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AROMATIC AMINE COUPLING OF *Aspergillus niger* LACTASE TO CONTROLLED-PORE SILICA WITH *o*-DIANISIDINE

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Lactose (β-galactosidase) derived from *Aspergillus niger* was immobilized on controlled-pore silica with an average pore diameter of 425 Å. The coupling of this enzyme to the surface of the silica was accomplished by reacting the surface of the silica with o-dianisidine followed by the functionalization of the residual amine with glutaraldehyde or with nitrite to form the diazonium salt. The PH profiles of the immobilized enzymes were determined and compared. Continuous reactor studies of the glutaraldehyde-functionalized, immobilized enzyme indicated a half-life of 52 days at 50°C with a 5% lactose feed at pH 3.5.

INTRODUCTION

This study was initiated with two objectives. The first was to further explore the reaction of aromatic amines with silanol surfaces; the second was to evolve a practical immobilized lactase system to be employed in a continuous reactor. Prior studies indicated that proteins were bound to the silanol surface by adsorption through an amine silanol bond and hydrogen bonds (1). A subsequent study indicated that if an amine was in the active site of an enzyme and that enzyme was bound to a silanol surface, the enzyme would become inactivated (2). A more recent report indicated that an aromatic diazonium salt was bound via a permanent bond to a silanol surface (3). Although the exact method by which this diazonium salt was bound to the silanol surface was not certain, a proposed mechanism for an ether linkage between the benzene ring and the silicon was offered. This mechanism is not at all satisfying, and can be justified only by hypothesizing a hydrophobic atmosphere surrounding the ether linkage. Although an alternate mechanism was proposed in that paper for a silicon-to-nitrogen bond, this mechanism was discarded as not being very likely. Another alternative mechanism, which was not cited in that paper, is the possibility that the diazonium was

converted to an amine during the adsorption process. To ascertain whether an amine of structure similar to that of the diazonium salt employed in the previous paper would bond permanently to a silanol surface, *o*-dianisidine was chosen. Preliminary studies with this molecule indicated that it was bound permanently to the silanol surface.

Although various lactases have been immobilized—i.e., yeast lactase on polyacrylamide polymer (4), *Escherichia coli* lactase on glass (5), and both yeast and fungal lactase on glass (6)—none of these preparations was remarkably active or stable. The enzymes that were immobilized on glass (5,6) employed the traditional silane coupling techniques, followed by functionalization with glutaraldehyde. We offer the following not as a competitive procedure, but rather as an alternative for producing an immobilized lactase.

MATERIALS AND METHODS

The *Aspergillus niger* enzyme, lactase-LP, was purchased from Wallerstein Laboratories. Controlled-pore silica, a Corning Glass Works product previously described (3), had an average pore diameter of 425 Å, a minimum pore diameter of 270 Å, a maximum pore diameter of 475 Å, a surface area of 40 m^2/g, and mesh size of 40/80.

The sources of reagents, including their grades, were: *o*-dianisidine (3,3'-dimethoxybenzidine), practical, Eastman Organic; $NaNO_2$, Baker Analyzed reagent grade; glutaraldehyde, practical, J. T. Baker. The lactose employed for the shaker bath assays was reagent-grade lactose obtained from ICN Pharmaceuticals, Inc. The lactose utilized in the column studies was a commercial grade of milk sugar sold under the designation of 30-S by Humko Sheffield. All other reagents employed in this study were of reagent grade.

Preparation of o-Dianisidine Silica Carrier. A 100-g quantity of controlled-pore silica was transferred to a coarse 350-ml fritted glass funnel. A 200-ml volume of solution containing 5 g *o*-dianisidine and 4 ml concentrated HCl diluted to 500 ml with water was delivered to the funnel at a rate of 1000 ml/h. The cake was drained, and a fresh 200-ml aliquot of the *o*-dianisidine solution was pumped through the cake. The controlled-pore silica cake was then washed with 100 ml isopropanol. The wash was intensely colored. A second wash using 100 ml isopropanol was circulated through the carrier for 1 h, and proved to be less intensely colored than the first wash. The derivative was then washed with 300 ml water, followed by another 100 ml isopropanol, and finally with 100 ml acetone. The product was then air-dried on the funnel with aspiration for 1 h. All the final washes

were colorless, and the dried carrier derivative had a pale, purple-pink hue.

