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SUBUNIT COMPOSITION OF ALCOHOL DEHYDROGENASE FROM *THEA SINENSIS* SEEDS AND ITS SUBSTRATE SPECIFICITY FOR MONOTERPENES

AKIKAZU HATANAKA, JIRO SEKIYA and TADAHIKO KAJIWARA
Department of Agricultural Chemistry, University of Yamaguchi, Yamaguchi 753, Japan

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Abstract—Alcohol dehydrogenase (alcohol:NAD oxidoreductase, E.C. 1.1.1.1.) was purified from *Thea sinensis* seeds. Its MW was 95000 and it was composed of two homogeneous subunits with MWs of 47000. The dissociation into subunits was caused by *o*-phenanthroline. Substrate specificity for monoterpene alcohols and aldehydes is discussed.

INTRODUCTION

Recently, several papers have reported partially purified alcohol dehydrogenases(ADHs) from plants [1–5]. These ADHs catalyze the oxidoreduction of aliphatic and/or aromatic alcohols and aldehydes.

We previously reported the purification and properties of ADH (alcohol:NAD oxidoreductase, E.C. 1.1.1.1) from tea seeds in connection with leaf alcohol biosynthesis [6]. We here describe the molecular weight, composition of subunits and substrate specificity for monoterpene alcohols and aldehydes, using purified tea seed ADH (TADH).

RESULTS AND DISCUSSION

Molecular weight and composition of subunits. The results of Sephadex G-200 gel filtration indicated a value of 95000 as the MW of TADH. Gel filtration with Sephadex G-150 also showed the same value. Polyacrylamide gel electrophoresis according to the Hedric-Smith method [13] also indicated the value of 95000. The sedimentation constant [7] of TADH, which was dissolved in 50 mM Tris–HCl buffer (pH 7·3) containing 10 mM mercaptoethanol at a protein concentration of 0·37%, was 5·03 S at 20°. A protein concentration dependency was not found (0·17–0·56%). The calculation of the MW from the sedimentation constant (5·03 S) and assumed values of $v = 0{\cdot}750$ ml/g and $D = 5{\cdot}95 \times 10^{-7}$ cm^2 sec^{-1} (for horse liver ADH [8]) resulted in a value of 85000, which supports the value of 95000 as the MW of TADH.*

Sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis showed a single band, which corresponded to a MW of 47000. This result indicates that TADH is composed of two homogeneous subunits with MWs of 47000.

Sephadex G-100 gel filtration of TADH treated with 10^{-3} M *o*-phenanthroline for 24 hr showed that 70% of its protein was eluted at a MW of 45000 and 30% at a MW of 95000. *o*-Phenanthroline treatment caused the dissociation of TADH into subunits and the loss of activity. Dialysis of dissociated TADH in the presence of both $ZnCl_2$ and NAD was the most effective for the recovery of enzyme activity (Table 1). The presence of only NAD or $ZnCl_2$ was not effective. Recovery of enzyme activity was probably due to the re-association of the subunits. The subunits of TADH may be partially bound by metal atoms, probably Zn.

Other plant ADHs have MWs similar to TADH [1,4], but their subunit compositions have not been reported. On the other hand, horse liver ADH (LADH, MW 80000) [9] and yeast ADH (YADH, MW 150000) [10] have been shown to be composed of two and four homogeneous subunits, respectively. Subunit composition of TADH is similar to that of LADH rather than that of YADH. The TADH as well as YADH [11] was dissociated into subunits on treatment with *o*-phenanthroline.

Substrate specificity. Monoterpene alcohols and their corresponding aldehydes were examined as substrates for the TADH as shown in Table 2. The acyclic terpenes were generally good substrates but the reactivities of the

Table 1. Dissociation and reactivation of *Thea sinensis* alcohol dehydrogenase

Treatment by *o*-phenanthroline (M)	Relative activity [%]		
	Before dialysis	After dialysis	
		None	$ZnCl_2$ + NAD
None	100	100	100
10^{-4}	17	20	50
5×10^{-4}	6	14	23

* From the above results, the MW of 150000 previously reported [6] must now be corrected to the value of 95000. The earlier value probably resulted from inappropriate conditions of gel filtration, for example, the use of partially crushed Sephadex G-200 gel.

