

EXTRACTION, PURIFICATION AND PARTIAL CHARACTERIZATION
OF THE SWEET MODIFYING GLYCOPROTEIN, MIRACULIN FROM
RICHARDELLA DULCIFICA.

A THESIS SUBMITTED

by

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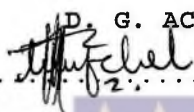
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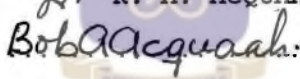
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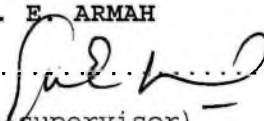
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D E C L A R A T I O N

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TO

My Supervisor and Mentor

Dr R. A. ACQUAAH



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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
A-25	Amberlite-25
BRI	Bio-Resources Incorporated
L-BApNA	L-benzoyl-D,L-arginine-p-nitroaniline
DEAE	Diethyl amino ethyl
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GDR	German Democratic Republic
GRAS	Generally regarded as safe
PAGE	Polyacrylamide gel electrophoresis
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
LD ₅₀	Lethal dose 50%
N.M.I.M.R	Noguchi Memorial Institute for Medical Research.
NPP	p-Nitroaniline
N.Y	New York
QAE	Quartinary amino ethyl
R ²	R-squared
SDS	Sodium dodecyl sulphate
TEMED	NNN'N'-tetramethylene diamine
Tris	Tris (hydroxymethyl) aminoethane
T _m	Transition (melting) temperature
V _e	Elution volume

ABSTRACT

In the present study miraculin, a non-nutritive taste modifier from the tropical plant, *Richardella dulcifica* has been purified to homogeneity. It was extracted from the pulp of the fruits in 0.5M NaCl and purified at ordinary temperatures by a three step chromatographic process namely; Hydrophobic interaction chromatography, (HIC), Ion exchange chromatography, (IEC), and Gel filtration chromatography (GFC) respectively. The relative molecular weight (M_r) of purified miraculin was about 43, 000 and 100 $\mu\text{g/ml}$ of the lyophilized product provoked sweetness with lemon. N-terminal amino acid analysis revealed two forms of miraculin, the major form being more than 70 % of the total miraculin population.

Protease activity was detected and measured in the pulp of fresh and aged fruits. The activity and specific protease activity in the pulp of freshly picked berries were 0.0756 units/mg pulp and 0.2414 units/mg protein respectively. Corresponding values for the aged (at least six months old) berries were 0.0369 unit/mg pulp and 0.1342 unit/mg protein. The difference was significant ($p \leq 0.05$).

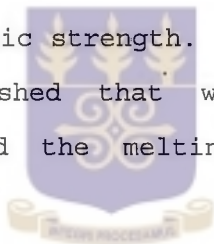
Total protein content measured for the fresh and aged berries were $31.3\% \pm 1.9\%$ and $27.4\% \pm 0.3\%$ (w/w) respectively. The difference was not significant ($p \leq 0.05$), in addition only approximately 10 % (w/w) of the fruits contained the active

principle, miraculin.

A predictive model:

$$Z = 0.027348 - 0.015177\mu - 0.003364\mu\text{pH} + 0.000128\text{pH} + 0.029960\mu^2$$

where Z is the solubility, was developed to explain the effect of ionic strength (μ) and pH on the solubility of miraculin. The model had an R^2 of 66.12%. Analysis of variance (ANOVA) showed ionic strength to be the most influential variable in the model. The protein had the maximum solubility at low pH and low ionic strength, with the minimum solubility recorded at high pH and high ionic strength. A thermal denaturation investigation, established that water-glycerol mixtures substantially increased the melting temperature of the purified product.



C H A P T E R 1

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

There is an increasing demand for non-nutritive sweeteners, and a growing departure from conventional nutritive sweeteners. This is probably due to the rising number of obese and/or diabetic people (Kurihara, 1992). Furthermore, some of the sweeteners have been implicated in the etiology of certain cancers and other malignancies (Higginbotham, 1979; Crosby, 1976).

Synthetic non-nutritive sweeteners which were commercially available until lately include aspartame, acesulfame K, xylitol, cyclamate and saccharin. These compounds are not good substitutes for conventional sweeteners in spite of their low calorie content. Studies suggest that the intake of these non-nutritive sweeteners induces insulin secretion with a resultant increase in appetite (Sardesai and Waldshan, 1991). Due to this and other adverse physiological effects induced by these synthetic sweeteners, their consumption has been restricted by legislation, and are not generally regarded as safe (Higginbotham, 1979). Saccharin, a popular non-nutritive sweetener, has a poor after-taste quality, and was once reported to be potentially hazardous to consumer health (Higginbotham and Hough, 1977). Aspartame consumption has been found to disturb the amino acid pool of individuals who suffer from phenylketonuria (Crosby, 1976). Xylitol has been reported to cause cancerous lesions when fed to rats in large

quantities (Higginbotham, 1979). The use of cyclamate in the food industry was banned on similar grounds (Holloway, 1977; Higginbotham, 1979; Higginbotham and Hough, 1977). Most other synthetic sweeteners are handled with uncertainty, and are usually passed through rigorous toxicological assays, before they are declared safe or otherwise, by the regulating agencies (Higginbotham, 1979).

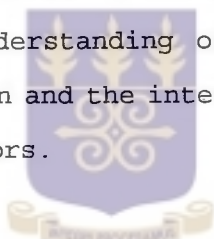
Natural sweeteners

There is an abundance of naturally occurring sweeteners and/or sweetener-enhancers from plant sources. These may be proteins or other macromolecules, and are much less hazardous. They may afford a solution to the search for low calorie sweeteners. Unlike the synthetic sweeteners, protein sweeteners are not known to disturb the balance of the amino acid pool in the body.

The taste of all known sweet proteins lingers on after stimulating the taste buds with them. This property of the sweet proteins suggests strong binding to the taste receptors on the tongue. The sweet proteins may therefore be used as suitable ligands to probe sweet taste perception. Being macromolecules, the sweet proteins possess more sites for labelling, without obstructing or influencing the sweetness domain, than smaller molecules. Isotope, photo-affinity and chemically labelled sweet proteins have been used to aid taste

receptor identification and isolation with encouraging results (Ming, 1994; Morris and Cagan, 1972).

The use of protein mutants generated by recombinant DNA technology, will provide an understanding of the interactive dynamics between sweet taste elicitors and taste receptors. Modification of the sweet proteins by recombinant DNA technology will also enhance the search for more desirable taste effectors as well as the design of new sweeteners. The sweet proteins/protein sweetener enhancers appear to be a key route to the proper understanding of the process of sweet taste signal transduction and the interaction of the mechanism with sweet taste receptors.



Six natural protein sweeteners/sweetener-enhancers are known: thaumatin, monellin, brazzein, mabinlin, curculin, and miraculin (Sardesai and Waldshan, 1991). Their high sweetening potential, relative to sucrose, makes the plant protein sweeteners a better alternative. Sucrose and other carbohydrate sweeteners are required in relatively large amounts (Higginbotham and Hough, 1979; Crosby, 1976; Pintauro, 1977) and therefore constitute a source of high calorie in the food.

Most of the plants from which the sweetness-enhancing proteins are obtained thrive in West Africa. For instance, monellin,

thaumatin, brazzein and miraculin are found in the fruit pulp of *Dioscoreophyllum cuminsii*, *Thaumatococcus danieli*, *Pentadiplandra brazzeana* and *Richardella dulcifica* respectively (Kurihara, 1992). Though all four plants thrive well in Ghana, *R. dulcifica* also known as the miracle fruit, appears to be the best adapted. In addition, amongst the list of protein sweeteners/ sweetener enhancers, the protein sweetener found in *R. dulcifica*, miraculin, has unique characteristics both structurally and functionally. It is the only glycoprotein and taste modifier with no intrinsic sweetness (Kurihara, 1992; Crosby, 1976). Miraculin will probably have a wider market than the other protein sweeteners for the reason that in addition to its sweetening ability, it can also be used to mask the sour taste of pharmaceutical products. Sweetening activity of the sweetener enhancer in the pulp of miracle fruits decreases with period of storage. Investigations seem to suggest that the presence of proteolytic enzymes in the pulp of the fruit may be one of the plausible explanations for this observation (Higginbotham, 1979; Crosby, 1976)

A lot of interest has been generated in miraculin ever since its rediscovery in the mid 1960s. Various attempts have been made by different groups of researchers to obtain it in pure form (Igeta et al, 1991; Kurihara and Terasaki 1982). There has been some success in the isolation and purification but

the methodologies employed are difficult to reproduce and the yields have been low (Brouwer et al, 1968; Kurihara, 1992). One other major problem has been the co-extraction of other pigments/compounds with miraculin. These compounds are very difficult but not impossible to separate from miraculin.

Miraculin is very sensitive to changes in ionic strength, temperature and pH. A knowledge of the optimum conditions for its stability and solubility will be of immense importance in its utilisation as a taste enhancer and food additive.

Objectives

In view of the problems enumerated above, the present study was undertaken to:

1. Determine the protease activity in the pulp of miracle fruit, both fresh and aged.
2. Purify miraculin to homogeneity at ordinary temperatures
3. Study the influence of pH and ionic strength on the solubility of miraculin.
4. Stabilise the purified miraculin (using a water-glycerol medium).
5. Determine the amino acid sequence of the N-terminal region of the purified product to ascertain purity or homogeneity.

1.2

LITERATURE REVIEW

Sweet tasting proteins.

Thaumatococcus danienii and Pentadipandra brazzeana are intrinsically sweet proteins obtained from the fruits of *Thaumatococcus danienii* and *Pentadipandra brazzeana* respectively. These are tropical plants from West Africa (Crosby, 1976; van der Wel, 1991 Ming and Hellekant, 1993; 1994). The plants thrive best in well drained alluvial soil in the rain forest zone. Both sweeteners are single chain non-glycosylated polypeptides and completely lack modified amino acid residues (Kim et al, 1988, Ming and Hellekant 1993; 1994, Ming 1994). Thaumatococcus has 207 amino acid residues, with an average relative molecular weight (M_r) of 22,000 and an isoelectric point range of 11.5 to 12.5. Brazzein on the other hand consist of 54 amino acid residues, has a M_r of 6,473, and an isoelectric point of 5.0. In comparative terms, brazzein elicits a higher degree of sweetness sensation than thaumatococcus. Thaumatococcus and brazzein are estimated to be 1600 and 2000 times sweeter than sucrose on weight basis respectively. Both proteins are exceptionally stable to heat. Kim et al. (1988 ; 1989), have shown that thaumatococcus, boiled for over an hour at low pH and cooled slowly, regains its sweetening activity. Attempts at expressing thaumatococcus in microorganisms have not been successful and have produced only non-functional proteins (Witty, 1990, Illingworth et al 1988: 1989).

Monellin and mabinlin are two very sweet proteins which are produced in the pulps of plants. Monellin is produced in the pulp/mucilage of the fruit of *Dioscoreophyllum cumminsii* and mabinlin in the fruits of *Cappris masaki*, a plant belonging to the family Capparidaceae and locally known in Chinese as "mabinlang". *C. Masaki* is indigenous to the Chinese province of Yunnan and Thailand in the Far East while *D. cumminsii* grows in tropical West Africa and also in Sudan and Equatorial Africa (Kim et al 1988; 1989, Liu et al, 1993, Bodani et al, 1993; van der Wel and Avidson, 1978). Both proteins in their native states, are made up of dimers of two dissimilar peptide chains bound tightly but non-covalently. Monellin has an average of 94 amino acid residues with the two peptide chains; A and B, consisting of 44 and 50 amino acid residues respectively (Bodani et al, 1993; Kim et al, 1988; Kohmura et al, 1990). Extensive hydrogen bonding exists between the two chains and this probably explains why the two chains of monellin can only be separated by very strong denaturation protocols (Kim et al, 1988). Separation of the chains leads to total loss of activity but partial activity is restored, albeit slowly, after reconstitution of the two chains (Bohak and Li, 1976; Crosby, 1976; Higginbotham, 1979). Monellin has an isoelectric point range of 8.7 to 9.4 and a M_r of approximately 11,000 (Kohmura et al, 1990; Morris et al, 1973). Mabinlin has a M_r of about 12,400 and an isoelectric point of 11.0. Monellin has a peculiar taste, and sweetness

takes several seconds to develop, gradually builds up to a peak intensity, lingers for minutes and slowly declines over a period of time. The sweetness survives rinsing of the mouth with water (Crosby, 1976; Morris and Cagan, 1972). Monellin is 1200 to 3200 times sweeter than sucrose on weight basis and approximately 100,000 times on molar basis (Crosby, 1976; Kim et al, 1988 and 1989; van der Wel, 1972). The sweetness of 0.8 mM mabinlin is equivalent to sweetness of 0.3 M sucrose suggesting that mabinlin is sweeter than monellin on molar basis. Unlike monellin, mabinlin is highly thermostable. The sweetening activity of mabinlin solution persists at high temperatures (80°C) in the absence of protein stabilizers for at least 48 hours (Kim et al, 1988; Liu et al, 1993).