Preparation of Immobilized Lactase for pH Profile Studies. A 1-g quantity of *o*-dianisidine silica carrier was transferred to each of two 9 × 150-mm water-jacketed columns. To prepare the diazonium derivative, 15 ml of a solution containing 375 mg $NaNO_2$ in water was circulated through the columns at 400 ml/h for 30 min. To prepare the glutaraldehyde derivative, 15 ml 2.5% glutaraldehyde solution was circulated through the other column at a rate of 400 ml/h for 30 min at room temperature. Each column was then washed with 150 ml water and drained.

The immobilized enzymes were prepared by circulating 15 ml of an aqueous solution containing 225 mg lactase (2520 lactase U) through each column at a rate of 400 ml/h for 20 h at room temperature. During this period, the direction of flow was changed twice, starting and terminating with an upward flow. The enzyme solutions were then removed from each column and retained separately for future assay. The immobilized enzymes in the columns were washed with 40 ml 0.5 M sodium chloride by employing an upward flow of solution. The wash from each column was combined with the previously retained enzyme solution from the same column. Each column was then washed with 150 ml water, which was then combined with each of the enzyme solutions and washes. Immobilized enzymes were then removed from the columns and retained for assay and pH studies.

Table 1 is a record of the enzyme balance for the diazonium and the glutaraldehyde immobilized enzyme IME preparations.

Preparation of Immobilized Enzyme for Continuous-Column Reactor Performance. A 2-g quantity of *o*-dianisidine silica carrier was transferred to a 9 × 150-mm water-jacketed column. A 30-ml volume of aqueous 2.5% glutaraldehyde solution was circulated through the carrier on the column at room temperature at a rate of 530 ml/h in a downward flow pattern for 10 min. At 10-min intervals, the direction of flow was reversed twice for a

TABLE 1. **Enzyme Balance for the Preparation of IMEs, Assayed at pH 4.5, 50°C, with Lactose As the Substrate**

	Diazonium IME	Glutaraldehyde IME
Enzyme offered	2520 U	2520 U
Recovered enzyme and wash solutions	600 U	820 U
IME activity	335 U/g	433 U/g
Total recovered enzyme activity	39%	52%
Coupling efficiency	17%	25%

total of 30 min of reaction with glutaraldehyde. The excess glutaraldehyde was removed from the column, and the carrier was washed with downward flow with 1 liter water.

The enzyme solution, 500 mg lactase diluted to 30 ml with water (5619 U lactase activity at pH 3.5), was circulated downward through the column at 530 ml/h at room temperature for 17 h. The enzyme solution was then removed from circulation, and the IME was washed with 100 ml water at a flow rate of 70 ml/h. This wash was followed by a 50-ml wash with 0.5 M sodium chloride, and finally with 20 ml water. The total volume of reacted enzyme solution and washes was 200 ml. This combined solution was saved for analysis and enzyme recovery determination.

The IME was evaluated in the column utilized for preparing the IME. This evaluation proceeded immediately without removal of the preparation from the column.

The enzyme activity recovery and coupling efficiency for this preparation are recorded in Table 2. The activity determination for the IME was performed by employing the in-column analysis utilizing a 5% lactose solution adjusted to pH 3.5 with HCl. This determination represents only the activity recorded during the first day of operation.

Assay Procedure. The substrate utilized for the soluble enzyme was 600 mM lactose in 12 mM acetate (Na^+) buffer, pH 4.5. After addition of the enzyme, the final concentration of substrate was 500 mM, and that of the acetate was 10 mM. These final concentrations of substrate and buffer were employed for the IME assay.

The specific conditions for the assays were as follows: 4 ml of substrate for the soluble enzyme (25 ml for the IME) was placed in a 25-ml Erlenmeyer flask (125 ml for the IME) and brought to temperature by immersing the flasks in a 50°C shaking water bath. Either 1 ml of soluble enzyme (approximately 10 μg/ml) or the IME sample, which contained 25–50 mg enzyme by dry weight, was added to the substrate. The reaction mixture was incubated at 50°C for 30 min in the case of the soluble enzyme, and 10 min for the IME. Heat-inactivated enzyme controls were included for the soluble enzyme assay. At the end of the incubation period, the soluble enzyme reaction was terminated by placing the sample in a boiling water

TABLE 2. **Activity Recovery and Coupling Efficiency for IME**

Enzyme offered	5619 U
Recovered enzyme in original solution and wash	1570 U
IME activity	620 U/g
Recovered enzyme activity	50%
Coupling efficiency	31%

bath for 5 min. The IME assay reaction was terminated by removing 1 ml substrate from the reaction mixture and transferring that sample to 9 ml water. This dilution was necessary to correspond to the range of the glucose determination. After the removal of the substrate for IME determinations, the dry weight of the IME was determined.

