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Purification and properties of an alcohol dehydrogenase (HUADHII) from *Hanseniaspora uvarum* K_5

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C. VENTURIN, J. ZULAIKA, H. BOZE, G. MOULIN AND P. GALZY. 1995. An alcohol dehydrogenase HUADHII was purified 43·2-fold from *Hanseniaspora uvarum* K_5. The enzyme was trimeric with subunits of mol. wt 42 kDa. The *N*-terminal amino acid sequence of HUADHII has between 45 and 75% identity with part of the sequence of isoenzymes related to group I from *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. C2–C4 alcohols and aldehydes were the preferred substrates. The presence of an 'α' double bond increased the enzyme activity both for alcohols and aldehydes. It was significantly inhibited by metal-binding agents and thiol reagents. Kinetic studies suggested that HUADHII catalyses the oxidation of ethanol by a random sequential mechanism. It appears that HUADHII, a cytoplasmic fermentative enzyme, is structurally and functionally similar to members of the group I alcohol dehydrogenases.

INTRODUCTION

It has been widely reported that the early stages of wine fermentation are characterized by the growth of non-*Saccharomyces* species, which consist mostly of apiculate yeasts of the genera *Kloeckera* and *Hanseniaspora* (Heard and Fleet 1988; Martini and Martini 1990). These yeasts can ferment the grape must up to a maximum ethanol content of 5–6% v/v (Mateo *et al.* 1991; Zironi *et al.* 1993), lower than the maximum value obtained for *Saccharomyces cerevisiae* (15% v/v) (Lafon-Lafourcade 1983; Mauricio and Ortega 1993). Alcohol dehydrogenases are responsible for the final step of the pathway: they catalyse the reversible oxidation of ethanol to acetaldehyde with reduction of a pyridine nucleotide.

NAD(P)-dependent alcohol dehydrogenases of three different protein types have been characterized in detail. The first type, exemplified by horse-liver ADH, contained zinc-dependent long chain ADHs of subunit mol. wt around 40 kDa; the second type, exemplified by *Drosophila* ADH, contained short chain zinc-independent ADHs of subunit mol. wt around 28 kDa; the third type, exemplified by *Zymomonas mobilis* ADHII, contained 'iron-activated' ADHs of subunit mol. wt around 40 kDa (Reid and Fewson 1994).

ADHs of *S. cerevisiae* received most attention and four isoenzymes have, so far, been found. Three isoenzymes are very homologous and related to the first group: SADHI, the major fermentative isoenzyme; SADHII, which is glucose repressed; and SADHIII, which is localized in mitochondria (Ciriacy 1975). The fourth isoenzyme, SADHIV, is a cytoplasmic form encoded by a normally cryptic gene, ADH4, and related to the group III (Drewke and Ciriacy 1988). Nevertheless, alcohol dehydrogenases of apiculate yeasts, like *Hanseniaspora uvarum* or *Kloeckera apiculata*, have been studied.

In previous works, this group have shown that the strain *Hanseniaspora uvarum* K_5 produced ethanol only in some conditions (Venturin *et al.* 1994, 1995). The present paper describes the purification and characterization of an alcohol dehydrogenase (HUADHII) from this strain.

MATERIALS AND METHODS

Micro-organism, medium and culture conditions

The strain *H. uvarum* K_5 was obtained from the INRA Guadeloupe research station. It was listed as 1–845 in the

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National Micro-organisms Collection at Institut Pasteur (France). The culture medium consisted of yeast extract (Difco) 5 g l^{-1}, peptone (Difco) 2 g l^{-1} to which was added glucose 10 g l^{-1} (Prolabo, France) in distilled water. Cultures were performed in Erlenmeyer flasks filled to one-fifth of their capacity and incubated at 28°C with reciprocal shaking (80 oscillations per min, amplitude 7 cm).

