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Using Natural Waste Material as a Matrix for the Immobilization of Enzymes: Chicken Eggshell Membrane Powder for β-Galactosidase Immobilization

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Abstract Avian eggshell membranes are good candidates as a matrix for immobilization procedures. Chicken eggshell, a waste material available from the poultry industry as a byproduct, is a very safe and cheap raw material. While pieces of eggshell membrane, or even particles from whole eggshell, have been previously used for these purposes, we report here the use of eggshell membrane powder for *E. coli* β-galactosidase immobilization with glutaraldehyde as cross-linker. A kinetic characterization is provided for eggshell membrane powder-bound enzyme compared to free enzyme. Results show a remarkable similarity between bound and free enzyme and also that the immobilized enzyme is stable and can be reused several times. Moreover, bound enzyme is able to produce glucose from skim milk serum.

Keywords Eggshell membrane powder · Enzyme immobilization · β-Galactosidase

Introduction

Sustainability and low environmental impact are two key demands for productive processes. Hence, the use of what is considered waste material has become not only desirable but necessary. Biomaterials represent an important opportunity because they are produced continuously, are environmentally friendly, and can enable business opportunities. Eggshells are a remarkable example of waste materials. Annual world egg production is about 65 million tons that is 1.3×10^{12} eggs [1]. Because it is estimated that half of this production is used not for

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direct human consumption but for industrial purposes, the accumulation of eggshell waste material is 6.5 million tons that must be discarded at considerable cost [2]. Avian eggshells are one type of many biomineralizing systems that are extensively represented in nature. The absence of cells renders avian eggshells a very stable material compared to other known systems. In the case of chicken eggshell, this material is available from the poultry industry as a byproduct, and it is, from microbiological and toxicological perspectives, a very safe starting (raw) material. Thus, eggshell is an inexpensive, microbe-resistant, and mechanically suitable material [3]. Over the last decade, much effort has been focused on using eggshell waste material for producing novel materials or devices for several applications [4–10]. For example, pieces of eggshells have been used to immobilize enzymes for diagnostic purposes. However, previous attempts to use powdered eggshells have proven unsuccessful, probably due to the presence of the mineral component (i.e., calcium carbonate). To circumvent this, isolation of the non-mineralized part of the eggshell has been tried.

Microscopically, chicken eggshell is a highly ordered array of two layers, consisting of calcified matrix overlying non-mineralized fibrillary membranes that serve as the foundation for the calcified matrix. There is evidence that the role of the fibrillary membranes is to control the shape and geometry of calcium carbonate mineralization [11–15]. The fibrillary membranes, which comprise the innermost layer of an eggshell, are composed of two non-mineralized sublayers, referred to as the inner and outer shell membranes. These membranes adhere to each other around almost the entire inner surface of the shell and can be easily isolated from the shell with mild procedures. These membranes are not membranes in the sense that is used to describe, for example, the cytoplasmic membrane of a cell. Rather, that name was probably used due to anatomical reasons to indicate the existence of a barrier between the cell inside the egg (i.e., the oocyte surrounded by egg white) and the calcified layer, or as a more mechanistic definition, such as a two-dimensional surface which separates two compartments or to which some substances can attach or through which they can selectively permeate. Eggshell membranes are composed of collagens (mainly type X), other proteins, and proteoglycans, and they contain lysine-derived cross-links, which are believed to be responsible for the insolubility and stability of eggshell membrane fibers [16–21]. In addition, eggshell membranes have a high surface area that contains functional groups such as hydroxyl (−OH), thiol (−SH), carboxyl (−COOH), amino ($-NH_2$), and amide ($-CONH_2$), which are both suitable and available for the attachment of macromolecules, including enzymes [8, 9, 15, 22–27].

Using enzymes for production and processing of products for human and animal consumption, among other applications, is a well-established approach [28]. The plethora of procedures that are available for enzyme production and purification has currently placed enzymes among the well-established and environmentally friendly products in biotechnology. However, from a sustainable and even economic perspective, the problem of reuse and stability of enzymes is an unresolved issue. In this connection, the need for natural, available, biocompatible, inexpensive, and safe materials for enzyme immobilization remains a relevant issue. The challenge of increasing both enzyme availability and enzyme stability may be addressed through enzyme immobilization, which provides an excellent approach for increasing availability of enzyme to the substrate with continued catalytic turnover over extended times. Currently, immobilized enzymes are preferred over their free counterpart due to their prolonged availability, which reduces redundant downstream and purification processes [29]. The selection of an appropriate matrix and method of immobilization are critical for retaining or improving enzyme activity and, when needed, to enhance enzyme stability.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. The *Escherichia coli* enzyme is a tetramer of identical subunits. Each subunit (MW 116.4 kDa) is composed of 1023 amino acid residues [30, 31]. Previous attempts have been made to immobilize β-galactosidase with enzyme from various sources; also, different matrices have been used, such as reversible hydrogel beads [32], agar gel [33], cellulose nanofibers [34], glyoxyl-modified supports [35], chitosan [36], and whole eggshell [3].

As mentioned before, β-galactosidase from different sources, mainly microorganisms, has been immobilized on numerous types of support, including the complete chicken eggshell [37, 38]. The results of these investigations have been variable both in terms of the conservation of the properties of the enzyme and its stability. Many of the support materials used are synthetic, and a few are natural products. The interest in immobilizing an enzyme clearly is based on the fact that production of that enzyme is a process that requires a great deal of effort, which makes its single use a very expensive one. If immobilization is done with a natural matrix which is, at the same time, a waste product, two problems are solved simultaneously. In this regard, using *E. coli* β-galactosidase as a model, we describe the immobilization of the enzyme on membrane powder from the eggshell of chicken eggs, not whole chicken eggshell as described elsewhere [3].

Materials and Methods

Materials

E. coli β-galactosidase (EC 3.2.1.23) and reagents for activity and protein assays were from Sigma-Aldrich. The enzyme preparation, used without further purification, was essentially pure as judged by PAGE (data not shown). Eggs and milk were from a domestic supplier. General reagents for eggshell membrane preparation and modification were analytical grade from Merck.