Glucose determinations were performed by utilizing either the Glucostat kit (Worthington Biochemical Corporation) or the Tris-glucose oxidase reagent described by Dahlqvist (7). A glucose standard (100 μg/ml) was included with every assay.

One unit of lactase activity is defined as the production of 1 μmol glucose/min at 50°C under the pH conditions of the assay.

All assay values in this report were obtained by incubating duplicate samples in lactose, which were further analyzed in duplicate for glucose content. In other words, each value is the average of 4 glucose determinations.

The Wallerstein lactase LP was assayed at pH 4.5 under the conditions described previously, and was found to contain 11,238 lactase U/g.

Column Reactor. The water-jacketed column, containing 2 g immobilized lactase previously described, was fed with a 5% lactose solution prepared by dissolving 200 g lactose in 3800 ml water and adjusting to pH 3.5 by adding approximately 1.3 ml 2 M HCl. The column was maintained at 50°C by a circulating water bath. The substrate feed rate to the column was maintained between 49 and 71 ml/h. The column and pump were washed twice a week with 100 ml 0.17 M acetic acid to remove growth within the pump and the column. The column was operated continuously for approximately 54 days, with the exception of the time that it was washed.

Samples of the product were collected periodically, the glucose content was determined with the Glucostat kit, the percent conversion of lactose was calculated, and the procedure of Weetall et al. (6) was employed for ascertaining column activity.

pH Profile Studies. The pH–activity relationships for the enzyme immobilized with glutaraldehyde and the enzyme immobilized with diazonium were studied in the shaking bath assay at 50°C. The buffer employed was 0.1 M Tris adjusted to pH with 0.1 M acetic acid.

RESULTS

pH Profile

Our intent in this study was to compare the *o*-dianisidine–glutaraldehyde- and the *o*-dianisidine–diazonium-coupled lactase preparations with respect to activity over the pH range for acid whey hydrolysis. The

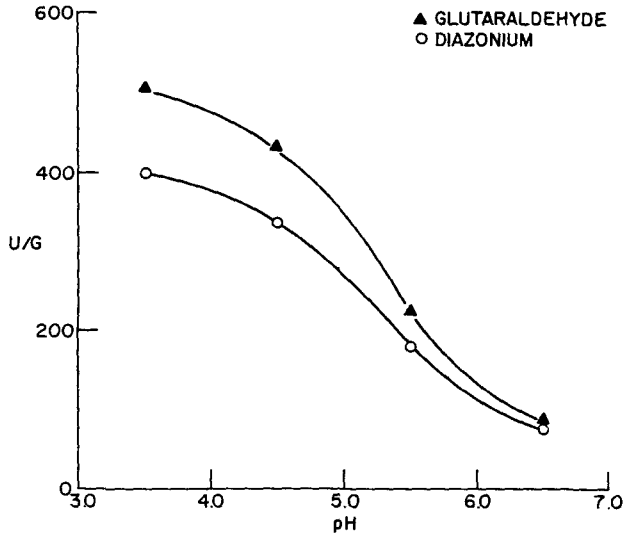

FIG. 1. pH profile for silica–*o*-dianisidine–glutaraldehyde lactase and silica–*o*-dianisidine–diazonium lactase at 50°C.

results recorded in Fig. 1 indicate that the *o*-dianisidine–glutaraldehyde lactase exhibits a higher activity over the pH range between 3.5 and 6.5. Since the pH optimum for these preparations appeared to be close to 3.5, we decided to operate our column reactor at that pH.

