LEGUME AND CEREAL STARCHES - CHEMICAL BASIS FOR DIFFERENCES IN THEIR DIGESTIBILITIES

Thesis

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November 1993

... To my

Parents,

Teachers,

Wife and Son

DECLARATION

I declare that the thesis entitled **LEGUME AND CEREAL STARCHES - CHEMICAL BASIS FOR** DIFFERENCES IN THEIR DIGESTIBILITY submitted to the University of Mysore for the award of Degree of DOCTOR OF **PHILOSOPHY** is the result of work carried out by me under the guidance of Dr.R.N.Tharanathan, scientist, Department of Biochemistry and Nutrition, during the period 1989-1993. I further declare that the results are not submitted for the award of any other degree or fellowship.

15/11/93

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CERTIFICATE

I certify that the thesis entitled **LEGUME AND CEREAL STARCHES - CHEMICAL BASIS FOR DIFFERENCES IN THEIR DIGESTIBILITY** submitted to the University of Mysore for the award of Degree of **DOCTOR OF PHILOSOPHY** by Mr.B.Madhusudhan, is the result of work carried out by him in the Department of Biochemistry and Nutrition under my guidance during the period 1989-1993.

15.11.93

R.N.THARANATHAN

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List of abbreviations and symbols

DMSO	- Dimethylsulfoxide
DEGS	- Diethylene glycol succinate
0V-225	- Phenyl cyanopropyl methyl silicone gum stationary phase
Rochelle salt	- Sodium potassium tartarate
Tris	- Tris (hydroxymethyl aminomethane)
TGO	- Tris-glucose oxidase
TCA	- Trichloroacetic acid
NSP	- Non-starchy polysaccharide
Con A	- Concanavalin A
Glc	- Glucose
B-insol.	- Butanol-insoluble
B-sol.	- Butanol-soluble
GLC	- Gas liquid chromatography
SE-HPLC	- Size Exclusion - high performance liquid chromatography
GPC	- Gel permeation chromatography
PC	- Paper chromatography
SEM	- Scanning Electron Microscopy
i.d.	- Internal diameter
O.D.	- Optical density
d.b.	- Dry basis
ss	- Stainless steel
GT	- Gelatinization temperature
rpm	- Revolutions per minute
eV	- Electron volts
min	- Minute(s)
sec	- Second(s)
h	- Hour(s)
nm	- Nanometer
mm	- Millimeter
cm	- Centimeter
dl	- Decilitre
ml	- Millilitre
L	- Litre
pmole	- Picomolar
µmoles	- Micromoles
mM	- Millimolar
M	- Molar
N	- Normal
µg	- Microgram
mg	- Milligram(s)
g	- Gram(s)
kg	- Kilogram(s)
kD	- KiloDalton (s)
^{o}C	- Degree centigrade

Å	- Angstrom unit
B.U.	- Brabender unit
B.V.	- Blue value
vol./v	- Volume
wt./w	- Weight
MW/mol.wt.	- Molecular weight
C/conc	- Concentration (concentrated)
Conc.	- Concentration
~	- About/approximately
<	- Less than
>	- Greater than
α	- Alpha
β	- Beta
λ	- Wavelength of maximum absorption
[η]	- Limiting viscosity number (ml/g)
DP	- Average degree of polymerisation
CL	- Average chain length
ECL	- Exterior chain length
ICL	- Interior chain length
LD	- Limit dextrin
RS	- Resistant starch
vs	- Versus
GG	- Greengram
BG	- Bengalgram
Rc	- Rice
Rg	- Ragi
Ala	- Alanine
Arg	- Arginine
Asp	- Aspartic acid
Cys/2	- Half cystine
Gly	- Glycine
Glu	- Glutamic acid
His	- Histidine
Ile	- Isoleucine
Leu	- Leucine
Lys	- Lysine
Met	- Methionine
Phe	- Phenylalanine
Pro	- Proline
Ser	- Serine
Thr	- Threonine
Tyr	- Tyrosine
Val	- Valine

SYNOPSIS

Legume's and cereals contribute a major share of both available and unavailable carbohydrates in any vegetarian diet. Among these, starch is the principle dietary carbohydrate (65-78% in cereals and 55-65% in legumes), and depending upon the source the isolated starch exhibits varied physicochemical and functionality characteristics. It is known from (our) earlier studies that the digestibility of legume starches, both in terms of absolute amount digested and also the rate at which it is hydrolysed, is lower (-65%) than that of cereal starches (>85%), whether in the native or gelatinized form, and that high consumption of legume-based foods leads to flatulence and other physiological discomforts. The various factors which affect starch digestibility in general, are the amylose content, starch-protein and starch-lipid interactions, extent of gelatinization and of retrogradation, shape and size (morphology) characteristics of granules and the presence or absence of any amylase inhibitors. Most commonly, the amylose content of cereal starches is 20-25% and of legume starches is -30-40%, while the remainder is due to amylopectin and other (starch) glucan fractions, if any. Thus, the overall differences in the *in vitro* digestibility values are primarily attributable to the nature and composition of starch per *se*. As there were no precise scientific data available to explain, at a molecular level, the digestibility differences, an attempt was made in the present investigation to look into these aspects more carefully.

Essentially, several debranching enzymes and SE-HPLC and GPC techniques were used to elucidate the subtle structural differences between the legume [Bengalgram *(Cicer arietinum)* and greengram *(Phaseolus aureus)*] and cereal rice, *(Oryza sativa)* and ragi *(Eleucine coracana)* starch fractions (amylose and amylopectin). It was shown that the DP/CL as well as the molecular weight values of these fractions differed significantly and thus formed a molecular basis to explain the *in vitro* digestibility differences.

The thesis presentation is done in five different chapters, viz., (1) **INTRODUCTION**, (2) **MATERIALS AND METHODS**, **(3) RESULTS AND DISCUSSION**, **(4) GENERAL DISCUSSION**, **and (5) SUMMARY AND CONCLUSIONS.** The collective **BIBLIOGRAPHY** is cited at the end.

In **Chapter 1 (Introduction)** is reviewed (extensive) literature data available on the starches of cereals and legumes with special•emphasis on their physico-chemical, morphological, digestibility, structural characteristics, and modification.

Chapter 2 (Materials and Methods) describes the overall experimental details of the present investigation. Concise experimental procedures and methods adopted, and sources of samples, chemicals and reagents used are given. Isolation of different starch isolates from Bengalgram, greengram, rice and ragi, determination of different constituents, fractionation of starches by classical and recent methods into their components, subfractionation, *in vitro* digestibility studies, chromatographic and electrophoretic techniques, and enzyme debranching methods are all described.

Chapter 3 (Results and Discussion) is the main part of the thesis, which separately describes the results obtained on a study of legume (Section A) and cereal (Section B) starches.

Four starch isolates were recovered from each source by differential sedimentation and centrifugation steps. All were studied for their size/shape, population density, starch content (varying from 48-88%) and some chemical and physico-chemical characteristics. It was revealed that starch isolates I, II and III were rich in (starch) carbohydrates, whereas the isolate IV was low in starch content (10.5-45.0%), but contained more of protein, lipid and other non-starchy carbohydrates. Some differences were also discernible in the qualitative and quantitative nature of starch granule proteins electrophoresed on SDS-PAGE. Analysis of the lipid fractions by GLC as FAME showed predominance of palmitic acid in all. The content of amylose was more (~42%) in legume than in cereal (~22%) starches. All the starch isolates were found to be non-ionic and exhibited single-stage swelling. The solubility pattern in DMSO (60 h) for legume starches was lower (76-88%) than for cereal starches (-100%), which might probably be due to the labile and heterogeneous bonding forces within the granule. Brabender amylograph studies indicated a low slurry viscosity and low set back viscosity in the case of Bengalgram starch I (170 B.U. and 320 B.U., respectively) compared to greengram starch I, which exhibited considerable paste (560 B.U.) as well as set back (retrogradation, 1080 B.U.) viscosities. The peak and cold paste viscosities of rice starch I were 250 and 360 B.U., respectively; whereas the corresponding values for ragi starch I were 330 and 420 B.U., respectively.

The *in vitro* digestibility of these starches was in the order of BG < GG < Rg < Rc. Rice starch digested most whereas Bengalgram starch digested least, suggesting that the lower digestibility could partly be attributable to its higher amylose content.

Out of the three different fractionation methods •employed, the Con A precipitation method yielded fairly homogeneous amylopectins. By hot butanol extraction, the crude amylose fraction was further resolved into pure amylose (BtíOH-insoluble) and intermediate (BUOH-soluble) fractions. GPC on Sepharose CL-2B and SE-HPLC of these fractions revealed homogeneity as well as molecular weight values. Legume starch fractions were of relatively very high molecular weight ($4.5x10^6$ to $1.7x10^7$) in comparison to those of ragi starch ($1.5x10^5$ to $1.5x10^7$) fractions.

β-Amylolysis studies revealed the branched nature of the presumed "linear" amylose fractions of legume starches. From the β-amylolysis values, it was inferred that the legume amylose was relatively rather more branched - 12-20% branching residues than the cereal amylose counterparts (only ~2-5% branching). This was supported by their debranching studies with pullulanase and iso-amylase. Enzyme debranching of both amylopectin and the derived p-limit dextrins indicated the preponderance of extended B-chains of CL 62-70 in legume starch fractions, which could also account for their relatively a higher blue value and possibly a higher amylose content. On the other hand, similar studies on ragi amylopectin showed higher proportions of short A- and B-chains (CL 17-48). Subtle variations in the degree of branching were also encountered in the intermediate fractions of these starches.

In the next **Chapter 4** a **General Discussion** is provided to compare and contrast the subtle variations existing, both at compositional and molecular levels, between the legume and cereal starches. Results obtained in this study are compared with the literature data.

Finally, the salient observations deduced from this •study are listed in Chapter 5 (Summary and Conclusions), followed by bibliographic citation.

INTRODUCTION

Starch is the principal food reserve polysaccharide of plants. It is widely distributed in nature, occurring in several parts of the plant, viz., leaves, seed grains, tubers, roots, ''etc. As a product of photosynthesis, starch is the biopolymer by which the solar energy is trapped for its subsequent depletion and later conversion to physiological energy. It is one of the abundant and renewable raw materials available on earth, and provides the major share of energy in any vegetarian diet. Starch is functionally a very important polysaccharide that has attracted the attention of chemists, biochemists and technologists all over, and literature is replete with studies of traditional and novel starches and their physicochemical characteristics[1,2]. Starch has innumerable applications both in food and non-food industries . The role of starch in most of the food systems, in addition to providing energy, is to contribute to the texture, and as a result to the organoleptic qualities of the food. Indirectly, starch acts as a temperature-triggered water sink in most of the food systems.

Granule Size and Shape Characteristics

In nature starch is present as tiny-discrete granules of varying size and shape characteristics. In wheat and a few other cereals, starches are shown to have a bimodal distribution of granules[4,5] , viz., small, spherical and big, lenticular granules, and such size variations are under strict genetic control[6,7]. As far as wrinkled-seed pea is concerned the differently sized granules are laid down at

different stages of development, small granules are formed early in the growth while larger-compound granules are formed at later stages of maturity[7] . Such an inference is suggestive of the larger granules not being formed by a simple apposition of starchy molecules on the small granules. In oat starch the large granules are indeed formed by aggregation of several small granules , akin to spherulitic crystallization initiated simultaneously at several nuclei . In corn, the two granule populations are reported to reside in two different types of endosperms, namely the translucent endosperm containing the closely arranged polygonal granules (7-8 urn in diameter) and the opaque endosperm containing large-smooth spherical granules . In barley over 90% of the granules are small (< 5pm) but they account for only 10% of the total weight of the starch[10]. Factors such as temperature[11], environmental conditions, soil agronomy[12] and grain maturity at harvest[13] do also govern the starch granule size distribution. It is therefore likely that during starch isolation and purification steps the small granules, because of their poor settling characteristics may inadvertently be discarded lost in the supernatant. There is also a tendency for the small starch granules to be associated with protein (non-specific adsorption because of their large surface area) and get lost during starch preparation steps.

In general, the granule size[2] ranges from sub-micron elongated granules of chloroplast (for eg. leaf starch) to the relatively large (100 µm) oval shaped granules of amyloplast. The granule shape includes nearly perfect spheres typical of small wheat[2] and pepper[14] starch granules; large lenticular granules of wheat, barley and rye; polyhedral granules as in rice[15] and millet starches;[16]

"oyster" shell-shaped irregular .granules of potato starch[17] ; very irregular shaped granule aggregates of pigweed starch[18] , etc. These differences in size and shape characteristics make it possible to recognize most of the starches[19]

Granule Morphology

The surface topography of starch granules has received considerable attention from microscopists all over. Due to •the very high resolving power electron microscopes reveal the intrinsic submicroscopic details of starch granules. In scanning electron microscope (SEM) the sample under study can be viewed in different angles by a suitable rotation device provided in the instrument, and hence it is possible to get a 3-dimensional picture' over a practical magnification range upto 20,000 X. Although the transmission electron microscope offers a much higher resolving power (< 5A) the specimen preparation steps are quite complex and tedious too. In SEM the specimen is simply mounted on a metal stub and viewed. For non-conducting biological materials a thin layer (~400A thick) of metal (usually gold) coating is made to carry off the charge arising from the primary electron beam.

In SEM the starch granules in their native form appear smooth with occasional surface indentations caused by the compression of small starch granules or protein bodies during the early stages of development in the amyloplast[5] . In wheat and barley starches the presence of equatorial groove, particularly around the big lenticular granules is conspicuous[20]. This is not merely an indentation but represents a manifestation of a medium plane of weakness, wherein the polarizing crosses intersect in the polarized light. The granule appears thin at this plane, and *in vitro*

the enzyme action and penetration starts from the equatorial region. The presence of an equatorial groove is seen persistent at all stages of swelling caused by gradual heating of starch in water, as evidenced by SEM analysis ' (Fig.l). The phenomenon of swelling is described as a two stage process, viz., the initial radial expansion to form a flattened disc followed by a tangential expansion to give a complex puckered granule. The fully swollen granule appears fragile and flexible.

The precise nature of the genetic and biochemical factors which control the number, size, shape and composition of starch granules remains still a mystery.

Physico-chemical Characteristics

Majority of starch granules when viewed through polarized light show birefringence, i.e., polarization cross passing through a point called hilum. Hilum probably represents the nucleus for the starch granule to grow and it is the point where the polarization crosses intersect. Birefringence is due to organization of the constituent polymeric molecules within the granule and its intensity is dependent on the degree of crystallinity as well as on the granule thickness[2].

With I_2~^{KI} solution starch, preferably in its gelatinized form gives a blue colour having a λ_{max} value of 530-630 nm, and the colour intensity is essentially dependent on the nature of starch (amylose content). Herein, the iodide ions are included in the helical amylose chain (Fig.2), which when treated with dilute sodium thíosulphate solution reacts with iodine at a molecular level and destroys

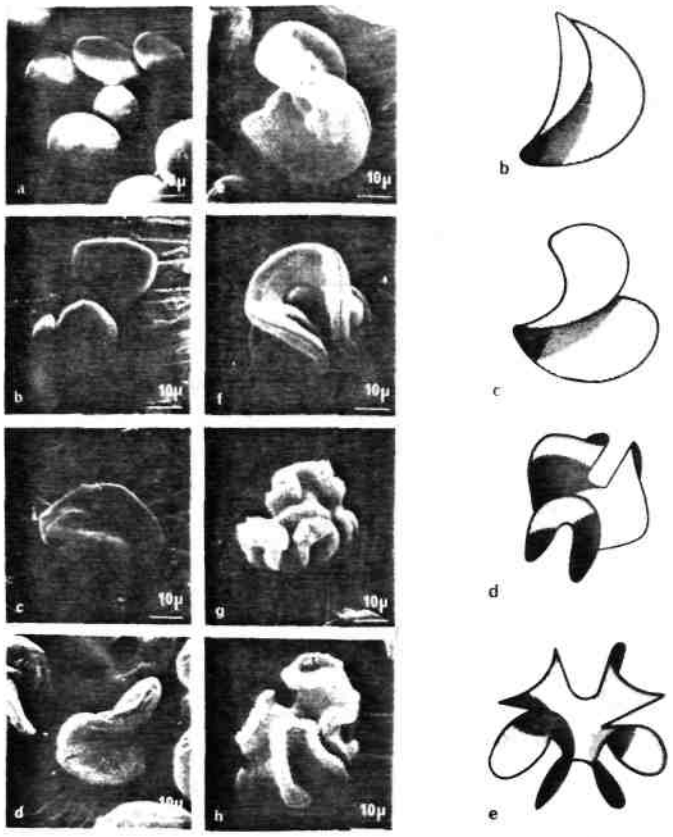
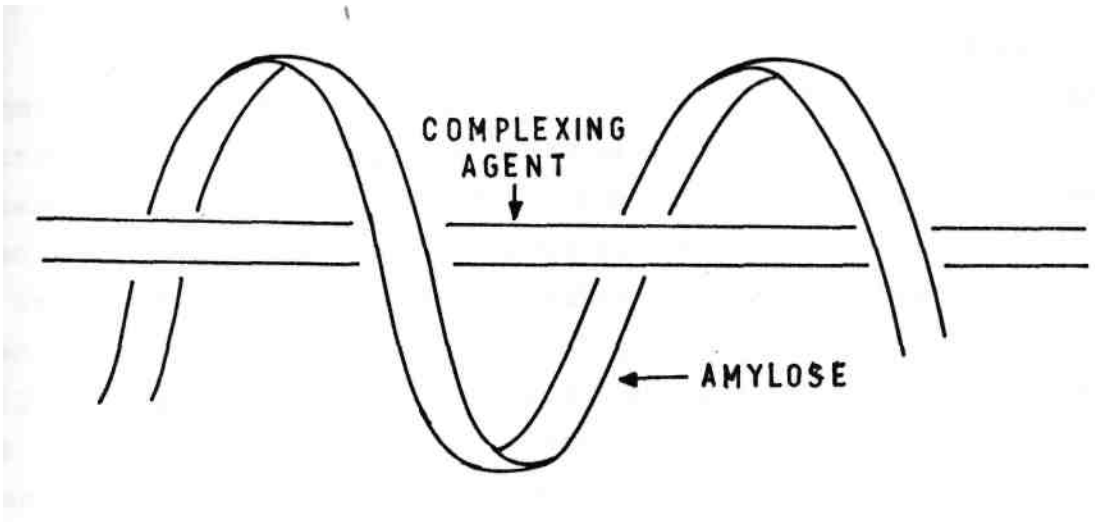
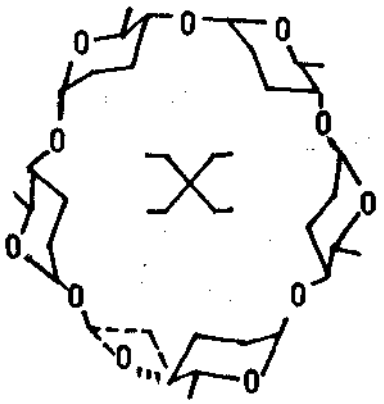


Fig.lSEM of wheat starch (A-type) granule heated in water, a, native; b, *0°; c, 50°; d, 60°; e, 70°; f, 80°; g, 90° and h, 97°. Adjacent to it are depicted the diagramatic representation of the granule morphological changes.

Fig.2. Amylose helix with the compiexing agent. | 6





the complex[23] . The excellent stoichiometry of this colour reaction has led to the development of a potentiometric titration method for the quantitative analysis of the amylose component of starch.

The characteristic property of most starches, in general, is the phenomenon of gelatinization and retrogradation taking place on progressive heating/cooling of aqueous starch suspensions[22] . The native granules which are insoluble in cold water on gradual heating swell enormously by imbibing a large excess of water until the granules burst open with the formation of starch pastes. As a result there will be a rapid and total loss of granule birefringence, and the hot paste viscosity of starch increases significantly[2]. Usually gelatinization takes place over a range of temperature, which is characteristic of the particular starch[2]. In gelatinized starch there is a many fold increase in the ease of digestion by amylolytic enzymes. On gradual cooling the gelatinized starch pastes undergo the phenomenon of retrogradation (see Fig.3). The later is due to the precipitation of amylose crystallites caused by extensive molecular associations (polymer-polymer interactions). Retrogradation is an attempt at crystallization by large unwieldy molecules, it is the fitting together of segments of starch chains.

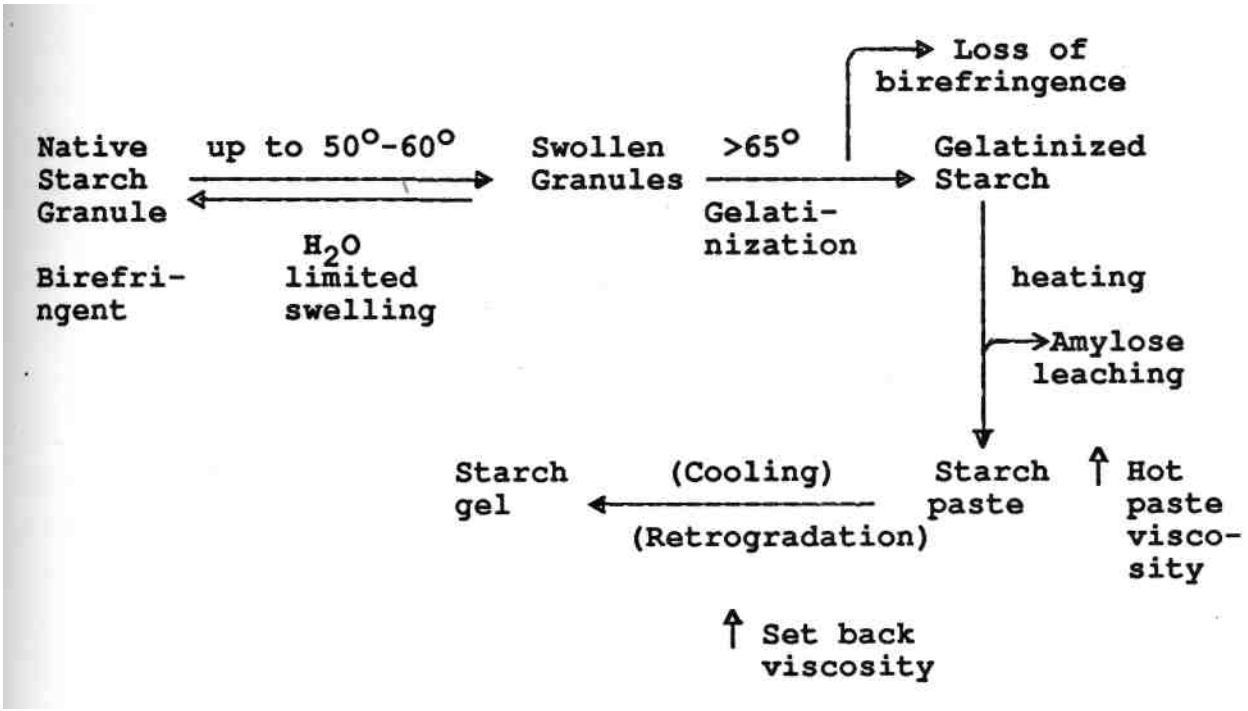


Fig.3: Schematic representation of starch swelling in water.

Staling of bread is in a way the crystallization of amylose that is leached out of the starch granules during gelatinization[24]. Based on the results of I_2-binding capacity, X-ray diffraction patterns, acid and enzymic hydrolyses a hypothetical model has been proposed • for the retrograded starch[25,26] . Three structural domains are identified in this model (Fig.4). Domain-A, mainly orginating from retrograded amylose and amylopectin chains, is relatively acid resistant but enzyme susceptible. The former is exemplified by several different modes of double stranded chains, namely (a) double helix formed by two amylopectin chains existing side by side, (b) double helix formation by two separate amylopectin chains situated far apart, (c) an amylose chain forming a helix by itself, (d) a parallel double helix formed by an amylose with two separate amylopectin chains, and (e) combination of c and d by a

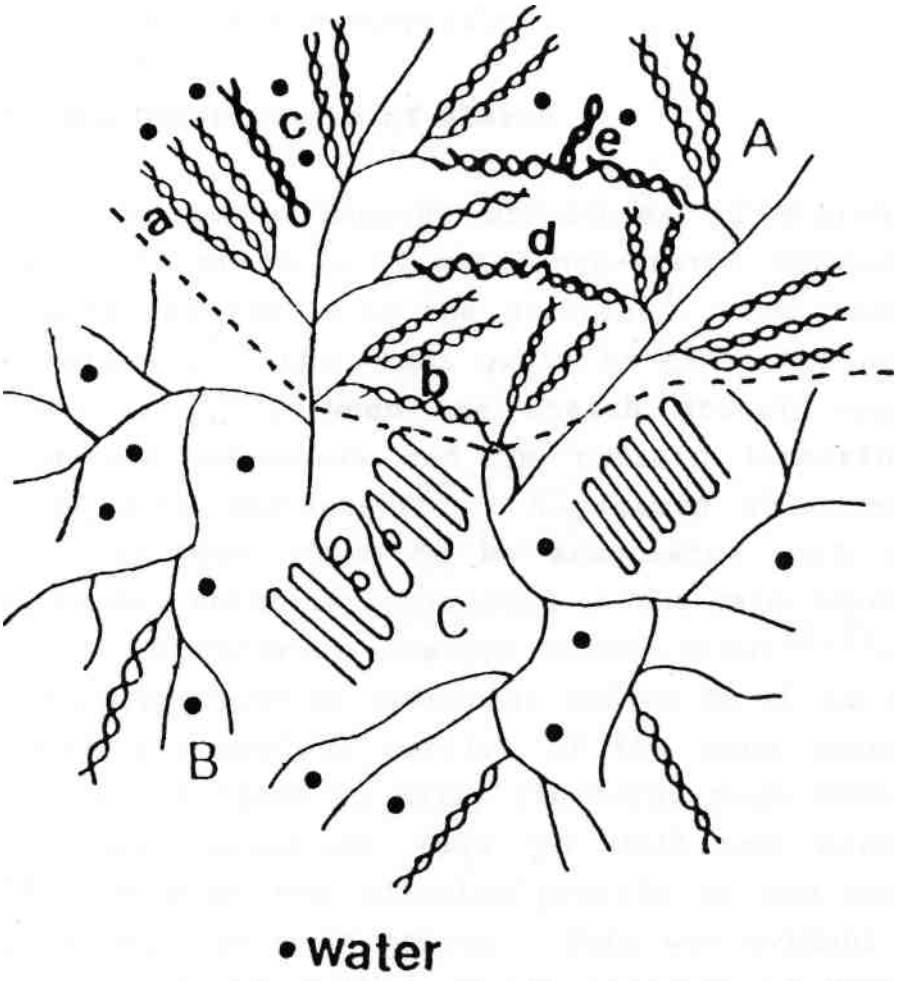


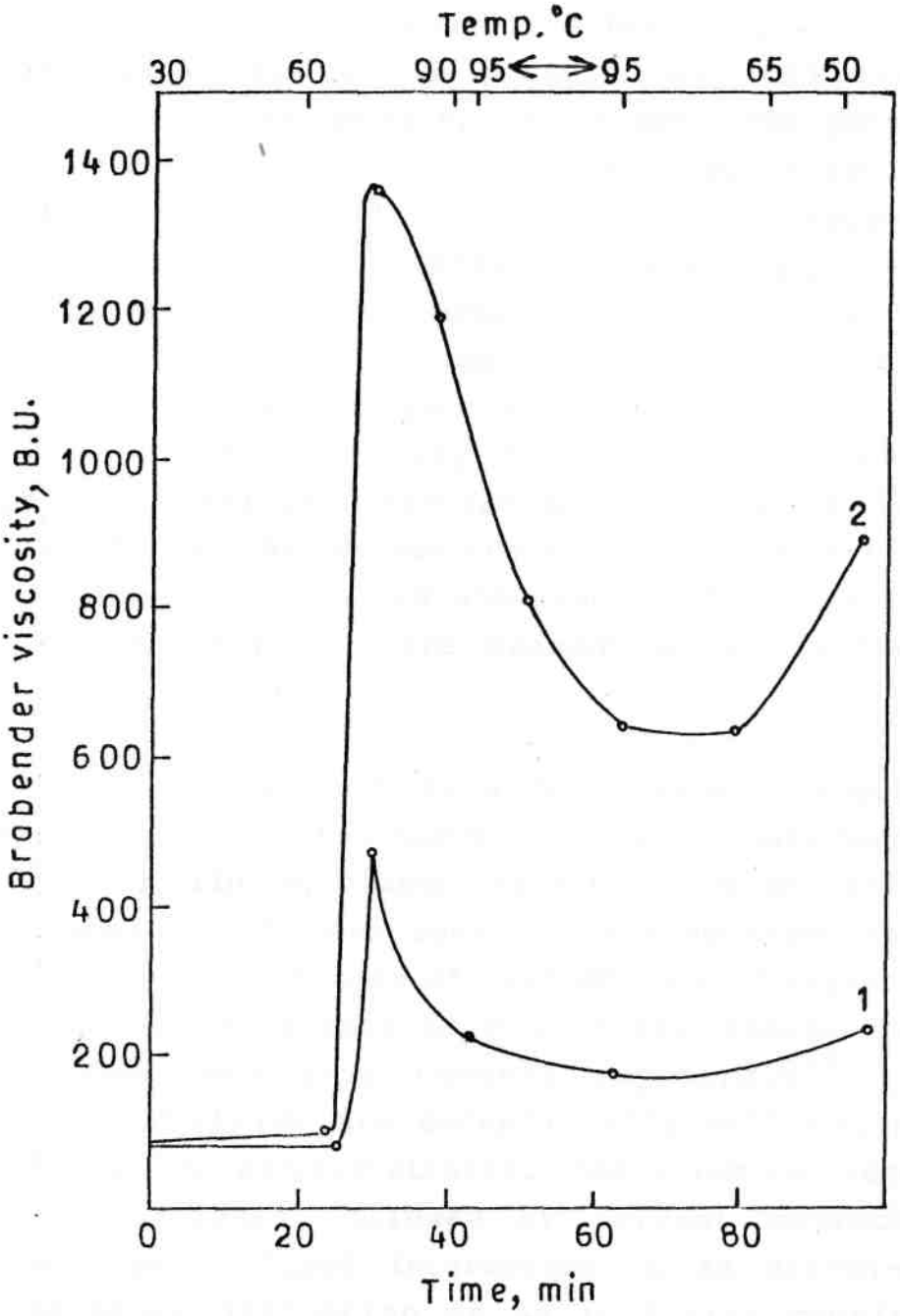
Fig.4. Plausible structural representation of retrograded starch.

single amylose chain. Syneresis is mainly caused in domain-A. Domain-B, representing a transition state, is usually found in aged starch gel. This domain is both acid and enzyme susceptible, whereas domain-C is the true representation of retrograded amylose and is very strongly enzyme resistant but acid susceptible.

Non-Carbohydrate Constituents of Starch

Some of the useful/unusual properties of starch are attributable to the presence of minor non-starch components, such as proteins and lipids in the granule[27]. For example, the milling hardness of the wheat grain is explained as due to the interactions between the starch granule surface (acting as an ion-exchanger) and the protein (adhering by virtue of its basic character)[29] . A protein of molecular weight 15 kD has been shown to be associated with wheat endosperm softness, which is implicated in the cake improving characteristics of chlorine treated wheat flour[30,31] . A tightly bound polypeptide of molecular weight 55 kD is found associated with the amylose portion of the corn starch . High protein content leads to melay flavours, foam formation and also colour formation (due to Maillard reaction products)[32a] . Some of the adhering protein on the granule surface may be amylolytic in nature. This was evident from the Brabender amylograph studies in the presence of HgCl2 of "prime" starch isolated from sweet potato[33] (see Fig.5). The significant increase in hot paste viscosity upon addition of HgCl2 was due to the inhibition of the highly active thermostable amylases present naturally in the tubers. These enzymes (α and β amylases) , probably involved in mobilising carbohydrates for respiration during storage, are evidently becoming active during the slow but gradual process of

Brabender amylograms of sweet potato "prime" starch without íl) and with (2) $HgCl_2$.



heating when the starch gets gelatinized. Because of their high thermal stability the enzymes quickly hydrolyse the starch paste into low molecular weight dextrins/maltooligosaccharides and thus lead to low viscosity values. However, some of the protein may still remain covalently bound to the granule, as evident from extensive amylolysis of maize and wheat starches to yield a resistant starch-like material, which contained significant amounts of protein and lipid in it[34] . Interestingly the glycoprotein nature of starch (through covalent linkage at the reducing end to protein) has been proposed and lends support to the above findings[31] . It is generally found that smaller granules have a higher affinity for protein. It has been shown that the bread loaf texture and loaf volume is the result of an interaction between the protein and starch[35] . It is not known whether the lipid and protein, both associated with the granule surface, are present as a lipoprotein complex.

