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BONE AS A SOLID SUPPORT FOR THE IMMOBILIZATION OF ENZYMES

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SUMMARY

Poultry bone residue was found to serve as a solid support matrix to which catalase, pepsin, pectinase, lactase and invertase could be insolubilized by covalent attachment and adsorption. Bone has great potential for enzyme immobilization since it is inexpensive, abundant, chemically functional, porous, non-toxic and mechanically strong.

INTRODUCTION

Despite the obvious technological advantages, the adaptation of immobilized enzyme systems to commercial food processes has been limited due to the cost of capital equipment and raw materials, instability of biocatalysts and the inability of many common support materials (alginates, agarose and polyacrylamide gels) to withstand high flow rates in continuous reactors (1). Solid supports that can withstand higher pressures, have difficulty handling fluid suspensions with particles larger than the pores (eg. fluid milk) without extensive fouling (2). Animal bone possesses many of the characteristics desired in a support matrix for immobilization. Bone is a strong porous material composed of relatively inert hydroxyapatite crystals imbedded in a stable protein matrix. This material is food grade, non-toxic, abundant and is an inexpensive product of mechanical separation of meat from

animal carcasses. These properties show promise in overcoming many of the industrial limitations confronting the development of commercial immobilized enzyme processes. The intent of this study was to assess the feasibility of using clean granular bone for this purpose.

MATERIALS AND METHODS

Materials: Bovine liver catalase, A. niger pectinase, porcine stomach pepsin, C. utilis invertase, cysteine-HCl, O-nitrophenyl-D-galactopyranoside (ONPG), C. histolyticum collagenase, and citrus fruit pectin were obtained from Sigma Chemicals (St. Louis, MO). Lactozym(TM) 3000L type HP (lactase) was a generous gift from Novo Industries (Denmark) and 2-cyanoacetamide was obtained from Aldrich Chemical (Milwaukee, WI). All other chemicals were reagent grade. Skim milk powder was a commercial product of Willow Grove Dairies (Toronto, ON). Clean granular chicken bone, which had been previously stripped using hot aqueous NaOH followed by washing and sizing to between 10 and 20 mesh, was supplied by Protein Foods Research (Guelph, ON). The wet bone was found to be 66% dry matter.

Enzyme Activity: Catalase was measured by the initial rate of oxygen evolution in the presence of 0.5 mM hydrogen peroxide in 0.5 M citrate-phosphate buffer (pH 5.0) using an oxygen electrode. Pectinase and invertase activities were measured by reducing group evolution using the 2-cyanoacetamide method (3). Milk clotting activity of the pepsin preparations was measured by timing the initial curd development of reconstituted skim milk 1:10 in 0.2 M acetate buffer (pH 5.8). Lactase (ß-galactosidase) activity was measured for ONPG (4). One unit of activity results in 1 µmol of substrate being reacted per minute at 25°C. All determinations were performed in duplicate.

Immobilization: Adsorption was achieved through the addition of the enzyme in buffer to the clean dry bone followed by incubation in vacuo for 1 hour at 0°C. The excess enzyme was removed by exhaustive washing with buffer prior to determination of enzymic activity. Glutaraldehyde derivatization (2% GHO at pH 5.5 to 6.5) with and without silanization was achieved as previously described (5). Collagenase pretreatment was accomplished by incubating bone in 0.2% collagenase - 0.2 M phosphate buffer (pH 7.0) for 8 hours at room temperature. Acyl-azide derivatization was carried out as previously described (6), except methylation was omitted. Carbodiimide derivatization was carried out by methods described elsewhere (7). Following derivatization, the enzyme was coupled using conditions employed for simple adsorption. The buffers used for coupling were as follows: catalase, 0.1 M phosphate-citrate (pH 7.0); invertase and pectinase 0.05 M acetate (pH 4.4); lactase, 0.2 M phosphate (0.2 M phosphate (pH 6.5) with 2 mM magnesium chloride and 5 mM cysteine; pepsin, 0.5 M citrate (pH 4.2).

RESULTS

The results of enzyme coupling to dry bone using the various techniques are presented in Table 1. The two most effective methods were adsorption and covalent coupling by acyl-azide derivatization. Catalase immobilization by acyl-azide derivatization was slightly superior to that by direct adsorption,

whereas the opposite appeared to be true for lactase. The observation that covalent crosslinking by glutaraldehyde (GHO) was ineffective (except for pectinase) is not surprising. GHO crosslinking requires free amino groups which are not abundant in the protein constitutents (largely collagen) of bone. It would be expected that immobilization methods that make use of carboxylic acid and hydroxyl functional groups would be more effective. This accounts for the relative success of acyl-azide derivatization. The acyl-azide method has previously been shown to be effective for coupling enzymes to collagen films (6). While there is not an abundance of free amino groups in bone, the effectiveness of GHO crosslinking (for lactase) could be enhanced by a preliminary derivatization step of silanization or by initially treating dried bone with collagenase, thereby liberating free amino groups.

Pectinase was the only enzyme observed to be more effectively immobilized by GHO coupling than the other methods explored. A limitation of the bone immobilized pectinase was gelling of the citrus pectin substrate about the bone matrix when assaying the preparation for activity. This may have been due to the high levels of calcium in bone or possibly pectinmethylesterase contamination of the stock enzyme and its presence on the immobilized catalyst.

Invertase provided the highest recoverable activity on the bone matrix. The acyl-azide technique was clearly superior to the other methods evaluated. Coupling by GHO and carbodiimide were also superior to simple adsorption.

With the exception of invertase, the low yields of activity observed for the immobilization of the enzymes to bone may be indicative of a relatively small number of functional sites available. However, the availability and economy of the granular bone matrix offers many advantages over conventional support materials. The process for preparing clean granular bone produces a sterile product that is non-toxic, porous and mechanically strong. Further studies into the application of bone in a continuous reactor system have shown that immobilized pepsin could clot 100 bed volumes of milk without loss of throughput or curd yield (unpublished results). This exemplifies the suitability of a granular bone support matrix for the continuous processing of milk, a colloidal fluid food material which chronically fouls conventional support materials.

Clean granular bone has many of the desired characteristics for immobilization of enzymes for continuous processing of fluid food and material systems. This material has also been successfully applied to the

immobilization of cells (unpublished data). Although this report represents a preliminary investigation into the potential uses of granular bone as a support matrix, the results are encouraging and further investigation is warranted.

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Table 1. Immobilization of enzymes to bone by various methods

Enzyme	Immobilization Method	Initial Enzyme Activity in Immobilizing Solution (U)	Activity Immobilized on Bone (U/g)
Invertase	Adsorption	143	2.3
	Acyl-Azide	104	38
	Glutaraldehyde	64	4.4
	Carbodiimide	28	1.0
Pepsin	Adsorption	43	0.68
	Acyl-Azide	38	0.63
Pectinase	Acyl-Azide	28	0.11
	Glutaraldehyde	32	0.27
	Carbodiimide	30	0.10
Lactase	Adsorption	12	0.12
	Acyl-Azide	12	0.11
	Glutaraldehyde	12	0.03
	Silanized GHO	24	0.10
	Collagenase GHO	24	0.16
Catalase	Adsorption	128	0.60
	Acyl-Azide	128	0.88