Food Chemistry **31** (1989) 243–250

Inhibition of Green Bean Lipoxygenase by Cyanide

J. B. Adams

Campden Food and Drink Research Association, Chipping Campden, Gloucestershire GL55 6LD, UK

(Received 11 March 1988; accepted 22 April 1988)

ABSTRACT

Cyanide, probably in the undissociated acid form, is shown to be a weak competitive inhibitor of green bean lipoxygenase (inhibitor constant, $K_i = 1{\cdot}54 \times 10^{-3}$ M KCN). Inhibition of the enzyme in a crude preparation was less sensitive to cyanide concentration (apparent $K_i = 1{\cdot}09 \times 10^{-2}$ M KCN). It is proposed that haem-proteins in the crude material preferentially react with cyanide, thereby lowering its effective concentration.

INTRODUCTION

The enzyme lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.12) catalyses the formation of hydroperoxides from polyunsaturated fatty acids containing a *cis*, *cis*-1,4-pentadiene moiety. Degradation of the hydroperoxides can cause unacceptable flavour changes (Sessa, 1979) and, by reaction with pigments, can lead to the loss of the characteristic colour of some products (Klein *et al.*, 1984; Hidaka *et al.*, 1986). In order to gain some understanding of the effect of lipoxygenase on quality, a number of inhibitors have been tried by various workers. These have included compounds which have been presumed to have a direct effect on the enzyme itself, compounds which block the formation of hydroperoxides (antioxidants) and compounds which cause a reduction in the available substrate concentration by increasing its concentration in micellar form.

To obtain information on the effect of inhibiting lipoxygenase, *per se*, it is essential that inhibitors are chosen which have a direct effect on the enzyme. The effect of cyanide on lipoxygenase activity has been frequently studied but, so far, the nature of the interaction has remained elusive. The reason for this probably lies in the presence of components in various preparations which react with the cyanide and reduce its effectiveness as a lipoxygenase inhibitor. Recently, a study on the chromatographic behaviour of lipoxygenase from green beans of similar maturity to those used by the freezing industry, has shown that a major proportion of the enzyme is in a hydrophobic form (Adams & Ongley, 1988). The purpose of the present work was to determine the nature of the effect of cyanide on this form of the enzyme and to compare it with the effect on the lipoxygenase activity of the crude starting material.

MATERIAL AND METHODS

Linoleic acid (99% pure), Tween 20, *n*-octyl β-D-glucopyranoside (*n*-octyl glucoside) and Tris (hydroxymethyl) aminomethane were purchased from Sigma Chemical Company Ltd. Polyethylene glycol 20 000 (Aquacide) was purchased from Calbiochem. All other reagents were of analytical grade and were purchased from BDH Chemicals.

Lipoxygenase assay

A stock substrate solution was first prepared containing $8{\cdot}02 \times 10^{-3}$ M linoleic acid and 0·25% Tween 20. This was then diluted with 0·18 M phosphate-citrate buffer (pH 7·0) to provide working substrates which give final linoleic acid concentrations of 8·42, 10·3, 13·2 and $18{\cdot}5 \times 10^{-5}$ M on assay of the lipoxygenase activity. Stock solutions of potassium cyanide were freshly prepared in the same buffer giving final cyanide concentrations of 0·96, 2·75, 4·63 and $9{\cdot}23 \times 10^{-3}$ M during lipoxygenase assay. Activity was determined by adding 0·1 ml of enzyme preparation to a mixture of 2·5 ml of working substrate and 0·1 ml of stock cyanide solution at 30°C. The rate of increase in absorbance at 234 nm due to the formation of hydroperoxides containing the conjugated diene chromophore was measured using a Pye Unicam PU 8800 UV/VIS recording spectrophotometer. The activity of the enzyme was taken to be the mean rate at the completion of 3 min reaction (rates every 20 s) and expressed as A_{234nm} min^{-1} 0·1 ml^{-1}.

Peroxidase assay

Peroxidase assays were performed using a solution of hydrogen peroxide ($3{\cdot}92 \times 10^{-3}$ M) and guaiacol ($5{\cdot}37 \times 10^{-3}$ M) in 0·2 M acetate buffer (pH 5·6).

The peroxidase activity was determined by adding 0·1 ml of enzyme preparation to 2·5 ml of substrate solution at 30°C. The rate of increase in absorbance at 420 nm due to the formation of brown guaiacol oxidation products was measured with the PU 8800 spectrophometer. The activity of the enzyme was taken to be the mean rate at the completion of 3 min reaction (rates every 20 s) and expressed as A_{420nm} min^{-1} 0·1 ml^{-1}.

