Biocatalytic Production of Glyoxylic Acid

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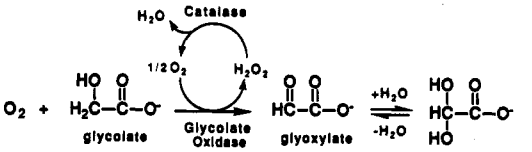
The production of glyoxylic acid from glycolic (hydroxyacetic) acid has been demonstrated using the soluble enzymes glycolate oxidase and catalase as catalysts. Catalase was included as cocatalyst to decompose byproduct hydrogen peroxide, thus limiting peroxide-dependent formate production and glycolate oxidase deactivation. The addition to reaction mixtures of a primary amine capable of forming the hemiaminal or imine of glyoxylate resulted in an increase in the yield of glyoxylate; hemiaminal/imine mixtures of glyoxylate were not as readily oxidized to formate and carbon dioxide by hydrogen peroxide and also limited product inhibition of glycolate oxidase at high glyoxylate concentrations. A synergistic effect was observed when using Tris or ethylenediamine (EDA) with catalase, where increases in selectivity to glyoxylate were much greater than the sum of selectivities to glyoxylate obtained when using amine or catalase alone. The inclusion of EDA in reactions produced selectivities to glyoxylic acid in excess of 98–99% at greater than 99% conversion of 0.25 M to 1.5 M glycolic acid. The technical feasibility of a biocatalytic process for the production of glyoxylic acid was demonstrated by the preparation of ca. 0.50 kg of the acid in a series of 2.0-L batch reactions.

Introduction

Glyoxylic acid is currently manufactured either by the nitric acid oxidation of glyoxal or via a three-step reaction which involves ozonolysis of dimethyl maleate, hydrogenation of the resulting hydroperoxide intermediate, and hydrolysis of the resulting methylglyoxylate hemiacetal.[1] The major use of glyoxylic acid is in the manufacture of vanillin, and the demand for vanillin manufactured from glyoxylic acid is expected to increase as the availability of vanillin from lignin (a byproduct of paper manufacture) decreases due to environmental concerns. Vanillin in turn is used as a flavor and fragrance ingredient, as well as in the manufacture of pharmaceuticals and agrochemicals. As an alternative to the current chemical processes for the production of glyoxylic acid, we now report the demonstration of a biocatalytic process which uses enzymes as catalysts and is run under the relatively mild reaction conditions normally associated with such catalysts. Additionally, this biocatalytic process produces a higher yield of glyoxylic acid and fewer undesirable waste streams (NaCl being the only reaction byproduct, generated by the ion-exchange separation step) than current methods of manufacture.

Glycolate oxidase (EC 1.1.3.15) is a peroxisomal enzyme commonly found in the leaves of many green plants which converts glycolic acid to glyoxylic acid (Scheme I). The enzyme has been isolated from a variety of sources, including spinach,[2,4] sugar beet,[4] pea,[5,6] pumpkin[7] or cucumber[8] cotyledens, lettuce,[9] and tobacco,[10-12] and an X-ray crystal structure of spinach glycolate oxidase has been determined.[13] The isolated spinach enzyme was reported to have a subunit molecular weight of 43 000 and was enzymatically active only as tetramers and/or octamers of identical subunits.[3] The enzyme activity was also found to be relatively unstable in solution; the protein tended to irreversibly aggregate in an inactive form, especially in the absence of added flavin mononucleotide (FMN), 1 equiv of which binds weakly and reversibly to each subunit. The optimum pH range for glycolate oxidase isolated from most plant sources was reported to be between pH 8 and 9.

Scheme I



The oxidation of glycolate to glyoxylate is believed to proceed via a mechanism similar to that observed for other flavoprotein oxidases:[14,15] binding of glycolate to the enzyme active site, two-electron transfer from glycolate to FMN, and reoxidation of the reduced FMN by reaction with oxygen to produce hydrogen peroxide. In subsequent undesirable reactions (Scheme II), enzyme-generated glyoxylic acid can react directly with byproduct hydrogen

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Scheme II

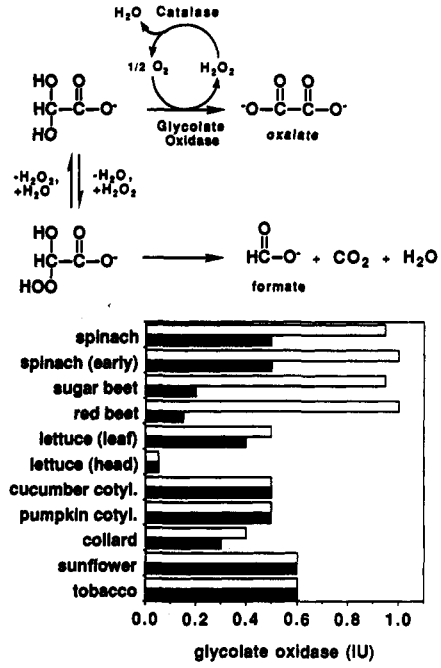

Figure 1. Total yield □, IU/g wet plant weight) and specific activity (■, IU/mg protein) of glycolate oxidase isolated from various plant sources. Glycolate oxidase activities from plant extracts were measured using the *o*-aminobenzaldehyde assay.