Lipids, though present to a minor extent, should no longer be regarded as contaminants or useless constituents of starches. Starch lipids, classified as surface and embraced (internal/bound) lipids have many interesting technological properties[36,37]. The presence of high amounts of lipids (and proteins) confer resistance to mechanical damage on the granule surface and also towards amylolysis[38] . The internally bound lipids are exceptionally well protected against chemical and enzymic attacks. Their removal requires prior acid hydrolysis followed by solvent extraction. Usually the mode of lipid interaction is as starch-ester bondage, as polar adsorption or as inclusion complex[39] . Nevertheless, the existence of amylose-lipid complex in the native state has not been unequivocally established-, although

the complex formation is inferred during starch processing steps.[40] Cereal starches, than others, contain appreciable amounts of lipids, and nutritionally they are beneficial as they provide a considerable proportion of essential fatty acids. This is especially advantageous in Indian and Oriental dietaries as a significant majority of population are in practice, vegetarian, wherein cereals such as wheat and rice constitute their staple food. It is suggested that the lipid molecules may act as a template for the constitution of the amylose helix[41] . It is also possible that the polar head groups of the included lysophospholipids, akin to those of phospholipid constituents of cell membrane, may orient themselves at the surface and further involve in intermolecular interactions with neighbouring molecules[42] . Thus, the polar head groups contribute to the net surface charge and also provide polar binding sites for the hydrolases released during germination. It appears very likely that these polar "caps" near the non-reducing end of the amylose chain may possibly inhibit the action of the branching enzyme on it, and thereby the amylopectin to amylose ratio in starches is governed. Evidence in favour of this is conceivable by the fact, that amylose in the presence of large amounts of phospholipids is virtually resistant to attack by phosphorylase and other amylases.[42]

Digestibility Characteristics

It is generally known that starch in the native granular form is poorly digested than their gelatinized counterparts. With the former the enzyme attacks from outside inwards (exocorrosion) throughout their structure rather than just at the surface. The characteristics of the action pattern of amylases on raw and gelatinized starch

suspensions have been the subject of numerous investigations[43-46] , and some properties of the enzyme are now well understood. High performance size exclusion chromatography (SE-HPLC) is gaining importance in the study of resulting amylolysis products[47,48]. However, *in vivo* most starches are better hydrolysed than *in vitro* for reasons not clearly understood. The enzyme degradation patterns seen on raw starch granules are multiple (Fig.6), such as surface pitting and erosion, wide and terraced punctures, selective peeling off the surface layers and resulting in characteristic "onion"-type layering[44,51]; multiple large hole tunnelling and some granules resisting any attack. As it is known that the starch granaule is not a static biosynthetic entity, it is plausible that each granule represents an entity of its own and differing in subtle structural-compositional details, which may be detrimental for the enzymatic attack.

Chemistry and Structure

Starch is a macromolecule composed essentially of D-glucopyranose residues. Structurally it is a heteropolymer consisting of a so-called linear (of course, not necessarily linear!) and a branched α-D-glucan, commonly designated as amylose and amylopectin, respectively (Fig.7). Both polysaccharides are α-(-1, 4-linked, but the latter contains, in addition, α-1, 6-branch points, which usually occur at every 20-25 glucose residues apart. There is now much evidence to show that even amylose contains a limited number of long-chain branchings involving α-1,6-linkages [52]. Support for this is deduced by the incomplete β-amylolysis of the amylose with β-amylase. The proportion of amylose varies from one starch to another, and most starches contain between 15 to

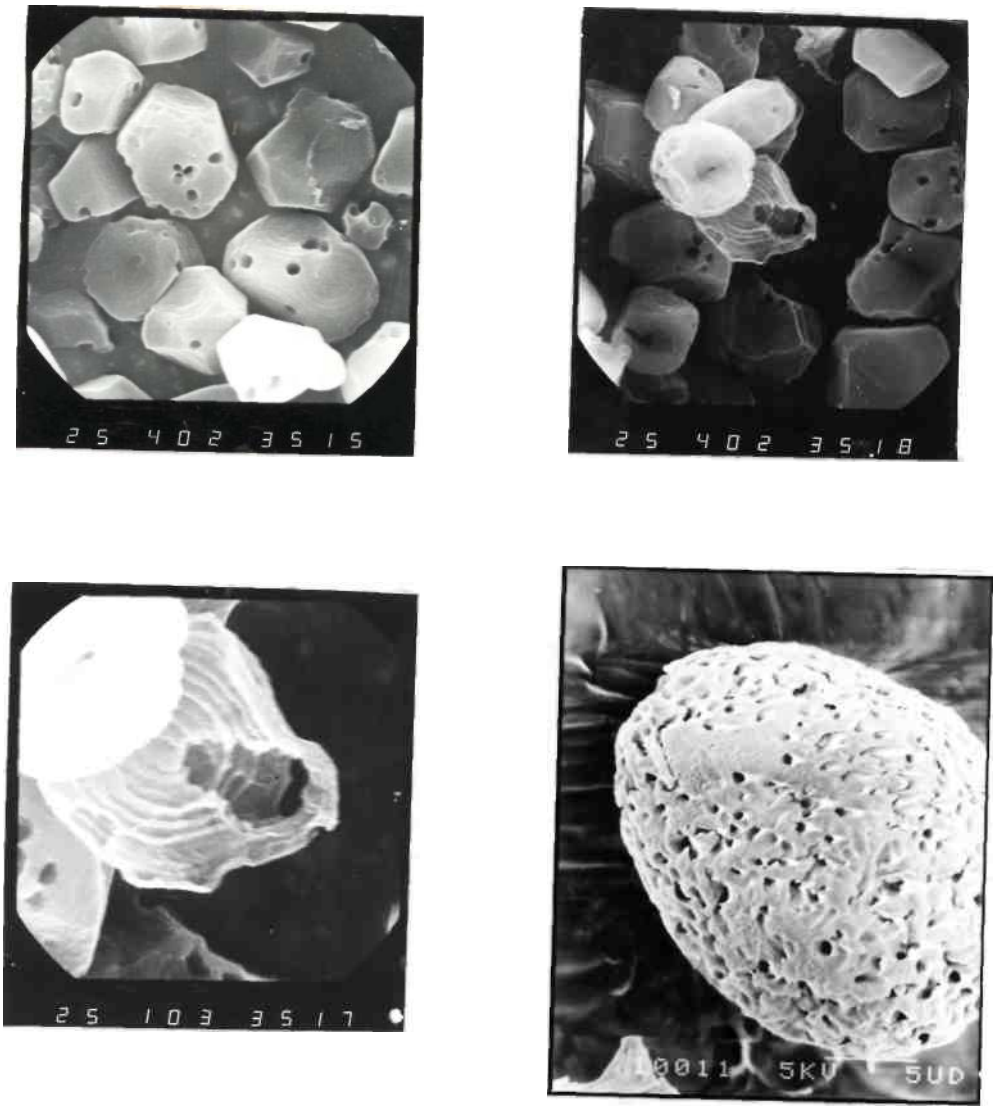
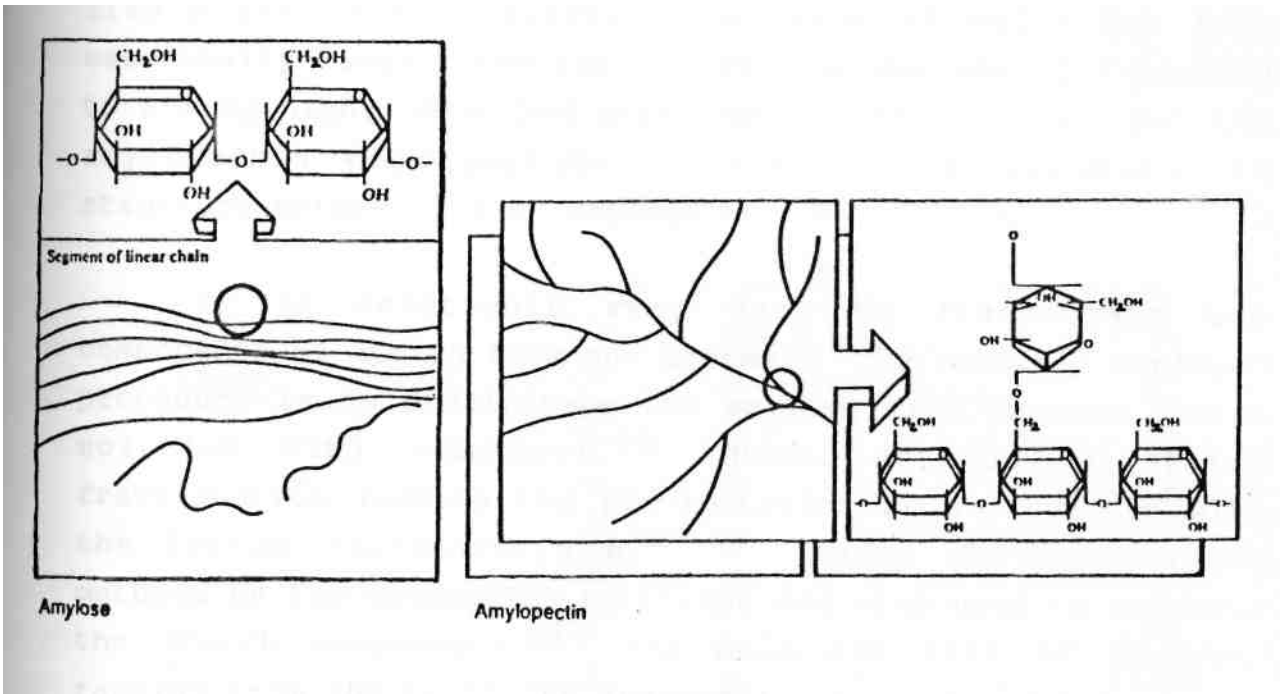


Fig.6. SEM of panivaragu starch granules attacked by, (a) glucoamylase (lOOh); b. salivary oc-amylase (100 min); c same as b, enlarged; and d. jn vivo digested horsegram starch granules isolated from the small intestine.

* Source: El Faki et^aL, Lebensm,W. Technol., 17(1983} 276 Bhat et a!., Staerke, 35 (1983) 261.

I am highly indebted, to Dr R.N.Tharanathan for providing me the original SEM photographs of their published data. Editor, Staerke, is thanked for permission.

Fig.7. Structural representation of linear ('amylose') and branched ('amylopectin') molecules of starch.



25% amylose[9]. However, some inbred varieties such as amylomaize starch possesses upto 85% amylose, whereas in waxy maize starch its content is as little as < 1%.

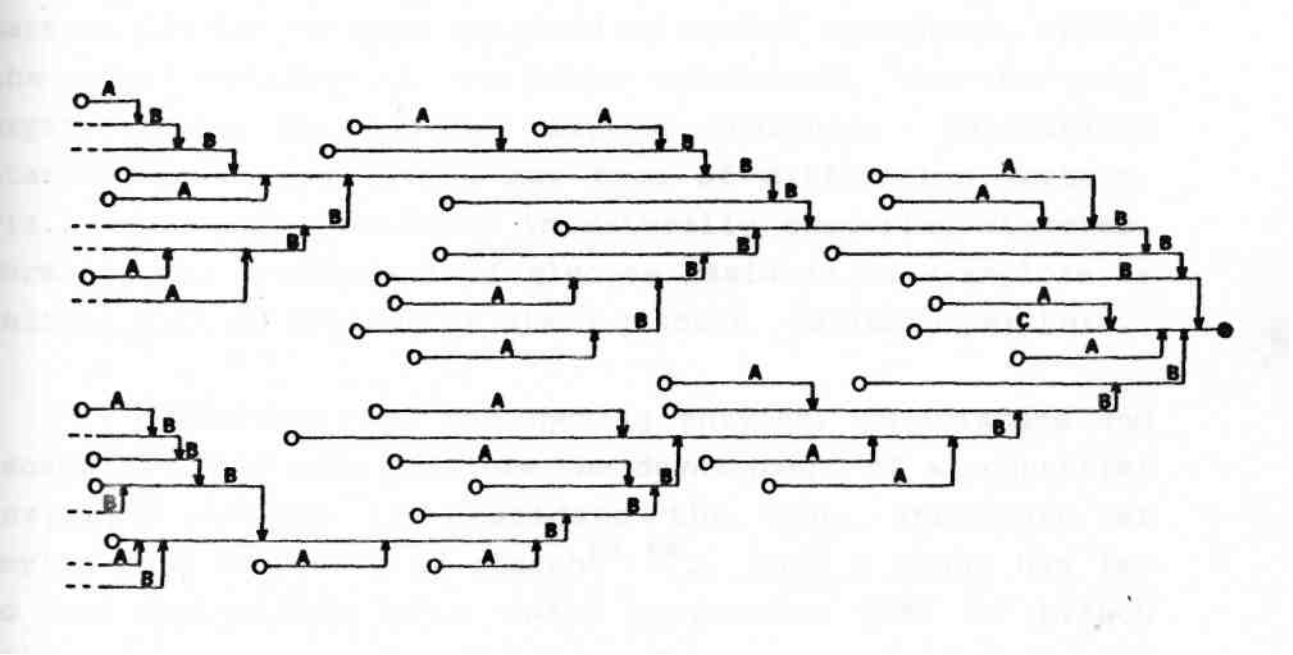
A fundamental study of starch necessiates fractionation of the constituent molecules. In addition to the classical amylose and amylopectin fractions, which constitute the bulk[53] ; starches from many other sources contain a third fraction, called intermediate or anamolous fraction (5-35%), which has some degree of short chain branching[54]. It is likely that starch exists as a range of molecules from essentially linear ones through various degrees of branching to a very highly branched amylopectin. It is shown that the amylopectin from amylomaize starch is intermediate in structure between "true" amylose and amylopectin[55].

It is relatively very easy to fractionate the components of starch from one another. The commonly employed procedure is to precipitate the amylose from aqueous starch solution with 1-butanol.[56] Newer methods of starch fractionation include the precipitation of amylopectin with the lectin concanavalin A.[57,58] Paper chromatographic methods by the descending technique are also used to separate the starch components.[59] The molecular size of amylose, ranging from 200 to 10,000 anhydroglucose units, is dependent on the starch source.

Amylopectin is the structure-ordering component of starch. It is highly branched and has a molecular weight between 10 to 500 million. The elucidation of the fine structure of amylopectin is still a topic of research at present. Among the several models proposed, the cluster

model of amylopectin put forth by French[60] and Robin et *al*[61]., has been substantiated by extensive enzyme debranching studies[62] . The results indicate a bimodal distribution of linear chains, short chains composed of 17-19 glucose residues (A-chains) and long-chains of 50-60 glucose residues (B-chains). In other words, A-chains are unsubstituted linear chains, whereas B-chains are substituted at one or several 0-6 groups by additional A-chains. The determination of the ratio of A- to B-chains, though very difficult, is very essential to understand the multiplicity of branching in the molecule. The A:B-chain ratio is usually determined by the amount of maltose and maltotriose liberated from β-amylolysis limit dextrin by the action of pullulanase. The A:B-chain ratio of amylopectin lies within the range of 1:1 to 1.5:1. The presently accepted cluster model structure of amylopectin is shown in Fig.8. The average DP of A-chains is assumed to be 15, i.e., 60A length, which results in a cluster diameter of around 100A, and as such the molecule measures 1,200-4000A. From the microscopic studies the occurrence of concentric layers, usually referred to as growth rings, which are composed of stacks of lamellae having both crystalline and amorphous regions is evident. The crystalline region is 50A thick and the amorphous region is 20A in the cluster model and further a minimum chain length of 10 glucose units is required for this model. In the starch granule the amylopectin, particularly the highly hydrogen-bonded bundles constitute the crystalline region, whereas the space between the clusters is being occupied by amylose molecules. It is observed that on amylolysis the enzyme attack initially takes place in the more open/accessible regions (amorphous regions) where the molecules are less densely spaced, followed by • a slower hydrolysis of the more resistant clusters. Nevertheless, the

Fig.8. Cluster model of amylopectin, o = nonreducing end; • = reducing end; f and * = oc-D-(l —► 6>-Unkage; — = [(1— ► 4>-oc-D-Glc 1



available evidence indicate that the amylopectins from normal and waxy maize starches differ considerably in their fine structure .

Due to crystallinity starches show characteristic X-ray diffraction patterns, which are useful in assigning the type of starch[2]. Cereal starches give A-pattern, tuber and root starches give B-pattern, whereas legume starches give C-pattern, which is in between the A- and B-patterns. The waxy variety of starch (composed of 100% amylopectin) gives a pattern similar to that obtained by normal starches, unlike the poor diffraction patterns exhibited, due to poor crystallinity, by the high amylose starches. Gelatinized starch suspensions give a new type of diffraction pattern, viz., V-pattern, not found in naturally occurring starches. Normally the arrangement of glucose residues in V-amylose is helical with an average of six D-glucose residues per turn.

The discovery of debranching enzymes, pullulanase and isoamylase, has made possible the development of a sequential enzymatic method to elucidate the fine structure of amylopectin component of starch[63,64] . Such a study has led to the recognition of a small proportion (1%) of branch points in the amylose molecule. This was supported by the incomplete p-amylolysis of the so called "linear" amylose fraction. Nevertheless, considerable amount of ambiguities still project in the various models, particularly with regard to the multiple branching phenomenon proposed for the amylopectin molecule. Methods are yet to be designed for selective cut-off of the A- and B-chains by the use of debranching enzymes, which can be later analysed for degree of polymerization (DP) and chain length (CL) values. The DP > CL in most of the amylose samples and DP »> CL for most of

the amylopectin. It is not clear whether the side chain branches of amylopectin are extended outward to form the limiting boundary of the granule.

Modified Starches

Starch is valued largely because of its contribution to the texture of the food. The starches can be pretreated, by chemical and enzymic means, to alter its properties in a predictable fashion[65]. The modifications are designed to change the gelatinization characteristics, gelling tendency, resistance to breakdown in viscosity by acids, mechanical shear, etc. A variety of chemically modified starches (see Fig.9) is used in both food and non-food industries. Cross-

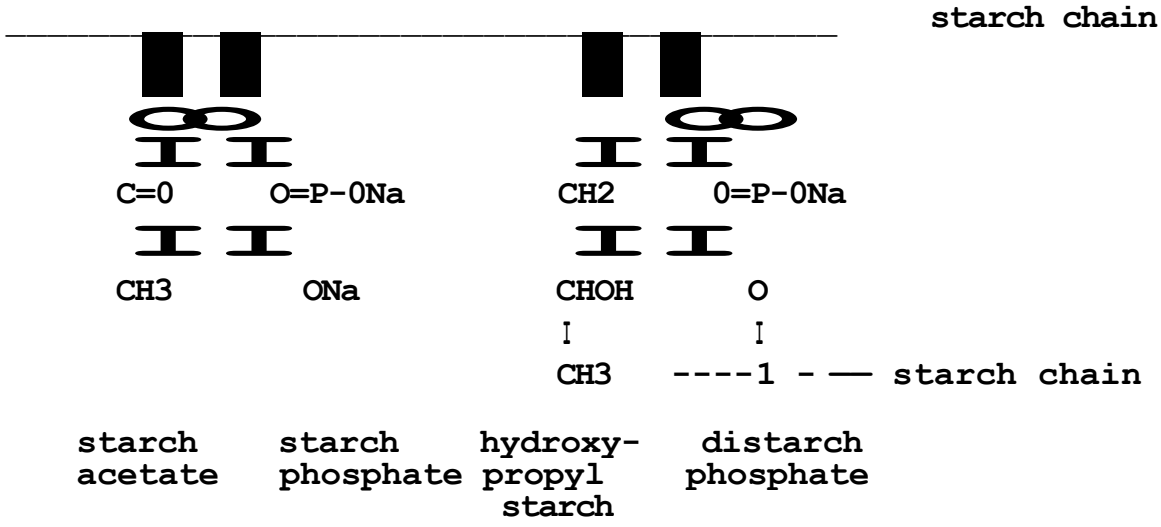


Fig.9. Schematic formulae of chemically modified starches.

bonded starches (by reacting with epichlorohydrin, POCI3, etc.) have greatly reduced swelling characteristics and find extensive use where stability at high temperatures is required. For example, a cross-bonding of 5 to 7 per 10,000 anhydroglucose units is used for making salad dressings, which contain a large amount of vinegar. Oxidised (using

NaOCl) starches, having low viscosity and low GT, are mainly used in paper industry for coating and surface sizing. These property changes are essentially dependent on the degree of modification and distribution of the substituent groups along the polymer chains.

In the light of declining oil supplies it is likely that starch may have a new future as a fermentable substrate in the alcohol-for-fuel industry, which may overtake its present role in the alcohol-for-drinks industry. Of late, thermoplastic starches are being manufactured which are biodegradable and therefore are environmentally friendly[66] .

Origin and Scope of the Present Investigation

Starchy foods have always been a dietary item of humans. Legumes and cereals constitute a major share of carbohydrates, both available (starch) and unavailable (dietry fibre), in any vegetarian diet. Inclusion of a wide variety of pulses in vegetarian diets is essential to ensure adequate dietary protein intake amongst such populations. The total carbohydrate content reported ranges from 65-80% for cereals and 55-65% for legumes. Starch is the major dietary carbohydrate. There is a popular belief that legume-based foods, in particular are less digestible and that their consumption leads to flatulence and other physiological discomforts[67]. From literature survey as well as from earlier findings from this laboratory, it is known that the digestibility, both *in vivo* and *in vitro,* of legume starches is lower than that of cereal starches, whether in the native or gelatinized form. Starches from different sources exhibit varied physico-chemical and functionality characteristics, and it is very likely that the differences in digestibility

could be attributed to subtle variations in the qualitative and quantitative makeup of starch per se. To a certain extent these variations are also manifested, in part in the wide morphological characteristics of starches. Antinutritional factors, such as saponins-tannins, glycosides-alkaloids, conjugates of protein with phytin-hemicellulose, and lectins, the haemagglutinating proteins, are also contributory to the poor digestibility of legumes as a whole[68,69]. As a result they exhibit low glycemic and insulin responses in both normal and diabetics. Substances like polyphenols and phytic acid are likely to retain inhibitory properties (for amylases) even after heat treatment of the food.

It was observed that during starch isolation steps the small starch granules, which constitute > 10% of total starch recovered, due to their poor settling characteristics would invariably be, lost. Because of the presence of finely hydrated fibre (both cellulosic and hemicellulosic) fraction the small starch granules may get associated with the protein matrix (during the NaCl-toluene step of purification) and later discarded as the supernatant[70] . The chemical nature as well as the *in vitro* digestibility profiles of these various starch isolates from legumes/cereals are not clearly known at the moment. It was therefore felt desirable to carry out a detailed systematic study of legume and cereal starches to understand, at a molecular level the precise scientific reasoning for their differences in the chemical nature and digestibility values. The proposed plan of work included the following:

1. Isolation and purification of starch granules of varying size and shape characteristics from two legume (Bengalgram and greengram) and two cereal (rice and ragi) sources.

2. Evaluation of the proximate makeup and some physico-chemical characteristics of different starch granule populations.

3. Rigorous fractionation of Bengalgram, greengram, rice and ragi starch isolates by the classical and modern methods and establishment of their homogeneity.

4. Enzyme debranching studies on the pure fractions and assessment of their DP, CL and molecular weight values.

5. *In vitro* digestibility profiles of native and gelatinized starch suspensions, and

6. Correlation of the (physico-) chemical - structural characteristics with the digestibility values.

The results indeed have shown a difinite and positive correlation between the starch digestibility and its chemical/structural makeup. The latter is supported by the results of enzyme debranching studies on pure homogeneous fractions.

MATERIALS AND METHODS

Chemicals

All the chemicals, organic solvents and acids used were of analytical grade. Sugar, fatty acid and amino acid standards, bovine serum albumin (99.9% pure), sodium borohydride, O-dianisidine, Tris (tris-hydroxymethyl aminomethane), Triton X-100, concanavalin A, tocopherol acetate, phytic acid, Coomassie Brilliant blue, acrylamide and N,N,N'N'-tetra-methyl ethylene diamine (TEMED) were purchased from Sigma Chemical Company, St. Louis, M.O., USA; 3% OV-225 on Chromosorb W (HP) , 80-100 mesh and 10% DEGS on Chromosorb W, 100-120 mesh and phenylisothiocyanate (PITC) were obtained from the Pierce Chemical company, Rockford, IL, USA; DMSO (99.5% pure, 0.04% H_20) of UV spectroscopic grade was from Spectrochem, Pvt. Ltd., Bombay, India. Enzymes such as glucoamylase (E.C. No. 3.2.1.2, 10 units/mg solid), *oc*~ amylase (E.C.No. 3.2.1.1, 695 units/mg solid), β-amylase (E.C.No. 3.2.1.2, 10 units/mg solid), pullulanase (E.C.No. 3.2.1.41, 35 units/0.58 ml), isoamylase (E.CNo. 3.2.1.68, 18,400 units/0.023 ml), glucose oxidase (E.CNo. 1.1.3.4, 19 units/mg solid) and peroxidase (E.CNo. 1.11.1.7, 100 units/mg solid) were procured from Sigma Chemical Co., USA. Sepharose CL-2B, Biogel P-10 and standard dextrans (T-10, T-20, T-40, T-70, T-500 and T-2000) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; PICO-TAG columns and SE-HPLC columns µ-Bondagel E-linear and E-1000 were from Waters Associates, Milford, USA; N,N'-methylene bisacrylamide (Bis-acrylamide) from Koch-Light laboratories, Coin-Brook, Bucks, England; and sodium dodecyl sulfate from Hindustan Lever Ltd., Bombay, India.

Raw Materials

Locally available vareities of chickpea (Bengalgram, *Cicer arietínum)* , mung bean (greengram, *Phaseolvs aureus)* cotyledons, rice *(Oryza sativa)* and ragi (finger millet, *Eleucíne coracana)* were purchased in the market. After cleaning they were thoroughly dried in the Sun and were finely ground in a standard plate mill to pass through a 60 mesh sieve. The 60-mesh flour was then passed through 170 mesh sieve and the final material was designated as crude "prime" starch.

General

1. The solvents were distilled using all glass apparatus before use. HPLC solvents were triple distilled, degassed and filtered through 2 µ millipore membranes.

2. All preparations, estimations and experiments were done using deionised double glass distilled water.

3. Samples were concentrated in a Buchi rotavapor RE-120 at 40° and at reduced pressure.

4. Solutions/suspensions were centrifuged using either HERMLE-Z 320 K refrigerated or Sigma 202-C bench top centrifuges.

5. Samples were incubated with enzymes in a shaking Julabo SW -20C incubator.

6. Samples were weighed in a Mettler AE-100 digital balance.

7. Samples were lyophilized in a Virtis freeze mobile 12 lyophilizer at -60° and 15 μ vacuum.

8. The O.D. values were read in either a Beckman (model 26 or DU-7) or Shimadzu UV 160 A or Spekol spectrophotometer.

9. Dry solid samples were sonicated for their efficient dissolution using Julabo USR I sonicator.

10. All enzymes were heat inactivated after digestion by placing in a boiling water bath for 10 min.

11. In all enzymatic hydrolysis blanks were prepared without the enzymes.

12. During enzymolysis, the surface of each preparation was covered with toluene to prevent external microbial growth.

Starch Isolation[33]

Starch from the -170 mesh flour (2 kg) was isolated by steeping in water (10 L) in the presence of $HgCl_2$ (0.1 M, 20ml) for 12 h and passing through 240 mesh sieve. The filtrate containing the crude starch was suspended in dilute NaOH (pH 9.0) for 10 min followed by neutralization with dilute HC1 to pH 7.0 and centrifugation. Further purification was done by repeated washings with 0.1 M NaCl-toluene (10:1, v/v) . After a brief centrifugation. (2000 rpm for 2 min) the floating scum layer (starch-protein-toluene complex, positive for KI-I2 solution) was removed by

centrifugation (4000 rpm for 5 min) and designated as isolate IV. The supernatant (positive again for KI-I2 solution) contained very small starch granules with poor settling characteristics and were removed by high speed centrifugation (10,000 rpm for 10 min) and designated as "isolate III". The firm starch sediment at the bottom contained an upper soft layer designated as "isolate II" and a lower hard layer designated as 'isolate I". All the four starch isolates (I to IV) were thoroughly washed with distilled water, ethanol (X 4), acetone (X 2), ether and finally air dried.

Microscopic Studies

Carl-Zeiss photomicroscope was used to determine the size (average diameter,µm) shape, and distribution of granules under ordinary light. Birefringence characteristics of starch granules in different isolates were observed at X 100 magnification with the help of polarized lenses attached.

Unless otherwise stated, a 0.1% aqueous suspension of starch was used.

Ionic Nature of Starches

To the starch granules (10 mg), suspended in water (5 ml) was added cationic (Safranine and methylene green) and anionic (Orange G and Fuchsin acid) dyes (10 mg) seperately and left shaking for about 20 min at room temperature. Excess dye was removed by a brief centrifugation and the granules were repeatedly washed with water till the washings were colourless, and finally observed under the light microscope.

Moisture Content [72,73]

Moisture content of the starch (2 g taken in a preweighed porcelain crucible) was determined by drying to constant weight in an oven at 105° for 14 h. Loss of weight was taken as the moisture content of the starch sample: % Moisture = Weight loss (g) x 100/Sample weight (g)

Phosphorus Content[74]

Starch (1 g) was weighed into a preweighed silica dish and carbonized, after adding a little ethanol for wetting, over a direct burner flame. After the smoking ceased, the sample was incinerated in a muffle furnace at 550° for 4 h into a white ash, samples not yielding white ash were treated with dil. HNO3 (1:2, 1 ml) and evaporated to dryness on a water bath, returned to furnace and ignited further to white ash. The inner sides of the dish were washed with 29% HNO3 (lml), mixed well, covered and kept at 105° for 30 min for conversion of phosphorus to orthophosphoric acid. The solution together with washings were transferred quantitatively into a 10 ml volumetric flask and the volume was made upto the mark, mixed well and filtered. To an aliquot (3 ml) of this solution (in a 10 ml volumetric flask) were added dil. HNO3 (29%, 1 ml), ammonium vanadate* (0.25%, 1 ml) and ammonium molybdate** (5%, 1 ml) reagents, mixed well and the solution was made upto the mark. The absorbance of the coloured solution was later (after 2 h) read at 4 60 nm against a reagent blank. Standard curve was prepared by dissolving 0.4390 g KH2PO4 in water (1 L) to obtain 10 mg phosphorus per 100 ml.

Ammonium vanadate was prepared by dissolving the compound, 2.5 g in boiling water (600 m1), cooled to 60°, and adding cone. HNO3 (20 ml) and finally diluted to 1 L with water.

Ammonium molybdate was prepared by dissolving the compound (50 g) in warm water (900 ml) and then dilution to 1 L.

$$\% \text{ Phosphorus} = \frac{P \times \text{Dilution volume} \times 100}{\text{Aliquot volume} \times \text{Sample weight in g} \times 1000}$$

$$\% PO_4^{2-} = P \% \times 3.065$$

Fractionation of Starch

I. The Butanol Complexation Method

a. Starch solubilization in alkali[56] **:** An aqueous slurry of starch (1 g in 6.5 ml water) was dissolved in 0.157 N NaOH (82.5 ml, 6.28 g NaOH in 1 L) by stirring for 20 min. 5% NaCl (22.8 ml) was added with continued stirring (5 min) and the contents were neutralized to pH 7.0 with IN HC1. After 16 h it was centrifuged (10,000 rpm for 5 min) and the supernatant was carefully filtered through a sintered glass G_4 filter. The insoluble residue was processed later for isolation of amylopectin.

Precipitation of amylose : The filtrate was added to excess 1-butanol, stirred for 1 h at room temperture, and the precipitate formed was allowed to settle (3 h). The contents were centrifuged (5000 rpm for 15 min) and the precipitate

was repeatedly washed with water-saturated butanol and centrifuged. The washings/supernatants were discarded. From the precipitated material butanol was removed by nitrogen flushing for 20 min at 40° in a water bath. The material was then freeze dried.

Purification of amylopectin : The residue from above (a) was treated with 1% NaCl (25 ml/g starch) solution, mixed well and left for 20 h at room temperature. The contents were then centrifuged for 15 min at 10,000 rpm and the supernatant was discarded. Washings with NaCl solution followed by 50% ethanol were repeated' (X 3) , and finally the material was suspended in water and freeze dried.

b. **Starch solubilization in aqueous DMSO (85%)**[75]**:** Initially the starch (1 g) was solubilized in aqueous DMSO (85%, 50 ml) by suspending for 24 h. Any insoluble non-starchy material was removed by centrifugation at 5000 rpm for 30 min. To the clear solution was added 1-butanol (2 volumes) and after 1 h the resulting non-granular precipitate (amylose-butanol complex) was centrifuged, and washed with butanol repeatedly to remove all traces of DMSO. The combined supernatants were stored separately.

Purification of amylose : The amylose-butanol complex was dissociated by boiling water (100 ml) for 1 h under oxygen-free conditions. The dispersion was allowed to cool to 60°. 'Excess butanol was added, mixed well and after 24 h centrifuged at 5000 rpm for 30 min. Finally butanol was removed by nitrogen flushing at 40° on a water bath. The residue was then lyophilized.

Purification of amylopectin : The combined supernatants were filtered through a G_4 filter funnel, concentrated to small volume (10 ml) and the polymeric material was precipitated by adding ethanol (3 vol). After evaporating the butanol by nitrogen flushing the amylopectin fraction was lyophilized.