Protein determination

Protein determinations were carried out using the commercial form of the dye-binding method of Bradford (1976). The assays were performed as recommended by the suppliers (Bio-Rad). A standard curve was constructed using soybean lipoxygenase (Type I) in the concentration range 200–1400 μg ml^{-1}. It was found to be non-linear.

Enzyme preparation

Whole green beans (*Phaseolus vulgaris* cv. Cascade) grown at Luddington Experimental Horticultural Station, were harvested at a maturity suitable for commercial freezing. The beans were extracted as described by Adams & Ongley (1988). Two lipoxygenase preparations were used in the present work, a crude 40–60% ammonium sulphate precipitate and a purified sample eluted from DEAE-Trisacryl M with 0·025 M *n*-octyl glucoside. The 40–60% precipitate was dissolved in 0·1 M Tris–HCl buffer (pH 8·0 at 4°C), dialysed to remove salts and then diluted with the same buffer to give an appropriate starting activity. The *n*-octyl glucoside eluate was concentrated by dialysis against solid Aquacide and the resulting solution used for the inhibition studies. During the course of the experiments, both preparations were stored at 4°C.

Data treatment

The double-reciprocal method of Lineweaver & Burk (1934) and linear regression by least squares was used to determine the Michaelis constants (K_m) and the maximum rate (V_{max}) in the absence of potassium cyanide. The inhibitor constant (K_i) was found by linear regression of the reciprocal-rates on potassium cyanide concentrations following the method of Dixon (1953). Reciprocal rates corresponding to the highest cyanide concentration ($9{\cdot}23 \times 10^{-3}$ M) were omitted from the regression as, generally, they did not fall on the best straight lines determined for the lower concentrations.

RESULTS AND DISCUSSION

Cyanide has been frequently investigated as an inhibitor of lipoxygenase activity but conflicting results have been obtained. Some workers have found little or no inhibitory effect (Hale *et al.*, 1969; Galliard & Phillips, 1971; Grossman *et al.*, 1972; Jadhav *et al.*, 1972; Eskin & Henderson, 1974; Bonnet & Crouzet, 1977), whilst others have observed a substantial inhibition (Chan, 1973; Haydar & Hadziyev, 1973; Flick *et al.*, 1975; de Lumen *et al.*, 1978; Truong *et al.*, 1982; Truong & Mendoza, 1982; Hidaka *et al.*, 1986). Sanders *et al.* (1975) showed that a peanut lipoxygenase isoenzyme with an alkaline pH optimum was not inhibited by sodium cyanide whereas an isoenzyme with an acidic optimum was. However, the studies, to date, have been carried out at one concentration of cyanide or at one concentration of substrate which is insufficient to determine the nature of any inhibitory effect. In addition, many of the enzyme preparations employed would probably have contained haemoproteins, such as the enzymes catalase and peroxidase. These have a high affinity for cyanide and, thus, would be expected to reduce its inhibitory effect on lipoxygenase (Kermasha & Metche, 1986).

In the present work, the effect of potassium cyanide and linoleic acid concentrations on the activity of a purified form of lipoxygenase was compared with their effect on lipoxygenase activity in the crude starting material (ammonium sulphate precipitate). The purified lipoxygenase preparation contained little or no haemoprotein as judged by the very low level of peroxidase activity ($< 0{\cdot}01\ A_{420nm}\ min^{-1}\ 0{\cdot}1\ ml^{-1}$) and the complete absence of any absorbance in the 400–450 nm region of its spectrum when measured at 1 mg protein ml^{-1}. The crude starting material gave an appreciable peroxidase activity ($0{\cdot}25\ A_{420nm}\ min^{-1}\ 0{\cdot}1\ ml^{-1}$).

Preliminary work, in the absence of potassium cyanide, showed an increasing degree of curvature of the doublereciprocal plots towards lower activities as the linoleic acid concentration was increased above 20×10^{-5} M. This indicated some substrate or product inhibition or possibly that the increasing concentration of Tween 20 was inhibiting the lipoxygenase as others have observed (Ben-Aziz *et al.*, 1970; Truong *et al.*, 1982). The investigations were, therefore, restricted to lower linoleic acid concentrations. K_m values for the crude and purified preparations were in reasonably good agreement with one another (see Table 1).

