Pergamon

Phytochemistry, Vol. 48, No. 1, pp. 35–40, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

PII: S0031-9422(97)01097-2

ALCOHOL DEHYDROGENASES FROM OLIVE (*OLEA EUROPAEA*) FRUIT

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(*Received in revised form* 27 *October* 1997)

Key Word Index—*Olea europaea*; Oleaceae; olive; fruit; oil aroma; alcohol dehydrogenase.

Abstract—Alcohol dehydrogenase activity was detected in extracts from the pericarp tissues of developing olive fruits using hexanal as the substrate. Total activity in the crude extract was 20-fold higher with NADPH than with NADH. Three discrete enzymes were resolved by means of a purification protocol involving ammonium sulfate fractionation followed by ion-exchange and affinity chromatography. One of the enzymes was NAD-dependent and displayed a high K_m for hexanal (K_m = 2.1 mM). Two NADP-dependent alcohol dehydrogenases were resolved, one showing a high K_m for hexanal (K_m = 1.9 mM) and the second with a lower K_m for the same substrate (K_m = 0.04 mM). The three enzymes have been partially purified and their kinetic parameters and specificities for various aldehydes determined. The involvement of these enzymes in the biogenesis of six carbon alcohols constituent of the aroma of olive oil is discussed. © 1998 Published by Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Alcohol dehydrogenases (ADHs), alcohol: NAD oxidoreductase (EC 1.1.1.1) and alcohol: NADP oxidoreductase (EC 1.1.1.2), catalyse the reversible reduction of aldehydes to alcohols, and are widespread in the plant kingdom [1–3]. The activity of these enzymes has been involved in processes such as resistance to prolonged anoxia [4], biosynthesis of cinnamic acid [5] and biogenesis of volatile constituents of the flavour of some fruits and vegetables [6–8]. In particular, ADHs have been reported as the enzymes responsible for the biosynthesis of certain six-carbon alcohols (hexanol, E-2-hexenol and Z-3-hexenol) constituents of the so-called "green odour" of some vegetables. These six-carbon alcohols, together with their acyl ester derivatives, are important constituents of the profile of volatiles of virgin olive oil [9, 10]. Because this oil is extracted by physical procedures (milling, malaxing and centrifugation) and moderate temperature (30°), the volatiles produced during the process are incorporated into the oil, and are responsible for its characteristic and priced aroma, which constitutes an important quality parameter of this product.

Labelling experiments with leaf tissues [11] have demonstrated that six-carbon alcohols are formed from polyunsaturated fatty acids through the lipoxygenase pathway. This pathway starts with the introduction of an oxygen molecule in the 1,4-pentadiene group of linoleic or linolenic acid to form the corresponding fatty acid hydroperoxide, a reaction catalysed by lipoxygenase. The resulting hydroperoxides are then split by the action of hydroperoxide lyase to yield a volatile aldehyde and a non-volatile oxoacid. The aldehydes so formed can be reduced to the corresponding alcohols by the ADHs.

This paper describes studies conducted to characterize ADHs present in the pulp of developing olives, which are responsible for the biogenesis of volatile alcohols constituent of the aroma of virgin olive oil.

RESULTS AND DISCUSSION

Previous investigations with ripening olives [12] failed to detect any ADH activity in cell free extracts from the pulp tissue, but tissue slices were able to reduce exogenously added six-carbon aldehydes. In the present study, ADH activity has been detected in cell free extracts from developing olives, using six-carbon aldehydes and either NADH or NADPH as the substrates.

The development of olive fruits is a slow process which lasts some eight months from anthesis to ripening [13] (Fig. 4), being one of the longest among crop fruits. It is common knowledge that enzyme activities vary during development of any living tissue

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or organ. It was important, therefore, to work out a protocol preserving enzyme activities of olives harvested at one particular stage of development. The acetone powder prepared from the pulp of developing olives was stable for months when stored in the freezer (−25°) under dry conditions. Moreover, the acetone powder was almost free of pigments and polyphenols, which are efficiently removed by acetone, making it a more suitable material for the preparation of active enzyme extracts. In fact, crude enzyme extracts prepared from fresh pulp tissues from developing olives by using conventional aqueous extraction protocols [14] displayed low NAD-dependent ADH (NAD-ADH) activity only, whereas extracts prepared from acetone powder as described in Experimental typically contained ADH activities equivalent to 10–20 nkat/mg protein when assayed with NADPH, as well as NAD-dependent activities which were some 20-fold lower (Table 1).

