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Hydration of 3-cyanopyridine to nicotinamide by crude extract nitrile hydratase

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SUMMARY

The kinetic and stability characteristics of crude extract nitrile hydratase from *Brevibacterium* R-312 were studied for the hydration of 3-cyanopyridine to nicotinamide. The enzyme was substrate and product inhibited and had the following kinetic constants: K_m = 28 mM; K_p = 36 mM; K_s = 155 mM; V_m = 5.8 μmol/min/mg protein (25 °C). Its maximum temperature and pH (phosphate buffer) were 35 °C and 8.0, respectively, and it had half-lives of 50 days, 10 days and 1 day at 4 °C, 10 °C and 25 °C, respectively. The crude extract also exhibited amidase activity on nicotinamide, but it became significant only at nicotinamide concentrations greater than 300 mM. Mathematical models for batch and fed-batch hydrations were developed to account for substrate and product inhibitions and for enzyme decay. They predicted to within 10% experimental results for initial substrate and final product concentrations up to 300 mM; the accuracies decreased at higher concentrations primarily because of the relatively rapid hydrolysis of nicotinamide.

INTRODUCTION

Enzymatic hydrations of nitriles appear to have potential for operating without the major problems encountered in commercial chemical syntheses of amides: wasteful side products, high energy demands, difficult purifications, salt formation, and optically-impure products. One candidate, and the subject of this study, is nicotinamide.

3-cyanopyridine is hydrated to nicotinamide by nitrile hydratase in whole cells of *Brevibacterium* R-312 [1–3]. But practical application probably will not be feasible economically because (1) the cells develop during fermentation unacceptably high levels of amidase activity well before peak activity of hydratase is reached, (2) the resting whole cells have very short hydratase half-lives (< 5 days) at practical operating temperatures and (3) the hydratase is inhibited strongly by nicotinamide [2,3].

Immobilization of the hydratase from crude extract may help to overcome at least the amidase and stability problems [2–3]. The kinetic study reported here for soluble crude extract was done to obtain the baseline data necessary for the development and evaluation of an immobilized (from crude extract) hydratase, and for preliminary designs and analyses of model processes. In particular, it was necessary to determine the basic nature of the hydration kinetics (e.g., types of inhibition), the kinetic constants, the pH and temperature profiles, and the stability characteristics. It also was important to obtain information about the level of amidase activity, and to develop mathematical models for the hydration.

The reader is referred to the references for further background [2,3] and for information [5,6] about *Rhodococcus rhodochrous* J1 which has been reported to give higher conversion of 3-cyanopyridine to nicotinamide at higher concentrations than does R-312. The work reported has been very limited and has been done only in shake flasks, but clearly *R. rhodochrous* does warrant much more study.

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MATERIALS AND METHODS

Microorganism. *Brevibacterium* R-312 was used throughout this study. Details of the organism's characteristics, fermentation and recovery are given in the references [2,3].

Preparation of crude extract. Concentrated cell paste (approx. 40 mg/ml dry weight) was sonified (Branson Sonic Power Co., Sonifier Model W-30) in an ice bath for 15 min. The sonified suspension was centrifuged at 18 000 rpm for 25 min; the supernatant was refrigerated for use in kinetic studies. Each stock so prepared was kept no longer than two weeks (see below).

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Protein assay. The Lowry method [4] was used for all protein assays. A Bausch and Lomb Spectronic 710 was used to measure absorbances.

Enzyme assays. The standard assay was conducted as follows: One part crude extract (approximately 10 mg/ml) was added to 10 parts of 8 g/l (77 mM) 3-cyanopyridine (0.05 M, pH 8 phosphate buffer) in a flask. The flask was sealed, and then was placed in a shaker bath operated at 125 strokes/min and 25 °C. 0.1 ml samples were taken at regular time intervals, and were diluted in 2 ml of distilled water at pH 3.0 (HCl) to stop the reaction. The diluted samples were kept in an ice bath until they could be analyzed by HPLC for cyanopyridine, nicotinamide and nicotinic acid [2,3].