Eggshell Membrane Preparation

The inner contents (egg white and oocyte) were removed from eggs that had been carefully punctured at one end, and the emptied eggs were washed several times with distilled water to completely remove the egg white. Then the emptied eggs were filled with 5% (*v*/*v*) acetic acid and allowed to stand for 40 min at room temperature. After the acetic acid was discarded, the emptied eggs were again washed with distilled water. The eggshell membranes were then obtained by carefully peeling them off the calcified part of the eggshell. The membrane preparation was washed several times with distilled water, washed three times with acetone, and dried at 40 °C overnight. Dried membranes were pulverized with a mortar and pestle in liquid nitrogen. The resulting powder was suspended in 50 mM sodium phosphate buffer, pH 7.0 and stored at 4 °C.

Eggshell Membrane Microscopy

Pieces of dried eggshell membranes were mounted on aluminum stubs with Scotch double-sided tape, coated with gold, and observed in a JEOL JSM-IT300 scanning electron

microscope at 20 kV. Eggshell membrane powder was mounted directly on a slide and observed under a Nikon E400 light microscope with a total magnification of ×400. Random chosen particles were used to estimate the corresponding surface.

Activity Assay

Enzyme activity was assayed by incubating appropriate quantities of enzyme (free or bound) in assay buffer, 40 mM sodium phosphate, pH 7.0 containing 3.3 mM *o*-nitrophenyl-galactoside (ONPG) at 25 °C in a final volume of 1.2 ml. Unless otherwise stated, activity assays were initiated by addition of substrate. The rate of *o*-nitrophenol (ONP) production was linear up to 60 min at room temperature. Furthermore, the initial rate, for both free and bound enzyme, varied linearly, as expected, with enzyme concentration (data not shown).

Reactions were stopped by the addition of 0.3 ml of 1 M sodium carbonate. Released ONP was spectrophotometrically determined at 420 nm. One unit (U) is the amount of enzyme that produces 1 μmol of ONP per minute under the conditions previously stated. When needed, the assay mix was centrifuged (16,000×*g* for 5 min) and ONP measured in the respective supernatant. Protein content of the enzyme solutions was determined by the Bradford assay with bovine serum albumin (BSA) as standard [39].

Immobilization of β-Galactosidase

Eggshell membrane powder (800 mg) was suspended in 50 mM sodium phosphate buffer, pH 7.0 and was incubated overnight at room temperature in the presence of 0.2% (*v*/*v*) glutaraldehyde with continuous stirring. To remove excess cross-linking reagent, the preparation was centrifuged (3900×*g* for 10 min) and resuspended in an appropriate volume of 50 mM sodium phosphate buffer, pH 7.0. After 15 min of stirring, the preparation was centrifuged again. This washing procedure was repeated four to five times.

The modified powder was then suspended in the presence of 24 units of β-galactosidase (0.3 mg of protein/ml). This preparation was incubated with continuous stirring overnight at 4 °C. To remove the free (unbound) enzyme, the preparation was centrifuged as indicated above. Enzyme activity was measured in the respective fractions as described before. Pellet fraction (bound enzyme) accounts for 30% of total enzyme activity.

Effect of Milk Serum on β-Galactosidase Activity

Milk serum was prepared by mixing 36 ml of skim milk with 4 ml of 10% acetic acid. After vigorous stirring, the preparation was immediately centrifuged (3900×*g* for 20 min). Supernatant neutralization to pH 7.1 was accomplished by dropwise addition of 5 N NaOH. The neutralized preparation was centrifuged again, as indicated above, and the supernatant stored at 4 °C until use. The effect of variable amounts of milk serum on free or eggshell membrane-bound enzyme was determined at 0.25 mM ONPG in 40 mM sodium phosphate buffer, pH 7.0.

Effect of pH

Appropriate amounts of enzyme (free or bound) were incubated at room temperature in 40 mM sodium phosphate buffer adjusted to different pH values. Reactions were stopped as indicated above, and ONP was determined spectrophotometrically.

Stability of Bound Enzyme

An enzyme-bound eggshell membrane powder preparation, stored at 4 °C, was sampled at various time intervals for β-galactosidase activity. Also, a preparation of free (i.e., unbound) enzyme with similar activity was tested in the same way. All measurements were performed at pH 7.0, room temperature, and saturating concentration of substrate.

Reuse of Bound Enzyme

An aliquot (2 ml) of eggshell membrane powder-bound enzyme was put into dialysis tubing, which remained sealed. The dialysis tubing was placed in a chamber containing the reaction mixture and stirred continuously. Measurements were conducted at room temperature. Unless otherwise stated, all measurements were made at saturating concentration of ONPG. Between measurements, the preparation was dialyzed against 50 mM phosphate buffer (50 ml, two to three changes every 30–40 min) to remove excess substrate and product formed in each reaction cycle. The dialysis tubing containing the preparation was stored at 4 °C. Measurements were done two to four times daily at defined days.

Glucose Determination

Glucose determination was done spectrophotometrically with a final point glucose oxidase assay. A commercial kit was used according to the instructions provided by the manufacturer (i.e., 10-min incubation time at room temperature and absorbance reading at 505 nm).

Structural Data

Structural data for *E. coli* β-galactosidase were taken from a public database (PDB ID 4V44). Lysine accessibility was assessed by means of WHAT IF software [40].

Results

Eggshell Membrane Powder Preparation

Figure 1 shows eggshell membrane as seen under SEM (1A) and eggshell membrane powder (1B) using light microscopy. The surface area of the particles is highly variable, ranging from 3 to 410 μm^2. The average value ($n = 12$) is 86.5 μm^2 with a standard error of ± 34.

Kinetic Studies

To characterize free and eggshell membrane powder-bound enzyme, we studied the dependence of initial rate on substrate (ONPG) concentration. Figure 2 shows that there is hyperbolic behavior for both of them. Accordingly, respective double reciprocal plots were linear, as expected. K_m values estimated from these data indicate values of 0.19 and 0.14 mM for free and eggshell membrane powder-bound enzyme, respectively.

