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The Kinetic Investigation of D-Hydroxyisovalerate Dehydrogenase from *Fusarium sambucinum*

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The steady-state investigation of the mechanism of D-hydroxyisovalerate dehydrogenase was performed in order to understand this type of kinetic patterns. The initial velocity was measured with various amounts of both substrates, NADPH and 2-ketoisovalerate. Double reciprocal plots gave patterns that conversed on or near the abscissa. Binding studies indicated that NADPH bound first to the enzyme. The product $NADP^+$ was found to be a competitive inhibitor with respect to NADPH at a constant concentration of 2-ketoisovalerate. However, it showed non-competitive inhibition against 2-ketoisovalerate at a fixed amount of NADPH. Another product, D-hydroxy-isovalerate, was a non-competitive inhibitor *versus* NADPH and 2-ketoisovalerate at constant levels of 2-ketoisovalerate and NADPH, respectively. These results were comparable with an ordered bi-bi mechanism, in which NADPH bound first to the enzyme, followed by the binding of 2-ketoisovalerate. $NADP^+$ is the last product to be released. The ordered reaction manner of D-hydroxyisovalerate dehydrogenase from 2-ketoisovalerate to D-hydroxy-isovalerate allows the accurate regulation of valine metabolism and it may lead to the regulation of total biosynthesis of enniatins in the *Fusarium* species.

Keywords: Eniatin, D-Hydroxyisovalerate dehydrogenase, Kinetic mechanism.

Introduction

D-hydroxyisovalerate dehydrogenase (D-HIVDH) catalyzes the reversible reduction of 2-ketoisovalerate (2-KIV), the intermediate of branched-chain amino acid metabolism (Duggleby and Pang, 2000), to D-hydroxyisovalerate (D-HIV) in the presence of NADPH, which cannot be replaced by NADH (Lee *et al.*, 1992, Lee and Zocher 1996). D-HIV is an intermediate in the biosynthesis of the enniatins in the *Fusarium* species (Fig. 1). D-HIVDH has an important role in the biosynthesis of some depsipeptides and peptolides, such as enniatins (Audhya and Russel, 1973), beauvericin (Peeters *et al.*, 1983), and cyclosporin like peptolide (Smith *et al.*, 1975) that contain D-HIV in a molecule structure. So far, D-HIVDH has been purified exclusively from the enniatin producer, *Fusarium sambucinum* (Lee *et al.*, 1992), but it may be distributed in other D-HIV containing peptide antibiotic producers. D-HIVDH was partially characterized in our previous work (Lee *et al.*, 1992, Lee and Zocher, 1996), but many of its biological properties are still unknown. Further, understanding of the kinetic mechanism of this enzyme is a matter of interest, because it catalyzes the production of D-HIV, which is the intermediate in the biosynthesis of various peptide antibiotics. It may also play an important role in the regulation of enniatin and other peptide antibiotic biosynthesis. Various kinetic methods are used for dehydrogenases, such as yeast aldehyde dehydrogenase (Bradbury and Jakoby, 1971), malate dehydrogenase (Hsu *et al.*, 1967) and NADH-quinone oxidoreductase (Kim and Suk, 1998) in order to obtain the enzymological and kinetic properties. These kinetic studies present more information on

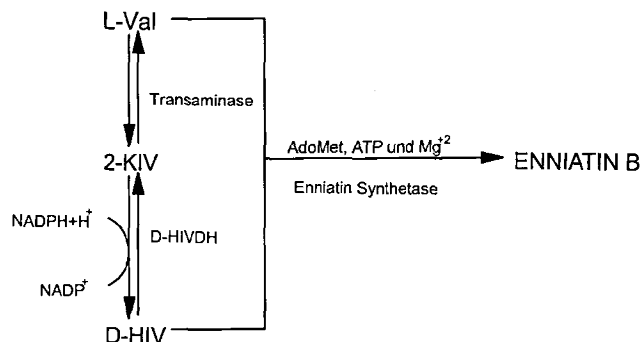

Fig. 1. Overall mechanism of enniatin biosynthesis in *Fusarium* species. AdoMet : S-adenosyl-L-methionine

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the function and structure of the enzymes. In this paper we described the kinetic mechanism of D-HIVDH and its possible role in the enniatin biosynthesis.

