C H A P T E R V.

INVERTASE INHIBITOR(S) IN UNRIPE MANGOES

Natural proteinic inhibitors for numerous enzymes generally belonging to the group of hydrolases have been reported.[227-236,242] Trypsin inhibitors have been found in soya beans,[227] potatoes,[228,229] sweet potatoes[237] and alfalfa;[236] those from soya bean,[231,232,238] potato[239,240] and alfalfa[241] have been isolated and purified. Two chymotrypsin inhibitors have been crystallised from potato tuber.[232,233,294] Chymotrypsin inhibitor has also been reported in the leaflets of potato and tomatoes.[234,235]

Spencer[18] brought out the fact that the changes in metabolic pathways in the activities of individual enzymes and in the rate limiting reactions can result due to many causes including synthesis or destruction of enzymes, substrates and cofactors, conditions of pH, temperature and presence of inhibitors.

Earlier from this laboratory natural proteinic inhibitors of the enzymes catalase, peroxidase and amylase from unripe mangoe have been reported.[153,154] These inhibitors were also shown to mask the activities of the enzymes in the unripe fruit and ethylene had the capacity to inactivate the inhibitors resulting in an enhancement in the activities of the affected enzymes and thereby initiating vigorous metabolic processes leading to the ripening of the fruit. Such inhibitors were also reported in papaya and banana.[252]

Considerable increase in the activities of enzymes has been found during ripening (chapters III and IV) in the present investigation. A survey was therefore made to see which other enzymes are inhibited by the dialysed extracts of unripe mango. The results in Table VII illustrates that the unripe fruit contains inhibitors for the enzymes cellulase, invertase, glucose-6-phosphatase, phosphofructokinase, sucrose synthetase and sucrose phosphate synthetase while the enzymes pectinesterase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase were not affected by the unripe fruit extract.

TABLE VII

Inhibitory Effect of Unripe Mango Extract on the Activities* of Enzymes From Ripe Fruit Extract

Enzymes tested	Ripe control	Unripe control	Ripe+ unripe	% Inhibition
Cellulase	12.20	2.6	12.0	20
Amylase	17.00	5.00	9,03	60
Glucose-6-phosphatase	0.20	**	0.033	83
Phospho-fructokinase	0.40	0.10	0.10	80
Sucrose synthetase	0.057	0.022	0.50	30
Sucrose phosphate synthetase	0.032	0.017	0.032	34
Invertase	4.50	1.60	3.00	50

* Results expressed are in units.

** Not detectable.

A number of investigations have revealed the presence of invertase inhibitor in potato tuber.[247-249,253] Pressey [248,249,251] isolated, purified and characterised the invertase inhibitor and showed its occurence in red beet, sugar beet and sweet potato. The double pH optima of potato invertase have been attributed to this inhibitor.[247,253]

We have studied the invertase inhibitor from unripe mango in greater detail. It was found that unripe extracts of other mango varieties like Sardar and Pachhatiyo also had the capacity to inhibit invertase indicating thereby the general nature of phenomena in mangoes (Table VIII). The inhibitor was partially purified and its kinetics with reference to invertase were studied.

TABLE VIII

Inhibition of Mango Invertase Activity* by Unripe Extracts of var. Sardar, var. Pachhatiyo.

	Extracts from		Unripe + Ripe**
	Unripe	Ripe	
Sardar	0.15	1.4	0.72 (43.6)
Pachhatiyo	0.38	1.25	0.60 (66.0)

* Values expressed are in units of enzyme activity.

** For all results dialysed unripe and ripe extracts in 1 ml. equal proportion were incubated for 10 minutes at 0° C before determining the residual activity. Ripe and unripe controls contained 1 ml. of buffer instead. Values in parenthesis are percentage inhibition.