Preparation of cell extracts

Cells were collected by centrifugation (5 min at 4000 *g*) at the end of the exponential phase, washed twice, and resuspended in sodium (Na_2HPO_4 . 12 H_2O)/potassium (KH_2PO_4) phosphate buffer 20 mmol l^{-1}, pH 7·50, containing 1 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} dithiothreitol (Boehringer, France) and 10% v/v glycerol (buffer A) in such a way as to obtain 100–200 g l^{-1} of dry cell material. Cell extract was obtained from crushed cells. Twelve ml of a cell suspension containing 1 g dry weight were homogenized in the presence of 8 g glass beads (diam. 0·45 mm) in a Braun MSK cell homogenizer for 2 min at speed two. The crushed material was separated to remove the glass beads and centrifuged for 10 min at 10 000 *g*. Protamine sulphate was added to the supernatant fluid (2·25 μg ml^{-1} of supernatant) to precipitate nucleic acids. After centrifugation for 90 min at 145 000 *g*, the supernatant fluid, containing ADH activity, was stored at −20°C.

Enzyme purification

All steps were carried out at 4°C, with the exception of the hydrophobic column chromatography, which was at 25°C.

Step 1. Cell extract (approx. 30 ml) was applied at 200 ml h^{-1} to a Q-Sepharose Fast Flow (Pharmacia) column (2·6 cm × 50 cm) pre-equilibrated with buffer A. Once loaded, the column was washed with 400 ml of the same buffer, followed by a linear gradient of 1200 ml of buffer to give finally, 0·3 mol l^{-1} NaCl. Buffer A, containing 0·5 mol l^{-1} NaCl, was used to desorb strongly adsorbed proteins. Fractions of 10 ml were collected. Active fractions, containing only HUADHII, were grouped, washed and concentrated by ultrafiltration (PM 10 000, Amicon, France) and resuspended in a 20 mmol l^{-1} pH 7·00 sodium-potassium phosphate buffer containing 1 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} dithiothreitol and 10% v/v glycerol (buffer B).

Step 2. The concentrate Q-Sepharose pool was applied at 60 ml h^{-1} to a Phenyl-Sepharose CL-4B (Pharmacia) column (1·6 cm × 18 cm). The column was washed with 100 ml of buffer B. The enzyme was eluted with 20% v/v glycerol in buffer B. Fractions of 3 ml were collected.

Step 3. The phenyl-Sepharose concentrated pool was applied at 30 ml h^{-1} to a 5'AMP-Sepharose (Pharmacia) column (1·6 cm × 18 cm). The column was washed with 60 ml of buffer B. The enzyme was eluted with 75 ml of 10 mmol l^{-1} NAD^+ in buffer B. Fractions of 3 ml were collected.

Step 4. The concentrated 5'AMP-Sepharose was applied at 30 ml h^{-1} to a Sephacryl S200-HR (Pharmacia) column (1·6 cm × 1 m) and then the enzyme was eluted with buffer B. Fractions of 2 ml were collected.

Analytical methods

Protein determination. The protein in the enzymatic extract and during purification was measured according to the method of Bradford (1976) after calibration with bovine serum albumin.

Enzyme assays. Activities were measured by monitoring the appearance of $NADH.H^+$ at 340 nm using a Beckman DU7 spectrophotometer according to the method of Bergmeyer *et al.* (1965). Ethanol oxidation activity was measured in the presence of 100 mmol l^{-1} ethanol and 3 mmol l^{-1} NAD^+ (Boehringer) in a medium buffered to pH 8·70 (sodium pyrophosphate buffer, 50 mmol l^{-1}) at 25°C. Acetaldehyde reduction activity was measured in the presence of 2 mmol l^{-1} acetaldehyde (Fluka, France) and 0·1 mmol l^{-1} $NADH.H^+$ (Boehringer) at pH 6·00 (sodium phosphate buffer, 50 mmol l^{-1}) at 20°C. Acetaldehyde was distilled before use. One enzymatic unit (U) was defined as the amount of enzyme converting 1 μmol of $NADH.H^+$ per min. Specific activity (U mg^{-1}) was the enzymatic activity related to the amount of proteins. Enzyme assays are means of, at least, triplicate determinations.

