C H A P T E R II.

MATERIALS AND METHODS

Alfanso mango (Mangifera indica) is one of the principal varieties of mangoes which are of commercial importance in India. Alfanso mangoes used for this study were obtained from the Bulsar district of Gujarat State (India). The mature unripe mangoes were picked from the trees and were transported to the laboratory within 24 hours. Immediately they were washed, cleaned and a few kept for ripening. Mangoes were usually ripened between 25°-30° C. The various stages of the fruit during ripening were marked by the colour development and appearance as indicated below:

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.

Banana, Papaya and other varieties of mangoes purchased from the local traders were also used in this study.

Estimation of Starch:

The pulp was ground with alcohol till the residue was snow white and then centrifuged. The alcohol insoluble residue was suspended in 0.02M phosphate buffer and digested with α amylase (obtained from M/s Sigma Chem.Co.; U.S.A.) at 37° C for 16 hours. This enzymatically digested solution was further hydrolysed with 0.1N HCl for two hours in a boiling water bath, neutralised with solid sodium

carbonate and estimated for free sugar content by Coles method.[155] The estimated free sugar was then calculated in terms of starch.

Determination of Cellulose:

Fruit pulp was extracted with alcohol followed by ether till the residue was snow white and then centrifuged. The residue was then suspended in acetate buffer (0.02M, pH 5.5) and digested with 10 mg. (1:2 w/w) cellulase (obtained from M/s Sigma Chem. Co.; U.S.A.) at 37° C for 16 hours. The mixture was further hydrolysed with 0.1N HCl in a boiling water bath for two hours, neutralised with solid sodium carbonate and the reducing sugar estimated by Cole's method.[155] The estimated free sugar was then calculated in terms of cellulose.

Estimation of Uridine - di - phosphate - glucose (UDPG):

UDPG was extracted by the method of Leloir and Paladini,[156] and estimated by the method of Strominger et al.[157] 30 gms. of mango pulp was extracted by grinding with 3 ml. of warm toluene and kept at 37° C for 3 minutes. To this solution 30 ml. of 95% ethanol was added, thoroughly mixed and kept in a boiling water bath for about 3-5 minutes and left at room temperature overnight. The following day it was filtered and aliquots taken were used for the estimation. The assay system contained 100 μ moles of glycine buffer (pH 8.7), 1 μ mole NAD excess of UDPG

dehydrogenase and aliquots of the sample. The change in optical density at 340 mμ was measured at an interval of one minute till no further reaction was detected difference in optical density of 12.0 per ml. corresponds to 1 micromole of UDPG.

Preparation of crude fatty acids from mangoes:

Crude fatty acids were prepared by extracting the mango pulp with acetone and ether (1:1 v/v) till the tissue was free from all the colouring material. After removing the moisture (sodium sulfate, one hour) the solvent was evaporated at 80°.C. Crude lipid extract was saponified in 1N alcoholic KOH and after acidification (pH 4.0) the fatty acids were extracted with ether. The etheral extracts were pooled and evaporated to dryness. The fatty acids were dissolved in a known volume of acetone and used. No attempt was made to quantitate the extracts. Qualitatively the extracts have been shown to contain palmitate, oleate, linoleic and linoleate.[266]

Preparation of cell free extracts of mangoes:

All the operations were carried out at 0-5° C. After removing the peel, the mango pulp was ground in a chilled mortar for 15 minutes with Tris-HCl buffer (0.1 M pH 7.2) to obtain a 30% extract. The extracts were neutralised with cold 0.1N NaOH during grinding. After squeezing through two layers of cheese cloth, the extracts were centrifuged

at 6000 x g for ten minutes. The supernatants so obtained were referred to as cell free extracts and were used for the assay of enzymes.