The partial purification was achieved by the following procedure. Fresh mature unripe mango pulp extract was prepared in the cold, with 0.1 M sodium phosphate buffer pH 7.5 and 2% carbowax (to remove plant phenols) by grinding without abrasive in an unglazed mortar precooled to 0° C. The extract was centrifuged at 5000 x g for 15 minutes (in a small super speed centrifuge) and the supernatant was collected. To the supernatant magnesium acetate was added to 0.016 M concentration to remove nucleic acid and pectins. After allowing it to stand for 30 minutes the precipitates were removed by centrifugation and to the supernatant powdered ammonium sulphate was added to bring it to 10% (0.1) saturation. The resulting precipitates were centrifuged and discarded. The inhibitor was then precipitated by adjusting the concentration of supernatant to 60% (0.6) with ammonium sulphate and left standing overnight. The precipitates were separated by centrifugation and dissolved in 0.1 M phosphate buffer (pH 7.0) and dialysed against distilled water for 12 hours. To the dialysate C^{γ}-alumina gel (1:18 protein to gel ratio) was added. The mixture was frequently stirred for 30 minutes. The gel was removed by centrifugation and the supernatant discarded. The gel was washed twice with distilled water followed by 0.05 M phosphate buffer (pH 7.0). The inhibitor was eluted from the gel with 0.1 M phosphate buffer (pH 7.0) and dialysed against distilled water for 12 hours.

By this method of purification a 27 fold purity could be achieved but a considerable loss in protein was encountered (Table IX). Further purification was achieved by column chromatography using DEAE cellulose and DEAE sephadex A 50 columns, since successful purification of proteinic inhibitors of other enzymes from mango[154] has been reported using these columns.

TABLE IX

Partial Purification of Invertase Inhibitor from Unripe Mango Extract

Fractions	Volume	Total units	Protein mg/ml	Specific activity units/mg. protein	Purification (fold)
Crude extract	100	45.4	1.02	0.24	-
0.016 M Magnesium acetate supernatant	80	47.5	0.79	0.40	1.7
0.1-0.6 M ammonium sulphate residue	20	11.0	0.25	2.20	10
0.1 M phosphate buffer eluate of CY-gel	10	9.8	0.15	6.53	27

DEAE cellulose (40 x 2 cms.) and DEAE saphadex A 50 (35 x 2.5 cms.) columns were equilibriated with 0.01 M and 0.05 M tris-HCl buffer, pH 7.5 respectively. The 27 fold purified invertase inhibitor from mango, obtained by the procedure as described earlier in this chapter (Table IX) was applied in 10 ml. portions to the columns separately (corresponding to about 5-8 mg. protein). The columns were developed with increasing gradient of NaCl, (0.05 to 0.2 M and 0.025 to 0.2 M for DEAE cellulose and DEAE saphadex A 50 columns respectively) after washing with the buffer used for equilibriation, as shown in Fig.8 and 9. Elution rate was maintained at 35 ml. per hour, the effluent fractions were collected in 10 ml. portions with help of an automatic fraction collector, the fractions were dialysed and then used. The typical elution profiles are given in Fig.8 and 9.

Four peaks in DEAE cellulose and five in DEAE saphadex columns were observed (Fig.8 and 9). The pattern of elution is very similar in both the columns. One fast moving inhibitor fraction is obtained. These results are almost the same as found for the amylase inhibitor.[154] Fractions 12 and 9 showed maximum specific activity of 52 and 50 on DEAE cellulose and DEAE saphadex columns respectively as compared to 0.24 of crude extract. These results account for a 200 fold purity. On subjecting these two fractions to poly acrylamide gel electrophoresis a single protein band was