peroxide to yield formate and carbon dioxide[16-18] or be further oxidized by glycolate oxidase and oxygen to produce oxalic acid[4] (although the enzymatic oxidation of glyoxylate is much slower than that of glycolate). Early studies of glycolate oxidase reported that the addition of certain amines, such as hydroxylamine,[11] ethylenediamine (EDA),[11] or tris(hydroxymethyl)aminomethane (Tris),[4] limited the further oxidation of glyoxylic acid; separately, the addition of catalase[19] (an enzyme that catalyzes the decomposition of H_2O_2) to reactions which did not employ an amine buffer was reported to improve the yield of glyoxylic acid by suppressing the formation of formic acid and CO_2.[2] The use of amine additive or catalase alone resulted in increases in the measurable amounts of glyoxylic acid produced, but significant quantities of formate were still generated.

The possibility that glycolate oxidase and catalase could be used as cocatalysts in a biocatalytic reaction which produces glyoxylic acid at significant (0.25–2.0 M) concentrations required that several potential problems be addressed. Previous studies of glycolate oxidase were typically performed at glycolic acid concentrations of about 3–40 mM, and oxalate[11] or carbonate[20] were each found to inhibit glycolate oxidase activity. The stability of glycolate oxidase with added FMN under reaction conditions was uncertain. Catalase has been widely reported to be deactivated in the process of decomposing hydrogen peroxide,[21,22] so the number of catalyst turnovers from either catalase or glycolate oxidase in reaction mixtures where 0.25–2.0 mol/L of hydrogen peroxide is generated remained to be determined. Optimization of reaction conditions to limit the production of byproducts (formate, carbonate, and oxalate) would simplify product isolation and purification. We now report the demonstration of a biocatalytic process for the production of glyoxylic acid, where the discovery of an unexpected synergistic effect when using EDA together with catalase has made it possible to oxidize glycolic acid to glyoxylic acid at concentrations of up to at least 1.5 M in almost quantitative yields.

Results and Discussion

Enzyme Sources. Two different commercially-available sources of glycolate oxidase (spinach or sugar beet leaves) and catalase (bovine liver or *Aspergillus niger*) were evaluated as catalysts for the oxidation of glycolate to glyoxylate. No significant difference in specific activity, enzyme stability, or selectivity was observed when using either glycolate oxidase. *A. niger* catalase was found to be superior to the catalase from bovine liver in the present application; higher recoveries of catalase activity were obtained when using *A. niger*, in agreement with the results of earlier comparisons of these two enzymes.[23-27] For the biocatalytic preparation of ca. 0.5 kg of glyoxylic acid, a variety of plant sources were screened for the enzyme. Figure 1 illustrates the total yield and specific activity of glycolate oxidase obtained from these sources, and of those examined, spinach leaves produced optimum yield and activity.

The method of isolation initially used to screen sources of enzyme, as well as produce sufficient enzyme for use as catalyst in the scale-up of the oxidation reaction, involved grinding and pressing of plant tissue to produce a protein-containing liquid, followed by acidification to pH 5.3, centrifugation, and isolation of the protein fraction from the resulting supernatant which precipitates between 20 and 35% $(NH_4)_2SO_4$. The glycolate oxidase specific activity of the resulting protein fraction was typically 0.7–0.8 IU/mg (an IU (international unit) is defined as the amount of enzyme which will catalyze the transformation of one μmol of substrate per min). An improved method of isolating the enzyme from fresh spinach leaves was also developed during the course of this work; the resulting procedure produced protein having significantly higher specific activity and was used to supply enzyme for optimization of oxidation reaction parameters. The results of this improved purification method (described in the Experimental Section) are listed in Table I.

Optimization of Reaction Parameters. In the absence of added catalase or amine, the enzymatic oxidation of 0.25–2.0 M solutions of glycolic acid by glycolate oxidase produced predominately formate. The addition of catalase improved the yield of glyoxylic acid, but a significant

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Table I. Summary of a Purification of Glycolate Oxidase from Spinach (16 kg)

purification step	vol (L)	total protein (g)	total activity[a] (IU)	specific activity (IU/mg)	recovery (%)	purification factor
crude extract	6.0	45.6	4800	0.1	100	1
ultrafiltration	1.13	9.04	4500	0.5	94	5
20–35% $(NH_4)_2SO_4$ fractionation	0.25	0.84	4200	5.0	88	50
DEAE-cellulose	0.35	0.18	1900	10.5	45	105

[a] Glycolate oxidase activities from plant extracts were measured using the *o*-aminobenzaldehyde assay.

