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Glyoxylic Acid Production Using Immobilized Glycolate Oxidase and Catalase

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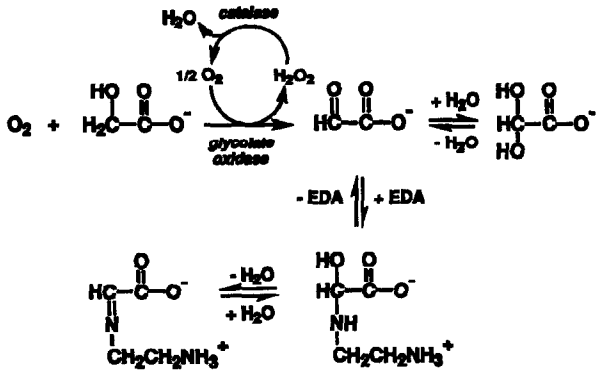
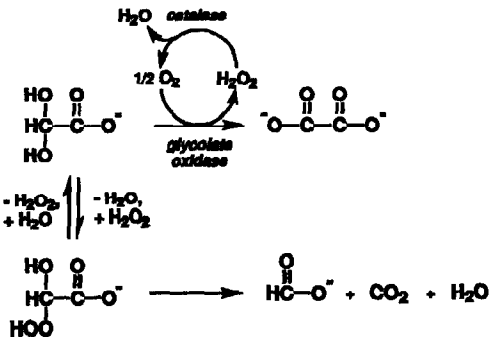
Abstract—A variety of methods for the immobilization of glycolate oxidase have been examined for the preparation of a catalyst for the oxidation of glycolic acid to glyoxylic acid. The co-immobilization of glycolate oxidase and catalase on oxirane acrylic beads produced a catalyst which was stable to the reaction conditions used for the oxidation, where glycolic acid and oxygen are reacted in aqueous solution in the presence of the immobilized enzyme catalyst and ethylenediamine. Under optimum reaction conditions, 99 % yields of glyoxylic acid were obtained at greater than 99 % conversion of glycolic acid, and the recovery and reuse of the co-immobilized enzyme catalyst was demonstrated.

Introduction

Although the enzyme-catalyzed oxidation of glycolic acid (hydroxyacetic acid) by oxygen has been known for many years,[1–5] high selectivities (> 99 %) to glyoxylic acid had not been previously obtained, and the oxidation of glycolic acid had been typically performed at glycolate concentrations of less than 50 mM. We have recently described a process (Scheme I) for the oxidation of 0.25–1.5 M solutions of glycolic acid to glyoxylic acid in the presence of oxygen, ethylenediamine (EDA), and the soluble enzymes glycolate oxidase (EC 1.1.3.15) and catalase (EC 1.11.1.6).[6] An unexpected synergistic effect was observed when using both a catalase (to destroy byproduct hydrogen peroxide) and an amine capable of reacting with glyoxylic acid to produce a mixture of the corresponding hemiaminal and imine (which limited further enzymatic oxidation of glyoxylic acid to oxalic acid, or its chemical oxidation to formate and carbonate by byproduct hydrogen peroxide; Scheme II). Neither the separate addition of catalase or EDA were found to produce the high selectivity to glyoxylic acid observed when both were present, and the almost quantitative yields obtained were greater than expected from a simple additive effect of using catalase or EDA alone. An improvement to the above process is now described, where an immobilized enzyme catalyst has been substituted for the soluble enzymes.



Scheme I.



Scheme II.

Results

Immobilization of glycolate oxidase and catalase

Protein crosslinking agents such as glutaraldehyde,[7] dimethyl adipimidate,[8] and dimethyl suberimidate,[8] were examined for their ability to crosslink multiple subunits of glycolate oxidase, and to determine the effect of intramolecular and intermolecular subunit crosslinking on enzyme activity and stability. Treatment of glycolate oxidase with glutaraldehyde at a pH of 5.0–10.0 indicated a major loss of enzyme activity above pH 7.0, and SDS gel electrophoresis of the glutaraldehyde-treated protein indicated no formation of crosslinked multiple subunits (data not shown). When using dimethyl suberimidate or dimethyl adipimidate, multiple-subunit crosslinking (formation of dimers, trimers and tetramers) increased with increasing pH (from pH 8.0 to 10.0) of the crosslinking reaction, but enzyme activity of the crosslinked protein decreased markedly with increasing multisubunit crosslinking.

Table 1 lists the immobilization yield (percentage of immobilized protein which still maintained enzymatic activity), specific activity and method of immobilization (physical adsorption, ionic binding, or covalent attachment) for a variety of solid supports evaluated for the preparation of immobilized glycolate oxidase. Physical adsorption of glycolate oxidase on to hydrophobic adsorption supports did not result in appreciable yields of immobilized enzyme activity. Benzylated poly(ethylenimine) (PEI) on silica gel, Amberlite® XAD-4 and XAD-8 (macroreticular polystyrene), and phenyl agarose irreversibly adsorbed glycolate oxidase from buffered aqueous solutions, but the yield of enzymatically-active immobilized protein was generally low (0–4 %), and the immobilized enzyme activity decreased with time upon storage in buffer at 5 °C. The physically-adsorbed enzyme also rapidly desorbed from these supports in reaction mixtures containing 0.75 M glycolic acid and 0.79 M EDA at pH 9.0.

