Phytochemistry, 1979, Vol 18, pp. 401 404. Pergamon Press. Printed in England.

PARTIAL PURIFICATION AND PROPERTIES OF A *CIS*-3:*TRANS*-2-ENAL ISOMERASE FROM CUCUMBER FRUIT

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(*Received* 17 *July* 1978)

Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber; flavour; volatile aldehydes; fatty acid hydroperoxide; cleavage enzyme; *cis*-3:*trans*-2-enal isomerase.

Abstract—An enzyme, which catalyses the isomerisation of *cis*-3-enals to *trans*-2-enals, has been partially purified from cucumber fruit. The isomerase activity has been resolved from significant contamination by the related activities, lipoxygenase and hydroperoxide cleavage enzymes. An examination of the substrate specificity of the isomerase enzyme showed it to be specific for the *cis*-3-enals. The most efficient isomerisation was achieved with *cis*-3-hexenal and *cis*-3-nonenal which are, physiologically, the two most significant substrates. The *trans*-3-enal and *cis*-3-enol were not suitable substrates for the enzyme.

INTRODUCTION

The 6-carbon chain length volatile compounds called 'leaf aldehyde' and 'leaf alcohol' are formed on disruption of leaves and other plant tissue [1]. Also, C_9 volatile compounds with characteristic odour properties are formed on disruption of some plant tissues, notably the fruits of cucumber [2–4], melon [5] and green bananas [6]. It has been established that both C_6 and C_9 volatiles are formed from polyunsaturated fatty acids (linoleic and linolenic acids). They are not present in the intact tissue but are produced by a sequence of very rapid enzyme reactions initiated by cellular disruption under aerobic conditions [6–21].

The characteristic flavour of cucumber fruit is due mainly to C_9 aldehydes produced on disruption by cutting or maceration. The major flavour component was first identified as 2,6 nonadienal [2] and the olefinic configurations were subsequently identified as *trans*-2,*cis*-6 [3, 22, 23]. Later, *trans*-2-nonenal and *trans*-2-hexenal were shown to contribute to the cucumber flavour profile [21] and, recently, the *cis*-3 isomers of these aldehydes (i.e. *cis*-3-hexenal, *cis*-3-nonenal and *cis*-3-*cis*-6-nonadienal) have also been identified in the volatile fraction from cucumber [5, 9, 24]. Radioactive labelling studies with cucumber have demonstrated that the nonenals are derived from linoleic acid whereas the hexenals and nonadienals come from linolenic acid [12, 25]. A biosynthetic pathway to the production of these volatile aldehydes in cucumber has recently been elucidated in this laboratory [13–15, 25]. Linoleic and linolenic acids, liberated from endogenous lipid by acyl hydrolases, are converted to their hydroperoxides by lipoxygenase enzyme; subsequent enzymic cleavage of the hydroperoxides forms C_6 and C_9 aldehydes. Unsaturated aldehydes as released from the cleavage reaction have *cis*-3 olefinic bonds but these can isomerise to form the conjugated *trans*-2 derivatives.

Similar pathways to that outlined above have been proposed for the formation of unsaturated aldehydes in other plant tissues, viz. bean [29], *Thea sinensis* [8] and *Farfugium japonicum* [27] leaves; banana [6] and tomato fruits [17, 19]. However, the formation of the *trans*-2-enal end products, found in many of these tissues, has not been characterised. Recently, good evidence for an enzymic isomerisation of *cis*-3-enals to the *trans*-2-enals has been obtained with crude extracts of cucumber fruit [14, 25] and of leaves from tea [7] and *Farfugium japonicum* [27] in which it was shown that the isomerisation was more rapid in crude extracts than the non-enzymic isomerisation reaction and was catalysed by a heat labile factor in the extracts [14].

In the present work, we demonstrate the enzymic nature of the isomerisation reaction by partial purification and characterisation of the enzyme.