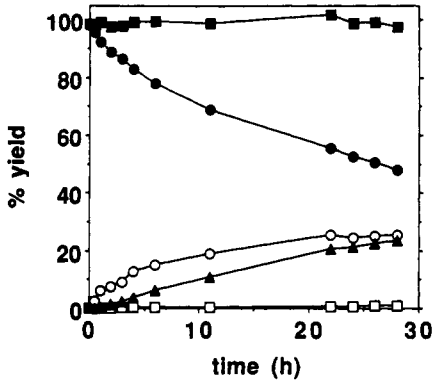
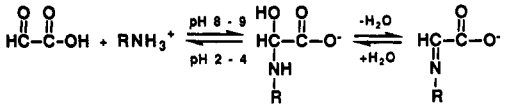


Figure 2. Time course for the oxidation of glycolic acid (0.25 M) in phosphate buffer (0.33 M, pH 8.3) containing FMN (0.2 mM) and propionate (75 mM) at 30 °C and 0.10 MPa O_2, using spinach glycolate oxidase (0.5 IU/mL) and bovine liver catalase (870 IU/mL): glycolate (●), glyoxylate (○), formate (▲), oxalate (□), mass balance (■).

Scheme III



fraction of glyoxylic acid was still further oxidized by hydrogen peroxide to formate. Figure 2 illustrates the production of glyoxylate, formate, and oxalate in a phosphate-buffered aqueous solution, where the ratio of units of catalase/glycolate oxidase was 1740:1. Where catalase alone was employed, product inhibition also produced a significant decrease in reaction rate at higher glycolic acid concentrations.

In the absence of added catalase, the use of either Tris or EDA as buffer in the enzymatic oxidation of glycolate has been reported to produce increased yields of glyoxylate by reacting with the aldehyde to form an oxidation-resistant N-substituted hemiaminal or imine (Scheme III). These two amines, as well as several other primary amines, were examined for their ability to increase glyoxylate yields in reactions which also included catalase to decompose byproduct hydrogen peroxide (Table II); control reactions were performed using phosphate or bicine buffer, which lack a primary amine functional group and so cannot react with glyoxylate to form a hemiaminal or imine. Improvements in the yield of glyoxylate produced by the substitution of a primary amine for phosphate buffer were found to be dependent on the pK_a of the protonated amine. The pH of reaction mixtures was adjusted to 8.3 to maximize the activity and stability of the glycolate oxidase catalyst, and of the amines examined, those with a pK_a approximately equal to or lower than the pH of the reaction mixture (i.e., EDA or Tris) produced much higher yields of glyoxylate (and less formate and oxalate) than amines whose pK_as were higher than the pH at which the reaction was performed.

Table II. Effect of Primary Amines on Glyoxylate Selectivity and Byproduct Formation during Enzymatic Oxidation of Glycolate[a]

buffer (pK_a)	% glyoxylate	% glycolate	% formate	% oxalate
ethylenediamine (6.85, 9.93)	86	1	2	7
Tris (8.08)	81	3	12	1
ammonium chloride (9.24)	40	38	19	1
2-propanolamine (9.43)	60	5	37	2
ethanolamine (9.50)	70	5	25	2
methylamine (10.62)	54	40	5	1
bicine (8.30)	25	26	44	1
phosphate (2.15, 7.10, 12.3)	25	52	21	1

[a] Reaction conditions: glycolate (0.25 M) in aqueous amine or buffer (0.33 M) containing FMN (0.2 mM), propionate (75 mM), spinach glycolate oxidase (0.5 IU/mL), and *A. niger* catalase (1400 IU/mL), at pH 8.3, 30 °C, and 0.10 MPa O_2 for 24 h.

These results are consistent with the expectation that an unprotonated amine is necessary to form an oxidation-resistant N-substituted hemiaminal and/or imine complex with glyoxylate;[28] an amine buffer whose pK_a is much higher than the pH of the reaction mixture would be present predominantly as the protonated amine in the reaction mixture and therefore be less likely to form such complexes with glyoxylate. Addition of hydrogen peroxide to an aqueous equimolar solution of glyoxylic acid and EDA at pH 9.0 produced significantly lower amounts of formate compared to that observed in the absence of added EDA, demonstrating the relative stability of this hemiaminal/imine mixture toward oxidation by hydrogen peroxide. A ^{13}C NMR spectroscopic analysis of an aqueous solution which was 0.25 M in both Tris and glyoxylic acid, and at pH 8.3, indicated the solution was predominately an equilibrium mixture of the hemiaminal and imine, with very little free glyoxylic acid present. Adjusting the pH of this solution to 2.0 completely shifted the equilibrium from an imine/hemiaminal mixture to protonated amine and glyoxylic acid.