The specific activity of crude extract was defined as: specific activity = μmol nicotinamide per minute/ml solution, divided by mg protein/ml solution. The conversions used for such calculations were always low enough to insure determination of initial rates.

Similar assays were performed to determine the Michaelis constant, the substrate and product inhibition constants and the maximum specific activity. These assays were performed using various initial concentrations of 3-cyanopyridine and/or nicotinamide.

Temperature and pH profiles were obtained by performing the standard assay at various temperatures and pHs.

Storage stability of crude extract. Crude extract was stored in pH 8 phosphate buffer (0.05 M) at 4 °C, 10 °C and 25 °C, and activities were measured periodically by the standard assay method. The hydratase half-life was 50 days at 4 °C (Fig. 1, RESULTS AND DISCUSSION); therefore, crude extract used for kinetic studies was stored at 4 °C no longer than two weeks.

Extensive hydrations. Batch and fed-batch hydrations were run to a high degree of conversion to simulate practical applications, and to test mathematical models (see below). Each batch hydration was performed using 100 cc of reaction mixture in a 500 cc sealed flask. Flasks were maintained at 25 °C in a shaker bath operated at 125 strokes per min. The initial 3-cyanopyridine and crude extract concentrations ranged from 50 to 300 mM and from 0.2 to 1.0 mg/ml, respectively, and the pH was 8.0 (0.05 M phosphate buffer). The initial concentration of nicotinamide was zero in all cases.

Fed-batch hydrolyses were performed in essentially the same manner with the following exceptions: the initial 3-cyanopyridine concentration was always between 10 and 100 mM; the initial reaction volume was 50 cc; concentrated substrate (up to 1 m) was fed (peristaltic pump) at rates calculated via the mathematical model to keep constant the substrate concentration in the reaction flask.

MATHEMATICAL MODELS

Note: It is assumed in all derivations to follow that the amidase hydrolysis of nicotinamide is negligible.

Determination of rate constants

The specific rate, v, for a substrate and product inhibited enzyme is, for competitive inhibition [2]:

$$v = V_m \cdot S/[S + K_m + (K_m \cdot P/K_p) + S^2/K_s] \quad (1)$$

where V_m = maximum specific rate (μmol or mmol/min/mg protein); K_m = Michaelis constant (mM); S = substrate concentration (mM); K_p = product inhibition constant (mM); K_s = substrate inhibition constant (mM).

The double-reciprocal form of Eqn. 1 is:

$$1/v = 1/V_m + (K_m/V_m) \cdot (1 + P/K_p)/S + S/(K_s \cdot V_m) \quad (2)$$

K_m and V_m can be determined from initial rates at very low substrate concentrations with no product present initially. Under these conditions, Eqn. 2 becomes:

$$1/v = 1/V_m + (K_m/V_m)/S \quad (3)$$

This is the equation of the low-concentration asymptote of Eqn. 2 from which V_m is obtained from the $1/v$-axis intercept, and K_m is obtained from the $1/S$-axis intercept.

K_p can be found from the asymptotes for various initial concentrations of product. For such cases, Eqn. 2 becomes:

$$1/v = 1/V_m + (K_m/V_m)((1 + P/K_p)/S \quad (4)$$

Eqn. 4 gives a separate asymptote for each product concentration. At the $1/S$-axis intercept ($1/S_{int}$), Eqn. 4 yields:

$$-S_{int}/K_m = 1 + P/K_p \quad (5)$$

K_p can then be determined from the slope of this equation.