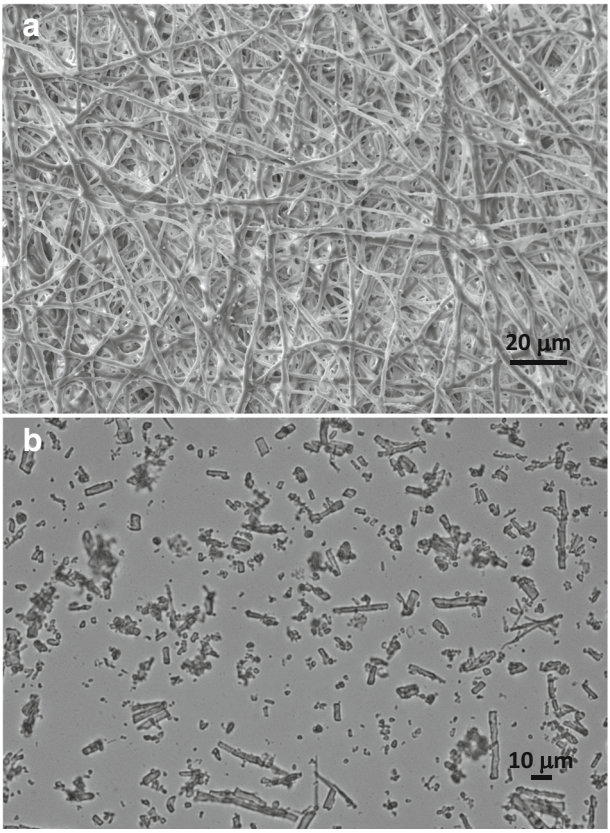

Fig. 1 Eggshell membranes and eggshell membrane powder. **a** Scanning electron micrograph of a piece of eggshell membrane (×550). **b** Light microscopy of eggshell membrane powder (×400). Both specimens were prepared as indicated in the "Materials and Methods" section

Effect of Milk Serum on β-Galactosidase Activity

For further comparison, the inhibitory effect of milk serum (which contains lactose) was assessed. As shown in Fig. 3a, the similarity of that effect on free and bound enzyme is evident. In both cases, the quantity of milk serum needed to obtain 50% inhibition (expressed as microliters of milk serum) is approximately 35 μl. Based on this result, a kinetic study with fixed quantities of milk serum (10 and 20 μl) and variable concentrations of substrate (ONPG) shows a competitive pattern of inhibition for both forms of the enzyme (Fig. 3b, c). Moreover, K_i values estimated from these data are in the same order of magnitude (as described in "Discussion" section).

Effect of pH

The pH dependence for both free and bound enzyme, measured in sodium phosphate buffer, is similar, as shown in Fig. 4. The optimum pH for free enzyme was about 7.0, a value slightly higher than the observed value of 6.8 for the eggshell membrane powder-bound enzyme.

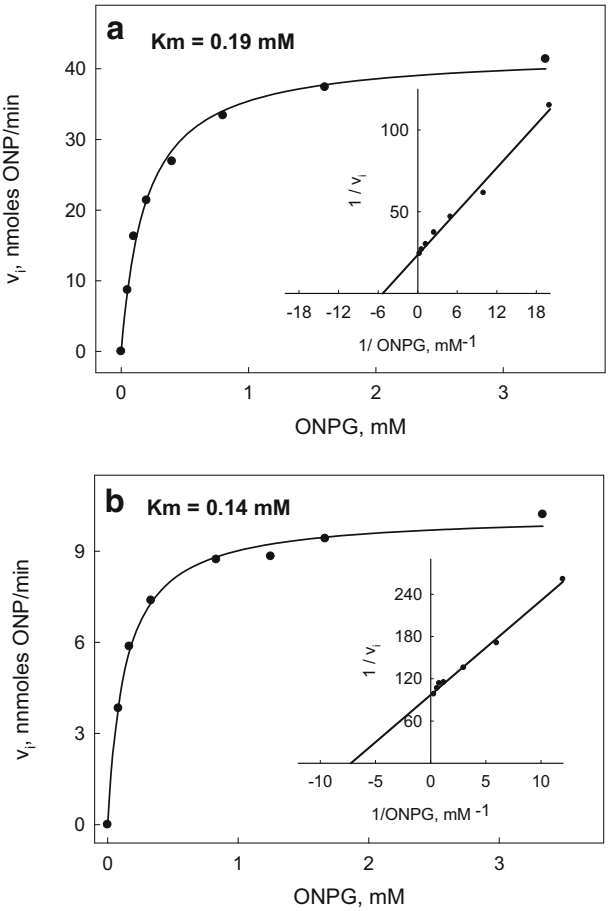

Fig. 2 Dependence of initial rate of free (**a**) and eggshell membrane powder-bound (**b**) β-galactosidase on ONPG concentration. Initial rates were measured in the presence of variable quantities of ONPG incubated with appropriate quantities of enzyme (free or bound) in assay buffer, 40 mM sodium phosphate, pH 7.0 at 25 °C as described in the "Material and Methods" section. Insets show double reciprocal plots of the data

Stability and Re-Utilization of Eggshell Membrane-Bound Enzyme

As shown in Fig. 5a, the activity of different aliquots of free and bound enzyme, sequentially measured at various times, indicated that the enzyme was stable up to at least 150 h (i.e., more than 6 days). Free enzyme, stored in the same conditions, behaved similarly. As shown in Fig. 5b, when one single aliquot of bound enzyme was sequentially used several times during a period of more than 90 days, the enzyme showed significant retention of its activity.