Enzyme fractionation

Enzyme extracts prepared from acetone powders were fractionated with ammonium sulfate and some 70% of the NADP-dependent ADH (NADP-ADH), the most abundant, was found in the 30–60% cut (Table 1). On the other hand, only 40% of the less abundant NAD-ADH activity was recovered in that fraction.

Ion-exchange chromatography resulted in the separation of two peaks of ADH activity (Fig. 1). The first peak (IE 1), which was not retained by the ionic exchanger, displayed NADP-dependent ADH activity only and accounted for about 40% of the total NADP-ADH activity. A second peak of activity (IE 2) was eluted from the column upon application of a NaCl gradient, this second peak contained the remaining 60% of the NADP-ADH activity together with the entire NAD-ADH activity.

Subsequent fractionation of the active pools obtained by ion-exchange chromatography was carried out by affinity chromatography on Cibacrom Blue-Sepharose. The first peak of activity (IE 1), containing NADP-ADH activity only, was tightly bound to the dye ligand but was eventually eluted from the column upon washing with 3 M NaCl (Fig. 2a). At this stage the enzyme (referred to as NADP-ADH I)

Table 1. Separation and partial purification of alcohol dehydrogenases from olive pulp

Fraction	NAD-ADH (nkat/mg prot)	NADP-ADH (nkat/mg prot)
Crude extract	0.5	11.6
30–60% $(NH_4)_2SO_4$ cut	0.5	19.3
DEAE-Sephadex-I		50.8
DEAE-Sephadex-II	3.3	30.0
Cibacrom Blue		
NAD-ADH	295	
NADP-ADH I		300
NADP-ADH II		337

ADH activity was measured by using hexanal (10 mM) and the stated pyridine nucleotide (0.2 mM) as substrates.

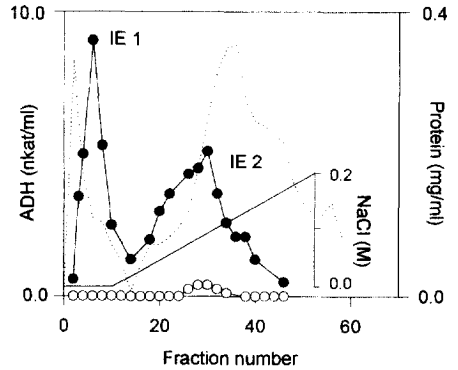

Fig. 1. Ion-exchange chromatography of the enzyme extract obtained after ammonium sulfate fractionation. —●—, NADP-ADH; —○— NAD-ADH; ··· protein.

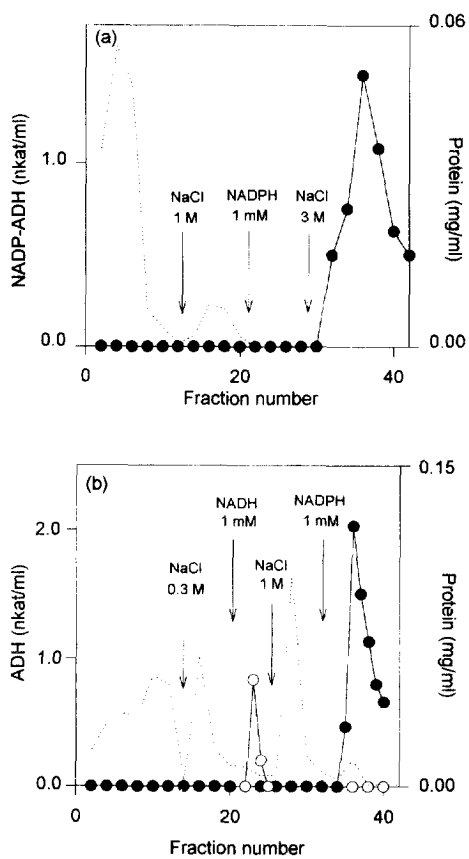

Fig. 2. Affinity chromatography of enzyme extracts obtained upon ion-exchange chromatography. (a) Elution profile corresponding to the IE 1 peak (Fig. 1) containing NADP-ADH activity only; (b) elution diagram of the IE 2 peak, which contained both NAD- and NADP-ADH activities. —●—, NADP-ADH; —○— NAD-ADH; ··· protein.

