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Comparative Studies on the Nutrient Content of Five Varieties of Rice Brans and Purification, Characterization and Structure Function Analysis of Proteins from the Best Quality Rice Bran

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Comparative Studies on the Nutrient Content of Five Varieties of Rice Brans and Purification, Characterization and Structure Function Analysis of Proteins from the Best Quality Rice Bran

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Molecular Biology
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BANGLADESH.

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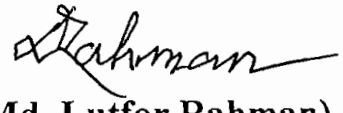
DEDICATED TO MY PARENTS AND TEACHERS WHO GAVE ME LIGHT OF KNOWLEDGE

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled **"Comparative studies on the nutrient content of five varieties of rice brans and purification, characterization and structure function analysis of proteins from the best quality rice bran"** prepared for submission to the university of Rajshahi, Bangladesh, for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology, are the original research works of mine and have not been previously submitted for the award of any degree or Diploma any where.



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CERTIFICATE

This is to certify that the thesis entitled **"Comparative studies on the nutrient content of five varieties of rice brans and purification characterization and structure function analysis of proteins from the best quality rice bran"** has been prepared by **Md. Lutfor Rahman** under my supervision for submission to the Department of **Biochemistry and Molecular Biology** university of Rajshahi, Bangladesh, for the Degree of **Doctor of Philosophy** in Biochemistry. It is also certified that the materials included in this thesis are the original work of the researcher.

I have gone through the draft of the thesis and found it acceptable for submission.

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The author

ABSTRACT

Five varieties of rice bran were analyzed to obtain comparative data on their chemical compositions and nutritive values. Rice bran oils were also characterized with respect to their physical and chemical properties.

The results indicated that moisture, ash, total soluble solid and crude fibre contents of the different varieties of rice bran were in the ranges of 9.98-10.90%, 9.45-11.12%, 10.06-11.25% and 9.18-10.35% respectively. The ranges in the values of total protein, water soluble protein, dry matter, polysaccharide, total sugar, reducing and non-reducing sugar compositions of rice brans were between12.45-14.06%, 3.20-4.71%, 89.10-90.02%, 10.95-12.86%, 4.35-5.04%, 1.08-1.45%, and 3.10-3.96% respectively. Some minerals such as potassium, calcium, magnesium, sodium, phosphorus, sulphur, iron, zinc and manganese are available in all the varieties of experimental rice brans. Vitamin B_1 and vitamin B_2 contents of rice brans were found to be ranged from 2.98-3.81 and 0.61-0.95 mg/100 g respectively.

Twelve amino acids were detected in all the five varieties of rice bran in the total form by paper chromatography. Among the variety IRRI-28 contained relatively higher amount of amino acids. Rice bran contained about 14.95-16.16% oil. The bran oils were also characterized with respect to their physico-chemical properties such as specific gravity, refractive index, smoke point, flash point, fire point, saponification value, iodine value, peroxide value, acid value, %FFA and unsaponifiable matter. Triglyceride diglyceride monoglyceride, and non-glyceride are the constituents of bran oils were found to be varied from 91.76-93.08%. 1.51-2.30%, 1.45-1.96% and 3.63-5.24% respectively.

Rice bran oils contained mostly unsaturated fatty acids which was varied from 71.06-74.95%. The total lipids were fractionated into lipid classes by silicic acid column chromatography and the bran oil contained triglyceride, hydrocarbon, free sterols, glycolipid, phospholipid and partial glyceride. GLC analysis of the oil indicated palmatic, stearic, oleic and linoleic acid, as a major fatty acids in all the varieties of rice bran oil. On the other hand linolenic, myristic and arachidic acid were also detected in small amount.

The quality of rice bran supplemented breads and market-made breads were compared by determining their physico-chemical characteristics such as moisture, ash, fibre, fat, protein, carbohydrate, sugar, reducing sugar etc. and solubility of proteins in different solvents. The bread available in the market (market made) has the highest amount of moisture, ash and fat contents than that of bread prepared from rice bran, but rice bran prepared bread contained highest amount of protein and carbohydrate than that of the market-made breads. The acid values of the market-made bread were found to be more than that of rice bran prepared breads. The extent of denaturation of protein, as determined by solubility in water and water insoluble mild and harsh solvents were found to be much higher in market made breads than rice bran prepared breads, which might be due to more denaturation of protein in market made breads. In relation to nutritional quality rice brans prepared breads might consider to be superior than the locally available market-made breads.