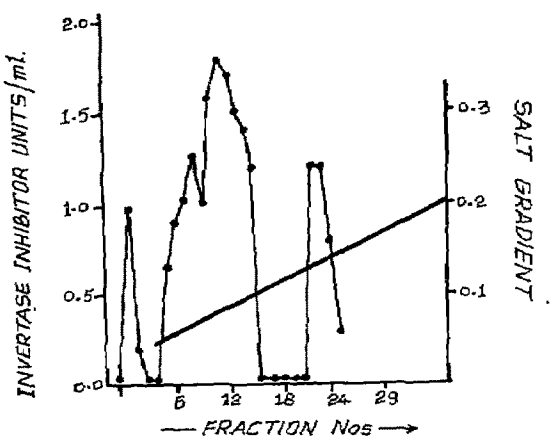

FIG. 8 - ELUTION PROFILE ON DEAE CELLULOSE

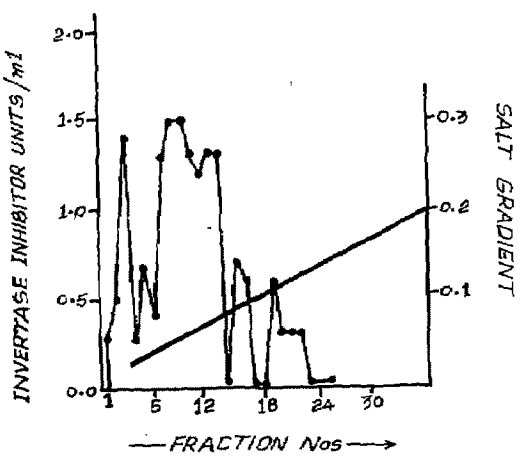

FIG. 9 - ELUTION PROFILE ON DEAE SAPHADEX

observed indicating the homogenity of the fraction. However, it should be mentioned that attempts to obtain a single fraction containing the inhibitory activity for invertase were not successful; invariably the activity was distributed amongst four or five peaks. Further invertase inhibitor was always associated with amylase inhibitor.

The partially purified inhibitor gave positive tests with folin phenol reagent, and was non-dialysable and heat labile indicating its proteinic nature. A linear relationship was obtained between the amount of inhibitor added and the % inhibition upto about 80-90% (Fig.10). The deviation from linearity above 90% inhibition has been observed for other inhibitors, and has been suggested to be due to the enzyme inhibitor complex having some activity.[247,257] The presence of residual free enzyme and the multimolecular forms of the enzyme, with different complexing abilities with inhibitors, are also possibilities.

Results in Table X indicate that the interaction of the inhibitor with the enzyme is a time dependant phenomena, the interaction reaching its maximum after 10 minutes of preincubation.

When the enzyme inhibitor complex was subjected to acrylamide electrophoresis a very faint band was observed (on staining for invertase),[298] on the other hand the free enzyme gave a discrete band. These results suggest

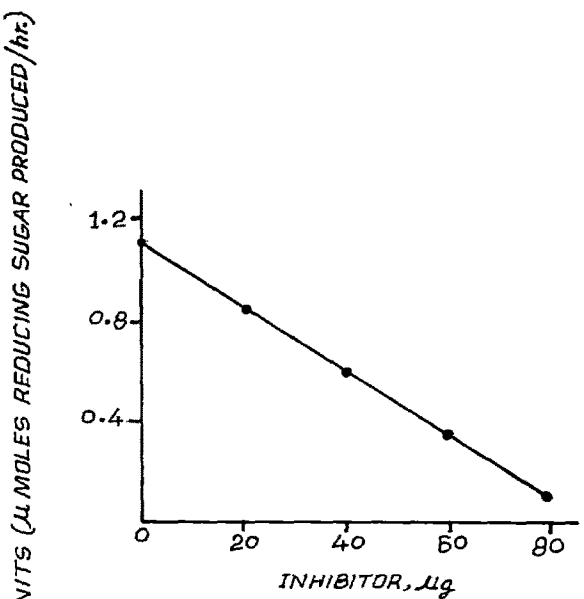

FIG. 10 – LINEARITY OF INVERTASE INHIBITION BY INCREASING INHIBITOR

that there is a complex formed between the enzyme and inhibitor.

TABLE X

Effect of Preincubation Time on Invertase Inhibition by the Inhibitor

Time of preincubation in minutes	Residual invertase activity (units)
0	0.775
2	0.715
5	0.651
10	0.500
15	0.501

It was also found that the invertase inhibitor was stable upto 72 hours at -15° C. On storing for 96 hours at the same temperature half of the activity was found to be lost (Table XI).