II. **Concanavalin A Precipitation Method**[57]

Preparation of sodium acetate buffer : This was prepared by dissolving anhydrous sodium acetate (4.92 g), sodium chloride (17,55 g) , $CaCl_2$. $2H_20$ (0.05 g) , $MgCl_2 \cdot 6H_20$ (0.07 g) and $MnCl_2 \cdot 4H_20$ (0.07 g) in water (90 ml) and adjusting the pH to 6.4 by adding glacial acetic acid.

Reagent buffer: Reagent buffer was prepared by diluting the sodium acetate buffer (30 ml to 100 ml with water) , just before use.

Concanavalin A solution: Concanavalin A (600 mg) was dissolved in reagent buffer (100 ml) just prior to use.

Starch solution: Starch (1 g) was dispersed in DMSO (85%, 50 ml), as before. The supernatant was mixed with ethanol (3 vol.) and stored for 16 h at 4° and centrifuged. The precipitated material was washed with ethanol, acetone, ether and dried.

The ethanol-precipitated starch (250 mg) was wetted with DMSO (85%, 2 ml) in a 250 ml volumetric flask, kept for 24 h and then mixed with 0.1 N NaOH (100 ml). After 30 min the mixture was neutralized with dil. acetic acid and the solution was made up to the mark with 0.1 M NaCl. Again to 150 ml of the above solution was added sodium acetate buffer

(75 ml) in a 250 ml volumetric flask and the volume was made up to the mark with water.

Precipitation of amylopectin with concanavalin A : The starch solution was mixed with concanavalin A solution in the ratio 1:1/ occasionally stirred for 2 h and the precipitate formed was centrifuged (10,000 rpm for 15 min). The sediment was dissolved in reagent buffer and the solution was heated in a boiling water bath to denature the protein, cooled and the protein contaminants were removed by $(NH_4)_2SO_4$ precipitation method and finally lyophilized.

Amylose recovery: The supernatant solution was concentrated and the solubles were ethanol precipitated, centrifuged and lyophilized to recover amylose fraction.

Subfractionation of Amylose Fraction[76]

Amylose (1 g) was dissolved in aqueous 10% butanol (100 ml) by heating (40° for 2 h) under nitrogen. The insoluble material was collected by centrifugation (10,000 rpm for 15 min) and dissolved again in 10% aqueous butanol as before. The procedure was repeated thrice. The final insoluble material (B-insol.) was washed with ethanol, ether and dried in vacuo over CaCl2. The combined supernatant solutions were filtered through G_4 filter and concentrated to small volume at < 40°. Excess butanol was removed by nitrogen flushing and the solution (B-sol.) was freeze dried. The yields of B-insol. and B-sol. were noted.

Total Carbohydrate

a. Phenol-H_2SO_4 method[77] : Aliquots (0.5 ml) of the fractions or sample solutions were taken in test tubes, 5% phenol (0.3 ml) was added and mixed well. Cone. **H_2SO_4** (1.8 ml, Sp. gr. 1.84) was added rapidly on to the liquid to obtain good mixing. The tubes were cooled to room temperature and the absorbance of the characteristic orange-yellow colour was read at 480 nm against a reagent blank.

b. **Modified Phenol-H_2SO_4 method**[78] : To the sample aliquot (0.5 ml) was added cone. **H_2SO_4** (1.8 ml) and mixed well. The tubes were cooled in ice bath for 2 min and 5% phenol was added (0.3 ml), shaken well and cooled to room temperature. The colour formed was read at 480 nm against a reagent blank.

The carbohydrate content, determined by reference to a standard glucose curve in the concentration range 5-25 µg, was multiplied by a factor 0.9 to express the starch content.

Reducing Sugar by Nelson-Somogyi Method [79]

Preparation of reagents; Mixed copper reagent was prepared by mixing 25 parts of reagent A [anhydrous Na_2CO_3 (25 g) , Rochelle salt (25 g) , $NaHCO_3$ (20 g) and anhydrous Na2SC>4 (200 g) dissolved in 1 L water] with one part of reagent B [$CuSO_4.5H_20$ (15 g) dissolved in 10 ml water containing a few drops of conc. **H_2SO_4**].

Arsenomolybdate reagent was prepared by dissolving ammonium molybdate (25 g) in water (450 ml) containing conc.

H_2SO_4 (21 ml) and Na_2HAsO_4 . $7H_2O$ (3 g in 25 ml water). The contents were incubated for 48 h at 37° and stored in dark brown bottles.

To an aliquot (1 ml, 50-500 µg of reducing sugar) of the sample was added the mixed copper reagent (1 ml) and the contents were heated in a boiling water bath for 20 min. To the cooled contents arsenomolybdate reagent (1 ml) was added and diluted to 25 ml with water. The O.D. was measured at 520 nm against the reagent blank. Standard curve was prepared by using either D-glucose or maltose.

Glucose by Glucose oxidase method[80]

Tris-glucose oxidase reagent: Glucose oxidase (2000 units, 125 mg) was taken in a 100 ml standard flask and 50 ml of Tris buffer [61 g of Tris dissolved in 5 N HC1 (85 ml) was diluted to 1 L with water and the pH was adjusted to 7.0] was added and mixed well. Peroxidase, (0.5 ml of 0.1% aqueous solution), O-dianisidine (0.5 ml; 10 mg/ml in 95% ethanol) and Triton X-100 (1 ml; 10 ml detergent in 40 ml ethanol) were added. The volume was made up to the mark with Tris buffer, filtered and used.

Aliquot (0.5 ml) of the sample, digested with glucoamylase/α-amylase was mixed with Tris-glucose oxidase reagent (3 ml) and incubated at 37° for 60 min. The reaction was stopped by placing the tubes in boiling water for 5 min. The purple colour developed was read at 420 nm against a reagent, blank. Standard curve was prepared by using D-glucose (10-50 µg) .

Amylose Content

a. McCready and Hassid method[81] **:** Starch (50 mg) was suspended in water (5 ml) containing 1 N NaOH (5 ml) . The contents were heated for 10 min, cooled, neutralized with IN HC1 (5 ml) and the volume was made up to 50 ml with water.

To an aliquot (5 ml equivalent to 5 mg) from the above was added iodine solution (0.1 ml of 0.2 % I2 and 2% KI in water) and the blue colour developed was read at 630 nm. Corn amylose (10 mg) was used as the standard.

b. **Joseph Chrastil method**[82] **:** Starch (20 mg) was suspended in water (4 ml) containing 1 N NaOH (2 ml) and the contents were heated for 30 min. An aliquot (0.1 ml) of starch solution was mixed with 0.5% TCA (5 ml), vortexed, and iodine reagent (0.01 N, 1.27 g of I2 and 3 g of KI in 1 L water) was added, and the blue colour formed was read after 30 min at 620 nm. Standard solutions of amylose and amylopectin (0-100%) were used for calibration.

Blue Value and Absorption Spectra

The absorbance of the blue colour produced in aqueous solutions of amylose on the addition of tri-iodide ion was studied according to the procedure of Gilbert and Spragg [83]. The blue value was determined by measuring the absorbance at 680 nm, according to the formula, B.V. = (Absorbance x 4)/C, where C (in mg/dl) , is the carbohydrate content of starch on dry basis.

Protein Content

a. Lowry method[84]: The sample solution (1 ml, 2% starch in 0.1 N NaOH) was mixed with 5 ml of mixed copper reagent (25 parts of 2% Na_2C0_3 in 0.1 N NaOH + 1 part of 0.5% CUSO4 . $5H_20$ in 1% Rochelle salt) and after 10 min the diluted Folin-Ciocalteu reagent (1:1/ 0.5 ml) was added and allowed to stand for 30 min. The blue colour was read at 660 nm. A standard curve was prepared using bovine serum albumin (10-100 ug/ml) .

b. **Micro-Kjeldahl method[85]** : The sample was digested in an automatic Büchi-430 digester and analyzer 322/342/645 provided with on-line titration and calculation systems. Protein content was calculated by N x 6.25.

Lipid Content

a. Extraction of surface lipids[86]: Starch (lg) was treated with water-saturated butanol (50 ml) containing tocopherol acetate (0.5 ml, 0.1 M solution) and left stirring at room temperature for 20 h followed by filtration through Whatman No.l filter circle. The residue on the filter circle was washed with alcohol and ether to dryness. The filtrate was carefully evaporated in a preweighed beaker, desiccated for 24 h and weighed.

b. **Extraction of internal lipids[86]:** The residue (0.5 g) from above was taken in 6 N HC1 (10 ml) and refluxed at

* Dr G.Changala Reddy of this Laboratory is thankfully acknowledged for help with the preparation of the reagent.

100° for 2 h in a vacuum sealed tube. After hydrolysis the liberated covalently bound lipids were repeatedly extracted with petroleum ether-solvent ether (1:1, v/v) mixture. The combined extracts were dried (anhydrous $Na2S0_4$) and evaporated to dryness. It was then transferred to a pre-weighed 5 ml beaker with chloroform, nitrogen flushed and desiccated.

Esterification of Lipids[87,88]

Methanolic-HCl (4M) was prepared by carefully adding acetyl chloride (1 ml) , with gentle stirring, to previously cooled methanol (19 ml) . To the surface and internal lipid fractions was added methanolic-HCl (2 ml) and the sealed tubes were refluxed at 100° for 3 h. Fatty acid methyl esters were extracted with n-hexane (X 5) , dried (anhydrous Na_2S0_4), concentrated (N_2) and analysed by GLC.

Viscosity Studies

Relative viscosity (η_r)[89] • The relative viscosity, η_r, of starch solutions in 1 M KOH at 0.5% concentration was measured at 25±0.5° in an Ostwald viscometer, $\eta_r = t_s/t_0$, where t_s and t_0 are the flow times for starch solution and solvent, respectively.

Limiting viscosity number [η] [90] : Limiting viscosity number [tj] was determined by extrapolation to zero concentration from a plot of reduced viscosity (**η** sp/c) *vs* concentration.

Brabender hot paste viscosity[91]

This was determined using the Brabender viskograph model E attached with temperature/torque programmer and auto-recorder fitted with 700 Cmg sensitivity cartridge. The following conditions were kept constant : torque speed, 75 rpm, thermoregulator setting at beginning of heating 40°, rate of heating as well as cooling 1.5°/min; highest temperature to which the slurry was heated 95°; holding time 20 min at 95°; and temperature to which finally cooled 50°. A 7% aqueous starch suspension was used and the viscosity in Brabender units (B-.U.) was recorded throughout the temperature range of gelatinization, holding as well as cooling. Chart speed was adjusted to 30 cm h-1

Swelling and Solubility

a. In Water[92] : Starch (2 g) , suspended in water (200 ml) was gelatinized by heating (with constant stirring) at different temperatures (50 to 95°) for 30 min. At each temperature interval contents were centrifuged (2,000 rpm for 15 min), the clear supernatant was carefully drawn off by suction into a porcelain dish and evaporated to dryness on a steam bath followed by vacuum oven drying at 105° for 4 h and weighed. The insoluble gelatinized residue was also weighed. The percentage of solubles and swelling power was calculated as mentioned below.

$$\% \text{ Solubility (on d.b.)} = \frac{\text{Weight of soluble starch}}{\text{Weight of starch (g)}} \text{r} \times 100$$

$$\text{Swelling power (corrected)} = \frac{\text{Weight of residue}}{\text{Weight of starch (100 - \% solubles)}} \times 100$$

b. **In DMSO[93]** : Starch (250 mg) was dispersed in DMSO (50 ml) in a stoppered 100 ml conical flask and placed in a shaker (100 rpm) to keep the starch granules continuously suspended. At regular time intervals (0, 2, 6, 12, 24, 36, 48, 60 and 72 h) an aliquot (5 ml) was removed and centrifuged (5000 rpm for 15 min). The supernatant was analysed for total carbohydrate by the modified phenol-H2SO4 method[79] . A graph was plotted with time (h) *vs* % digestion.

Acid Hydrolysis[94]

Starch and its fractions were hydrolysed with 1 N H2SO4 at 100° for 4-6 h followed by neutralization with solid $BaCO_3$, deionization with Amberlite IR-120 (H^+) resin and concentration under reduced pressure. Sugars in the hydrolysates were resolved by PC and GLC of the derived alditol acetates.

Chromatography

Paper Chromatography (PC)

This was done by the descending method using Whatman No.l filter paper sheets. A definite amount (usually 5 µl containing 50 µg of sugars) of the neutralized hydrolysate was spotted with the help of a micro syringe/capillary tube, dried and irrigated with n-propanolrethanolrwater (7:1:2, v/v/v) as solvent system. Glucose, maltose and maltotriose were the standards used.

Staining[95]**:** Sugars on the paper chromatogram were visualized by staining with aniline phthalate reagent, prepared by dissolving phthalic acid (1.66 g) in water-saturated butanol (100 ml) and aniline (1 ml) . The reagent was sprayed on the dry chromatogram by the "dip" method, air dried and finally dried at 110° for 5 min.

Gas Liquid Chromatography (GLC)

A. Preparation of alditol acetates[96]**:** The starch hydrolysate after neutralization, deionization (Amberlite IR-120 H^+) and concentration was mixed with sodium carbonate (0.1 M, 0.5 ml). After 30 min at room temperature, the contents were reduced with sodium borohydride (10 mg) overnight. Dilute acetic acid was added dropwise to destroy the excess borohydride and the liberated boric acid was removed by co-distillation with methanol (1 ml, x 5) . The derived sugar alcohols were O-acetylated by adding pyridine-acetic anhydride (0.5 ml, 1:1) and heating in a boiling water bath for 1 h. The alditol acetates formed were evaporated with water and toluene (1 ml, twice each) to remove the excess reagents. The derivatives were taken in chloroform, filtered (glass wool) and used for GLC analysis.

B. Operating conditions: For alditol acetates GLC was done in a Packard model 437 GC fitted with a flame ionization detector and a 5 ft x 1/8 inch stainless steel column containing 3% OV-225 on Chromosorb W (HP, 80-100 mesh). The column was operated by the isothermal mode at 190°. The injector and detector port temperatures were 230° and 250°, respectively, and nitrogen was the carrier gas' used (15 ml/min). The quantitation of the resolved components was done by the. attached Packard model 604 recording data

processor. Myoinositol was used as an internal standard. A typical gas chromatogram of standard alditol acetates is shown in Fig.10.

For fatty acid methyl esters (FAME)[88] the column (ss, 6 ft x 1/8 inch) used was DEGS, 10% on Chromosorb W (100-120 mesh) operating at 180° isothermal. The injector and detector port temperatures were 250° and nitrogen was the carrier gas used (15 ml/min).

Gel Permeation Chromatography (GPC)[97,98]

Sepharose CL-2B was packed into a glass column (1.7 x 92 cm) and equilibrated with the running eluent overnight. The sample (amylose, amylopectin or the intermediate fraction, 10 mg) , dispersed in 85% DMSO (2 ml) , was applied over the column bed and eluted by the descending method with water containing 0.02% sodium azide, at a constant flow rate (18 ml/h). Fractions (1.5 ml) were collected and an aliquot (0.2 ml) of the fraction was analysed for total sugar as well as for starch-iodine blue colour O.D. at 630 nm.

Molecular Weight Determination^0

The approximate molecular weight (MW) of the starch fractions was determined from a calibration curve prepared for standard dextrans (T-10, T-20, T-40, T-70, T-500 and T-2000) of known molecular weight on the same GPC column. The void volume (V_0) was determined by using a predialyzed blue dextran (5 mg/0.5 ml water). The molecular weight values were computed from the standard plot (Fig. 11) of logMW *vs* $V_e/V"_0$, where V_e was the elution volume of the respective fractions.

Fig. 10. GLC of standard alditol acetates.

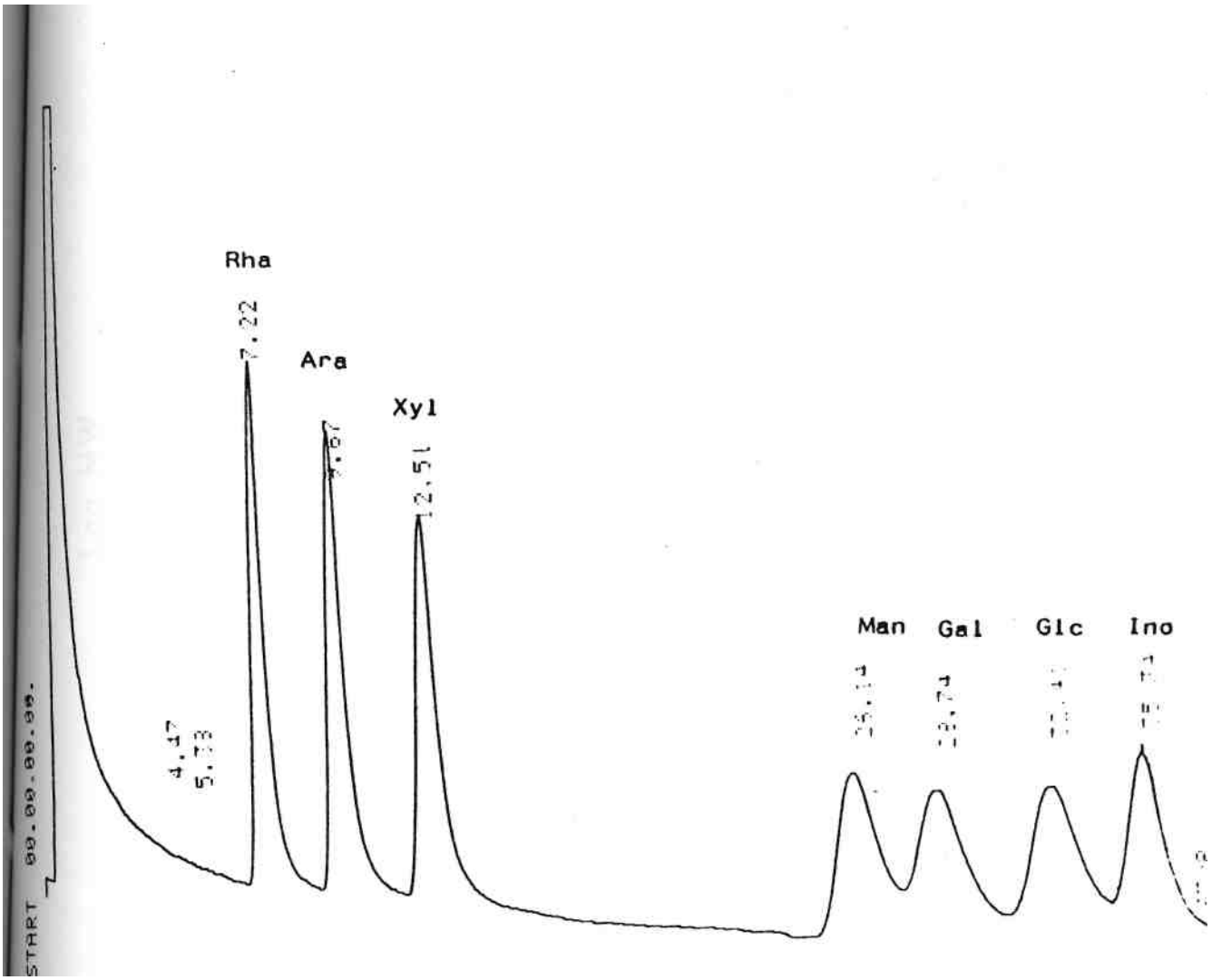
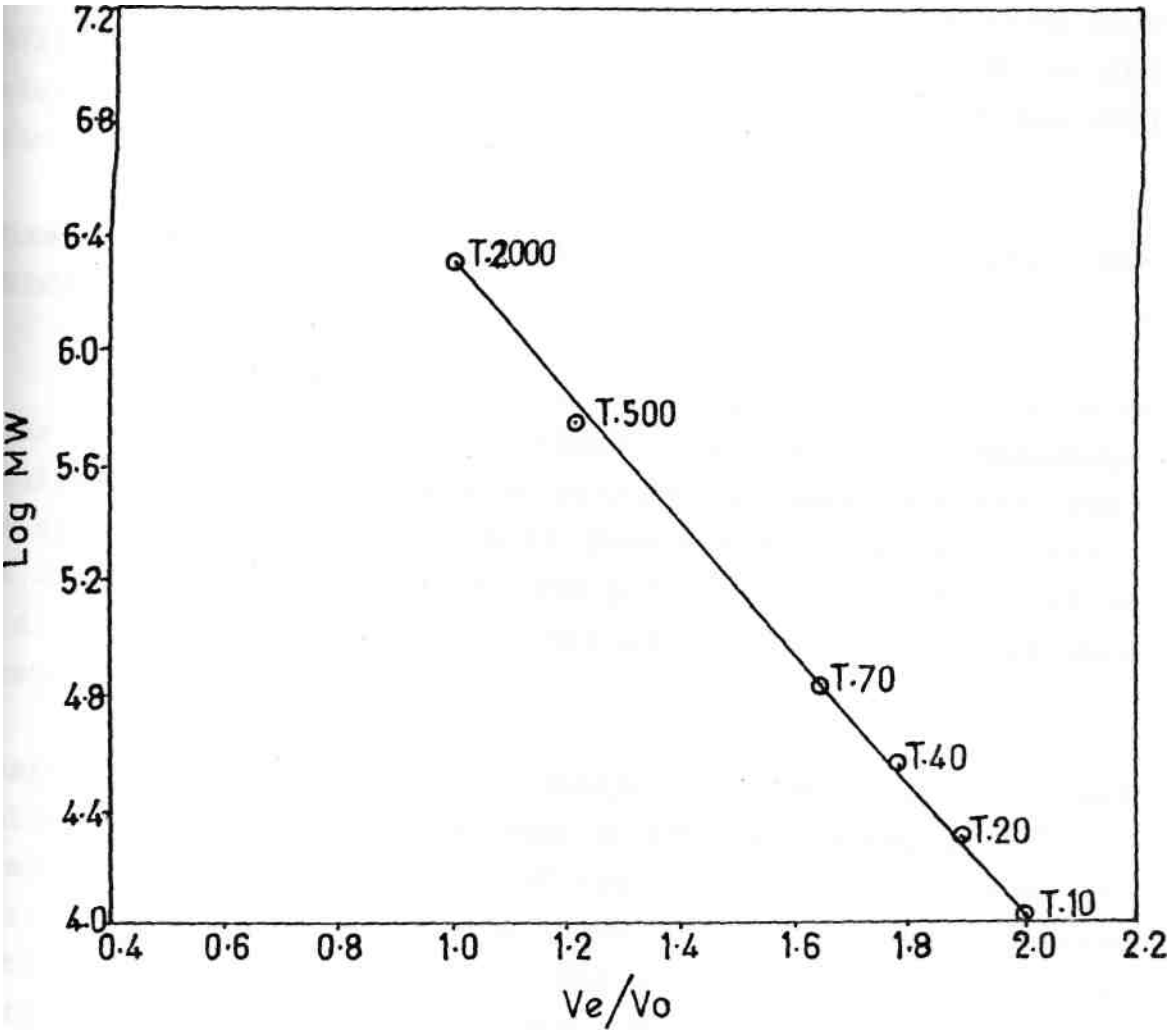


Fig. 11. Calibration curve for the MW determination by GPC on Sepharose CL-2B.



Fractionation of β-Limit Dextrins/Debranched Polysaccharides

The sample, digested either with β-amylase or pullulanase/isoamylase was applied on a Biogel P-10 column (1.7 x 80 cm) . The column was run in the descending mode with distilled water containing 0.02% sodium azide as the eluent (15 ml/h) and the fractions (3 ml each) were analysed for total[78] and reducing sugar[79] contents.

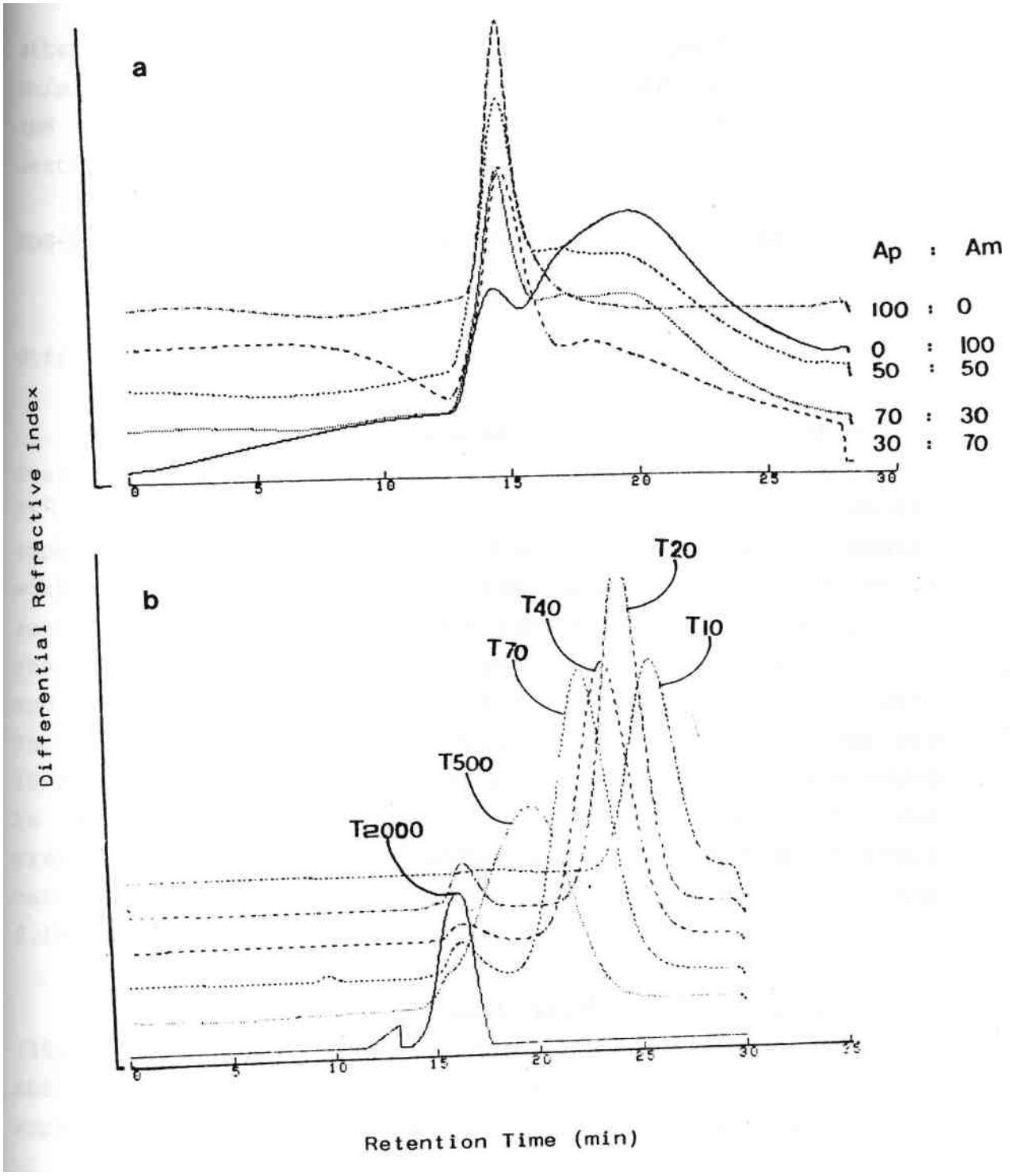
Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)[48]

Amylose, amylopectin and intermediate fractions were also analysed by SE-HPLC in Shimadzu HIC-6A ion chromatograph equipped with Shimadzu RID-6A refractive index detector, SCL-6A system controller and CR-4A Chromatopac integrator units. As columns, E-linear and E-1000 μ-Bondagel (30 cm x 3.9 mm i.d. ss columns) connected in series with a guard column were used.

Sample preparation: The polysaccharide (20 mg) was solubilized in 85% aqueous DMSO (1 ml) by heating at 95° for 5 min. It was then spun (2000 rpm for 5 min) to remove any insolubles, and 10 ul of the clear supernatant was injected into SE-HPLC. Corn amylose and amylopectin were used as reference materials in different ratio, 0:100, 30:70, 50:50, 70:30 and 100:0, respectively, to determine the retention time characteristics (see Fig.12a).

Operating conditions: The columns were eluted with distilled water (filtered and degassed) at a flow rate of 0:2 ml/min. The column temperature was maintained at 40°. Internal pressure was kept at 450 Kgf/cm^2. The refractive index

Fig. 12. SE-HPLC profiles of, a. mixtures of amylose and amylopectiπ and b. standard T-series dextrans.



attennuation was adjusted to 8 and chart speed was set to 5 mm/min. The void volume (V_Q) using Sesbanium mosaic virus (MW = 60,00,000 , 10 mg/ml) and V_e of standard T-series dextrans (Fig.12b) were measured.

SDS-PAGE Electrophoresis of Starch Granule Proteins

The starch granule proteins were extracted by three different methods[99].

a. **Extraction of surface proteins with sodium chloride:** Starch (500 mg) was suspended in 0.1 M NaCl (4 ml) for 2 h at 25° and centrifuged (12,000 rpm for 15 min) to obtain supernate I and sediment I. The latter was again treated with 0.1 M NaCl (twice) under the same conditions to obtain supernate II, sediment II and supernate III, sediment III, respectively. All the three supernatants were mixed together and acetone (3 vol.) was added to precipitate the protein. The precipitate obtained by centrifugation, (12,000 rpm for 15 min) was washed with water (2 ml) , lyophilized and added to treatment buffer (0.5 ml). The treatment buffer was prepared using 2-mercaptoethanol (1 ml), 0.5 M Tris-HCl buffer (2.5 ml, pH 6.8), 10% SDS (4 ml), glycerol (2 ml) and 0.1% bromophenol blue (0.5 ml).

b. **Extraction of residual surface proteins with SDS (1%, W/V)** : The sediment III from above was digested with 1% SDS (4 ml) at 50° for 30 min and centrifuged to obtain supernate IV and sediment IV. The extraction was repeated to

* A gift from **Dr M.R.N.Murthy** (Molecular Biophysics Unit), Indian Institute of Science, Bangalore.

obtain supernate V and sediment V. The supernatants IV and V were mixed together, the solubles were acetone precipitated, processed and dissolved in treatment buffer as before.

c. **Extraction of internal proteins with 1% SDS:** The sediment V from (b) was dispersed in 0.1 M NaOH (1 ml) for 10 min, neutralized and then mixed with 1% SDS (3 ml) . The •contents were heated at 95° for 30 min and centrifuged to obtain supernatant and sediment. The solubles in the supernatants were precipitated and taken to treatment buffer. The final sediment was discarded.

SDS-PAGE was done with 12.5% separating gel according to the procedure described by Laemmlis[99a]. Electrophoresis was carried out at a constant voltage (100 volts) for 3 h. After run the gel was stained with Coomassie brilliant blue R-250 for 16 h and then destained with several changes of methanol:acetic acidrwater (25:10:65 v/v).

Molecular weights were determined by comparison with proteins of known molecular weight, viz., Bovine serum albumin (MW.66,000), egg white albumin (MW. 43,000), glyceraldehyde-3-phosphate dehydroginase (MW. 36,000), carbonic anhydrase (MW. 29,000), trypsinogen (MW. 24,000) and soyabean trypsin inhibitor (MW. 20,100).

Amino acid analysis by Reverse phase - HPLC[99b]

Amino acids, liberated by vapour phase and hydrolysis with 6N HCl at 110° for 24 h were derivatized with phenylisothiocyanate and separated/quantified by reverse phase HPLC using PICO-TAG columns.

Enzymatic Studies

Glucoamylase Activity[80]

Corn starch (120 mg) was gelatinized with water (3 ml) for 30 min and diluted with sodium acetate buffer (3 ml, 0.1 M, pH 4.6). The suspension was incubated with glucoamylase (0.4 ml, 40 units) for 15 min at 60°. The reaction was stopped by keeping it in a boiling water bath for 10 min. The supernatant.obtained after centrifugation (5000 rpm for 15 min) was made up to 8 ml and analysed for glucose by the Tris-glucose oxidase method. Suitable controls were prepared by omitting either the enzyme or the substrate in the procedure. One unit 'of enzyme acitivity is defined as the amount of enzyme required to release 1 μ mole of glucose per min under the above conditions.

α-Amylase Activity[99c]

Corn starch (120 mg) was gelatinized with water (3 ml) for 30 min and diluted with phosphate buffer (3 ml, 0.02 M, pH 6.9). The mixture was incubated with hog pancreatic α-amylase (0.4 ml, 278 units) at 37° for 15 min. The enzyme was inactivated by heating in a boiling water bath for 10 min. The digest was centrifuged (5000 rpm for 15 min) and the supernatant was made up to 8 ml with water. Maltose released was estimated by the Nelson-Somogyi method[79]. Controls were prepared as described. One unit of enzyme activity is defined as the amount of enzyme required to release 1 u mole of reducing sugar expressed as maltose per min under the experimental conditions.