Three proteins have been isolated and purified from IRRI-28 variety by 100% ammonium sulfate saturation followed by gel filtration on Sephadex G-50 and then ion-exchange chromagrophy on DEAE-cellulose. All the three "proteins are homogeneous as judged by SDS- Polyacrylamide slab gel electrophoresis. The molecular weight of the proteins RBP-1, RBP-2 and RBP-3 as determined by gel filtration on Sephadex G-150 as well as by SDS-PAGE were calculated to be about 46-47,000, 33-35,000 and 26-28,000 respectively. All the three proteins RBP-1, RBP-2 and RBP-3 are dimer in nature and their molecular weight were calculated to be 24-25KDa, 17-18, KDa and 13-15KDa respectively.

The proteins are glycoprotein in nature and they contained about 2-4% neutral sugar. The proteins showed hemagglutinating activities against rat red blood cells and the agglutination by all the three proteins were inhibited by D-glucose and D-glucosamine. Further the hemagglutinating activities of RBP-2 and RBP-3 were also inhibited in the presence of N-acetyl D-glucosamine. The proteins gave absorption maxima between 275-280 nm.

All the three protein showed strong toxicity as detected by brine shrimp lethality bio-assay and the LC_{50} of RBP-1, RBP-2 and RBP-3 against brine shrimp eggs were found to be 9.12, 6.91 and 11.22 μg/ml respectively.

Effect of physical and chemical treatments on the cytoagglutinating activities of RBL-1. RBL-2 and RBL-3 were also investigated. The lectins RBL-1, RBL-2 and RBL-3 gave maximum hemagglutinating activities around P^H 7.2 and between 30 to 40°C. The activities of all the three lectins were decreased sequentially with increasing of acetic acid concentration and they destroyed their activities completely in the presence 15% acetic acid. The lectins are slightly more sensitive to the denaturing agent Guanidine-HCl than urea and the hemagglutinating activities were abolished completely in the presence of 6M guanidine-HCl and 8 M-urea. The activities of RBL-1, RBL-2 and RBL-3 were enhanced in the presence of $Ca^{2+}$and Cu^{2+}, while the activities were completely lost in the presence of 100 mM EDTA.

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CHAPTER-I

General Introduction

GENERAL INTRODUCTION

RICE BRAN

Rice (*Oryza sativa* L.) apparently originated more than 6,000 years ago in southeast Asia. More than 3,000 varieties have been collected by the IRRI (International Rice Research Institute). The plant *Oryza sativa* is the major source of food for nearly one-half of the world's population. In some countries of the orient the consumption of rice per capita is estimated at 40-120 kg per year. Over 95% of the world rice crop is used as human food. In Bangladesh, the rice crop occupies the largest area among all the crops and accounts for as much as 30% of the total crop area. Rice bran is a valuable by-product of the rice milling industry which is now poorly utilized. It offers a potential source for exploitation as human food. Vast quantity of rice brans become available as the by-product both as a potential source material of valuable edible oil and for better utilization as human food.

In Bangladesh the diet of the masses consists largely of rice and is more or less deficient in fat, protein, minerals, and vitamins. The process of husking rice, apart from traditional dhenki husking, can be accomplished in two types of rice mills, namely the cottage type huller and automatic rice mils. The automatic rice mills have larger capacities, yield more head rice and causes less loss. In these mills hull is first removed leaving the kernel which is some what coloured and commonly called unpolished rice, which is then passed through a combined scrapping and polishing process. This removes the outer portion of kernel which is called 'Rice Bran', locally known as 'Kura' or 'Gura'. Rice bran consists of seed coat, aleurone layer, germ and some portion of the sub-aleurone layer of the starchy endosperm (Sivala, K., *et al.,* 1991).