Table 1. Immobilization of glycolate oxidase on solid supports

Immobilization support	Immobilization yield (%)[a]	Immobilized specific activity[b]	Immobilization type
PEI/silica gel (benzylated)	0	0	physical
Amberlite® XAD-4	0	0	physical
Amberlite® XAD-8	4	0.76 IU/g	physical
phenyl agarose	3	0.62 IU/g	physical
Bio-Rex® 70	0	0	ionic
CH Sepharose® 4B	0	0	ionic
CPG-120 glass beads	16	20.0 IU/g	ionic
CPG-240 glass beads	19	21.0 IU/g	ionic
Celite® R-650	7	9.7 IU/g	ionic
Celite® R-640	6	12.1 IU/g	ionic
CNBr-4% agarose	20	5.0 IU/mL	covalent
CNBr-Sepharose® 4B	25	4.3 IU/mL	covalent
CNBr-Sepharose® 6MB	44	0.68 IU/mL	covalent
PAN-500	19	0.14 IU/mL	covalent
Emphaze™ azlactone beads	10	6.1 IU/mL	covalent
Eupergit® C oxirane acrylic beads	17	7.8 IU/g	covalent
Eupergit® C250L	8	5.0 IU/g	covalent
Eupergit® C1Z	0	0	covalent
epoxy-activated 6% agarose	3	0.14 IU/g	covalent
epoxy-activated Sepharose® 6B	2	0.14 IU/g	covalent
PEI/silica gel/glutaraldehyde	0	0	covalent

[a]yields based on percentage of immobilized protein that retains enzymatic activity. [b]IU (International Unit) is the quanitity of enzyme which converts 1 μmol/min of substrate under standard assay conditions; specific activity is equal to IU per weight or volume of immobilized enzyme.

At a pH less than the isoelectric point (pI) of glycolate oxidase (pI > 10.25), the protein has a net positive charge, and weakly acidic cation exchangers (CH Sepharose® 4B, Bio-Rex® 70) adsorbed the protein, but the adsorption was reversible and the protein was easily washed from the support. Controlled-pore glasses (CPG) are available in a variety of pore diameters, and have a slight negative charge in aqueous solutions due to the presence of surface hydroxyl groups and Lewis acid sites which preferentially bind proteins with a high pI.[9,10] The immobilization of glycolate oxidase (ca 4 % w/w) on CPG having nominal pore diameters of 1000 Å, 500 Å, 240 Å, and 120 Å resulted in decreasing specific activities of enzyme when using a nominal pore size > 500 Å; the specific activity on CPG-1000 was typically half that obtained on CPG-120 or CPG-240 at a similar protein loading. The best CPG support, CPG 240-200 (200 micron beads, 24 nm pore size), produced a catalyst which had a specific activity of 21 IU glycolate oxidase/g CPG beads. This immobilized enzyme activity was not stable upon storage in buffer, and in reaction mixtures the protein slowly desorbed from the support.

Diatomaceous catalyst supports (Celite® catalyst carriers)[11] have properties similar to those of CPG, therefore a variety of controlled-pore diatomaceous catalyst supports were screened for glycolate oxidase immobilization via ionic binding. Supports having nominal pore diameters of 3800 Å, 600 Å, 500 Å and 300 Å were examined, and optimum specific activities of immobilized glycolate oxidase were obtained using Celite® R-640 (300 Å pore diameter, 87 % SiO_2) and R-650 (500 Å pore diameter, 85 % SiO_2). The ionically-bound enzyme was stable upon storage in bicine buffer at 5 °C; after 48 h, the recovered glycolate oxidase activities on R-640 and R-650 were 98 and 92 %, respectively. The adsorbed enzyme was not stable in 0.25 M EDA at pH 9.0; after 4 h, only *ca* 40 % of adsorbed glycolate oxidase activity remained.

Covalent attachment of the protein to oxirane acrylic beads[12] (Eupergit® C and C250L), cyanogen bromide-activated agarose or Sepharose®,[13] and bis-acrylamide/azlactone copolymer beads[14] (Emphaze™) produced immobilized enzymes having useful specific activities, but only catalysts prepared using oxirane acrylic beads proved to be suitable for the present application. Supports which relied upon immobilization via the formation of ester bonds between the support and protein-produced catalysts which were unstable to the reaction conditions employed for the enzymatic oxidation. Immobilized enzyme catalysts prepared using cyanogen bromide-activated agarose or Sepharose® reacted with the EDA in reaction mixtures, releasing the covalently-bound enzyme from the support into the surrounding solution (determined by enzymatic assay). Glycolate oxidase immobilized on azlactone beads retained only 48 % of its initial activity when placed in 0.25 M EDA for 6 h (pH 8.3, 25 °C), compared to a 95 % recovery when maintained in 0.1 M bicine buffer under the same conditions. The specific activity of the enzyme attached to PAN-500 poly(acrylamide-co-*N*-acryloxysuccinimide) gel crosslinked with triethylenetetramine[15] was too low to be useful as a practical catalyst in the reaction. Covalent attachment of glycolate oxidase to PEI/silica gel with glutaraldehyde[16] resulted in complete loss of enzyme activity.

Immobilization of glycolate oxidase via reaction of nucleophilic groups on the protein surface (amine, hydroxyl, thiol groups) with epoxy groups on a support surface produced an immobilized enzyme stable in glycolate/EDA reaction mixtures. Oxirane acrylic beads Eupergit® C (150 μm particle size, 40 nm average pore size, 5 atom spacer linkage) and Eupergit® C250L (250 μm particle size, 160 nm average pore size, 5 atom spacer linkage), and epoxy-activated Sepharose® or agarose (12 atom spacer linkages), were examined for their ability to immobilize glycolate oxidase. Immobilization yields were optimized by varying buffer, buffer concentrations, pH, temperature, reaction time, protein loading and surface derivatization prior to or post-immobilization. Protein

loadings of from 0.02 to 5 % were examined, and immobilization yields were observed to decrease with increasing protein loading. The best immobilization yields on Eupergit® C were in the range of 15–20 % immobilized enzyme activity at a protein loading of *ca* 1 % (weight/weight of support), with 90–100 % protein immobilization. Screening of a variety of reaction conditions for the immobilization of glycolate oxidase on porous oxirane acrylic beads did not produce major increases or decreases in the specific activity of the immobilized enzyme at optimum protein loadings. By comparison, epoxy-activated agarose or Sepharose gave 0–3 % yields at 20–30 % protein immobilization under similar conditions. Immobilization of glycolate oxidase on non-porous oxirane acrylic beads (Eupergit C1Z, 1 μm particle size, 5 atom spacer linkage) produced no measurable enzyme activity at protein loadings similar to that used for the porous beads.