TABLE XI

Stability of Invertase Inhibitor

Time in hours	Invertase inhibitory activity
0	0.45
72	0.42
96	0.19

To understand the mechanism of inhibition, the effect of varying concentrations of substrates was studied on a constant amount of inhibitor and the enzyme. It is evident from the results in Fig.11 that the inhibitor is of the competitive type, the Km and Ki being 5.0×10^{-2} and 1×10^{-1} (M) respectively.

The partially purified mango inhibitor was found to inhibit both papaya and banana invertase and not the yeast enzyme to a significant extent indicating the specificity of this inhibitor towards the fruit invertase (Table XII).

TABLE XII

Effect of Partially Purified Mango Inhibitor on Banana, Papaya and Yeast Invertase

System	Invertase activity	% Inhibition
Extract from ripe banana (control A)	0.850	-
Control A + Inhibitor (100 µg.)	0.600	30
Extract from ripe papaya (control B)	24.000	-
Control B + Inhibitor (100 µg.)	19.000	20
Yeast invertase* (control C)	1.80	-
Control C + Inhibitor (100 µg.)	1.7	5

* Obtained from Sigma Chem. Co., U.S.A.

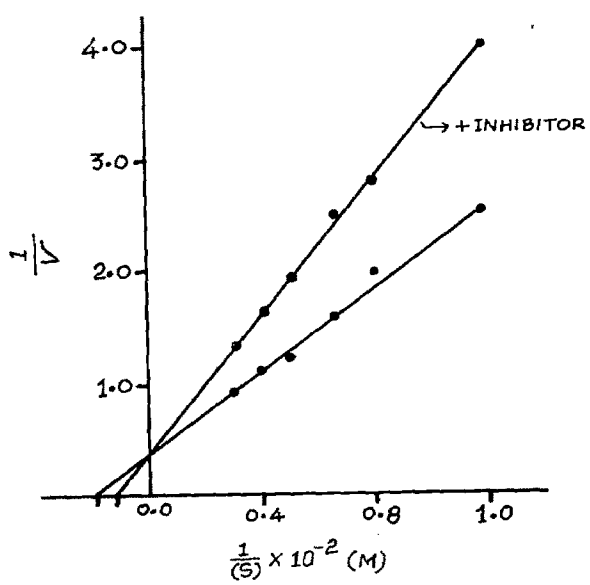

FIG. 11 - LINE WEAVER BURK PLOT OF INVERTASE AND IT'S INHIBITOR

To obtain a comparative data, studies were extended using the climacteric fruits banana and papaya. In both the fruits considerable increase in invertase activity during ripening was found (Table XIII). It was seen that in these fruits also the unripe extract had an inhibitory effect on the ripe fruit invertase.

TABLE XIII

Invertase Activity in Banana and Papaya Extract and the Inhibitory Effect of Unripe Extract

Stage of fruit	Invertase activity (units/mg. protein)	
	Banana	Papaya
Unripe	0.74	3.1
Ripe	9.1	30.0

System	Enzyme activity (units)	
	Banana	Papaya
Unripe (control)	0.17	3.38
Ripe (control)	5.16	33.5
Ripe + Unripe	3.25 (20)	22.3 (34)

Values in parenthesis are % inhibition.

The partially purified inhibitor was found to be heat labile. On heating the inhibitor in a boiling water bath for 3 minutes, the capacity to inhibit invertase was completely lost, thus resembling the mango invertase inhibitor.

It was of interest to study the change in the inhibitor and in invertase during the ripening period in mango. For this study a group of 12 fruits almost of the same maturity were selected. Each day the fruits were weighed and the invertase activity and the inhibitory activity towards this enzyme were studied. The reducing sugars were also measured. The results presented in Fig.12 indicate that the increase in invertase activity and the decrease in the inhibitory activity coincide with the maximum weight loss which is on the 6th day. These results are further substantiated by the increase in reducing sugars during ripening. Mattoo[154] has reported that ethylene production and respiration is maximum on the 6th day for Alfanso mangoes. These results suggest that ethylene might be playing a part in the inactivation of the inhibitor.