In Vitro digestibility of native and gelatinized starches

Starch (100 mg), suspended in sodium acetate buffer (pH 4.8, 0.05 M, 4 ml) was gelatinized, cooled to 60° and incubated with glucoamylase for 30 min at 60°. The enzyme was inactivated by heating the digest in a boiling water bath for 10 min. The mixture was centrifuged (5000 rpm for 15 min) and the residue was washed with water. The supernatant was made upto 15 ml with all washings and analysed for released glucose by the glucose oxidase method.[80]

Structural Studies by Enzymatic Methods

A. β-Amylolysis[100]

Preparation of β-Limit Dextrins (β-LD) : The pure amylose or amylopectin fraction (50 mg) was solubilized in DMSO (85%, 2 ml) by heating in a boiling water bath for 10 min, cooled to room temperature and centrifuged (2000 rpm for 2 min) to remove any insoluble material. The clear supernatant was made up to 10 ml with acetate buffer (0.1 M, pH 4.8), β-amylase (1500 units) was added and incubated at 37° for 24 h. When the reducing power became constant (Nelson-Somogyi method)[79] the solution was boiled for 20 min to inactivate the enzyme and the percent β-amylolysis was calculated.

$$\% \text{ p-amylolysis} = \frac{\text{Reducing Power (as maltose)}}{\text{Total Sugar (as glucose)}} \times 100$$

One unit of β-amylase is defined as the amount of enzyme releasing 1 µmole of maltose from soluble starch per min at pH 4.8 and at 37°.

Fractionation of β-LD : After dialysis against water at room temperature (16 h) to eliminate maltose, the resulting β-LD was subjected to a second β-amylolysis. The second dialysed β-LD was then precipitated by the addition of methanol (3 vol.)- The precipitated β-LD was subjected to GPC on a precalibrated Biogel P-10 column.

B. **Debranching Studies**[101,102]

Debranching of β-LD by Pullulanase : β-LD (20 mg) was debrached with pullulanase (3.2 units in 2 ml of 0.1 M acetate buffer, pH 5.5) for 24 h at 37°. The digest was heated in a boiling water bath for 10 min and the insoluble material/heat coagulated enzyme were thus removed by a brief centrifugation (10,000 rpm for 5 min). The hydrolysate was analysed by PC, GPC (Biogel P-10 column) and SE-HPLC.

Debranching of amylopectin/amylose and intermediate fraction by isoamylase

The respective sample (50 mg) was dissolved in 85% DMSO (1 ml) and the material was precipitated with methanol (3 vol.) and redissolved in water (9 ml) by heating in a boiling water bath for 30 min. The solution was cooled to room temperature, acetate buffer (1 ml, 0.1 M, pH 3.5) and crystalline isoamylase (9000 units, *Pseudomonas* sp.) were added and the mixture was incubated in a shaker bath at 40° for 48 h to complete the debranching reaction. The enzyme was heat inactivated and the digest centrifuged (8000 rpm for 15 min). The clear supernatant was subjected to GPC (Biogel P-10) and SE-HPLC analyses.

RESULTS AND DISCUSSION

SECTION A: LEGUME STARCHES

a. Physicochemical properties

The filtrate obtained by sieving (-240 mesh) wet flour suspensions represented the crude starch, which upon purifications by successive treatments with dil. alkali and NaCl-toluene gave four starch isolates as schematically shown in Fig.13. Except for starch isolate I, which was recovered in major amounts from greengram (GG) and Bengalgram (BG) flours, the recovery of starch isolates II to IV was considerably different (Table 1) The relative granule size distribution or the population density profile of starch isolates I to IV is given in Fig.14. It may be inferred that the population density of larger granules was relatively more in BG than GG starch. The latter was, however rich in small sized granules. This was also evident from the photomicrographs of respective starch isolates (Figs.15 and 16) . Starch isolates I and II were comparatively rich in bigger granules, whereas the isolate IV was especially rich in non-starch/fibrous matter. Their granule size varied from 7.5 to 16.0 µm (spherical) and 7.0 to 30.0 *pm* (oval) for GG starch; and 9.0 to 14.0 µm (spherical) and 15.0 to 25 µm (oval) for BG starch. Some of the granules showed hilum. All the granules, big or small, showed characteristic birefringence under polarised light, and the polarization cross passed through the hilum.

The proximate composition of the different starch isolates is given in Table 1. It is seen that the protein content of isolate IV was very high (-14%) which could not be brought down even after several purification steps. Isolate I was very low in protein but correspondingly it was rich in

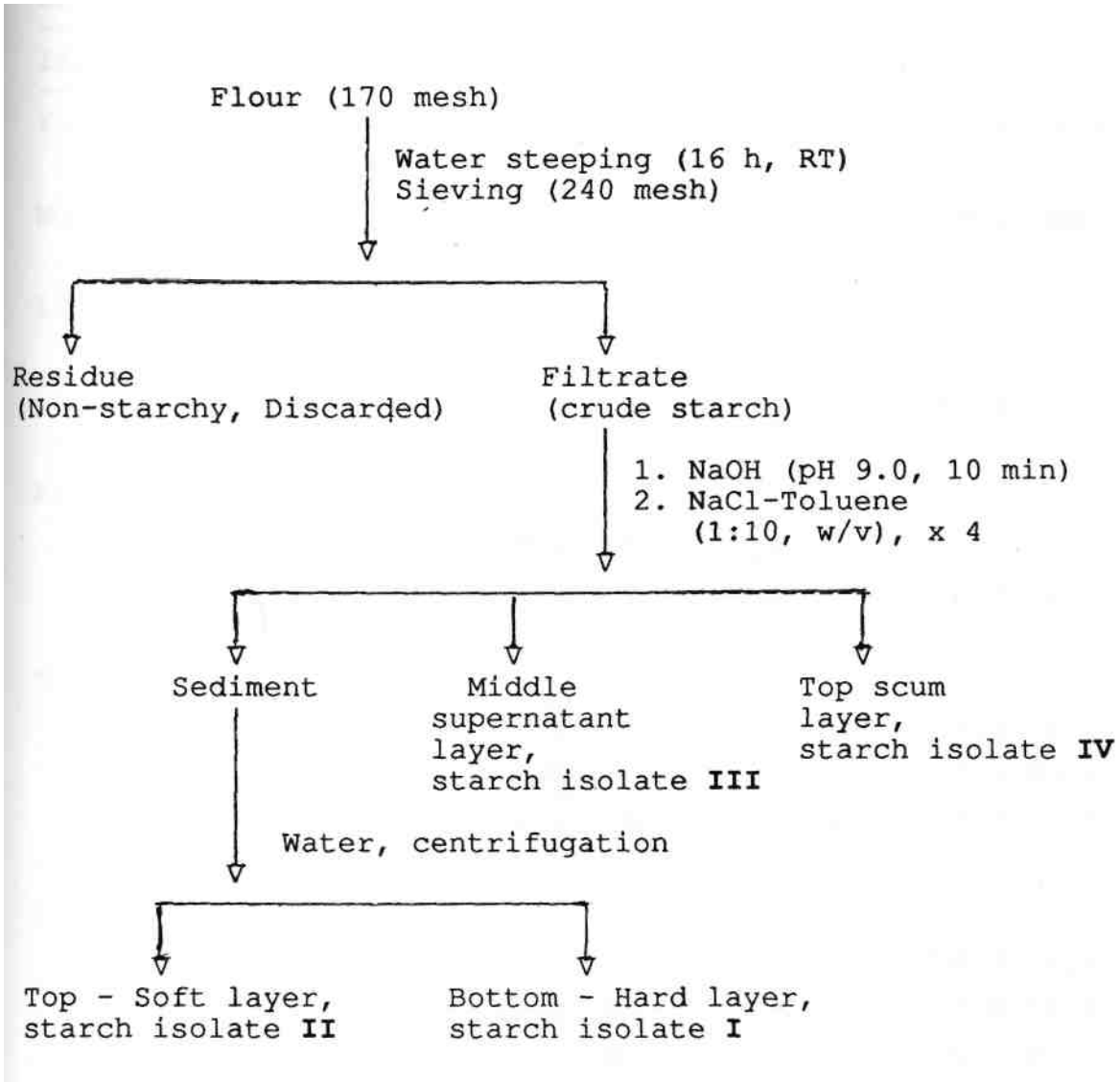


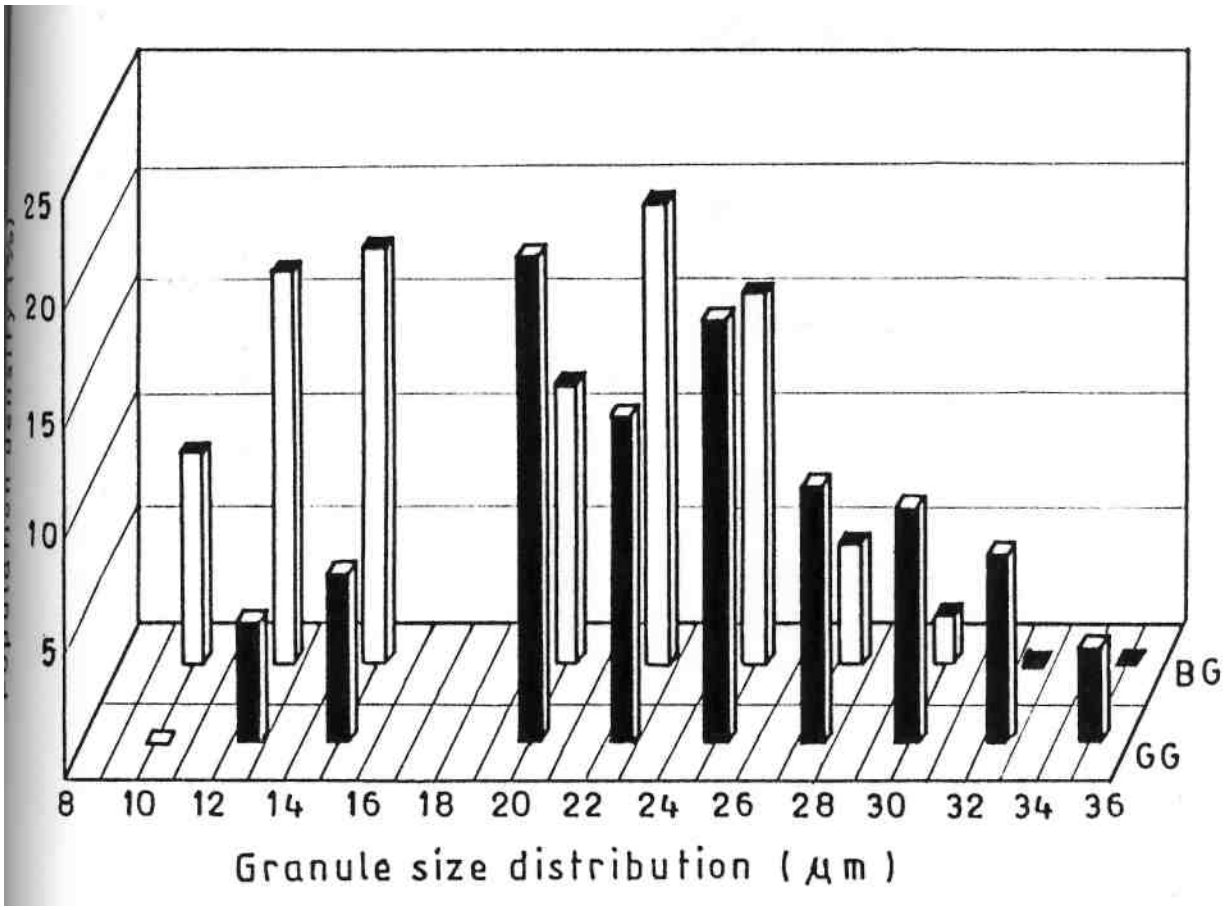
Fig.13. Recovery of starch isolates I-IV from legume/cereal flours

Table 1. Proximate composition (%) of the starch isolates of greengram and Bengalgram

Source	Greengram starches				Bengalgram starches			
Isolates	I	II	III	IV	I	II	III	IV
Yield*1	72.5	16.5	6.7	3.8	76.4	4.4	8.4	5.1
Moisture	9.3	9.4	ND	ND	9.1	9.2	ND	ND
Lipid								
Surface	0.8	1.0	0.5	0.4	0.9	1.2	0.5	0.4
Internal	0.3	0.4	0.2	0.1	0.8	0.8	0.3	0.1
Protein								
Nitrogen (N)*2	0.05	0.1	1.0	2.2	0.1	0.1	0.4	2.3
N x 6.25	0.3	0.6	3.9	14.1	0.7	0.9	2.3	14.3
Total carbo-hydrate*3	89.0	79.8	78.0	60.0	86.0	78.8	70.0	52.5
Starch by TGO*4	74.0	71.5	67.8	34.7	72.0	69.1	60.8	35.4
NSP*5	15.0	8.3	10.2	26.3	14.0	9.7	9.2	17.1
Starch components								
Am	60.0	64.0	66.0	83.0	58.0	68.0	75.0	80.0
Ap*6	40.0	36.0	34.0	17.0	42.0	32.0	25.0	20.0
λ max (nm)	620	ND	ND	ND	626	ND	ND	ND

*1 Out of 48 and 46% net yield of starch from GG and BG flours, respectively *2 Micro-Kjeldahl method *3 Phenol-H_2S0_4 method *4 Glucose oxiαase method (GJ.C x 0.9) *5 NSP (non-starch polysaccharide) = Total carbohydrate - starch content *6 100-amylose ND, not determined

Fig.l*. Granule size distribution of GG and BG starches.



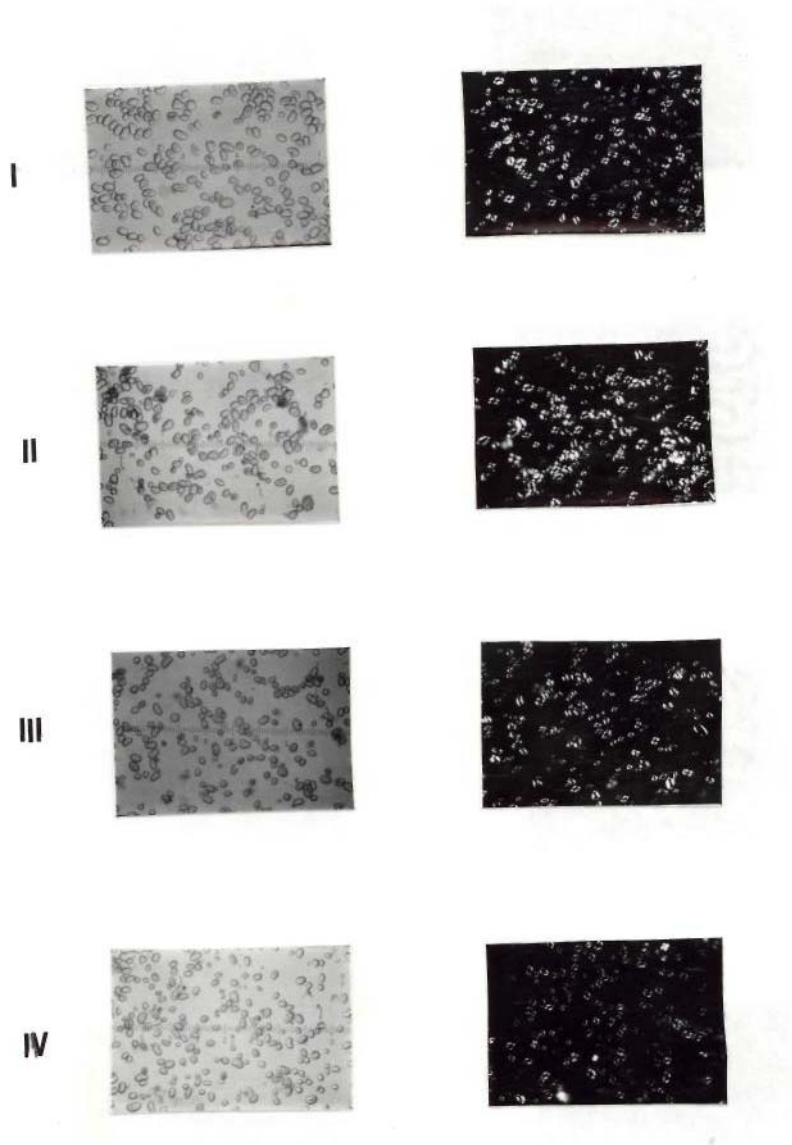


Fig. 15. Light and polarized photomicrographs of GG starch isolates.

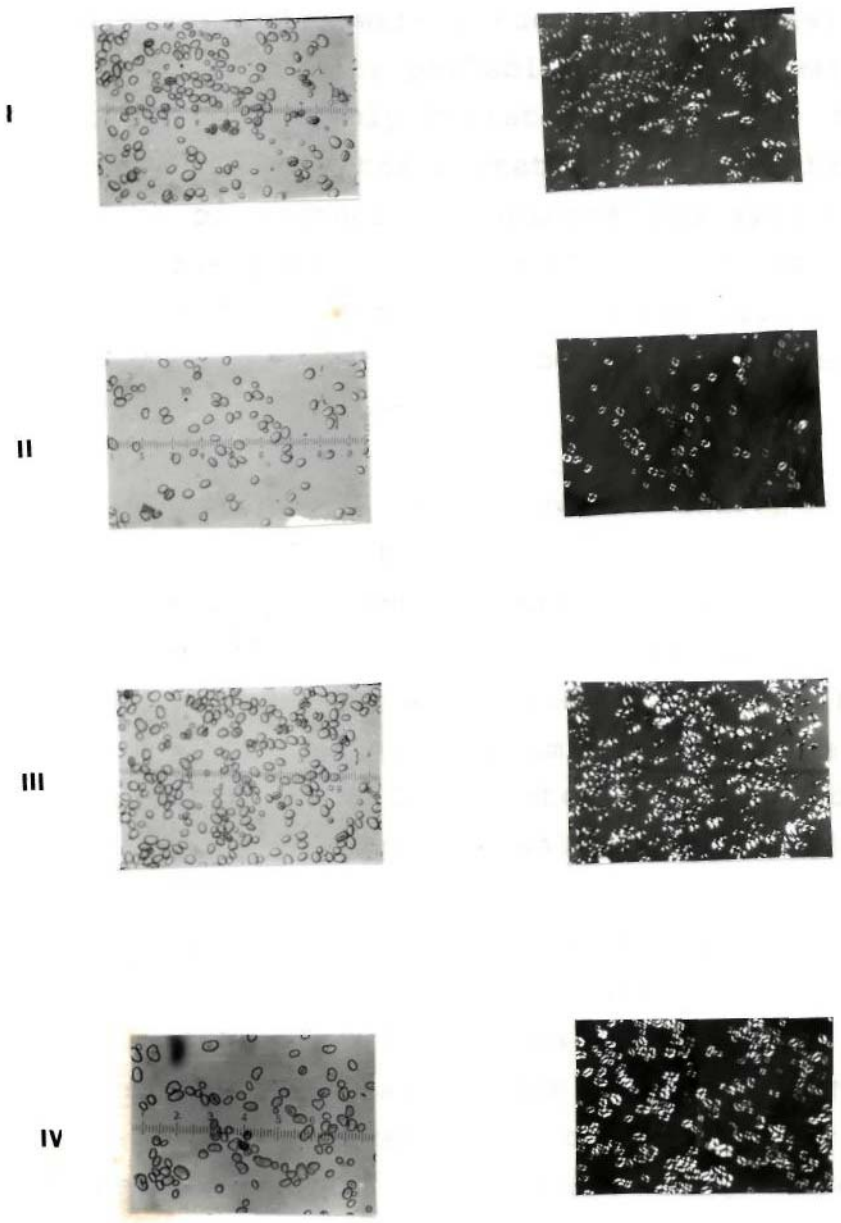


Fig. 16. Light and polarized photomicrographs of BG starch isolates.

starch carbohydrates. The slightly higher value of total sugar obtained by the chemical method could probably be attributed to the presence of non-starch carbohydrates (eg.hemicelluloses) which were predominant especially in the isolates IV. The latter are probably associated with more of insoluble protein and highly hydrated fine fibre fractions. Their GLC analysis as alditol acetates revealed the presence of small amounts of rhamnose, arabinose and xylose together with a huge glucose peak. The starch content as determined by the enzymatic method[80] ranged -70-74% for isolates I/II of GG and BG starches. Isolate IV in both the sources, was low in starch (-35%), as expected.

Total lipid contents of GG and BG starch isolates I varied from -1.2 to 1.6%. These were further subdivided into surface and internal-covalently bound lipids based on their extraction methods. Quantitatively the internal lipids (IL) were rather less than the surface lipids (SL) (Table 2). Palmitic acid (C16:0) was predominant in these lipid fractions, except the SL of GG isolate IV (9.1%) and IL of BG isolate I (5.1%), which were unusually low in its content. The content of C16:0 was highest in IL of GG isolate IV (52%). Invariably all the isolates were rich in C18:2, and C18:3 was found in considerable amounts in the SL of BG starch. Especially, the SL fractions of GG and IL fractions of BG starch isolates contained high amounts of unsaturated fatty acids. From their retention time characteristics on GLC they appeared to be long chain fatty acids. The occasional presence of C12:0, C14:0 and other unsaturated fatty acids in some isolates needs careful consideration. The ratio of unsaturated to saturated fatty acids was 1:1.75 in GG starch isolate I compared to 1:0.95 in BG starch isolate I. Though present in variable amounts their

Table 2. Major fatty acids (%) in surface (SL) and internal (IL) lipid fractions of starches

5.0%; -/ not present

Source	Fraction		C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	Unidentified fatty acids
GG starch	SL	I	*	*	26.9	6.6	9.5	11.2	*	7.1
		II	*	*	16.7	*	5.0	*	21.5	39.8
		III	*	*	34.0	6.0	12.7	15.6	*	*
		IV	*	*	9.1	*	*	*	*	69.1
	IL	I	7.7	7.8	39.5	-	-	16.4	*	10.3
		II	*	6.9	36.8	-	-	21.9	12.2	5.8
		III	18.8	11.9	28.3	-	-	6.4	-	15.9
		IV	*	*	52.0	-	7.2	9.9	9.5	13.5
BG starch	SL	I	*	*	23.7	5.0	*	21.8	13.8	11.2
		II	5.3	7.9	31.7	*	*	32.8	13.9	*
		III	*	*	27.7	9.1	*	29.2	10.4	*
		IV	*	5.6	28.7	*	*	29.0	10.4	*
	IL	I	-	-	5.1	-	-	29.3	-	64.1
		II	6.3	7.9	40.1	-	7.4	14.4	*	14.7
		III	*	*	46.1	-	*	18.7	*	16.2
		IV	*	*	42.5	-	-	31.5	*	7.8

biochemical-technological significance is not clearly known at the moment. Considerable qualitative and quantitative variations were also seen in the profile of minor (< 5%)fatty acids (Fig.17), which consisted of a few unsaturated fatty acids, viz., C16:l C18:l and C18:3, in addition to the common saturated fatty acids.

Both starches were found to be non-ionic as they did not adsorb either cationic or anionic dyes and thus behaved like other legume starches.[103] The amylose content, as determined by the I2-^{KI} blue colour reaction (see Table 1) of *GG* starch isolates ranged between 17-43% and of BG starch between 20-46%. The amylose content of isolate I was much higher than others, probably because of contamination of the latter with various non-starch components. In accordance with their high amylose content the λ_{max} value was -625 nm for isolates I and II, whereas isolates III and IV showed some variations. Subtle variations were also observed in the starch-iodine λ_{max} values between GG and BG starches for reasons not clearly known at present. Starches in general have a λ_{max} of 580-620 nm, whereas those of waxy-type (i.e., high amylopectin starches) have a λ_{max} of -520-550 nm. Pure amylose fraction shows a λ_{max} of -630-650 nm[105] .

The isolates I and II of both GG and BG starches exhibited single stage swelling and low solubility in water (see Fig.18). The former indicated relaxation of homogeneous and strong bonding forces within the granule at temperatures above gelatinization, similar to those of other leguminosae starches. The solubility pattern of starches in DMSO is given in Fig.19. GG starch showed complete solubility in DMSO in -60 h, compared to BG starch which showed only -70% solubilization in 60 h.

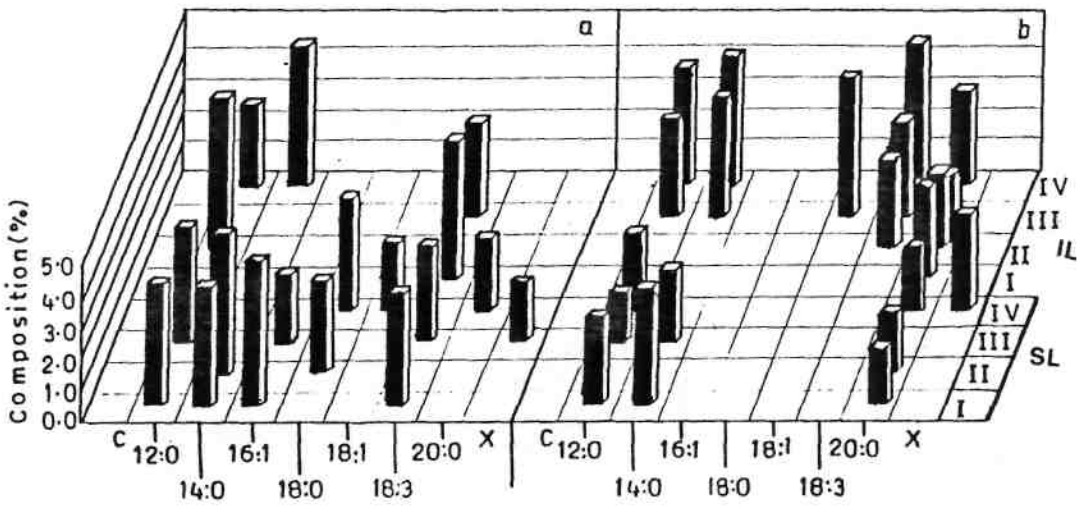


Fig. 17. Minor fatty acids of SL and DL fractions of, a. GG and b. BG starches; X = unidentified fatty acids.

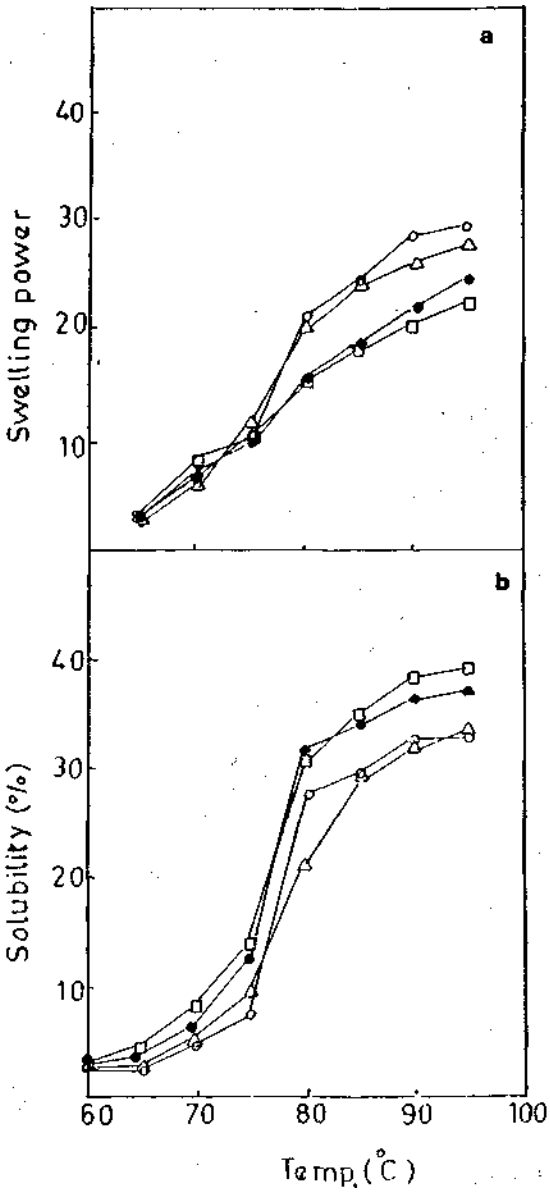
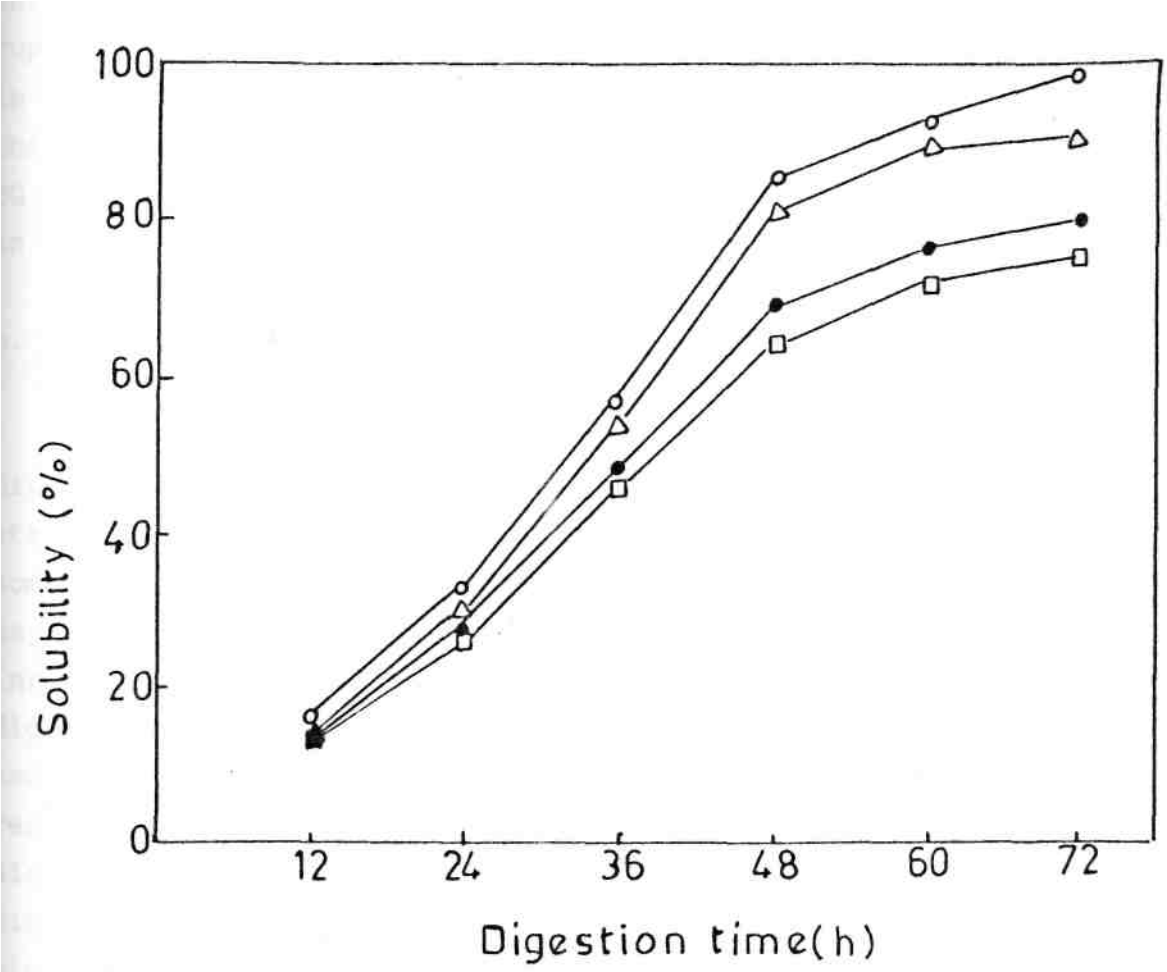


Fig. 18. Swelling power (a) and % solubility (b) in water of GG and BG starch isolates; GGS-I, -o-; GGS-II. -A-: BGS-I, -+-; and BGS-II, -D-.

Fig. 19. Solubility in DMSO of GG and BG starch isolates; GGS-I, GGS-II, -A-; BGS-I, -*Φ*-; and BGS-II, - D-.



