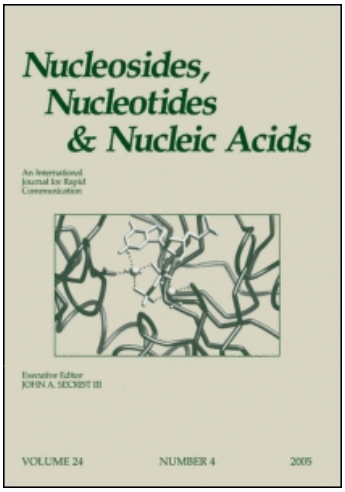

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Beach, Carol M. , Evans, Robert K. and Coleman, Mary Sue(1991) 'Strategy for Industrial Scale Production of Dideoxyinosine: Enzymatic Deamination of Dideoxyadenosine by Adenosine Deaminase', Nucleosides, Nucleotides and Nucleic Acids, 10: 7, 1499 — 1505
To link to this Article: DOI: 10.1080/07328319108046678
URL: http://dx.doi.org/10.1080/07328319108046678

NUCLEOSIDES & NUCLEOTIDES, 10(7), 1499-1505 (1991)

STRATEGY FOR INDUSTRIAL SCALE PRODUCTION OF DIDEOXYINOSINE: ENZYMATIC DEAMINATION OF DIDEOXYADENOSINE BY ADENOSINE DEAMINASE

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Abstract. A procedure has been devised for the production of dideoxyinosine (ddI) in high yield and purity. The method employs adenosine deaminase to deaminate 2',3'-dideoxyadenosine (ddA) to ddI. On the basis of systematic evaluation of various reaction conditions, a substrate concentration of 0.5 M ddA and an enzyme concentration of 5.6 units per ml were selected, and all buffers were eliminated. Under these conditions ddA was quantitatively converted to ddI within 4 hrs. Addition of 25% DMSO to the reaction facilitated the isolation of ddI as a result of the differential solubilities of the product and substrate, but did not affect the enzymatic reaction. An industrial scale reaction has been designed on the basis of the data presented.

The 2',3'-dideoxynucleoside, 3'-azido-2',3'-dideoxythymidine (AZT), has been demonstrated to inhibit the infectivity and cytopathy of human immunodeficiency virus (HIV). AZT is being used successfully in clinical settings to extend and improve the quality of life in both children and adults infected with HIV[3]. A potential problem with any antiviral drug is the emergence of drug resistant virus strains. In cancer therapy the emergence of drug resistance has been successfully suppressed by the administration of combinations of drugs. A closely related 2',3'-dideoxynucleoside, ddI, has been recently demonstrated to inhibit HIV in vitro[4], and has been shown to exhibit antiviral activity in patients.[5] Moreover, initial studies have shown that AZT-resistant strains of HIV selected in vitro retained susceptibility to ddI.[6,7] The apparent absence of cross-

resistance between AZT and ddI offers the promise of combination therapy with these drugs in HIV-infected patients. These theoretical clinical considerations have stimulated interest in the development of industrial scale procedures for the production of pure ddI.

Several procedures for the chemical synthesis of ddI have been reported.[8-11] We have investigated an alternate procedure based on the observation that ddA can be deaminated to ddI by the enzyme adenosine deaminase[11]. We describe herein the substrate properties of ddA, the differential solubilities of ddA and ddI in several solvents, and the reaction conditions suitable for the efficient production of kilogram quantities of ddI by enzymatic deamination of ddA.

EXPERIMENTAL

The efficiency of adenosine deaminase in deaminating ddA was assessed by comparing the specific activities of the enzyme determined with the standard substrate, adenosine, to that obtained with ddA as substrate. In this initial experiment standard reaction conditions for deamination of adenosine, 50 mM potassium phosphate buffer, pH 7.4, were employed, and 2.0 mg/ml bovine serum albumin was added to stabilize the diluted enzyme.[12] Conversion of substrate to product was monitored during the course of the reaction by chromatography on a Spherisorb S5ODS2 reverse phase HPLC column using a linear gradient consisting of 95% 0.05 M KH_2PO_4, pH 4.0, 5% methanol and 50% 0.05 M KH_2PO_4, pH 4.0, 50% methanol.[13] The specific activity of pure adenosine deaminase obtained with adenosine was 600-700 units per mg, versus with ddA was 500 units per mg. The high catalytic efficiency exhibited by this deamination reaction is ideally suited for industrial applications.

In order to tailor the standard reaction conditions to industrial scale product purification, each reaction component was systematically evaluated. First, the phosphate buffer was replaced by a volatile buffer, 50 mM NH_4HCO_3, pH 7.4. No decrease in the enzyme specific activity was observed. In order to minimize the reaction volume, conditions were sought for increasing the substrate concentration. Therefore the

TABLE 1. Solubility of ddA and ddI

Compound	Concentration	DMSO%	Buffer	Solubility[1]
ddA	2M	100%	-	S
ddA	1M	50%	phosphate	S
ddA	0.5M	25%	NH_4HCO_3	S
ddA	0.5M	0%	NH_4HCO_3	S
ddI	2M	100%	-	I
ddI	1M	50%	NH_4HCO_3	I
ddI	0.5M	50%	NH_4HCO_3	I
ddI	0.5M	0%	NH_4HCO_3	S

[1] The dideoxynucleosides were dissolved in solvent at 90°C, cooled to 37°C and maintained for several hours. S = soluble(100%), I = insoluble(100%)

solubility of ddA and the activity of adenosine deaminase were monitored in several organic solvents. Addition of dimethylsulfoxide (DMSO) up to 25% in either buffer had no effect on the reaction rate or extent. However, at 50% DMSO in either buffer, only 50% of the substrate was converted to product regardless of the time span of the reaction. Other organic solvents, for example dimethylformamide, were inhibitory to adenosine deaminase even at concentrations of less than 10% (data not shown). Under ideal conditions, the solvent used would maximize the solubility of the substrate, ddA, and minimize the solubility of the product, ddI. The differential solubilities of ddA and ddI in DMSO were therefore determined. At virtually all concentrations tested ddA was more soluble than ddI (Table 1).