Eggshell Membrane Powder-Bound Enzyme Produces Glucose from Skim Milk Serum

Previous data were obtained with an unnatural substrate (ONPG) under strongly controlled conditions. Therefore, to further test the activity of eggshell membrane powder-bound enzyme, experiments were done with a source of native substrate. Briefly, a piece of dialysis tubing

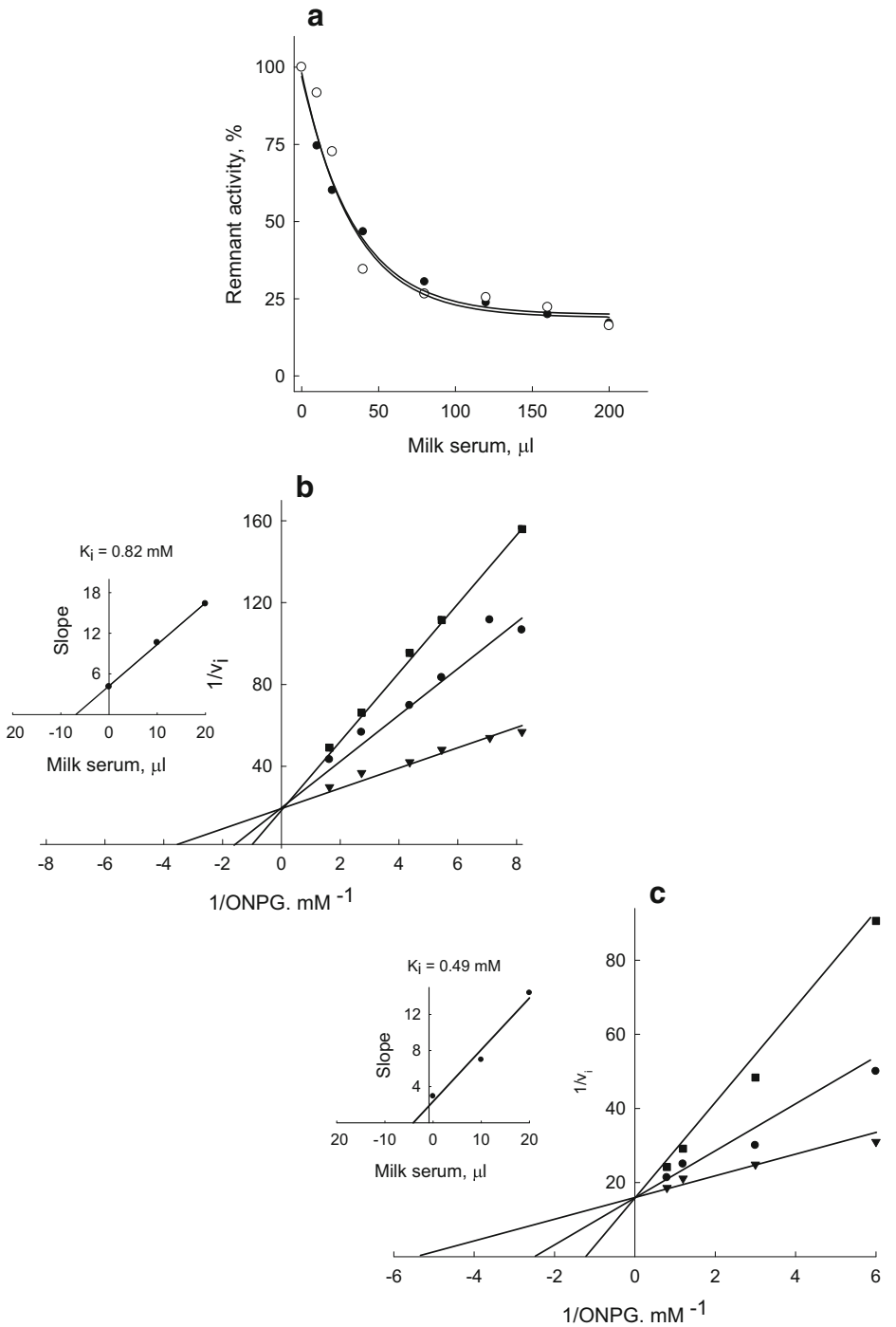
a
Remnant activity, %
Milk serum, μl
b
Ki = 0.82 mM
Slope
Milk serum, μl
1/vi
1/ONPG. mM -1
c
Ki = 0.49 mM
Slope
Milk serum, μl
1/vi
1/ONPG. mM -1

Fig. 3 Effect of milk serum on free and bound β-galactosidase activity. **a** Initial rate was measured for both free enzyme (filled circles) and eggshell membrane powder-bound enzyme (open circles) in the presence of variable quantities of milk serum. For both preparations, the initial rate measured in the absence of milk serum was taken as 100%. All determinations were done at 0.25 mM ONPG. For free (**b**) and bound (**c**) enzyme, initial rates for control (triangles) and 10 μl (circles) or 20 μl (squares) of milk serum were measured in the presence of variable quantities of ONPG. Insets show slope replots as a function of the amount of milk serum. K_i values were estimated from lactose in milk serum, as measured with a glucose oxidase assay

(3500 CO) filled with 5 ml of eggshell membrane-bound enzyme suspension (approximately 35 mU) was incubated in the presence of 10 ml of skim milk serum (diluted 1/10) for 16 h at 4 °C with continuous stirring. A diluted milk serum control (10 ml) was incubated under the same conditions, but in the absence of enzyme-bound suspension. After the indicated time, a glucose oxidase assay was used to determine the glucose content for both the enzyme-treated and untreated milk serum. The quantity of glucose measured in the enzyme-treated milk serum was 418 μg, while in the control glucose was, as expected, undetectable. The total carbohydrate content for that milk was reported as 49 g/l. Assuming that all of the reported carbohydrate content was lactose (or at least 95% of that value), then the diluted milk serum contained about 2450 μg of glucose. Thus, this enzyme-bound preparation produces about 17% of the expected glucose.

Based on the previous result, the rest of the suspension (15 ml) was tested directly on milk serum (diluted 1/10). To this end, the preparation was centrifuged as described in "Materials and Methods" section and then suspended in 10 ml of milk serum. This mixture was incubated for 120 min at room temperature with continuous stirring. Bound enzyme was removed by centrifugation, and glucose in the supernatant was measured as described above. Under these conditions, 476 μg of glucose was detected, which represents about 19% of the total value expected. Finally, after extensive washing, the enzyme-bound preparation was again suspended in diluted milk serum and incubated for 24 h at 4 °C with continuous stirring. Under these conditions, 1286 μg of glucose were detected, which is about 52% of the total value expected.