Finally: the derivative with respect to $1/S$ of Eqn. 2 at zero initial product concentration is:

$$d(1/v)/d(1/S) = K_m/V_m - S^2/(K_s \cdot V_m) \quad (6)$$

The minimum in the double-reciprocal plot occurs where the derivative is zero at which point Eqn. 6 becomes:

$$K_s = S^2_{min}/K_m \quad (7)$$

Integrated rate equations

For the batch hydration of 3-cyanopyridine, the basic rate equation is:

$$dS/dt = V_m \cdot E \cdot S/(S + K_m \cdot (1 + P/K_p) + S^2/K_s) \quad (8)$$

where E is the crude-extract concentration (mg protein/l) in the reactor. Assuming a first order decay of hydratase activity,

$$V_m = V_{mo} \cdot \mathrm{Exp}\,(-kt) \quad (9)$$

where V_{mo} = maximum specific activity at time = 0 (μmol or mmol/min/mg protein); k = denaturation rate constant (1/min); t = time (min).
The product concentration, P, is (assuming zero initial product concentration):

$$P = S_o - S \quad (10)$$

where S_o is the initial substrate concentration.

Combining Eqns. 8 through 10 and integrating yields:

$$\frac{K_m}{k \cdot V_{mc} \cdot E} \cdot (1 + S_o/K_p) \cdot L_n\,(S_o/S) +$$

$$\frac{1}{k \cdot V_{mo} \cdot E} \cdot (1 - K_m/K_p) \cdot (S_o - S) +$$

$$\frac{1}{2 \cdot k \cdot V_{mo} \cdot K_s \cdot \mathrm{E}} \cdot (S_o{}^2 - S^2) = 1 - \mathrm{Exp}\,(-kt) \quad (11)$$

The value of k can be predicted from an Arrhenius relationship:

$$k = k_o \cdot \mathrm{Exp}\,(-E_d/RT) \quad (12)$$

where E_d = thermal decay activation energy (kcal/mol); R = gas constant (1.987 kcal/mol (K)); T = absolute temperature (K); k_o = Arrhenius constant (1/min).
Similarly, the value of $V_{mo}\,(T)$ at any temperature, T, can be obtained from

$$V_{mo}\,(T) = V_{mo}\,(T_o) \cdot \mathrm{Exp}\,(-(E_a/R) \cdot (1/T - 1/T_o)) \quad (13)$$

where $V_{mo}(T_o)$ = initial maximum specific activity at the assay temperature, T_o; E_a = activation energy for hydratase (kcal/mol (K)).

Product and substrate concentrations as functions of time can be predicted by substituting Eqns. 12 and 13 into Eqn. 11. The material balance equation for a fed-batch hydration is:

$$d\,(SV)/dt = Q \cdot S_f - V_m \cdot S \cdot E \cdot V \cdot \mathrm{Exp}\,(-kt)/ (K_m + S + P \cdot (K_m/K_p) + S^2/K_s) \quad (14)$$

where V is the reaction volume (l), Q is the volumetric feed rate of substrate (l/min) and S_f(mM) is the substrate concentration in the feed.

Regardless of the feed rate schedule, the product material balance will be:

$$P \cdot V = S_o \cdot V_o + S_f \cdot V_f - P_o \cdot V_o \quad (15)$$

where V_f is the volume of feed added, V_o is the initial reaction volume and P_o is the initial product concentration.

Substituting $V_f = V - V_o$ into Eqn. 15 and rearranging, one gets:

$$P \cdot V = V_o \cdot (S_o - S_f) + V \cdot (S_f - S_o) \quad (16)$$

For the special case in which the feed rate is controlled so as to keep the substrate concentration constant at S_o (as done in this work), Eqn. 16 is:

$$P = ((V_o - V)/V) \cdot (S_f - S_o) \quad (17)$$

The weight of crude extract protein remains constant; therefore,

$$E \cdot V = E_o \cdot V_o \quad (18)$$

Combining Eqns. 14, 17 and 18, and noting that $dS/dt = 0$ (substrate concentration assumed constant), we get:

$$Q = dV/dt =$$

$$\frac{V_{mo} \cdot S_o \cdot E_o \cdot V_o \cdot \mathrm{Exp}\,(-kt)/(S_f - S_c)}{K_m + S_o + S_o{}^2/K_s + (K_m/K_p) \cdot (S_f - S_o) \cdot (1 - V_o/V)} \quad 19$$