An immobilized enzyme catalyst for the oxidation of glycolic acid to glyoxylic acid in the presence of a stoichiometric amount of EDA was prepared by co-immobilizing spinach glycolate oxidase and *Aspergillus niger* catalase on porous oxirane acrylic beads; Eupergit® C was used for all co-immobilizations. The best co-immobilization yields of catalase on this support were in the range of 15–20 % at a catalase loading of 1 % (w/w); the specific activity was typically 6000–8000 IU/g support. Co-immobilization of catalase and glycolate oxidase at 1 % protein loadings, respectively, only slightly decreased the immobilization yield and specific activity of glycolate oxidase when compared to that obtained in the absence of catalase. The stability of the co-immobilized enzyme activities was checked by storing the catalyst in 0.1 M bicine/0.02 mM flavin mononucleotide (FMN) buffer (pH 8.3) at 5 °C and monitoring the enzyme activities with time (Figure 1). The stability of unimmobilized (soluble) glycolate oxidase under similar storage conditions was poor; only 50 % activity remained after 24 h, and no activity was measured after 48 h. The stability of glycolate oxidase precipitated in 3.2 M ammonium sulfate/2.0 mM FMN (pH 8.3) and stored at 5 °C was also monitored; 85 % of the original activity remained after six months.

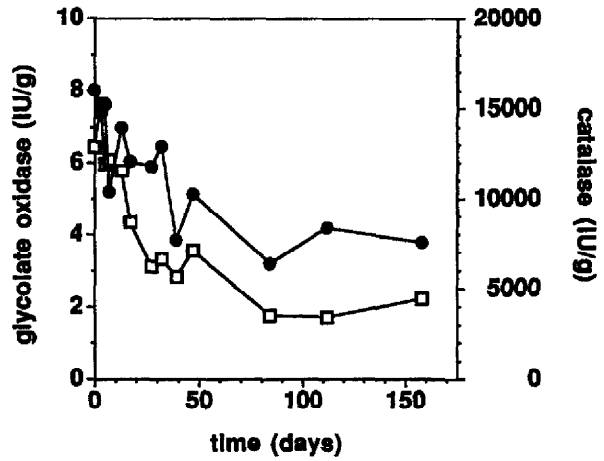


Figure 1. Stability of spinach glycolate oxidase (●) and *Aspergillus niger* catalase (□) co-immobilized on oxirane acrylic beads and stored in bicine (0.10 M)/FMN (0.02 mM) buffer at pH 8.3 and 5 °C.

The pH dependence of the enzyme activities of spinach glycolate oxidase and *A. niger* catalase when co-immobilized on oxirane acrylic beads were determined and are illustrated in Figure 2. A comparison of the pH dependence of the activities of the immobilized enzymes indicated little or no change from those reported for soluble spinach glycolate oxidase[4] or *A. niger* catalase.[17]

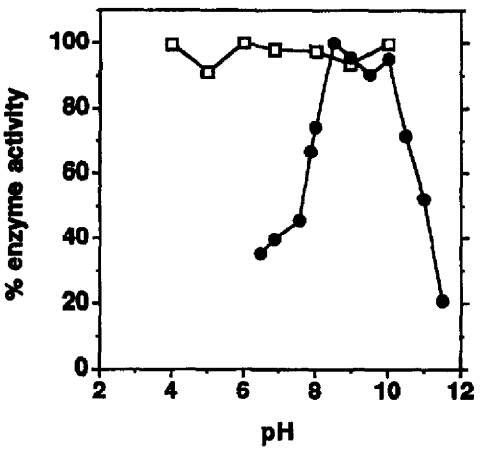


Figure 2. Dependence of enzyme activity on pH for spinach glycolate oxidase (●) and *Aspergillus niger* catalase (□) co-immobilized on oxirane acrylic beads.

Glycolic acid oxidation using immobilized enzymes

The stability of the co-immobilized enzyme catalyst to oxygen sparging was demonstrated by sparging an aqueous mixture containing glycolic acid, EDA, FMN, and, as catalyst, glycolate oxidase and catalase co-immobilized on oxirane acrylic beads (Figure 3). After 5 h, the yields of glyoxylate, oxalate and formate were 99.3, 0.3 and 0 %, respectively, at 100 % conversion of glycolate; the recovered activity of immobilized glycolate oxidase was 95 %. Repeating the reaction, but using the same amounts of soluble, unimmobilized glycolate oxidase and catalase, the reaction stopped at 3 h after only 46 % conversion of glycolate to glyoxylate; only 2 % of the initial glycolate oxidase activity was detected in the product mixture.