Discussion

As shown by Makkar and Sharma [3], whole eggshell can be effectively used for β-galactosidase immobilization of *Lactobacillus bulgaricus* enzyme. However, in that short

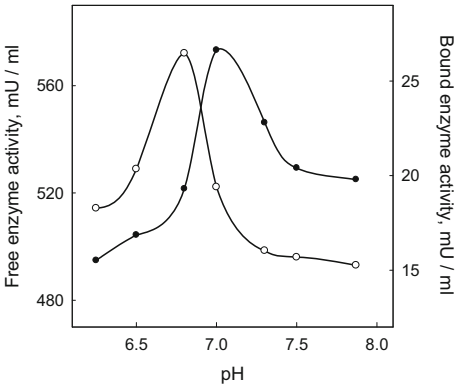

Fig. 4 Effect of pH on free and eggshell membrane powder-bound β-galactosidase activity. The activity of free (filled circles) and eggshell membrane powder-bound (open circles) β-galactosidase was measured at various pH values. The final concentration of sodium phosphate buffer was 40 mM. Initial rates are given as milliunits of enzyme per milliliter

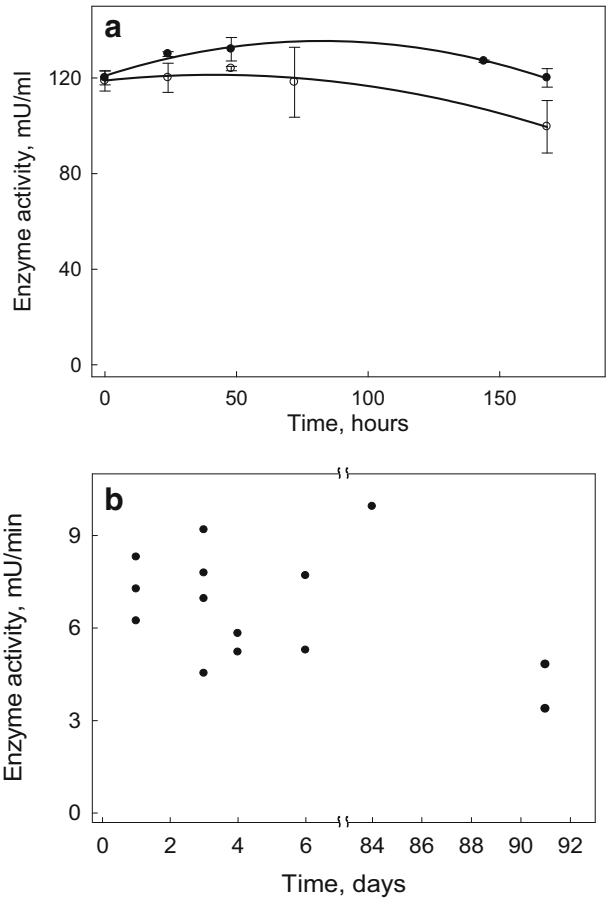

Fig. 5 Stability and re-utilization of eggshell membrane powder-bound β-galactosidase over time. **a** The activity of different aliquots of free (filled circles) and eggshell membrane powder-bound (open circles) β-galactosidase was measured at the indicated times. Values are the average of four determinations. **b** The activity of one single aliquot of eggshell membrane-bound β-galactosidase was measured on the indicated days. All data in the graph were obtained with the same aliquot of bound enzyme, which was measured two to four times daily (except on day 84). All measurements were done with a 10-min incubation time at a saturating concentration of ONPG

communication using whole eggshell, not eggshell membranes, a final concentration of 2% glutaraldehyde was used. In our conditions, 0.5% glutaraldehyde was enough to inactivate 50% of the enzyme activity (data not shown), which may be understood considering the enzyme source or the pH conditions that they used. Moreover, although kinetic parameters are mentioned, no supporting data are shown, as well as no explanation for the reported similarity between free and bound enzyme. Other studies used eggshell membranes for the immobilization of enzymes other than β-galactosidase with a concentration of cross-linking agent of 2% [41]. Finally, in other studies undiluted cross-linking agent has been directly used on eggshell membranes [42].

Our data show that the effect of glutaraldehyde on β-galactosidase activity was marginal at the concentration that we used (i.e., 0.2%). The dependence of the initial velocity of the reaction on the ONPG substrate was remarkably similar for free and bound enzyme. In fact, the K_m values for both preparations are similar and comparable to that reported previously [43].

Moreover, when the effect of different amounts of milk serum as a source of lactose was tested on both preparations, it was observed that, as expected, the inhibition pattern was competitive and the K_i values are again in the same order of magnitude. From the data of Kuby and Lardy [43], we calculated a value of K_i that is similar to the one calculated from our data for the free enzyme. Finally, we found that the optimal pH for both preparations is similar.

The above results suggest that the remarkable similarity of the properties we report should be a consequence of at least partial conservation of the quaternary and tertiary structure of the enzyme after immobilization. The rather low concentration of cross-linking agent that was used at least partly explains this assertion. Most notably, the available structural information accounts for the results described. In fact, the evaluation of the lysine residues exposed to the solvent shows that two of them (773 and 774) are on the surface rather far from the active site of the enzyme (Fig. 6). Because the active site of *E. coli* β-galactosidase is shared between subunits [30, 31, 44], we suggest that the immobilization compromises the indicated lysine residues without greatly affecting the quaternary or tertiary protein structure. This would explain, at least in part, the similarity of kinetic behavior between free and immobilized enzymes.