The results of hot paste viscosity characteristics,as determined by the Brabender viscograph, are presented in Table 3 and Fig.20. The overall peak viscosity as well as set back viscosity (C-H) of BG starch isolates I and II are very low in comparison to the corresponding GG starch isolates. The low viscosity (170 B.Ü.)of the former was due to restricted swelling, and further their pasting temperature (i.e., gelatinization temperature) was also slightly higher (-80°) than that of GG starch (-73°). Accordingly, BG starch showed very low breakdown indicating that the granules do not rupture during continued heating with stirring. The increase in viscosity of GG starch isolates on cooling to 50° reflects their better retrogradation tendency. The low viscosity of BG starches corroborates well with their low swelling power in water.

b. ***In Vitro*** **Digestibility**

Comparatively, BG starch granules were little less digestible with glucoamylase than the corresponding GG starches (see Table 4) . In the starch isolates III and IV some amount of fibrous materials (non-starch carbohydrate or may be even resistant starch) were left behind after incubation, which may partly account for their low digestibility values. In addition, high contents of protein and may be also internally bound lipids account for the restricted enzyme susceptibility and as a result reduced digestibility of the latter isolates. The better digestibility of GG starch granules can also be correlated with their high solubility in DMSO (over 95% solubilization in -60 h) and their slightly lower amylose content. Because of a positive correlation between the amylose content and the formation of resistant starch[106a], it is possible that these

Table 3.
Hot Paste Viscosity (B.U.) Characteristics of Major Starch Isolates

Starch isolate	PT °C	P	H	C	C-H	H/P	C/P	C/H
GG_I	73.0	560	600	1080	480	1.07	1.93	1.80
GG_{II}	73.0	560	560	920	360	1.00	1.64	1.64
BG_I	79.0	170	240	330	90	1.41	1.94	1.38
BG_{II}	80.5	170	240	320	80	1.41	1.88	1.33

PT = Pasting temperature
P = Peak viscosity
H = Hot paste viscosity
C = Cold paste viscosity
C-H= Set back
C/P = Set back ratio
C/H = Total set back ratio
H/P = Break down ratio

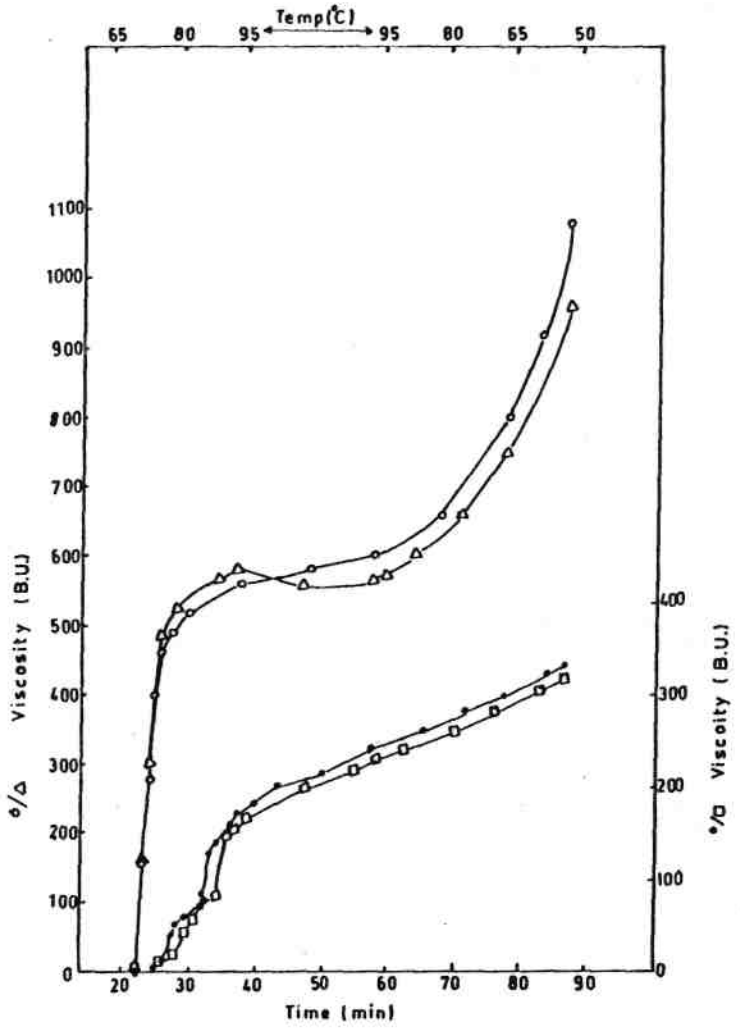


Fig. 20. Brabender amyiogram of GG and BG starch isolates; GGS-I, -o-; GGS-II, -A-; BGS-I, -•- and BGS-II, -o-.

Table 4. *In vitro* digestibility of legume starch isolates

Starch		% hydrolysis		
		Native		
		Glucoamylase*	Pancreatic α-amylase**	Gelatinized***
Corn starch		5.7	57.1	72.9
Greengram starch	I	3.7	38.9	67.5
	II	ND	ND	57.1
	III	ND	ND	48.6
	IV	ND	ND	23.1
Bengalgram starch	I	3.5	30.1	63.4
	II	ND	ND	54.0
	III	ND	ND	43.3
	IV	ND	ND	20.9

* At 37°C, pH 4.6, 120 min
** At 37°C, pH 6.9, 120 min
*** At 60°C, pH 4.6, 30 min

ND, not determined

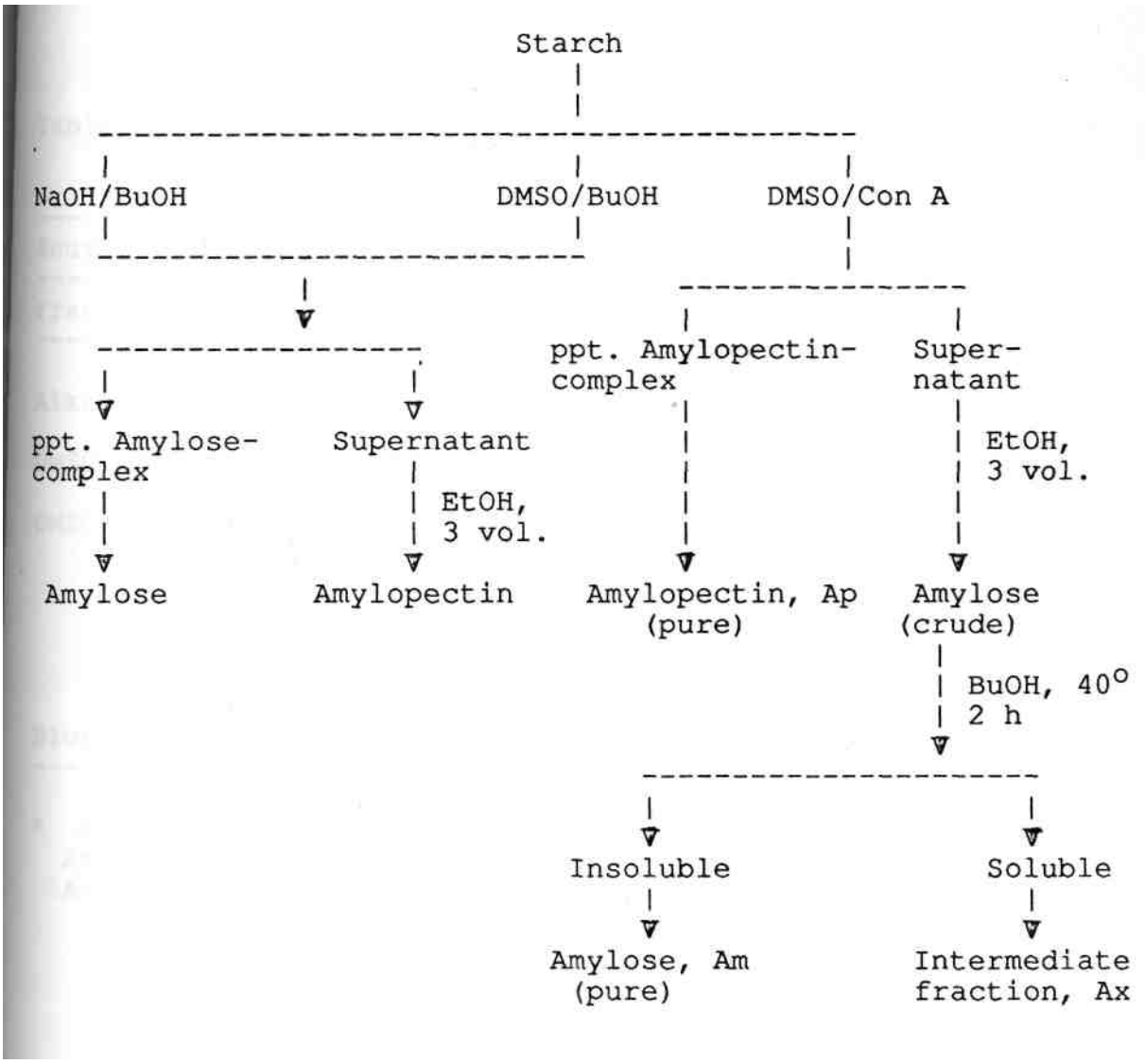
high amylose-containing legume starches are relatively less digestible, *in vitro*. Nevertheless, the gelatinized starch suspensions were better hydrolysed than the native granules.

c. Fractionation

Three different methods of fractionation, as shown in Fig.21 were employed to separate the constituent molecules in isolate I of GG and BG starches. In methods 1 and 2 1-butanol was the complexing agent used to separate fairly pure amylose;[56,75] whereas in method 3 con A was used to precipitate the amylopectin (Ap) in a pure form. [56] The crude amylose fraction was subjected to further fractionation by extraction with hot butanol[76] into pure amylose (Am, B-insol.) and an intermediate fraction (Ax, B-sol.)

The % yield of various fractions obtained by these fractionation methods are given in Table 5. For comparison the % amylose values as deduced by the blue value method are also given in Table 5.

As could be seen from Table 5, the % yield of total amylose compared well with that determined by the $^{\lambda}$blue Value' method.[83] However, the "true" amylose content, as deduced by hot butanol extraction method, was comparatively much less, which indicated the contribution of extended long B-chains of amylopectin molecule in starch-iodine colour reaction. The intermediate fraction, Ax was present in comparable amounts (-6.5%) in both starches. The presence of Ax was discernible in SE-HPLC analysis of crude amylose, which indicated a minor peak of Ax eluting at -16.26 min and a major peak of amylose (-22.21 min); whereas purified amylose and the amylopectin (-14.32 min) as obtained by the



Fig, 21. Fractionation scheme employed to separate the constituent molecules in isolate I of legume/ cereal starches

Table 5. Starch fractionation into amylose, amylopectin and intermediate fraction (% values)

Source	GGS-I		BGS-I	
Fraction	Amy	Ap	Amy	Ap
Alkali-BuOH	27.0	65.0	26.7	51.2
DMSO-BuOH	39.5	60.3	38.2	60.0
DMSO-Con A	36.5	61.0	39.0	60.0
	Am = 29.7 Ax = 6.8		Am = 32.6 Ax = 6.5	
Blue value*	40.0	60.0	42.0	58.0

* Ap = 100 - amylose
Amy = Amylose (crude)
Am = Amylose (pure)

con A method gave single symmetrical peaks on SE-HPLC, showing their homogeneity (Fig.22). The width of amylose peak was relatively more broader, indicating its probable intrinsic polydispersity nature.[101] GPC analysis using Sepharose CL-2B also substantiated their homogeneity criteria. The λ_{max} values and the average molecular weight of these fractions, as determined by precalibration of the column with standard dextrans of known molecular weight, are given in Table 6.

Table 6. Average molecular weight and λmax value of starch fractions

Source	GGS-I			BGS-I		
Fraction	Am	Ax	Ap	Am	Ax	Ap
Molecular weight	4.5×10^6	9.1×10^6	1.7×10^7	6.3×10^6	1.5×10^7	2.2×10
λ max (nm)	642	609	560	646	602	566

It is clear that amylose has the highest λ_{max} value whereas the Ax fraction has a λ_{max} value in between those of Am and Ap fractions. A similar trend is noticeable in their molecular weight values too, Ax has a molecular weight in between those of Am and Ap. In all probabilities, it appears that Ax is not essentially a linear molecule, instead it may have some amount of sparcely distributed short chain branches. Further insight into their molecular structure was deducible by enzyme debranching studies, as described later.

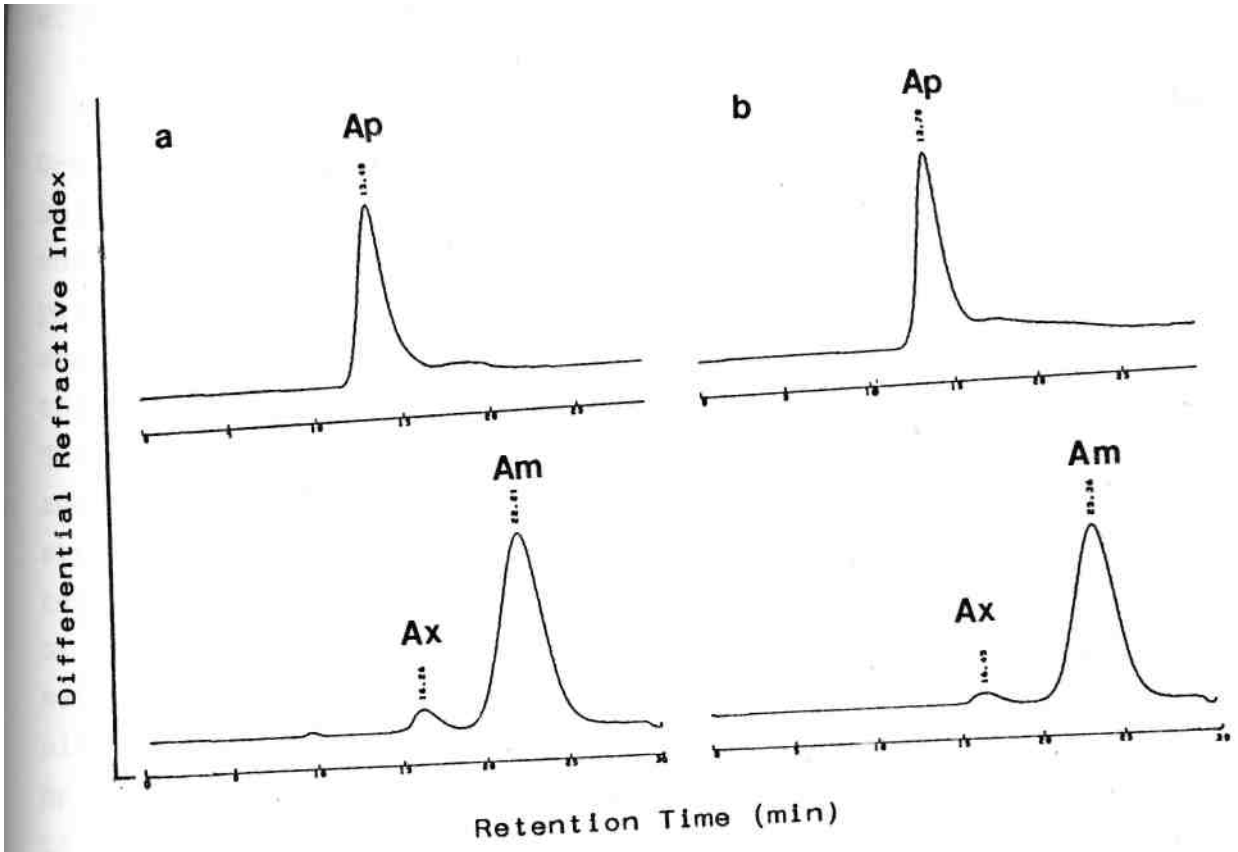


Fig.22. SE-HPLC profiles of Ap and Ax/Am of, a. BG and b. GG starches, respectively.

d. Limiting viscosity[90]

The limiting viscosity number of BG amylose and amylopectin fractions showed higher value than those of GG starch fractions (see Fig.23).

e. Fine structure of starch components

The enzyme β-amylase is capable of attacking an α-1,4-D-glucan from the non-reducing end in a sequential manner splitting off β-maltose units.[90,107] Its action stops whenever it comes across a 1,6-linkage, due to a branch point, as in the case of amylopectin, and leaves behind eventually a β-limit dextrin, which can be precipitated by the addition of alcohol. The latter is nothing but a "modified" amylopectin devoid of all the long and short A- and B-chains, instead possessing maltosyl and maltotriosyl stubs, and shortened B-chains at the branch points with the remaining part of the molecule being intact. Hence, a study of p-amylolysis reaction is of great value in elucidating the nature of a branched α(-glucan macromolecule. [108] In order to elicit such an information, all the three fractions, viz., Ap, Ax and Am of GG and BG starch isolates I were subjected to the actions of β-amylase and pullulanase in a sequential manner. In both the cases the action of B-amylase was completed at the first β-amylolysis, since repeated *β*-amylolysis of the first B-limit dextrins, freed from maltose, did not occur. The absence of any free glucose in the digests revealed the purity of β-amylase used. An α-glucosidase is the usual contaminating enzyme found in some β-amylase preperations.[109] The β-amylolysis limit values of these starch fractions are given in Table 7. From the values presented, it may be inferred that none of the so called

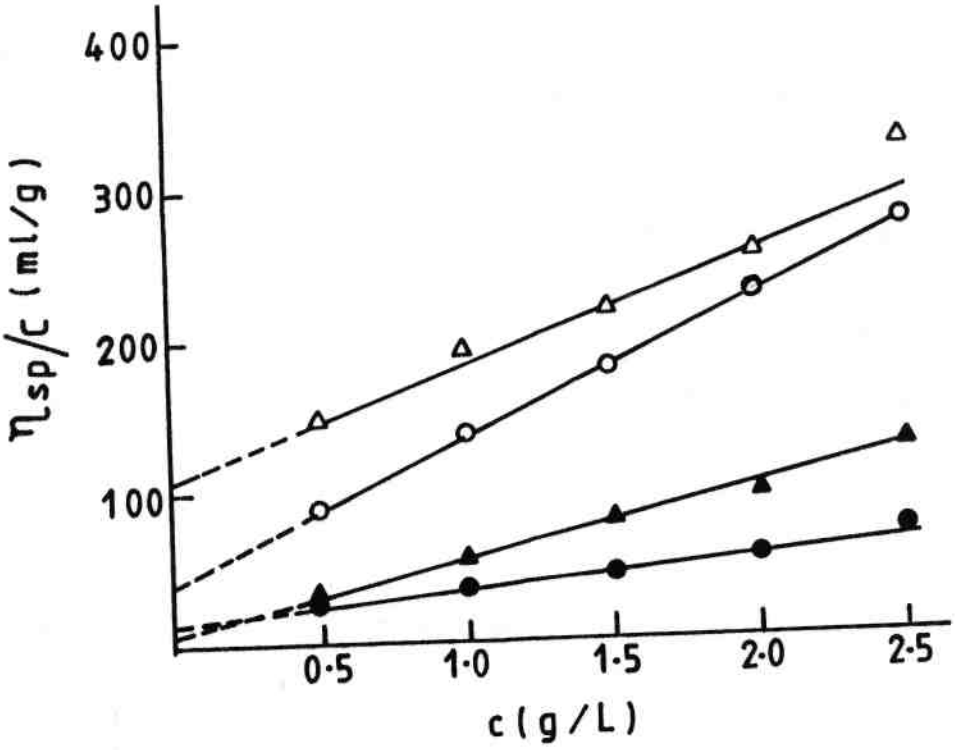


Fig.23. Limiting viscosity of, -△- =BGS-Ap; -o- = GGS-Ap; -▲- = BGS-Am; and -●- = GGS-Am.

'linear' amylose fractions gave a theoretical % β-amylolysis value of -98%, and hence it is implied that these two legume amylose fractions are not essentially linear, instead they do possess some amount of sparcely distributed short chain branches. However, such a branching was relatively slightly more in the case of BG starch than in GG starch. On the otherhand Ax and Ap fractions of both starches had almost .comparable β-amylolysis limit values. The value for Ap was very much within the range like those of other starch amylopectins (-52-56%).[109]

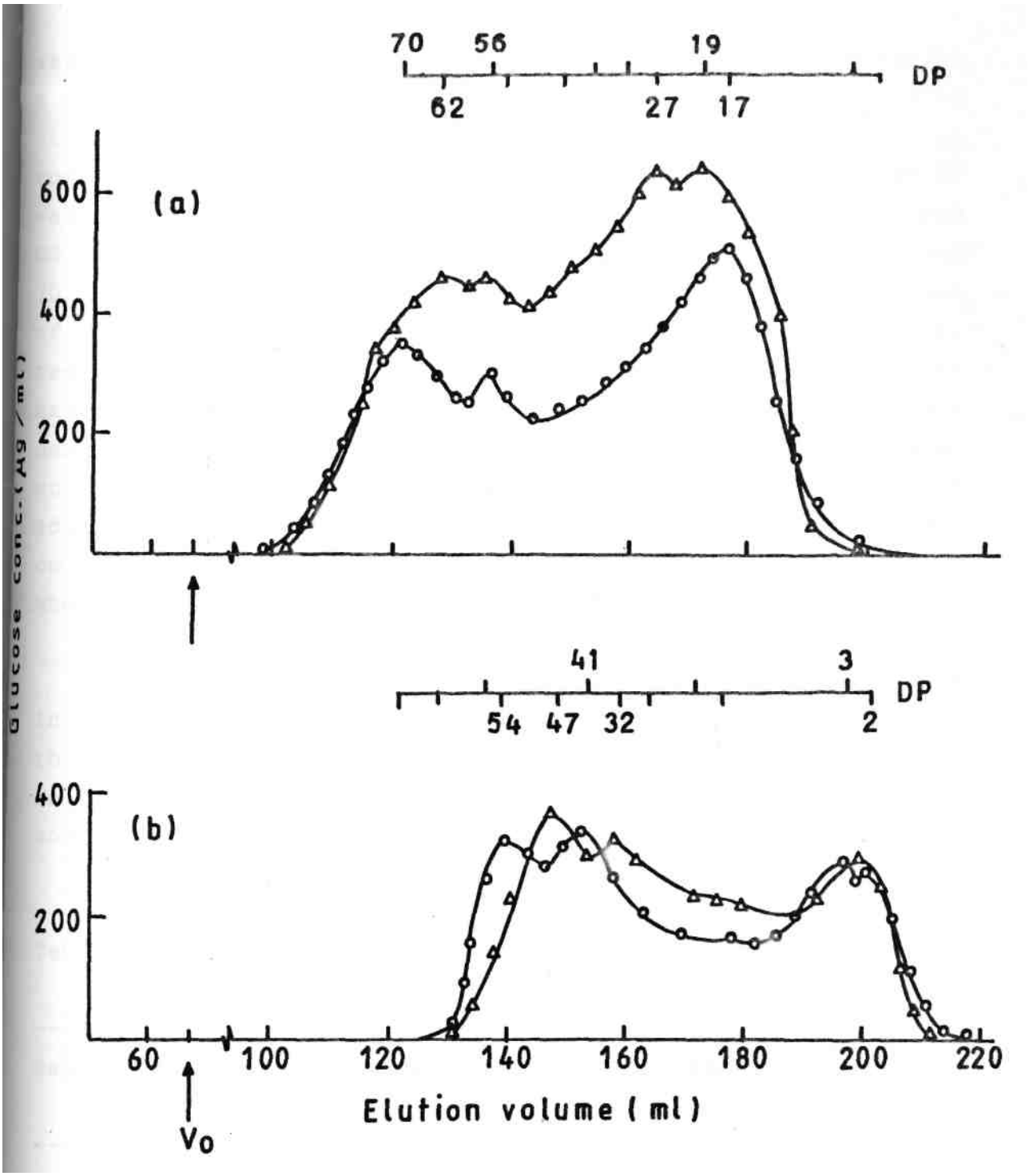
Table 7. p-amylolysis (%) values of starch fractions

Source	GGS-I			BGS-I		
Fraction	Am	Ax	Ap	Am	Ax	Ap
β-amylolysis	87.5	62.5	54.2	79.1	65.8	56.3

f. **Examination of chain profile**

To understand further their molecular architecture the β-LDs, obtained as above, and/or the respective native fractions as well were subjected to complete debranching by pullulanase. The enzyme selectively knocks off the left over isomaltosyl and other short chain branch points from the *β*-LDs and resulting in the formation of different A-and B-chains. These were quantitatively then seperated by GPC on Biogel P-10 to determine the size distribution of the constituent chains, i.e., the chain profile.[110,111] The elution profiles of debranched native amylopectin and the derived β-LDs of GG and BG starches are shown in Fig.24. Some differences discernible in the relative proportions of

Fig. 2*. GPC profiles on Biogel P-10 of debranched Ap (a) and its p-LD *(bY.* GGS (-0-) and BGS (-Δ-).



various peaks indicate these two starches to be dissimilar to a certain extent. The fact that the void volume (Vo) peak is virtually absent indicated clean fractionation as well as complete debranching of the branched molecules. From the DP values shown in the Fig.24a, it was clear that the Ap from GG starch has three types of chains having a DP 17, 56 and 70, respectively; whereas the BG-Ap has at least five types of chains with DP values of 19, 27, 33, 56 and 62, respectively. Interestingly no low molecular weight peaks of maltose or maltotriose were seen in them. On the otherhand debranching of the respective β-LDs (see Fig.24b) revealed considerable amounts of both maltose and maltotriose in addition to large peaks due to chains of DP 32-56, The latter could probably represent long, medium and short β-chains; whereas chains of DP -17 would constitute normal A-chains.

Assuming equal number of A- and B-chains the information, obtained on the average chain length (CL) and the β-amylolysis limit values of amylopectin, was used to calculate the exterior (ECL) and interior (ICL) chain lengths and the results are presented in Table 8. It may be seen

Table 8. Chain length values of amylopectins

Sample	CL	β-amylolysis limit, %	ECL	ICL
GGS-Ap	22.2	54.2	14.0	7.0
BGS-Ap	26.0	56.3	16.6	8.4

that both ECL and ICL values of BG amylopectin are higher than those of GG amylopectin which may invariably have an implication on the overall starch digestibility. If so, the ECL is therefore the number of glucose residues removed by β-amylase plus two (the size of the stub) and the ICL is given by the equation ICL • CL - ECL - 1.[112,113] The actual branch point residue is regarded as separate from the exterior and interior chains.

A similar set of experiments was performed on both Am and Ax fractions of these starches and the results obtained are shown in Figs.25 and 26, respectively. Contrary to amylopectin, which could be debranched completely and which gave no Vo peak, the amylose fraction on pullulanase treatment gave a major Vo peak together with small amounts of peaks with low DP values (2 to 11) in both GG and BG samples (Fig.25a) . The Vo peak is indicative of a high molecular weight linear molecule, whereas the latter minor peaks are the result of debranching of the side chain stubs. These chains were, at least, of three different types (DPs 9,15 and 21) in BG amylose, and in GG amylose only two chains of DP values 11 and 19, respectively were found. These stubs probably represent short and long A-chains and some short B-chains, atypical of those in amylopectin. On the otherhand, a prior β-amylolysis of the amylose fractions gave β-LDs which on subsequent pullulanase debranching and GPC gave a pattern as shown in Fig.25b. The results indicated that the B-LD of BG Am to have apparently a major single side chain stub of DP = 2 (i.e., maltose) and a minor chain of DP = 9, whereas the β-LD of GG-Am to have possibly two chains of DP = 3 (i.e., maltotriose, the major peak) and DP = 6, respectively. Both maltose and maltotriose are released from

Fig. 25. GPC profiles on Biogel P-10 of debranched Am (a) and its 3-LD (b): GGS (-o-) and BGS (-M; V_0 = high MW peak.

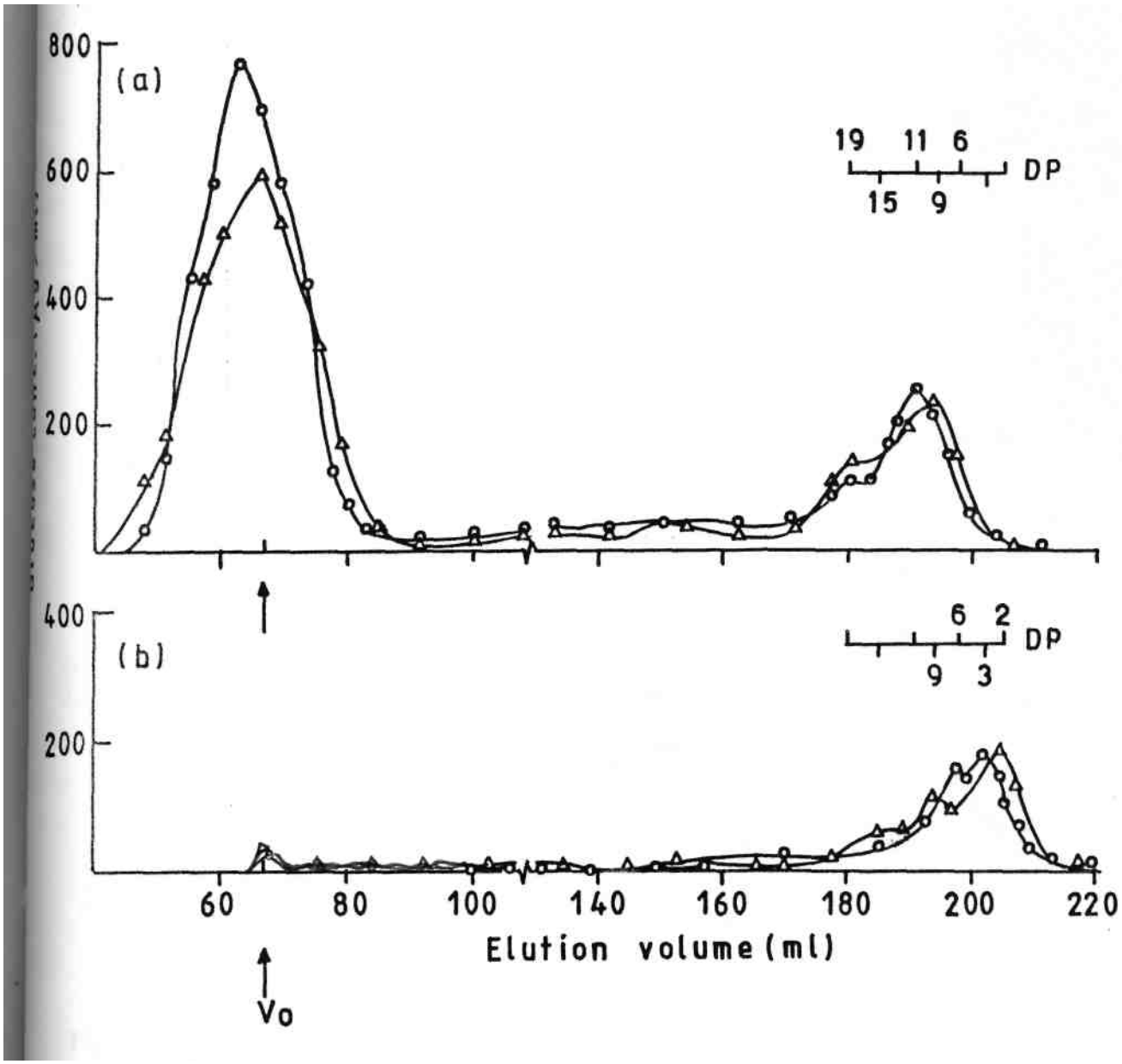
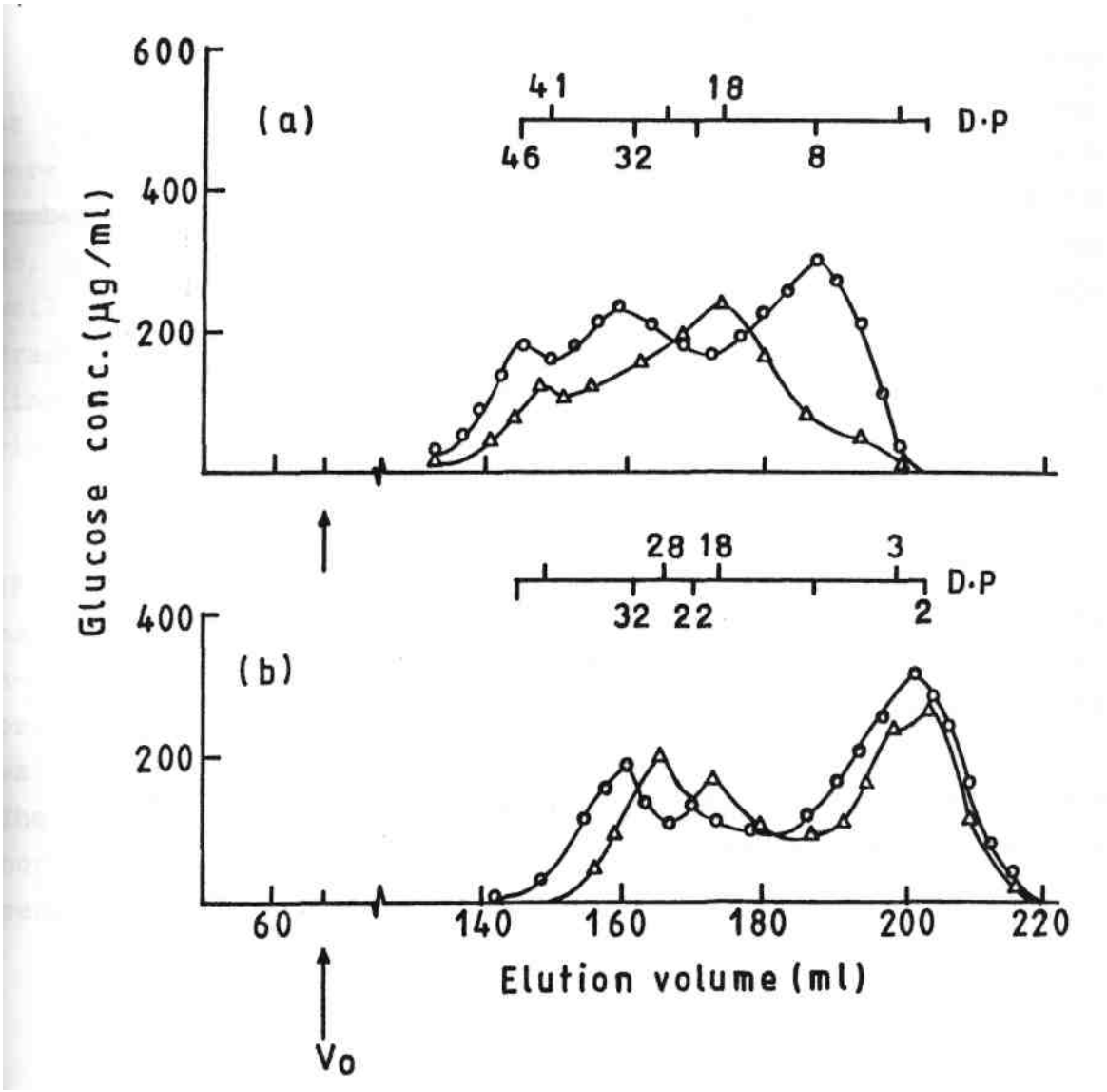


Fig. 26. GPC profiles on Biogel P-10 of debranched Ax (a) and its J3-LD (b): GGS (-o-) and BGS (-A-).