Rice bran is the most important by-product of rice milling industry and obtained to the extent of 5-8% of milled rice, depending on the degree of polish (The wealth of Indian 1966). It comprises the germ, the pericarp and aleurone layer and often found mixed with varing quantities of husk. The bran, obtained from cottage type huller is a mixture of maximum amount of husk. Apparently the amount of bran obtained from the cottage type huller is less than that of the automatic huller. The extent of recovery of the bran depends upon the process of husking and the degree of polishing, variety of paddy rice milled and area of cultivation. It is the most nutritious part of the grain since it contains proteins, minerals and vitamins (Gnanasambandam, R, *et al.,* 1995; Carangian, D.D., *et al.,* 1970). It is also a good source of lipid, 15-25% (Sarkar, S., *et al.,* 1989; Raghavendra Rao, *et al.,* 1965; Sayre, R.N., *et al.,* 1985).

The sole use of rice bran in Bangladesh is as livestock feedstuff. Rice bran produced at present in Bangladesh is not taken care of properly and much of it is allowed to spoil and wasted. Rice bran has a much higher food value (calories) than either the wheat or the flours. This is due to the fact that the rice bran has a much higher fat content than the wheat bran or flour and rice bran also contained high class protein which might serve as supplement in human diet. Out of the world's total production of bran about four million tons of proteins, five million tons of edible oil and 75,000 billion calories can be utilized (Sarker, S., *et al.,* 1989). The protein content in the true bran ranges from 9.8 to 15.4% and that in defated bran from 10.4 to 21%. Rice protein contains about 80% glutelin, 5% albumin, 10% globulin, and less than 5% prolamin. (Sherman, H.C., *et al.,* 1931). In our country beriberi is a very common and fatal disease among the poorer classes who live on a diet which is deficient mainly in vitamin B_1. Rice bran contains the water soluble vitamin B_1 (Bhatia, I.S., 1969) and nicotinic acid which is available at a very low price. The rice bran lipid contains the fat soluble vitamin A and E (Rice bran

utilization 1974). Rice bran is used for the production of alcoholic beverage. People, live on a mixed diet required the rice bran for its medicinal value who really need it and for others who would benefit by using it. Little attention has been given in the nutritional qualities of rice bran for human foods, though such use has long been suggested.

PROTEIN

Proteins are highly complex substances that are universally present in living organisms. The term protein is derived from the Greek word proteose, meaning "Primary". Proteins were first recognized as a unique molecule by Gerardus J. Mulder in the 1830s and were named Proteins on July 10, 1838 (Hartley, H., 1961). At that time eight protein were recognized (Mulder, G.J., 1938), who first used the named in print at the suggestion of Berzelius. Four were animal (fibrin, serum, albumin and casein) and four were plant proteins (soluble albumin, coagulated albumin, legumin and gluten). By 1871 animals had overhauled plants with 24 proteins in 12, though plant "proteins" included perphaps the most complex mixture over categorized as a single substance yeast (Gmelin, L., 1871). In the 1860s major advances in the chemistry of proteins were made by Ritthausn, who also published the first table of the amino acid composition of proteins (Ritthausn, H., *et al.,* 1872). His work was followed by other distinguished workers among whom Osborne, Fischer, Vickery and Chibnall were outstanding. Osborne's work at connecticut Agricultural Experiment station on seed proteins was remarkable in that not only did he transfrom our knowledge of the chemical composition of proteins by their meticulous analysis, often by methods developed by himself, but in that he also made studies of their behaviour in solution and thus laid the foundation for much fruitful development of the physical chemistry of protein. The chemical structure and the peptide bond of amino acids were elucidated in 1902 by Emil Fischer (The Macmilian Family Encyclopedia 1980), his early synthesis of large polypeptides. Cohn and his collaborators (Cohn 1953) at

Harvard were bringing to a peak the method of proteins separation based on differences in solubility induced by changes in temperature, pH, ionic strength and dielectric constant and by the presence of specific divalent cations.

CLASSIFICATION

The first universal classification of proteins was that laid down in Great Britain (1907) and America (1908) by the respective physiological societies (Anon, J., 1907). It was based in its initial division on chemistry first, on the presence or absence of prosthetic groups and then, for finer distinctions, entirely on solubility properties. It was a useful pragmatic framework and served well for many years. Today a multitude classifications are possible. Other classification of proteins based on their structure or function have been advanced, but have not as yet proved useful or detailed enough for wide adoption. It is perhaps significant that a recent four volumes treatise on proteins contain neither a definition of its subject nor a classification of the compounds to be described.