Integration of Eqn. 19 yields:

$$(K_m + S_o + S_o{}^2/K_s + (K_m/K_p) \cdot (S_f - S_o)) \cdot (V - V_o) +$$

$$\frac{V_o \cdot (K_m/K_p) \cdot (S_f - S_o) \cdot \mathrm{Ln}\,(V/V_o)}{(V_{mo} \cdot S_o \cdot E_o \cdot V_o)/(k \cdot (S_f \cdot S_o))} =$$

$$1 - \mathrm{Exp}\,(-kt) \quad (20)$$

The value of V_{mo} at the reaction temperature can be obtained from Eqn. 13, and the reaction volume as a function of time can be calculated from Eqn. 20. These results can be sued in Eqns. 17 and 19 to obtain the product concentration and the feed rate (as functions of time), respectively.

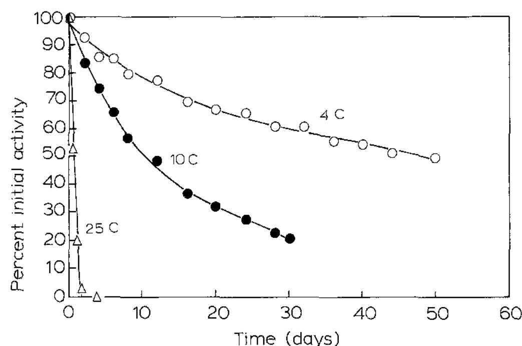

Fig. 1. Stability of crude extract hydratase (pH 8 phosphate buffer).

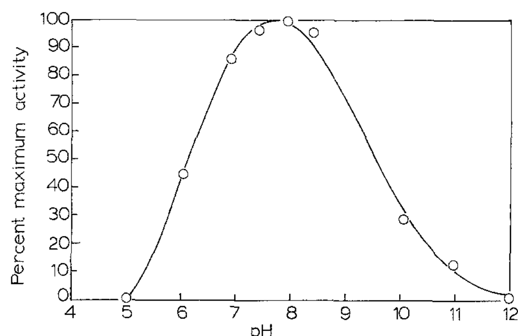

Fig. 3. Crude extract hydratase pH profile (25 °C).

RESULTS AND DISCUSSION

Thermal stability

Crude extract hydratase half-lives were 50 days, 10 days and 1 day at 4, 10 and 25 °C, respectively (Fig. 1). An Arrhenius plot of this data shows that the thermal decay activation energy, E_d, and the Arrhenius constant, k_o, are 41.78 kcal/mol and 8.84×10^{27} min^{-1}, respectively.

The crude extract hydratase half-lives were lower than those reported for whole-cell hydratase [2]. The reasons are not clear; we can speculate only that the cellular environment provides some protection against inherent thermal decay and/or degradation by *Brevibacterium* proteases. (See refs. 1 and 3 for further discussion of this point.) It also should be noted that this uncertainty causes some ambiguity in the exact meaning of the activation energy, E_d.

If the proteases are a significant problem in the crude extract, substantive increases in hydratase half-life might be effected by a fairly crude separation (or selective denaturation) followed by immobilization (which might also be done in a partially selective manner). Addition of protease inhibitors is another possibility, but this might be too costly for practical applications and might cause product purity problems.

Temperature and pH profiles

Crude extract hydratase temperature and pH profiles are given in Figs. 2 and 3, respectively. The maximum temperature (at pH 8.0) is 35 °C, but activity drops rapidly at higher temperatures: The hydratase activation energy, E_a is 7.307 kcal/mol. It should be noted also that although the maximum temperature was 35 °C, standard assays (see MATERIALS AND METHODS) were done at 25 °C because the enzyme half-life was less than 0.5 h at 35 °C.