Scale-up of the co-immobilized enzyme-catalyzed oxidation of glycolic acid was performed in a 300-mL stirred autoclave reactor, which allowed control of stirring rate, pressure, temperature and sparge rate of oxygen while under pressure. Reactions were typically performed at 15 °C using 100 mL of an aqueous solution of glycolic acid (0.75 M), EDA (0.86 M), FMN (0.01 mM), and propionic acid (75 mM) as HPLC internal standard; for reactions run in the absence of added internal standard, the EDA concentration employed was 0.788 M (1.05 mol EDA/mol glycolate). Operating in the temperature range of 5–15 °C resulted in maximum recovery of immobilized enzyme activity. The pH of the reaction mixture usually decreased by *ca* 0.5 pH units over the course of the reaction, so it was preferred to start the reaction near the upper end of the maximum activity pH range of glycolate oxidase and allow it to decrease as the reaction proceeded with no pH control. The initial pH of the reaction mixture was adjusted to

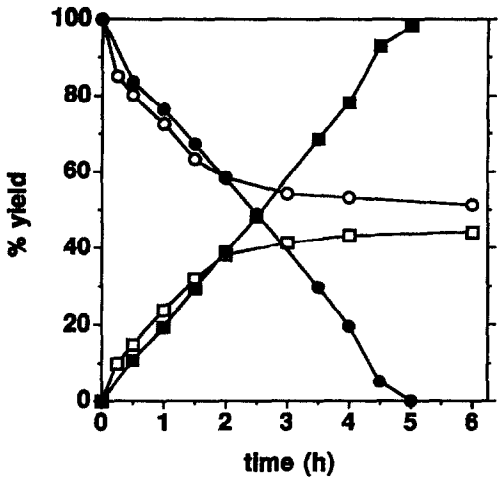


Figure 3. Time course for oxidations of glycolic acid (0.25 M) in aqueous EDA (0.33 M, pH 8.7)/FMN (0.2 mM) at 15 °C and with 1.0 mL/mL/min oxygen sparging at 0.10 MPa O_2, when using as catalyst: a) soluble glycolate oxidase (0.25 IU/mL) and soluble catalase (270 IU/mL) (glycolate, ○; glyoxylate, □), and b) glycolate oxidase (0.25 IU/mL) and catalase (270 IU/mL) co-immobilized on 0.5 g oxirane acrylic beads (glycolate, ●; glyoxylate, ■).

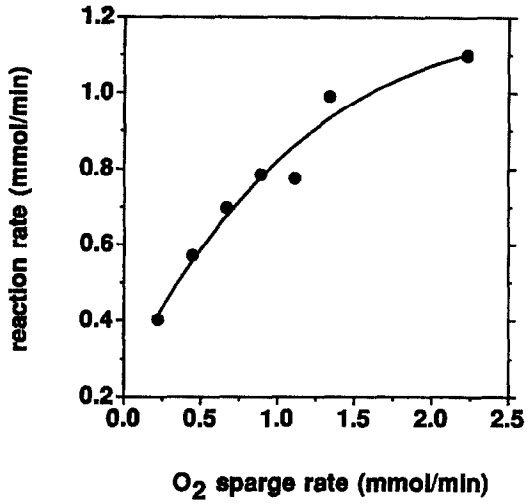


Figure 4. Reaction rate dependence on oxygen sparge rate for the oxidation of glycolic acid (0.75 M) in aqueous EDA (0.86 M, pH 9.2)/FMN (0.01 mM) at 15 °C and 0.58 MPa O_2 using glycolate oxidase (0.52 IU/mL) and catalase (950 IU/mL) co-immobilized on oxirane acrylic beads.

between 8.9 and 9.1 by the addition of ethylenediamine to the glycolate solution; no additional acid or base addition was required.

The minimum initial concentration of co-immobilized catalase employed in reaction mixtures to produce high selectivity to glyoxylic acid and high recovery of both immobilized glycolate oxidase and catalase activities was *ca* 350 IU/mL. Although the reaction could be performed by the separate addition of immobilized catalase and immobilized glycolate oxidase, co-immobilization limited the amount of solid catalyst added to the reaction. The ratio of catalase activity to glycolate oxidase activity when co-immobilized was usually at least 250:1.

Reaction parameters were varied to optimize catalyst concentration, recovery of both glycolate oxidase and catalase activity for reuse, reaction rate and number of catalyst recycles. Reaction rates were independent of stirring rates at or above 400 rpm when using the 300-mL stirred autoclave reactor. The dependence of reaction rate on the concentration of immobilized glycolate oxidase activity was approximately first order for reactions run with from 0.2 IU/mL to 0.8 IU/mL of co-immobilized glycolate oxidase at 0.58 MPa (70 psig) oxygen and sparged with oxygen at 0.2 mL/mL reaction volume/min. The effect of oxygen sparge rate on reaction rate at 0.58 MPa oxygen and 0.52 IU/mL of co-immobilized glycolate oxidase activity is illustrated by Figure 4. The dependence of reaction rate on oxygen pressure, either with no oxygen sparging or with oxygen sparging at 0.5 mL/mL/min, is depicted in Figure 5.

Using co-immobilized glycolate oxidase (80 IU) and *A. niger* catalase (72,600 IU) as a recyclable catalyst, the oxidation of 10 consecutive 100-mL volumes of 0.75 M glycolic acid was performed at 0.58 MPa oxygen (sparged