RESULTS

Enzyme purification and MW estimation

The enzyme could be kept in a soluble state only by maintaining the detergent level at all stages of the purification, since the protein was prone to aggregation and gelling in its absence [25]. A more efficient and less time-consuming concentration procedure, however, was developed. On gel filtration with Ultrogel AcA 34, the cleavage activity was eluted just after the void volume so, although an accurate MW cannot be quoted, it is *ca* 200000. The peak of isomerase activity followed closely behind and had a MW *ca* 150000. The fractions were also assayed by the oxygen electrode for lipoxygenase and a peak of activity was found to be following the isomerase. Table 1 shows the degree of purification achieved by this procedure. The pooled fractions were called the partially purified enzyme preparation and this was used in all further studies.

Substrate specificity

The purified enzyme was incubated against a series of *cis*-3-unsaturated aldehydes of different chain length

Table 1. Partial purification of *cis*-3:*trans*-2-enal isomerase from cucumber fruit

Enzyme preparation	Total protein (mg)	Isomerase activity (μmol/min/ml)	Specific activity (μmol/min/mg)	Recovery (%)
Crude supernatant	60	0.049	0.123	100
Ammonium sulphate concentrate	46	1.400	0.142	95
Ultrogel AcA34 (pooled fractions)	1.1	0.060	1.364	20

Incubation mixtures contained substrate, *cis*-3-nonenal (2.35×10^{-4} M), and enzyme preparations as indicated, in total volume of 3 ml of 0.1 M borate buffer pH 8.5. Incubations were run at 25° for 15 min. The reaction products were extracted with 1 ml of pentane containing 20 μg octanal as internal standard and analysed by GLC.

from 6 to 9 carbons. The non-volatile product of cleavage of the 13-hydroperoxide of linoleic acid, 12-oxo-*cis*-9-dodecenoic acid, was also included in the survey. The enzyme showed activity against each of these substrates. In addition *trans*-3-nonenal and *cis*-3-hexenol were also incubated with the partially purified isomerase activity but these were ineffective as substrates (Table 2).

The opportunity was taken after the purification to check the substrate specificity of the purified cleavage enzyme. Under conditions which produced 50% cleavage of both 9- and 13-hydroperoxy-linoleic acid, the purified cleavage enzyme showed no evidence of cleavage of linoleic acid nor linolenic acid, above the control level.

pH Optimum

The purified isomerase enzyme had an optimum activity at pH 8.5 with half-maximal values at pH 5.5 and 9.5. The crude enzyme had shown a further peak of activity at pH 4 but this had been shown to be present in a boiled extract, due to a non-enzymic factor.

Stability

The preparation showed loss of activity during the purification procedure and the enzyme was unstable even when stored at 0° in 2 mM dithiothreitol (DTT) and 0.1% Triton X-100, losing *ca* 50% of its activity in 24 hr. The partially purified enzyme lost 70% of its activity after boiling for 5 min.

Studies on the response of the partially purified enzyme to possible inhibitors or activators showed no significant changes. No inhibition of activity was achieved by incubating with 10^{-2} M KCN or 10^{-2} M EDTA, or in the presence of 10^{-2} M *p*-chloromercuribenzoate. No change was noted when the isomerisation was carried out in the presence of several different SH- compounds or with a range of transition metals including Mn^{2+}, Mg^{2+} and Fe^{2+} all at 10^{-2} M.

Enzyme kinetics

Using the partially purified enzyme preparation, a Lineweaver–Burk plot of V^{-1} against $[S]^{-1}$ was linear and the apparent K_m value obtained for *cis*-3-nonenal as substrate was 10^{-4} M. A plot of enzyme concentration versus product formed was linear over the range used for these experiments.