Experiments were therefore conducted to see the effect of ethylene on the unripe mango slices. The unripe mangoes used for these experiments were surface sterilized and then cut into uniform slices (2 gm. fresh weight). These slices were placed in a desicator with a capacity of 3 litres, ethylene (50 ppm and 100 ppm) was

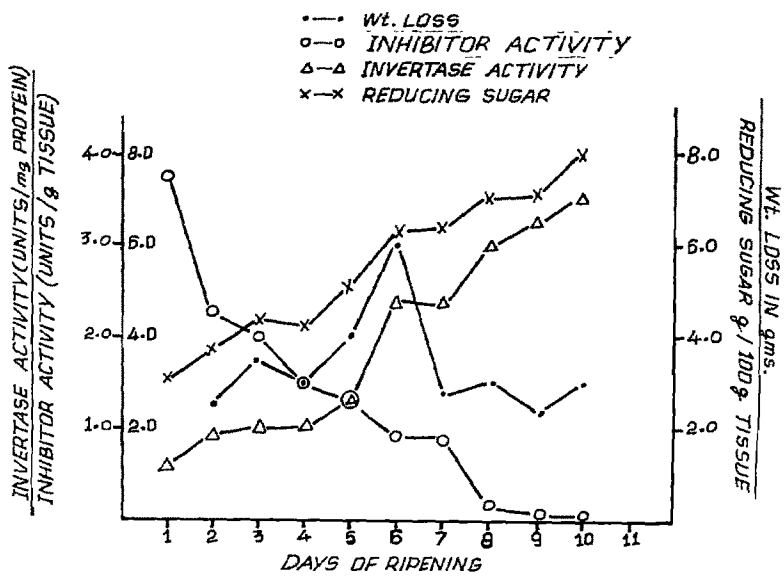

Fig. 12. ACTIVITY OF INVERTASE & INHIBITOR DURING RIPENING OF MANGOES

introduced into these chambers using the evacuation method.[40] The chambers were washed and rinsed with alcohol before use. A beaker containing solid NaOH was kept in the centre of the container to absorb the carbon dioxide evolved. Control slices were kept in a similar chamber without ethylene. The containers were sealed and incubated at 25° C. At various time intervals (17 hrs; 23 hrs; 45 hrs; and 70 hrs) the slices were removed for analysis. The containers were unsealed at indicated intervals and exposed to air for an hour and again the stipulated amount of ethylene introduced and the chambers were sealed. The treated slices and their controls, after removal, were cooled at 0° C and extracted for the determination of the activities for the enzyme and its inhibitor.

In the treated slices invertase activity rose from 0.175 to 1.2 and as in the untreated slices an insignificant increase to 0.20 was observed (Fig. 13). Ethylene stimulation of the enzyme activity reached the maximum level at 45 hours in 100 ppm treated slices and at 70 hours in 50 ppm treated slices; in controls it increased slightly till 23 hours and then remained constant upto 70 hours. During this period it was observed that the ethylene treated mango tissue softened; with the increase in incubation time though slight, but significant change in colour development of the pulp from white to yellow occured thus suggesting the appearance of the symptoms characteristic of ripening. The

disappearance of the invertase inhibitor in the treated slices coincided with the increase in the enzyme activity (Fig. 14). In the control slices there was a slight decrease in the inhibitor concentration upto 23 hours and then the inhibitor concentration remained almost constant till 70 hours, which substantiated the result that the invertase activity did not increase significantly in the control slices. In the 50 ppm ethylene treated slices a sharp fall was noted in the first 17 hours and then a steady but sharp decrease upto 70 hours in 100 ppm ethylene treated slices the drop in inhibitor was found to be sharp upto 45 hours and then no further decrease was observed upto 70 hours. These results gave additional evidence for the activity of the enzyme, invertase, which remained steady from 45 to 70 hours in the 100 ppm treated slices. During these studies it was found that even though the inhibitor decrease was well marked in 23 hours the increase in invertase activity was not very steep (Fig. 13 and 14). Similar results have been reported in the whole fruit (Fig. 12) where a marked loss of the inhibitor activity was found between the 1st and 2nd day of ripening whereas the activity of invertase did not increase markedly during this stage. These results indicate that the inhibitor (with reference to invertase) concentration has to decrease to a certain level in the tissue to effect a considerable increase in the invertase activity. These findings are similar to the earlier finding from this laboratory on the inactivation of the inhibitor(s) of catalase, peroxidase and amylase by ethylene.[252]

