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L-Asparaginase Production by the Rumen Anaerobe *Vibrio succinogenes*

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The rumen anaerobe *Vibrio succinogenes* possesses a constitutive L-asparaginase. The amount of enzyme produced is affected by the compound supplied to the organism to generate the fumaric acid it requires as a terminal electron acceptor. When nitrate is provided as the terminal electron acceptor, the amount of enzyme produced is affected by the compound provided to satisfy the nutritional requirement of the organism for succinic acid. Specific activities of up to 8.4 IU/mg of protein in cell-free extracts have been obtained. This specific activity is higher than has been previously reported for any organism. The enzyme has an apparent K_m of 1.7×10^{-5} M and low activity towards L-glutamine when assayed at pH 8.5.

Vibrio succinogenes is a rumen anaerobe that obtains its energy by coupling the oxidation of either hydrogen or formate to the reduction of either fumarate or nitrate. Wolin et al. (14) found that malate could serve as a substitute for fumarate or nitrate, presumably by being converted to fumarate intracellularly. Recently Niederman and Wolin (7) showed that, when nitrate is provided as the terminal electron acceptor, the organism exhibits an absolute nutritional requirement for small amounts of succinate (3 μmol/ml). This requirement can be satisfied by succinate, malate, or fumarate. It was also shown that L-aspartate or L-asparagine can satisfy this requirement, which suggests that the organism can convert these compounds to fumarate. Maloney and Wolin (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 143, 1972) demonstrated the presence of L-aspartase (EC 4.3.1.1) in cell-free extracts. The ability of L-asparagine to satisfy the succinate requirement suggests the presence of L-asparaginase (EC 3.5.1.1). The relationship of these compounds and enzyme activities is summarized in Fig. 1.

There has been much interest in the antileukemic properties of L-asparaginase. The L-asparaginases currently in use are obtained from various members of the enterobacteria. Unlike the enterobacteria in which the enzyme apparently has a scavenger function, *V. succinogenes* can be grown under conditions such that the L-asparaginase reaction is potentially growth limiting. Therefore, this organism might have the potential to produce large quantities of enzyme with a low K_m and high substrate specificity.

MATERIALS AND METHODS

Organism. *V. succinogenes* was obtained from M. J. Wolin, University of Illinois, and was maintained as described by Wolin et al. (14).

Cultivation methods: complex medium. The basal medium consisted of $(NH_4)_2SO_4$, 0.1% (wt/vol); K_2HPO_4, 0.25% (wt/vol); yeast extract, 0.1% (wt/vol); sodium formate, 0.27% (wt/vol) (0.04 M); and $FeSO_4 \cdot 7H_2O$, 0.002% (wt/vol). When organic electron acceptors were used, one of the following compounds was included in the medium at a final concentration of 0.04 M: Na fumarate, L-aspartate, L-malate, or L-asparagine. When nitrate was used as the electron acceptor, KNO_3 was added to a final concentration of

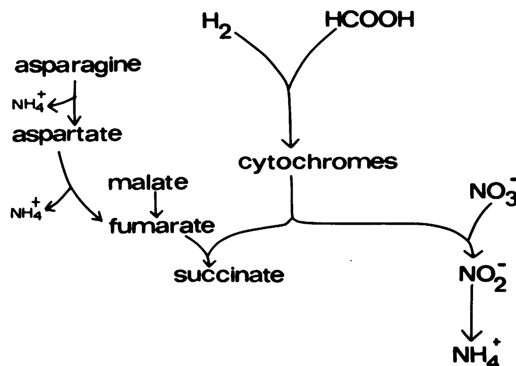

FIG. 1. *Electron transport and generation of terminal electron acceptors in V. succinogenes.*

0.07 M, and the succinate requirement was satisfied by one of the following compounds at a final concentration of 0.004 M: sodium succinate, sodium fumarate, L-aspartate, L-malate, or L-asparagine.

Two-liter cultures were prepared by autoclaving 1,900 ml of media (adjusted to pH 7.0 to 7.2), cooling the medium in cold water while gassing with N_2, adding sterile $MgCl_2 \cdot 6H_2O$ solution (0.4 g in 10 ml of water) and sterile sodium thioglycolate solution (0.5 g in 90 ml of water), inoculating with 1 ml of a 24-h culture grown in the medium of Wolin et al. (14), and sealing under N_2. After 18 to 22 h of incubation at 37 C, the cells were harvested by centrifugation at 4 C and washed twice with a solution containing (per liter): NaCl, 8.7 g; $MgCl_2 \cdot 6H_2O$, 2.03 g; and β-mercaptoethanol, 0.1 ml. The washed cell pellet was stored at −70 C until it was needed.

Defined medium cultivation. Yeast extract was deleted from the medium and replaced by a mixture of L-glutamic acid, L-alanine, and L-cysteine-hydrochloride, each at a concentration of 100 mg/liter. The medium was prepared as above, except that a 95:5 mixture of N_2-CO_2 was used to provide anaerobic conditions. The cultures were incubated for 48 h at 37 C.