The following buffers were used to observe the influence of the pH on activity: citric acid–dibasic sodium phosphate buffer, 50 mmol l^{-1}, pH 4·00–6·00; sodium phosphate buffer, 50 mmol l^{-1}, pH 6·00–8·00; sodium pyrophosphate buffer, 50 mmol l^{-1}, pH 8·00–10·00.

Measurement of molecular weight

Gel exclusion-diffusion chromatography. The molecular weight of the native enzyme was determined by gel exclusion-diffusion on a Sephacryl S200-HR column during the last step of purification. The column was calibrated with molecular weight markers (Sigma) by the method of Andrews (1964): cytochrome c (mol. wt 12 400), carbonic anhydrase (mol. wt 29 000), bovine serum albumin (mol. wt 66 000), alcohol dehydrogenase (mol. wt 150 000),

β-amylase (mol. wt 200 000) and blue dextran (mol. wt 2 000 000).

SDS-PAGE electrophoresis. The molecular weight of HUADHII subunits was determined using SDS-PAGE denaturing conditions (12% in acrylamide) according to the method of Laemmli (1970). Low molecular weight standard proteins (BioRad, France) were used for calibration: lysozyme (mol. wt 14 400), soybean trypsin inhibitor (mol. wt 21 500), carbonic anhydrase (mol. wt 31 000), ovalbumin (mol. wt 45 000), bovine serum albumin (mol. wt 66 200) and phosphorylase b (mol. wt 97 400). Proteins were stained by the silver method (BioRad) for quantities of proteins ranging from 0·1 to 10 μg.

Protein sequencing

The *N*-terminus of alcohol dehydrogenase of *H. uvarum* K_5 was sequenced by J.C. Huet at INRA (Versailles) (93% sequence repetition). Sample preparation was carried out on the pure HUADHII extract by adsorption under PVDF membrane by centrifugation (PROSPIN Applied Biosystems, France) according to Sheer (1990). The sequence data presented in this paper have been submitted to GenBank and assigned accession number 94102.

RESULTS

Purification of HUADHII

During the first step of Q-Sepharose chromatography, two alcohol dehydrogenase activity peaks, non-separated, were eluted at 0·12 mol l^{-1} NaCl in buffer A (Fig. 1). The first peak contained two ADH bands (HUADHI and HUADHII) on gel electrophoresis under non-denaturing conditions, but a part of the second peak contained only one band (HUADHII) (data not shown). This last part was grouped, concentrated by ultrafiltration to be subjected to other chromatographies. The purification results of HUADHII are summarized in Table 1. After gel exclusion-diffusion, the alcohol dehydrogenase HUADHII was pure. The enzyme was purified 43·2-fold with a final specific activity of 186 U mg^{-1}.

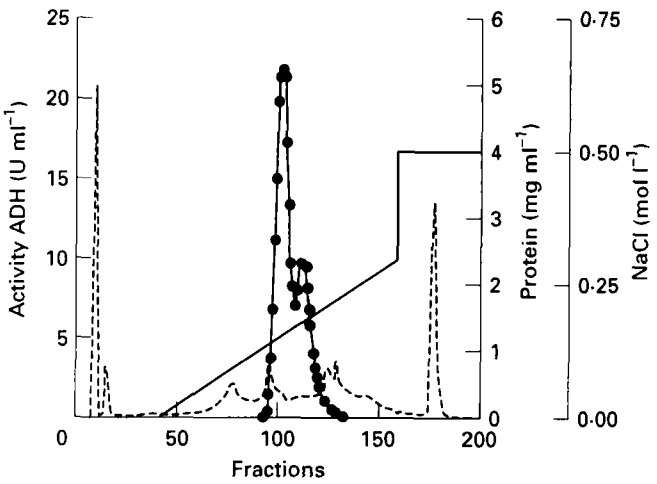

Fig. 1 Elution profile of *Hanseniaspora uvarum* K_5 in ion exchange chromatography on Q-Sepharose (column size, 2·6 cm × 50 cm; flow rate: 200 ml h^{-1}). ●, Activity alcohol dehydrogenase (U ml^{-1}); ..., proteins (mg ml^{-1}); —, NaCl