An unexpectedly large increase in the yield of glyoxylic acid was observed when using catalase together with either EDA or Tris for the enzymatic oxidation of glycolic acid. In order to demonstrate this synergistic effect of catalase and amine, reactions were run using catalase and three different buffers: bicine, which does not react with and protect glyoxylate from further oxidation, and either EDA or Tris. Additionally, the reactions with either EDA or Tris were performed without added catalase. Figure 3 illustrates the optimum yields of glyoxylic acid produced via the glycolate oxidase-catalyzed oxidation of glycolic acid using glycolic acid concentrations of 0.25, 0.75, 1.0, and 1.5 M. A comparison of the yields of glyoxylate produced when using (1) bicine and catalase, (2) either Tris or EDA with no added catalase, and (3) either Tris

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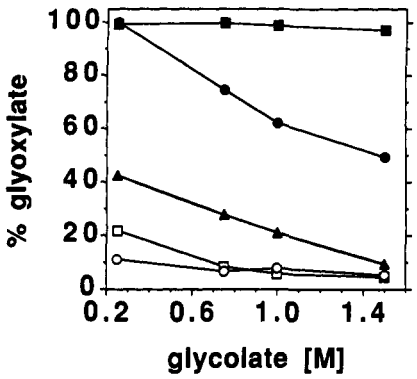

Figure 3. Glyoxylate yield dependence on glycolate concentration for reactions run with bicine and catalase (▲), Tris and no catalase (○), EDA and no catalase (□), Tris and catalase (●), and EDA and catalase (■). Buffer or amine concentrations were 105% (mol/mol) of glycolate; *A. niger* catalase concentration was 1400 IU/mL (reaction conditions as described in text).

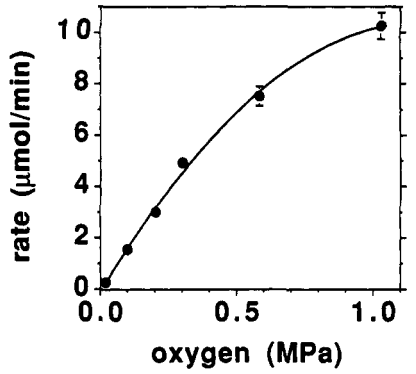

Figure 4. Reaction rate dependence on oxygen pressure for the oxidation of glycolic acid (0.250 M) in Tris buffer (0.33 M, pH 8.3) containing FMN (0.2 mM) and propionate (75 mM) at 30 °C, using spinach glycolate oxidase (0.25 IU/mL) and *A. niger* catalase (1400 IU/mL).

or EDA with added catalase illustrates the synergistic effect of using catalase together with either Tris or EDA. The yields of glyoxylic acid obtained when using catalase together with either Tris or EDA are not only greater than those obtained when catalase and either Tris or EDA are employed separately, but are also much greater than the sum of the yields of glyoxylic acid obtained from reactions run using, separately, catalase and either Tris or EDA.

The almost quantitative yields of glyoxylic acid obtained when using EDA with catalase were particularly striking, especially at high glycolate concentrations. The nature of this synergistic effect has not been determined. An activation of catalase activity by EDA, together with the oxidation-resistance of imine/hemiaminal mixtures of EDA and glyoxylate, might have accounted for the lack of formate production, but an assay of catalase in the presence or absence of 0.25 M EDA at pH 7.2 indicated no significant difference in enzyme activity, while at pH 8.5 there was a 36% decrease in catalase activity in the presence of 0.25 M EDA.

The dependence of reaction rate on oxygen concentration was determined by stirring soluble enzyme-containing solutions under oxygen at various pressures. The rate of oxidation of glycolic acid increased with increasing pressure of oxygen over the reaction mixture (Figure 4); the increase in reaction rate was not linear with increasing oxygen pressure above 0.3 MPa of oxygen (oxygen K_m = 0.68 mM for spinach glycolate oxidase[14]). Increasing the concentration of glycolate oxidase from 0.2 IU/mL to 4.0 U/mL at 0.58 MPa O_2 did not result in an increase in reaction rate, indicating that within this concentration range of enzyme, the rate was only dependent on the concentration of oxygen in the solution (mass-transport limited in oxygen). In order to increase the reaction rate for reactions run under an oxygen atmosphere, oxygen was sparged into a reaction mixture containing soluble spinach glycolate oxidase and *A. niger* catalase at ca. 0.5 vol of O_2/vol of reaction mixture/min at atmospheric pressure. A short time after the reaction was started, no further oxidation of glycolate was observed, and an assay of the solution indicated that no detectable glycolate oxidase activity remained. The soluble glycolate oxidase was rapidly deactivated by sparging (possibly by dissociation of subunit multimers, followed by irreversible unfolding of the individual subunits).

Table III. Enzyme Catalyst Recovery Dependence on Reaction Temperature[a]

temp (°C)	catalase recvry (%)	glycolate oxidase recvry (‰)	glyoxylate yield (%)
40	38	87	92
30	60	100	96
15	100	100	99
5	100	100	99

[a] Reaction conditions: glycolate (0.25 M) in aqueous EDA (0.33 M) containing FMN (0.01 mM), propionate (75 mM), spinach glycolate oxidase (0.5 IU/mL), and *A. niger* catalase (1400 IU/mL), at pH 8.3 and 0.10 MPa O_2.