Sweetness modifying proteins

Curculin is obtained from the fruit of *Curculigo latifolia*, a stemless herb that grows wild in Western Malaysia and the southern part of Thailand (Yamashita et al, 1990; Kurihara, 1992). The fruit is eaten by natives to give a sweet taste to sour food. Additionally, it elicits a sweet taste when taken after water and black tea. Curculin is a dimer of two similar peptides and consists of one hundred and fourteen amino acid residues. It has an isoelectric point of 7.1 and a M_r of 12,500 (Kurihara, 1992; Yamashita et al, 1990; Nakajo et al, 1990). Purified curculin is only sparingly soluble in water,

but dissolves readily in aqueous sodium chloride solution. The sweetness of 10 μ M pure curculin is equivalent to the sweetness elicited by 0.2 M sucrose. It is therefore 20,000 times sweeter than sucrose on molar and 550 times on weight basis (Kurihara, 1992; Nakajo et al, 1992). After administration of 10 μ M of pure curculin, 0.02 M citric acid elicits a sweetness equivalent to 0.35 M sucrose while water elicits an equivalent sweetness of 0.20 M sucrose (Yamashita et al, 1990).

Miraculin is obtained from the plant *R. dulcifica*. It has the unusual property of changing sour taste to sweet taste after the mouth has been exposed to the sweetness inducing principle (Crosby, 1976; Higginbotham 1979, Kurihara and Beidler, 1968). In places where it is found, people usually sensitize their mouths with the fruit before eating oranges. It is also used to render acidulated bread, locally made beers and wines more delightful (Kurihara and Beidler, 1968). Daniell (1852), was first to document the unusual property of the berry and called it miraculous berry. However, the chemical nature of the active principle was not investigated (Crosby, 1976). The active principle is not known to influence the taste of bitter and salty substances (Kurihara and Beidler, 1968). Miraculin is also reported to modify overall flavour perception (Crosby, 1976; Kurihara 1992).

The plant *Richardella dulcifica* otherwise known as *Sensypalum dulcificum* belongs to the family Sapotaceae (Higginbotham, 1979; Irvine, 1961). It grows to a height of 1.52 - 3.81 m and is characterized by slender branches with clusters of dense slender foliage at the tips (plate 1). The leaves, which have very short petioles, measure 5.1-10.2 x 2.0-3.8 cm. The plant is propagated mainly by seed and fruiting begins 3 to 4 years after planting the seedling (Irvine, 1961 and Higginbotham, 1979). Although *R. dulcifica* grows at a very slow pace, it has been reported to have a long life span. Plants of over 40 years have been reported to be capable of bearing fruits (Dastoli and Harvey, 1974). The plant is indigenous to Africa and stretches from Ghana to Congo. It normally grows wild on farmlands, around dwellings, forest fringes and along the coast (Higginbotham, 1979; Dastoli and Harvey, 1974). In Ghana, it is found mainly as a bush in citrus farms/plantations.

Flowering and fruiting times vary from place to place. However, in general it flowers and fruits twice a year (Higginbotham, 1979; Dastoli and Harvey, 1974). In Ghana, it flowers and fruits between the months of May and June, and August to October. The flowers which are initially brown,

occur in small axillary sub-sessile clusters with ribbed calyx. These eventually develop into red ellipsoidal berries which measure between 1 cm to 2 cm long with a thin jelly-like pulp surrounding a single large olive shaped seed (plate 2).



plate 1: A tree of *R. dulcifica*

R. dulcifica has low aesthetic value if it has at all. The use of the plant or plant parts in the field of medicine has not been documented. However, its stems and twigs are used as chewsticks and as a source of fuel. Miraculin is found in the pulp of the fruit of *R. dulcifica* (Higginbotham, 1979; Kurihara, 1992).



Plate 2: The Miracle fruits

Ever since its rediscovery in the mid 1960s, it has been extremely difficult to obtain commercial quantities of purified miraculin from the pulp of miracle fruits. Most of the seemingly successful reported methods of purification are not reproducible. The first attempt at purification was made in 1965, but the active principle failed to solubilize in either aqueous or organic media (Brouwer *et al*, 1968; Crosby, 1976; Kurihara and Beidler, 1968). This characteristic of the active principle was later confirmed, when varying conditions of pH and ionic strength also failed to extract it from the pulp of the fruit (van der Wel, 1974). However, solubilization of the taste modifying principle was achieved at pH 7 and at slightly higher pH values when conditions prevailing in the mouth were simulated using whole human saliva (Brouwer *et al* 1968, van der Wel 1974). Similar results were also obtained when the aqueous extracting medium was enriched with neuraminic acid derivatives or tanning binding agents like polyethylene glycol, gelatin, Tween 80 and caffeine at neutral or slightly higher pH (Brouwer *et al*, 1968, van der Wel, 1974). Further work revealed that an aqueous solution of slightly basic compounds such as salamine, protamine, naturally occurring spermine and polyamine can be used to extract the active principle, producing a better yield than previous methods (Brouwer *et al* 1968; van der Wel, 1974).

Kurihara and Baidler (1968), reported an alternative protocol

for the isolation and purification of miraculin. It was extracted using carbonate buffer at pH 10.5 and purified successively through DEAE-Sephadex A-25 and CM-sepharose columns. Their results showed that ion exchange chromatography resulted in a partial loss of the sweetening activity of miraculin. Moreover, the carbonate buffer co-extracted various other substances which were difficult to separate (Crosby, 1976; Kurihara and Terasaki, 1982; Theerasilp *et al*, 1989). Miraculin has also been purified using Bio-Gel, CM-30, and QAE-Sephadex columns. However, it was found to have half maximal activity (Kurihara and Terasaki, 1982). Three active homologues of miraculin were isolated using the method of electrofocusing (Kurihara and Terasaki, 1982). The method is reported to be expensive and therefore not suitable for commercial scale purification. Theerasilp and Kurihara, (1988) reported a new method of isolating and purifying the active principle from the pulp of the fruit. In their protocol, the active principle was solubilized by homogenizing the washed pulp of the fruit in 0.5 M aqueous sodium chloride, followed by ammonium sulphate fractionation. The precipitated protein was purified at 4°C by first passing through an ion exchange resin and subsequently by affinity chromatography on a concanavalin A-sepharose column. (Takahasi *et al*, 1990, Theerasilp *et al*, 1989).

Chemical properties

The sweetening activity of miraculin is destroyed by boiling while pH changes profoundly affect it. Studies have shown that, it retains its sweetening activity within the pH range of 2.5 and 12.0 at room temperatures but loses activity outside this range (Brouwer *et al*, 1968; Kurihara and Beidler, 1968). Exposure of miraculin to increasing concentrations of organic solvents at room temperature leads to complete loss of activity. Incubation with proteolytic enzymes such as pronase and trypsin or exposure to UV radiation leads to a complete loss of sweetening activity (Brouwer *et al*, 1968; Kurihara and Beidler, 1968). It was found to have a similar behaviour pattern of proteins; it had an absorption maxima at 278 nm, responded positive to the biuret test and was precipitated by iodoacetic acid and acetone. Electrophoretic analysis on polyacrylamide gel at pH 4.5 caused the active principle to migrate to the cathode, while no net migration was observed at pH 8.3 suggesting a basic active principle (Kurihara and Beidler 1968; 1969, van der Wel and Loeve, 1972). Acid hydrolysis followed by chemical analysis revealed that the hydrolysate consisted of a mixture of amino acids and carbohydrates (Kurihara and Beidler, 1968; Theeraslip *et al*, 1989; van der Wel, 1974). This was further confirmed when a sample of miraculin tested positive for sugars before and after hydrolysis (Brouwer *et al*, 1968). These results depicted miraculin as a glycoprotein.

Three homologues of miraculin with similar sweetness modifying activity have been isolated and named as miraculin I, II and III. These homologues have identical chemical properties and only differ in their carbohydrate content and isoelectric points. Miraculin I has a pI value of 9.3; II has a value of 9.0; and III a value of 8.0 (Kurihara and Terasaki, 1982). However, using starch gel electrophoretic analysis, the protein (miraculin) runs as one single band and has a pI of approximately 9.0 (Brouwer et al 1968; Higginbotham, 1979; van der wel, 1974).

Miraculin homologue II, is the most abundant. Like the other two homologues, it has a total of 191 amino acid residues in its primary structure (Takahashi et al, 1990; Theeraslip et al, 1989). There does not seem to be much difference between the amino acid compositions of the three homologues (Kurihara and Terasaki, 1982). van der Wel, 1972 found the most common amino acids present in miraculin to be glycine, arginine and lysine. On the other hand, Theerasilp et al. (1989) reported that the amino acids of highest frequency in miraculin are aspartic acid, valine, glycine, proline, threonine and phenylalanine. The relative amounts of the various amino acids present in miraculin has also been estimated and reported (Kurihara, 1992). Takahasi et al (1990) and Theeraslip et al (1989) have shown that, the N- and C- termini of the protein are aspartic acid and phenylalanine

respectively. It has a proline rich N-terminal region. Of the total of fourteen proline residues present in miraculin ten are located in this region of the protein. A total of seven cysteine residues have been found in miraculin, five of which are located between positions 138 and 159 while the remaining two are at positions 47 and 92. These cysteine residues form three intrachain and one interchain disulphide bridges. Cysteine 47-92, cysteine 143-159, and cysteine 152-155 form the three intrachain bridges while the last cysteine residue (cysteine 138), forms an interchain disulphide bridge between miraculin monomers (Theeraslip *et al*, 1989; Theeraslip and Kurihara, 1988). Carbohydrate-linked asparagine residues are located at positions 42 and 186 of miraculin homologue II, while valine and lysine are at positions 157 and 158 respectively (Igeta *et al* 1991; Takahashi *et al*, 1990). Results gathered from amino acid analysis by the various workers reveal the presence of all the essential amino acids in the primary structure of miraculin.

Brouwer *et al* (1968), found the total carbohydrate content of miraculin to be 7.5% and 21% by the anthrone and carbazole methods respectively. The sugars that constitute total carbohydrates in miraculin are glucose, arabinose, ribose, galactose, and rhamnose. These results differed markedly from those reported by Kurihara and Beidler, (1969) who found the carbohydrate content to be 6.7% of the total mass of the

glycoprotein. Sugar analysis by paper chromatography on a proteolytic hydrolysate of miraculin established the presence of only two residues; D-xylose and L-arabinose (Kurihara and Beidler, 1968 and 1969; Kurihara *et al*, 1969). Later research established 6.3% sugar in miraculin comprising fucose, mannose and galactose (Giroux and Henkin, 1974, Higginbotham, 1979). These analytical differences probably reflect the difficulty of purification and the likely presence of residual contaminants. With increasing improvements in the sensitivity of analytical techniques, more consistent values for miraculin carbohydrates have been reported. Theerasilp and Kurihara (1988) found the total carbohydrate content of miraculin to be 13.9% and identified five species of sugars namely glucosamine, mannose, galactose, xylose, and fucose in relative molar amounts of 3.03:3.00:0.69:0.96:2.12 respectively. These results compare with the findings of Takahashi *et al* (1990) who reported the presence of five sugar residues; N-acetylglucosamine, mannose, fucose, galactose and xylose in relative molar proportions of 6.06:6.00:4.24:1.34:1.92 respectively. The three homologues of miraculin were found to differ significantly in their total carbohydrate content (Kurihara and Terasaki, 1982). The percentage of carbohydrate based on w/w for proteins I, II, and III was 16%, 20% and 41% respectively. Geographic distribution and/or climatic conditions seem to play a significant role in carbohydrate biogenesis of the protein, miraculin, and may

explain the reported differences in carbohydrate content (Kurihara and Terasaki, 1982).

Relative molecular weight (M_r)

Various attempts have been made to determine the relative molecular weight of miraculin. Kurihara and Beidler (1968) estimated the M_r of miraculin to be 44,000 by comparing its elution volume to that of standard marker proteins. However, using analytical ultracentrifugation, a molecular weight of $42,000 \pm 3,000$ was obtained (Pintauro, 1977). On sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and in the presence of a reducing agent, a M_r of 28,000 was reported, while a value of 24,000 was obtained from calculations based on the amino acid sequence and total carbohydrate content of the glycoprotein (Theerasilp *et al*, 1989; Theerasilp and Kurihara, 1988). Furthermore, miraculin was found to have a M_r of 21,200 following hydrolysis of the carbohydrate moiety (Theerasilp and Kurihara, 1988). These results suggest that miraculin exists as a dimer of 43 kDa in the native state, (Igeta *et al* 1991; Kurihara and Terasaki 1982).

It is widely known that substances with high molecular weights do not normally stimulate the taste buds/cells and hence cannot elicit sweetness (Kurihara, 1992; Higginbotham 1979). However, miraculin and other protein sweeteners and/or

sweetener-enhancers appear to be exceptions to this rule.

Mechanism of action

Miraculin is not intrinsically sweet, but is believed to modify or alter the action of appropriate taste receptors in the mouth to perceive sweet taste when exposed to sour substances. It has therefore been assigned to the class of gustatory chemoreceptors (Dastoli and Harvey; 1974 Vinetskii, 1987). It is by far the largest known molecule that can cause a sweet sensation. However, its mechanism of action has not been well elucidated (Higginbotham 1979). When mixed with a sour substance, the mixture initially tastes sour but gradually turns sweet if held in the mouth for a while. This over-rules the claim that the protein modifies the sour substance itself and suggests that the sweetening effect is due to the action of the protein on some specific taste receptors (Kurihara and Beidler, 1968 and 1969).