Molecular weight

The result of Sephacryl S200 HR gel exclusion-diffusion indicated a mol. wt value of about 120 ± 3 kDa for HUADHII. Maximum activity was a single sharp symmetrical peak. SDS-PAGE polyacrylamide gel electrophoresis showed a single band corresponding to a mol. wt value of 42 ± 5 kDa when compared with standard proteins (Fig. 2). The equivalence of the molecular weight found using two methods showed that the enzyme was trimeric.

N-terminal amino acid sequence

The sequence of the first 20 amino acids of the alcohol dehydrogenase from *H. uvarum* K_5 was determined and

Table 1 Purification of *Hanseniaspora uvarum* K_5 HUADHII

Purification step	Protein mg	Protein %	Activity U	Activity %	Specific activity (U mg^{-1})	PF
Crude extract	840	100	3584	100	4·3	1
Q-Sepharose (HUADHII)	86	10·2	1320	36·8	15·3	3·6
Phenyl Sepharose	11·3	1·35	1220	34	108	25·1
5'AMP Sepharose	4·05	0·48	664	18·5	164	38·2
Sephacryl S200-HR	1·64	0·20	305	8·5	186	43·2

PF, Purification factor.

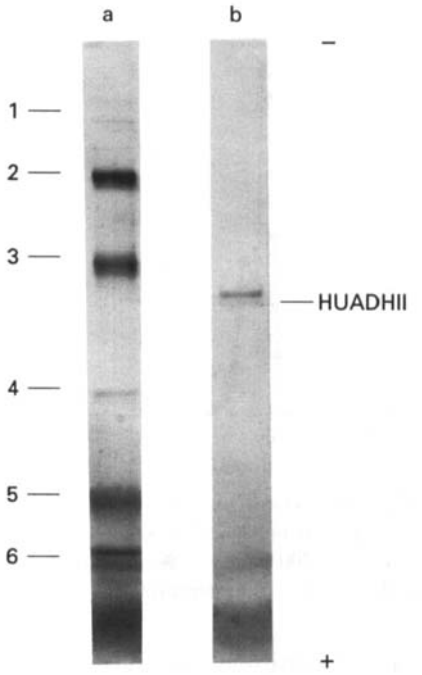

Fig. 2 Molecular weight measurement of HUADHII subunit by SDS-PAGE electrophoresis. Silver staining: a, standard proteins (BioRad); b, purified enzyme

then compared with other protein sequences. The other alcohol dehydrogenases to which any significant homology was observed, were those of yeasts related to group I (Table 2). HUADHII has between 45 and 75% homology with isoenzymes of *S. cerevisiae*, between 65 and 70% with isoenzymes of *K. lactis* and 25% with ADH of *Schizos. pombe*.

Effects of pH and temperature on enzyme activities

HUADHII was active in acetaldehyde reduction at pH values between 4·50 and 10·00, with an apparent optimum value at 6·50. In ethanol oxidation, the enzyme was active at pH values between 6·50 and 10·00, with an apparent optimum value at 8·00.

Effects of temperature (between 10° and 85°C) on the enzyme activity were examined in 50 mmol l^{-1} sodium pyrophosphate buffer (pH 8·00), under the standard assay conditions, with ethanol as substrate. Variation of activity in relation to temperature displayed an apparent optimum value at 55°C. The Arrhenius plot made it possible to calculate the apparent activation energy value for the reaction, $E = 25{\cdot}6$ kJ mol^{-1}.

Thermic denaturation of the enzyme was studied at various temperatures (between 25 and 60°C) for several times (0–50 min). The residual activity was determined under the standard assay conditions with ethanol as substrate at 25°C. A decrease of 50% in activity was observed after 5 min of treatment at 45°C. The apparent activation energy value of denaturation (E = 139 kJ mol^{-1}) was estimated using the Arrhenius plot.