The immobilized enzyme reported here was shown to be reasonably stable over time. Indeed, as mentioned above, a single sampled preparation with different aliquots was stable for

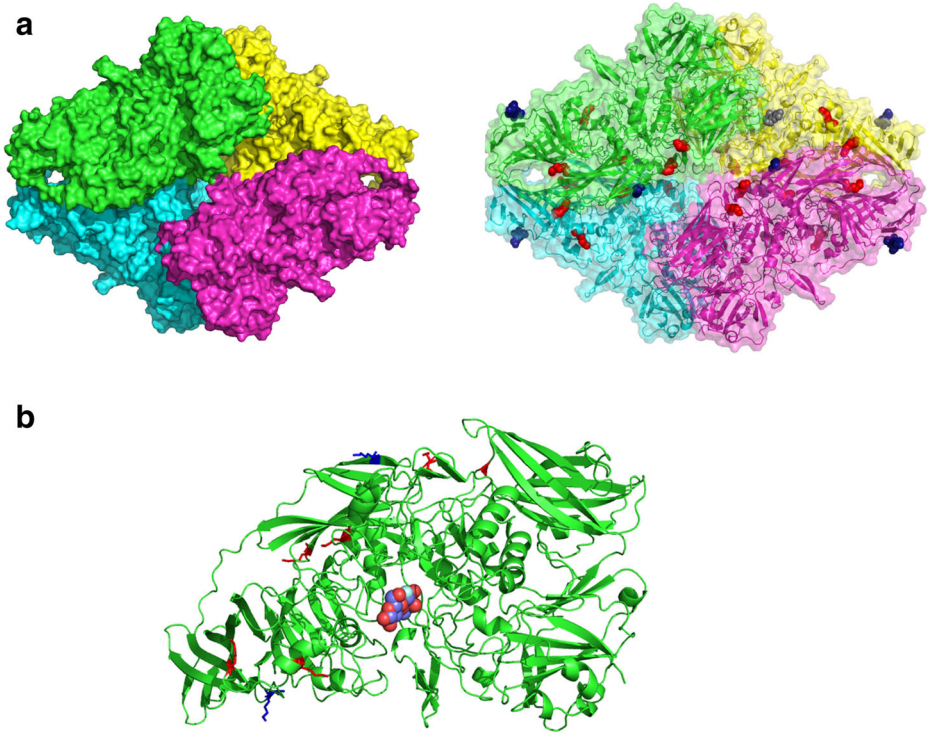

Fig. 6 Structural analysis of β-galactosidase from *E. coli* (PDB ID 4V44). **a** The quaternary structure of *E. coli* β-galactosidase with the molecular surface colored by monomers. **b** Ribbon representation of a monomer with 2-fluoro-2-deoxy-lactose in its active site (shown as spheres). Lysines with total accessibility values between 20 and 40 are colored in red (residues 229, 347, 551, 661, and 811), while lysines with accessibility over 40 are shown in blue (residues 677, 773, and 774). Accessibility was assessed by means of WHAT IF software

up to 150 h and a single aliquot showed significant retention of its activity for up to 91 days. This result contrasts with those previously reported by Makkar and Sharma [3], who showed a rapid loss of enzyme activity. The difference may be explained again in terms of the enzyme source (i.e., structural differences), the concentration of cross-linking agent used, or a combination of both. In this regard, an inspection of the primary structure of the *L. bulgaris* and *E. coli* enzymes shows only 33% identity, and the lysines in positions 667, 773, and 774 of the *E. coli* enzyme are replaced with threonine, arginine, and glutamate, respectively, in *L. bulgaris*. While the stability of the free and immobilized enzyme is similar, the immobilization of the enzyme allows its reuse for a long time. We think that this is partly because the interaction of the enzyme with the matrix stabilizes the quaternary structure that is necessary for the activity of the enzyme since the active sites are at the interfaces of the subunits. In this connection, it has been proposed that the stability of immobilized urease using eggshell membrane as a support is due to the cross-linking agent (polyethyleneimine) providing a high concentration of amino groups [45].

Finally, our preparation was capable of producing glucose when we used skim milk as a source of lactose at 4 or 25 °C. Certainly, as was reasonable to expect, it only took 120 min to produce, at 25 °C, a quantity of glucose comparable to that produced when the preparation was incubated at 4 °C overnight. Moreover, when incubating the same preparation for 24 h at 4 °C the amount of glucose produced reached a value close to 50% of the expected value. We think that any potential application of this immobilized enzyme should consider its use under the conditions provided by a standard refrigerator (i.e., 4 °C).

We believe the results reported here are useful in at least two ways. First, the use, with very little manipulation and manipulation at low cost, of a byproduct of the poultry industry as a carrier for the immobilization has evident benefits from an economic and environmental point of view. Second, lactose intolerance has been recognized as a health problem for a long time. Lactose intolerance is a condition in which people, due to a deficiency of lactase activity, have symptoms that result from a decreased ability to digest lactose, a sugar found in milk products. Lactose-intolerant people require lactose-free milk products, which are produced by treatment of milk with lactase (β-galactosidase) from different sources. This procedure requires large amounts of enzyme, which are lost in the process. Immobilization of β-galactosidase to a suitable matrix appears to be a useful approach to improve this process.

While the production of milk without lactose by the addition of soluble β-galactosidase (from various sources) has been the usual solution, an obvious, sustainable, and better solution is the use of an immobilized enzyme. In this regard, understanding the conservation of the properties of the immobilized enzyme is basic for the design of new solutions for different problems from both the production and health sectors.

Conclusion

In summary, the use of eggshell membrane powder for immobilization of β-galactosidase not only maintains the enzyme properties relative to unbound enzyme but also allows the reutilization of a single aliquot several times over an extended period of time. Also, the immobilized enzyme is able to hydrolyze lactose in the presence of skim milk serum. Our results suggest that eggshell membrane powder is a very promising material for the immobilization of enzymes, which allows reuse of the enzymes and thus makes the process beneficial both from an environmental and economic point of view.