The maximum pH (25 °C) is 8.0, and the activity drops to zero at pH 5.0 and at pH 12.0.

Maxima reported here are in close agreement with those published by Eyal and Charles [3] for whole-cell hydratase, and by Arnaud et al. [1] for free enzyme.

Rate constants

Fig. 4 contains double reciprocal plots for various product concentrations. Applying Eqn. 3 to the low substrate concentration asymptote for zero product concentration, we get a K_m = 28 mM and V_m = 5.8 μmol/min/mg protein

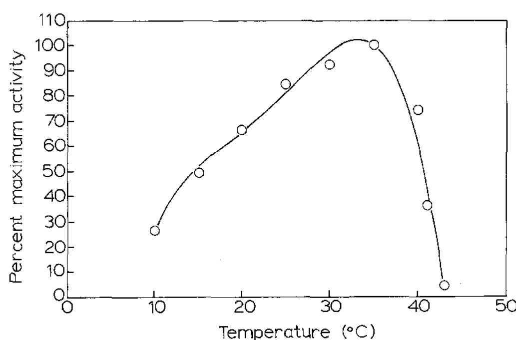

Fig. 2. Crude extract hydratase temperature profile (pH 8 phosphate buffer).

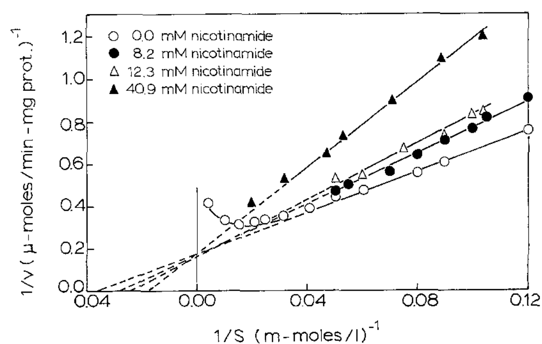

Fig. 4. Double reciprocal plots at various initial concentrations of nicotinamide (pH 8, 25 °C).

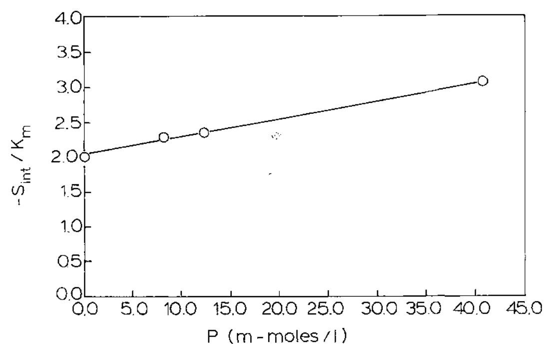

Fig. 5. Slopes of double reciprocal asymptotes vs. nicotinamide concentration.

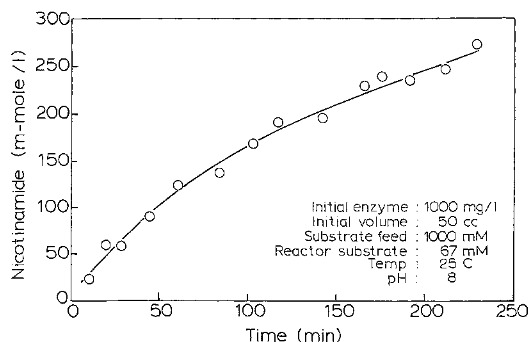

Fig. 7. Extended fed-batch hydration (——— model prediction).

(25 °C). The minimum in this curve is at $1/S = 0.015$; therefore; Eqn. 7 gives $K_s = 155$ mM.

The product inhibition constant, K_p, is obtained by plotting Eqn. 5 for the asymptotes of all the curves in Fig. 4. The result is given in Fig. 5. The slope (P/K_p) of this plot gives $K_p = 36$ mM. The inhibition appears to be competitive.