Action of inhibitors

Several metal ions and other substances (0·05–10 mmol l^{-1}) were examined for their effects on HUADHII activity (Table 3) which was strongly inhibited by Cu^{2+} and Zn^{2+}, but not by Co^{2+}, Mn^{2+}, Ca^{2+}, Na^{+} and K^{+}. Mg^{2+} (10 mmol l^{-1}) reduced the initial reaction rate to 56%. Bromosuccinimide and pyrazole inhibited HUADHII. Metal-binding agents such as *o*-phenanthroline, potassium ferricyanide and EDTA and thiol reagents such as pCMB inhibited HUADHII activity. Inhibition by pCMB can be eliminated by addition of cysteine. DTT and cysteine, reducing agents for disulphide bonds, did not activate the enzyme at a concentration of 5 mmol l^{-1}. These studies as a whole showed that it was a thiol metallo-enzyme.

Substrate specificity

The highest activities were obtained with ethanol and acetaldehyde (100%). When primary alcohols or aldehydes

Table 2 Comparison of the *N*-terminal amino acid sequence of the NAD-dependent alcohol dehydrogenase (HUADHII) from *Hanseniaspora uvarum* K_5 with those of yeast alcohol dehydrogenases group I (Reid and Fewson 1994)

	10	20	
HUADHII	. . . . A V P K T Q	K G V T F Y E N N G	. . Q L K Y
SADHI	. . . M S I P E T Q	K G V I F Y E S H G	. . K L E H
SADHII	. . . M S I P E T Q	K A I I F Y E S H G	. . K L E Y
SADHIII	Q S T A A I P K T Q	K G V I F Y E N K G	. . K L E Y
KADHI	. M A A S I P E T Q	K G V I F Y E N G G	. . E L E Y
KADHII	. . . M S I P E T Q	K G V I F Y E N G G	. . E L Q Y
KADHIII	. L A T S V P E T Q	K G V I F Y E N G G	. . K L E Y
KADHIV	N S S F A I P E T Q	K G V I F Y E N G G	. . K L E Y
SPADH	. . . M T I P D K Q	L A A V F H T H G G	P E N V K F

SADHI, *S. cerevisiae* ADHI; SADHII, *S. cerevisiae* ADHII; SADHIII, *S. cerevisiae* ADHIII; KADHI, *K. lactis* ADHI; KADHII, *K. lactis* ADHII; KADHIII, *K. lactis* ADHIII; KADHIV, *K. lactis* ADHIV; SPADH, *Schizos. pombe* ADH.

Table 3 Influence of inhibitors on activity of HUADHII

Inhibitors	Concentration (mmol l^{-1})	Relative activity (%)
None	—	100
Metal ion		
$CuCl_2$	0·10	83
	1·00	10
$ZnCl_2$	0·10	70
	1·00	28
$MgCl_2$	1·00	90
	10·00	44
Other agents		
p-CMB	0·05	0
p-CMB + cysteine 2·5 mmol l^{-1}	0·05	83
EDTA	5·00	73
Potassium ferricyanide	1·00	0
o-phenanthroline	5·00	8
DTT	5·00	100
Cysteine	5·00	80
Bromosuccinimide	0·10	0
Pyrazole	5·00	4

Enzyme assay was performed at 25°C in the presence of 500 mmol l^{-1} ethanol, 3 mmol l^{-1} NAD^+ and 50 mmol l^{-1} sodium pyrophosphate buffer (pH 8·00).

were used as substrates, HUADHII activity decreased as the carbon chain of the alkyl group was elongated (Table 4). The enzyme showed low activities with secondary alcohols, sugar alcohols, cyclic alcohols or aldehydes. The enzyme was inactive on $NADP^+$ or on methanol, but it displayed slight activity on NADPH and formaldehyde. Its activity increased with substrates having an 'α' double bond (allyl alcohol and aldehyde, cinnamyl alcohol and aldehyde).