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References

1. Windhorst, H.-W., Grabkowsky, B., & Wilke, A. (2013). Atlas of the Global Egg Industry, 1–36. Retrieved from http://www.internationalegg.com/wp-content/uploads/2015/08/atlas_2013_web.pdf
2. Walton, H. V, Cotterill, O. J., & Vandepopuliere, J. M. (1973). Composition of shell waste from egg breaking plants. Poultry Science, 52(5), 1836–1841. Retrieved from https://doi.org/10.3382/ps.0521836
3. Makkar, H. P. S., & Sharma, O. P. (1983). Egg shell as a carrier for enzyme immobilization. *Biotechnology and Bioengineering, 25*(2), 595–597. https://doi.org/10.1002/bit.260250222.
4. Guru, P. S., & Dash, S. (2014). Sorption on eggshell waste—a review on ultrastructure, biomineralization and other applications. *Advances in Colloid and Interface Science, 209*, 49–67. https://doi.org/10.1016/j.cis.2013.12.013.
5. Arias JL, Arias JI, Fernandez, M. (2007). Arias JL, Arias JI, Fernandez MS. 2007; Avian eggshell as a template for biomimetic synthesis of new materials. In Handbook of biomineralization, vol 2, E Baeuerlein, P Behrens, M Epple (eds). Wiley-VCH: Weinheim, Germany; 109–117. In Handbook of biomineralization*:* *biological aspects and structure formation* (pp. 100–117).
6. King'ori, A. (2011). Review of the factors that influence egg fertility and hatchability in poultry. *International Journal of Poultry Science., 10*(6), 483–492. https://doi.org/10.3923/ijps.2011.483.492.
7. Mittal, A., Teotia, M., Soni, R. K., & Mittal, J. (2016). Applications of egg shell and egg shell membrane as adsorbents: a review. *Journal of Molecular Liquids, 223*, 376–387. https://doi.org/10.1016/j.molliq.2016.08.065.
8. Park, S., Choi, K. S., Lee, D., Kim, D., Lim, K. T., Lee, K. H., Seonwoo, H., & Kim, J. (2016). Eggshell membrane: review and impact on engineering. *Biosystems Engineering, 151*, 446–463. https://doi.org/10.1016/j.biosystemseng.2016.10.014.
9. Baláz, M. (2014). Eggshell membrane biomaterial as a platform for applications in materials science. *Acta Biomaterialia, 10*(9), 3827–3843. https://doi.org/10.1016/j.actbio.2014.03.020.
10. Cordeiro, C. M., &. Hincke, M. T. (2011). Recent patents on eggshell: Shell and membrane applications. *Recent Patents on Food, Nutrition & Agriculture, 3* (1), 1–8. https://doi.org/10.2174/2212798411103010001.
11. Arias, J. L., Fink, D., Xiao, S.-Q., Heuer, A. H., & Caplan, A. I. (1993). Biomineralization and eggshells: Cell-mediated acellular compartments of mineralized extracellular matrix. *International Review of Cytology*, 145, 217–250. https://doi.org/10.1016/S0074-7696(08)60428-3.
12. Dennis, J. E., Xiao, S. Q., Agarwal, M., Fink, D. J., Heuer, A. H., & Caplan, A. I. (1996). Microstructure of matrix and mineral components of eggshells from white leghorn chickens (Gallus gallus). *Journal of Morphology, 228*(3), 287–306. https://doi.org/10.1002/(SICI)1097-4687(199606)228:3<287::AID-JMOR2>3.0.CO;2-#.
13. Arias, J. L., Cataldo, M., Fernandez, M. S., & Kessi, E. (1997). Effect of beta-aminoproprionitrile on eggshell formation. *British Poultry Science, 38*(4), 349–354. https://doi.org/10.1080/00071669708418001.
14. Fernandez, M. S., Araya, M., & Arias, J. L. (1997). Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. *Matrix Biology, 16*(1), 13–20. https://doi.org/10.1016/S0945-053X(97)90112-8.
15. Soledad Fernandez, M., Moya, A., Lopez, L., & Arias, J. L. (2001). Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. *Matrix Biology, 19*(8), 793–803. https://doi.org/10.1016/S0945-053X(00)00128-1.
16. Arias, J. L., Fernandez, M. S., Dennis, J. E., & Caplan, A. I. (1991). Collagens of the chicken eggshell membranes. *Connective Tissue Research, 26*(1–2), 37–45. https://doi.org/10.3109/03008209109152162.
17. Arias, J. L., Carrino, D. A., Fernandez, M. S., Rodriguez, J. P., Dennis, J. E., & Caplan, A. I. (1992). Partial biochemical and immunohistochemical characterization of avian eggshell extracellular matrices. *Archives of Biochemistry and Biophysics, 298*, 293–302.
18. Nys, Y., Hincke, M. T., Arias, J. L., Garcia-Ruiz, J. M., & Solomon, S. E. (1999). Avian eggshell mineralization. *Poultry and Avian Biology Reviews, 10*(3), 143–166.
19. Du, J., Hincke, M. T., Rose-Martel, M., Hennequet-Antier, C., Brionne, A., Cogburn, L. A., et al. (2015). Identifying specific proteins involved in eggshell membrane formation using gene expression analysis and bioinformatics. *BMC Genomics, 16*(1), 1–13. https://doi.org/10.1186/s12864-015-2013-3.