Kurihara and Beidler (1968) showed that the taste function of miraculin proceeds slowly, attaining maximum sweetness after three minutes. At higher protein concentrations, the sweetening effect induced by miraculin persists longer than at lower concentrations. Further, it was demonstrated that inducing secretion of saliva either by chewing gum or eating foods after application of miraculin caused the sweetening effect to decline rapidly (Kurihara and Beidler, 1968 and

1969). Dzendolet (1969), proposed that anions of some acids such as citrate ions of citric acid, are sweet but are normally inhibited by sour taste. He postulated that miraculin blocks the sour receptor sites allowing a sweet taste to be generated by the anionic group of the acid molecule. Others are of the view that miraculin, being a glycoprotein, gets hydrolysed under mild acid conditions thereby releasing hexoses which in turn elicit sweetness (Kurihara and Beidler, 1968; 1969). Using gymnamic acid, a known suppressor of sweetness, whose effect is reportedly less persistent than miraculin, it was demonstrated that sourness of an acid solution is not depressed after application of miraculin to the mouth (Kurihara and Beidler 1968; 1969). Chemical analysis by the same group of workers revealed L-arabinose and D-xylose as the only carbohydrate species present in the glycopeptide. It is known that these sugars are not as sweet as sucrose. The sweetness of 1 M arabinose and 1 M xylose is found to be equivalent to the sweetness of $0.35 \pm 0.07\text{M}$ and $0.34 \pm 0.06\text{M}$ sucrose solutions respectively (Kurihara and Beidler 1968; 1969). On establishing this fact, the notion that the sugar component of the glycoprotein gets hydrolysed under mild acid conditions setting free the hexoses which then stimulate the taste buds to perceive sweetness was discarded. Under such mild acid conditions, the linkage between the protein and sugar components is stable and therefore the sugar component will not be easily hydrolysed.

Moreover, it has been shown that 2×10^{-8} M solution of the protein gives a measurable sweetening effect and one molecule of the taste modifying protein is found to contain approximately 20 sugar residues. Therefore, nearly 4×10^{-7} M of the sugar will be contained in 2×10^{-8} M protein solution. The sweet receptors are not sensitive enough to detect arabinose and/or xylose at such low concentrations (Kurihara and Beidler 1968; 1969).

An induced fit model was proposed by Kurihara and Beidler (1969) to explain the mechanism by which the active principle acts to elicit the sweet sensation. It was proposed that:

- i. Due to the high molecular weight of the protein, it is unlikely that the protein penetrates the taste (receptor) cells, and probably binds to the membrane of the taste cells.
- ii. The taste modifying protein contains two allosteric domains, one domain binds to the receptor surface of the taste cells and the other to the active site of the receptor. The carbohydrate groups present in the glycoprotein are believed to bind to the sweet receptor membrane. When the mouth is sensitized with a sour substance (ie. low pH), the taste receptor membrane changes its conformation. The conformational change induced leads to the fitting of the sugar moiety of the protein into the sweet site of the sweet taste receptor thus eliciting sweetness (Kurihara and Beidler 1968; 1969).

Limitation to miraculin production

Frantic efforts have been made to extract and purify the protein by researchers in some temperate countries like Japan and USA. However, since the natural habitat of *R. dulcifica* is tropical West Africa, work done so far has been on fruits grown in green houses (Kurihara, 1992). The protein however, loses its sweetening properties with long storage at normal temperatures. The taste modifying activity quickly declines especially when exposed to the air once the skin has been broken. In fact, the active principle begins to degrade after the fruit has been picked even prior to breaking the skin, but at a slower rate (Crosby, 1976; Fennel and Harvey, 1972; Pintauro, 1977). Efforts geared at synthesizing functional protein sweeteners/sweetener enhancers like miraculin by recombinant DNA technology have not been very successful. Although miraculin has been successfully expressed in *E. coli*, the expressed protein was not active (Kurihara 1992). The difficulty in expressing active miraculin in micro-organisms is attributed in part to its glycoprotein nature and the presence of disulphide bridges. It appears that the oxidation/reduction potential in bacteria and yeast is not suitable for correctly forming disulphide bridges in proteins from eukaryotes. This probably explains why it has so far been possible to express only monellin in micro-organisms (Kim et al, 1991). Biologically active thaumatin and monellin have been successfully expressed in transgenic plants (Penarrubia

et al, 1992 ; Witty et al 1992). It is therefore hoped that the same can be done for miraculin, to afford the production of the protein all-year round thus surmounting the problem of seasonal production from the fruit.

Stability

The decline in sweetening ability of the miracle fruit appears to be accelerated by increased temperature and by contact with air. The degradation of miraculin in the frozen fruit occurs even when stored at -15°C (Dastoli and Harvey, 1974; Fennel and Harvey, 1972) suggesting that it cannot be preserved satisfactorily even at low temperatures. The stability of miraculin in aqueous media is not entirely satisfactory. It is very unstable at normal room temperatures and atmospheric conditions. This property of miraculin poses serious problems especially when it is incorporated into certain foods such as carbonated and still beverages which are often subjected to many months of storage prior to consumption (Fennel and Harvey, 1972). Miraculin is appreciably stable in dairy products at pHs close to 3.0 (Pintauro, 1977). A miraculin- NaHCO_3 concentrate has been found to be stable at pH 4-5 and at room temperature (Kurihara 1992). The lyophilized protein is reportedly stable for several years at room temperature and is equally as effective as the natural fruit in terms of quality response and more effective than the natural fruit on the basis of equivalent weight or volume (Harvey 1973, Fennel

and Harvey, 1972).

Quantization

There are two methods of miraculin quantization. The first method is very subjective and hence biased. In this method, a solution of the protein is held in the mouth for at least three minutes after which it is spat out and the mouth rinsed with water. Citric acid solution, 0.02M, is then administered and the sweetness elicited compared to standard sucrose solutions of varying concentrations (Kurihara and Beidler, 1968; 1969). This method, however, does not quantify the miraculin concentration in the sample. A more accurate and specific method of miraculin quantization is based on the enzyme-linked immunosorbent assay (ELISA) method reported by Nakajo et al, 1988. The method is able to quantify picogram (pg) quantities of the protein in the crude extract accurately. By this method the amount of miraculin in the miracle fruit was found to increase greatly seven weeks after cross fertilization and attained a maximum value of 102 $\mu\text{g}/\text{mg}$ protein after eight (8) weeks. The ELISA method, though very accurate and sensitive, does not tell whether the protein still remain in the active form or not. Hence the two methods of quantifying the protein appear to be required simultaneously.

Safety and toxicity assay

No adverse reports associated with the long term human consumption of miraculin has been reported. Oral toxicity tests conducted for miraculin on mice and rats showed that miraculin is safe to consume (Dastoli and Harvey, 1974).

The Mason Research Institute of Worcester based in Massachussets U.S.A concluded after a series of experiments that there is no actual LD_{50} for miraculin as no mice died at the maximum achievable single-dose rate of 5 mg/kg body weight. Acute oral toxicological test in hamsters on a maximum level of 3,000 times the level for human consumption, showed no effect on any pathological, biochemical, physiological or cytological parameter measured (Dastoli and Harvey, 1974). Consequently miraculin was considered to be safe (Dastoli and Harvey, 1974).

Potency

Although miraculin contributes to calories by virtue of the presence in miraculin of amino acids and carbohydrates, its induced sweetness relative to glucose makes it a better choice as a sweetener. Hundred micrograms (100 μ g) of miraculin has been reported to elicit sweetness for one to two hours (Higginbotham, 1979 : van der Wel, 1974). A pure miraculin solution at 2.0×10^{-6} M level elicits sweetness after applying 0.02 M citric acid and lingers for three hours. Similarly, a pure miraculin solution of 2.0×10^{-8} M also elicits sweetness

but has a shorter lingering effect of about twenty minutes (Kurihara and Beidler, 1969; Pintauro, 1977).

Applications

The lingering taste of miraculin is a serious drawback to its use as a sweetener or food additive. In a mixed meal, grape fruit would be very pleasant as a starter but lemon squeezed on fish or any vinegar based dressing or sauce will taste revolting (Higginbotham, 1979; van der Wel, 1974). Another limitation to practical application of miraculin in foods and drinks is its cumulative action. When miraculin is added to a sour food such as yoghurt, it initially tastes sour, but the more yoghurt one eats the sweeter it becomes (van der Wel, 1974).

The properties of miraculin such as thermolability, pH sensitivity and ease of hydrolysis in aqueous solutions complicates its use as a food additive. In spite of these drawbacks, miraculin has so far found applications in chewing gum formulations, popsicles, candy and straw coatings, and in droplets which are intended to be chewed before eating sour foods such as unsweetened iced lemon tea, and unsweetened lemon chiffon (Johnson, 1972; Harvey, 1973). Plans are advanced to incorporate it into yoghurts and oral care products such as toothpaste and other pharmaceuticals (Fennel and Harvey, 1974; Johnson, 1972; Dastoli and Harvey, 1974).

Some researchers claim that if administered orally five times daily, at a dose of 10 to 400 μg , miraculin can be used to control obesity (Pintauro, 1977).

C H A P T E R 2

2.0 MATERIALS AND METHODS

2.1 MATERIALS/REAGENTS.

Acetone, sodium chloride, Tris (hydroxymethyl) aminoethane, hydrochloric acid, sodium dodecyl sulphate (SDS), sucrose, bromophenol blue, Coomassie brilliant blue R-250, glacial acetic acid, ammonium bicarbonate, sodium potassium tatarate, sodium hydroxide and sodium carbonate were from Fluka Chemical Company (Buchs) and were of analytical grade. Sodium dihydrogen phosphate, dipotassium hydrogen phosphate, NNN'N'-tetramethelenene diamine (TEMED), ammonium persulphate and glycine were obtained from Wako Chemical Company (Japan).

N'N'Methelenebisacrylamide was from Serva Fienbiochemica Company Ltd. (N.Y.). Analytical grade P-Nitroaniline was obtained from Hopkin and Williams Ltd. Essex (England).

Ethanol, disodium hydrogen phosphate and ethanoic acid were obtained from Riedel-deHaën (ph.Eur). 2-mercaptoethanol and Folin-Ciocalteau reagent were from BDH Chemical Company Ltd. (England) and were of analytical grade. Hydrated copper sulphate was obtained from Merck Company Ltd. (Germany). Pumps, HIC columns 1 and 2 (pre-loaded with column resins), the column resins; SP-sepharose FF, sephacryl S-100, sephadex G-25 and standard protein markers namely, transferrin, ovalbumin, α -chymotrypsinogen and carbonic anhydrase were obtained from Pharmacia Biotech. AB (Sweden). The ovalbumin and transferrin were premixed. Unless otherwise indicated,

all other chemicals were from Sigma Chemical Company St. Louis, MO., and were of analytical grade.

The fruits of *Richardella dulcifica* were kindly supplied by BioResources Incorporated (Ghana) Ltd. (BRI) from their out growers in the Eastern region of Ghana and stored at 4°C until when needed.

2.2 METHODOLOGY

2.2.1 Depulping Of Fruits.

The fruits were washed and depulped on ice with a depulper (Magnetek, USA). The seeds and the fruit coat were weighed and discarded and the pulp pelleted by centrifugation at 8,500 rpm at 4°C for 10 minutes in a high speed centrifuge (Seiko Co. Ltd, Japan). The intense red coloured supernatant (fruit juice) was discarded and the pellet resuspended in double distilled water and centrifuged again for 10 minutes at 4°C. This procedure was repeated until a colourless supernatant was obtained. The washing supernatant and the washed pellet were examined for proteolytic activity and total protein content.

2.2.2 Estimation of extractable proportion of the miracle fruits.

The mass of the washed and unwashed pellet were determined and the extractable proportion of the miracle fruits estimated as the ratio of the mass of the washed pellet to the total

mass of miracle fruits used. This was expressed as a percentage.

2.2.3 Total Proteins.

Total protein was determined for each of the various samples viz;

- i. Crude pulp of fresh fruits (Hof)
- ii. Crude pulp of aged fruits (Hoa)
- iii. Washed pulp of fresh fruits (Wpf) and
- iv. Washed pulp of aged fruits (Wpa).

"Fresh" refers to fruits stored for less than a fortnight while the aged fruits had been in storage for more than six months.

The protein concentration of each sample was determined by the method of Lowry et al (1951). Protein standards were prepared from Bovine Serum Albumin (BSA). A calibration linear regression equation was developed from the standard BSA solutions (0.01, 0.05, 0.10, 0.15 and 0.20) mg/ml and used to determine the amount of protein in the four samples mentioned. The absorbance at 750nm was measured in a double split beam Shimadzu spectrophotometer (Kyoto, Japan). The linear regression model developed for the calibration was as follows:

$$Z = -3.212 \times 10^{-2} + 1.652C$$

where; Z = Absorbance at 750nm

and C = protein Conc in mg/ml

The model had an R^2 of 98.73%, a standard error of estimate of 0.0165 and a correlation coefficient of 0.994.

2.2.4 Protease activity.

The protease activity was determined at pH 4.3 on unwashed as well as washed pulp of fresh and aged berries using L-Benzoyl D, L-Arginine-p-Nitroaniline (L-BAPNA) as substrate. The method was a modification of the method of Erlangler et al. (1961). Each of the reaction mixtures containing 1 ml of 50 mM Tris-HCl buffer (pH 4.3), 0.253 mM L-BAPNA and 20 mM CaCl_2 , was mixed with 10 μl of test solution (crude pulp) and allowed to stand. The reaction was stopped after 1, 2 and 3 minutes, by adding 3 ml of 10% trichloroacetic acid. The mixture was allowed to stand for a further 30 minutes at room temperature. It was then centrifuged for 10 minutes at 3,000 rpm in Kubota 1120 centrifuge (Kubota Co. Ltd. Japan) for 10 minutes and the supernatant assayed. Control samples containing trichloroacetic acid added prior to enzyme addition were included in each assay. One unit of activity was defined as the amount of extract that catalysed the release of 1 $\mu\text{M}/\text{ml}$ p-Nitroaniline (NPP)/min at 25°C from L-BAPNA.