The temperature stabilities of spinach glycolate oxidase and *A. niger* catalase under reaction conditions were determined over the temperature range of 5–40 °C (Table III). Catalase was found to be much less stable at higher reaction temperatures than was glycolate oxidase; recovery of activity of both enzymes was optimal between the temperatures of 5 and 15 °C. The dependence of enzyme activity recovery on the concentration of FMN in reaction mixtures was also determined by performing oxidations with 0.01, 0.10, and 1.0 mM added FMN; no difference in glycolate oxidase activity recoveries was observed over this concentration range, and 0.01 mM FMN was routinely used in oxidation reactions to stabilize glycolate oxidase activity.

Maintaining a relatively high concentration of catalase activity in the reaction mixture was important for both high selectivity to glyoxylic acid and good stability of both glycolate oxidase and catalase. By varying the concentration of catalase between 50 IU/mL and 14,000 IU/mL for oxidations of either 0.25 or 0.75 M glycolic acid (and using EDA as buffer), it was determined that a minimum concentration of catalase of ca. 350 IU/mL was required to obtain yields of glyoxylic acid of greater than 99%, especially at higher glycolate concentrations. Typically, reactions were run using 1400 IU/mL of catalase (to ensure good enzyme activity recoveries) and 0.2–1.0 IU/mL of glycolate oxidase; higher recoveries of glycolate oxidase activity were usually obtained when using 0.5–1.0 IU/mL of this enzyme.

Oxidations of glycolic acid were performed over a range of concentrations (0.25–2.5 M). The molar ratio of EDA or Tris to glycolic acid used in the oxidations ranged from 1.05:1 to 1.33:1. Within this range, the exact ratio of amine of glycolic acid was usually adjusted to obtain the desired pH. Reactions run at glycolic acid concentrations as high as 2.0 M went to completion, while those run at concentrations greater than 2.0 M showed significant substrate

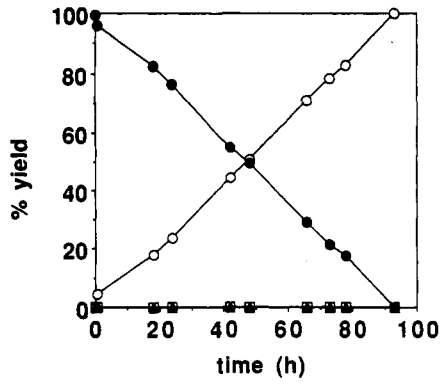

Figure 5. Time course for the 2.0-L batch oxidation of glycolic acid (0.75 M) in aqueous EDA (0.788 M, pH 8.9) and FMN (0.01 mM) at 15 °C and 0.58 MPa O_2, using spinach glycolate oxidase (1.0 IU/mL) and *A. niger* catalase (1400 IU/mL): glycolate (●), glyoxylate (○), formate (▲), and oxalate (□) (run no. 3, Table IV).

Table IV. Product Analysis and Remaining Catalyst Activities from 2.0-L Batch Oxidations of Glycolic Acid[a]

run no.	1	2	3	4	5
glyoxylate (%)	99.4	99.3	99.7	99.8	99.2
glycolate (%)	0.4	0.6	0.0	0.0	0.6
formate (%)	0.1	0.1	0.0	0.0	0.2
oxalate (%)	0.1	0.0	0.3	0.2	0.0
reaction time (h)	107	87	93	77	74
glycolate oxidase (%)	58	29	34	74	62
catalase (%)	94	86	94	87	90

[a] Reaction conditions: glycolate (0.75 M) in aqueous EDA (0.788 M) containing FMN (0.01 mM), spinach glycolate oxidase (1.0 IU/mL), and *A. niger* catalase (1400 IU/mL), at pH 8.9, 15 °C, and 0.58 MPa O_2.

inhibition. Increasing the concentration of glycolic acid within the range of 0.25–2.0 M did not result in an increase in the reaction rate (glycolate K_m for spinach glycolate oxidase = 0.38 mM[14]). The dependence of reaction rate, enzyme recovery, and product selectivity was studied over the range of pH 7–9.5, and optimal glyoxylic acid yields and glycolate oxidase activity were observed between pH 8.0 and 9.1. It was also found that the pH of the reaction mixture decreased by ca. 0.5 pH units over the course of the reaction, so the initial pH of reaction mixtures was adjusted to the high end of the maximum enzyme activity pH range (8.9–9.1) and allowed to decrease without pH control.

Scale-Up of Glycolic Acid Oxidation. The biocatalytic production of glyoxylic acid comparable in purity to commerically available material was demonstrated by the preparation of a 0.5-kg sample, using soluble glycolate oxidase and catalase as enzyme cocatalysts. EDA was employed as amine additive because of its lower cost relative to Tris, as well as the higher selectivity to glyoxylate obtained when oxidizing glycolate at concentrations of greater than 0.25 M (relative to Tris). Approximately 2000 IU of glycolate oxidase (isolated from fresh spinach leaves) was added to each 2.0-L reaction mixture; Figure 5 illustrates the time course and describes the reaction conditions for a typical reaction. Five 2.0-L reactions were run, each producing ca. 100 g of glyoxylic acid; Table IV lists the reaction times, HPLC analyses of reaction mixtures, and remaining enzyme activities for each of the five reactions.