Materials and Methods

Materials All of the chemicals were obtained from commercial sources and were of the highest purity available. 2-KIV was purchased from Sigma. Radioactive 2-[^{14}C]KIV was prepared from radioactive L-[^{14}C]Val by L-amino acid oxidase and catalase (Ruediger *et al.*, 1972). D-HIV was the product of Serva (Heidelberg, Germany). NADP$^+$ and NADPH were obtained from Boehringer (Mannheim, FRG). Mono-Q and S-Sepharose Fast Flow were from Pharmacia (Uppsala, Sweden).

Enzyme preparation The maintenance and the cultures of *F. sambucinum* followed our previous procedures (Madry *et al.*, 1983). D-HIVDH was obtained by the purification methods of Lee *et al.* (1992). The rapid NaCl gradient (0-1 M) on S-Sepharose Fast Flow HR 5/5 cation exchange FPLC chromatography (Pharmacia, Uppsala, Sweden) was used for the final concentration of D-HIVDH instead of lyophilization. Enniatin synthetase (ESYN) was purified as described by Zocher *et al.* (1982).

Protein determination Protein concentrations were determined by a modified Bradford procedure (1976) with bovine serum albumin as a standard and the purity of the protein was analyzed as described by Hwang and Lim (1999).

Reaction conditions for the biological activities The enzyme activity was measured as previously described (Lee *et al.*, 1992). The standard D-HIV dehydrogenase assay mixture contained 50 mM potassium phosphate buffers pH 7.0, 0.5 mM 2-KIV, 0.54 mM NADPH, and enzyme in a final volume of 0.35 ml. The reaction was initiated by the addition of a substrate (2-KIV) and the decrease in absorbency of NADPH at 340 nm was measured at 35 by using an UVICON 930 spectrophotometer. A molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ of NADPH was used for the calculation of enzyme activity. One unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH/min under the standard assay conditions. Specific activity was expressed as units of enzyme activity per mg of protein.

ESYN activity was measured by the formation of radioactively labeled enniatin (Zocher *et al.*, 1982). [Methyl-^{14}C]-S-adenosyl-L-methionine served as a radio label.

Binding studies The binding of both substrates to D-HIVDH were tested by gel filtration on Ultrogel AcA 202 at 35 in 50 mM potassium phosphate buffer containing 4 mM DTE (pH 7.0). 2-[^{14}C]KIV (0.1 μCi) was added to 200 μl of D-HIVDH (20 μg) and loaded on Ultrogel AcA 202 column (0.5 × 7.0 cm). It was eluted by 50 mM potassium phosphate buffer containing 1mM of 2-KIV. The fractions were collected and the radioactivity in the enzyme fractions was measured.

The binding of NADPH to D-HIVDH was investigated by the techniques of fluorescent enhancement (Dugan and Porter, 1971) and by a modified method of Dean *et al.* (1989). D-HIVDH (0.5 μM) was mixed with NADPH (0.5 μM) in 50 mM potassium phosphate buffer (pH 7.0) in a final volume of 1 ml. After incubation for 5 min, the fluorescence emission spectra of NADPH were measured at 340 nm excitation by using a Perkin-Elmer fluorospectrophotometer MPF 44. Fluorescence excitation spectra were obtained at 450 nm emission.

Kinetic studies The initial reaction rate was measured as described in the reaction conditions for biological activities. In the product inhibition study fixed amounts of NADP$^+$ and D-HIV were added to the assay mixture at various concentrations of substrates, 2-KIV and NADPH, respectively. The measurement of the reaction was started 1 min later by the addition of 2-KIV. A modification of the spectrophotometer allowed the reaction to be followed starting 3 sec after the addition of 2-KIV. The values for the initial rate shown in the figures were obtained by means of duplicate measurements and the results were proportional to the enzyme amount at all concentrations of the substrate used.

Treatment of kinetic data The theory and nomenclature of Cleland (Segel, 1975a) were used for the treatment of kinetic data. The studies on the substrate binding and product inhibitions were performed in order to obtain the binding sequence of substrates and release of the products from the enzyme. Rate equations of the different possible bi-bi mechanism were derived. Denominators of the rate equations were generated by the method of King and Altmann (Segel, 1975b). The complete rate equations and the kinetic constants were defined in an analogous way that is described by Segel (1975c). The results were presented as double reciprocal plots and the kinetic constants were determined from replots of slopes and intercepts. The lines were fitted to the points by the methods of least-squares.