DISCUSSION

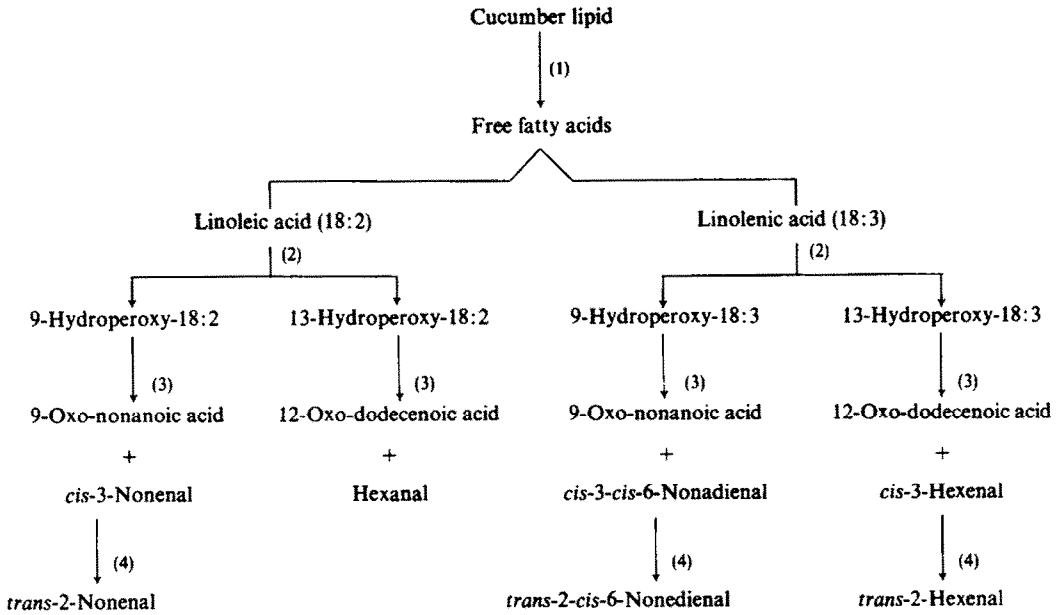
The enzymic biosynthetic pathway to the production of the major volatile cucumber flavour components has how been elucidated (Scheme 1). It has been shown that the isomerisation of the *cis*-3-enals to *trans*-2-enals is effected by a non-dialysable, heat labile enzyme activity and this enzyme has been purified free of other related activities. Despite the instability of the enzyme, even under the influence of protecting agents at 0°, a 55-fold purification with respect to protein content was achieved.

The purification described showed the cleavage enzyme and the isomerase to be associated with two distinct proteins. This differs with evidence presented for a similar enzyme complex in watermelon seedlings [28], where

Table 2. Substrate specificity of the partially purified isomerase enzyme from cucumber fruit

Substrate	Volatile products Mean % conversion of substrate	Identity
cis-3-Nonenal	80	*trans*-2-nonenal
cis-3-Octenal	48	*trans*-2-octenal
cis-3-Heptenal	40	*trans*-2-heptenal
cis-3-Hexenal	80	*trans*-2-hexenal
cis-3-Hexenol	—	—
trans-3-Nonenal	—	—
12-Oxo-*cis*-9-dodecenoic acid	72	12-oxo-*trans*-10-dodecenoic acid

Incubations containing 100 μg of aldehyde and an amount of enzyme equivalent to 2 g fr. wt of tissue in a total volume of 3 ml of 0.1 M borate pH 8.5 buffer were run at 25° for 15 min. The reaction products were extracted in 1 ml pentane and analysed by GLC.



Scheme 1. Proposed pathway for the formation of C_6 and C_9 volatile compounds in cucumber fruits. The enzyme reactions are catalysed by (1) lipid acyl hydrolase, (2) lipoxygenase, (3) hydroperoxide cleavage enzyme, (4) *cis*-3; *trans*-2-enal isomerase.

it was stated that the cleavage and isomerase activities were both associated with the same protein. The substrate for the isomerase in that instance was the 12-oxo-*cis*-9-dodecenoic acid, the product from the cleavage of 13-hydroperoxy-linoleic acid, and the product was the 12-oxo-*trans*-10-dodecenoic acid. The examination of the substrate specificity of the purified cucumber isomerase has shown that the enzyme effects the *cis*-3 to *trans*-2 isomerisation in the unsaturated aldehydes at various chain lengths as well as of the 12-oxo-*cis*-9-dodecenoic acid. It was of note that the most efficient isomerisation was achieved with the C_6 and C_9 aldehydes, physiologically the two most significant substrates.