A-chain stubs. However, in the both β-LDs a very small peak of high molecular weight material was excluded from the gel.

The results obtained on the debranching of both native as well as derived β-LDs of the intermidiate fraction, Ax, were of different nature, in that the native fractions gave a number of peaks of varying DP values, ranging from DP 8 to 46, along with the Vo peak (Fig.26a) . The latter could as well be the amylose impurity resulting from an incomplete fractionation of crude amylose or it may represent a truely linear high molecular weight material. The Vo peak was virtually absent in the debranched β-LD profiles (Fig.26b).

The GPC profiles of debranched β-LDs of Ax fraction (Fig.26b) showed predominantly a large amount of maltose and maltooligosaccharides (DP~5) originating from the short/long A- and some B-chains, as before. In addition, the GPC profile indicated some incompletely debranched peaks with DP values corresponding to 22-31 of short and medium B-chains. The results clearly demonstrated that Ax was neither amylose nor amylopectin in its structure, instead it was in its true sence an intermediate fraction.

SECTION B: CEREAL STARCHES

As before, both rice and ragi flours furnished four starch isolates of varying size/shape characteristics (Figs.27 and 28 and Table 9) . Rice (Re) starch granules (Fig.29) were relatively of smaller size than ragi (Rg) starch isolates (Fig.2 9) and these small starch granules were poorly birefringent. From the data presented in Table 10 it may be inferred that the starch isolate I, in both the cereals, are relatively of much higher purity. However, the isolates still contain some amount of non-starch matter (-10-12%) as judged by the differences in carbohydrate content (as glucose) between the chemical and enzymatic methods. The isolate IV of rice starch was especially very low in carbohydrate and its purification was found to be extremely difficult. The isolates (I/II) were rich in surface lipids (~0.6%) than internal lipids (~0.2%), and their GLC analysis as FAME derivatives revealed the predominance of C16:0 in rice starch lipids and both Cl6:0 and C18:2 in ragi starch lipids (Table 11) . The ratio of saturated to unsaturated fatty acids was 1:0.77 and 1:0.81, respectively in rice and ragi starch isolate I. The profile of minor (< 5%) fatty acids of these lipid fractions is shown in Fig.30. The total protein content of isolates I was rather very low (< 0.5%), whereas that of isolates IV was -5.0%. The amylose content of isolates I to III as determined by the blue value method,[81] ranged between 22-30%. The $\lambda._{max}$ of the blue coloured complex was -620 nm.

Like several other cereal/millet starches, both rice and ragi starches were non-ionic and exhibited a single stage swelling and solubility pattern in water (Fig.31). The isolate II in both starches showed a lower solubility and

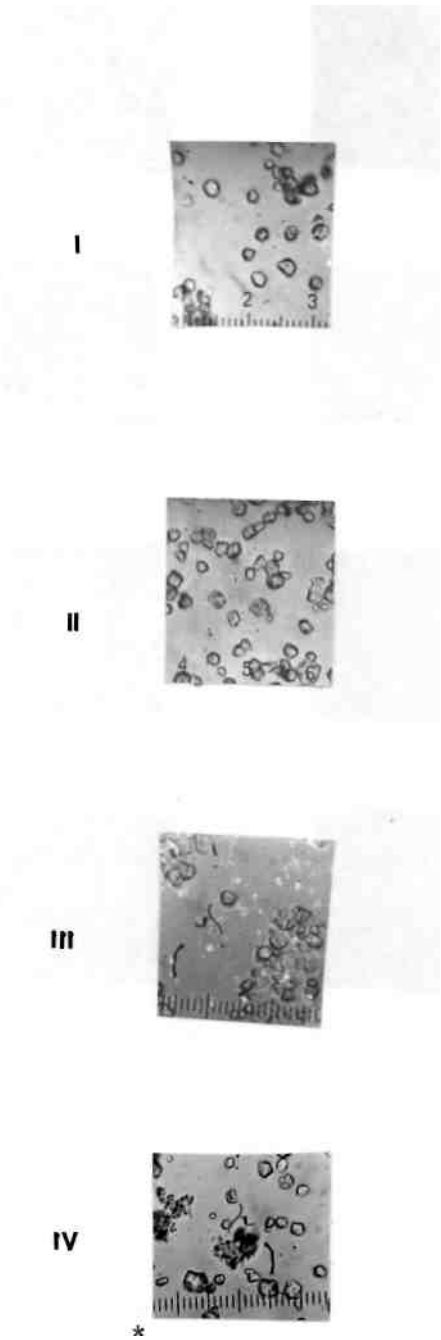


Fig. 27. Light and polarized photomicrographs of Re starch isolates.

* was not possible

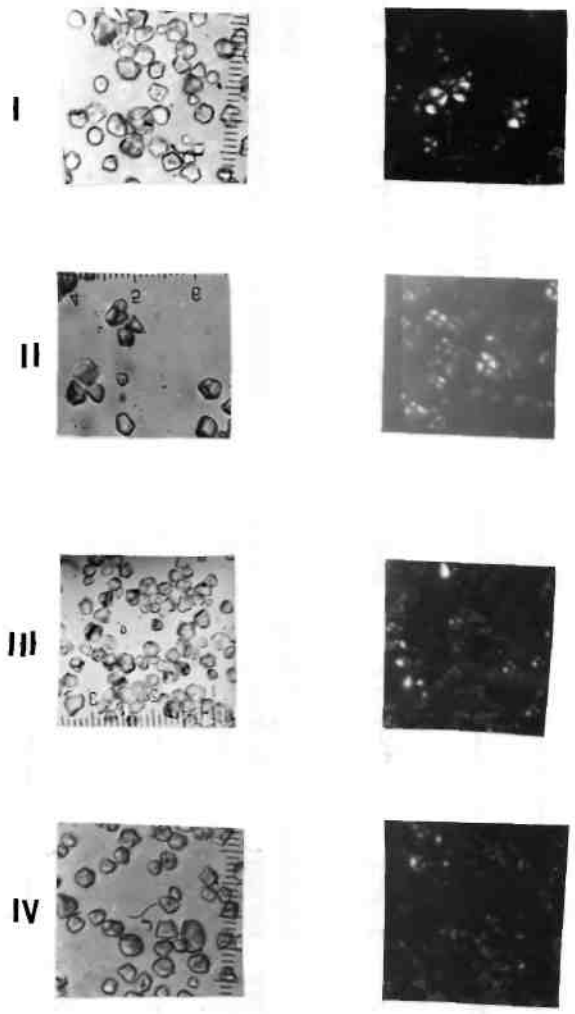


Fig. 28. Light and polarized photomicrographs of Rg starch isolates.

Table 9. Morphological characteristics of starch isolates

Source	Rice starches				Ragi starches			
Isolates	I	II	III	IV	I	II	III	IV
Shape	Polygonal	Small polygonal	Small round/ polygonal	Small round/ polygonal	Big hexagonal	Big/small hexagonal	Small hexagonal	Small oval/ hexagonal
Size (μ)	3-4.5	2-3	1.5-2	1-1.15	5-9	3-5	2-4.5	1.5-3
Hilum	Faint no fissures				Concentric no fissures			

Fig.29. Granule size distribution of Re and Rg starches. 85

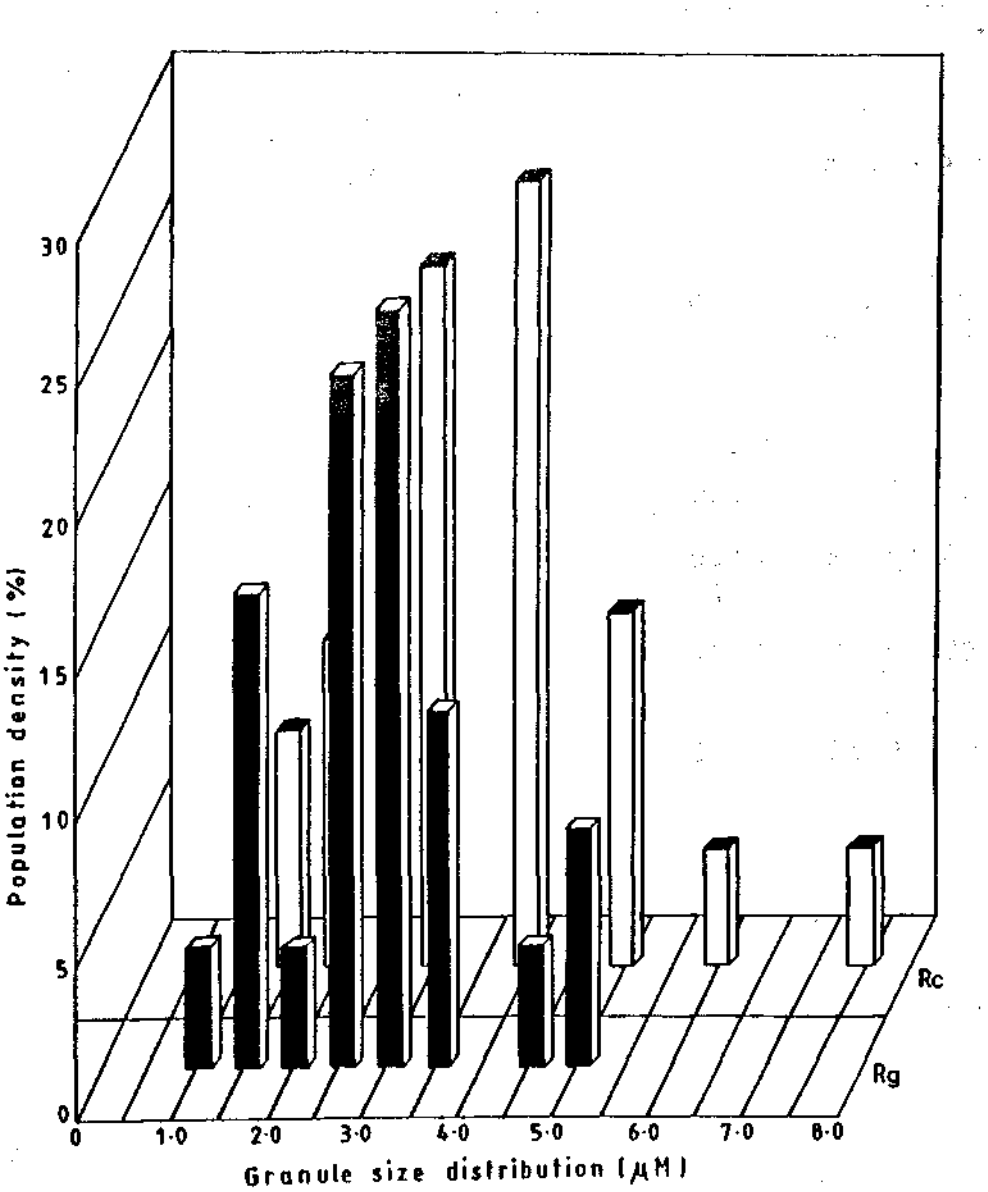


Table 10. Proximate composition (%) of the starch **isolaltes of rice and r** acii

Source	Rice starches				Ragi starches			
Isolates	I	II	III	IV	I	II	III	IV
Yield	45.4	9.6	7.3	15.2	41.1	8.2	18.3	7.3
Moisture	8.9	8.9	9.7	9.9	8.9	8.9	9.1	9.4
Lipid								
Surface	0.6	0.6	ND	ND	0.7	0.5	ND	ND
Internal	0.2	0.2	ND	ND	0.2	0.2	ND	ND
Protein								
Nitrogen (N)	0.05	0.05	0.60	0.70	0.14	0.17	0.40	0.60
N x 6.25	0.32	0.33	3.75	4.40	0.87	1.06	2.50	3.75
Total carbo-hydrates	91.0	85.0	90.0	30.0	90.0	89.5	83.0	45.0
Starch by TGO[*1]	79.2	72.9	70.8	10.5	77.4	75.5	72.1	35.0
NSP[*2]	11.8	12.1	19.2	19.5	12.6	14.0	10.9	10.0
Starch Components								
Am	30.0	28.0	25.0	8.0	28.0	26.0	22.0	9.0
Ap[*3]	70.0	72.0	75.0	92.0	72.0	74.0	78.0	91.0
λ max (nm)	619	ND	ND	ND	616	ND	ND	ND

***1 (GLc x 0.9)**

NSP = Total carbohydrate - starch content

° 100 - amylose; ND, not determined.

Table 11. Fatty acids (%) in surface and Internal lipid fractions of starches

Source	Rice starches								Ragi starches							
Fraction	Surface lipids				Internal lipids				Surface lipids				Internal lipids			
Isolates	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
C14:0	5.3	*	*	*	*	*	5.5	5.2	-	-	-	-	*	*	*	-
C16:0	44.1	40.0	46.1	38.0	26.9	50.8	51.8	42.8	35.8	29.8	23.4	35.0	27.7	39.0	21.0	39.2
C18:0	-	-	-	-	-	-	-	12.3	-	-	-	-	-	-	-	-
C18:1	-	-	-	-	-	-	7.6	13.7	-	-	*	-	-	-	-	-
C18:2	20.4	23.8	35.4	24.0	35.5	29.4	10.8	17.3	52.2	50.5	49.0	54.0	25.5	39.2	38.2	49.2
C18:3	27.0	23.1	22.1	27.4	22.5	16.3	*	-	7.5	7.1	12.2	7.8	27.0	*	20.1	5.7
C20:0	-	-	-	-	-	-	5.8	-	-	10.2	7.0	-	8.6	-	-	-
Unidentified fatty acids	-	-	-	-	8.8	-	8.7	*	-	-	*	-	-	*	9.5	16.4

* < 5.0%

-, not present

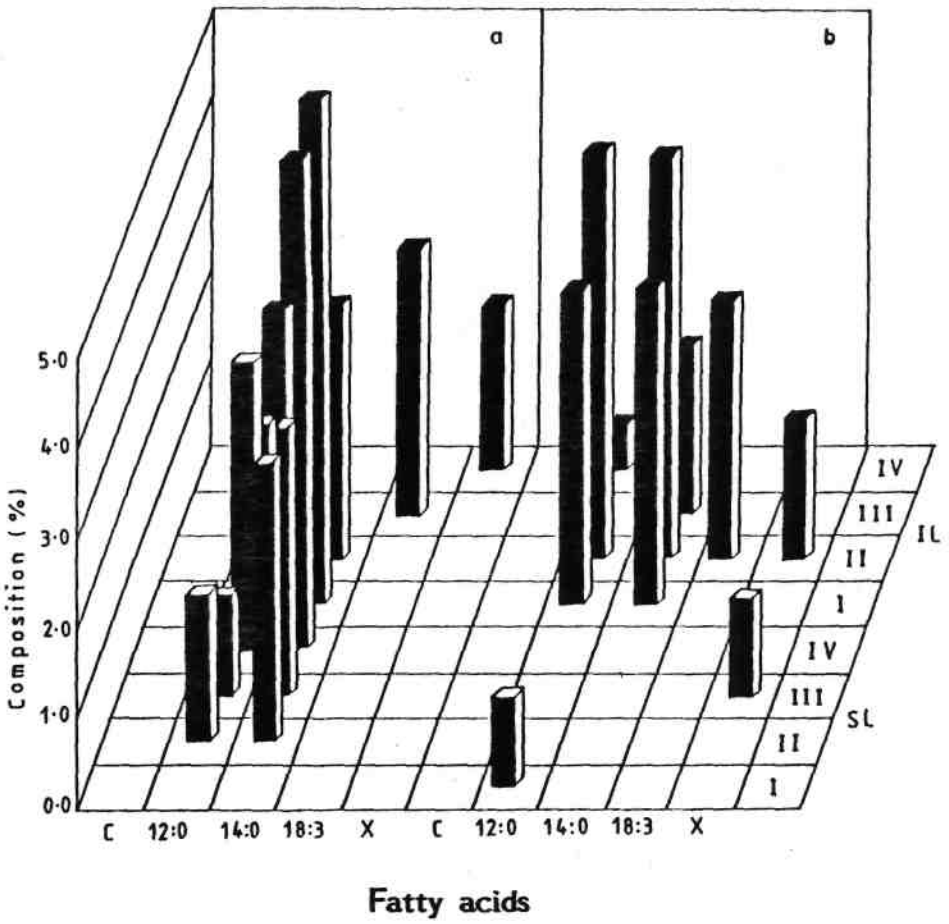


Fig.30V Minor fatty acids of SL and EL fractions of Re (a) and Rg (b) starches; X, unidentified fatty acids.

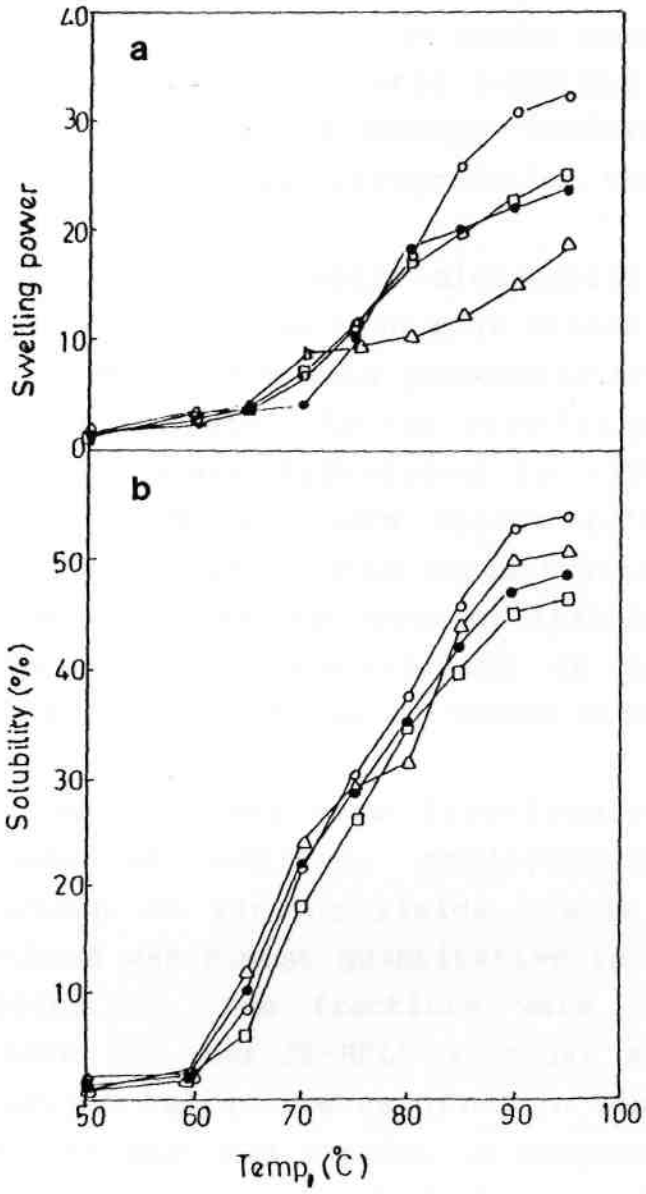


Fig.31. Swelling power (a) and % solubility (b) in water of Re and Rg starch isolates; RcS-I, -o-; RcS-II, -*-; RgS-I, -•- and RgS-II, -O-.

swelling power than those of isolate I. In DMSO the rice starch attained almost a total solubility in just -60 h compared to only -61% (and 45%) solubility for ragi starch isolate I (and isolate II, see Fig.32).

In the Brabender amylograph the ragi starch isolates (I/II) exhibited a slightly higher hot paste viscosity (-300 .B.U.) than those of rice starch isolates (-200 B.U.) [Table 12 and Fig.33]. Their set back viscosity increase was also minimal indicating a rather low retrogradation tendency.

With regard to their *in vitro* digestibility values, rice starch was more susceptible than ragi starch (Table 13). In the native state amylolysis with pancreatic α-amylase was better than with glucoamylase. In the gelatinized state the rice starch isolate I was hydrolysed to -78%, a value slightly higher than that for corn starch (-73%) used as control. However, at uniform starch concentration, probably all the isolates may get digested more or less to a similar extent. Nevertheless, the digestibility of cereal/millet starches was much better than those of legume starches.

As before the starches were fractionated by three different methods to amylose, amylopectin and the intermediate fraction in varying yields (Table 14) . The recovery of fractions was almost quantitative in the case of ragi starch isolate I. The fractions were found to be homogeneous by both GPC and SE-HPLC (Fig. 34) methods, and their molecular weight values are as given in Table 14. The intermediate fraction (Ax) was present in comparable amounts (-5.5%) in both ragi and rice starch isolates. The $^{}_{max}$ and the mol. wt. values of Ax fraction were in between those of pure amylose and amylopectin (see Table 14) . The λ_{mav} of Ax

Fig. 32. Solubility in DMSO of Re and Rg starch isolates; RcS-I, -o-; RcS-II, -Δ-; RgS-I, -•- and RgS-II, -*a*-.

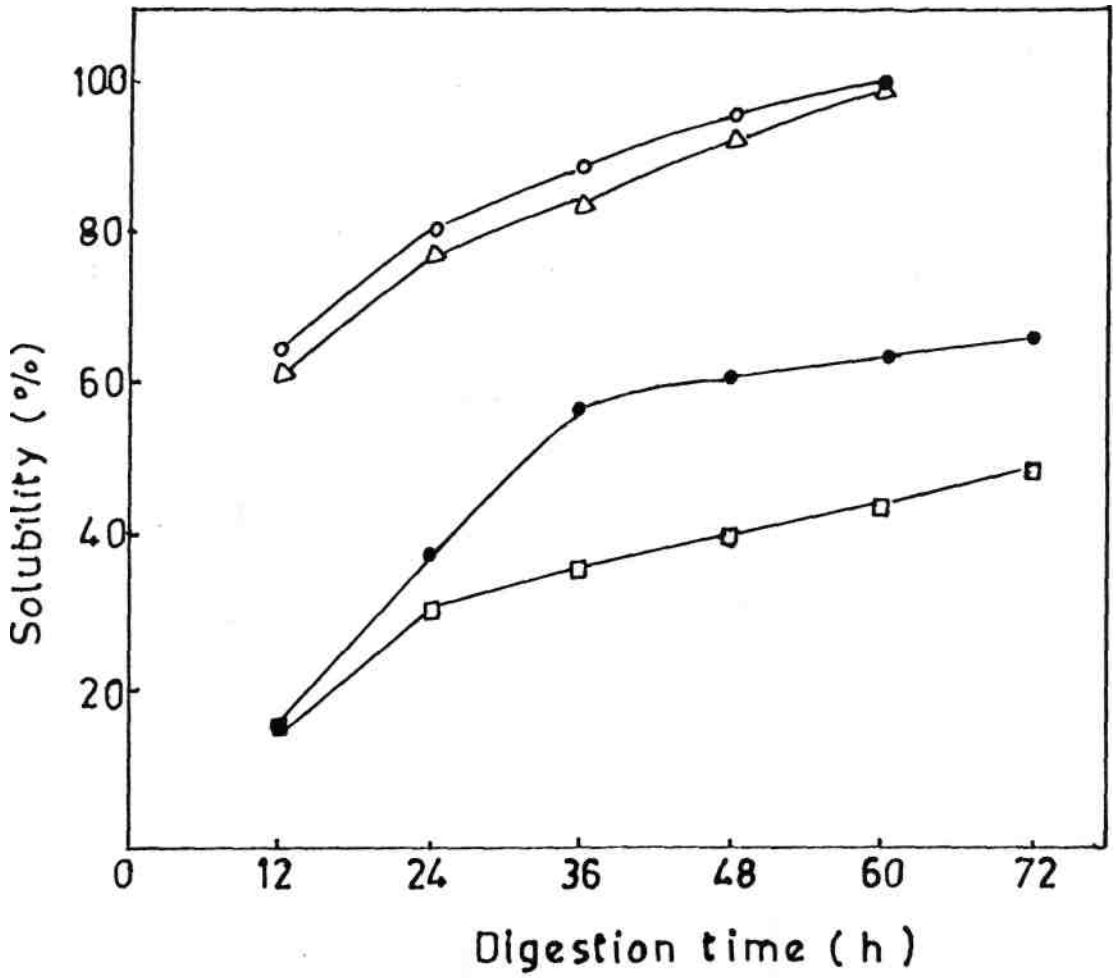


Table 12. Hot paste viscosity (B.U.) characteristics of major starch isolates

Isolate	PT, (°C)	P	H	C	C-H	H/P	C/P	C/H
Rc I	76.0	250	220	360	140	0.88	1.44	1.64
Rc II	83.5	200	200	320	120	1.00	1.60	1.60
Rg I	86.5	330	310	420	110	0.94	1.27	1.35
Rg II	80.5	280	250	380	130	0.89	1.36	1.52

PT = Pasting temperature
P = Peak viscosity
H = Hot paste viscosity
C = Cold paste viscosity
C-H = Set back
C/P = Set back ratio
C/H = Total set back ratio
H/P = Break down ratio

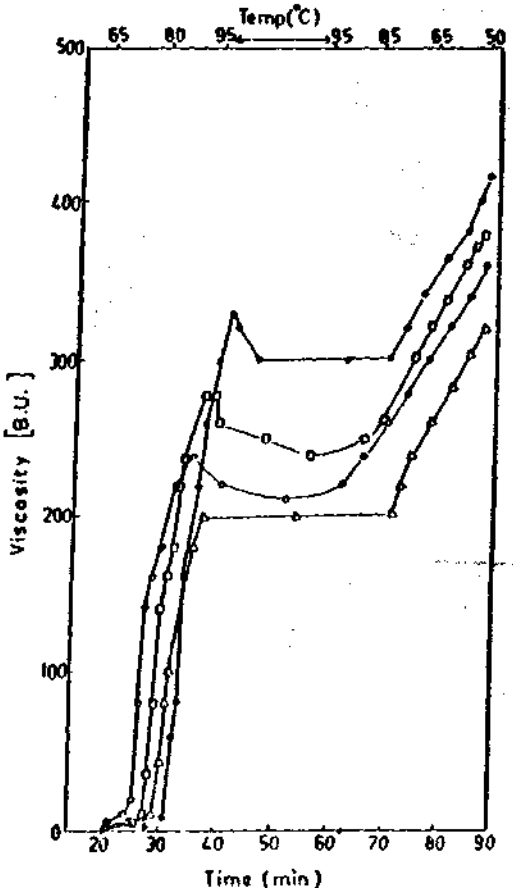


Fig. 33. Brabender amyíograph oí Rc and Rg starch isolates RcS-I, -o-; RcS-II, -*-; RgS-I, -«_ and RgS-Il, -D-.

Tabxe 13. *In vitro* digestibility of cereal starch isolates

Starch		% hydrolysis		
		Native		Gelatinized***
		Glucoamylase*	Pancreatic α-amylase**	
Corn starch		5.7	57.1	72.9
Rice starch	I	6.6	59.2	78.3
	II	ND	ND	71.5
	III	ND	ND	63.1
	IV	ND	ND	44.3
Ragi starch	I	5.8	55.8	70.2
	II	ND	ND	66.1
	III	ND	ND	51.0
	IV	ND	ND	30.8

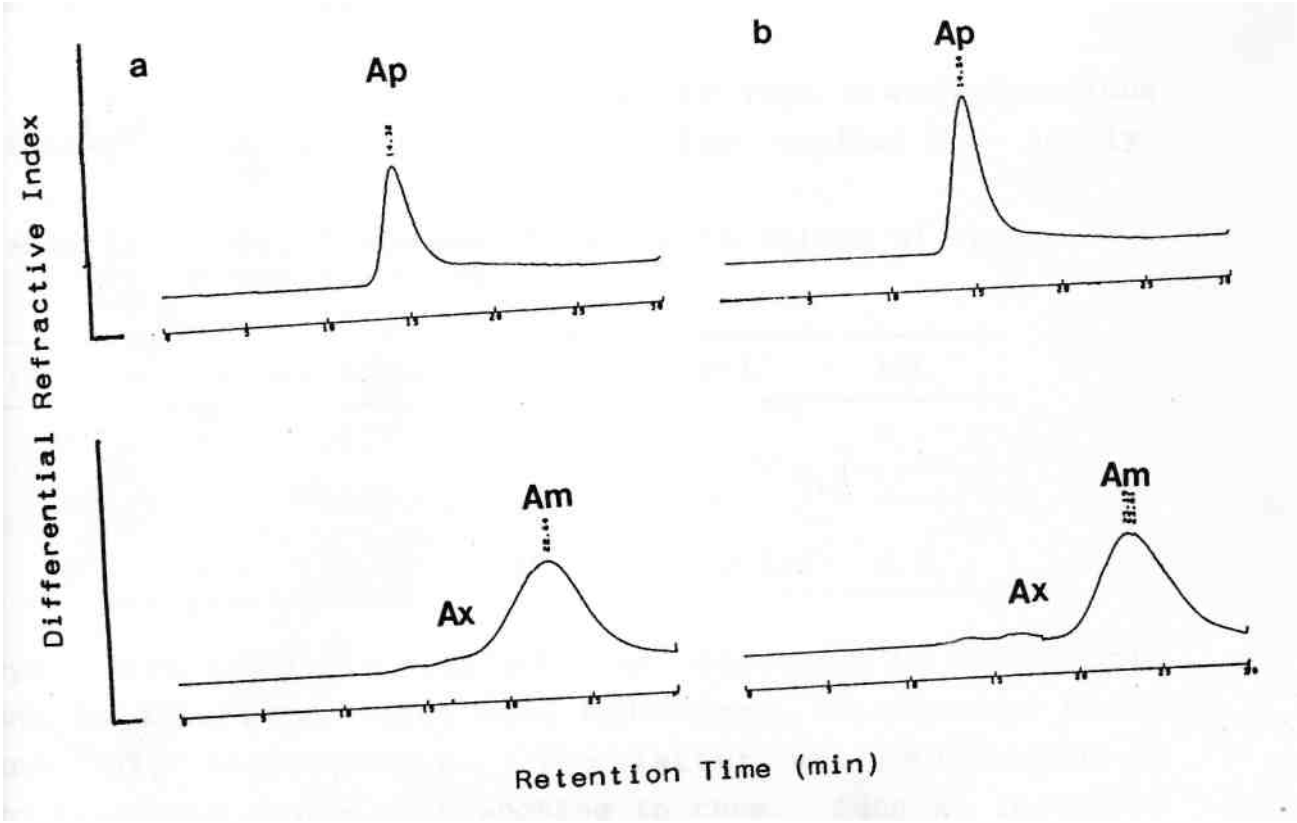
See Table 4 for details
ND, not determined

Table 14. Starch fractionation into amylose, amylopectin and intermediate fraction (%)

Source	RcS-I		RgS-I	
Fraction	Amy	Ap	Amy	Ap
Alkali-BuOH	17.4	60.7	16.0	77.0
DMSO-BuOH	24.2	63.0	19.3	73.0
DMSO-Con A	27.0	62.0	28.0	70.0
	Am = 21.5 Ax = 5.5		Am = 22.1 Ax = 5.9	
λ max (nm)	635	574 556	632	591 554
Mol. wt.	ND	ND ND	1.6×10^6	4.5×10^6 1.3×10^7
Blue value*	30.0	70.0	28.0	72.0

* Ap = 100 amylose
Amy = Amylose (crude)
Am = Amylose (pure)

ι. , , n« and b. Re starches, Fig. **3».SE-HPLC profiles ol Ap and Ax/Am of, a. Rg and respectively.**



derived from ragi starch was slightly higher (591 nm) than that of rice fraction (574 nm), and this might be attributed to a slightly higher proportion of long-extended B-chains in the former.

The limiting viscosity number of rice Am and Ap fractions was much smaller than those of ragi starch .fractions (see Fig. 35). This corroborated well with the amylography data of ragi starch.

From the β-amylolysis values of ragi starch fractions (Table 15) it was inferred that the amylose was nearly

Table 15. β-amylolysis and chain length values of ragi starch fractions

Fraction	β-amylolysis	CL	ECL	1CL
Am	93.7	-		
Ax	75.0	-		
Ap	52.1	20.7	12.1	6.3

hydrolysed (-94%) to completion in comparison to amylopectin and Ax fractions; which were hydrolysed, as expected to 52 and 75%, respectively. The latter was indicative of considerable degree of branching in them. Such an inference was substantiated by debranching the native fraction as well as their B-LDs with pullulanase. From the average chain length value of 19.4 for ragi amylopectin fraction its ECL and ICL values were deduced to be 12.1 and 6.3, respectively. Examination of the chain profiles after debranching revealed (Fig.36) multiple chains of wide DP value ranging from 17-48 for native (Fig.36a) and 3-30 for the derived β-LD (Fig.36b).