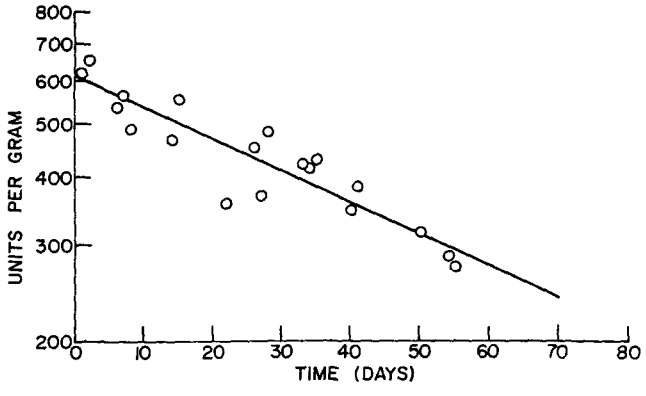

FIG. 2. Continuous-reactor performance of silica–*o*-dianisidine–glutaraldehyde-coupled *A. niger* lactase at 50°C employing a 5% lactose feed at pH 3.5.

Continuous-Reactor Half-life

The data obtained from the column performance of the *o*-dianisidine–glutaraldehyde-coupled lactase are plotted in Fig. 2. The half-life of the column and the initial enzyme loading were calculated from a linear regression of ln E vs. time. The initial loading of the enzyme, E_0, from the column performance, was estimated to be 609 U/g. The half-life of the column at 95% confidence limit was found to be 52 days with an LCL of 43 days and a UCL of 65 days.

DISCUSSION

The continuous-reactor performance of the silica–*o*-dianisidine–glutaraldehyde-coupled lactase indicates that we have achieved both satisfactory loading of the enzyme and a reasonable half-life. We do not mean to indicate that this system has been optimized with respect to either the coupling agent or the enzyme; however, the indications are that this system warrants further optimization and investigation for acid whey hydrolysis.

The prime objective of this study was the investigation of aromatic amine bonding with silanol surfaces. Although we are not at all certain of the mechanism by which the *o*-dianisidine is attached to the surface of either silica or glass, we believe that the most likely mechanism is the formation of an ionic amine bond at the silanol surface, as indicated in Fig. 3. In our preliminary studies, we attempted to remove *o*-dianisidine from the surface of glass by eluting with 0.5 M sodium chloride, boiling water, and urea. None of these solutions was capable of removing the attached *o*-dianisidine from the glass or silica surface.

The issue as to whether an ether linkage is formed by reacting the diazonium salt with silica, as reported previously (3), has not been clarified by this study; however, we now have an alternate mechanism to offer as a possible candidate, namely, the ionic bond. A single nitrogen may have been hydrolyzed from the diazonium salt, resulting in an amine terminal group

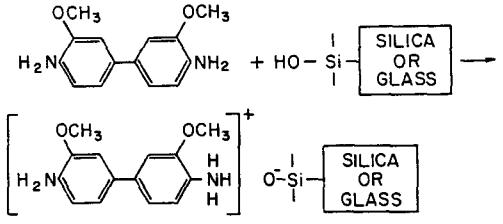

FIG. 3. Proposed mechanism for the bonding of *o*-dianisidine to the surface of silica or glass.

that formed an ionic bond on the silanol surface; however, it is doubtful that the bonding is the same, since the methoxy group in the previous study (3) was in the *meta* position, while in the current study it is *ortho*.

There is a dramatic difference between the silane bonding and the aromatic amine bonding. The silane traditionally employed, γ-aminopropyltriethoxysilane, is capable of polymerization. Thus, in fact, a polymer surface with localized concentrations is to be found under certain conditions of applications on the glass surfaces. This polymerization is due to the reaction between the amine and the silanols of the molecule. The polymerization may or may not be of advantage when enzymes are bound to the surface. The *o*-dianisidine does not readily polymerize; therefore, in all probability, this molecule is deposited as an individual entity on the surface of the glass or silica, and reacts on a one-to-one basis with the surface silanol. Again, this does not imply that there is an advantage in employing either of these coupling agents, but the differences may become more apparent when more enzymes are investigated. There is a further difference to be noted between the silane and the *o*-dianisidine, in that there is a major contribution of aromatic structures with the *o*-dianisidine, while the contribution of the silane is primarily aliphatic. This, again, may or may not have an effect on specific enzymes and their enzyme substrate reactions. Further characterization and comparative studies must be performed to clarify these issues, and to ascertain where it is advantageous to employ one system in preference to the other. It is fairly certain, on the basis of our previous studies, that each will find its advantage with particular enzyme systems.

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