At the end of each 2.0-L reaction the protein catalysts were heat precipitated and the resulting denatured protein removed by centrifugation and discarded. Numerous attempts were made to recover soluble glycolate oxidase activity from reaction mixtures by using membrane concentrators or membrane filtration with 10 000 molecular weight cutoff filters, but very low recoveries of active glycolate oxidase were obtained. After removal of the protein catalyst and FMN, glyoxylic acid was separated from EDA by ion-exchange chromatography using a strong acid ion-exchange resin. By starting with the resin in an acid form, EDA was completely retained on the column, while glyoxylic acid was recovered in the eluent. EDA was subsequently eluted from the column in its dibasic form using a caustic wash, which allows for the possibility of recycling this EDA solution into a subsequent reaction. The resulting aqueous solutions of glyoxylic acid recovered from the ion-exchange step were concentrated to 50 wt % product, which is the form in which bulk quantities of glyoxylic acid is commercially available. Overall yields of glyoxylic acid for the five separate reactions ranged from 91 to 94% (unoptimized), and recovery of EDA from the ion-exchange step was typically 99%. The number of catalyst turnovers for glycolate oxidase and catalase in a single 2.0-L reaction were estimated to be ca. 1×10^6 and 9×10^5, respectively, which is quite respectable for an oxidation that generates 0.75 mol/L of hydrogen peroxide over the course of the reaction.

Conclusions

The technical feasibility of using enzymes as catalysts for the preparation of glyoxylic acid has been demonstrated by the biocatalytic oxidation of 0.75 M (5.7% w/v) solutions of glycolic acid; greater than 99% selectivity to the desired product at greater than 99% conversion of starting material has been obtained. The ability to perform an almost quantitative conversion of glycolate to glyoxylate was primarily due to the unexpected synergistic effect of using catalase in combination with EDA, where catalase destroyed byproduct hydrogen peroxide and EDA both protected glyoxylate from further oxidation by hydrogen peroxide and limited product inhibition of glycolate oxidase. The final product, a 50 wt % solution of glyoxylic acid in water, was chemically equivalent to existing commercially-available material.

Glycolate oxidase and catalase activity remained at the conclusion of each of the five 2.0-L reactions, but significant glycolate oxidase activity could not be recovered for reuse in subsequent reactions. Decreasing the reaction time would also result in an improvement in the present process, and this would most easily be accomplished by sparging the reaction mixture with oxygen under pressure. Immobilization of the enzymes on a nonsoluble support should stabilize the enzymes against denaturation by the action of sparging, resulting in an increase in reaction rate, decrease in reaction time, and simplification of recovery and recycle of the enzyme catalysts. An examination of the immobilization of these two enzymes and their use in the present biocatalytic process is underway.

Experimental Section

General. Chemicals were purchased from Aldrich, Sigma, or Baker and used as received. Glycolate oxidase (from spinach or beet leaves) and catalase (from bovine liver or *A. niger*) were purchased from Sigma and used without any further purification; additionally, glycolate oxidase was isolated from a variety of plant sources as described below. Reactions were generally run within

a pH range of 8.0–9.0. Samples were prepared for HPLC analysis by mixing 0.100 mL of a reaction mixture with 0.300 mL of 0.10 N H_2SO_4 and then filtering the resulting solution 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 × 7.8-mm) at 50 °C, using as solvent 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.

Enzyme Assays. Glycolate oxidase activity in plant extracts was measured using a reported enzyme assay[29] where glyoxylate reacts with *o*-aminobenzaldehyde (OAB) and glycine to yield a yellow product with an absorbance maximum at 440 nm. Glycolate oxidase activity in reaction mixtures was assayed with 2,6-dichloroindophenol (DCIP) as electron acceptor, using a modification of a reported procedure.[2] Into a 3-mL quartz cuvette was placed 2.0 mL of a solution containing 2,6-dichloroindophenol (0.12 mM), glycolic acid (20 mM), and Tris buffer (80 mM, pH 8.3). The cuvette was capped with a rubber septum, and then nitrogen was bubbled through the solution for 5 min. To the solution was then added an enzyme sample containing ca. 0.5 IU (international units) of glycolate oxidase activity, the assay solution briefly mixed, and the change in absorbance at 606 nm measured for 30 s ($\epsilon = 22\,000$ L mol^{-1} cm^{-1})[30] at room temperature. One DCIP IU was equivalent to ca. 2 OAB IU of glycolate oxidase activity; except for Table I and Figure 1, all glycolate oxidase activities are reported in DCIP IU.