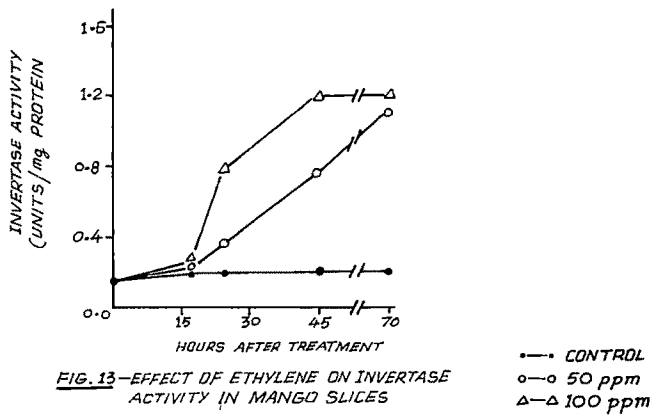

FIG. 13—EFFECT OF ETHYLENE ON INVERTASE ACTIVITY IN MANGO SLICES

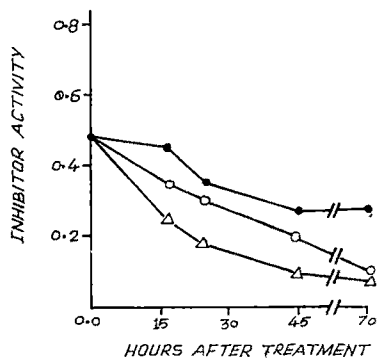

FIG. 14 - EFFECT OF ETHYLENE ON INVERTASE INHIBITOR IN MANGO SLICES.

It was of interest to study further the effect of ethylene on the partially purified inhibitor. For this study the ethylene gas was passed through the inhibitor solution while maintaining air tight conditions at the calculated concentrations (of 100 to 150 ppm). During this process the temperature was maintained at 0-5° C. The gas was allowed to bubble through the solution by a capillary at the rate of 20 bubbles per minute till the required concentration of ethylene was attained. This solution was immediately assayed for the inhibitory activity. In controls a stream of air was passed under the same conditions. From the results in Table XVI it is evident that in the presence of ethylene the partially purified mango inhibitor gets inactivated.

TABLE XVI

Effect of Ethylene Treatment on the Activity of Partially Purified Invertase Inhibitor from Unripe Mango

System	Residual invertase activity (units)
Ripe mango extract (control)	0.52
Ripe mango extract (ethylene treated)	0.50
Ripe + Inhibitor (60 µg.)	0.36
Ripe + Inhibitor (100 µg.)	0.25
Ripe + Inhibitor (60 µg.) (ethylene treated)	0.502
Ripe + Inhibitor (100 µg.) (ethylene treated)	0.51

Earlier results in this chapter show that the inhibitor is proteinic in nature. The protein complement of a given fruit may be expected to be a function of genetic setup, stage of maturation pre and post harvest history. An attempt has been made to study qualitative changes occurring in the soluble protein during ripening in mangoes. Figures 15 and 16 are the diagramatic representation of the profiles obtained. The unripe extract gave a profile with 6 discrete bands. If higher concentrations of protein were spotted two secondary bands were visible but the nature of the bands were diffused. In the ripe fruit extract two of the primary bands clearly visible in the unripe stage, disappeared. The general profile of the ripe fruit protein became diffused. The fastest moving band was strong and sharp throughout the course of ripening. On staining with PAS for glycoprotein identification two bands were visible which correspond to the first and sixth bands stained with amido black. In the glycoproteins the first band became diffused as the fruit ripened. Clements[209] has found a similar profile in the avocado and many other fruits.

