CITRATE CLEAVAGE ENZYME IN MANGO FRUIT

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Summary: Citrate cleavage enzyme has been detected in mango fruit. The enzyme activity increases considerably during ripening of the fruit and is stimulated by glucose, fructose and fatty acids (oleic and palmitic). This enzyme appears to be of major importance in the ripening fruit in making available acetyl CoA and oxaloacetate, from citrate, for synthetic processes.

There is now considerable evidence regarding the presence of citrate cleavage enzyme (EC. 4.1.3.8, ATP:citrate oxaloacetate-lyase) in mammalian tissues cytoplasma (1-4). This enzyme has been considered important in fatty acid biosynthesis, since citrate may be the immediate source of acetyl CoA in the cytoplasm (2-5). Very little information regarding this enzyme is available in plants.

It has been shown that during ripening in mango fruit, the increases in carotene (6) and fatty acids (7) is accompanied by a considerable increase in the activity of enzymes generating reduced NADP, viz., the pentose phosphate cycle enzymes and malic enzyme along with ten fold decrease in citrate (6). The availability of sugars for high respiration (8) and synthetic reactions is limited by the fact that sugars accumulate in the ripening mango (6). It was therefore suspected that citrate might be cleaved by citrate cleavage enzyme to yield acetyl CoA and oxaloacetate for synthetic reactions, as for example, the carotene and fatty acid syntheses. This communication reports

the occurrence and quantitative changes in the activity of citrate cleavage enzyme during ripening in mango fruit and the factors which activate this enzyme.

MATERIALS AND METHODS

Plant material : Mangoes (Mangifera indica L. ev. Alfonse) used for this study were purchased from local farm; immediately after picking the mature unripe mangoes were kept for ripening at 20°-25°C. The various stages of the fruit during ripening were marked by the colour development and appearance, in addition to the criteria of increasing respiratory activity (7) :-

Unripe : green peel,firm when touched and white pulp;

Partly ripe : green to yellow peel, slightly soft and faint yellow pulp; Ripe : golden yellow peel, soft when touched and yellow to golden yellow pulp.

Enzyme preparation : All the procedures were carried out at 0°-5°C. The fruits were peeled and a known weight of the pulp was manually ground for 20 min in a mortar with 0.1 M KCl (in 0.05 M Tris-HCl buffer at pH 7.5) to make a 30 % extract (w/v). During grinding, when necessary, the pH was maintained by careful, dropwise addition of 0.1 N NaOH. The homogenate was centrifuged for 15 min at 1,500 x g and the supernatant recentrifuged at 15,000 x g for 15 min. The clear supernatant obtained was examined for the citrate cleavage enzyme activity. Dialysis against 0.005 M Tris-HCl buffer (pH 7.5) for 2 hours with intermittent changes of buffer was carried out to remove dialysable substrates; dialysis for more than 2 hours was found to result in a considerable loss of the enzyme activity.

Acetone powder of the ripe fruit pulp was prepared by

overnight immersion of the crushed tissue in acetone at (-)20°C and carefully grinding it the next day till the residue was snow-white. The dried acetone powder was then extracted for enzyme preparation by the procedure described above. Protein in the enzyme extracts was determined by the method of Lowry et al (9) using serum albumin as the standard.

Assay of enzyme activity : Unless otherwise stated, the assay system contained, in micromoles : Tris buffer (pH 7.0), 100; $MgCl_2$, 10; CoA, 0.5; glutathione, 10; sodium citrate, 20; ATP, 5; hydroxylamine-hydrochloride (neutralised), 200 and an appropriate concentration of the enzyme extract in a total volume of 1 ml. Enzyme reaction was started by the addition of ATP. Blank tubes contained all components except CoA. The enzyme mixtures were incubated at 30°C for 30 min after which the acetyl CoA formed was estimated as acetyl hydroxamate at 540 mμ (1). One unit of enzyme activity is defined as the amount of enzyme which produces 1 micromole of acetyl-hydroxamic acid per 30 min under the experimental conditons.