Catalase activity was assayed using a modification of a reported procedure.[31] Into a 3-mL quartz cuvette was placed 2.0 mL of phosphate buffer (16.7 mM, pH 7.0) and 1.0 mL of hydrogen peroxide (59 mM) in the same phosphate buffer. To the solution was then added an enzyme sample containing ca. 5.0 IU of catalase activity, the assay solution briefly mixed, and the rate of decomposition of H_2O_2 measured by the change in absorbance at 240 nm ($\epsilon = 39.4$ L mol^{-1} cm^{-1})[31] for 30 s at room temperature.

Purification of Glycolate Oxidase from Spinach Leaves. Glycolate oxidase from spinach was purified using selective ammonium sulfate fractionation followed by batch adsorption of the extract using DEAE cellulose. The latter step resulted in the adsorption of all plant proteins except glycolate oxidase. All steps in the purification were performed at 4 °C unless otherwise stated. At 25 °C, two bushels (16 kg) of fresh spinach were chopped into fine particles using a Fitz Mill grinder fitted with a 0.5-in. mesh screen. The liquid fraction (ca. 6 L) of the resulting pulp was isolated by squeezing through four layers of cheesecloth; alternatively, a juice extractor (Vitantonio) was used. To the liquid fraction was added 5.6 g of dithiothreitol (5 mM final concentration), and then the pH was adjusted to 5.2 by adding 20% (v/v) aqueous acetic acid. After a 10-min incubation, the resulting mixture was centrifuged at 13000*g* for 25 min at 4 °C. The pellet was discarded and the pH of the supernatant adjusted to 7.5–8.0 using 6 N potassium hydroxide. The supernatant (approximately 5.5 L) was then concentrated 5-fold using a Pelicon ultrafiltration apparatus fitted with a 100 000 MW membrane cassette; the final volume of concentrate was approximately 1.1 L. To the concentrate was then added solid ammonium sulfate (154 g) slowly over 10 min. After all the ammonium sulfate dissolved, the resulting precipitate was removed by centrifugation at 13000*g* for 15 min. The pellet was discarded, and 77 g of ammonium sulfate was added to the supernatant (approximately 1.1 L), which was then centrifuged as before. The resulting protein pellet was collected and the supernatant discarded.

The protein pellet was dissolved in ca. 200 mL of 20 mM bicine buffer (pH 8.0) and dialysed (12 000–14 000 MWCO) for 16 h vs 4 L of 20 mM bicine buffer (pH 8.0) containing 2 mM FMN. The conductivity of the protein solution was measured relative to the conductivity of fresh bicine buffer using a conductivity meter, and if the readings were not equivalent, the protein solution was dialysed an additional 4 h and then tested as before. The dialysed protein solution (ca. 250 mL) was stirred in a beaker using either a magnetic stir bar or overhead stirrer and then 25 g of preswollen DEAE cellulose added and the resulting mixture incubated for 10 min. Protein binding to the resin was monitored by following the decrease in protein concentration of the solution using the Bradford assay (Bio-Rad). When the protein concentration of the supernatant was reduced to trace levels (<0.2 mg/mL), the unbound protein was recovered from the mixture by vacuum filtration through a 11-cm Whatman no. 1 filter disk. To maximize enzyme recovery, the resin cake was washed with 100 mL of 20 mM bicine buffer (pH 8.0). FMN was added to the enzyme solution (400 mL) to a 2 mM final concentration, and then 240 g of solid ammonium sulfate was added gradually over 15 min with stirring while the pH was maintained at 8.0 by the dropwise addition of 5 N potassium hydroxide. The resulting precipitated glycolate oxidase was collected by centrifugation and stored at 4 °C.

Glyoxylate Selectivity Dependence on Primary Amine Addition. Into a 25-mL round-bottom flask were placed a magnetic stirring bar and 10 mL of an aqueous solution containing glycolic acid (0.250 M), primary amine (Table II, 0.330 M), propionic acid (HPLC internal standard 75 mM), and FMN (0.2 mM), at pH 8.3. To this solution was added spinach glycolate oxidase (0.5 IU/mL) and *A. niger* catalase (1400 IU/mL), and the resulting solutions were stirred at 30 °C under oxygen at atmospheric pressure. Similar reactions were performed with nonprimary amine buffers (phosphate and bicine) for comparison purposes. The yields of oxalate, glyoxylate, and formate and the recovery of unreacted glycolate from reactions using the amines or buffers were determined by HPLC analysis after 24 h.

Determination of Synergistic Effect when Using Amine Additive and Catalase. Into a 3-oz Fischer-Porter glass aerosol reaction vessel were placed a magnetic stirring bar and 10 mL of an aqueous solution containing glycolic acid (either 0.250, 0.75, 1.0, or 1.5 M), a 5% molar excess of EDA (0.263, 0.788, 1.05, or 1.65 M, respectively), FMN (0.01 mM), propionic acid (HPLC internal standard, 75 mM), spinach glycolate oxidase (2.0 IU/mL), and *A. niger* catalase (1400 IU/mL) at a pH of 8.9. The reaction vessel was sealed, the reaction mixture was cooled to 15 °C, and then the vessel was flushed with oxygen by pressurizing to 0.58 MPa and venting to atmospheric pressure five times with stirring. The vessel was then pressurized to 0.58 MPa of oxygen and the mixture stirred. Aliquots (0.10 mL) were removed through a sampling port (without loss of pressure in the vessel) at regular intervals for analysis by HPLC to determine the optimum yield of glyoxylate. The reactions were repeated with EDA and no catalase, Tris and catalase, Tris and no catalase, and bicine and catalase.