Five p-Nitroaniline (NPP) standards were prepared by dissolving NPP in Tris-HCl buffer (pH 4.3). A calibration linear regression model was developed from the standard NPP solutions (0.4, 0.6, 0.8, 1.0 and 1.2) μM and used to

determine the activity of protease in the extracts in 2.2.3 above. The simple linear regression model that was generated to relate absorbance at 405 nm to enzyme activity was:

$$Z_1 = -4.12 \times 10^{-2} + 0.0818c_2$$

Where, Z_1 = Absorbance at 405 nm

and c_2 = NPP concentration in μM .

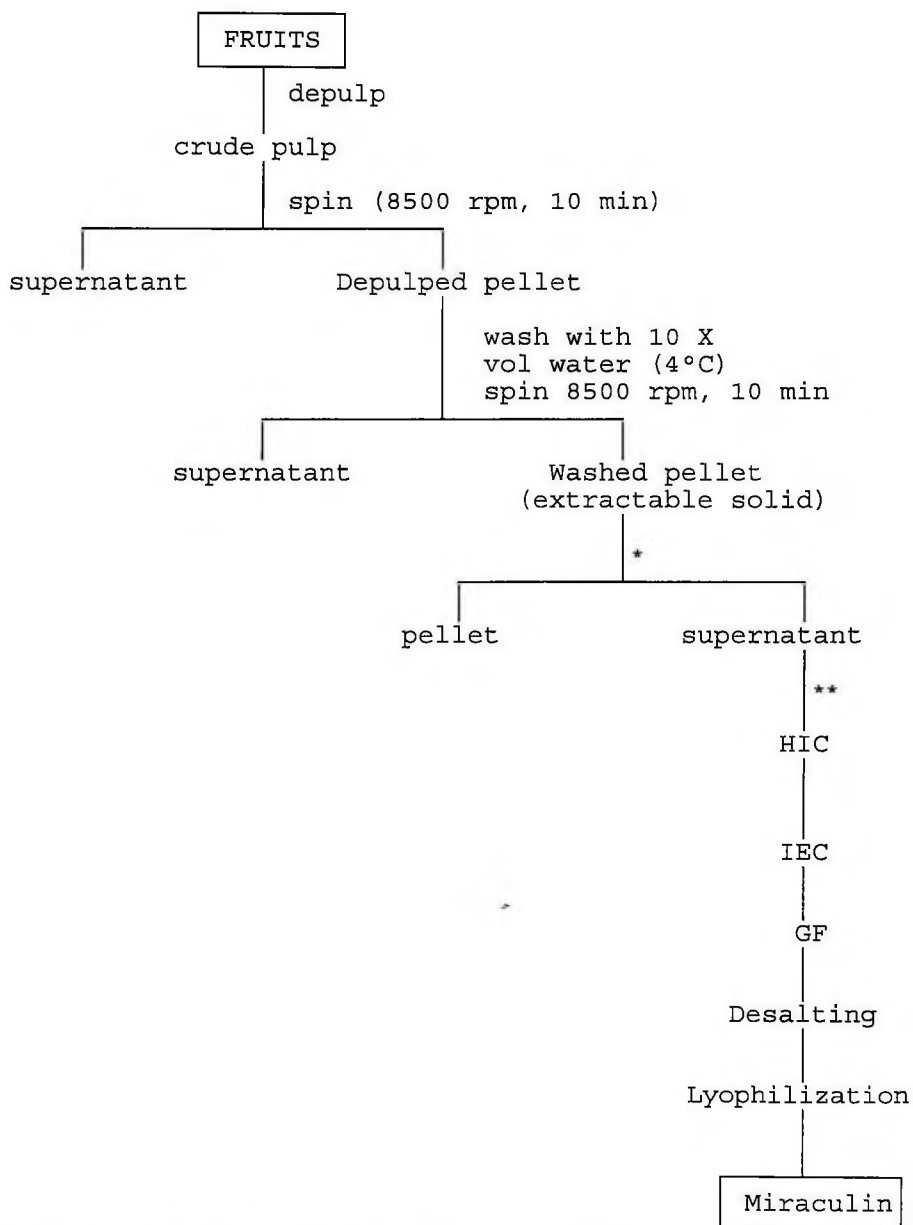
The model had an $R^2 = 99.77$, a correlation coefficient of 0.9986 and a standard error of estimate of 7.8×10^{-6} .

2.2.5 Extraction and purification of miraculin.

The extraction and purification procedure was a modification of the method of Theerasilp and Kurihara (1988) (*Fig. 1*).

The washed pellet was re-suspended in 0.5M NaCl solution and homogenized for 15 to 20 minutes at 4°C in a Bransonic-92 sonication bath (Yamato Co. Ltd., Japan). The homogenate was centrifuged at 8,500 rpm in a Hitachi 20PR-52D centrifuge (Hitachi Koki Co. Ltd. Japan) at 4°C for 10 minutes and the supernatant fraction was recovered. From this stage onwards, the rest of the experiment was carried out at room temperature.

Fig 1: Flow chart for the extraction and purification of miraculin from Fruits of *R. dulcifica*



* Homogenise in 0.5 M NaCl for 30 min, spin at 8,500rpm, 15 min, 4°C

** Add $\text{NH}_4(\text{SO}_4)_2$ to 0.6 M.

2.2.6 Chromatographic separation

Miraculin was purified from the crude extract by three chromatographic methods namely: Hydrophobic interaction, ion exchange, and gel filtration chromatographies.

A. Hydrophobic interaction chromatography (HIC)

The unique properties of HIC columns, that is, separating proteins on the basis of their surface hydrophobicity was utilized as the first step in the chromatographic separation process (Pharmacia 1993, Wilson 1994).

A HIC column (26 x 8.0cm) containing S-butyl sepharose in tandem arrangement with another HIC column (26 x 13.5cm) containing butyl sepharose (Pharmacia, Sweden) was equilibrated with three column volumes of 20 mM sodium phosphate buffer (pH 6.8) containing 0.6M ammonium sulphate (buffer A). The crude extract was applied to the tandem columns at a rate of 10 ml/min. Elution of protein from this and all other columns was monitored continuously at 280 nm. After washing with buffer A (appendix A), the tandem arrangement was then disconnected and the proteins bound on the butyl sepharose column eluted as fraction 1B with 20 mM sodium phosphate buffer pH 6.8 (buffer B, appendix A).

B. Ion exchange chromatography (IEC)

Ion-exchange chromatography which separates proteins based on

molecular charge (Himmilhoch, 1971; Wilson 1994) was employed as the next tool in the purification scheme.

SP-Sepharose-FF column (Pharmacia, Sweden) of column dimensions 26 x 14 cm was equilibrated with four column volumes of 20 mM sodium phosphate buffer pH 6.8 containing 50 mM NaCl (buffer C₁, appendix A) and loaded with fraction 1B diluted 1 in 2 with buffer C₁. The flow through (unbound sample) was collected as fraction 2A. The bound proteins were eluted step wise first with buffer C₂ (20 mM sodium phosphate buffer, pH 6.8 containing 0.12 M NaCl, appendix A) followed by buffer D (20 mM sodium phosphate buffer, pH 6.8 containing 0.35 M NaCl, appendix A) to obtain fractions 2B and 2C respectively. Fraction 2C which contained the eluted miraculin was concentrated using Amicon concentrator (molecular weight cut-off 10 kDa) and purified by gel filtration chromatography.

C. Gel filtration chromatography (GFC).

Gel filtration chromatography which separates molecules on the basis of size (Reiland 1971, Wilson 1974), was finally employed to achieve full scale purification.

The concentrated sample (8 ml) was loaded on to a 26 x 60 cm Sephacryl S-100 column (Pharmacia, Sweden) pre-washed with 20 mM Na-phosphate buffer pH 6.8 containing 0.35 M NaCl (buffer

D, appendix A) and the protein was eluted as fraction 3B with two void volumes of buffer D at a flow rate of 1.4 ml/min.

2.2.7 Desalting and lyophilization

The fraction from the gel filtration column was desalted by applying it on to a 26 x 13.5 cm sephadex G-25 column (pharmacia Sweden) pre-equilibrated with 10mM ammonium ethanoate (pH 6.8, buffer E) at a rate of 1.4 ml/min and eluted with the same buffer (buffer E). The desalted pure proteins were then frozen in standard freeze drying bottles and dried using an Eyela freeze drier FD-1 (Tokyo Japan)

2.2.8 Psychometric studies

Purified miraculin at a concentration of 100 μ g/ml was used for the taste evaluation as described by Kurihara and Beidler (1968). However, in this work, lemon was substituted for graded sucrose solutions. The sweetening strength of the purified product was also not determined since the essence of this study was only to determine whether the purified product was functional or not.

2.2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Each stage of the purification process was monitored by SDS-PAGE. The process was carried out as described previously (Chung, 1987: Laemmli, 1970: Weber and Osborn, 1975).

Aliquot samples at each stage of purification were concentrated and dissolved in 2X sample buffer (see appendix A). The samples were then heated to boiling and applied to a 10% acrylamide vertical slab gel. After electrophoresis at 10 V/cm for 90 minutes, the gel was stained with shaking in Coomassie brilliant blue R-250 for 40 minutes followed by destaining in a destaining solution (see appendix A) to locate protein bands. Protein markers (Pharmacia) and a purified miraculin sample (Kurihara, Japan) were included in the run.

2.2.10 Estimation of molecular weight of purified miraculin by gel filtration.

Standard calibrating proteins (Table 1) at concentration of 1 mg/ml were applied to a 26 x 60cm Sephacryl S-100 column of packed bed volume 318 ml, equilibrated with buffer D. 100 µg/ml of the purified miraculin was also applied to the column and run as previously described (section 2.2.6, C).

Table 1. Molecular weights of calibrating proteins

Protein	Relative Molecular weight
α-chymotrypsinogen	25,000
carbonic anhydrase	30,000
ovalbumin & transferrin	43,000, 78,000

The molecular weight of purified miraculin was estimated by comparing its elution volume (V_e) determined as the apex of the miraculin chromatogram with that of the standard proteins.

2.2.11 N-terminal amino acid analysis of purified miraculin.

A unique N-terminal sequence (or at least N-terminal amino acid) is excellent confirmation that a protein or peptide preparation is homogeneous. Besides, comparison of a short N-terminal sequence with a published sequence can aid positive identification of the protein.

The purified protein was incubated with a solution of 1M HCl/methanol (1:11 v/v) at 35°C for 24hr and the solution was lyophilized. Using the lyophilized sample, N-terminal amino acid residues were determined as reported previously (Yarwood, 1989).

2.2.12 Miraculin solubility.

A. Experimental design and statistical analysis

The central composite rotatable design for $K = 2$ (Cochran and Cox, 1957) was used to study the effects of independent experimental variables (pH and ionic strength, μ) on miraculin solubility. The levels of the independent variables that were used are summarized in Table 2.

Table 2. Variables used in the central composite rotatable design

Independent Variable	Variable levels				
	-1.41	-1.00	0.00	1.00	+1.41
pH	2.50	3.60	6.20	8.90	9.98
ionic strength	0.00	0.15	0.50	0.85	1.00

14 sample combinations were generated (Table 3). The effects of the independent variables on solubility of miraculin were investigated. Stepwise regression analyses was performed using stat graphics computer software (statistical graphics co-operation Stsc. Inc. USA). Models developed for each index were examined and a response surface plot generated.

Table 3. Design matrix and variable combination for experiments

	<u>Level Codes</u>		<u>Actual values</u>	
	Ionic Strength	pH	Ionic strength	pH
1	-1.00	-1.00	0.15	3.60
2	-1.00	1.00	0.15	8.90
3	1.00	-1.00	0.85	3.60
4	1.00	1.00	0.85	8.90
5	0.00	0.00	0.50	6.20
6	0.00	0.00	0.50	6.20
7	0.00	0.00	0.50	6.20
8	1.41	0.00	1.00	6.20
9	-1.41	0.00	0.00	6.20
10	0.00	1.41	0.50	1.00
11	0.00	-1.41	0.50	2.50
12	0.00	0.00	0.50	6.20
13	0.00	0.00	0.50	6.20
14	0.00	0.00	0.50	6.20

B. Sample preparation

Fourteen different solutions (samples) were prepared according to the pre-determined ionic strength in Table 3. Each sample was made to a concentration of 0.2 (mg/ml) of miraculin. The solutions were agitated for 30 minutes at 21°C followed by

centrifugation at 3000 rpm in a Kubota 1120 centrifuge (Kubota cooperation, Tokyo, Japan) for 5 minutes. The protein concentrations in the supernatants was analyzed by the Lowry method (1951) using Bovine Serum Albumin as standard. The linear regression model developed for the calibration was:

$$Y_3 = 5.048 \times 10^{-3} \times 1.813 c_3$$

Where Y_3 = Absorbance at 750nm,

and c_3 = concentration of protein mg/ml.

The model had an $R^2 = 96.87\%$, a standard error of 0.0286 and a correlation coefficient of 0.9842.

2.2.13. Stabilization by glycerol.

The thermal denaturation of pure miraculin was monitored by difference-UV spectroscopy at 278 nm using varying concentrations of glycerol and following the method of Gekko and Timasheff (1981). The UV difference spectra were measured with a Shimadzu double-beam spectrophotometer (UV-190 Kyoto, Japan) using 1-mm quartz jacketed cells. The temperature of the cell in the sample beam was controlled by circulating water of a given temperature through the cell housing from a *Medingen Sitz Freitai-48* (GDR) circulating bath. The reference cell was controlled at 20°C using a second water bath. Since experimental results of denaturation are very sensitive to protein concentration and pH of solution (Tsong et al, 1970) extreme care was exercised in preparing the solutions. Using a miraculin stock solution of 0.5 mg/ml,

five solutions of identical miraculin concentration (0.2 mg/ml) and varying glycerol conc 0, 10, 20, 30, 40 (v/v)% were prepared according to the protocol in Table 4. Each had a final pH of 4.5

Table 4: Sample preparation for glycerol stabilization.