Results and Discussion

Initial velocity patterns in absence of products The kinetic mechanisms of the reaction catalyzed by the bireactant enzyme have been studied extensively. It can be largely classified into two possible classes; a sequential and ping-pong mechanism. The main difference is the formation of the enzyme-substrate complex. To form a ternary complex (enzyme·substrateA·substrate B) the substrate A and substrate B bind to the enzyme in an ordered manner, or a random manner in a sequential mechanism. In a ping-pong mechanism the enzyme reacts covalently with only one of the substrates to form the complex. After dissociation of the first product, the second substrate binds to the modified enzyme.

A series of steady-state kinetic analysis was carried out to investigate the type of reaction order of D-HIVDH for a reductive reaction. Initial velocity studies give some information about kinetic patterns of bireactant enzymes. Initial velocity studies for the reductive reaction of D-HIVDH were performed with pure D-HIVDH having a specific activity of 9,000 unit per mg of protein. Intersecting straight

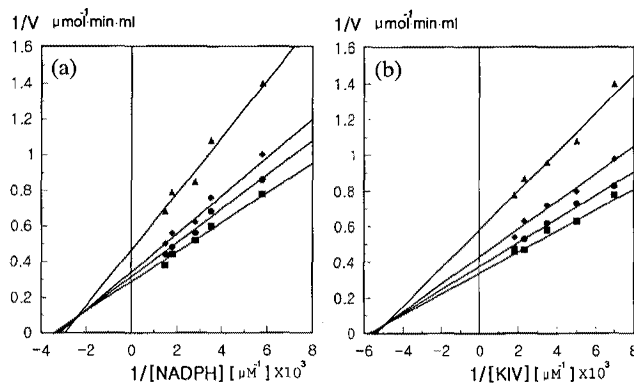

Fig. 2. Double-reciprocal plots of initial velocity.
(a) $1/v$ vs 1/[2-NADPH] at various concentrations of 2-KIV
▲: 570 μM, ◆: 430 μM, ●: 280 μM, ■: 140 μM
(b) $1/v$ vs 1/[2-KIV] at various concentrations of NADPH
▲: 570 μM, ◆: 354 μM, ●: 280 μM, ■: 170 μM

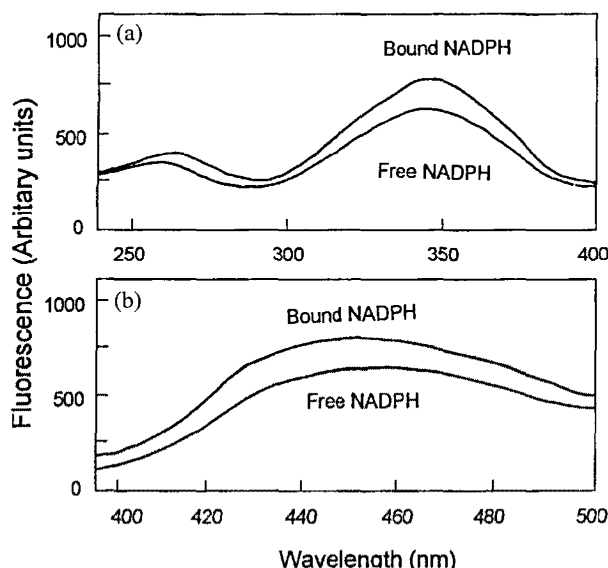

Fig. 3. Determination of enzyme-bound NADPH. 5 μM of NADPH in 50 mM potassium phosphate buffer pH 7.0, and 0.5 μM of D-HIVDH were used. Spectra are not corrected.
(a) Fluorescence emission spectra of NADPH, free and bound to D-HIVDH (340 nm excitation)
(b) Fluorescence excitation spectra of NADPH, free and bound to D-HIVDH (450 nm emission)

