 and 10. The color changed the most at pH 4 compared with the residue of treated melanin from both sources under all pH conditions (Fig. 2).

Enhanced Immobilization Efficiency of Lysosomal Enzymes Extracted from Hen's Egg White on TiO_2 Pretreated with Monovalent and Divalent Positive Ions under Different pH Conditions

On the basis of the pH condition and cations treatment results, in order to obtain more effective immobilization efficiency and melanin treatment, we examined to add the

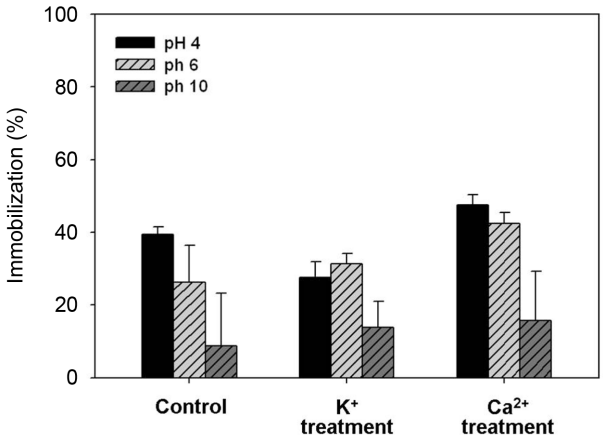

Fig. 3. Immobilization efficiency of lysosomal enzymes, extracted from Hen's egg white, immobilized on TiO_2 pretreated with monovalent and divalent positively charged ions under different pH conditions.

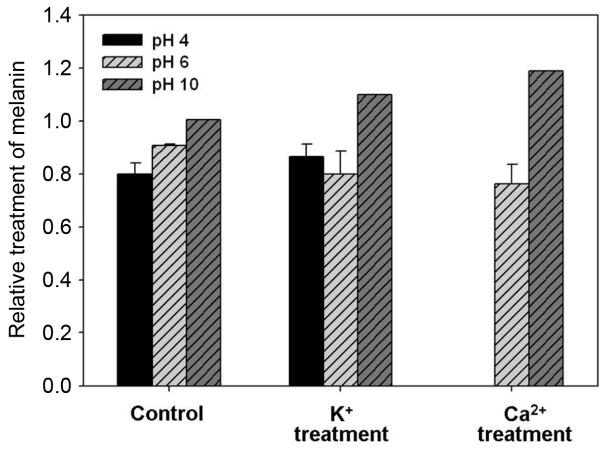

Fig. 4. Effect of melanin treatment using lysosomal enzymes, extracted from Hen's egg white, immobilized on TiO_2 pretreated with monovalent and divalent positively charged ions under different pH conditions.

This data corresponds to the treatment efficiency relative to the melanin dissolved in phosphate buffer under each pH condition.

cations such as KCl and $CaCl_2$ under various pH conditions. Collectively, we found that the immobilization efficiency of untreated and pretreated lysosomal enzymes under different pH conditions was highest when treated with $CaCl_2$ at pH 4. Immobilized lysosomal enzymes on TiO_2 untreated with cations showed that the more the pH condition increased, the less the immobilization efficiency decreased. However, immobilized lysosomal enzymes on TiO_2 treated with KCl indicated that there was a similar tendency at pH 4 and 6, whereas efficiency was lowest of all conditions. Lastly, immobilized lysosomal enzymes on TiO_2 treated with $CaCl_2$ showed similar results as with KCl, but the acidic condition was a little higher than at neutral pH.

We evaluated the immobilization efficiency of lysosomal enzymes with treated cations under different pH conditions. We found that treatment with divalent ions under the lower pH increased the immobilization efficiency. Similarly, for increasing the melanin treatment, we used the immobilized lysosomal enzymes extracted from Hen`s egg white on TiO_2 pretreated with a positive ion like K^+ and Ca^{2+} under different pH conditions. Totally, we found that the melanin treatment was the highest for immobilized lysosomal enzyme pretreated with Ca^{2+} under pH 6 condition among all conditions. In the case of the control (untreated TiO_2), the result indicated that the lower the pH was, the more melanin treatment increased. Melanin treatment was highest pretreated with K^+ under pH 6 condition. However, we could not measure the melanin treatment pretreated with Ca^{2+} under pH 4, because the Ca^{2+} in the buffer returned to a solid state. We suggest that to treat melanin is good to pretreat with Ca^{2+} under neutral condition.

Immobilization experiments were conducted to maintain the activity of the lysosomal enzymes, which may show sensitivity to pH changes because the lysosomes are in unstable pH conditions [17]. To sustain the activity of lysosomal enzymes and adapt them in the human body, we immobilized them on TiO_2, and then we performed to treat the melanin with lysosomal enzymes immobilized on TiO_2 to confirm the possibility that they can adapt in the human body (Fig. 4). We suggest that lysosomal enzymes immobilized on TiO_2 have a higher potential for melanin treatment in the human body.

Acknowledgments

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