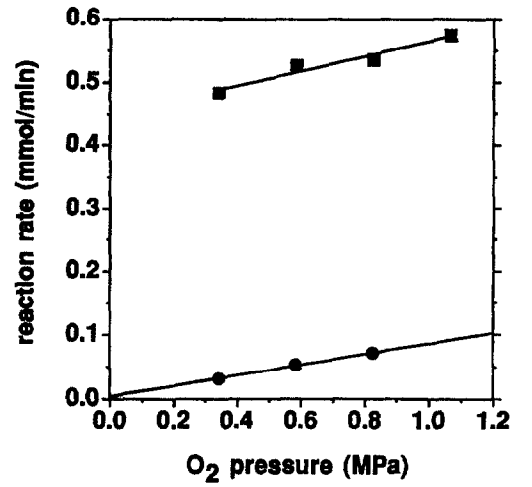


Figure 5. Reaction rate dependence on oxygen pressure for the oxidations of glycolic acid (0.75 M) in aqueous EDA (0.86 M, pH 9.2)/FMN (0.01 mM) at 15 °C, using glycolate oxidase (0.42 IU/mL) and catalase (420 IU/mL) co-immobilized on oxirane acrylic beads, without oxygen sparging (●), or with oxygen sparging (■) at 0.5 mL/mL/min and at the indicated pressure.

at 1.0 mL/mL/min) and at 15 °C (Figure 6). Over the course of the 10 reactions, the reaction time ranged from 2 h to 3 h, and the final enzyme activities of the co-immobilized catalase and glycolate oxidase were 100 and 85 %, respectively, of their initial values. The average yield of glyoxylic acid produced for the 10 reactions was 99 %. Lowering the concentration of immobilized glycolate oxidase below 0.8 IU/mL resulted in lower recoveries of immobilized glycolate oxidase activity after each run, and the reaction times for each consecutive run increased markedly after a fewer number of catalyst recycles.

A soluble enzyme hollow-fiber reactor and a fixed-bed immobilized enzyme reactor were each examined as a possible alternative to the stirred autoclave reactor described

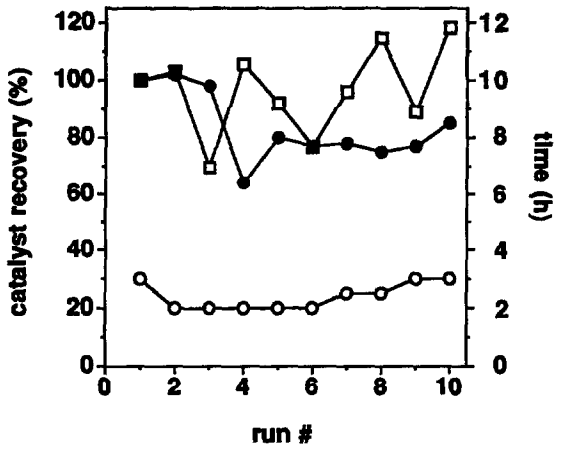


Figure 6. Reaction time (○), and recovery of glycolate oxidase (●) and catalase (□) activities, for the oxidation of glycolic acid (0.75 M) in aqueous EDA (0.86 M, pH 9.2)/FMN (0.01 mM) at 15 °C and 1.0 mL/mL/min oxygen sparging at 0.58 MPa O_2, for ten consecutive 100 mL batch reactions with catalyst recycle using glycolate oxidase (0.89 IU/mL) and catalase (726 IU/mL) co-immobilized on oxirane acrylic beads.

above. A 400-mL aqueous solution of 0.75 M glycolate reaction mixture was cooled to 15 °C, and oxygen was bubbled through this mixture at *ca* 100 mL/min. This oxygenated solution was then pumped through either a hollow-fiber reactor or a fixed-bed immobilized enzyme reactor. For the hollow fiber reactor, an Amicon H1 P30-20 (30,000 mw cutoff, 0.5 mm internal fiber i.d.) hollow fiber cartridge was employed. Soluble glycolate oxidase (66 IU) and *A. niger* catalase (357,000 IU) were contained inside the hollow fibers, and the oxygenated solution recirculated through the outer shell of the cartridge at 200 mL/min. For the fixed bed immobilized enzyme reactor, glycolate oxidase (14 IU) and catalase (56,000 IU) co-immobilized on oxirane acrylic beads (21 mL packed wet beads) were placed in a 24 mL jacketed chromatography column, and 15 °C coolant circulated through the column jacket. The reaction mixture was recirculated through the packed column at 40 mL/min. The hollow fiber reaction was completed in 550 h, and the recovery of glycolate oxidase and catalase was 30 and 18 %, respectively. The fixed-bed reaction was completed in 370 h, and the corresponding recovery of co-immobilized glycolate oxidase and catalase was 69 and 48 %, respectively.

Discussion

Spinach glycolate oxidase (*ca* 37 kDa) is a membrane-associated protein located in the peroxisomes of leaf cells.[4,5] The enzyme has been reported to be active only as homo-octamers or homotetramers in solution; dissociation of protein multimers and subsequent irreversible unfolding of the monomeric protein has been postulated to result in enzyme inactivation.[5] An X-ray crystallographic determination of the structure of octameric glycolate oxidase indicated that that the octamer has an approximate shape of a cube with a side length of *ca* 100 Å.[18] In the present study, the highest yields and specific activities for glycolate oxidase immobilization have been obtained using supports and immobilization methods which would favor the maintenance of the active tetrameric or octameric structure of the enzyme.

Porous supports capable of stable multipoint attachment of the octameric or tetrameric form of glycolate oxidase produced immobilized enzyme catalysts with the highest specific activities. CPG, diatomaceous catalyst carriers, oxirane acrylic beads and bis-acrylamide/azlactone copolymer beads having pore sizes two to five times the mean diameter of the octameric enzyme all produced immobilized enzyme catalysts having useful specific activities, whereas for the non-porous form of oxirane acrylic beads (C1Z in Table 1), a complete loss of enzyme activity was obtained at a comparable loading of protein on this support. A similar relationship between pore size and specific activity has been observed for several enzymes immobilized on CPG.[19] Cyanogen bromide-activated 4 % agarose and Sepharose® 4B (4 % agarose) gels also produced acceptable yields and specific activities of immobilized glycolate oxidase, while CNBr-Sepharose® 6MB (6 % agarose) produced a catalyst with a significantly lower specific activity; the lower activity on the 6MB support could be due to inefficient multipoint attachment of the protein tetramer (or octamer).