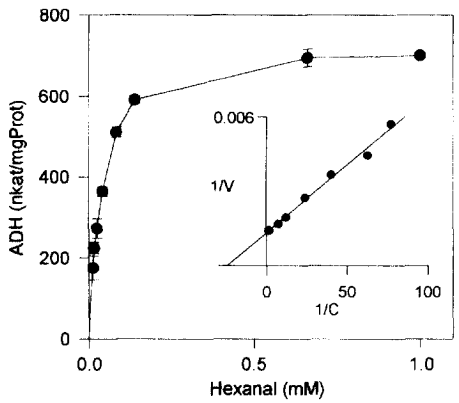

Fig. 3. Effect of hexanal concentration on the activity of NADP-ADH II. The inset shows the double-reciprocal plot of the data, which yielded a K_m value of 0.04 mM and a V_{max} of 740 nkat/mg prot.

had been purified 26-fold (Table 1) and was stable when stored at −25° in 50% glycerol.

The second peak of activity (IE 2), containing both NAD- and NADP-ADH activity, was also chromatographed on the dye column (Fig. 2b). The enzyme activity was bound to the ligand and could not be eluted by washing with 0.3 M NaCl. A first peak of activity was eluted when washing the column with 1 mM NADH. The column was then washed with 1 M NaCl, resulting in the elution of a peak of protein with no ADH activity, and subsequently with 1 mM NADPH, which eluted a peak displaying NADP-ADH activity only (NADP-ADH II). Thus, three different ADHs have been separated and partially purified by two successive chromatographic steps (Table 1).

Kinetic parameters and substrate specificity

Since six carbon alcohols are the most prominent components of the volatile profile of virgin olive oil [9, 10], hexanal was chosen as the standard substrate for the determination of kinetic parameters of the three ADHs isolated from the pulp tissue of developing olives. All the three enzymes displayed typical saturation kinetics when assayed with increasing concentrations of hexanal (Fig. 3).

The kinetic parameters of the three ADHs isolated from olive pulp are listed in Table 2. Among them NADP-ADH II displayed the lowest K_m for hexanal

Table 2. Kinetic parameters for hexanal of the three different ADHs from olive pulp

Enzyme	K_m (mM)	V_{max}(nkat/mg prot)
NAD-ADH	2.10	320
NADP-ADH I	1.90	280
NADP-ADH II	0.04	740

Table 3. Kinetic parameters of the low K_m NADP-ADH II

Substrate	K_m (mM)	V_{max} (nkat/mgprot)
Propionaldehyde	4.400	190
E-2-hexenal	0.012	520
Nonanal	0.030	560
NADPH	0.006	660

In the case of NADPH, hexanal was used as the second substrate at saturating concentration (1 mM).

and the highest V_{max}, suggesting that this might be the isoform actually involved in the generation of volatile alcohols during grinding and malaxing of olives in the process of olive oil extraction. The enzyme was further characterized by determining kinetic parameters for other carbonyl substrates and the reductant NADPH (Table 3). Among the substrates tested, the enzyme showed the lowest K_m for the unsaturated, six-carbon aldehyde E-2-hexenal (K_m = 12 μM), which is an intermediate of the lipoxygenase pathway from linolenic acid [15]. The enzyme also had a low K_m for the nine-carbon, saturated aldehyde nonanal (K_m = 30 μM), which is not an intermediate of the lipoxygenase pathway, and a very high K_m for propanal (K_m = 4.4 mM). The enzyme showed a low K_m for the reductant NADPH (K_m = 6 μM).

To further characterize the isolated enzymes, and to try to define their role in the biosynthesis of volatile alcohols, they were assayed with a number of aldehydes ranging from 2–9 carbon atoms, among them some intermediates of the lipoxygenase pathway from either linoleic or linolenic acids, such as hexanal, E-2-hexenal, E-2-nonenal and E-2-Z-6-nonadienal. As shown in Table 4 the isoform NADP-ADH II displayed the highest activities when assayed with those aldehydes involved in the lipoxygenase pathway, pointing again to its direct participation in the biogenesis of volatile alcohols. The other NADP-dependent ADH, NADP-ADH I, showed no activity with short chain aldehydes and the highest activity for nonanal, indicating specificity for long chain saturated aldehydes. The NAD-ADH, on the contrary, showed preference for shorter chained aldehydes, a high K_m for hexanal (Table 2) as well as low specificity for other aldehydes of six or nine carbon atoms (Table 4), thus precluding any significant role of this enzyme in the biogenesis of six-carbon alcohols and esters constituent of the aroma of olive oil.