Using the optimal conditions determined above; 50 mM NH_4HCO_3, and 25% DMSO, the time course of deamination of ddA to ddI was determined over a substrate concentration range of 0.5-0.9 M and at three enzyme concentrations. Deamination reactions contained 25% DMSO in 50 mM NH_4HCO_3, highly purified adenosine deaminase (including 2.0 mg/ml bovine serum albumin), and ddA at the concentrations indicated. Reactions

TABLE 2. Enzymatic Conversion of ddA to ddI

Substrate ddA	Enzyme (Units/ml)[2]	Conversion of Substrate to Product[1] 1.75hr	4hr	15hr	44hr
0.5 M	5.6	87%	99%[3]	99%	99%
0.5 M	0.56	9%	20%	37%	---
0.7 M	5.6	---	---	75%	77%
0.7 M	1.18	---	---	46%	37%
0.9 M	5.6	50%	77%[4]	---	---

[1] The reaction products were analyzed by HPLC on a 4.1 mm x 25 cm Spherisorb S5ODS2 + guard column. The mobile phase consisted of A: 95% 0.05 M KH_2PO_4, pH 4.0 + 5% methanol and B: 50% 0.05 M KH_2PO_4, pH 4.0 + 50% methanol with a linear gradient of 25% to 75% B in 20 minutes at 37°C. Under these conditions ddI elutes at 5.5 min and ddA elutes at 10 min.
[2] Units of adenosine deaminase are calculated based on the substrate adenosine.
[3] When precipitation occurred in the reaction, samples were heated prior to dilution (1:25) for HPLC analysis.
4 This reaction was completely precipitated by 8 hr and did not progress beyond 80% conversion of substrate to product.

were incubated at 37°C for the times indicated. At a substrate concentration of 0.5 M ddA, and an enzyme concentration of 5.6 units adenosine deaminase per ml, virtually complete deamination occurred within 4 hr. (Table 2). At higher concentrations of ddA (0.7 and 0.9M), complete conversion (99%) of ddA was not achieved at the enzyme concentrations tested.

In order to facilitate subsequent product purification, the dependence of the enzyme reaction upon the presence of buffer, bovine serum albumin (BSA), and DMSO was assessed by sequential elimination. During this reaction, the large quantities of NH_4^+ generated would change the pH of the solution from 7 to 9 or 10 in the absence of a buffer. Almost

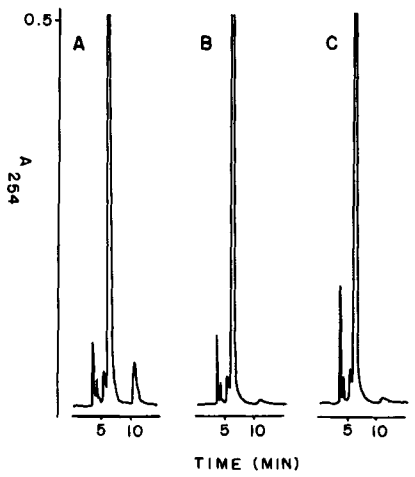

FIGURE 1

total conversion to ddI was observed after a 3 hr. reaction in the absence of BSA (Fig. 1A), indicating that the enzyme remained stable after dilution in the reaction. Likewise, elimination of DMSO did not result in substrate precipitation or in a loss of enzyme activity (4.5 hr reaction) (Fig. 1B). Finally, complete conversion of substrate to product (17 hr. reaction) was obtained in the absence of DMSO, buffer, and BSA (Fig. 1C) indicating that the basic pH of the reaction did not inhibit enzyme activity. The product was virtually 100% pure on the basis of the HPLC analyses.

Figure 1. Analysis of reaction products by HPLC. Aliquots of the reaction were heated and diluted 1:25 in water. The sample was analyzed by HPLC as decribed in the legend to Table 2. A. Reaction (3 hr.) contained 0.5 M ddA, 5.6 units/ml adenosine deaminase and 25% DMSO in ammonium bicarbonate buffer. B. Reaction (4.5 hr) contained 0.5 M ddA, 5.6 units/ml adenosine deaminase in ammonium bicarbonate buffer. C. Reaction (17 hr) contained 0.5 M ddA, 5.6 units/ml adenosine deaminase in water.

Based on the data presented above, an industrial scale reaction, designed for the production of 1 kilogram of ddI and extrapolated from pilot reactions, would contain 0.5 M ddA,

+/- 25% DMSO, and 50,000/units (or 72 mg) adenosine deaminase in 9.1 liters.[12,14] The reaction will be complete in less than 4 hours, however the data in Table 2 indicate that even at a 1/10 dilution the enzyme is still active up to 15 hours. These results suggest that it may be possible to reduce the enzyme concentration several fold and carry out the reaction overnight. Reactions carried out in 25% DMSO have the advantage that 33% of the product will precipitate if the reaction is cooled to 0°C. Additional precipitation occurs upon concentration for final product yields in excess of 90%. When the reaction is carried out in pure water, the product can be concentrated and recrystallized from methanol[11] to give ddI in greater than 90% yield. The product of the enzyme reaction was identical to an authentic sample of ddI by the criterion of coelution on reverse phase HPLC.

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Received 11/26/90
Accepted 2/15/91