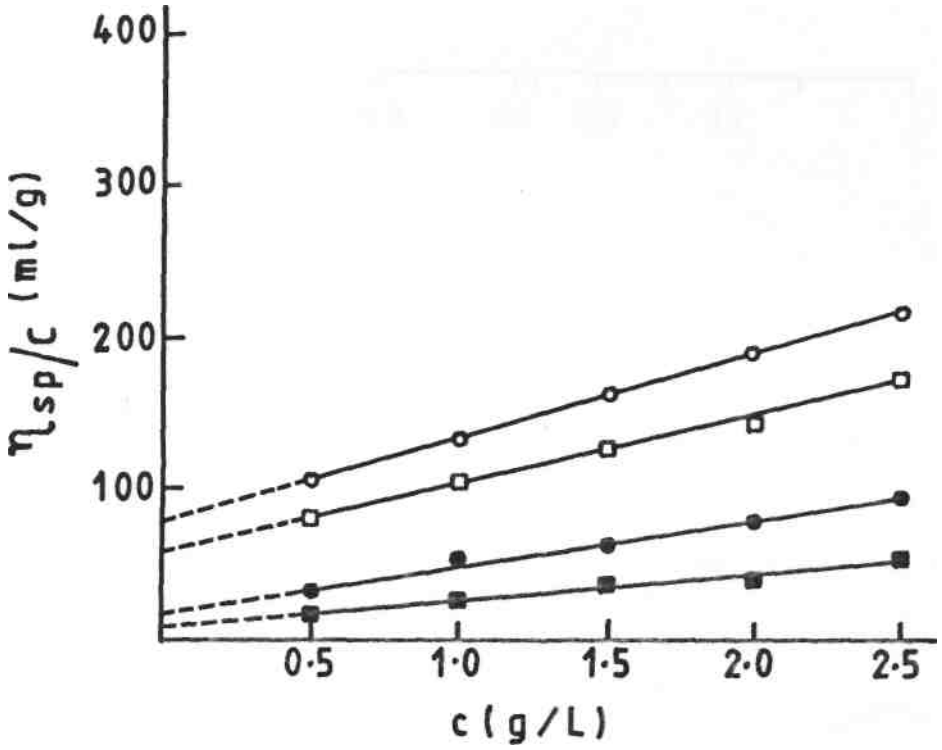


Fig-35. Limiting viscosity of, -o- = RgS-Ap; - o - = RcS-Ap; -+- - RgS-Am; and -■ - = RcS-Am.

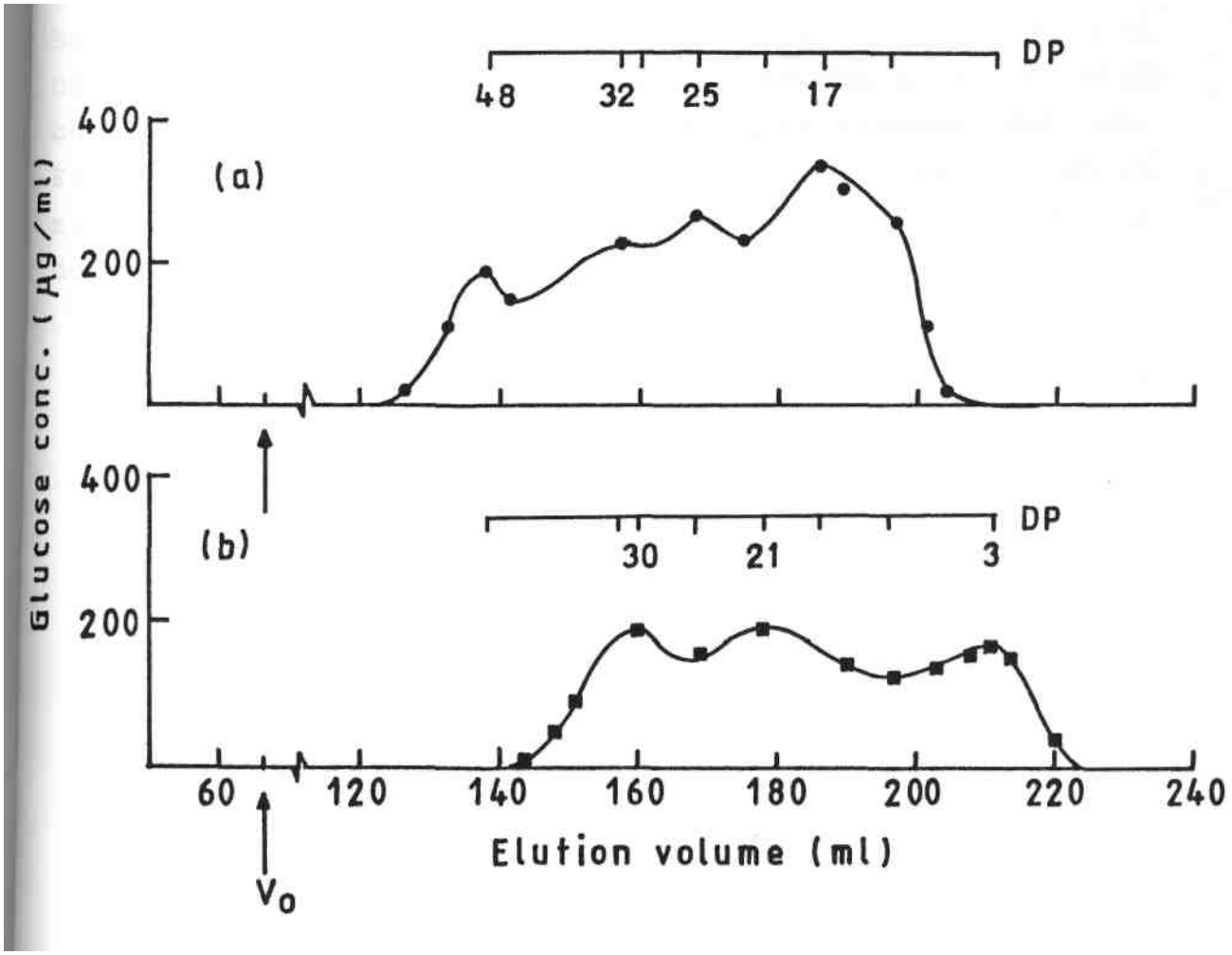
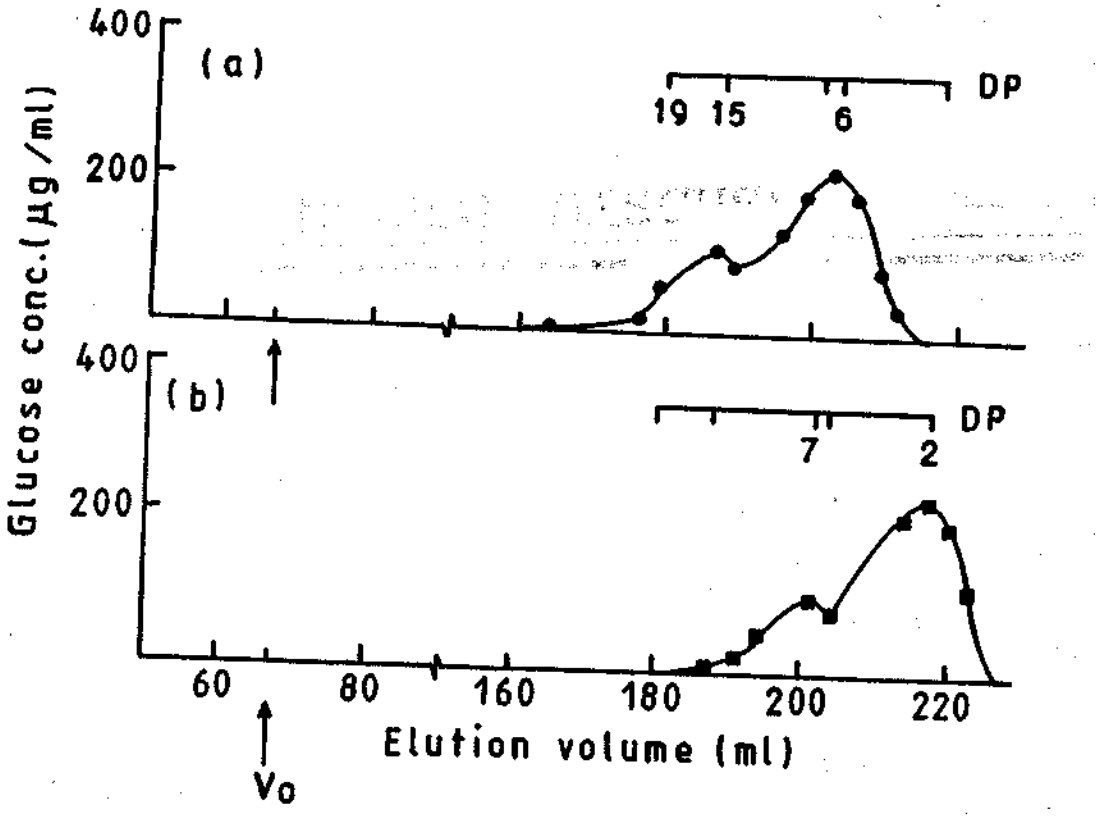


Fig.36. GPC profiles on Biogel P-10 of debranched Apia) and its 3-LD(b) of Rg starch.

The results are in consonance with the existence of short A-long A- / short B-, long B-chains in the ragi amylopectin molecule. On the otherhand, debranching of native as well as derived β-LD of Ax fraction gave profiles as shown in Fig.37a and 37b, respectively. In accordance with its limited branching the Ax molecule contained only short A-/B-chains of DP values 2-20. The absence of Vo peak in here indicated completion of both β-amylase and pullulanase actions. Especially, its β-LD showed on debranching a major peak of maltose (>80%) and a small peak of DP 7, which are possibly derived from short A-chain stubs.

Fig 37: GPC profiles on Biogel p-10 of debranched Ax(a) and its 3-LD (b) of Rg starch



GENERAL DISCUSSION

Among the storage carbohydrates starch is of prime importance because of quantity and distribution in the plant kingdom, and its uninterupted abundance relatively at a low cost. Starch is functionally an important biopolymer, in that it is generally composed of a range of α-D-glucans, i.e., from essentially linear to highly branched ones, and associated with varying amounts of non-starch constitutents. Accordingly there exists a characteristic distribution of starch granules of differing size and shape, and as such the granule distribution, has marked effects on the overall starch functionality attributes. It is likely that if due care is not taken starch granules of smaller sizes (especially of isolates III and IV) would have been lost during starch preparation and purification steps. The small granules are reported to contain a slightly higher amount of protein and amylose, and to have a better *in vitro* digestibility.[10] The latter could be attributed to their higher surface area and therefore increased accessibility to enzymes. It is not clear whether the small spherical granules are incompletely biosynthesized product of regular normal starch granules or indeed true small granules. In wheat[115] and a few other cereals a bimodal distribution of starch granule populations has been reported and it is stated that these various granule populations are under separate genetic control.[6,114,115] The birefringence characteristics exhibited by all the granules were indicative of a high degree of molecular orientation by the crystalline (and amorphous) regions within the granule.

Polymer-polymer interactions/associations are generally common in any naturally occuring macromolecular systems. The slightly higher content of glucose found by phenol-H_2SO_4 versus that by the glucose oxidase method[80] supports such

starch-non-starch carbohydrate association in the recovered starch isolates of GG, BG, Re and Rg. However, the possibility of the formation of any resistant starch[117,118] during starch preparative steps cannot be discounted at this stage. Invariably a portion of starch may get physically modified during storage (preisolation) and processing (starch isolation from the mill-ground flour) steps, and become inaccessible for *in vitro* α-amylolysis. Unlike in isolate IV wherein the GLC analysis of the starch hydrolyzate showed, in addition to a very big glucose peak, small peaks due to rhamnose, arabinose and xylose, no such sugar peaks could be detected in isolates I and II of all starches.

The protein content of legume starches was slightly higher than that of cereal starches. It is suggested that the starch granule surface may act as ion-exchanger to which the protein by virtue of its basic character may adhere.[29] Rigarous conditions are required indeed to extract the the firmly bound protein(s) in the granule.[99] Nevertheless, glycoprotein nature of starch proposed by Thorn et *al*[109] cannot be ruled out and lends support to the findings of small amount of protein even after repeated purification steps. Srivastava et *al*[119] report considerably a higher protein content (0.89%) in unfractionated total Bengalgram starch, whereas 0.68% protein is reported in greengram starch.[120,121]

Proteins, especially in the form of matrix encapsulating (some) starch granules, are shown to restrict *in vitro* digestibility of both raw starch and cooked wheat flour.[122] Starch-protein interactions have been- shown to reduce the overall digestibility of wheat in the human gastro-intestinal tract.[123] Nevertheless, a preliminary

incubation with pepsin, however has resulted in ready hydrolysis of swollen starch by a subsequent incubation with α-amylase. An improved correlation between the *in vitro* and *in vivo* digestibility results was thus possible when both pepsin and α(-amylase were used in the former assay, instead of α-amylase alone. The presence of protein structures encapsulating starch and restricting enzymic digestibility, has been reported for barley,[124] maize[125] and sorghum.[126] Such a situation has also been encountered in some legume starches, and prior denaturation or predigestion of the protein increases accessbility of α-amylase to the substrate. [127]

From the SDS-PAGE patterns it is evident that the granule-bound protein is atleast qualitatively similar in both Bg and Rg starches (Fig. 38) . Though less, SDS at 90° removed high molecular weight protains, -62-80 kD, contrary to what was observed in wheat starch where SDS treatment left proteins of ~59 kD and larger unextracted which later were removed by pronase treatment[99] . Very faint protein bands were seen in the Nacl and SDS at 50° extracts. Data shown in Table 16 indicate that quantitatively the aminoacid composition of legume and cereal starch-bound proteins is different. On a relative mole percent basis Bg starch protein contained more of aspartic and glutamic acids, whereas the Rg starch protein contained, in addition, high amounts of glycine. Conspicuously, methionine and cysteine were absent in Bg and histidine was absent in Rg starch protein fractions. The content of hydrophobic amino acid was high in Rg starch-protein, than in BG starch-protein, which was rich in polar amino acids.

Table 16. Amino acid composition (pmole %) of starch-bound proteins

Amino acid	Source BGS-I	Source RgS-I
Asp	20.5	10.5
Glu	21.7	12.8
Ser	6.7	7.7
Gly	7.3	13.5
Arg	5.7	3.7
Thr	3.3	4.0
Ala	5.2	9.1
Pro	4.9	7.2
Tyr	1.0	2.8
Val	4.4	6.0
Met	-	3.2
Cys	-	3.7
His	1.5	-
Ileu	3.5	2.3
Leu	6.3	5.8
Lys	3.9	4.7
Phe	3.9	2.6

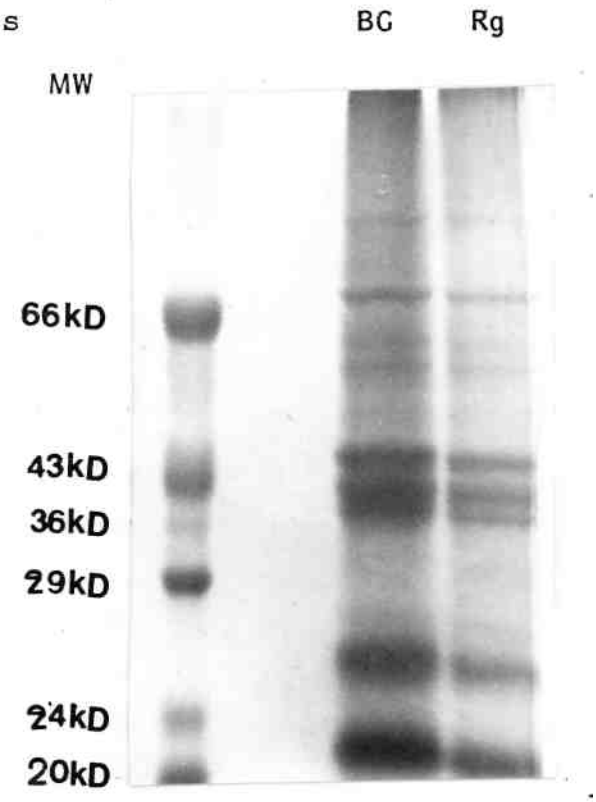


Fig.38. SDS-PAGE patterns of starch granule proteins extracted from BG and Rg starches.

On the contrary, the legume starch isolates contained more lipids (-1.5%) than the cereal starches (~0.5%). It is reported that high amylose starches (eg. legume starches) contain more lipids than normal starches, [37] and thus a positive correlation between the amylose and lipid contents has been proposed. The presence of high amounts of lipids confers resistance to mechanical damage on the granular surface, and possibly also towards amylolysis, [128] (see later), both *in vitro*[129,130] and *in vivo*[131] . In model studies, [131] potato amylose complexed with either lysolecithin (palmitic acid) or oleic acid was shown to be highly resistant to α(-amylase *in vitro*. Such amylose-lipid complexes are generally regarded as dietary fibre because of their low enzyme susceptibilities. The lower glucose and insulin responses in man after ingestion of high amylose rice compared to rice with no amylose has been attributed to the presence of amylose-lipid complexes in the former.[123] Though complexed amylose has low susceptibility to amyloglucosidase it is readily hydrolysable by the thermostable α-amylase, Termamyl, at high incubation temperatures. The existence of amylose-lipid complex within the granule, [133] though not unequivocally established, cannot be ruled out. Some times, the processing conditions (flour milling and drum drying) favour lipid-complex formation and thus restrict the enzymatic attack. [130] Nutritionally, the starch lipids are beneficial as they provide a considerable proportion of essential fatty acids, especially for vegetarians whose staple food constitutes cereals and legumes.

Analysis of lipids, surface and internally bound, as FAME indicated the predominance of palmitic acid in all starches. Occasionally other saturated and unsaturated fatty acids were also seen in these isolates. The biochemical

significance for the qualitative and quantitative differences descernible in these lipid fractions is not yet clearly understood. In a sense the surface lipids are considered as artefacts, whereas the internal lipids may act as templates for the synthesis of amylose helix and further that the lipid protects amylose chains during biosynthesis and that branching can only occur in chains that are not thus complexed.

Starch granules, in the presence of water and heat, swell enormously by imbibing water and finally burst open resulting in profound increase in solubility-viscosity. The measure of solubility and swelling power of starch is useful to understand the nature of associative bonding forces within the granule, lower the solubility the stronger are the bonding forces. In the present study both legume and cereal starches exhibited a single stage swelling, indicating relaxation of homogeneous and strong bonding forces within the granule at (one) higher temperature range. In comparison to ragi and rice starches (47-54% at 95°) , starches from Bengalgram and greengram showed a low solubility (33-38% at 95°) in water, like those of other leguminosae starches. [135,136] The solubility pattern of starch in DMSO is yet another method of knowing about the nature of granular bonding forces. [137] Re and Rg starch isolates I/II showed nearly a complete solubilization (~100% in 60 h) in comparison to BG and GG starch isolates (-72-88% in 60 h) . However, GG starch showed a better solubility than BG starch. Poor solubility of the latter is indicative of a strong resistant micellar structure, probably due to homogeneous bonding forces within the granules; whereas the higher solubility of cereal starches shows an easy penetration of solvent due to heterogenous bonding forces. A very high

solubility of ~90% in 20 h is reported for pigweed starch. [138] The solubility data in DMSO is also a measure of susceptibility to amylase action, higher the solubility better is the amylolytic digestibility. [93] With their low DMSO solubility legume starches were found to be less digestible, especially so in the case of Bengalgram starch.

Brabender viscograph is an instrument used to measure the hot paste viscosity of starches as a function of temperature, including both heating and cooling cycles and time. During a gradual but progressive heating the amylose molecules get disentangled because of hydrogen bond breaking by the thermal energy and leach out into the medium. Correspondingly there will be a steady increase in the starch slurry viscosity until it reaches a high value at the gelatinization temperature. During the subsequent cooling phase the linear amylose molecules are reassociated[139] by extensive hydrogen bonding and the viscosity again further increases. The latter phenomenon is known as retrogradation and the viscosity gain is refered to as set back viscosity. [140,141] The overall hot paste as well as the set back viscosities of isolates I/II of GG, BG, Re and Rg starches were generally low; the values for BG isolates were especially very low. Such a low value is indicative of restricted swelling due to a strong granular matrix of BG starch. All starches showed very low or negligible break down (P-H) suggesting that the granules do not rupture during continued heating and stirring. Their set back viscosities (C-H) were also minimal indicating a rather low retrogradation tendency. The relative break down ratio (H/P) for these isolates was negligible and the total set back ratio (C/H) of BG isolates was less in comparison to GG starch isolates. The low peak and set back viscosities of

the former corroborates well with its low swelling power in water/ DMSO and poor digestibility values (see later) . Differences in the pasting temperature and viscosity of starches, in general are attributable also to the adherance of hydrophobic proteins and surface lipids on the granules, which would affect the ability of granules to swell. [142] Especially lipids may be present as inclusion compounds in the form of amylose-lipid complexes and modify the physicochemical characteristics.[143] Infact removal of surface lipids by either propanol or 1-butanol extraction permitted substantial granule swelling at even low pasting temperatures[144]

BG and GG starches, because of their higher amylose content (~42%) showed a slightly higher λmax value (-625 nm) than the Re and Rg starches (-28% amylose having a λmax of -616 nm) (see Fig.39). Possibly the higher amylose content of legume starches, particularly the BG starch, may be attributed not only to the 'true' amylose content, but also to the contribution by the long extended/not much branched B-chains of amylopectin molecule to the overall colour yield. The intermediate fraction Ax should also have its contribution to the starch-I2 blue colour. Precise correlation between the chain length, molecular weight and the intensity (λmax) of the starch-I2 blue colour is not fully available.

Of the four starches studied, rice starch was more digestible and Bengalgram starch least digestible *in vitro.* Greengram starch was slightly better than BG starch, and this result was in agreement with its high solubility in DMSO and also its slightly lower 'total' amylose content. In the native state, amylolysis with pancreatic α-amylase was

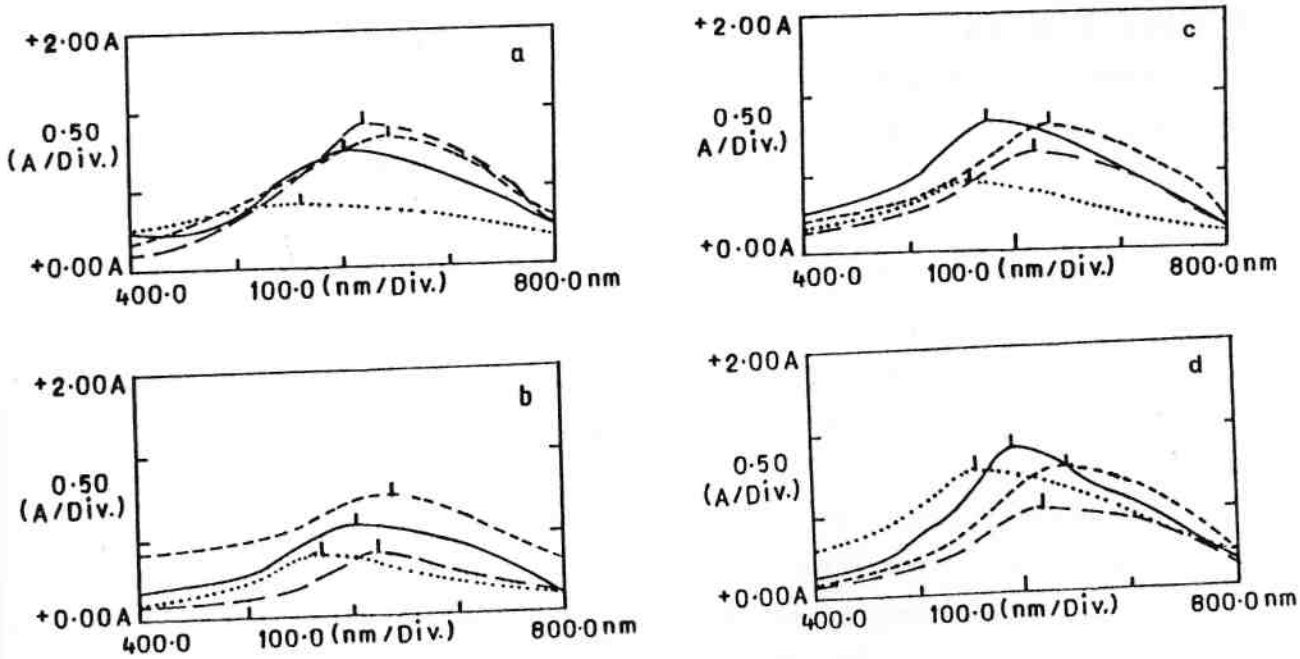


Fig. 39. λ_{max} of starch-I_2 blue colour of GG (a), BG (b), Rc (c) and Rg (d) starches and their fractions; —·— = starch; ... = Ap; – – – = Am and —— = Ax.

better than with glucoamylase. Legume starches, in general are known to be poorly digested and this could be related to their relatively high total amylose content,[120,145] as is the case in the present investigation. Their low solubility and swelling power in water, low cold and hot paste viscosity values, and their high content of non-starch constituents, viz., proteins, lipids, enzyme inhibitors, lectins, phytate, phenolic compounds, etc, would all contribute to the low *in vitro* (as well *in vivo)* digestibility values. [146] Partly the latter could also be accounted for by the resistant starch formed during sample preparation/purification steps. From the dietary energy point of view the role of resistant starch (RS) is very important. [128,147] RS behaves like dietary fibre, but its exact physiological significance/involvement is not clearly understood. RS is nothing but retrograded amylose and thus it appears reasonable that higher the content of amylose lower is the digestibility. [149,150] Indeed there exists a positive correlation between the amylose content and the formation of RS *in vitro.* [34,151] The fibrous material found after enzyme incubation of starch may likely constitute the dietary fibre (non-starch polysaccharide and RS) fraction associated with the starch. Nevertheless, the gelatinized starch suspensions were better hydrolysed than their native counterparts. This enhancement in starch digestibility of cooked starches may be attributed to the swelling and rupturing of starch granules, which facilitate a more randomised configuration for the enzyme to effect hydrolysis.[152] Increased *in vitro* digestibility of carbohydrates after cooking has been reported for several other legume starches such as chickpea, cowpea and horsegram. [153]

High amylose varieties of maize are poorly digested in both humans and mice.[154] It is shown that amylomaize starch is far more resistant to swelling and gelatinization than normal starches. Accordingly, it is reported that high amylose rice produces lower glucose and insulin responses in man than rice free of amylose. In legumes too starch availability has shown to be related to the amylose content.[155,156]

In general, the starch digestibility is dependent on the source as well as on the nature of starch per se. Some granular starches resist starch digestion much more strongly than others.' Factors that were shown to affect digestion in foods (starch) include degree of gelatinization, granule size, amylose-amylopectin ratio, starch-protein interaction, amylose-lipid complexing and availability of retrograded starch (i.e., RS). The lowering of digestion by the non-starchy carbohydrates is probably due to the non-specific adsorption of the enzyme molecules on to dietary fibre and/or entrapment of starch in the fibrous matrix. [122]

Further understanding, at a molecular level of the gross differences in the digestibility of legume and cereal starches is possible only after a rigarous fractionation followed by enzyme debranching studies. Classically starch fractionation is effected by the addition to starch solution in either alkali or DMSO of a polar organic solvent such as thymol or 1-butanol, which gives an insoluble complex with the amylose; and the amylopectin remaining in the supernatant solution.[56,75] These methods are, however, imperfect and calls for repeated precipitations to obtain pure fractions. This difficulty has been circumvented by utilizing newer methods of separation. One such method described recently

was to utilize the precipitation of amylopectin with the lectin concanavalin A. [57,157] From studies carried out on a number of polysaccharides, Goldstein et *al.*, [158] have suggested that all branched polysaccharides possessing multiple, terminal (non-reducing) α<-glucopyranosyl (and α<-mannopyranosyl or fructofuranosyl) groups would precipitate with Con A. A "chain-end" mechanism in which tetravalent •Con A, interacts with specific glycosyl residues of polysaccharide is shown in Fig.40. However, the reaction of Con A with starch is shown to be dependent on the lectin concentration, molecular weight, frequency and nature of branching residues and the exterior chain length. Amylose, with a single (or few) non-reducing end group per molecule does not allow multivalent association and produces no turbidity.

Though starch is generally known to be a mixture of amylose and amylopectin, there appears to be a range of structures with overlapping molecular sizes and possibly an intermediate fraction, which appears to be neither amylose nor amylopectin.[101] The actual content of these fractions vary with the source of the starch. The separated amylose, amylopectin and the intermediate fraction from all the starches were found to be pure by SE-HPLC as well as GPC on Sepharose CL-2B.

Limiting viscosity, which is a characteristic property of a molecule in a given solvent, is a vital parameter for interpreting the flow behaviour of polysaccharide solutions. Limiting viscosity depends essentially on the molecular weight and chain rigidity of the polymer as well "as on the nature of the solvent used. The amylose from Re and Rg starch isolate I showed relatively a lower limiting viscosity value than those of BG and GG amylose fractions.

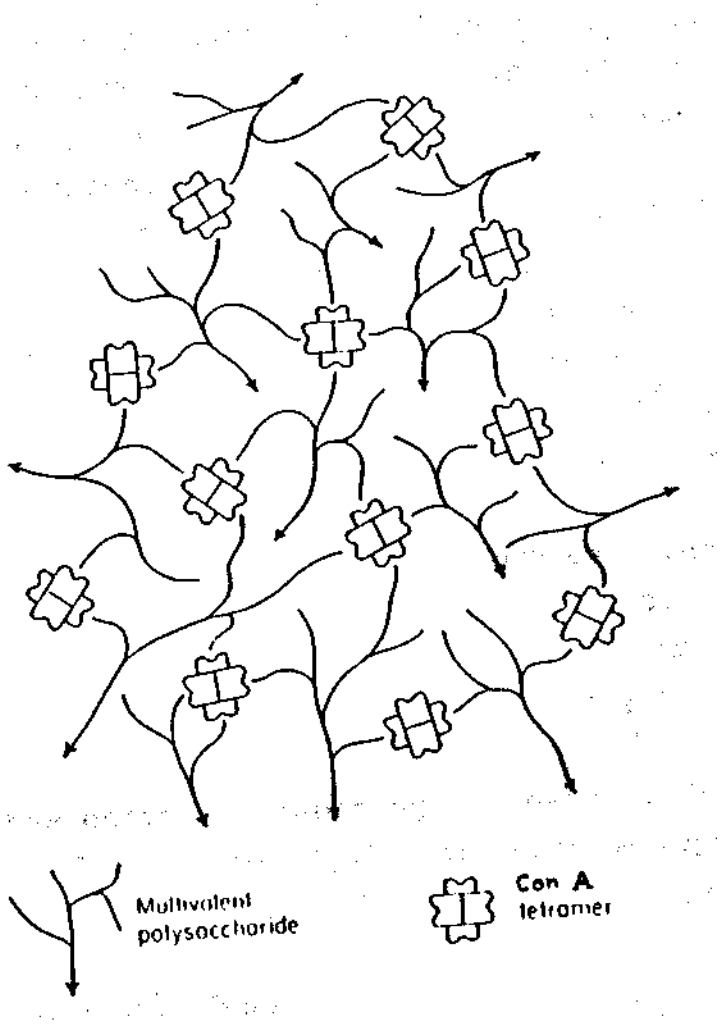


Fig.40. Diagrammatic representation of the chain-end mechanism of con A-polysaccharide interaction

The results of enzyme debranching studies of the native fractions as well as their derived β-limit dextrins of BG,GG and Rg starches appeared to be very dissimilar, both qualitatively and quantitatively. The low β-amylolysis limit value of Bengalgram amylopectin is in consonance with the values generally reported for a few other amylopectins, [159] and suggests extensive branching in the molecule than so in •cereal (ragi) starch fraction. Considerable differences were discernible also in the nature of branching in the intermediate fraction. The β-amylolysis limits of GG (88%) and BG (79%) amyloses do indicate them to be no more linear molecules, instead possess limited degree of branching. Unlike the amyloses of wheat, triticale and rye which had low jS-amylolysis value (77 to 82%),[102] the ragi amylose had a value of ~ 94%, which was more towards linearity. The incomplete conversion to maltose by β-amylase is attributed to the presence of (1 —>6) α-D-glucosidic linkages (branching) in the amylose molecule (see Fig.41).