Kinetic studies

The kinetic analysis of HUADHII is summarized in Table 5. The kinetic constants (V_m, K_m (Michaelis constant) and K_i (inhibition or dissociation constant)) were calculated graphically from primary plots of the reciprocal of initial rates of reaction against the reciprocals of cofactor concentration for various substrates at constant concentrations, followed by secondary plots of their slopes and intercepts in the primary plots as a function of the reciprocals of the substrate concentrations. HUADHII operated by a sequential mechanism according to Cleland's Classification (Cleland 1963a, b).

The type of sequential mechanism was specified by study of inhibition by the products of the reaction (Cleland 1963c). The ethanol oxidation reaction was studied using acetaldehyde and $NADH.H^+$ as inhibitors (Fig. 3). Inhibition by acetaldehyde was non-competitive in relation to ethanol (acetaldehyde inhibition constants K_{ii} = 0·49 mmol l^{-1} and K_{is} = 0·071 mmol l^{-1}). Inhibition by $NADH.H^+$ was non-competitive in comparison with NAD^+ (inhibition constants of $NADH.H^+$, K_{ii} = 0·030 mmol l^{-1}, K_{is} = 0·022 mmol l^{-1}). These results are only compatible with a random sequential mechanism.

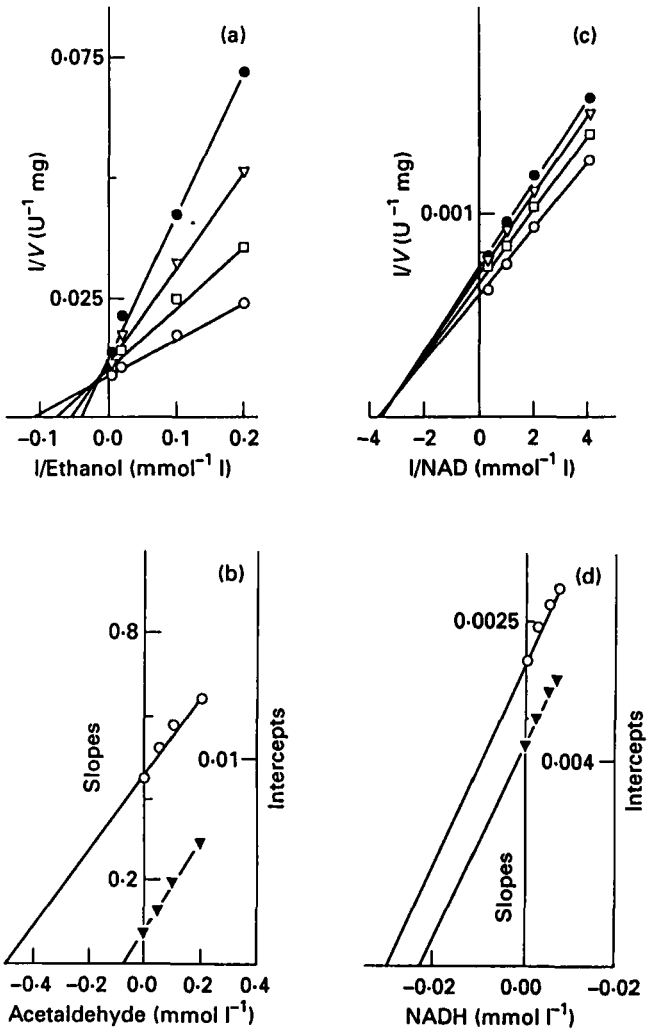

Fig. 3 Product inhibition patterns of ethanol oxidation of HUADHII from *Hanseniaspora uvarum* K_5. (a) Primary plot: inhibition by acetaldehyde with ethanol as variable substrate at constant NAD concentration (3 mmol l^{-1}). Acetaldehyde concentrations (mmol l^{-1}) were: 0 (○), 0·05 (□), 0·1 (▽), 0·2 (●). (b) Secondary plot: replots of slopes (▽) and intercepts (○) from (a) against the acetaldehyde concentration. (c) Primary plot: inhibition by $NADH.H^+$ with NAD^+ as variable substrate at constant ethanol concentration (500 mmol l^{-1}). $NADH.H^+$ concentrations (mmol l^{-1}) were: 0 (○), 0·0025 (□), 0·005 (▽), 0·007 (●). (d) Secondary plot: replots of slopes (▽) and intercepts (○) from (c) against the $NADH.H^+$ concentration. Standard errors ranged from 20 to 40%