The purified lipoxygenase was found to be less stable than the crude enzyme and the cyanide inhibition studies were performed as quickly as possible to minimise the effect of activity loss on the type of inhibition. The double-reciprocal plots for both lipoxygenase preparations, in the presence of potassium cyanide, converged near to the reciprocal-rate axis which

TABLE 1
The Michaelis Constants (K_m) and Maximal Rates (V_{max}) for Two Preparations of Green Bean Lipoxygenase

Lipoxygenase preparation	*Protein concentration* ($mg\,ml^{-1}$)	K_m ($M \times 10^{-4}$)	V_{max} ($A_{234nm}\ min^{-1}\ 0{\cdot}1\,ml^{-1}$)
Crude	2·2	3·7	0·55
Purified	0·4	2·9	0·19

indicated competititive inhibition (Laidler & Bunting, 1973). This was further confirmed by plotting reciprocal rates against potassium cyanide concentrations (Dixon plots). In the case of the purified enzyme, the regression lines converged at $1/V_{max}$ (Fig. 1). This gave a mean $K_i = 1{\cdot}54 \times 10^{-3}$ M (standard error $= 0{\cdot}090 \times 10^{-3}$ M) which is relatively high compared with K_m, implying that the lipoxygenase has a lower affinity for cyanide than for linoleic acid.

In the case of the crude enzyme preparation, the regression lines did not converge at $1/V_{max}$ (Fig. 2). However, the estimate of K_i ($1{\cdot}09 \times 10^{-2}$ M,

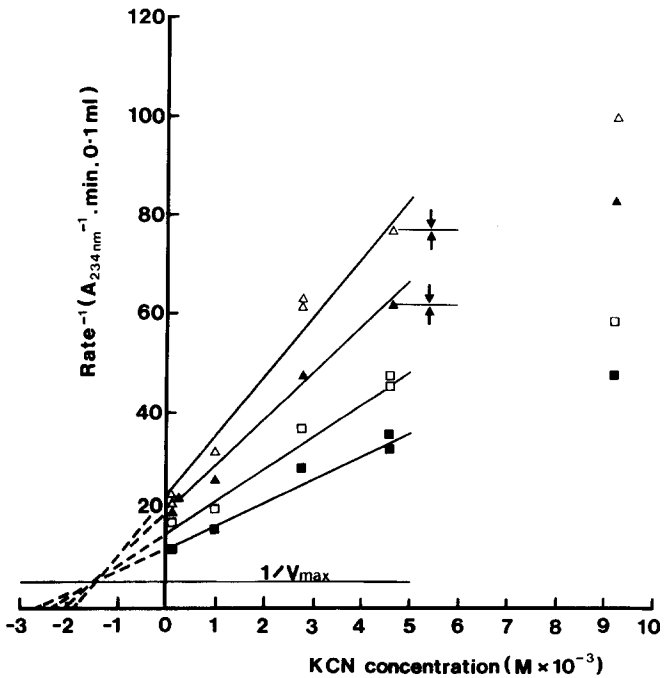

Fig. 1. The effect of potassium cyanide on the activity of purified green bean lipoxygenase (Dixon plot). △, $8{\cdot}42 \times 10^{-5}$ M linoleic acid; ▲, $10{\cdot}3 \times 10^{-5}$ M linoleic acid; □, $13{\cdot}2 \times 10^{-5}$ M linoleic acid; ■, $18{\cdot}5 \times 10^{-5}$ M linoleic acid. ↓↑, Two identical rates.