From the enzyme debranching followed by GPC studies the multiplicity of A- and B- chains in both amylopectin and the intermediate fraction of starches could be understood. In the calculation of ECL and ICL of amylopectins an equal number of A- and B-chains has been assumed, but it may not be so as envisaged from the examination of their chain profiles after pullulanase debranching. Since A-chains are unbranched and B-chains are those to which one or more A-chains are attached, increased A-chain to B-chain ratio indicates increased multiplicity of branching. The amylopectin of triticale [102] with a A:B-chain ratio of 2.1 has greater extent of branching than other cereal starches. In the present study, the amylose fractions were found to contain only short, sparcely distributed A-chains, more so in the

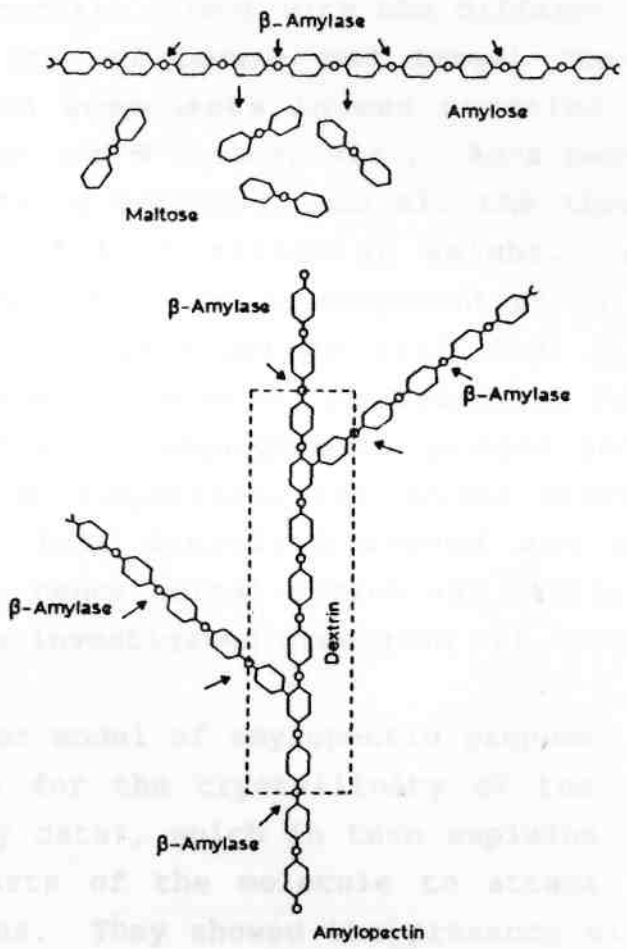


Fig. *1. Action pattern of S-amylase on starch fractions.

Source: G.Tegge, Glucose syrups - the raw material in glucose syrups; Science and Technology, S.Z.Dziedzie and M.W.Kearsley (Eds.), (Elsivier, London), 198^, pp. 9.

Bengalgram fraction. Pullulanase was the debranching enzyme mainly used in these studies. Another debranching enzyme commonly used is isoamylase, which is shown to readily and completely debranch both glycogen and amylopectin. [160]

The implications of these molecular details could be correlated to a certain extent with the differences in the *in vitro* digestibility of legume and cereal starches. The Bengalgram starch components indeed revealed a polymodal distribution of A- and B-chains, viz., Ap-a pentamodal, Am-a bimodal and Ax- a trimodal, and all the three fractions were relatively of high molecular weight. A plausible structural representation of Ax component of GG,BG and Rg is shown in Fig.42. These together with its high amylose content explains well the molecular reasoning for the low *in vitro* digestibility of Bengalgram in general and its starch in particular. In comparison, the cereal starch fractions were relatively less densely branched and also of low molecular weight; hence cereal starch was easily digestible. Of the two pulses investigated greengram was more digestible.

The cluster model of amylopectin proposed by Robin et *al* ., [61] accounts for the crystallinity of the molecule (as revealed by X-ray data), which in turn explains the relative resistance of parts of the molecule to attack by acid and amylolytic enzymes. They showed the presence of populations of chains having CL values of 15-20 in highly ordered clusters, which were linked additionally by long, extended B-chains. Variants to cluster model have also been proposed. Possibly these clusters, due to their extensive inter- and intra-hydrogen bonding capabilities, may constitute the strong crystalline or the junction zone and thus account for a rather slow accessibility for acid or

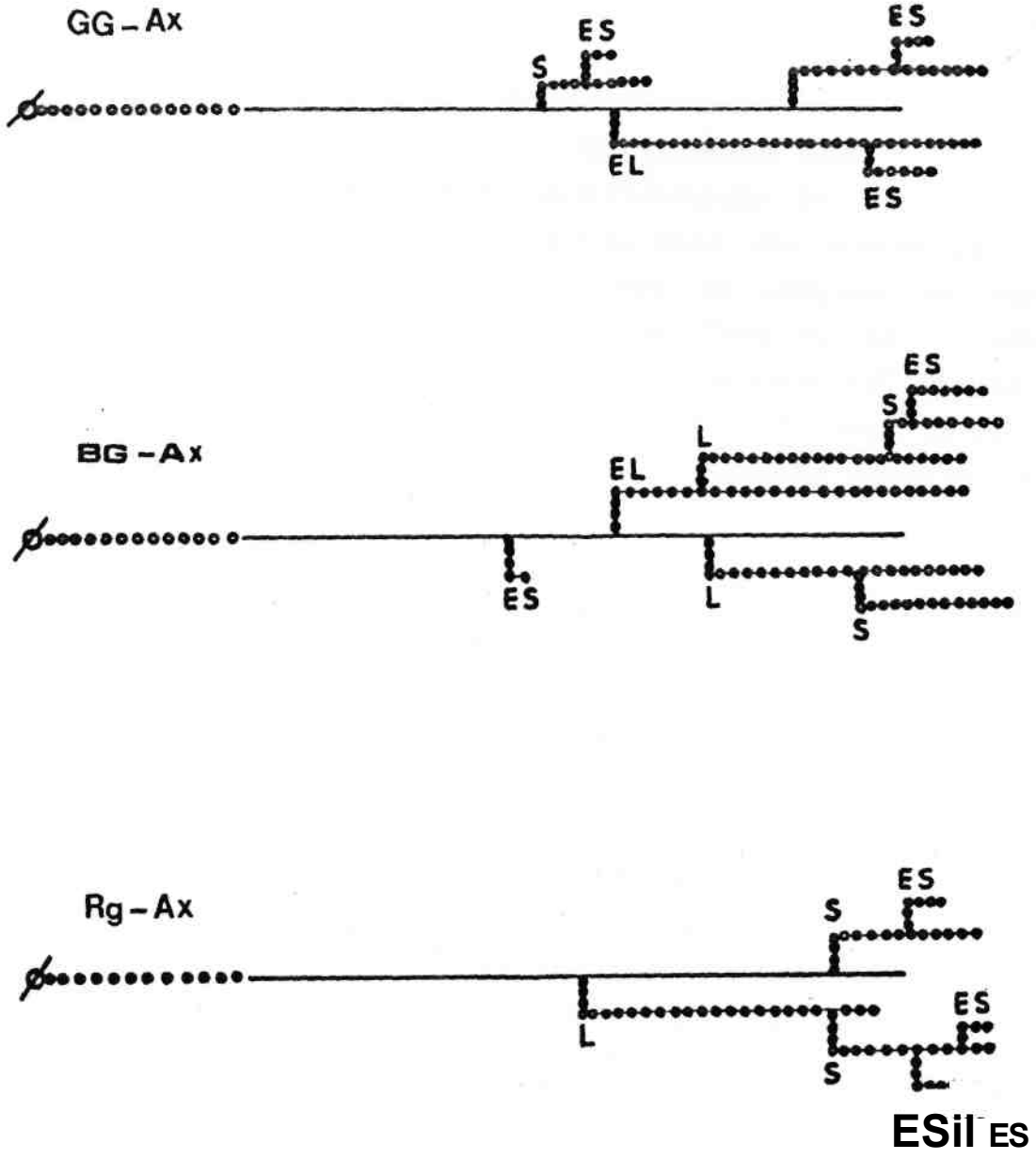


Fig. 42. Plausible structure of Ax derived from GG, BG and Rg starches.

enzyme to attack, in comparison to loose, amorphous zones, which are easily susceptible for hydrolysis.

The digestibility per *se* of the starchy foods in general could as well be due to the various other factors, viz., the presence of enzyme inhibitors, content of tannins, phytic acid, other non-starch constituents and also the .presence of any unusual substituent/linkages in the starch molecules. Several studies have shown that the structure of cell walls in legumes restrict access of enzymes to the starch. Dietary fibre present in the form of an intact structural network acts as a steric hinderance to enzymic attack. The nutritional significance of heat stable amylase inhibitors and antinutrients is probably greater in legumes than in cereals. [61]

In a study aimed at understanding the nature and utilization of carbohydrates four commonly consumed pulses, such as Bengalgram, greengram, redgram and blackgram were studied for *in vitro* digestibility as well as the role of gastric factors in enhancing the rate of digestibility. [155] In accordance with the present investigation carbohydrates of greengram were hydrolysed more rapidly as compared to other pulses. Both in the raw and cooked form greengram maintained its superiority over other pulses interms of rapid digestion. *In vivo* the enzymatic digestion of starchy foods takes place essentially by pancreatic α-amylase after a preliminary gastric digestion. [155] To simulate such a gastric digestion an experiment was designed to pretreat the pulse flour with hydrochloric acid and pepsin, prior to the action of pancreatic juice. [155] The results showed that the presence of such gastric factors greatly enhanced the low digestibility of Bengalgram carbohydrates, and thus the earlier observed

differences in digestion were considerably narrowed down. Their product analysis showed the most digested mass to be comprised of higher dextrins and maltotriose. Further studies showed the involvement, at least partly, of amylose, both qualitatively and quantitatively, in such digestibility values. [155] Greengram had -35% amylose of DP 667 (number of glucose residues per molecule) , whereas Bengalgram had -45% amylose of much higher DP (1667). These findings talley well with those observed in the present study.

The poor digestibility of Bengalgram is the primary cause for flatulence, which leads to bacterial fermentation by the gut bacteria. [162,163] Not only the non-reducing sugars such as raffinose, stachyose and verbascose present in Bengalgram are implicated in flatus production, but even the lower digestibility of Bengalgram starch could contribute to flatulence. [121] , This was also shown earlier by El Faki et *al* ., [164] who also reported the presence of an anti-flatus factor in legume husk fractions.

In conclusion, it may be argued that amylose plays a crucial role as far as the starch digestibility per *se* is concerned. Amylose does involve in a variety of *in situ* modifications, such as complexing with lipid, retrogradation and resistant starch formation, in addition to the differences in molecular size and molecular architecture, which all together decrease the starch digestibility as a whole. The subtle architectural variation due to side chain branches in the otherwise linear amylose molecule is another contributing factor for the low digestibility of legume starch. Such a phenomenon is not observed in waxy-starch varieties which are naturally low in or devoid of amylose content.

SUMMARY AND CONCLUSIONS

Starches were isolated from Bengalgram, greengram, rice and ragi flours with a view to study, at a molecular level the differences, in their *in vitro* digestibility values. The results revealed the following salient features.

* All the starch granules varied in size and shape characteristics; isolates I, II and III were rich in starch- carbohydrates, whereas the isolate IV was low in starch content, but contained rhamnose, arabinose and xylose in addition to glucose; the protein content of the isolates I and II was considerably lower than the latter isolates (III and IV); quantitatively internal lipids were more than surface lipids in legume than in cereal starches; palmitic acid was the predominent fatty acid identified; the amylose content of legume starches was more (-42%) than that of cereal starches (~28%); the starches were non-ionic.

* Legume starches, because of their high amylose content, showed considerably an increased absorption, λmax 625 nm in the starch-I2 blue colour test.

* Starch isolates I and II of both legumes and cereals exhibited a single stage swelling (in water); legume starches in comparison to cereal starches showed a lower solubility both in water and DMSO; Brabender viscography revealed a decreased paste viscosity as well as low retrogradation tendency; the limiting viscosity of legume starch amylose and amylopectin fractions was much more than those of cereal starch components.

* **In vitro** digestibility with glucoamylase and pancreatic α-amylase of both native and gelatinized starch suspensions varied considerably; Bengalgram starch digested least, whereas rice starch digested most.

* Of the three fractionation methods employed concanavalin A precipitation gave pure amylopectin (Ap) . The crude amylose fraction on subfractionation with hot 1- butanol yielded pure amylose (Am) and intermediate fraction (Ax); the latter was more in legume than in cereal starches; Ap, Am, and Ax fractions of all starches were homogeneous by SE-HPLC and GPC techniques; the molecular weight of legume starch fractions was much higher than their cereal counterparts.

* **The** β-amylolysis limit values of amylopectins were in the range of -52-56%; those of GG, BG and Rg amyloses were 88, 79 and 94%, respectively; whereas the intermediate fractions had β-amylolysis of 66, 62 and 75%, respectively; the results indicated some degree of branching in amylose fractions, more so in BG amylose.

* Pullulanase debranching of native Ap, Am and Ax as well as their β-LDs followed by the examination of chain profiles indicated polymodal distribution of chains; the Ap of BG had a very high molecular weight (2.2×10^7) and the molecule was comprised of very long B-chains (DP 70), long B-(DP 56), long A-/short B-(DP 25) and short A-(DP 17) chains; whereas the Ap of Rg was comparatively of low molecular weight (1.3×10^7) and having long B-(DP 48), short B-(DP 25) and short A-(DP 17) chains; gross dissimilarities were also observed in debranched Am and Ax

fractions of these starches; interestingly, Rg amylose was more or less a linear molecule unlike Am of BG and GG, which had some sparcely distributed side chain branches.

In conclusion, at a molecular level the scientific reasoning for the low digestibility of BG starch was (1) its high amylose content, (2) low solubility in water/DMSO, (3) more of non-starch contaminants, (4) high molecular weight of the component fractions Ap, Am, and Ax, (5) multiplicity of branching due to chains of different CL/DP values and (6) a sparcely branched Am molecule.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. R.N.Tharanathan, G.Muralikrishna, P.V.Salimath and M.R.Raghavendra Rao, *Indian. Acad. Sci. (Plant Sci.,* **97** (1987) 81.

2. R.L.Whistler, J.N.BeMiller and E.F.Paschall (Eds.), Starch-Chemistry and Technology (Academic Press, New York), 1984, 2nd edition.

3. J.C.Johnson, Industrial Starch Technology, Noyes Data Corp., New Jersey, USA, 1979.

4. P.Meredith, D.W.Baruch and L.D.Jenkins, *Staerke,* 29 (1977) 217.

5. J.L.Robutti, R.C.Hoseney and C.E.Wassom, *Cereal Chem.,* 51 (1974) 173.

6. D.Paton, *Staerke,* 31 (1979) 184.

7. W.Banks, C.T.Greenwood and D.D.Muir, *Staerke,* 26 (1974) 46.

8. A.C.Eliasson, K.Larsson, S.Andersson, S.T.Hyde, R.Nesper and H.G.Von schnering, Staerλe, 39 (1987) 147.

9. W.Banks and D.D.Muir, Structure and Chemistry of Starch Granule, in the Biochemistry of Plants, J.Preiss (Ed.,) (Academic press, New York), 3, 1980, pp.321.

10. J.R.Stark and X.S.Yin, *Staerke,* 38 (1986) 369.

11. L.C.Macleod and C.M.Duffus, J. *Cereal Sci.,* 8 (1988) 29.

12. R.Karlsson, R.Olered and A.C.Eliasson, *Staerke,* 35 (1983) 335.

13. S.N.Moorthy and T.Ramanujam, *Staerke,* 38 (1986) 58.

14. U.Ramadas Bhat and R.N.Tharanathan, Staerλe, 35 (1983) 189.

15. S.Hizukuri, Y.Takeda, N.Maruta and B.O.Juliano, *Carbohydr. Res.,* **189** (1989) 227.

16. S.V.Paramahans and R.N.Tharanathan, *Staerke,* 34 (1982) 73.

17. K.H.Moledina, P.Fedec, D.Hadziyev and B.Oovaikul, *Staerke,* 30 (1978) 191.

18. K.J.Goering, P.V.Subba Rao, D.H-.Fritts and J.Carroll, *Staerke,* 22 (1970) 217.

19. A.Th.Czaja, *Taxon.,* 27 (1978) 463.

20. A.D.Evers and E.E.McDermott, *Staerke, 22* (1970) 23.

21. P.Bowler, M.R.Williams and R.E.Angold, *Staerke,* 32 (1980) 186.

22. K.Ghiasi, R.C.Hoseney and E.V.Maiston, Cereal *Chem.,* 59 (1982) 81.

23. S.P.Moulik and S.Gupta, *Carbohydr. Res.,* **125** (1984) 340.

24. V.J.Morris, in Functional Properties of Food Macromolecules, J.R.Mitchell and D.A.Ledward (Eds.,). (Elsevier, London), 1986, pp. 121; J.R.Katz, *Baker's Weekly,* 81 (1934) 34 .

25. U.Matsukura, A.Matsunaga and K.Kainuma, *J. Jpn. Soc. Starch Sci.,* 30 (1983) 106.

26. A.Matsunaga and K.Kainuma, Staerke, 38 (1986) 1.

27. P.Bowler, P.J.Towersey, S.G.Waight and T.Galliard, Minor Components of Wheat Starch and Their Technological Significance, in New Approaches to Research on Cereal Carbohydrates, R.D.Hill and L.Munck (Eds.), (Elsevier, Amsterdam), 1985, pp-71.

28. D.H.Simmonds, K.K.Barlow and C.W.Wringley, Cereal *Chem.,* 50 (1973) 553.

29. G.D.A.Lowy, J.G.Sargeant and J.D.Schofield, *J. Sci. Food Agric,* 32 (1981) 371.

30. R.C.Hoseney and P.A.Seib, Bakers Digest, 47 (1973) 26.

31. P.Greenwell and J.D.Schofield, Cereal *Chem.,* 63 (1986) 379.

32. S.H.Imam, *J. Cereal Sci.,* 9 (1989) 231.

32a. J.O.Brieu and P.A.Morrissey, *Critical Reviews in Food Sci. Nutr.,* 28 (1989) 211.

33. B.Madhusudhan, N.S.Susheelamma and R.N.Tharanathan, *Staerke,* 45 (1993) 8.

34. C.S.Berry, *J. Cereal Sci.,* 4 (1986) 301.

35. M.Maleki, R.C.Hoseney and P.J.Mattern, *Cereal Chem.,* 57 (1980) 138.

36. S.P.Cauvain, B.M.Gough and M.E.Whitehouse, *Staerke,* 29 (1977) 91.

37. W.R.Morrison, *J.* Cereal *Sci.,* 8 (1988) 1.

38 A.C.Eliasson, T.L.G.Carlson, K.Larsson and Y.Meizis, Staerλe, 33 (1981) 130.

39. T.L.G.Carlson, N.D-Nguyen, N.Krog and K.Larsson, *Staerke,* 31 (1979) 222.

40. J.Karkalas and S .Raphaelides, Carbohydr. *Res.,* 147 (1986) 215.

41. W.R.Morrison, *J. Sci. Food Agric,* 29 (1978) 365.

42. D.J.Baisted, Starch Lipids in Barley and Malt, in Lipids in Cereal Technology. P.J.Humes (Ed.)., (Academic Press, New York), 1983, pp-93.

43. A.W.MacGregor and J.E.Morgan, Cereal *Foods World,* **31** (1986) 638.

44. H.Fuwa, Y.Sugimoto, T.Takeya and Z.Nikuni, *Carbohydr. Res.,* 70 (1979) 233.

45. T.A.Kuracina, K.Lorenz and K.Kulp, Cereal *Chem.,* 64 (1987) 182.

46. J.S.Smith and D.R.Lineback, *Staerke,* 28 (1976) 243.

47. P.L.Chang Rupp and S.J.Schwartz, *J. Food Biochem.,* 12 (1988) 191.

48. S.Kobayashi, S.J.Schwartz and D.R.Lineback, *J. Chromatog.,* **319** (1985) 205.

49. R.N.Tharanathan, S.V.Paramahans and D.B.Wankhede, Staerλe, 32 (1980) 158.

50. H.Fuwa, D.V.Glover, Y.Sugimoto, R.Nishimura and M.Tanaka, *Staerke,* 30 (1978) 367.

51. R.N.Tharanathan and U.Ramadas Bhat, *Staerke,* 40 (1988) 378.

52. W.Banks and C.T.Greenwood, *Staerke,* 19 (1967) 197.

53. J.G.Sargeant, Staerke, 34 (1982) 89.

54. Y.Takeda, C.Takeda, A.Hizukuri and S.Hizukuri, *J. Food Sci.,* 54 (1989) 177.

55. D.J.Manners, The Enzymic Degradation of Starches, in Polysaccharides in Food, J.M.V.Blanshard and J.R.Mitchell (Eds.), (Butterworths, London), 1979, pp.75.

56. T.J.Schoch, J. Aril. *Chem. Soc,* 64 (1942) 2957.

57. S.H.Yun and N.K.Matheson, Staerke, 42 (1990) 302.

58. N.K.Matheson, *Carbohydr. Res.,* **180** (1988) 301.

59. M.Taki, *Agric. Biol. Chem.,* 26 (1962) 1.

60. D.French. Organization of Starch Granules, in Starch: Chemistry and Technology, R.L.Whistler, J.N.BeMiller and E.F.Paschall (Eds.), (Academic Press, New York), 1984, 2nd edition Chapter 7.

61. J.P.Robin, C.Mercier, R.Charbonniere and A.Guilbot, Cereal *Chem.,* 51 (1974) 389.

62. D.J.Manners, Structural Analysis of Starch Components by Debranching Enzymes, in New Approaches to Research on Cereal Carbohydrates, R.D.Hill and L.Munck (Eds.), (Elsevier, Amsterdam), 1985, pp.45.

63. S.Peat, W.J.Whelan and G.J.Thomas, J. *Chem. Soc,* (1956) 3025.

64. D.J.Manners, *Carbohydr. Polym.,* 16 (1991) 37.

65. N.B.Petersen, Edible starches and Starch Derived Syrups, Noyes Data Corp., New Jersy, USA, 1975.

66. Anonymous, *Food Technol. Newzealand,* 27 (1992) 9.

67. T.V.Jaya, H.S.Naxk and L.V.Venkataraman, *Nutr. Rep. Internat.,* 20 (1979) 393 and references cited therein.

68. Guo-Hua Feng, M.Chen, K.J.Kramer and G.R.Reeck, Cereal *Chem.,* 68 (1991) 95.

69. B.C.Fish and L.U.Thompson, *J. Agric. Food Chem.,* **39** (1991) 727.

70. R.D.M.Prentice, J.R.Stark and M.J.Gidley, *Carbohydr. Res.,* **227** (1992) 121.

71. T.J.Schoch and E.C.Maywald, *Anal. Chem.,* **28** (1956) 382.

72. D.G.Medcalf and K.A.Gilles, Cereal *Chem.,* 42 (1965) 558.

73. A.O.A.C Official Methods of Analysis, 1975, 12th edition, Washington D.C., USA

74. R.J.Smith and J.L.Caruso, *Methods Carbohydr. Chem.,* 4 (1964) 42.

75. W.Banks and C.T.Greenwood, *Staerke,* **19** (1967) 394.

76. Y.Takeda, T.Shitaozono and S.Hizukuri, *Carbohydr. Res.,* **199** (1990) 207.

77. M.Dubois, K.A.Gilles, J.K.Hamilton, P.A.Rebers and F.Smith, *Anal. Chem.,* **28** (1956) 350.

78. P.Rao and T.N.Pattabiraman, *Anal. Biochem.,* **181** (1989) 18.

79. N.Nelson, *J. Biol. Chem.,* **153** (1944) 375.

80. A.Dahlqvist, *Anal. Biochem.,* 7 (1964) 18.

81. R.M.McCready and W.E.Hassid, J. Am. *Chem. Soc,* 65 (1943) 1154.

82. J.Chrastil, *Carbohydr. Res.,* **159** (1987)154.

83. G.A.Gilbert and S.P.Spragg, *Methods Carbohydr. Chem.,* 4 (1964) 168.

84. O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, *J.Biol. Chem.,* 193 (1951) 265.

85. A.O.A.O.C Official Methods of Analysis, 1960, 9th Edition, Washington D.C., USA

86. D.B.Wankhede, R.Saroja and M.R.Raghavendra Rao, *Staerke,* 29 (1977) 223.

87. L.D.Metcalfe, A.A.Schmitz and J.R.Pelka, *Anal. Chem.,* 38 (1966) 514.

88. D.Van Wijngaarden, *Anal. Chem.,* 39 (1967) 848.

89. R.R.Myers and R.J.Smith, *Methods Carbohydr. Chem.,* 4 (1964) 124.

90. A.Suzuki, S.Hizukuri and Y.Takeda, Cereal *Chem.,* 58 (1981) 286.

91. E.G.Mazurs, T.J.Schoch and F.E.Kite, Cereal *Chem.,* 34 (1957) **141.**

92. H.W.Leach, L.D.McCowen and T.J.Schoch, *Cereal Chem.,* 36 (1959) 534.

93. H.W.Leach and T.J.Schoch, Cereal *Chem.,* 39 (1962) 318.

94. S.V.Paramahans and R.N.Tharanathan, *Stareke,* 32 (1980) 73.

95. S.M.Partridge, Nature, **164** (19) 443.

96. J.S.Sawardekar, J.H.Sloneker and A.Jeanes, *Anal. Chem.,* 37 (1965) 1602.

97. H.Bruun and H.Henriksnae, *Staerke,* 29 (1977) 122.

98. G.A.Brown and J.J.Volenec, *Staerke,* 41 (1989) 247.

99. R.B.Malouf, W.D.A.Lin and R.C.Hoseney, Cereal *Chem.,* 69 (1992) 169.

99a. U.K.Laemmlis, *Nature,* **227** (1970) 680.

99b. Waters, PICO-TAG Amino acid analy-sis system operation mannual (1986), No.88140, Waters Publications, Massachusetts, USA

99c. W.J.Whelan, *Methods Carbohydr. Chem.,* 4 (1964) 252.

100. W.A.Atwell, R.C.Hoseney and D.R.Lineback, Cereal Chejn., **57** (1980) 12.

101. C.G.Biliaderis, D.R.Grant and J.R.Vose, Cereal *Chem.,* 58 (1981) 496.

102. C.Y.Lii and D.R.Lineback, *Cereal Chem.,* **54** (1977) 138.

103. F.R.T.Rosenthal, T.Nakamura, A.M.C.Espindola and M.R.Jochimek, *Staerke,* 26 (1974) 50.

104. Y.Sugimoto, K.Yamada, S.Sakamoto and H.Fuwa, *Staerke,* 33 (1981) 112.

105. B.O.Juliano, R.M.Villareal, C.M.Perez and C.P.Villareal, Y.Takeda and S.Hizukuri, *Staerke,* 39 (1987) 390.

106. G.Changala Reddy, N.S.Susheelamma and R.N.Tharanathan, *Staerke,* **41** (1989) 84.

106a. J.Szczodrak and Y.Pomeranz, Cereal *Chem.,* 68 (1991) 589.

107. R.S.Tipson and D.Horton, Adv. *Carbohydr. Chem. Biochem.,* 30 (1974) 257.

108. W.Thorn and S.Mohazzeb, Staerke, **42** (1990) 455.

109. W.Thorn and S.Mohazzeb, *Staerke,* **42** (1990) 373.

110. E.Y.C.Lee, C.Mercier and W.J.Whelan, Arch. *Biochem. Biopys.,* **125** (1968) 1028.

111. S.Hizukuri, Carbohydr. *Res.,* **147** (1986) 342.

112. N.Inouchi, D.V.Glover and H.Fuwa, Staerke, 39 (1987) 259.

113. D.J.Manners, *Carbohydr. Polym.,* **11** (1989) 87.

114. V.Rasper, *J. Sci. Food* Agric, **22** (1971) 572.

115. H.Dengate and P.Meredith, J. Cereal *Sci.,* 2 (1984) 83.

116. I.Larsson and A.C.Eliasson, Staerice, 43 (1991) 227.

117. F.S-Calixto, I.Goni, L.Bravo and E.Manas, J. *Food Sci.,* 58 (1993) 642.

118. C.S.Berry, K.I.Anson, M.J.Miles, V.J.Morris and P.L.Russell, *J. Cereal Sci.,* 8 (1988) 203.

119. H.C.Srivastava, S.N.Harshe, M.M.Gharia and G.P.Mudia, *Staerke,* 22 (1970) 162.

120. K.Ganesh Kumar and L.V.Venkataraman, *Nutr. Rep. Internat.,* **13** (1976) 115.

121. K.S.Shurpalekar, O.E.Sundaravalli and M.Narayana Rao, *Nutr. Rep. Internat.,* 19 (1979) 111.

122. J.Holm, I.Bjorck, N.G.Asp, L.B.Sjoberg and I.Lundquist, *J. Ceral Sci.,* 3 (1985) 193.

123.I.H.Anderson, A.S.Levine and M.D.Levitt, *New England* J» *Med.,* **304** (1981) 891.

124. P.T.Slack, E.D.Baxter and T.Wainwright, *J. Inst. Brew.,* 85 (1979) 112.

125. V.P.V.Twisk, *Staerke,* 22 (1970) 228.

126. J.W.McNeill, G.D.Potter, J.K.Riggs and L.W.Rooney, J. *Anim. Sci.,* **40** (1975) 335.

127. P.Wursch, S.Del Vedovo and B.Koellreutter, *Am. J. Clin. Nutr.,* **43** (1986) 25.

128. S.G.Ring, J.M.Gee, W.Whittam, P.Orford and I.T.Johnson, *Food Chem.,* 28 (1988) 97.

129. H.Van Lonkhuysen and J.Blankestijn, *Staerke,* 28 (1976) 227

130. K.Larsson and Y.Miezis, *Staerke,* **31** (1979) 301.

131. J.Holm, I.Bjorck, O.Ostrowska, A.C.Elisson, N.G.Asp, K.Larsson and I.Lundquist, *Staerke,* 35 (1983) 294.

132. M.S.Goddard, G.Young and R.Marcus, Am. *J. Clin. Nutr.,* 39 (1984) 388.

133. J.W.Cone and M.G.E.Wolters, *Staerke,* 42 (1990) 298.

134. F.R.T. Rosenthal, L.Espindola, M.I.S.Serapiao and S.M.O.Silva, *Staerke,* 23 (71) 18.

135. K.Minagawa T.Ohkura, G.Goshima, Y.Miwa and H.Tsuge, *Staerke,* 39 (1987) 241.

136. C.C.Lai and E.V.Marston, J. *Food Sci.,* 44 (1979) 528.

137. L.E.Butler, D.D.Christianson, J.C.Scheerens and J.W.Berry, Starelle, 38 (1986) 156.

138. K.J.Goering, StaerJce, 30 (1978) 181.

139. M.L.Martin, K.J.Zeleznak and R.C.Hoseney, Cereal *Chem.,* 68 (1991) 498.

140. Y.C.Shi and P.A.Seib, Carbohydr. *Res.,* **227** (1992) 131.

141. T.Noel, S.G.Ring and M.Whittam., *Food Sci.Technol. Today,* 6 (1992) 159.

142. L.M.Hansen, R.C.Hoseney and J.M.Faubion, Cereal *Chem.,* 68 (1991) 347.

143. W.R.Morrison, Staerle, 33 (1981) 408.

144. F.Sosulski, W.Waczkowski and R.Hoover, *Staerke,* 41 (1989) 135.

145. C.W.Glennie, Staerke, 39 (1987) 273.

146. Y.P.Gupta, *Plant Foods Hum. Nutr.,* 37 (1987) 201.

147. J.H.Cummings, Lancet, 1 (1983) 1206.

148. Anonymous, *Food Sci. Technol. Today,* 7 (1993) 77.

149. L.Gruchala and Y.Pomeranz, *Cereal Chem.,* 70 (1993) 163.

150. J.Szczodrak and Y.Pomeranz, *Cereal Chem.,* 69 (1992) 626.

151. D.Sievert and Y.Pomeranz, Cereal *Chem.,* 66 (1989) 342.

152. P.C.Lee, S.P.Brooks, O.Kim, L.A.Heitlinger and E.Lebenthal, J. *Nutr.,* **115** (1985) 93.

153. H.A.E1 Faki, Ph.D.Thesis, University of Mysore, (1981).

154. M.J.Wolf, U.Khoo and G.E.Inglett, StaerJce, 29 (1977) 401.

155. P.Srinivasa Rao, *Nutrition News,* NIN, Hyderabad, (India), 9 (1988) 1.

156. E.Jyothi and P.R.Reddy, Nutr. *Rep.Internat.*, 23 (1981) 799.

157. R.J.Doyle, *Staerke,* 22 (1970) 317.

158. I.J.Goldstein, C.E.Hollerman and J.M.Merrick. *Biochem. Biophys. Acta.,* 97 (1965) 68.

159. L.Slominska and M.Maczynski, *Staerke,* 37 (1985) 386.

160. H.Akai, K.Yokobayashi, A.Misaki and T.Harada, Biochem. *Biophys. Acta.,* 252 (1971) 427.

161. L.V.Thompson, J.H.Yoon, D.J.A.Jenkins, T.M.S.Wolever and A.L.Jenkins, Am. .J. *Clin. Nutr.,* 39 (1984) 745.

162. I.A.Nnanna and R.D.Phillips, *J. Food Sci.,* 55 (1990) 151.

163. P.Srinivasa Rao, *J. Agric. Food Chem.,* 24 (1976) 958.

164. H.A.El Faki, T.N.Bhavanishankar, R.N.Tharanathan and H.S.R.Desikachar, Nutr. *Rep. Internat.,* 27 (1983) 921.

165. H.A.El Faki, T.N.Bhavanishankar, L.V.Venkataraman, R.N.Tharanathan and H.S.R.Desikachar, J. *Food Sci. Technol.,* 21 (1984) 259.

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