Immobilization yields for the porous oxirane acrylic beads were sensitive to the amount of protein loading on the support. Increasing the protein loading from 0.02 to 0.6 % (w/w) resulted in a decrease in the yield of immobilized active enzyme, although 100 % of the protein was bound to the support over this range of protein loading. The decrease in immobilization of active enzyme with increasing protein loading could be due to the need for multipoint covalent attachment of the tetrameric enzyme within the porous structure of the support; as the loading of the protein on the support increases, sites capable of binding an active enzyme tetramer may become fully occupied, and additional protein then binds to sites which do not result in the attachment of an active enzyme tetramer.

The previously reported[6] use of soluble glycolate oxidase and catalase as catalysts for the oxidation of glycolic acid to glyoxylic acid posed several problems: glycolate oxidase activity was not very stable in the aqueous reaction mixture, recovery of both glycolate oxidase and catalase from reaction mixtures for reuse was not easily performed, and the soluble enzymes were rapidly inactivated if a reaction mixture was sparged with oxygen. Co-immobilization of the two enzymes on porous oxirane acrylic beads has resulted in a catalyst which is stable in the reaction mixture and to the reaction conditions, and has an acceptable specific activity of both glycolate oxidase and catalase for use in this application. Catalase and glycolate oxidase could each be immobilized separately on oxirane acrylic beads and the two separate catalysts used together, but less immobilization support was needed when the two enzymes were co-immobilized, and less solids were added to reaction mixtures, thereby limiting catalyst attrition.

Many of the deficiencies of the soluble enzymes were eliminated by employing an immobilized enzyme catalyst

for the production of glyoxylic acid. The co-immobilized enzyme catalyst exhibited good stability and attrition-resistance under reaction conditions employing stirring and sparging with oxygen under pressure, and the yield of glyoxylic acid at complete conversion of glycolic acid was typically 98–99 %. Recovery and reuse of the co-immobilized enzyme catalyst was easily performed by filtering the catalyst from the reaction mixture and recycling it in consecutive batch reactions; in this manner, a total turnover number for co-immobilized glycolate oxidase of *ca* 1 x 10^7 was obtained after 10 cycles of the enzyme.

The ability to sparge oxygen through the reaction mixture without denaturing the enzyme catalyst resulted in a significant improvement in reaction rate compared to reactions run without sparging. The increase in reaction rate produced by increasing the rate of oxygen sparging indicated that the reaction was mass transport limited by oxygen dissolution into the reaction mixture. A 10-fold increase in the reaction rate at a given oxygen pressure was obtained by sparging a 100 mL reaction mixture with 50 mL/min of oxygen; performing the reaction under increasing oxygen pressure alone did not increase the reaction rate markedly when compared to the increase in rate obtained with oxygen sparging. An even larger difference in reaction rates for sparged and non-sparged reactions would be expected as one increases the reaction volume, where the increase in the ratio of reaction volume to surface area of the mixture would severely limit the rate of oxygen dissolution into the mixture in the absence of sparging. The need for efficient oxygen dissolution was also demonstrated by oxidations performed in fixed-bed co-immobilized enzyme or soluble enzyme, hollow-fiber reactors, where reaction rates were *ca* two orders of magnitude less than those obtained in the sparged, stirred autoclave reactor.

Experimental

General

Chemicals were purchased from Aldrich, Sigma or Baker and used as received. Spinach glycolate oxidase (Sigma) and *Aspergillus niger* catalase (Sigma) were used without any further purification; additionally, glycolate oxidase was isolated from fresh spinach leaves as previously described.[6] Samples for HPLC analysis were prepared by filtering an aliquot of a reaction mixture using a Millipore Ultrafree MC filter unit (10,000 mw cutoff). Analyses for glycolic acid, glyoxylic acid, oxalic acid and formic acid were run on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) at 50 °C, using propionic acid as internal standard and, as mobile phase, an aqueous solution of H_2SO_4 (0.010 N) and 1-hydroxyethane-1,1-diphosphonic acid (0.1 mM) at 1.0 mL/min. Soluble enzyme assays were performed as previously described.[6]

Poly(ethylenimine) (PEI) on silica gel and PEI on silica gel, benzylated (20–400 mesh beads, Aldrich), Amberlite® XAD-4 and XAD-8 (20–60 mesh, Sigma), phenyl agarose (crosslinked 4 % beaded agarose, Sigma), Bio-Rex® 70 (100–200 mesh, Bio-Rad), CH Sepharose® 4B (Pharmacia), CPG-120 and CPG-240 glass beads (120–200 mesh, Sigma), Celite® R-650 and R-640 (Manville), cyanogen bromide (CNBr)-activated 4 % agarose (Sigma), CNBr-activated Sepharose® 4B (Sigma), CNBr-activated Sepharose® 6MB (Sigma), Emphaze™ azlactone beads (3M), Eupergit® C, C250L, and C1Z oxirane acrylic beads (Röhm Pharma), and epoxy-activated 6 % beaded agarose and epoxy-activated Sepharose® 6B (Sigma) were commercially available and used as received, except for Celite® R-650 and R-640, which were obtained as 1/8 " pellets, ground using a mortar and pestle, and then sieved to a particle size range of 75–125 μm. PAN-500 poly(acrylamide-co-*N*-acryloxysuccinimide) gel crosslinked with triethylenetetramine) was prepared according to a literature procedure.[15]

Oxidation reactions performed under oxygen pressure were run in an Autoclave Engineers EZE-Seal 300-mL stirred autoclave reactor, which was equipped with a sparge tube and back pressure regulator to allow sparging of oxygen while performing a reaction under oxygen pressure. Oxygen flow rates were controlled using a Brooks mass flow controller.