Table 2. Substrate specificity of *Thea sinensis* alcohol dehydrogenase

Substrate	K_m (mM)	Relative V_{max}(%)	Substrate	K_m (mM)	Relative V_{max}(%)
Ethanol	14·30	100	Acetaldehyde	1·04	100
Acyclic					
geraniol	6·25	50	Citral*	0·67	3
Nerol	33·0	3			
Citronellol	1·83	10	Citronellal	0·44	396
Alicyclic					
Perillylalcohol	−†	+‡	Perillylaldehyde	−	+
Carveol	−	+	Carvone	−	+
α-Cyclogeraniol	−	+	α-Cyclocitral	−	+
β-Cyclogeraniol	−	+	β-Cyclocitral	−	+
l-Menthol	−	−	*l*-Menthone	−	+

* Mixture of citral a and b. † Calculation of the K_m value was not possible. ‡ The symbols + and − indicate slightly active (less than 1%) and inactive, respectively.

cyclic ones were slight or nonexistent. Of the 2-enoic alcohols, the *trans*-isomer (geraniol) was a much better substrate than the *cis*-isomer (nerol) as judged by the relative V_{max}. In contrast, for reduction, citronellal was extremely reactive and a mixture of citral a (*cis*-form) and b (*trans*-form) was slightly active.

The redox reaction of acyclic monoterpene alcohols and aldehydes was catalyzed by the TADH, but not that of cyclic ones. Potty and Bruemmer [1] have reported that geranyl dehydrogenase (GeDH) from the orange catalyzes the oxidation of geraniol and nerol. However the substrate specificity of GeDH differs from that of TADH. Another difference is that GeDH requires NADP and TADH requires NAD as a cofactor.

EXPERIMENTAL

Materials. Seeds of tea (*Thea sinensis* var. Yabukita), grown in the commercial tea garden near Yamaguchi, were used. Terpenoid compounds (purity > 99%) were purchased from Tokyo Chemical Co.

Purification of TADH and the enzyme assay. TADH was purified by the previously reported method [6] with a slight modification: 20 mM Ca^{2+} treatment for 30 min and centrifugation to remove the resultant precipitate were inserted before acetone fractionation. TADH was purified 1600-fold with a sp. act. of 13·4 units/mg protein. It was proved to be a single protein both by disc electrophoresis and ultracentrifugation. Enzyme activity was determined by the method previously described [6].

MW determination. MW of TADH was determined by Sephadex G-200 gel filtration according to the method of Andrews [12] and by polyacrylamide gel electrophoresis [13]. The following crystalline proteins served as MW markers: beef liver catalase (MW 230 000), soybean lipoxygenase (MW 102 000), bovine haemoglobin (MW 68 000) and Blue Dextran for gel filtration and soybean lipoxygenase, bovine haemoglobin and bovine serum albumin (MW 67 000) for disc electrophoresis.

SDS-polyacrylamide gel electrophoresis. MW of subunit of TADH was determined using SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn [14]. The following crystalline proteins served as MW markers: beef liver catalase (MW 60000 with SDS), ovalbumin (MW 43000), pepsin (MW 35500) and trypsin (MW 24000).

Dissociation and reactivation. TADH incubated with 10^{-4} M or 5×10^{-4} M *o*-phenanthroline at 4° for 6 hr. After the enzyme soln had been diluted 10-fold with 50 mM Tris–HCl (pH 7·2) containing 10 mM mercaptoethanol and 10% glycerol, and the enzyme activity was measured (indicated as activity "before dialysis" in Table 1). The diluted enzyme soln was dialyzed at 7° for 24 hr against the above buffer containing 10^{-5} M $ZnCl_2$, 5×10^{-5} M NAD, both $ZnCl_2$ and NAD, or no additions. After dialysis, the enzyme activity was measured (indicated as activity "after dialysis" in Table 1).

Substrate specificity. Each substrate was dissolved in dioxane. The substrate soln was added to a mixture of NAD (or NADH) in a final vol of 3 ml 10 mM pyrophosphate buffer (pH 7·5) and the enzyme activity was determined. The NAD concentration was 0·5 mM and NADH was 0·15 mM. The Michaelis constant (K_m) and maximum reaction velocity for each substrate were determined from Lineweaver–Burk plots [15].

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