PROPERTIES

The properties of proteins are, as would be expected, very varied. Most proteins are water soluble in solutions of moderate ionic strength, though some are insoluble under all normal conditions and others are soluble in organic solvents either in their natural state or as a result of salt formation with organic acids. Most are denatured by heat, that is, they lose their solubility in water and their specific properties, though some are apparently unaffected by heating when in solution, even upto 100°C and for considerable periods. The solubility of some proteins increases with temperature, that of others decreases. Most enzymes show a high substrate specificity; in others it is very broad. Some proteins have unusual amino acids as part of their structure, for example one residue of o-tyrosine sulfate per molecule in fibrinogen (Altschul, A.M., 1965), but most are confined to the usual 18 to 20 amino acids in their makeup.

The properties of proteins are also determined in part by their amino acid composition. For example, the charge on the macromolecule at any given hydrogen-ion concentration is largely a function of the relative number of basic (lysine, histidine and arginine) and dicarboxylic amino acids (aspartic and glutamic acids). This net charge strongly influences the solubility of protein at different pH values, since the solubility depends in part on the proportion of polar groupings on the macromolecule. When the hydrogen-ion concentration is high (low pH), the net charge is positive charge. The pH at which the net charge of protein is zero and defined as the iso-electric point.

As macromolecules that contain many side chains that can be protonated and unprotonated depending upon the pH of the medium, so proteins are excellent buffers. The fact that the pH of blood varies only very slightly in spite of the numerous metabolic processes in which it participates, is due to the very large buffering capacity of the blood proteins.

ROLE OF PROTEIN

Green plants use energy from the sun to synthesized over the surface of the earth about 200 billion tons of organic material per annum, of which some 10 billion (metric) tons is protein. Green plant and many microorganisms do not require protein as food. For man and all animals, however, protein is an essential constituent of the diet. Without this death is inevitable, stricly speaking, it is not protein itself that is require, but the blocks of protein, the amino acids. There are 20 different amino acids that occur naturally in proteins. About half of these can be synthesized in the cells of the animal body. Proteins that lack one or more essential amino acids are called incomplete. If an essential amino acids occurs only in small amounts it may be a limiting factor in respect to the nutritional value of the protein (Encyclopedia Britanica 1973). If on the other hand, another protein should contain large amount of the same

amino acid, the two proteins probably would supplement each other when ingested together. For example most animal proteins are fairly high in lysine, an essential amino acid, while cereal proteins tend to be low in lysine. Animal proteins therefore effectively supplement most cereal proteins. In general, as food for man, proteins of animal origin are nutritionally superior to those from plants.

Deficiency of protein in the diet has many harmful effects, including impairment of growth, interference with reproduction, and lowered resistance to many diseases.

RICE BRAN PROTEINS AND OILS

Since our interests have mainly centered around proteins of rice bran, a short review of literature available on this proteins and oils are given here.

Rice bran which is an industrially and economically useful by- product of rice milling have not been the subject of much work in our country with respect to their potentiality as a source of proteins. On milling paddy, the main components obtained are rice, hull and bran which includes pericarp, seedcoat, germ and aleurone layer. As may be expected, it is a repository of many enzymes (Kitamura. S. Ida., I. *et al.,* 1970; Kanamori T., *et al.,* 1972) such as lipases, phosphatases, peroxidases, protease, lecithinase, dehydrogenase, glucosidase etc.

Some infromation are available regarding the fat-degrading enzymes in rice bran. Considerable changes in the oil composition occur soon after milling and during storage of rice bran (Hussain M.G., *et al.,* 1995). There is a rapid release of FFAs from the lipid which lead to the deterioration of the lipid. The deteriorative changes of the rice bran lipid are due to the presence of enzyme which causes hydrolytic rancidity. Lipid is a major and potential component of this waste raw material.

Fats, oils and fat-like substances, because of similar solubilities, are classified as lipid. Most natural lipids (Kause, M.V., 1972) are composed of about 98 to 99 percent triglycerides. The remaining 1 or 2 percent include traces of mono and diglycerides, free fatty acids, phospholipids and unsaponifiable matter containing sterols.