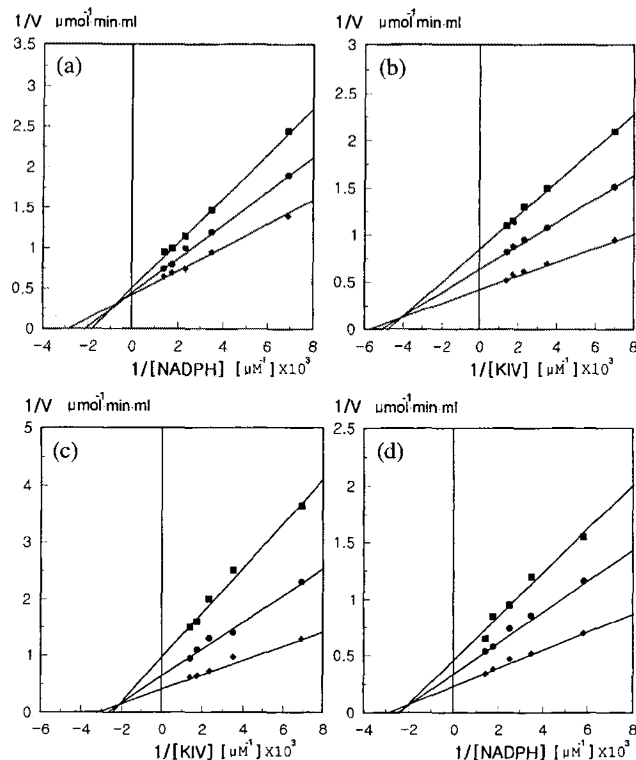

Fig. 4. Product inhibition analysis. 1.7 U of D-HIVDH in 50 mM potassium phosphate buffer (pH7.0) was used for the product inhibition study. Inhibition patterns by $NADP^+$ and D-HIV were analyzed with NADPH and 2-KIV as the substrate varied.
(a) $1/v$ vs [NADPH] with $NADP^+$: $NADP^+$ showed competitive inhibition with respect to NADPH.
■: 2.5 mM, ●: 1.2 mM, ▲: 0 mM
(b) $1/v$ vs [2-KIV] with $NADP^+$: $NADP^+$ gave non-competitive inhibition with respect to 2-KIV.
■: 2.5 mM, ●: 1.2 mM, ▲: 0 mM
(c) $1/v$ vs [NADPH] with D-HIV: D-HIV exhibited non-competitive inhibition with respect to NADPH.
■: 8.5 mM, ●: 5.5 mM, ▲: 0 mM
(d) $1/v$ vs [2-KIV] with D-HIV: D-HIV gave non-competitive inhibition with respect to 2-KIV.
■: 8.5 mM, ●: 5.5 mM, ▲: 0 mM

lines were shown in the double reciprocal plots of the initial velocity against NADPH in the presence of various fixed amounts of 2-KIV (Fig. 2a). The reciprocal plots of initial velocity against 2-KIV also exhibited intersecting straight lines in the presence of NADPH (Fig. 2b). The conversing patterns indicate that the binding of one ligand decreases the affinity of enzyme-substrate complex for the second substrate. This rules out the possibility of a ping-pong type of kinetic mechanism, since a ping-pong mechanism exhibits parallel lines and no crossing points in the double reciprocal plots. Since conversing initial velocity patterns are given by the ordered bi-bi, random bi-bi as well as theorell-chance mechanism, the reaction mechanism of D-HIVDH follows one of these rapid bireactant mechanisms. Binding and product inhibition studies were used to distinguish between these possibilities.

Binding studies Binding studies were performed to confirm which substrate binds first to the enzyme. The ordered addition of substrates is well established for many dehydrogenases. In the case of UDP-glucose dehydrogenase (Ordmann and Kirkwood, 1977), yeast aldehyde dehydrogenase (Bradbury, and Jakoby, 1971) and histidinol dehydrogenase (Buerger and Goerisch, 1981) the substrate binds first to the enzyme, followed by the binding of the

coenzyme. The other dehydrogenase binds first to the pyridine nucleotide, rather than the substrate. This binding pattern is shown for formate dehydrogenase (Peacock and Boulter, 1970), glyceraldehyde-3-phosphate dehydrogenase (Orsi and Cleland, 1972), and horse liver aldehyde dehydrogenase (Felden and Weiner, 1972). Binding of 2-KIV to D-HIVDH was examined by gel filtration chromatography. After gel chromatography on Ultrogel AcA 202 column, no radioactivity could be detected in the enzyme fractions (data not shown). This means that the substrate, 2-KIV, did not bind to the enzyme. It was completely separated from the reaction mixture by gel chromatography.

