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-
porosity 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 continu-
ous 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 mechan-
ism was proposed in that paper for a silicon-to-nitrogen bond, this mechan-
ism was discarded as not being very likely. Another alternative mechanism,
which was not cited in that paper, is the possibility that the diazonium was

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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 Wal-lerstein 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 m²/g, and mesh size of 40/80.

The sources of reagents, including their grades, were: o-dianisidine (3,3'-dimethoxybenzidine), practical, Eastman Organic; NaNO₂, 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