Variable	Glycerol concentration (v/v)%				
	0	10	20	30	40
	Volume of variable /ml				
Glycerol	0.0	0.5	1.0	1.5	2.0
Stock miraculin (0.5 mg/ml)	0.2	0.2	0.2	0.2	0.2
0.01M Tris-HCl (pH 4.5)	4.8	4.3	3.8	3.3	2.8
Total vol./ml	5.0	5.0	5.0	5.0	5.0

In the temperature studies, two 1 mm-quartz matched cells were filled with aliquots of the same solution and sealed tightly with teflon stopper to avoid evaporation during the thermal denaturation experiment. The progress of the reaction and attainment of equilibrium were monitored by change of the difference in absorbance at 278 nm. After attainment of equilibrium, difference spectra were recorded from 250 to 360 nm using a Kipp and Zonen B41 (Holland) printer to check baseline shifts. The difference in absorbance at 278 nm were plotted as a function of temperature.

C H A P T E R 3

3.0 RESULTS

3.1 TOTAL PROTEINS

The results (means of three determinations) of total protein assays are presented in Table 5. See section 2.2.3 for details of analytical procedure.

Table 5: Amount of protein in the crude pulp of fresh and aged miracle fruits.

<u>sample</u>	<u>% mass of protein in pulp</u>
Unwashed pulp of fresh fruits	31.3 ± 1.9
Washed pulp of fresh fruits	27.4 ± 0.2
Unwashed pulp of aged fruits	27.5 ± 0.3
Washed pulp of aged fruits	26.8 ± 0.1

The protein concentrations were calculated from the linear regression model (section 2.2.3) based on the average absorbance at 750nm value obtained for each sample. The means of the total protein content of the treated (washed) and untreated (unwashed) pulps were compared using the student t-test and the observed differences were found not to be statistically significant ($p \leq 0.05$) (appendix B, Table B1). A similar comparison was made on the pulps of aged and fresh fruits and again, the differences were observed to be

statistically insignificant ($P \leq 0.05$) (appendix B, Table B2).

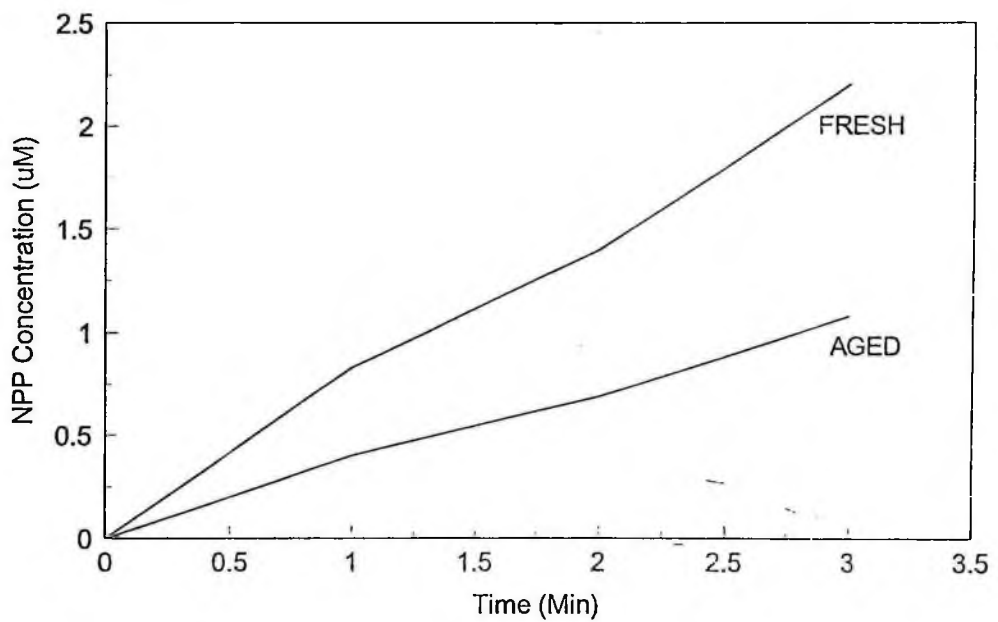
3.2 PROTEASE ACTIVITY

A. Catalytic activity of protease.

The protease activity and specific protease activity at pH 4.3 in the pulp of fresh miracle fruits were found to be 0.0756 units/mg pulp and 0.2414 units/mg protein respectively. On the other hand, the protease activity and specific protease activity in the pulp of aged miracle berries were estimated to be 0.0369 units/mg pulp, 0.1340 units/mg protein in respective order (appendix D).

Figure 2 shows the protease activity in the pulp of fresh and aged fruits. Clearly, the figure show the protease activity in the pulp of fresh fruits to be higher than that of aged berries. This was further confirmed by statistical analysis using the students t-test ($P \leq 0.05$). No protease activity was detected in the washed pulps of both aged and fresh fruits.

Fig. 2: Comparison Of Protease Activity In The Pulps Of Fresh And Aged Miracle Fruits.



3.3 EXTRACTABLE SOLIDS (% W/W) IN THE MIRACLE FRUITS.

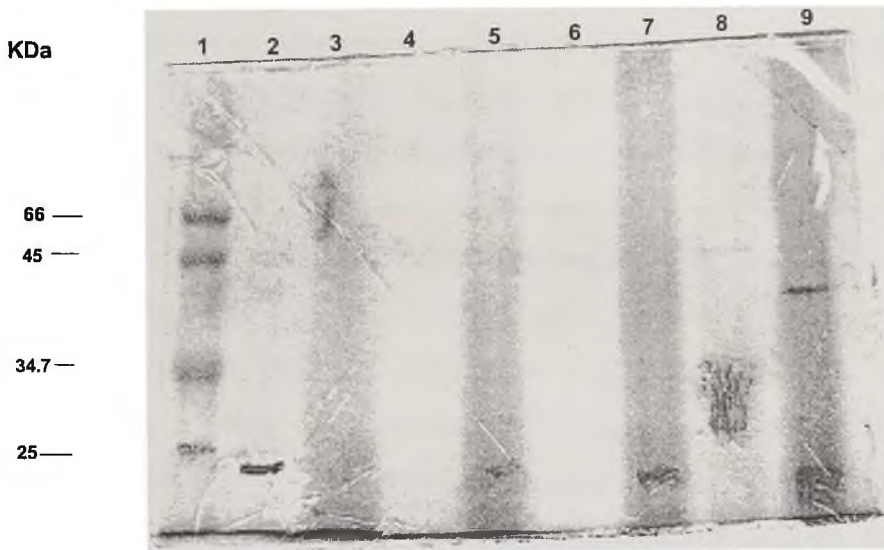
Table 6: Percentage of miracle fruit from which miraculin is obtainable.

For each batch of miracle fruits taken, the seed and coat, fruit juice and pulp (extractable solids) were separated from each other and the mass of each portion obtained expressed as a percentage of the starting material used (section 2.2.2). The results are shown in Table 6 below.

<u>Portion</u>	<u>Percent (%) by mass</u>
Seed and coat	47.8
Liquid waste	42.2
<u>Extractable solids</u>	<u>10.0</u>

Figure 3 shows the extraction and the various stages of purification (purification profile) of miraculin from miracle fruits. In the figure, except for samples in lanes four and six which were prepared by acetone precipitation of protein from solutions, all the other samples in the various lanes were prepared as described in section previously (section 2.2.8). A Tris-glycine-HCl buffering system was used for the electrophoresis at 10V/cm which lasted for 90 minutes.

Figure 4: Reducing SDS-PAGE Showing Extraction Profile Of Miraculin From Miracle Fruits.



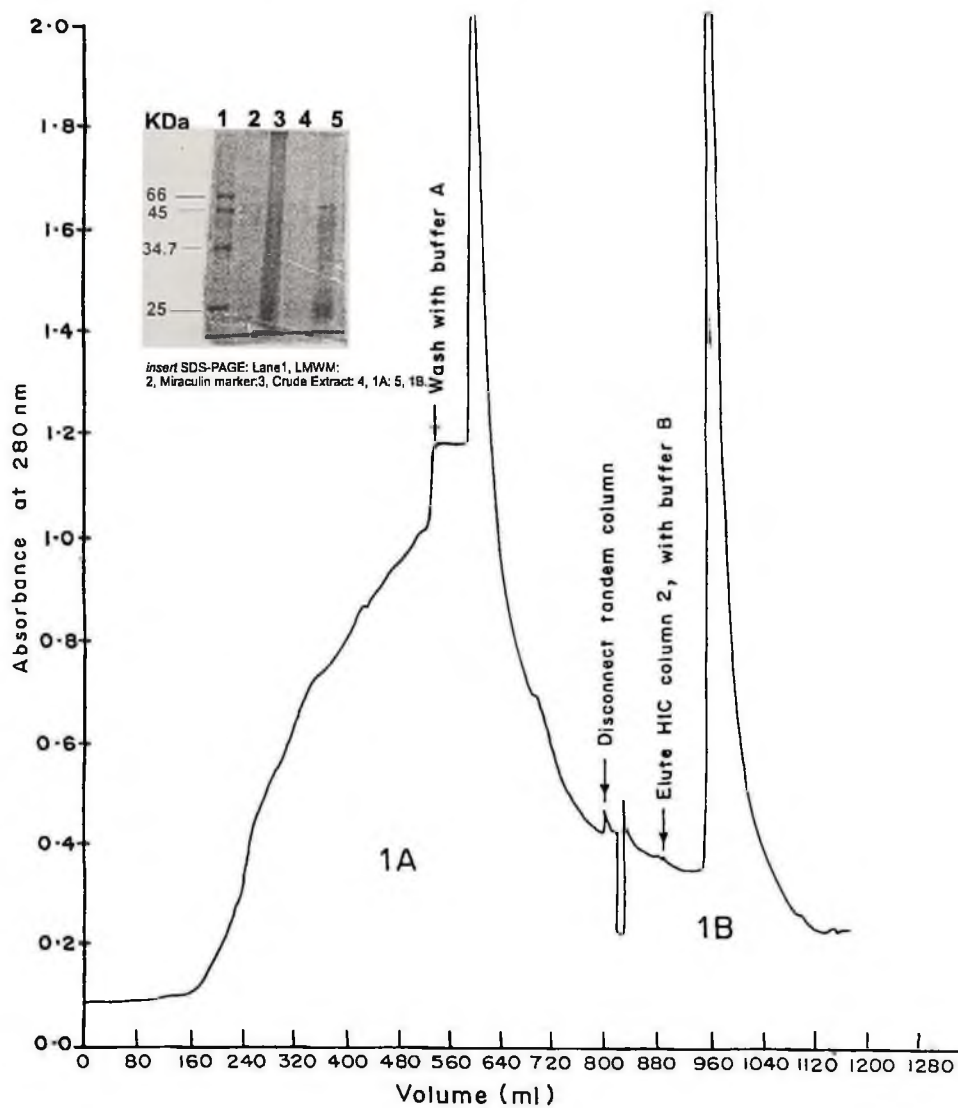
Lane: 1, LMWM: 2, standard Miraculin: 3, crude pulp:
4, fruit juice: 5, depulped pellet: 6, washing effluent:
7, washed pellet: 8, spent pulp (S.P): 9, crude extract (C.E).

3.4 CHROMATOGRAPHY

3.4.1 Hydrophobic Interaction Chromatography.

Figure 4, shows the elution profile of the crude miraculin extract on the tandem HIC columns (see section 2.2.6 A, for experimental details). The flow through (peak 1A), constituted approximately 60.0% of the total absorbance of the material which was applied on the column. This fraction however contained no miraculin as can be seen in PAGE (insert, lane 4) . It was very coloured with no sweetness modifying activity. Peak 1B was desorbed from the butyl sepharose column by elution with buffer B. 12.8% of the total absorbance applied contained miraculin (insert, lane 5) and had a taste modifying activity. It had a pale yellow coloration and elicited sweetness. The S-butyl column, which acted as a guard column was highly discoloured (dark brown) and bound no miraculin.