From these results it is evident that ripening is accompanied by a change in the protein complement. The unripe fruit contains inhibitor(s) capable of inactivating a number of enzymes.

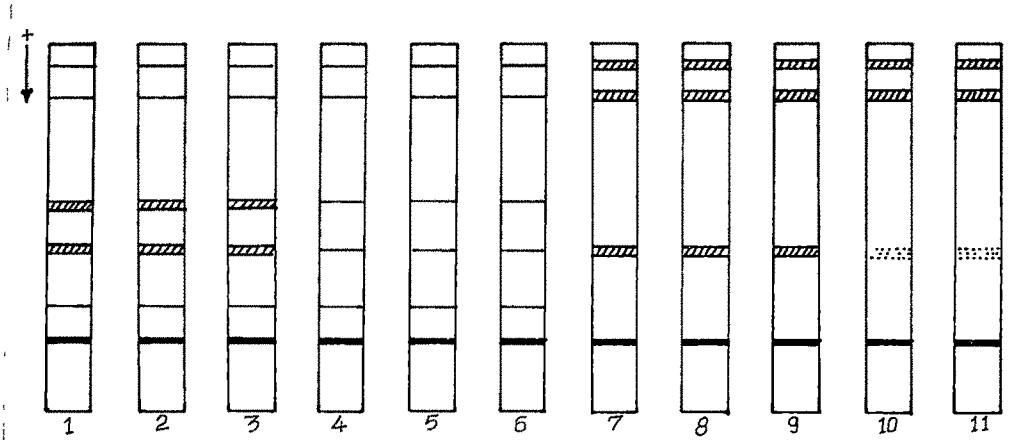

FIG. 15 – DISC ELECTROPHORETOGRAM OF THE SOLUBLE PROTEINS IN THE PULP (STAINED WITH AMIDOBLACK)

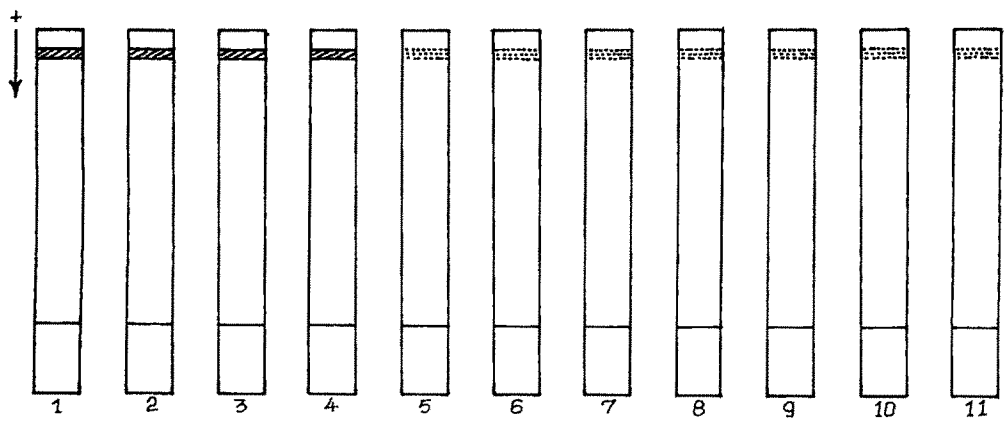

FIG. 16 — DISC ELECTROPHORETOGRAM OF THE SOLUBLE GLYCOPROTEINS IN THE PULP (STAINED WITH PAS)

—— ····· DISCRETE & DARK

▨ ·· ··· LIGHT

⁘ ····· DIFFUSED

Invertase inhibitor which has been partially purified and the kinetic details studied is found to be inactivated by ethylene as the mango fruit ripens.

The mango invertase inhibitor has been found to inhibit papaya and banana invertase also. These results suggest the regulatory role played by the inhibitor during the ripening of mangoes.