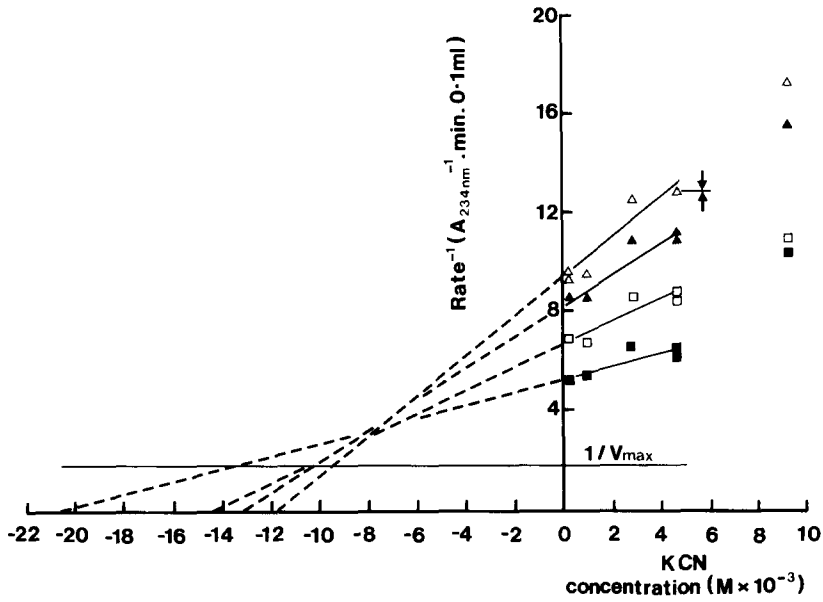

Fig. 2. The effect of potassium cyanide on the activity of a crude preparation of green bean lipoxygenase (Dixon plot). Symbols as in Fig. 1.

standard error $= 0{\cdot}18 \times 10^{-2}$ M) showed that potassium cyanide was a much less effective inhibitor of lipoxygenase activity in the crude preparation than in the purified material. As suggested earlier, this may be because the cyanide binds to haemoproteins so that the effective concentration is reduced. de Lumen *et al.* (1978) have shown that 0·1 M sodium cyanide was required to prevent volatiles being formed in green beans during blending. The requirement for this high level is an indication that components other than lipoxygenase are binding the cyanide. Heidrich *et al.* (1983) have reported K_i values for cyanide inhibition of peroxidase of $(0{\cdot}15\text{–}3{\cdot}3) \times 10^{-6}$ M (pH 5–6) which show that peroxidase is considerably more sensitive to cyanide than the lipoxygenase used in the present work. The fact that cyanide has been used traditionally to differentiate between lipoxygenase-catalysed and haem-catalysed oxidation of lipids (Hale *et al.*, 1969) is probably a reflection of the different affinities of the two types of catalyst for cyanide and not that lipoxygenase does not bind the inhibitor.

It has been demonstrated that iron is required at the active site in soy-bean lipoxygenase (Chan, 1973; Pistorius & Axelrod, 1974) and its presence may explain the inhibitory effect of cyanide on green bean lipoxygenase. However, the large difference in K_i values for peroxidase and lipoxygenase inhibition strongly suggests that the iron is present in different oxidation or spin states in the two enzymes under the conditions of measurement. The

pK_a of hydrogen cyanide is 9·14 (Dawson *et al.*, 1986) which implies that the undissociated acid (HCN) is the major species at the pH employed in the present studies (pH 7·0). Thus, the inhibiting species is probably hydrogen cyanide rather than the cyanide anion. A working hypothesis would be that the nitrogen of the hydrogen cyanide forms a coordination bond with the iron at the lipoxygenase active site, thus preventing interaction with the fatty acid.

Evidence has been presented by St. Angelo & Kuck (1977) that cyanide inhibition of peanut lipoxygenase was caused by the increase in pH value obtained on addition of the inhibitor to buffered solutions. Under the present conditions, however, the pH increase was less than 0·1 for potassium cyanide concentrations up to 1×10^{-2} M and this was considered to be too small to have a significant influence on enzyme activity. In addition, if the inhibition had been caused by pH changes, it would have been expected to be non-competitive rather than the competitive type actually observed.

In conclusion, it has been shown that cyanide is a weak competitive inhibitor of green bean lipoxygenase. It is possible that other, non-toxic, nitrogen-containing compounds may also have lipoxygenase inhibiting properties. Such compounds could have applications in reducing the blanch treatments used by the frozen vegetable industry.

ACKNOWLEDGEMENTS

The author thanks the Ministry of Agriculture, Fisheries and Food for financial support for this work and the Director of Campden Food and Drink Research Association for permission to publish it.

REFERENCES

Adams, J. B. & Ongley, M. H. (1989). The behaviour of green bean lipoxygenase on chromatography and isoelectric focusing. *Food Chem.*, **31**, 57–71.

Ben-Aziz, A., Grossman, S., Ascarelli, I. & Budowski, P. (1970). Linoleate oxidation induced by lipoxygenase and heme proteins: A direct spectrophotometric assay. *Analyt. Biochem.*, **34**, 88–100.

Bonnet, J. L. & Crouzet, J. (1977). Lipoxygenase from tomato fruit: Partial purification and study of some properties. *J. Food Sci.*, **42**, 625–8.