Preparation of crude fatty acids : Mango pulp was extracted with acetone and ether (1:1, v/v) till the tissue was free from all the colouring material. After removing the moisture (sodium sulphate, 1 hour) the solvent was evaporated at 80°C. Crude lipid extract was saponified in 1 N alcoholic KOH, and after acidification (pH 4), the fatty acids were extracted with ether. The ethereal extracts were pooled and evaporated to dryness. Before use the fatty acids were dissolved in a known volume of acetone and used in the activation experiments. When fatty acid mixture was added in the enzyme assay system, corresponding amount of acetone was added in the control tubes; the amount of acetone added (< 0.3 ml) was found to

have no effect on the enzyme activity.

RESULTS AND DISCUSSION

During preliminary studies samples of mango tissue at different stages of ripening were examined for possible changes in the levels of citrate cleavage enzyme. The results are summarised in Table I. The table shows the average of triplicate assays of three separate samples of the fruit tissue. It can be seen that the activity of citrate cleavage enzyme increases considerably during ripening. These overall changes in the enzyme activity suggest that the enzyme may either be synthesized or activated during ripening process.

TABLE I

Citrate cleavage enzyme : changes during ripening in mango fruit

Stage of the fruit	Specific activity*
Unripe	0.094 - 0.141
Partly ripe	0.188 - 0.282
Ripe	0.423 - 0.564

Details of the experiment are described in the text.

* μ moles product formed per mg protein per 30 min.

The activity of the enzyme was affected by pH as shown in Fig 1. The enzyme displayed maximum activity around pH 7. Deletion studies showed that the fruit enzyme is dependent on ATP to the extent of > 97%, thereby disproving the possibility of citrate breakdown by the reversal of condensing enzyme.

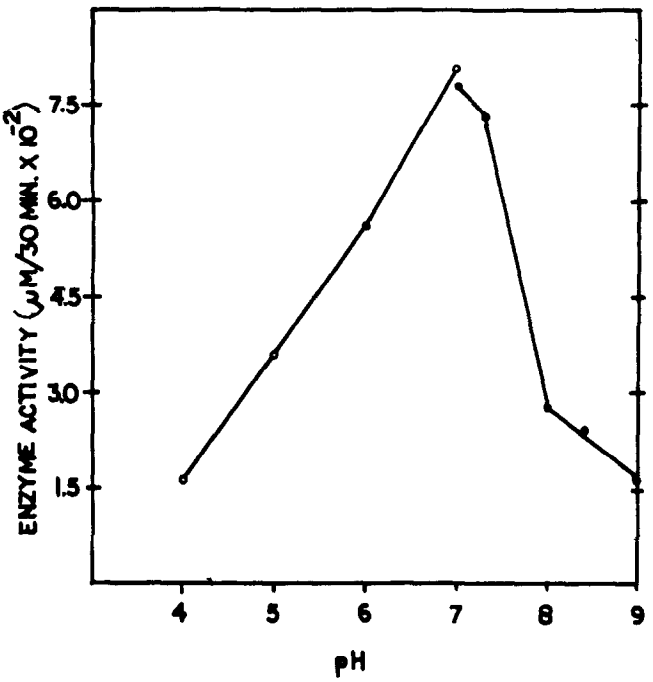

Fig.1 Effect of pH on citrate cleavage enzyme. Incubation was carried out at 30° for 30 min. Other experimental details are described in the text. o–o, Succinate-NaOH buffer; •–•, Tris-HCl buffer.

Regulation of cellular metabolism on the basis of ''cross-feedback'' inhibitors and effectors is well known (10). Using mango fruit, Mattoo et al. (8) found that in the ripe fruit carotenogenesis is regulated by phosphatase which seems to dephosphorylate the intermediates; β-carotene stimulates the activity of this enzyme. Attempts were therefore made to study the effect, on citrate cleavage enzyme, of glucose, fructose, oleate, and palmitic acid, the cellular constituents which accumulate during ripening in mango. The protein content does not change quantitatively during ripening (7). From our observations it has been found that the individual ratio of glucose, fructose and fatty acids with respect to protein varies in unripe mango between 1.3 to 2.6, 1.7 to 3.3, and