On the other hand, the NADP-dependent ADH measured in crude extracts was over 20-times higher than that determined with NADH (Table 1). Two distinct NADP-dependent ADHs, displaying different kinetic parameters and substrate specificities, have been resolved. The first one, NADP-ADH I, showed high K_m for hexanal (Table 2) and high specificity for nine-carbon aldehydes (Table 4). Among the substrates used, the enzyme displayed the highest activity

Table 4. Substrate specificities of the three ADHs isolated from olive pulp

Substrate	NAD-ADH (nkat/mg prot)	%	NADP-ADH I (nkat/mg prot)	%	NADP-ADH II (nkat/mg prot)	%
Acetaldehyde	138±17	49	nd	0	nd	0
Propionaldehyde	262±2	92	nd	0	85±8	12
Butyraldehyde	270±2	95	nd	0	130±12	17
Hexanal	280±17	100	178±20	100	730±10	100
E-2-Hexenal	58±7	21	103±18	58	500±33	68
Nonanal	75±3	27	778±20	437	590±50	80
E-2-Nonenal	88±3	31	345±8	194	295±21	40
E-2-Z-6-Nonadienal	55±2	19	305±33	171	512±15	70

Aldehydes were assayed at 10 mM concentrations in the case of NAD-ADH and 1 mM concentrations for the two NADP-dependent ADH. In all cases the concentration of reduced pyridine nucleotide was 0.2 mM. Results are means of three determinations.

with nonanal, an aldehyde which is not an intermediate of the lipoxygenase pathway. These results suggest that this enzyme is not actually involved in the formation of six carbon alcohols present in the aroma of olive oil, and might be involved in the formation of both medium- or long-chain alcohols needed for the biosynthesis of some structural lipids.

The second NADP-dependent ADH resolved from olive pulp extract showed both kinetic parameters and substrate specificities which pointed to its actual participation in the biogenesis of volatile alcohols. As shown in Table 2, among the three resolved ADHs, NADP-ADH II displayed the lowest K_m for hexanal, with a K_m value 50-times lower than the other two enzymes, as well as the highest maximum velocity. The enzyme also showed a low K_m for E-2-hexenal, another intermediate of the lipoxygenase pathway, and for the reductant NADPH (Table 3), as well as a high K_m for propanal. Moreover, this enzyme displayed a clear preference for six and nine-carbon aldehydes (Table 4), which are intermediates of the lipoxygenase pathway leading to the formation of volatile alcohols. In the case of olives, however, the high activity of the enzyme when assayed with nine-carbon unsaturated aldehydes does not fit with the scheme, because neither nine-carbon unsaturated alcohols nor their corresponding esters are present in significant amounts in the aroma of olive oil [9, 10]. This apparent paradox is stronger in the case of nonanal, considering that this saturated aldehyde, although reported to be present in the aroma of olive oil [9], is not an intermediate of the lipoxygenase pathway [15].

Developmental profile of NADP-dependent ADH activity

To determine developmental variations of NADP-ADH activity, acetone powders were prepared from olives collected at different stages of development. The activity was determined in crude, non-fractionated extracts and using hexanal as the substrate at 1 mM concentration. Thus, results in Fig. 4 represent mainly activity of the low K_m NADP-ADH II, and show how it increased steadily from the time when lignification of the endocarp takes place, 13 weeks after flowering, which coincides with the onset of triacylglycerol synthesis and accumulation [12], up to 25 weeks after flowering, before the beginning of the ripening process, when more than half of the final oil content has been reached and the fruits are still green. After that point, a decline in ADH activity was observed when the fruit colour changed to purple along the ripening process. This observation fits in well with the analytical data reported by ref. [9], which showed that the content of six carbon alcohols in the aroma of olive oils underwent an overall decrease in parallel with the degree of fruit ripeness.

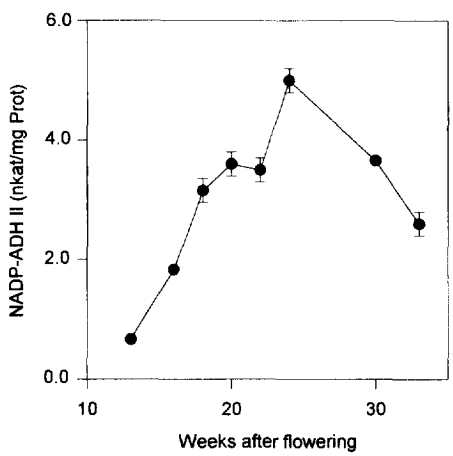

Fig. 4. Developmental profile of the NADP-ADH. Activities were measured using 1 mM hexanal and 0.2 mM NADPH.