Table 4 Substrate specificity of HUADHII

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Alcohol oxidation (a)		*Aldehyde reduction (b)*	
Ethanol (500)	100	Acetaldehyde (2)	100
Methanol (100)	0	Formaldehyde (7)	12
n-Propanol (250)	40	Propanol (3)	96
n-Butanol (33)	23	Butanal (1·5)	80
n-Pentanol (16)	9	Pentanal (0·7)	10·6
n-Hexanol (6)	3	Hexanal (0·1)	16
n-Octanol (0·6)	7	Octanal (0·1)	10
n-Dodecanol (1·2)	1	—	—
2-Propanol (12)	1	—	—
2-Butanol (50)	2	—	—
—	—	Isobutanal (0·2)	11
Isopentanol (0·8)	2	Isopentanal (0·2)	1
Benzyl alcohol (0·1)	3	Benzaldehyde (1·5)	0
Furfuryl alcohol (82)	0	Furfuraldehyde (5)	8
Allyl alcohol (250)	82	Acrolein (5)	69
Cinnamyl alcohol (3·3)	73	Cinnamaldehyde (0·2)	28
Glycerol (160)	2	Glyceraldehyde (1·5)	2
Sorbitol (30)	1	—	—
Galactitol (1)	2	—	—
NAD^+ (3)	100	$NADH.H^+$ (0·1)	100
$NADP^+$ (0·2)	0	$NADPH.H^+$ (0·15)	1

Percent activity was measured on 500 mmol l^{-1} ethanol and 3 mmol l^{-1} NAD^+ (a) and on 2 mmol l^{-1} acetaldehyde and 0·1 mmol l^{-1} $NADH.H^+$ (b).
For alcohol oxidation (a), activities were measured at 25°C in 50 mmol l^{-1} sodium pyrophosphate buffer (pH 8·00). NAD^+ concentration was fixed at 3 mmol l^{-1}. The alcohol concentrations used for this enzyme, expressed in mmol l^{-1}, were indicated in brackets.
For aldehyde reduction (b), activities were measured at 20°C in 50 mmol l^{-1} sodium phosphate buffer (pH 6·50). $NADH.H^+$ concentration was fixed at 0·1 mmol l^{-1}. The aldehyde concentrations used for this enzyme, expressed in mmol l^{-1}, were indicated in brackets.

Table 5 Kinetic constants of HUADHII

Kinetic constants	Ethanol oxidation (1)	Acetaldehyde reduction (2)
V_{max} (U mg^{-1})	126·7	817
K_m substrate (mmol l^{-1})	1·10	0·31
K_m cofactor (mmol l^{-1})	0·25	0·19
K_i substrate (mmol l^{-1})	23·9	0·020
K_i cofactor (mmol l^{-1})	0·94	0·0059

For ethanol oxidation (1), the activities were determined at 25°C in 50 mmol l^{-1} sodium pyrophosphate buffer (pH 8·00) as a function of different concentrations of NAD^+ (0·12, 0·25, 0·50, 1·00 and 2·00 mmol l^{-1}) with different concentrations of ethanol (5, 10, 50, 100, 200 and 500 mmol l^{-1}) and reciprocally.
For aldehyde reduction (2), the activities were determined at 20°C in 50 mmol l^{-1} sodium phosphate buffer (pH 6·50) as a function of different concentrations of $NADH.H^+$ (0·01, 0·25, 0·05, 0·10 and 0·25 mmol l^{-1}) with different concentrations of acetaldehyde (0·05, 0·10, 0·20, 0·50, 1·00 and 2·00 mmol l^{-1}) and reciprocally.
Cofactors were NAD^+ for ethanol oxidation and NADH for acetaldehyde reduction. Standard errors ranged from 8 to 37%.