Immobilization of glycolate oxidase on solid supports

Methods for the immobilization of proteins on most commercial supports which bound enzyme by covalent attachment or ionic adsorption were available from the manufacturers. For those supports which bound protein by physical adsorption (Table 1), immobilizations were performed by washing the support with an aqueous buffer at pH 5–10 as appropriate, then exposing the support to a buffered solution of the enzymes for a predetermined time at either 5 or 25 °C, then washing the support with fresh buffer three to four times to remove any unadsorbed enzyme and assaying the support and combined washes for protein concentration (Bio-Rad protein assay) and soluble glycolate oxidase activity. For supports used in conjunction with glutaraldehyde, the procedure outlined above was repeated except that prior to addition of the enzyme, the supports were treated with 5 % aqueous glutaraldehyde. The yields of immobilized glycolate oxidase listed in Table 1 were obtained by optimizing the immobilization conditions for each support, and are based on the amount of enzyme activity relative to the amount of total protein bound to the support.

Co-immobilization of glycolate oxidase and catalase on oxirane acrylic beads

Into a 125-mL Erlenmeyer flask was weighed 10.0 g of oxirane acrylic beads (Eupergit® C). To the flask was then added *ca* 75 mL of a solution containing bicine buffer (50 mM, pH 8.0) and FMN (0.02 mM), and the oxirane acrylic beads suspended in the buffer by briefly mixing the contents of the flask. After the beads had settled to the bottom of the flask, the fine particles which floated to the top of the mixture were removed by pipette, along with as much of the supernatant which could be removed without

disturbing the settled beads. This washing procedure was repeated a second time.

An ammonium sulfate-precipitated glycolate oxidase mixture containing 327 IU of glycolate oxidase activity (100 mL, isolated from fresh spinach leaves) was centrifuged at 12,000 rpm for 20 min (Sorvall GSA rotor at 4 °C). The supernatant was discarded and the pellet dissolved in 50 mL of bicine (50 mM, pH 8.0)/FMN (0.02 mM) buffer. A 10 mL mixture containing 100 mg (715,000 IU) of ammonium sulfate-precipitated *A. niger* catalase was centrifuged at 15,000 rpm for 10 min (Sorvall SS-34 rotor). The supernatant was discarded and the pellet dissolved in the buffer containing the glycolate oxidase. This enzyme solution was then added to the flask containing the washed oxirane acrylic beads, and the final volume adjusted to 125 mL with additional buffer. The resulting mixture was transferred to a 250 mL polypropylene bottle, which was capped and placed on a bottle roller at 4–5 rpm for 16 h at 15 °C. The mixture was then transferred to a chromatography column equipped with a fritted bed support, and the co-immobilized enzymes were washed three times with 30 mL of the bicine/FMN buffer and stored at 5 °C in this same buffer. The co-immobilized enzyme catalyst had 7.2 IU of glycolate oxidase activity/g and 5680 IU of catalase activity/g.

Enyzme assays for glycolate oxidase and catalase immobilized on oxirane acrylic beads

Glycolate oxidase immobilized on oxirane acrylic beads was assayed by accurately weighing *ca* 5–10 mg of the beads into a 3-mL quartz cuvette containing a magnetic stirring bar and 2.0 mL of 0.12 mM 2,6-dichlorophenol-indophenol/80 mM TRIS buffer (pH 8.3). The cuvette was capped with a rubber septum and the solution deoxygenated by bubbling with nitrogen for 5 min. To the cuvette was then added by syringe 40 μL of 1.0 M glycolic acid/1.0 M TRIS (pH 8.3) with stirring, while measuring the change in absorption with time at 605 nm (ε = 22,000).[20]

Catalase activity was assayed by accurately weighing *ca* 2–5 mg of the treated beads into a 3-mL quartz cuvette containing a magnetic stirring bar and 2.0 mL of 16.7 mM phosphate buffer (pH 7.0), then adding 1.0 mL of 59 mM hydrogen peroxide in 16.7 mM phosphate buffer (pH 7.0) with stirring and measuring the change in absorption with time at 240 nm (ε = 39.4).[21]

Stability of immobilized or soluble enzymes to oxygen sparging

Into a 2.5-cm I.D. x 20 cm glass column equipped with a 20-mm diam. porous polyethylene bed support was placed 10 mL of a solution containing glycolic acid (0.25 M), EDA (0.33 M), propionic acid (0.075 M, HPLC internal standard) and FMN (0.2 mM). The column and its contents were cooled to 15 °C, then 2.5 IU of spinach glycolate oxidase and 2700 IU of *A. niger* catalase (co-immobilized on 0.5 g dry weight of oxirane acrylic beads) were added to the solution. Oxygen was then passed through the porous bed support and bubbled through the reaction mixture at a rate of 10 mL/min. The reaction was monitored by taking a 0.100 mL aliquot of the reaction mixture at regular intervals, mixing the aliquot with 0.300 mL of 0.1 N sulfuric acid to quench the reaction, filtering the aliquot and analyzing by HPLC. After 5.5 h, the yields of glyoxylic acid, oxalic acid, and formic acid when using the immobilized enzyme catalyst were 98, 2, and 0 %, respectively, with complete conversion of glycolic acid. The final activities of glycolate oxidase and catalase were 95 and 65 % of their initial values. The reaction was repeated using the same amounts of soluble glycolate oxidase and catalase and, after 4 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 43, 0, and 0 %, respectively, with a 46 % conversion of glycolic acid. The final activities of glycolate oxidase and catalase were < 2 and 82 % of their initial values, respectively; no further reaction was observed at longer reaction times.