Cell free extracts for the estimation of pectinesterase were prepared in 10% NaCl as recommended by Kertesz.[158] Protein was estimated in the trichloro acetic acid precipitates of the cell free extracts by the method of Lowry et al.[159]

Disc electrophoresis:

The cell free extracts, after concentration with sucrose by reverse dialysis were used for this study. Disc electrophoresis was performed using 7.5% and 10% bis acrylamide gel in Tris glycine buffer (pH 8.3)[215] at 4 mA. per tube for 40 minutes.[216] The protein bands were stained with amido black as well as with periodic acid schiff's reagent (PAS).[3]

Enzyme assay:

The constituents in the following assays are expressed in micromoles unless otherwise stated.

Amylase:

The method was based on the colorimetric determination of the reducing groups of sugars by 3, 5, dinitro salicyclic acid. The assay system used is similar to that of Bernfield.[161] The system contained

0.5 ml. of 1% soluble starch, 50 potassium phosphate buffer (pH 7.0) and an appropriate concentration of enzyme extract (230-650 µ g protein) in a final volume of 2.0 ml. Blank tubes contained boiled enzyme. The tubes were incubated at 37° C for one hour and the reducing groups assayed.

A unit of enzyme is that amount which liberates one micromole of reducing group calculated as maltose per hour at 37° C.

Pectinesterase:

This enzyme was essentially determined by following the method of Kertesz.[170,171] The assay system contained 20 ml. of 1% pectin solution containing 0.1 M NaCl adjusted to a pH of 7.5 and enzyme solution containing 100-600 µg protein was added and the time noted. 0.02 N alkali was added at the rate required to keep the mixture at pH 7.5 for 30 minutes (with a pH meter).

One unit of enzyme is that amount of which removes one micromole of methoxy group per hour at 25° C.

Cellulase:

Cellulase was estimated by the method of Trage.[172] The assay mixture contained 100 mg. of carboxy methyl cellulose in 2 ml. of 0.1 M phosphate buffer (pH 5.5) and an appropriate amount of enzyme (50-300 µg protein).

Blank tubes contained boiled enzyme. The system was incubated for 24 hours at 37° C on a shaker. The reaction was terminated with 5 ml. of 0.1N NaOH and the liberated glucose was estimated by the method of Willstater-Schudel.[173]

A unit of enzyme is defined as that amount which liberates one micromole of glucose per hour under the experimental conditions.

Invertase:

The enzyme was estimated by measuring quantitatively the hexose formed by the method of Nelson.[162] The reaction mixture contained 100 sodium acetate buffer (pH 5.0-5.5), 25 sucrose, and an appropriate concentration of the enzyme extract (50-300 µg protein). Enzyme blanks were run. Incubation was carried out at 37° C for one hour. The reaction was terminated by adding 3.0 ml. of 0.5 M dibasic sodium phosphate and heating in a boiling water bath for two minutes. Precipitated proteins were filtered and various aliquots were assayed for reducing sugars liberated.

A unit of invertase is that amount which liberates one micromole of reducing sugar per hour under the experimental conditions.

Fructose-1:6-diphosphatase (FDPase):

The method was that of McGilvery[163] with a slight modification that cysteine and $MnCl_2$ were omitted

where as, $MgCl_2$ was added. The assay system containing 5, fructose diphosphate, 50, Tris HCl (pH 7.0), 0.5, $MgCl_2$ and mango extract containing 50-100 µg protein (along with the substrate blanks) was incubated at 37° C for one hour.

Glucose-6-phosphatase (G-6-Pase):

The method described by Swason[164] was followed. The test system contained 5, glucose-6-phosphate, 50, acetate buffer (pH 9.0), 1 $MgCl_2$ and enzyme extract in a final volume of 2.0 ml. was incubated for one hour at 37° C. Substrate blanks were run along with experimental. The reaction was terminated with 15% trichloro acetic acid.

Phosphofructokinase (PFK):

Phosphofructokinase was estimated by the method of Ling et al[166] after a slight modification that the alkali labile inorganic phosphate of triose phosphate was estimated. The test system containing 100 Tris-HCl buffer (pH 8.0), 20 ATP, 20 fructose-6-phosphate, 1 $MgCl_2$, 20 KCN, and excess of aldolase (obtained from M/s Sigma Chem. Co., U.S.A.) along with the enzyme extract (80-150 µg protein) in a final volume of 2.5 ml. was incubated at 37° C for one hour. Boiled enzyme blanks were run along with the experimental. The reaction was terminated with 1 ml. of 1N NaOH and kept for 15 minutes at 35±2° C. The reaction mixture was then neutralised with 1 ml. of 1N HCl.