DISCUSSION

This work was aimed at characterizing an NAD-dependent alcohol dehydrogenase of *H. uvarum* K_5, and the authors found apparent similar homology with yeast ADHs related to group I.

Comparisons of the *N*-terminal amino acid sequence obtained for the enzyme from *H. uvarum* K_5 with those of the other alcohol dehydrogenases described in the literature, revealed significant sequence identities with ADHs of group I. This enzyme showed very high homologies with yeast ADHs (KADHs and SADHs), up to 75% identical to the isoenzyme SADHIII of *S. cerevisiae*. In contrast, HUADHII share only 5–20% identity with the *N*-terminal amino acid sequence of bacterial enzyme, like other group I yeast ADHs (Reid and Fewson 1994). Many sequences have been aligned to that of horse liver (HLADH) isoenzyme (group I archetype). HLADH has little identity with HUADHII (10%). The most highly conserved part of the *N*-terminal sequence from group I yeast ADHs is the strech TQKGV.

The subunit mol. wt value of *H. uvarum* K_5 enzyme (mol. wt 42 kDa) were consistent with enzymes belonging to either group I or group III NAD(P)-dependent alcohol dehydrogenases. However, the apparent trimeric structure of the enzyme was unusual among alcohol dehydrogenases, most being monomers like ADHc of *Schwanniomyces castellii* (Mouillet-Loevenbruck *et al.* 1989), dimers like horse liver ADH, human ADHs (Sun and Plapp 1992) and ADH2 of *Candida guillermondii* (Indrati and Ohta 1993) or tetramers like isoenzymes of *S. cerevisiae* or *K. lactis* (Reid and Fewson 1994) and ADH1 of *Candida guillermondii* (Indrati and Ohta 1992). The only trimeric ADH reported of mol. wt 49 kDa has been isolated from *Amycolatopsis methanolica* and related to the group I alcohol dehydrogenases (Reid and Fewson 1994).

HUADHII has a narrow substrate specificity, like group I yeast ADHs, as compared to animal ADHs (Tsai *et al.* 1987). HUADHII appeared to prefer C2–C4 alcohols as substrates. The enzyme HUADHII showed very similar substrate specificities to SADHI, the cytoplasmic fermentative enzyme, of *S. cerevisiae* (Lustorf and Megnet 1968); similar substrate specificities to KADHI and KADHII, cytoplasmic fermentative enzymes, of *K. lactis* (Shain *et al.* 1992).

There was more evidence for the existence of a metal ion and thiol groups in this enzyme as judged by its high inhibition respectively by metal-binding agents and oxidants of SH terminals (pCMB). In the group I ADHs, a zinc atom and thiol group have been described as playing catalytic roles (Brändén *et al.* 1975; Reid and Fewson 1994).

Studies of reactional mechanism catalysed by HUADHII lead to the conclusion that the enzyme acts essentially according to a random sequential mechanism. Dickinson and Monger (1973) described mechanisms which have some characteristics of the random order type for yeast alcohol dehydrogenases. But, generally, the catalytic mechanism found most in the literature is ordered with the NAD^+ binding first: like SADHI of *S. cerevisiae* (Wratten and Cleland 1963; Ganzhorn *et al.* 1987), ADHc of *Schw. castellii* (Mouillet-Loevenbruck 1989) and ADH1 of *C. guillermondii* (Indrati and Ohta 1993).

Overall, there are enough structural and functional similarities between the alcohol dehydrogenases HUADHII of *H. uvarum* K_5 and SADHI of *S. cerevisiae* to suggest that the enzymes may be homologous and that they may have the same physiological role. HUADHII was proposed to be related to group I ADHs.

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