Enzyme methods: routine assay. Cell-free extracts were prepared by sonicating cell pellets suspended in 0.1 M H_3BO_3 buffer (adjusted to pH 8.5 with NaOH and containing 2 mM ethylenediaminetetraacetate [BE buffer]). Cell debris was removed by centrifugation for 30 min at 24,000 × *g* at 4 C. Extracts were assayed immediately for L-aspartase, L-asparaginase, and L-glutaminase (EC 3.5.1.2) activity. Substrates were provided at a final concentration of 0.04 M in BE buffer, and the assays were conducted at 37 C. Liberated ammonia was determined with Nessler reagent. Protein was determined by the Lowry method using bovine serum albumin (fraction V) as the standard. Activities are reported as international units per milligram of protein (micromoles of ammonia liberated per minute per milligram of protein).

K_m determination. L-Asparaginase activity at low substrate concentrations was measured by using radioactive L-asparagine. L-[U-^{14}C]asparagine was diluted with carrier to a specific activity of 7.65 mCi/mmol. Samples containing from 0.2 to 8.0 nm were diluted to 40 μliters in BE buffer. Ten microliters of cell extract diluted in BE buffer (containing 5 mg of bovine serum albumin per ml) to contain 0.005 unit of L-asparaginase activity was added, and 10-μliter samples were withdrawn at appropriate time intervals. The samples were quickly spotted on strips of Whatman 3MM paper that had been previously spotted with 10 μliters of 5% trichloroacetic acid. The strips were subjected to electrophoresis in 0.04 M sodium acetate (pH 5.5) for 2 h at 200 V. The separated L-aspartate and L-asparagine were determined by liquid scintillation counting of the appropriate portions of the strips. The assays were performed in a 37 C constant-temperature room to avoid temperature changes in the small volumes of assay mixture. Control experiments showed that bovine serum albumin had no detectable L-asparaginase activity.

RESULTS

L-Asparaginase is constitutive. Table 1 presents the data obtained with cells grown in defined medium in the absence of L-asparagine, L-aspartate, or L-glutamine. The enzyme specific activity in fumarate culture is higher than that reported by Wade et al. (13) for all but a few of some 200 strains of bacteria representing 78 species.

Complex media studies. The relatively high specific activity of L-asparaginase obtained with cells grown on a defined medium led us to investigate the effects of the various organic compounds that can serve as electron acceptors or succinate sources on L-asparaginase levels. Because the enzyme is constitutive and yeast extract is highly stimulatory both in terms of growth rate and cell yields, these experiments were performed with complex media. Table 2 presents data obtained when organic electron acceptors were provided. Table 3 presents data obtained when nitrate was the electron acceptor and organic compounds were provided to satisfy the succinate requirement. The data presented are for at least duplicate cultures. The specific activities obtained were in some conditions the highest yet reported (13). The L-aspartase activ-

TABLE 1. *Enzyme specific activities in L-asparagine- and L-aspartate-free defined medium*

Electron acceptor	Enzyme activity (IU/mg of protein)	
	L-Asparaginase	L-Glutaminase
Fumarate	2.18	0.053
Nitrate + fumarate	0.88	0.033

TABLE 2. *Specific activity in cultures grown on basal medium with organic electron acceptors*

Electron acceptor	Enzyme activity (IU/mg of protein)	
	L-Asparaginase	L-Glutaminase
Fumarate	4.6	0.00
	4.0	0.07
L-Malate	5.5	0.09
	5.0	0.09
L-Aspartate	6.3	0.11
	6.6	0.10
L-Asparagine	7.8	0.12
	7.4	0.11

TABLE 3. *Specific activities in cultures grown on basal medium with nitrate as electron acceptor and on organic succinate source*

Succinate source	Enzyme activity (IU/mg of protein)	
	L-Asparaginase	L-Glutaminase
Succinate	8.4	0.13
	7.9	0.11
Fumarate	4.5	0.07
	4.1	0.07
L-Malate	6.5	0.09
	4.8	0.08
L-Aspartate	6.8	0.10
	7.8	0.10
L-Asparagine	3.5	0.06
	5.6	0.07
	2.7	0.05
	2.4	0.06

ity in the extracts was also determined since this enzyme liberates NH_4^+ from the product of the L-asparaginase reaction and can thus lead to an overestimation of L-asparaginase activity. Maloney and Wolin (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 143, 1972) reported that *V. succinogenes* L-aspartase requires divalent cations and sulfhydryl reagents. Our assay conditions included ethylenediaminetetraacetate and no added reducing reagents. L-Aspartase activity was never more than 1% of the L-asparaginase activity.

K_m determination. The finding that *V. succinogenes* can, under the appropriate conditions, produce high specific activities of L-asparaginase suggested that a determination of the K_m of the enzyme was warranted, since this is one of the parameters of importance in evaluating the potential usefulness of the enzyme for antileukemic therapy. Because this parameter can be estimated in crude cell-free extracts, we measured the initial velocity at L-asparagine concentrations varying from 4 to 160 μM. A Lineweaver-Burk plot fitted by the least squares method yielded an apparent K_m of 1.7×10^{-5} M (Fig. 2).