In all the above cases the liberated inorganic phosphate was estimated by Fiske and Subbarow method.[165] One micromole of inorganic phosphate liberated under the conditions stated was termed as one unit.

Hexokinase:

Hexokinase was determined by following the method of Pakoskey et al.[218] The method was based on the measurement of the reduction of NADP by glucose-6-phosphate dehydrogenase in presence of glucose-6-phosphate, the latter being formed by the action of hexokinase.

The system contained 100, Tris-HCl buffer (pH 7.5), 50, $MgSO_4$, 5, nicotinamide, 0.4 NADP, 5, ATP, 5, glucose and excess of glucose-6-phosphate dehydrogenase in a final volume of 3.0 ml. The reaction was started by adding glucose, blank contained all the components except glucose.

Pyruvate Kinase:

The method was essentially that of Theoder and Peleiderer.[219] The system contained 5, NADH, 5, ADP, 10, phosphoenol pyruvate, 50, $MgSO_4 \cdot 7H_2O$, 10, KCl, 100, Triethanol amine hydrochloride buffer (pH 7.5) an excess of lactic dehydrogenase and mango extract containing 100-200 µg protein, in a final volume of 3.0 ml. The reaction was started by adding phosphoenol pyruvate. Substrates blanks were run along with experimental.

Isocitrate-dehydrogenase:

The enzyme was estimated by the method of Kornberg.[169] The system in a total volume of 3.0 ml. contained 100, phosphate buffer (pH 7.0), 15, NAD or NADP, 5, $MgCl_2$, 6, DL isocitrate and the enzyme extract.

α-ketoglutarate dehydrogenase:

Kaufman's[220] method was followed for the estimation of this enzyme with slight modifications. The assay system contained 0.26, coenzyme A, 100, phosphate buffer (pH 7.4), 0.27, NAD, 8.3, cysteine, 25 potassium salt of α-ketoglutarate with an appropriate concentration of the enzyme extract in a total volume of 3.0 ml.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase:

These two enzymes were determined by the method of Kornberg and Horecker.[168] The test system contained 200, glycyl glycine buffer (pH 7.4), 25, $MgCl_2$, 6, glucose-6-phosphate, 1.5 NADP and an appropriate concentration of mango extract in a final volume of 3.0 ml. For the estimation of 6-phosphogluconate dehydrogenase $MgCl_2$ and glucose-6-phosphate were omitted and 6-phosphogluconate was added in the test system.

Malic enzyme:

Malic enzyme was estimated by the method of Ochoa.[167] The assay system contained 200, Tris-HCl

buffer (pH 7.4), 5, $MnCl_2$) 20, L-malate, 1.5, NADP, and an appropriate concentration of enzyme in a final volume of 3.0 ml.

In the above mentioned seven enzymic assays the increase in optical density at 340 mμ was measured against the respective substrate blanks.

One unit of enzyme is termed as that amount which causes a change in optical density of 0.001 per minute.

Sucrose synthetase and Sucrose phosphate synthetase:

These two enzymes were estimated by the method of Leloir[150] with the modification that the sucrose formed was estimated by the method of Emil-Van-Mandel[174]. The system contained 12.0, phosphate buffer (pH 7.0), 0.02, $MgCl_2$, 0.5, UDPG, 2.0 frutose, and mango extract containing 30-60 μg protein in total volume of 0.3 ml. was incubated at 37° C for one hour. In case of sucrose phosphate synthetase fructose-6-phosphate was used instead of fructose, and after the reaction was completed the pH was adjusted to 9.7 and the system treated with alkaline phosphatase for ten minutes.

One unit of the enzyme is that amount which synthesis one micromole of sucrose per hour under the experimental conditions.