Reaction rate dependence on oxygen pressure for sparged and non-sparged enzymatic oxidations of glycolic acid in a stirred autoclave reactor

A 300-mL stirred autoclave reactor was charged with 100 mL of a solution containing glycolic acid (0.75 M), EDA (0.86 M, pH 9.0), propionic acid (0.075 M, HPLC internal standard), and FMN (0.01 mM), and the solution cooled to 15 °C. To the autoclave was then added 41 IU of spinach glycolate oxidase and 42,800 IU of *A. niger* catalase co-immobilized on 15 g of oxirane acrylic beads. The resulting mixture was stirred at 400 rpm and 15 °C under 0.34, 0.58, 0.82 or 1.06 MPa (35, 70, 105 or 140 psig) of oxygen, while bubbling oxygen through the mixture at 50 mL/min. The reaction was monitored by taking a 100 μL aliquot of the reaction mixture at regular intervals, mixing the aliquot with 300 μL of 0.1 N sulfuric acid to quench the reaction, filtering the aliquot and analyzing by HPLC. The rates of glycolic acid oxidation for reactions run under 0.34, 0.58, 0.82 or 1.06 MPa of oxygen were 0.48, 0.54, 0.53 and 0.57 mmol/min, respectively.

The reactions described above were repeated, except that no oxygen was sparged through the reaction mixtures. The rates of glycolic acid oxidation for reactions run under 0.34, 0.58 or 0.82 MPa of oxygen were 0.032, 0.053 and 0.071 mmol/min, respectively.

Dependence of the rate of glycolic acid oxidation on oxygen sparge rate

The procedure describing the measurement of the reaction rate dependence of glycolic acid oxidation on oxygen pressure was repeated using 52 IU of spinach glycolate oxidase and 95,000 IU of *A. niger* catalase co-immobilized on 18 g of oxirane acrylic beads. The reaction mixture was stirred at 500 rpm and 15 °C under 0.58 MPa (70 psig) of oxygen, while sparging oxygen through the mixture at 5,10,15, 20, 25, 30 or 50 mL/min (0.22, 0.45, 0.67, 0.89,1.12,1.34 or 2.23 mmol/min); the corresponding rates of glycolic acid oxidation were 0.40, 0.57, 0.70, 0.79, 0.78, 0.99 and 1.10 mmol/min, respectively.

Glyoxylic acid production using co-immobilized glycolate oxidase and catalase in a stirred autoclave reactor

A 300-mL stirred autoclave was charged with 100 mL of a solution containing glycolic acid (0.75 M), EDA (0.86 M, pH 9.0), propionic acid (0.075 M, HPLC internal standard) and FMN (0.01 mM), and the solution cooled to 15 °C. To the autoclave was then added 89 IU of spinach glycolate oxidase and 72,600 IU of *A. niger* catalase co-immobilized on 28 g of oxirane acrylic beads. The resulting mixture was stirred at 500 rpm and 15 °C under 0.58 MPa (70 psig) of oxygen, while bubbling oxygen through the mixture at 100 mL/min. The reaction was monitored by taking a 100 μL aliquot of the reaction mixture at regular intervals, mixing the aliquot with 300 μL of 0.1 N sulfuric acid to quench the reaction, filtering the aliquot and analyzing by HPLC. After 3 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 100, 0, and 0 %, respectively, with complete conversion of glycolic acid. The final activities of glycolate oxidase and catalase were 100 and 100 % of their initial values, respectively.

The co-immobilized enzyme catalyst was recovered from the reaction described above by filtering the reaction mixture through a 2.5-cm I.D. x 20 cm glass column equipped with a 20-mm diam. porous polyethylene bed support. The remaining liquid adsorbed on the catalyst was removed by briefly passing a stream of nitrogen through the column, then the catalyst was resuspended in 100 mL of a fresh 15 °C solution containing glycolic acid (0.75 M), EDA (0.86 M, pH 9.0), propionic acid (0.075 M, HPLC internal standard) and FMN (0.01 mM). The 300-mL autoclave reactor was charged with this reaction mixture, and the reaction described above repeated. This catalyst recovery procedure was performed for 10 consecutive batch reactions; the reaction time for complete conversion of glycolate to glyoxylate in 98–99 % yield, and the recovered activities of co-immobilized glycolate oxidase and catalase, are depicted in Figure 6.

Fixed-bed co-immobilized enzyme reactor

Into a Kontes Airlift Bioreactor was placed 400 mL of a solution of 0.75 M glycolic acid, 0.86 M ethylenediamine, 0.075 M propionic acid (HPLC internal standard) and 0.01 mM flavin mononucleotide (pH 9.0). Wet oxygen was bubbled through the solution in the bioreactor, and a peristaltic pump was used to recirculate the oxygenated solution (at 40 mL/min) from the bioreactor through a jacketed 1-cm I.D. x 30 cm chromatography column containing spinach glycolate oxidase (13.9 IU) and *A. niger* catalase (56,000 IU) co-immobilized on oxirane acrylic beads (21-mL fixed bed volume). The contents of the bioreactor and jacketed chromatography column were maintained at 15 °C by recirculating 50:50 ethylene glycol:water through the jacket of the reactor and column using a refrigerated bath/circulator. After 377 h, the yields of glyoxylic acid, oxalic acid and formic acid were 93, 0, and 0.3 %, respectively, with a 94 % conversion of glycolic acid. The final activities of glycolate oxidase and catalase were 48 and 69 % of their initial values.

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