The binding of NADPH was tested by the method of fluorescence detection. Binding of NADPH to dehydrogenase changes the conformation of NADPH from a closed to an open form. The nicotinamide ring then associates with the hydrophobic groups of the protein. This effect results in the change of fluorescence spectra. A difference was observed in the fluorescence emission spectra of free NADPH and NADPH coupled with D-HIVDH (Fig. 3a). The wavelength of the maximum emission is shifted from 460 to 450 nm. These effects were not shown by NADH (data not shown). The excitation spectra for fluorescence by free NADPH and NADPH bound enzyme are shown in Fig. 3b. A marked difference was observed in the excitation spectra. These differences in the fluorescence properties between free NADPH and NADPH bound D-HIVDH means that NADPH binds to the enzyme without 2-KIV.

These binding assays suggest that only one substrate, NADPH, binds to the free enzyme. Therefore, the random bi-bi mechanism can be ruled out from possible kinetic patterns for D-HIVDH. Also, the reaction mechanism of D-HIVDH follows one of the ordered bi-bi and thorell-chance mechanisms.

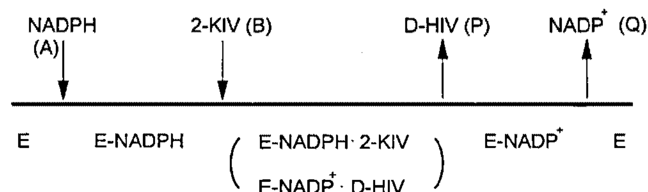

Fig. 5. Kinetic mechanism for the reductive reaction of D-HIVDH

The patterns of end product inhibition The patterns of end product inhibition give more information on the order of substrate binding. Product inhibition studies in the reductive reactions were performed to determine the order of substrate addition and product release (Fig. 4). One of the substrates was varied and the other was kept fixed in the presence of a constant amount of one of the products (D-HIV or $NADP^+$). The initial velocity of reductive reaction versus NADPH was determined at a fixed concentration of 2-KIV. With $NADP^+$ as an inhibitor, the double reciprocal plots of velocities showed a competitive inhibition versus NADPH at a constant level of 2-KIV (Fig. 4a). On the other hand, $NADP^+$ gave a non-competitive inhibition with respect to 2-KIV (Fig. 4b). These results suggest that NADPH and 2-KIV bind to the enzyme sequentially in the reductive reaction and $NADP^+$ is released from the enzyme at the last stage. Another product, D-HIV, showed non-competitive inhibition against NADPH at a fixed level of 2-KIV. It also exhibited non-competitive inhibition related to 2-KIV at a constant amount of NADPH (Fig. 4c, 4d). Since an ordered type of reaction would have led to one competitive and the other non-competitive, the results from the binding and kinetic studies (NADP+ *versus* NADPH, $NADP^+$ *versus* 2-KIV, D-HIV *versus* NADPH, and D-HIV *versus* 2-KIV) were identical with those predicted for the

$$v = \frac{V_{max}^{AB} \cdot V_{max}^{PQ}\left[A \cdot B - \frac{P \cdot Q}{K_{eq}}\right]}{K^A{}_i \cdot K^A{}_m \cdot V^{PQ}{}_{max} + K^A{}_m \cdot V^{PQ}{}_{max} \cdot B + K^B{}_m \cdot V^{PQ}{}_{max} \cdot A + V^{PQ}{}_{max} \cdot A \cdot B + \frac{K^Q{}_m \cdot V^{AB}{}_{max}}{K_{eq}} \cdot P + \frac{K^P{}_m \cdot V^{AB}{}_{max}}{K_{eq}} \cdot Q + \frac{V^{AB}{}_{max}}{K_{eq}} \cdot P \cdot Q + \frac{V^{AB}{}_{max} \cdot K^Q{}_m}{K_{eq} \cdot K^A{}_i} \cdot A \cdot P + \frac{V^{PQ}{}_{max} \cdot K^A{}_m}{K^Q{}_i} \cdot B \cdot Q + \frac{V^{PQ}{}_{max}}{K^P{}_i} \cdot A \cdot B \cdot P + \frac{V^{AB}{}_{max}}{K_{eq} \cdot K_i^B} \cdot B \cdot P \cdot Q}$$