Reaction Rate Dependence on Oxygen Pressure. Into a 3-oz Fischer-Porter glass aerosol reaction vessel were placed a magnetic stirring bar and an aqueous solution (10 mL) containing glycolic acid (0.250 M), Tris buffer (0.330 M, pH 8.3), propionic acid (HPLC internal standard, 75 mM), spinach glycolate oxidase (0.25 IU/mL), *A. niger* catalase (1400 IU/mL), and FMN (0.2 mM). The solution was stirred at 30 °C in air (0.02 MPa O_2) or oxygen (0.10 MPa O_2) at atmospheric pressure or at 0.20, 0.30, 0.58, or 1.03 MPa O_2, and the corresponding initial rates of production of glyoxylic acid (for the first 15% of the reaction) were 0.276, 1.56, 3.01, 4.94, 7.52, and 10.3 μmol glyoxylate/min, respectively.

Reaction Rate Dependence on Glycolate Oxidase Concentration. Aqueous solutions (10 mL) containing glycolate (0.250 M), EDA (0.330 M), propionic acid (HPLC internal standard, 75 mM), sugar beet glycolate oxidase (0.20, 0.40, or 4.0 IU/mL), *A. niger* catalase (1,400 IU/mL), and FMN (0.01 mM) were stirred at 15 °C and pH 8.9 under 0.58 MPa O_2. The initial rates of production of glyoxylic acid (first 15% of reaction) when using 0.20, 0.40, or 4.0 IU/mL of sugar beet glycolate oxidase were 1.37, 1.33, and 1.32 μmol/mL/min, respectively.

Scale-Up of Biocatalytic Synthesis of Glyoxylic Acid. Reactions were performed in an Amicon Model 2000 2.5-L high-output stirred cell, where a $^1/_{16}$-in. Teflon sheet was substituted for the filtration membrane. In a typical reaction, glycolate oxidase (2000 IU, isolated from spinach) was added to a 2.0-L solution containing glycolic acid (113 g, 0.75 M), EDA (95 g,

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0.788 M), FMN (9.7 mg, 0.01 mM), and *A. niger* catalase (2.8×10^6 IU, from *A. niger*) at 15 °C, and the resulting mixture (final pH = 8.9) was stirred at 15 °C under 0.58 MPa O_2. Aliquots were removed at regular intervals for analysis by HPLC (using 75 mM propionate as external standard) to monitor the progress of the reaction. After 77 h, glyoxylic acid (99.8% yield), and oxalic acid (0.2% yield) were the only reaction products; no formate was detected, and complete conversion of glycolic acid was attained. Glycolate oxidase and catalase activities were 74% and 87% of their initial activities, respectively. The reaction was terminated by sparging the solution with nitrogen and then heating the reaction mixture to 70 °C for 5 min under a nitrogen blanket. Precipitated protein was removed by centrifugation, and FMN was subsequently removed by filtration of the reaction mixture through a bed of activated carbon. Any remaining soluble protein was removed by filtration using ultrafiltration with a 10000-MW cut-off filter.

Glyoxylic acid and EDA were next separated by ion-exchange chromatography. Amberlite CG-120 (900 g, Rohm & Haas, 100–200 mesh, 4.5 mequiv/g) was suspended in 1.0 N HCl to yield ca. 2.0 L of swollen resin, which was then rinsed with distilled water to remove excess HCl. A 50- × 100-cm column was packed with 1900 mL of washed resin, 2.0 L of distilled water was pumped through the column at 8.0 mL/min, and then half of the glyoxylic acid/EDA filtrate (1.1 L, containing 0.788 mol of EDA and 0.75 mol of glyoxylic acid) was loaded onto the column at a flow rate of 8 mL/min. Glyoxylic acid was collected during an initial water elution phase, which was monitored by absorbance at 254 nm. Approximately 2.2 L of glyoxylic acid-containing eluent was collected. EDA was then eluted with 3.4 L of 1 N NaOH, yielding 0.77 mol of dibasic EDA (99% recovery). The column was reequilibrated by washing with 1 N HCl (2.4 L) followed by 3 L of distilled water to remove chloride. The combined column fractions containing glyoxylic acid from the ion-exchange separation of two 1.1-L fractions of the glyoxylic acid/EDA filtrate were combined and concentrated by rotary evaporation at 40 °C to produce a 50 wt % solution containing 104 g (1.40 mol, 94% yield) of glyoxylic acid; the purity of the glyoxylic acid was greater than 99.5% as determined by ^1H and ^{13}C NMR spectroscopy and HPLC analysis.