DISCUSSION

The data presented in this paper indicate that *V. succinogenes* produces a constitutive L-asparaginase. Synthesis of the enzyme is affected by electron acceptor or succinate source provided in the growth medium. The data obtained with organic electron acceptors (Table 2) follow the pattern that one would predict on the basis of the pathways outlined in Fig. 1. The nitrate culture data (Table 3) are anomalous with respect to the succinate and L-asparagine cultures. Succinate cultures have been found to have the highest specific activities, and asparagine cultures have been found to have variable but generally low levels of activity, the opposite of what would have been predicted. Distasio and Niederman have found that L-asparagine-nitrate cultures harvested at 15 h have high activities (approximately 8 IU/mg; personal communication). It is therefore possible that the details of the cultivation and harvesting procedure are important in determining the final yield of enzyme.

Wade et al. (13) reported that, of 200 strains of bacteria from 78 species, strains of *Erwinia carotovora* were the best L-asparaginase producers, yielding extracts that contained 2.9 to 5.3 IU/mg of protein. Examination of published purification schemes for L-asparaginases yielded the following initial specific activities in crude cell-free extracts: *Proteus vulgaris*, 1.1 (12); *Serratia marcescens*, 0.7 (2); *Erwinia aroideae*, 1.53 (6); *Escherichia coli*, 1.0 (4), 0.5 (8); *Bacillus coagulans*, 0.033 (5); *Achromobacter* sp., 1.8 (9); and *Mycobacterium bovis*, 2.74 (11). The data presented in this paper indicate that crude cell-free extracts of *V. succinogenes* grown under the appropriate condition can contain as much as 8.4 IU/mg of protein. Thus, *V. succinogenes* extracts possess an obvious advantage as far as enzyme purification is

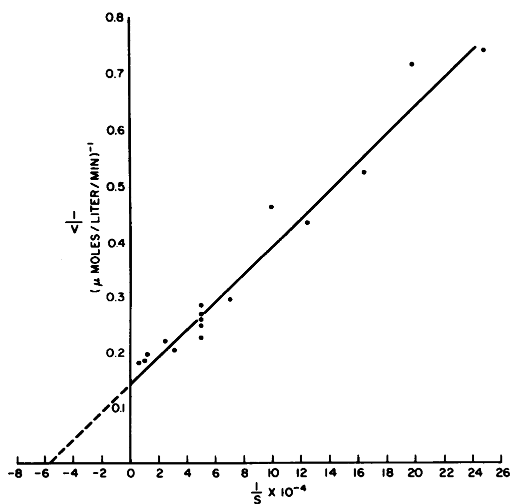

FIG. 2. *Lineweaver-Burk plot of V. succinogenes L-asparaginase.*

concerned. The composition of the growth media used in this study is the result of essentially arbitrarily chosen modifications of the medium described by Wolin et al. (14). The cell yield obtained with the medium of Wolin et al. (14) and with our modification of this medium is very low—usually 1.0 g (wet weight) per liter. The low yields are due to the pH rise accompanying the oxidation of formate. The CO_3^{2-} produced causes the pH to rise to 8.5, at which point growth stops. Preliminary experiments with a tris(hydroxymethyl)aminomethane-buffered, low-phosphate, complex medium have resulted in cell yields (estimated turbidimetrically) about four times as great as obtained in the medium of Wolin et al. (14). The final pH in the new medium is 7.8 (unpublished data). It is evident that a systematic study of the nutrition of this organism can yield media that will produce greater cell yields and possibly higher enzyme levels. There has been much work reported on the effects of the growth medium on enzyme production by members of the enterobacteria. Although the yields of enzyme can be improved by various medium modifications, it has been reported that *E. coli* does not produce larger quantities of enzyme when L-asparagine is added to either a complex medium or an amino acid-free defined medium (13). *V. succinogenes* does respond to the presence of L-asparagine in the medium, thus lending support to our belief that manipulation of the medium composition might yield greater enzyme production.

The K_m determination of the enzyme in diluted cell-free extracts yielded an apparent value of 1.7×10^{-5} M. This value is comparable to the values found for *E. coli* (1.25×10^{-5} M) (10) and *P. vulgaris* (2.6×10^{-5} M) (12). Since both these enzymes have been found to be clinically effective agents, it is possible that the *V. succinogenes* enzyme might also be effective. Among other parameters that must be considered are rate of clearance of the enzyme from the blood stream and immunological relatedness to other L-asparaginases. We have presented data for the L-glutaminase activity observed in cell extracts, since L-glutaminase activity is believed to be responsible for some of the toxic side effects of L-asparaginase therapy (1). Wade et al. (13) reported that specific L-glutaminases have a pH optimum of about 5. Thus, at pH 8.5, the standard pH for L-asparaginase assays, the L-glutaminase measured is presumed to be the activity of L-asparaginase on L-glutamine. The data we obtained indicate that *V. succinogenes* L-asparaginase has as low an L-glutaminase activity as most of the L-asparaginases yet described.

We suggest, on the basis of our data, that metabolically unusual bacteria may well be an unexploited source of potentially valuable products and that a careful examination of the literature, particularly with regard to anaerobes, may be preferable to mass screening of common bacteria.

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