To summarize, the results reported here suggest that volatile alcohols present in the aroma of olive oil, which have been identified as responsible for its characteristic and valued "green notes" [10], are formed from the corresponding aldehydes by the action

of a NADP-dependent ADH present in the pulp tissue of developing olives.

EXPERIMENTAL

Materials

Developing olives (24 weeks after flowering) were harvested from 25-year-old trees (cv Picual) growing in an orchard near Seville (Spain), which were endowed with drop irrigation and fertirrigation after full bloom.

Preparation of extracts and enzyme purification

An Me_2CO powder was prepared from the pulp tissue of olive fruits after removing the endocarps. Typically 100 g of pulp tissue was ground in 750 ml of cold (−20°) Me_2CO, using a domestic blender. The operation was performed in 3 successive steps using 250 ml of Me_2CO and filtering through paper under vacuum. The whitish powder was finally rinsed with dry Et_2O, sieved to remove coarse particles, and stored in a freezer (−25°) in flasks fitted with silica gel desiccator.

Enzyme extracts were prepared from 3 g of Me_2CO powder in 90 ml of a buffer consisting of 50 mM K-Pi, pH 7.2, 14 mM 2-mercaptoethanol (2-ME), 2 mM DTE and 10% glycerol (Buffer A), by using a manual glass homogenizer. The resulting homogenate was centrifuged at 27 000 *g* for 10 min. The clear supernatant was submitted to $(NH_4)_2SO_4$ fractionation, the 30–60% cut, containing most of the alcohol dehydrogenase activity, was desalted with Sephadex G-25 and submitted to ion-exchange chromatography.

A DEAE-Sepharose column (13 × 150 mm) equilibrated with 25 mM K-Pi, pH 7.2, buffer containing 7 mM 2-ME, 0.5 mM DTE and 10 % glycerol (buffer B) was used. After loading the desalted enzyme extract (9 ml) the column was eluted with 50 ml of buffer B, resulting in the elution of a first peak of activity which was found to be a NADP-dependent ADH, hereafter referred to as NADP-ADH I. Subsequently the column was eluted with a linear gradient of NaCl, from 0 to 0.2 M, in buffer B, which resulted in the elution of a second peak displaying both NAD- and NADP-dependent ADH activity (see Fig. 1 for elution profile and further details). Active fractions corresponding to both peaks of activities were pooled, concd by $(NH_4)_2SO_4$ precipitation and desalted with Sephadex G-25. Then they were submitted to affinity chromatography using a Cibacrom Blue-Sepharose column (10 × 50 mm) equilibrated with buffer B. The first peak of activity from the ion-exchange chromatography (NADP-ADH-I) was eluted from the affinity column by increasing the ionic strength of the mobile phase up to 3 M NaCl (see Fig. 2). The second peak of activity from the DEAE-Sepharose column, displaying both NAD- and NADP-activity, was eluted successively with 50 ml of buffer B and 50 ml of 0.3 M NaCl in buffer B. Then the column was eluted with 50 ml of 1 mM NADH in buffer B, which resulted in the elution of a peak displaying NAD-ADH activity only. Subsequently the column was washed with 50 ml of 1 M NaCl in buffer B and then with 1 mM NADPH in buffer B, which resulted in the elution of a peak displaying NADP-ADH activity and no NAD-dependent activity (see Fig. 3). The three fractions thus obtained were stored at −25° after addition of glycerol to 50%.

Enzyme assay

ADH activity was assayed in the reverse direction by measuring aldehyde dependent oxidation of pyridine nucleotides. The standard assay mixture contained 50 mM K-Pi, pH 7.2, 7 mM 2-ME, 0.5 mM DTE, 0.2 mM NADH (or NADPH), 10 mM hexanal and enzyme extract equivalent to 10–50 μg of protein, in a vol. of 1 ml. The reaction was started by addition of 10 μl of 1 M hexanol in EtOH. When studying substrate specificity the assay mixture was supplemented with 0.5% Tween-20 to the solution of aldehydes of nine carbon atoms.

Protein measurement

Protein was assayed according to ref. [16] using BSA as standard.

Acknowledgement—Supported by the European Commission (AIR3-CT94-1967).

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