Although rice bran has considerable potential as a contributor to world lipid supply (Table-1), it is seldom considered in the list of edible oil raw material sources. In some countries, production of edible grade rice bran oil has been increasing steadily.

The edibility of rice bran oil is comparable to other oils including groundnut (Peanut), cottonseed, soybean and rapeseed oils (Gupta H.P., 1989). Moreover, rice bran oil has better keeping qualities due to the presence of natural antioxidants, tocopherol (Rice bran utilization, 1974). The physico-chemical constants of this oils are also comparable with a number of edible oils except for unsaponifiable matter, which is rather high due to the presence of wax. Rice bran oil is seen as a superior oil, rich in vitamins and low in ingredients responsible for cholesterol. More recently rice bran oil has been reported to have hypocholesterolemic activity (Seetharamaiah G.S., *et al.,* 1989; Kahlon, T.S., *et al.,* 1993; Kahlon T.S., *et al.,* 1996) and may lower heart disease risk (Hegested, M., *et al.,* 1994; Hegested, M., *et al.,* 1993). Rice bran oil is absorbed less as foods during cooking. In fact, 25% more food can be cooked with a fixed quantity of this oil compared to other vegetable oil such as groundnut and sunflower (Ghosh chowdhury, P. 1987).

The prime problem in producing quality grade oil is the presence of enzyme (Aoyagi, Y., *et al.* 1985) in rice bran which splits the oil into FFA and glycerol (Hiroyamma, O. *et al,* 1975). The rate of increase of FFA depends on the moisture content of the bran, relative humidity and temperature during storage of the bran. The FFA should not exceed 6% for the economic production of edible oil.

Table-1: Data available on paddy rice, bran and this oil in major paddy rice growing countries (Million tons).

Country	Paddy rice	Rice bran	Rice bran oil
Bangladesh	27.9	1.8414	0.2946
China	198.5	13.1010	2.0962
India	123.1	8.1246	1.2999
Indonesia	49.1	3.2406	0.5185
Japan	25.5	0.8250	0.1320
Myanmar	17.3	1.1418	0.1827
Pakistan	6.9	0.4554	0.0729
Philippines	10.7	0.7062	0.1130
Thailand	22.1	1.4586	0.2334
Vietnam	27.5	1.8150	0.2904

Source: Food and Agriculture Organization of the United Nations.

DIFFERENT VARIETIES OF RICE BRAN

There are many varieties of paddy rice grown in Bangladesh. According to habitat, method of cultivation, and general properties, these numerous varieties are usually classified in terms such as up land rice and low land rice, glutinous and nonglutinous. They are dentified by certain local names, seaasonal names or trade names such as BR-5, BR-10, IRRI-28, BRRI-39, Kalijira, Sarna, Parija, IR-50, IRRI-20, Aus, Aman, paijam, Bora, Balam, IRRI-8, BR-11, BR-14, Dadkhani etc. The different varieties are distinguished by their principal physical characteristics such as length, width and thickness of the grain, the colour of the hull, flavour and other properties. BR-5, BR-10, IRRI-28, BRRI-39 and Kalijira are the extensively cultivated paddy rice of Bangladesh. IRRI-28 varieties is being cultivated in, more or less every place of Bangladesh.

The paddy rice of these varieties are taken to the automatic rice mills by the growers. In order to get the rice bran of the appropriate varieties, it is a very common practice to distinguish definite variety of paddy rice before milling.

USES OF RICE BRAN IN BAKERY FOOD PRODUCTS

Rice bran as it comes from the polisher, has a fresh and some what sweet order. It is a very nutritious cereal by-product of rice milling. Rice bran contains high percent of proteins, vitamins, fats, carbohydrate etc. The proteins in bran contain the amino acids which is necessary for the maintenance and growth of human body. Rice bran contains substantial amount of water soluble vitamin B_1 and also fat soluble vitamin A and E. So rice bran can play an important roll to reduce the malnutritions of the children as well as the people of our country. Bran when used alone, lacks gluten and does not make bread or any other bakery products. The addition of wheat flour serves not only to dilute the bran but also to supply the gluten necessary for making bread or biscuits. So a mixture of certain percent of wheat flour and rice bran were mixed thoroughly, required amount of other ingredients were also added to the mixture and a nutritious whole bread and biscuits were made.