0.14 to 0.23; in partly ripe fruit from 3.3 to 3.7, 4.5 to 5.0, and 0.4 to 0.5; and in ripe fruit from 4.0 to 6.0, 6.0 to 9.3, and 0.62 to 0.91, respectively. In the present experiments the concentrations of the above constituents in the assay

TABLE II

Citrate cleavage enzyme : stimulating effect of various effectors on enzyme activity

Addition to the assay system	Concentration (M)	Specific Enzymatic Activity*	Activation (% change)
Nil	-	0.423	-
Glucose	1.0×10^{-6}	2.287	440
Glucose	2.5×10^{-6}	2.650	526
Fructose	1.0×10^{-6}	0.721	70.4
Fructose	2.5×10^{-6}	1.457	244
Sodium oleate	1.0×10^{-7}	0.501	18.5
- do -	2.0×10^{-7}	0.627	48.1
- do -	5.0×10^{-7}	0.830	96.3
Palmitic acid	1.0×10^{-7}	0.893	111
- do -	2.0×10^{-7}	1.049	148
- do -	5.0×10^{-7}	1.065	151

The 15,000 x g supernatant of ripe mango was assayed for enzymatic activity in the presence of indicated effectors under otherwise standard conditions. Results recorded are an average of three sets of experiments.

* μ moles product formed per mg protein per 30 min.

system were so chosen that these represent a close approximation to the ratios reported above for partly ripe and ripe fruit. Results of such a study are recorded in Table II. As is evident, all the compounds tested stimulated the enzyme activity and the activation is found to be almost linear with increasing concentrations of free hexoses and fatty acids. It should be noted that palmitic acid and glucose can bring about enzyme activation at a much lower molar concentration than sodium oleate and fructose.

TABLE III

Effect of crude fatty acids on citrate cleavage enzyme activity

System	Specific Enzymatic Activity*
1. Extract from mango tissue (control A)	0.470
2. Control A+Fatty acid mixture**	0.893 (90)
3. Extract from acetone powder (control B)	0.320
4. Control B + Fatty acid mixture**	0.705 (120)

Number in parentheses denotes activation (% change).

* μ moles of acetylhydroxamate formed per mg protein per 30 min.

** The concentration was 1×10^{-7} M with respect to palmitic acid.

Finally, crude fatty acid extract of the mango exerts the same effect (Table III) thus demonstrating that a naturally occurring lipid product from the fruit could be of some physiological significance in the control of the activity of this enzyme. No attempt was made to quantitate the extract. Qualitatively, however, the extract was found to contain palmitate, oleate, linoleic and linoleneate. Similar activation of the enzyme was observed when the enzyme preparation from acetone powders was used (Table III). It may be noted that the enzyme extracts from acetone powder showed lesser activity which might be due to the removal of some factors, and remains to be studied.

TABLE IV

Citrate cleavage enzyme : effect of crude fatty acids at different pH values on enzyme activity

System	Specific Enzymatic Activity	
	pH 7.0	pH 8.4
1. Control - A	0.548	0.235
2. Control - A + Fatty acid mixture	1.253	0.366
3. Control - B	0.329	0.216
4. Control - B + Fatty acid mixture	0.652	0.251

Experimental conditions same as in Table III except the pH as indicated.

As a counter check, the activities of acid phosphatase and glutamine synthetase were found to be same in both the extracts. It is interesting to note that the activation of citrate cleavage enzyme by crude fatty acids is maximum at pH 7 (Table IV), the pH at which the enzyme is optimally active (Fig 1). Further investigations on the mechanism of activation are in progress.

These results suggest that the rise in citrate cleavage enzyme during ripening in mango fruit provides more acetyl CoA and oxaloacetate to meet heavy demands mainly for synthetic processes, and that the necessary concentrations of free hexoses and fatty acids in ripe fruit activate this process. Supply of reduced NADP can be met by the increased activity of pentose phosphate cycle enzymes (6); also the increase in malic enzyme (6) together with malic dehydrogenase and oxaloacetate could possibly comprise a transhydrogenase system from NADH and NADP to form NADPH as has been proposed earlier in animal tissues, liver (11) and heart (12).

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