$K^A{}_m \cdot K^B{}_m \cdot K^P{}_m$ and $K^Q{}_m$: *Michalis-Menten constant*

$K^A{}_i \cdot K^B{}_i \cdot K^P{}_i$ and $K^Q{}_i$: *dissociation constant*

K_{eq} : *equilibrium constant*

A, B, P, and Q : *concentrations of NADPH, 2-KIV, D-HIV and $NADP^+$*

Equation 1. Velocity equation of ordered bi-bi reaction mechanism for D-HIVDH

kinetic mechanisms as an ordered bi-bi mechanism as shown in Fig. 5. Eqn. 1 gives the rate equation of the ordered bi-bi reaction according to Segel (1975) in a kinetic constant form for D-HIVDH. A, B, P, and Q represented NADPH, 2-KIV, D-HIV, and $NADP^+$, respectively. As the reverse reaction could not be measured in a standard assay system, the kinetic constants for an oxidative reaction were not calculated. According to the mechanism NADPH is first bound to the enzyme, which lead to the enzyme-substrate binary complex. The second substrate, D-HIV, binds to the enzyme-NADPH complex following the formation of the ternary enzyme NADPH 2-KIV complex. The reduction equivalent is then transferred to the enzyme and continuously to 2-KIV. The product, D-HIV, is released and the enzyme $NADP^+$ complex is separated to the enzyme and the last product, $NADP^+$.

The role of reaction mechanism of D-HIVDH in the enniatin biosynthesis In the biosynthetic pathway of enniatins there may be a rate limiting step for the regulation of overall biosynthesis of enniatins. The valine metabolism in *Fusarium* for enniatin biosynthesis is a matter of interest. L-Valine itself is a substrate of ESYN and it is also deaminated to 2-KIV by valine dehydrogenase or transaminase. 2-KIV is then reduced to D-HIV by D-HIVDH for the biosynthesis of enniatins. At last ESYN synthesizes enniatin B from L-valine and its metabolite, D-HIV under the consumption of ATP, Mg^{2+} and AdoMet. Therefore, if the valine metabolism in *Fusarium* can be regulated, the production of enniatin may be controlled. No evidence exists for the possible control mechanism in the biosynthetic pathway of enniatins in the *Fusarium* species. One possible mechanism is the product inhibition. To see the feed back inhibition of enniatins to enniatin biosynthesis, we examined the inhibition of enniatins on the activity of ESYN and D-HIVDH. Up to 1 mM of enniatin A, B and C were added to ESYN and D-HIVDH assay system, respectively. They exhibited no inhibition on the activity of both ESYN and D-HIVDH. We also added each enniatin (1 mM) to the culture media and examined the level of D-HIVDH and ESYN in the cell of *F. sambucinum* during fermentation. Adding enniatins to the culture made no significant change in the amount of both enzymes (data not shown). There was also no change in the growth or biomass accumulation. These results rule out the possibility of feed-back inhibition by enniatins on the pathway of enniatin biosynthesis during fermentation. Therefore, the most possible regulation system in the *Fusarium* species is to control valine metabolism, especially from 2-KIV to D-HIV. From this aspect the ordered sequence of substrate addition for D-HIVDH may be beneficial for the biosynthetic system, since the unique site on the enzyme is modified by the ordered manner. This modification allows the accurate regulation of valine metabolism. This may lead to the regulation of total biosynthesis of enniatins in the *Fusarium* species.

The content of ESYN and D-HIVDH during fermentation suggests another role of D-HIVDH in *F. sambucinum*. As previously described in our paper (Lee *et al.*, 1992), ESYN and D-HIVDH behave like a constitutive of primary metabolism since they are present in the early log phase as well as in the stationary phase. The maximal activity of soluble D-HIVDH in cell-free extracts did not coincide with that of ESYN during the fermentation. The maximum ESYN activity was in the middle of the log period of the growth. Maximal activity of D-HIVDH was detected in the stationary phase, even though no increase in the enniatin level can be detected in this period. From this fact it can be supposed that D-HIVDH may play another role in the *Fusarium* species. According to Akimenko *et al.* (1982), two processes are associated with the discharge of excess reducing equivalents during the last growth phase of the fungus *F. sambucinum*. One is enniatin biosynthesis for the enniatin producers. Another is a cyanide resistant respiration for the enniatin non-producing strains. Only a small portion of ESYN activity can be detected during the stationary phase (Lee *et al.*, 1992). However, the maximal activity of D-HIVDH can be found in this period and it is the only highly activated enzyme related to enniatin biosynthesis. The D-HIVDH transfers the reducing equivalent from NADPH to 2-KIV for the enniatin biosynthesis. This phenomenon may lead to the discharge of a reducing equivalent in the stationary growth phase of the enniatin producers.

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