In their recent publication, Vick and Zimmerman [28] suggest that the 12-oxo-*trans*-10-dodecenoic acid, formed on cleavage of 13-hydroperoxy-linoleic acid, is involved in the biosynthetic pathway to the production of the wound hormone 'traumatic acid' which has the structure *trans*-10-dodecendioic acid. This would require only an an ω-oxo-acid dehydrogenase and the presence of such a specific enzyme has been demonstrated in the leaves of *Vicia faba* [30]. This would provide a more reasonable physiological justification for the existence of this biological system in plant tissue.

EXPERIMENTAL

Cucumbers were purchased locally; the origin varied throughout the season and varieties were not known.

Enzyme preparation and purification. An aq. extract of cucumber was prepared in a Moulinex juice extractor from 2 parts of diced cucumber tissue with 1 part of 0.2 M Pi buffer pH 7 containing 0.2% Triton X-100 and 4 mM DTT. The extract was filtered through Miracloth and centrifuged at 60000 *g* for 1 hr. The supernatant was called the crude supernatant. The crude supernatant was precipitated with $(NH_4)_2SO_4$ at 80% satn. Since the detergent present in the soln prevented the sedimentation of the precipitated protein by centrifugation, the activity was concd on to a glass fibre filter disk. The ppt. was dissolved from the glass fibre in a small vol. of buffer and dialysed against the eluting buffer for 1 hr. The whole sample was loaded on to a column of LKB Ultrogel AcA34 (100 × 2 cm) in the eluting buffer 0.1 M Pi pH 7 containing 0.2 mM DTT and 0.1% Triton X-100. The activity was eluted and *ca* 2.5 ml fractions were collected at 3.5 ml/cm/hr flow rate. The fractions were

Substrate preparation. The *cis*-3-alkenals were prepared by the method of ref. [31] via the 1-methoxy-alk-1-en-3-yne formed by reaction of 1-methoxybut-1-en-3-yne with the appropriate alkyl halide in the presence of liquid NH_3 (method B, *loc. cit.*). It was found that generation of the free aldehyde from the dimethylacetal was accompanied by formation of an impurity containing a conjugated enone system (IR 1695 cm^{-1}), probably the *trans*-2-alkenal. The extent of this impurity ranged from 10% in the *cis*-3-nonenal and *cis*-3-octenal preparations to 40% in the prepn of the *cis*-3-hexenal. A degree of purification could be achieved by high vacuum distillation but, to obtain samples of the purity required for this study, it was necessary to use prep-GLC. *trans*-3-Nonenal was prepared in a similar manner to the *cis*-isomer, except that the intermediate 1,1-dimethoxynon-3-yne was reduced in an unexpectedly low yield (*ca* 30%) using Na and liquid NH_3. The sample was again purified by prep-GLC which was carried out on a column (2.1 m × 9 mm) of 10% 20M on 90–100 mesh CQ-celite. The carrier gas was Ar and the temp. was programmed from 75° to 160° at 2°/min. The 12-oxo-*cis*-9-dodecenoic acid was prepared from vernolic acid as described previously [17]. All compounds were characterised by PMR, IR and GC–MS.

Assay methods. The incubation conditions are to be found in the figure legends. The volatile carbonyls were analysed by GLC as in [14]. Lipoxygenase was assayed using an O_2 electrode at pH 5.5 with NH_4 linoleate as substrate [32]. Protein was assayed by the method of ref. [33] after dialysis against H_2O.

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