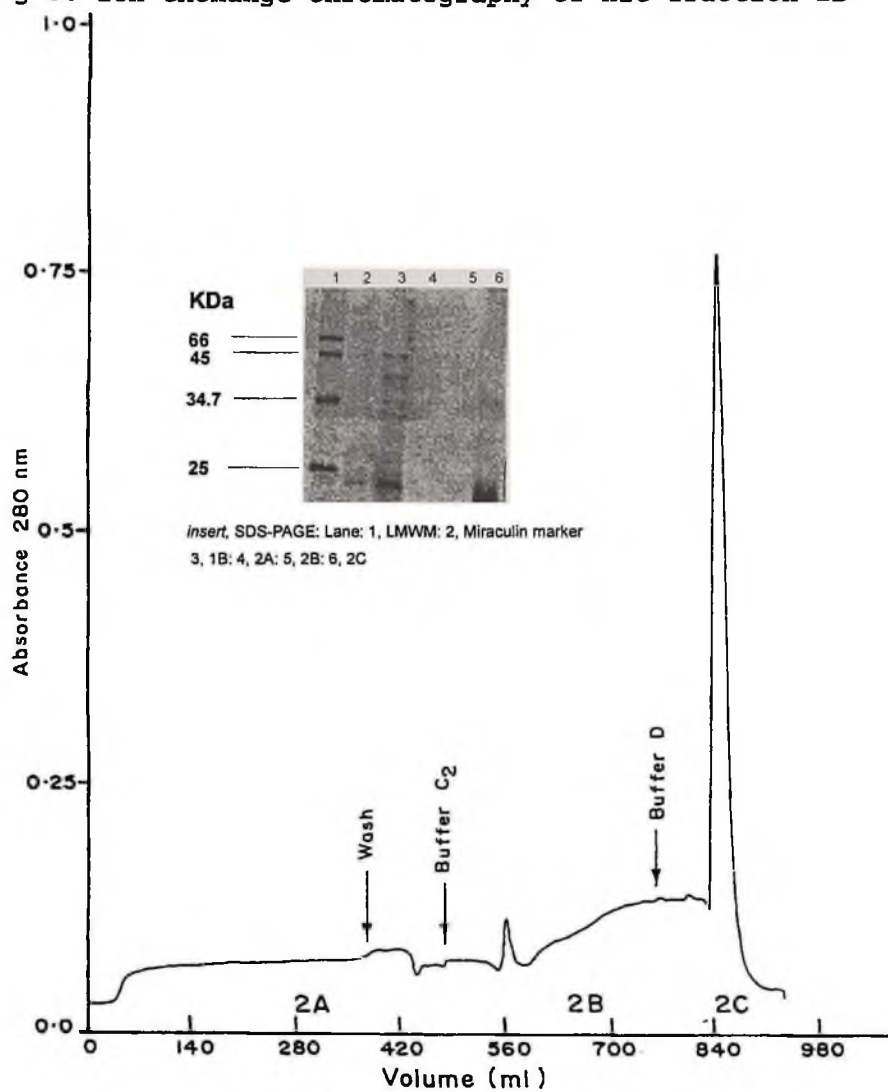
Fig. .5: Crude Miraculin Extract on Tandem HIC columns.



3.4.2 Ion Exchange Chromatography.

Fig 5 shows the results of stepwise elution of the HIC fraction (fraction 1B) from the ion exchange resin (section 2.2.6 B) with buffer C₂ followed by buffer D (see appendix E). Peak 2A, which accounted for 20.4% of the total applied absorbance represents the unbound fraction. It contained no miraculin as revealed by PAGE (insert, lane 4) and had no taste modifying properties. Peak 2B, desorbed from the IE resin with a buffer of low ionic strength (buffer C₂), represented 23.6% of the total absorbance applied on the IE column. Fraction 2B (insert, lane 5), barely elicited sweetness. The main peak 2C, was highly purified (> 80%) miraculin (insert, lane 6). It accounted for 37.2% of total absorbance applied on the column and was found to have a high sweetening activity.

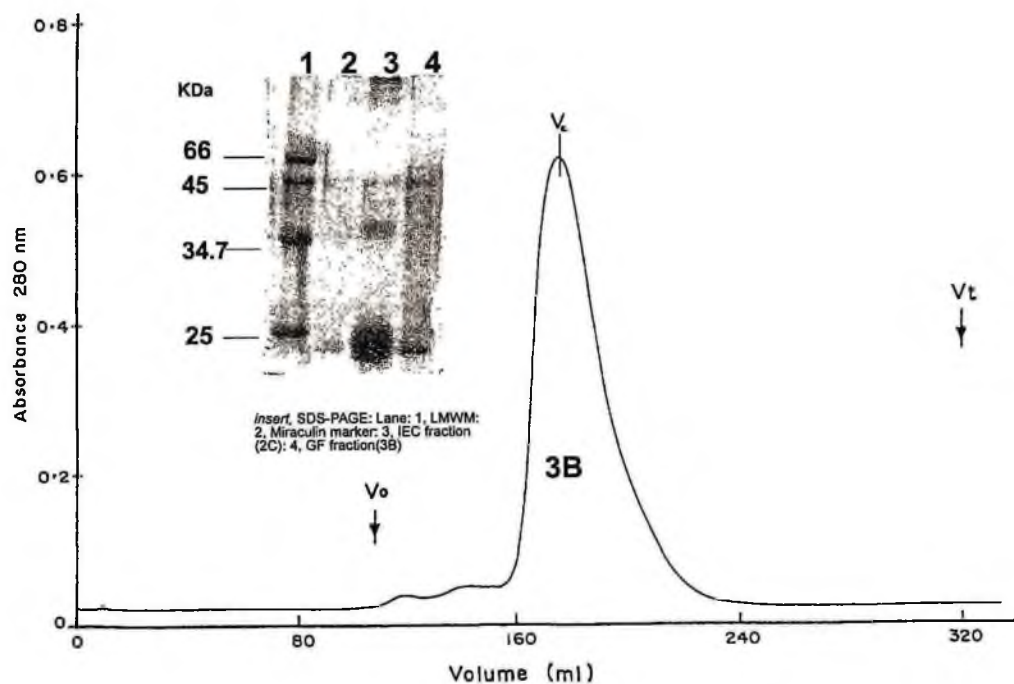
Fig 5: Ion exchange chromatography of HIC fraction 1B



3.4.3 Gel filtration chromatography.

Gel filtration (section 2.2.6 C) showed a single peak which accounted for 94.0% of the total absorbance applied on the column. The eluted fraction, 3A was highly purified miraculin (insert, lane 4) and elicited a high degree of taste modifying activity.

Fig. 6: Gel filtration chromatography of IEC fraction (2C).



3.5. PSYCHOMETRIC ANALYSIS

Following purification of miraculin, sensory evaluation was conducted using a solution of 100 $\mu\text{g/ml}$ of purified miraculin and fresh lemon fruits as described previously (Kurihara and Beidler 1968), to assess whether the product was functional or not. However, it was not compared to the sweetness elicited by standard sucrose solutions as a trained panel was lacking. Table 7, shows the response of some panellist (untrained) to the taste of the fruit before and after administration of the miraculin.

Table 7: Taste Modifying Test (n=20)

	<u>Sour n(%)</u>	<u>Sweet n(%)</u>	<u>very sweet n(%)</u>
TLBM	20 (100)	00 (0)	00 (0)
TLAM	00 (0)	02 (10)	18 (90)

TLBM: Taste of lemon before miraculin.

TLAM: Taste of lemon after miraculin.

3.6 ESTIMATION OF THE MOLECULAR WEIGHT

Table 8: Comparison of the elution volumes of standard proteins used to that of miraculin.

Table 8 shows the elution profile of purified miraculin and standard protein markers. The elution volumes (V_e) were determined as the apex of each elution peak. The V_e s were then compared to estimate the molecular weight (M) of the purified miraculin, with the molecular weight of miraculin estimated to be 43 kDa.

Standard Protein	M (kDa)	V_e (ml)
α -Chymotrypsinogen	25	192.7
Carbonic anhydrase	30	187.0
Ovalbumin	43	159.6
Transferrin	78	142.3
Miraculin	43	160.0

3.7 N-TERMINAL SEQUENCE ANALYSIS OF PURIFIED MIRACULIN.

In the N-terminal sequence studies, two forms of pure miraculin were detected one being more dominant than the other and were accordingly named major and minor forms respectively. The major form constituted more than 70% of the total purified miraculin population and was identical to the published sequence by Theerasilp et al 1988, up-to the first ten amino acid residues. The amino acid sequence data base identified miraculin as the parent protein to which this sequence belonged. The minor form had the same N-terminal sequence of miraculin but lacked the NH₂-terminal aspartic acid. The N-terminal sequences of the two forms of miraculin are shown in Figure 7 below.

Figure 7: The partial NH₂-terminal sequence of the major (> 70%) and minor forms of purified miraculin.

Major form:

NH₂-Asp-Ser-Ala-Pro-Asn⁵-Pro-Val-Leu-Asp-Ile¹⁰....

Minor form:

NH₂-Ser-Ala-Pro-Asn-Pro⁵-Val-Leu-Asp-Ile⁹....

3.8 EFFECT OF PH AND IONIC STRENGTH ON THE SOLUBILITY OF PURIFIED MIRACULIN.

Following the methodology of section 2.2.11, the results obtained were analyzed using the stats graphics computer software (statistical graphics co-operation Stsc. Inc. U.S.A) to develop a model that will explain the solubility trend of miraculin with respect to pH and ionic strength (μ). The results obtained from the statistical investigation are displayed in Table 9.

Table 9: Coefficients of variables used to develop model.

Inde. variable	Coefficient	Std. error	t-value
CONSTANT	0.027348	0.00342	*7.9848
μ	-0.015177	0.01174	-1.2925
$\mu \times \text{pH}$	-0.003364	0.00131	*-2.5680
pH^2	0.000128	0.00065	1.9766
μ^2	0.029963	0.00785	*3.8177

$R^2 = 0.6612$, * Significant ≤ 0.05

From Table 9, the coefficients of the variables used to describe the solubility of miraculin were obtained. The predictive model established was:

$$Z = 0.027348 - 0.015177\mu + 0.000128\text{pH} + 0.029963\mu^2 - 0.003364\text{pH}.\mu$$

where Z = solubility.

The equation developed could explain 66.12% of the observed variation in miraculin solubility (ie $R^2 = 0.6612$).

Further statistical analysis was performed on the model developed in Table 9 above, to determine the most and least influential contributors to the model. Again the stats graphics computer software (statistical graphics co-operation Stsc Inc. U.S.A) was used to carry out the investigation and the results shown in Table 10.

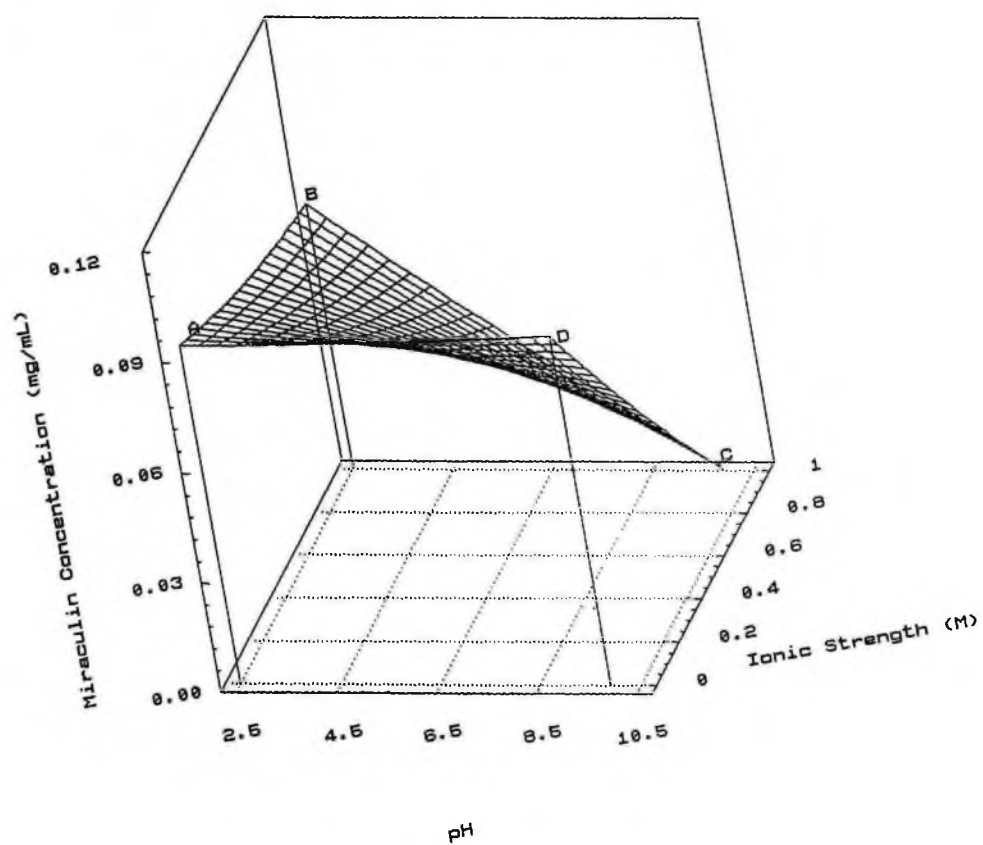
Table 10: Further ANOVA for variables in the order fitted for the miraculin solubility model.

Source	% cont. to R^2	sum of squares.	D.F	F-Ratio
μ	0.137	3.752×10^{-5}	1	*5.70
$\mu \times \text{pH}$	0.035	9.600×10^{-6}	1	1.46
pH^2	0.137	3.745×10^{-5}	1	*5.69
μ^2	0.3515	9.599×10^{-5}	1	*14.57
Total R^2	0.6612	1.806×10^{-4}	4	

μ = ionic strength. * Significant $p \leq 0.05$

Figure 8, is the result of the measurement of the amount of miraculin left in standard miraculin solutions with variation in ionic strength and pH. For experimental details, see section 2.2.11 B.

Fig.8: Effect of pH and ionic strength on miraculin solubility.



3.9 STABILIZATION OF PURIFIED MIRACULIN IN WATER-GLYCEROL MIXTURE.

Figure 9a shows the thermal denaturation of purified miraculin at various levels of glycerol concentration. Each point represents a mid point of triplicate values. The transition temperature (T_m) of the protein at each of the glycerol levels used was taken as the mid point of the transition zone (range of drastic change in absorbance). The transition temperatures were then plotted as a function of glycerol concentration (% v/v) to study the relationship between the two parameters mentioned, *Figure 9b*.

Fig. 9a: Thermal denaturation of purified miraculin in 10mM Sodium Phosphate buffer as a function of glycerol concentration (%v/v).