RICE CULTIVATION

Rice, the major food crop of about twenty percent of the worlds population and the dominant food grain (90%) in Asia, is grown under diverse irrigated, rained and deep water condition (Bangladesh, J. Ag., 1986). The geographical as well as the agroclimatic conditions of Bangladesh are favourable for cultivation of rice all the year round (Bangladesh, J. Ag., 1980).

Cultivation of rice can be considered under two main heads: **low land rice** (wet paddy cultivation) and **upland rice** (dry paddy cultivation). There is

also a semi-wet rice where the crop is raised as a dry one to begin wit, but gets flooded with rain during growing period. Low land rice forms the major portion of the rice cultivated in Bangladesh and confined to lowlands fully inundated with water from the time of planting or transplanting until harvest approaches.

The seeds are sown either broad cast or by drilling or dibbling in plough furrows. Upland rice are generally of short maturation period (90-120 days) and have a well developed and larger root system and a significant tolerance to drought. The grain is generally course, broad and bold and also have a red coloured kernal, though their chatting quality may not be poor. In fact some upland rice of Thailand and Orissa (India) are considered to be of high chatting quality.

SPECIES AND VARIETIES

Rice belongs to the genus *Oryza* of the subtribe oryzinea in the family graminea. The genus *oryza* includes 23 species, of which 21 are wild and two are only cultivated namely *O.sativa* and *O.glaveriman All* the rice varieties of Asia, Europe, Australia and America belong to the species *O.sativa,* while many of the cultivated varieties of West Africa belong to the species of glaberima. All the species are hygrophilous and the genus is distributed in most tropics. In Indian subcontinent five species are found. They are *O.coarctatea, O.granulata, O.officinalis, O.perennis* and *O.sativa.* The common wild rice, *O.sativa* which occurs as a weed in deep water rice field in Bangladesh. Twenty six varieties of rice are produced in Bangladesh now. Among them, the twenty two varieties have been developed by Bangladesh Rice Research Institute (BRRI) and the rest four varieties are developed by International Rice Research Institute (IRRI).

AREA AND PRODUCTION

Rice is the most extensively cultivated cereal crop in the World after wheat. Its cultivation is particularly concentrated in Asian countries which together include about 90% of the World area (Bangladesh, J. Ag., 1986).

The two most important rice producing countries are India and China which account for about 28% and 26% respectively of the total world area. The other important rice producing countries are Bangladesh, Indonesia, Thailand, Burma, Japan and Philippines in Asia, Brazil and U.S.A. in America and Malagasy (Madagascar) and Egypt in Africa. Small quantities are also grown in a few southern European countries like Italy and Spain.

In Bangladesh, the rice crop occupies the largest area among all crops and accounts for as much as 30% of the total cropped area. The net cropped area of Bangladesh is 117.01 lakh acres for cultivation.

NUTRITIONAL IMPORTANCE OF RICE BRAN

Rice bran is the most nutritious part of the rice since it contains fats proteins and vitamins (Hammond, N., 1994). Van veen (1941) reported that the rice bran is rich in protein, fat and in calcium, magnesium and phosphorus and also suggested that it may be used locally as a supplementary human food.

Carangia D.O. *et al.* (1970) examined the seven varieties of rice bran in the Philippines. In this study, the vitamins investigated were found to be vitamin A, thaimin, riboflavin, niacin, ascorbic acid, carotene, tocopherol and vitamin K. They found that phillippino rice bran contained an average of 1.8 mg% thiamin, 0.232 mg% riboflavin, 41.79 mg% niacin, 0.377 mg% ascorbic acid, 7.42 mg% tocopherol and 12.41 mg% carotene.

At present rice bran is very cheap and is used for feeding cattle and poultry. Bran flours are considered quite nutritious and are very popular in United States and other countries. A mixture consisting of 3 parts of wheat flour and 1 part of bran makes a good combination for bakery products such as bread and cookies (West, A.P., *et al.,* 1933).