Bradford, M. N. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analyt. Biochem.*, **72**, 248–54.

Chan, H. W.-S. (1973). Soya bean lipoxygenase: An iron containing dioxygenase. *Biochim. Biophys. Acta.*, **327**, 32–35.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1986). *Data for Biochemical Research.* (3rd edn) Clarendon Press, Oxford.

de Lumen, B. O., Stone, E. J., Kazeniac, S. J. & Forsythe, R. H. (1978). Formation of volatile flavor compounds in green beans from linoleic and linolenic acids. *J. Food Sci.*, **43**, 698–702.

Dixon, M. (1953). The determination of enzyme inhibitor constants. *Biochem. J.*, **55**, 170–1.

Eskin, N. A. M. & Henderson, H. M. (1974). Lipoxygenase in *Vicia faba minor*. *Phytochem.*, **13**, 2713–16.

Flick, G. J., St. Angelo, A. J. & Ory, R. L. (1975). KCN inhibition of lipoxygenase in purple, green and white eggplants. *Lipids*, **10**, 564–6.

Galliard, T. & Phillips, D. R. (1971). Lipoxygenase from potato tubers. Partial purification and properties of an enzyme that specifically oxygenates the 9-position of linoleic acid. *Biochem. J.*, **124**, 431–8.

Grossman, S., Trop, M., Avtalion, R. & Pinsky, A. (1972). Egg plant lipoxygenase: Isolation and partial characterisation. *Lipids*, **7**, 467–73.

Hale, S. A., Richardson, T., von Elbe, J. H. & Hagedorn, D. J. (1969). Isoenzymes of lipoxidase. *Lipids*, **4**, 209–15.

Haydar, M. & Hadziyev, D. (1973). A study of lipoxidase in pea seeds and seedlings. *J. Sci. Food Agric.*, **24**, 1039–53.

Heidrich, E., Lorenz, G. & Schreier, P. (1983). Ultrathin-layer isoelectric focusing of partially purified peroxidase from tomato fruit. *Food Chem.*, **10**, 285–96.

Hidaka, T., Katsuki, S., Nagata, I. & Nakatau, S. (1986). Partial purification and properties of pumpkin lipoxygenase with carotene bleaching activity. *J. Food Biochem.*, **10**, 55–73.

Jadhav, S., Singh, B. & Salunkhe, D. K. (1972). Metabolism of unsaturated fatty acids in tomato fruit: Linoleic and linolenic acid as precursors of hexanal. *Plant and Cell Physiol.*, **13**, 449–59.

Kermasha, S. & Metche, M. (1986). Characterisation of seed lipoxygenase of *Phaseolus vulgaris* cv. Haricot. *J. Food Sci.*, **51**, 1224–7.

Klein, B. P., Grossman, S., King, D., Cohen, B. S. & Pinsky, A. (1984). Pigment bleaching, carbonyl production and antioxidant effects during the anaerobic lipoxygenase reaction. *Biochim. Biophys. Acta*, **793**, 72–79.

Laidler, K. J. & Bunting, P. S. (1973). *The Chemical Kinetics of Enzyme Action*, 2nd edn. Clarendon Press, Oxford.

Lineweaver, H. & Burk, D. J. (1934). Determination of enzyme dissociation constants. *J. Amer. Chem. Soc.*, **56**, 658–66.

Pistorius, E. K. & Axelrod, B. (1974). Iron, an essential component of lipoxygenase. *J. Biol. Chem.*, **249**, 3183–6.

Sanders, T. H., Pattee, H. E. & Singleton, J. A. (1975). Lipoxygenase isozymes of peanut. *Lipids*, **10**, 681–5.

Sessa, D. J. (1979). Biochemical aspects of lipid-derived flavors in legumes. *J. Agric. Food Chem.*, **27**, 234–9.

St. Angelo, A. J. & Kuck, J. C. (1977). Effects of cyanide on peanut lipoxygenase. *Lipids*, **12**, 682–3.

Truong, Van Den & Mendoza, E. M. T. (1982). Purification and characterisation of two lipoxygenase isoenzymes from Cowpea (*Vigna unguiculata* (L.) Walp.). *J. Agric. Food Chem.*, **30**, 54–60.

Truong, Van Den, Raymundo, L. C. & Mendoza, E. M. T. (1982). Winged bean lipoxygenase—Part 2: Physicochemical properties. *Food Chem.*, **8**, 277–89.