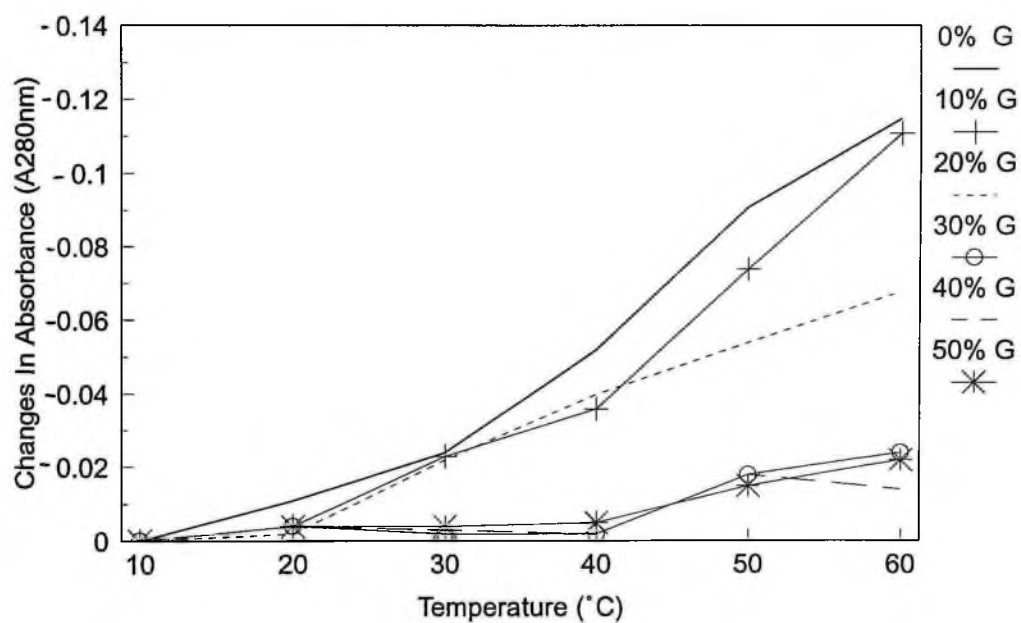
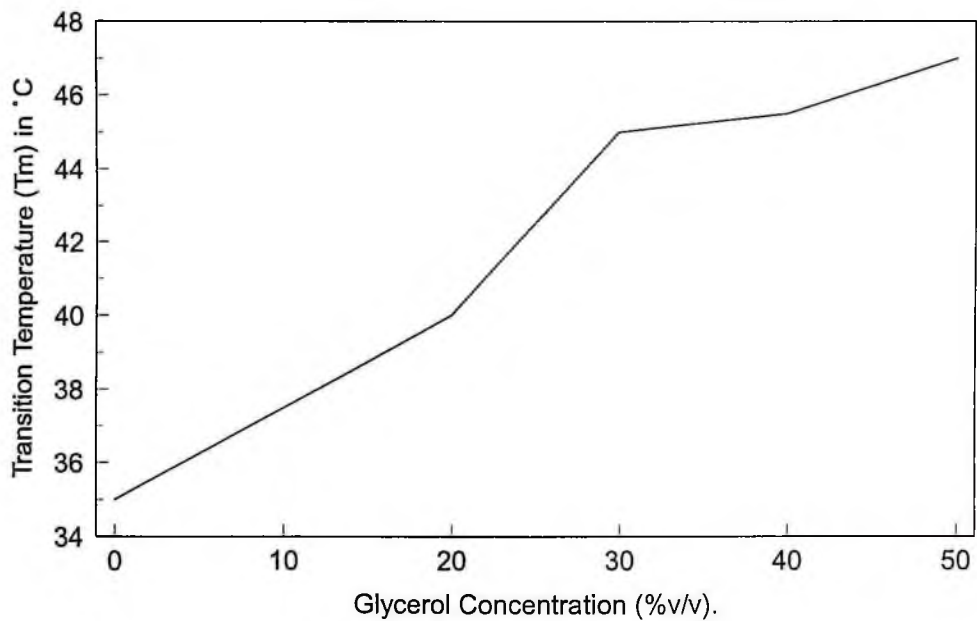


Fig. 9b: Dependence of transition temperature (T_m) of purified miraculin on glycerol concentration.



C H A P T E R 4

4.0 DISCUSSION AND CONCLUSION.

In this study, the possible extraction and purification of miraculin with sweetening ability from the fruits of *R. dulcifica* to homogeneity at room temperature was investigated. Prior to this, total protein determination and assay for proteases in the pulp of fresh and stored fruits were performed. The N-terminal sequence of the purified product was also investigated to establish its purity and possible proteolytic degradation. Finally, the effects of varying pH and ionic strength on the solubility of the purified product as well as its thermal stability in water-glycerol mixture were analyzed.

As can be seen in Table 3, the crude protein content of the treated (washed) and untreated (unwashed) pulps of the fresh and aged berries are $31.3 \pm 1.9\%$ and $27.4 \pm 0.3\%$ respectively. After treatment, the total protein content decreased to $27.4 \pm 0.3\%$ and $26.8\% \pm 0.1\%$ for the fresh and aged fruits respectively. A two sample analysis (Appendix B table B1) conducted on the results showed that the differences observed were not statistically significant ($P \leq 0.05$). This is because miraculin is membrane bound and hence not accessible to water which is consistent with the findings of earlier workers (Inglett and May 1968, and Kurihara and Beidler 1968).

The etiology of the persistent decline of the sweetening effect of miracle fruits with storage has been traced to proteases in the pulp. (Crosby, 1976, and Higginbotham, 1977). There was evidence for the existence of proteases in the crude pulps. As the specificities of proteases vary considerably, there is no single synthetic substrate which will be hydrolysed by all proteases (Dalling, 1986). Therefore, the protease activity measured in this study may be an underestimation of the overall proteolytic activity in the pulp. Results of the enzyme assay are, discussed based on the assumption that the measurable protease activity is the "total" proteolytic activity in the pulp of the fruits. In particular, protease activity were detected in the unwashed pulps of both fresh and aged berries but was absent in the washed pulps. From *Fig 2*, it is seen that the protease activity in the pulp of fresh fruit is higher than that in aged fruits. This was found to be statistically significant ($P \leq 0.0014$) (Appendix C, table B3). The decline in protease activity in the pulp of the fruit with age is not surprising. Proteases, like all proteins, gradually get denatured due to slight changes in environmental conditions such as temperature, and undergo auto-digestion as well. After at least six months in ice storage, a specific protease activity of 0.1342 unit/mg protein, is quite significant to have serious repercussions. Though the proteolytic activity at storage temperature may not be the same as that at the

experimental temperature, the low storage temperature may only slow down but not altogether arrest enzyme activity, allowing degradation of miraculin to proceed albeit slowly when the fruits are stored. Since no protease activity was detected in the pulp after washing with water, it appears that the proteolytic enzymes are easily removed by washing. It was thus not necessary to include protease inhibitors to the extraction buffer.

From these results, it seems logical to argue that, although washing the pulp does not seem to decrease the total protein content in the pulp, it does not mean that there are no cytosolic proteins in the pulp. Since this is a relative comparison, their presence may be so minute as to go undetected by the method employed for data analysis, but their effect may rather be devastating. The insignificant decrease in total protein concentration with storage reported earlier may be due to the fact that, hydrolysis of miraculin by the proteases occurs as anticipated, but the active principle is probably only hydrolysed to smaller peptides or protein fragments with no ability to elicit sweetening. These fragments of miraculin are still quantitated as proteins by the analytical method employed for the determination.

Purification of functional miraculin was achieved at ordinary temperatures by a three-step chromatographic procedure. It is

pertinent to note that, contrary to the claim by other workers that all aspects of the extraction and purification of miraculin be done at 4°C, (Pintauro, 1977) in this study most of the work was done at room temperature. Only the depulping and washing stages were performed at 4°C. It is perhaps obligatory to perform the very initial stages of the process at very low temperatures to help reduce the protease activity to its barest minimum. Since the proteases are separated from the miraculin by washing the pulp, degradation of the protein will be significantly reduced after washing.

It can be seen from Table 6 that, of the total mass of the miracle fruit, approximately 10% can actually be exploited for miraculin. The seed and fruit coat constitutes some 47.8% of total mass while roughly 42.2% of it is composed of the fruit juice.

Fig. 3, shows the initial purification and extraction stages of miraculin from the pulp of miracle fruits. For all practical purposes, lanes 4, and 6, which are representative samples of the fruit juice and washing effluent respectively, do not contain miraculin bands. These results further confirm that while in the pulp, miraculin is not soluble in aqueous medium in the absence of added salts (Inglett and May 1968, Kurihara and Beidler 1968). Lanes 2, 3, 5, and 7, identified below the *Fig.* all show smeared bands of miraculin but

sharpness of the band increase with washing. The extraction of miraculin from the pulp is represented by lanes 8, and 9. The spent pulp, (Lane 8), is seen only as a smear on the gel with practically no miraculin, while the crude extracts, lane 9, contain miraculin.

As can be seen in *Fig. 4*, HIC partially purified miraculin in the crude extract. In spite of the high absorbance value of fraction 1A (60% of applied absorbance), SDS-PAGE analysis reveals that it contains practically no miraculin (*Fig. 4*, insert, lane 4). The first HIC column which contained the weaker hydrophobic resin, S-butyl sepharose FF was highly discoloured after the sample application. These results strongly suggest that during the extraction of miraculin from the pulp of the miracle fruits, a lot of other very hydrophobic, UV-absorbing compounds (high A_{280} Value of 1A) are co-extracted. The HIC column, containing butyl sepharose FF a much stronger hydrophobic resin, bound the miraculin, (fraction 1B) (*Fig. 4*, insert lane 5) and was hardly discoloured. This also indicates that most of the co-extracted coloured pigments/compounds either got bound to the first resin (S-butyl sepharose) or had a poor affinity for the butyl sepharose resin. By implication therefore, the highly coloured co-extracted compounds in the crude extract of miraculin are strongly hydrophobic since they got bound to the weaker hydrophobic resin while the miraculin and other

proteins are relatively weakly hydrophobic as they were retained by the stronger hydrophobic resin. The S-butyl sepharose resin thus appear to be very crucial in the separation of the coloured co-extracted hydrophobic pigments/compounds from the crude extract. Fraction, 1B, contained partially purified miraculin (*Fig. 4*, insert, lane 5).

From *Fig. 5*, it is seen that, IEC further improved the purity of fraction 1B. SDS-PAGE analysis of the flow through (fraction 2A) from the ion exchange (IE) column revealed practically no protein bands (*Fig. 5*, insert lane 4) indicating that, most of the applied proteins got bound to the column resin. This notwithstanding, the unbound fraction had a total A_{280} of approximately 20.4% of the total absorbance applied on the IE column. The high total absorbance then, can not be attributed solely to unbound proteins, it seems likely that fraction 2A contains almost colourless UV-active chromophores accounting for the high A_{280} value. Thus, in addition to coloured compounds, it appears that the extraction treatment also co-extracts UV-absorbing compounds which are apparently not brightly coloured. These UV-active chromophores are separated from miraculin by the IEC column. The proposed chromophores, like miraculin, should be relatively weakly hydrophobic as they are retained by the stronger hydrophobic resin. Application of buffer C_1 to the loaded IEC column eluted fraction 2B containing practically no

miraculin (*fig. 5*, insert lane 5). For all practical purposes fraction 2C, eluted by buffer C₂ from the IEC, contains highly purified miraculin (*fig. 5*, insert lane 6). Organoleptic analysis revealed that 2C contained active miraculin.

Gel filtration chromatography yielded pure miraculin as only one peak and only one protein band are observed on the GF elution profile and gel analysis respectively (*fig. 6*, insert lane 4). The purification profile of miraculin shown in the flow chart (*Fig. 1*) is basically a modification of the method of Kurihara and Beidler (1988).

It can be inferred from Table 7 that, the standard miraculin solution elicited sweetness in spite of its very low strength of 100 µg/ml. This result tentatively affirm that the purification of miraculin can be effectively carried out at room temperature.

The results of the M_r determination (Table 8) show that purified miraculin has a M_r approximately equivalent to that of ovalbumin (M_r Ca. 43,000). This corresponds very well to the dimer molecular weight of purified miraculin (Kurihara, 1992). It is thus apparent that miraculin occurs as a dimer in its natural state, which is not surprising. This protein contains seven cysteine residues of which six form three intrachain disulphide bonds leaving one cysteine residue to

form an intermolecular disulphide bond thus giving a dimer form of miraculin.

The result of N-terminal sequence of the purified miraculin Fig. 7, reveal that the miraculin preparation is a pure one. It further portrays two populations of miraculin molecules due to heterogeneity in the N-terminal amino acid. The minor form also had the same sequence as miraculin but lacked the NH₂-terminal aspartic acid. This might either be due to a proteolytic removal of just this amino acid or a heterogeneity on the NH₂-terminal due to a natural variant of the same miraculin protein in the purified sample.

There is no tangible evidence for a proteolytic cleavage of the molecule at sites other than the NH₂-terminal end. This is because the sequence of the minor fraction could not be localized any where on the molecule. Further clarification is needed to confirm that the heterogeneity is caused by post translational modification, or partial damage by proteases in the process of storage or purification. It is likely that a family of proteins exist in *R. dulcifica* and share most of the protein sequence and may also come from the same gene. The heterogeneity of the N-terminal may be speculated at the gene or translational level.

A model for predicting the effect of ionic strength and pH on

the solubility of miraculin was developed using multiple regression procedures (Table 9). The model was:

$$Z = 0.027348 - 0.015177\mu - 0.003364\mu X + 0.000128X + 0.029960\mu^2 \dots \text{eqn.1}$$

Where Z = solubility.