20. Cordeiro, C. M. M., & Hincke, M. T. (2015). Quantitative proteomics analysis of eggshell membrane proteins during chick embryonic development. *Journal of Proteomics, 130*, 11–25. https://doi.org/10.1016/j.jprot.2015.08.014.
21. Rao, A., Arias, J., & Cölfen, H. (2017). On mineral retrosynthesis of a complex biogenic scaffold. *Inorganics, 5*(1), 16. https://doi.org/10.3390/inorganics5010016.
22. Arias, J. L., Arias, J. I., & Fernandez, M. S. (2008). Avian eggshell as a template for biomimetic synthesis of new materials. *Handbook of Biomineralization: Biological Aspects and Structure Formation, 2*, 109–117. https://doi.org/10.1002/9783527619443.ch30.
23. Joshi, P., Joshi, H. C., Sanghi, S. K., & Kundu, S. (2010). Immobilization of monoamine oxidase on eggshell membrane and its application in designing an amperometric biosensor for dopamine. *Microchimica Acta, 169*(3), 383–388. https://doi.org/10.1007/s00604-010-0346-9.
24. Yang, H. T., Tan, Q., & Zhao, H. (2014). Progress in various crosslinking modification for acellular matrix. *Chinese Medical Journal, 127*(17), 3156–3164. https://doi.org/10.3760/cma.j.issn.0366-6999.20140900.
25. Zheng, B., Xie, S., Qian, L., Yuan, H., Xiao, D., & Choi, M. M. F. (2011). Gold nanoparticles-coated eggshell membrane with immobilized glucose oxidase for fabrication of glucose biosensor. *Sensors and Actuators, B: Chemical, 152*(1), 49–55. https://doi.org/10.1016/j.snb.2010.09.051.
26. Chattopadhyay, S., & Sen, R. (2012). A comparative performance evaluation of jute and eggshell matrices to immobilize pancreatic lipase. *Process Biochemistry, 47*(5), 749–757. https://doi.org/10.1016/j.procbio.2012.02.003.
27. Fernández, M. S., Valenzuela, F., Arias, J. I., Neira-Carrillo, A., & Arias, J. L. (2016). Is the snail shell repair process really influenced by eggshell membrane as a template of foreign scaffold? *Journal of Structural Biology, 196*(2), 187–196. https://doi.org/10.1016/j.jsb.2016.10.001.
28. Fernandes, P. (2010). Enzymes in food processing: a condensed overview on strategies for better biocatalysts. *Enzyme Research, 2010*, 1–19. https://doi.org/10.4061/2010/862537.
29. Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech, 3*(1), 1–9. https://doi.org/10.1007/s13205-012-0071-7.
30. Juers, D. H., Jacobson, R. H., Wigley, D., Zhang, X. J., Huber, R. E., Tronrud, D. E., & Matthews, B. W. (2000). High resolution refinement of beta-galactosidase in a new crystal form reveals multiple metal-binding sites and provides a structural basis for alpha-complementation. *Protein science : a Publication of the Protein Society, 9*(9), 1685–1699. https://doi.org/10.1110/ps.9.9.1685.
31. Juers, D. H., Matthews, B. W., & Huber, R. E. (2012). LacZ β-galactosidase: structure and function of an enzyme of historical and molecular biological importance. *Protein Science, 21*(12), 1792–1807. https://doi.org/10.1002/pro.2165.
32. Park, T. G., & Hoffman, A. S. (1990). Immobilization and characterization of beta-galactosidase in thermally reversible hydrogel beads. *Journal of Biomedical Materials Research, 24*(1), 21–38. https://doi.org/10.1002/jbm.820240104.
33. Sen, S., Ray, L., & Chattopadhyay, P. (2012). Production, purification, immobilization, and characterization of a thermostable beta-galactosidase from Aspergillus alliaceus. *Applied Biochemistry and Biotechnology, 167*(7), 1938–1953. https://doi.org/10.1007/s12010-012-9732-6.
34. Sulaiman, S., Mokhtar, M. N., Naim, M. N., Baharuddin, A. S., & Sulaiman, A. (2014). A review: Potential usage of cellulose nanofibers (CNF) for enzyme immobilization via covalent interactions. *Applied Biochemistry and Biotechnology, 175*(4), 1817–1842. https://doi.org/10.1007/s12010-014-1417-x.
35. Calandri, C., Marques, D. P., Cesar Mateo, A., Carrascosa, V., Guisán, J. M., Lorente, G. F., & Pessela, B. C. (2013). Purification, immobilization, stabilization and characterization of commercial extract with β-galactosidase activity. *Journal of Biocatalysis & Biotransformation, 02*(01), 1–7. https://doi.org/10.4172/2324-9099.1000104.
36. Klein, M. P., Fallavena, L. P., Schöffer, J. D. N., Ayub, M. A. Z., Rodrigues, R. C., Ninow, J. L., & Hertz, P. F. (2013). High stability of immobilized β-d-galactosidase for lactose hydrolysis and galacto-oligosaccharides synthesis. *Carbohydrate Polymers, 95*(1), 465–470. https://doi.org/10.1016/j.carbpol.2013.02.044.
37. Panesar, P. S., Kumari, S., & Panesar, R. (2010). Potential applications of immobilized β-galactosidase in food processing industries. *Enzyme Research, 2010*, 1–16. https://doi.org/10.4061/2010/473137.
38. Kishore, D., Talat, M., Srivastava, O. N., & Kayastha, A. M. (2012). Immobilization of β-galactosidase onto functionalized graphene nano-sheets using response surface methodology and its analytical applications. *PLoS One, 7*(7), e40708. https://doi.org/10.1371/journal.pone.0040708.
39. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
40. Vriend, G. (1990). WHAT IF: A molecular modeling and drug design program. *Journal of Molecular Graphics, 8*(1), 52–56. https://doi.org/10.1016/0263-7855(90)80070-V.

41. Vemuri, G., Banerjee, R., & Bhattacharyya, B. C. (1998). Immobilization of lipase using egg shell and alginate as carriers: optimization of reaction conditions. *Bioprocess Engineering, 19*(2), 111–114. https://doi.org/10.1007/s004490050490.
42. Choi, M. M. F., & Yiu, T. P. (2004). Immobilization of beef liver catalase on eggshell membrane for fabrication of hydrogen peroxide biosensor. *Enzyme and Microbial Technology, 34*(1), 41–47. https://doi.org/10.1016/j.enzmictec.2003.08.005.
43. Kuby, S. A., & Lardy, H. A. (1953). Purification and kinetics of β-D-galactosidase from Escherichia coli, strain K-12. *Journal of the American Chemical Society, 75*(4), 890–896. https://doi.org/10.1021/ja01100a035.
44. Matthews, B. W. (2005). The structure of *E. coli* β-galactosidase. *Comptes Rendus - Biologies, 328*(6 SPEC. ISS), 549–556. https://doi.org/10.1016/j.crvi.2005.03.006.
45. D'Souza, S. F., Kumar, J., Jha, S. K., & Kubal, B. S. (2013). Immobilization of the urease on eggshell membrane and its application in biosensor. *Materials Science and Engineering C, 33*(2), 850–854. https://doi.org/10.1016/j.msec.2012.11.010.