Nicotinic acid (produced by amidase hydrolysis of nicotinamide) was found to act as a mild competitive inhibitor of hydratase; the inhibition constant, K_i, was found to be 295 mM (determined in essentially the same manner used for K_p but at zero initial concentration of nicotinamide and at various initial concentrations of nicotinic acid). Inhibition of hydratase by nicotinic acid was not a significant factor in any of the hydration experiments reported here.

Extended hydrations

Model predictions agreed well with experimental results for batch and fed-batch hydrations (typical results are given in Fig. 6 and 7). In general, agreement was within 10% for product concentrations up to 300 mM: at higher concentrations agreement became poorer because of the increasing rate of hydrolysis of nicotinamide to nicotinic acid.

Simulations based on the kinetic model were done to evaluate the relative importances of substrate and product inhibitions. Results of one such study for batch hydration are given in Fig. 8. For this case, elimination of substrate inhibition, of product inhibition and of both might increase productivity 20, 40 and 60%, respectively. Other simulations for batch and fed-batch hydrations yielded similar results.

CONCLUSIONS

(1) Crude extract hydratase half-lives were 50 days, 10 days and 1 day at 4, 10 and 25 °C, respectively. Practical applications probably will require more stable enzymes. For strain R-312 hydratase, genetic modification to increase inherent thermal stability and/or to eliminate protese activity, selective denaturation of protease, partial purification of crude extract and selective immobilization of hydratase are routes which should be explored.

(2) The enzyme has a temperature maximum of 35 °C (pH 8), and has a pH maximum of 8.0 (25 °C).

(3) *Brevibacterium* R-312 hydratase is product/substrate inhibited. The kinetic constants are: $V_m = 5.8$ µmol/min/mg protein (25 °C); $K_m = 28$ mM; $K_p = 36$ mM; $K_s = 155$ mM. The enzyme also is inhibited

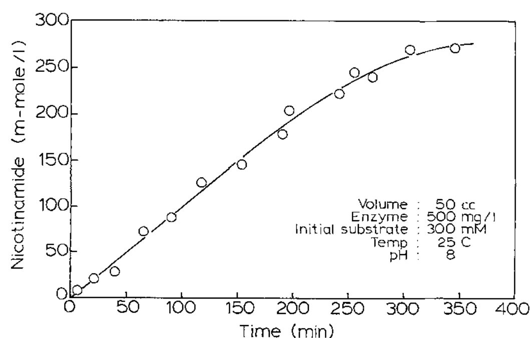

Fig. 6. Extended batch hydration (——— model prediction).

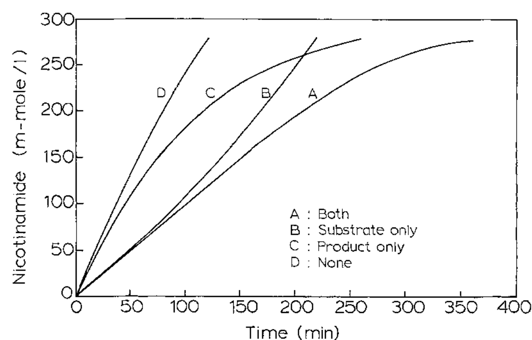

Fig. 8. Hypothetical extended batch hydrations after removal of either or both inhibitions.

mildly by nicotinic acid which gives an inhibition constant of 295 mM.

(4) The crude preparation contains amidase which hydrolyzes nicotinamide to nicotinic acid. Rates of hydrolysis do not become appreciable until the nicotinamide concentration reaches approximately 300 mM. This may be a problem in practical applications.

(5) A kinetic model incorporating the effects of product and substrate inhibitions and of enzyme decay predicts to within 10% experimental data for both batch and fed-batch hydrations for product concentrations up to 300 mM. Predictions at higher concentrations are less accurate primarily because of increased rates of nicotinamide hydrolysis by amidase.

(6) Product inhibition is a much more serious problem than is substrate inhibition which can be overcome by judicious feeding of substrate. Methods to minimize or to eliminate the product inhibition should be investigated.

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