From Table 10, it is seen that the quadratic term of ionic strength was the most influential factor in the predictive model. It contributed 35.15% of the explainable portion of the model. The linear and quadratic terms of ionic strength and pH were the next most significant contributors to the model, contributing 13.74% and 13.70% respectively. The interactive term of pH and ionic strength was the least significant contributor (3.50%).

The predictive model (eqn. 1) was used to generate a response surface plot (*Fig. 8*) which showed that the effect of ionic strength on miraculin solubility depended largely on the pH of the medium. Miraculin had a high solubility at low pH (2.5) and low ionic strength (A). The observed high solubility of miraculin at low ionic strength (salting-in) is possibly due to Debye-Hückle screening (Timasheff, 1993). When pH was maintained at 2.5, increasing the ionic strength caused only a slight decrease in the solubility of miraculin (B). Increasing ionic strength at high pH (pH 10), however, considerably decreased the solubility of miraculin (D). This

may largely be due to the fact that the solubility of native proteins is influenced by pH, being a minimum at their isoelectric point. The high ionic strength of the solution may have had the greatest influence on the solubility of the protein at its isoelectric point of 9.1 leading to the minimum solubility at that point.

One of the basic observations of introducing polyhydric alcohols and sugars into solvent media containing biological macromolecules is that these additions increase the thermal transition temperature (T_m) of the molecules thus stabilising them (Gekko and Timasheff 1981; Lee and Timasheff 1981). This phenomenon was examined in this study. Taking the mid point of the transition to be the transition temperature, T_m , it can be seen from *Figure 9a* that the T_m of the protein is a function of glycerol concentration. It is seen that, T_m increases with glycerol concentration in an approximately linear manner. It is thus evident that, the presence of glycerol caused an elevation of the thermal transition temperature of purified miraculin. The stabilizing effect of these additions may be attributed to the decreased hydrogen bond-rupturing capacity of the medium or an increase in the apparent energy of activation of the unfolding reaction thus hindering protein unfolding. However, further work is needed to establish whether the thermal stabilization still leaves the miraculin in its active state.

The work reported here shows that, full scale purification of functional miraculin can be done at ordinary temperatures by a simple three step chromatographic process: HIC, IEC, and GFC. However, in view of the presence of cytosolic proteases it is obligatory to perform the initial purification stages at low temperatures (4°C) to reduce proteolytic activity. Alternatively, soon after disruption of the berries, protease inhibitors which are compatible with the biological system could be added to the pulp to arrest enzyme activity.

The M_r of the purified miraculin was found to be approximately 43,000. The N-terminal analysis seems to suggest that two forms of miraculin molecules exist in a natural population of miraculin. Also, it has been established that miraculin is most soluble at low pH in the ionic strength range of 0-1.0 M aqueous NaCl solution while the solubility is minimal at high pH for the range reported. Finally, water-glycerol mixtures were observed to stabilize the protein by increasing its melting temperature.

5.0

APPENDICES

APPENDIX A

BUFFERS

1. Extracting buffer:

0.50 M NaCl pH 7.0.

2. Purification Buffers:

STOCK BUFFERS;

(i) 0.20 M Na_2HPO_4 (ii) 0.20 M NaH_2PO_4

Buffer A (pH 6.8);

20 mM sodium phosphate buffer composed of;

85 ml 0.20 M Na_2HPO_4 15 ml 0.20 M NaH_2PO_4 80 g $(\text{NH}_4)_2\text{SO}_4$ add dH_2O to 1000 ml

Buffer B; 20 mM sodium phosphate (pH 6.8)

composed of;

50 ml 0.20M Na_2HPO_4 50 ml 0.20M NaH_2PO_4 add dH_2O to 1000 mlBuffer C₁ pH 6.8;

0.05M NaCl in buffer B

Buffer C₂ pH 6.8;

0.12M NaCl in buffer B

Buffer D pH 6.8;

0.35M NaCl in buffer B

Buffer E pH 6.8;

0.01M $\text{CH}_3\text{COONH}_4$ pH 6.8

3. Electrophoresis buffers:

(i) 3M Tris-HCl pH 8.8 (solution 1)

To 60 ml dH_2O add;

36.34 g tris base

adjust pH with 1M HCl to 8.8

and add dH_2O to 100 ml

store at 4°C.

(ii) 10% SDS (solution 2)

Dissolve 5 g of SDS in 50 ml dH_2O

store at room temperature.

(iii) 1M Tris-HCl pH 6.8 (solution 3)

To 20 ml of solution 1 add;

20 ml dH_2O and adjust pH to 6.8 with 1M HCl

top to 60 ml with dH_2O .

(iv) 30% acrylamide/0.8% bisacrylamide (solution 4)

30 g acrylamide,

0.8 g N,N'-methylene-bisacrylamide,

add dH_2O to 100 ml.

filter through a 0.45 μM filter.

store in dark bottle at 4°C.

(v) Stacking solution (solution 5)

8 ml solution 4,

6.25 ml solution 3,

0.50 ml solution 2,

dH_2O to 50 ml.

store in dark bottle at 4°C.

(vi) 10% Stacking gel

10.63 ml dH₂O,
2.50 ml solution 1,
6.67 ml solution 4,
20 mg ammonium persulphate,
13.34 µl TEMED.

(vii) 5% Stacking solution or soft gel

5 ml solution 5,
5 mg ammonium persulphate,
5 µl TEMED.

(viii) 5X SDS Electrophoresis buffer pH 8.3

15.1 g tris base,
72.0 g glycine,
5.0 g SDS,
add dH₂O to 1000 ml.

Do not adjust pH to 8.3 since pH changes automatically to 8.3 when diluted to 1X for use.

(ix) 2X SDS/sample buffer

To 40 ml dH₂O add;
1.52 g tris base,
20 ml glycerol,
2.00 g SDS,
2 ml 2-mercaptoethanol,
1.00 mg bromophenol blue.
Adjust pH to 6.8 with 1M HCl.

(x) Staining solution

1.20 g Coomassie brilliant blue R-250,
500 ml absolute ethanol,
200 ml glacial acetic acid,
600 ml dH₂O.

(xi) Destaining solution

300 ml absolute ethanol,
100 ml glacial acetic acid,
600 ml dH₂O.

4. Reagents used for Folin-Lowry protein assay:

Reagent A; 2% Na₂CO₃ in 0.1M NaOH

Reagent B; 0.5% CuSO₄·5H₂O in 1% sodium,potassium
tartarate

Reagent C; alkaline copper solution prepared by mixing 50
ml of reagent A with 1 ml of reagent B on day of
experiment.

Reagent D; Folin-Ciocalteau reagent.

Prepared by diluting commercial reagent with an equal
volume of dH₂O on day of use.

Standard Protein; 0.20 mg BSA stock solution.

STATISTICAL ANALYSIS 1

Table B1: Comparison of protein concentration in the pulps of washed and unwashed fruits using the t distribution.

Sample statistic	Variable		
	Washed	Unwashed	Pooled
No. of Obs.	6	6	12
Average	0.2705	0.2927	0.2816
Variance	1.74×10^{-4}	6.48×10^{-4}	4.11×10^{-4}
Std. deviation	1.32×10^{-2}	2.55×10^{-2}	2.03×10^{-2}
Median	0.2705	0.281	0.2755

Difference between means = -0.0221667

Conf. Interval for Diff. in means = 95%

Equal variance) Sample (1-2); - 0.04825 3.9163×10^{-3} 10 D.F.

(Unequal Vars) sample (1-2); -0.0494755 5.1422×10^{-3} 7.5 D.F

Ratio of variance = 0.268419

Conf. Interval for ratio of variable; 0%

(Sample 1 / sample 2)

Hypothesis testing

$H_0: \mu_1 = \mu_2$

$H_1: \mu_1 \neq \mu_2$ (two tailed).

$\alpha = 0.05$

computed statistic, $t = -1.89409$

Sig. level = 0.0874698.

Conclusion:

A significant value of t at 5% with $v^* = 6 + 6 - 2 = 10$ is roughly 0.1, therefore value $t(-1.89409)$ is insignificant, hence H_0 rejected, and the conclusion that there is no significant difference between the protein conc. of washed and unwashed pulp of miracle fruit is drawn.

Table B2: Comparison of protein concentration in the pulp of fresh and aged miracle fruits using t distribution.

Sample Statistic	Fresh	Variable	
		Aged	Pooled
No. of Obs.	6	6	12
Average	0.271	0.292	0.282
Variance	4.52×10^{-5}	8.026×10^{-4}	4.239×10^{-4}
Std. dev.	6.72×10^{-3}	2.833×10^{-2}	2.059×10^{-2}
Median	0.272	0.288	0.276

Difference between means = 0.0211667

Conf. Interval for Diff. in means; 95%

(Equal Vars). Sample (1 - 2); -0.047659 5.326×10^{-3} 10 D.F.

(Unequal Vars) sample (1 - 2); -0.050827 8.494×10^{-3} 5.6 D.F.

ratio of variance = 0.0563193

Conf. Interval for Ratio of Variance: 0%

(Sample 1 / sample 2).

Hypothesis testing;

$H_0: \mu_1 = \mu_2$

$H_1: \mu_1 \neq \mu_2$

computed statistic, $t = -1.7807$

Conclusion:

A significant value of t at 5% with $v^* = 6 + 6 - 2 = 10$ is roughly 0.1, therefore value $t(-1.89409)$ is insignificant, hence H_1 rejected, and the conclusion that there is no significant difference between the protein conc. of washed and unwashed pulp of miracle fruit is drawn.

Table B2: Comparison of protein concentration in the pulp of fresh and aged miracle fruits using t distribution.

Sample Statistic	Fresh	Variable	
		Aged	Pooled
No. of Obs.	6	6	12
Average	0.271	0.292	0.282
Variance	4.52×10^{-5}	8.026×10^{-4}	4.239×10^{-4}
Std. dev.	6.72×10^{-3}	2.833×10^{-2}	2.059×10^{-2}
Median	0.272	0.288	0.276

Difference between means = 0.0211667

Conf. Interval for Diff. in means; 95%

(Equal Vars). Sample (1 2); -0.047659 5.326 x 10⁻³ 10 D.F.

(Unequal Vars) sample (1 2); -0.050827 8.494 x 10⁻³ 5.6 D.F.

ratio of variance = 0.0563193

Conf. Interval for Ratio of Variance: 0%

(Sample 1 / sample 2).

Hypothesis testing;

$$H_0: \mu_1 = \mu_2$$

$$H_1: \mu_1 \neq \mu_2$$

computed statistic, $t = -1.7807$

$$\alpha = 0.05$$

$$\text{Sig. level} = 0.105308$$

Conclusion:

A significant value of t at 5% with $v = 6 + 6 - 2 = 10$ is roughly 0.1, therefore value $t(-1.89409)$ is insignificant, hence H_1 rejected, and the conclusion that there is no significant difference between the protein concentration of washed and unwashed pulp of miracle fruit is drawn.

APPENDIX C

STATISTICAL ANALYSIS 2

Table B3: Comparison of Protease Activity in pulps of fresh and aged fruits.

Sample Statistic	AGED	Variable FRESH	POOLED
No. of Obs.	3	3	6
Average	0.1342	0.2414	0.1878
Variance	1.09×10^{-4}	4.54×10^{-4}	2.82×10^{-4}
Std Dev.	0.01046	0.02132	0.01679
Median	0.1313	0.2351	0.1849

Difference between means = 0.107233

Conf. intervals for diff. in means ; 95%

(Equal Vars) Sample (1-2); -0.145308 - 0.069159 4 D.F.

(Unequal Vars) Sample (1-2); -0.151636 - 0.062830 2.9 D.F

ratio of variances; 0%

(sample 1/ sample 2)

Hypothesis testing:

$$H_0: \mu_1 = \mu_2$$

$$H_1: \mu_1 \neq \mu_2$$

computed statistic, $t = -7.82237$

$$\alpha = 0.05$$

$$\text{Sig. level} = 1.44179 \times 10^{-3}$$

Conclusion.

A significant value of t at 5% with $v = 3 + 3 - 2 = 4$ is roughly 0.0014 therefore value $t(-7.89409)$ is significant, hence H_0 rejected, and the conclusion that there is a significant difference between the protease activities in the two samples drawn.

APPENDIX D

ACTIVITY CALCULATIONS.

1. Protease activity in fresh
pulp (10 mg)

$$= \frac{0.830 + 0.701 + 0.736}{3}$$

$$= 0.7557 \mu\text{M NPP/ml/min.}$$

From total protein analysis, 1 mg of fresh pulp contains 0.313 mg of protein

Thus 10 mg of fresh pulp will contain 0.313×10 mg protein

specific protease activity = $\frac{0.7557 \mu\text{M NPP/ml/min}}{3.313 \text{ mg}}$
in fresh pulp of Miracle fruit

$$= 0.2414 \mu\text{M NPP/ml/min/mg protein}$$

2. Protease activity in aged pulp = $\frac{0.401 + 0.345 + 0.361}{3}$
(10 mg)

$$= 0.369 \mu\text{M NPP/ml/min}$$

From total protein analysis, 1 mg of aged pulp contains 0.275 mg of protein

Thus 10 mg of aged pulp contains 0.275×10 mg of protein

Specific protease activity in = $\frac{0.369 \mu\text{M NPP/ml/min}}{2.75 \text{ mg}}$
aged pulp of miracle fruit

$$= 0.1342 \mu\text{M NPP/ml/min/mg protein}$$

6.0

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