INDUSTRIAL APPLICATION AND OTHERS IMPORTANCE

The oil, containing high percentage of linolenic acid are valued industrial use. The oil, which content more oleic and linoleic acids are considered good for edible purpose. An antioxidant for pharmaceuticals and foods has been extracted from rice bran. Bran exhibits protective effect against experimental polyneuritic in chicken. It is stated to be effective larvicide against mosquito larvae (Paddy, J., Ind 1955).

Rice starch is used commercially for various purposes such as laundering, preparing foods, making paste and making medicinal tablets. It is also employed extensively in the cosmetic industry for making face powders (West, A.P., *et al.,* 1933). Rice bran wax can be used in the preparation of candles, polishes, cosmetics, emulsifies and other industrial preparation (Zachariassen and Giasotta, 1964).

REVIEW OF LITERATURE

All over the world much works were performed on the chemical composition, biochemical importance, nutritive values and industrial uses of rice bran. Some works also carried out on the enzymatic activity of rice bran. A few reports are summarized below:

Saunders R.M. (1986) made a study on composition and showed that rice bran contains 6.7-17.2% protein, 4.7-22.6% fat, 6.2-26.9% fibre and 8.0-22.2% ash. It was reported that Indian rice bran contained the following nutrients: fat 10.6-22.4%, protein 10.6-14.8%, fibre 9.6-14%, ash 9.3-15.0%, moisture 8.9-12.5%, sucrose, 3-5%, reducing sugar 1.3%, calcium 0.13 ppm, phosphorus 2.39 ppm, potassium 0.14 ppm, sodium 0.24 ppm, magnesium 0.14 ppm and iron 224 ppm.

Tei Hidaka (1939) reported that Japanese rice contained 6.43-8.95% moisture, 5.28-12.89% oil, 13.51-38.63% starch, 12.27-17.63% protein and 9.70-24.95% ash.

Funatsu, M., *et al.,* (1971) stated that rice bran contained several types of lipases and isolated basic protein lipases from rice bran. Aizona and co-workers (1971) reported that the optimal pH values of rice bran lipases were ranged from 7.5-8.0.

As reported by Aoyagi *et al.,* (1985), the utilization of rice bran as a source of oil is limited by deterioration in storage, due to enzymic hydrolysis of triglycerides. Rice bran stored at higher temperature (30-40°C) showed a considerable increase in acid value while peroxide value remained stable. Levels of free fatty acids (plamitic, oleic and linoleic acid) increase during storage with increasing time.

Panduranga B. Rao, *et al.* (1967) also reported that heating the bran lowers the moisture to <2% and stabilizes the bran. The fine particle size of rice bran in which oil is exposed over a large surface area appears to be primarily responsible for the rapid rise of free fatty acid in the bran during storage.

Bernardini E. (1973) determined the fatty acid composition of the rice bran oil and the results are given in the Table (2).

Table-2: Fatty acid composition of rice bran oil.

Fatty acid	Amount %
Myristic acid, $C_{14:0}$	< 1%
Palmitic aicd, $C_{16:0}$	12-18%
Palmitoleic acid, $C_{16:1}$	< 0.4%
Stearic acid, $C_{18:0}$	< 3.0%
Oleic acid, $C_{18:1}$	40-50%
Linoleic acid, $C_{18:2}$	29-42%
Linolenic acid, $C_{18:3}$	< 1.0%
Arachidic acid, $C_{20:0}$	< 1.0%

In Bangladesh, it was reported that the oil content of rice bran varied between 23 to 26% when the bran extracted inmediately after milling. BR-6 has the highest and BR-9 has the lowest oil content. The F.F.A. (free fatty acid) content in the bran was varied from 1.7 to 3.0% inmediately after milling.

PLANT SEEDS AS A RICH SOURCE OF PROTEIN

Although animal kindom is the main source of protein but plant kindom also possesses a lot of protein. Green leaves, barks, roots stems etc. contain small amount of protein; while seeds are the main sources of protein in plants. Pulses contained about 18-26% which oil seeds contain approximately 30-35% protein (Rutkawaski, A., 1970). In addition, wheat barley, rice bran, maize etc. also contained significant amount of protein. Some plant seed proteins are toxic and some are non-toxic.

MOST OF THE PLANT SEED PROTEINS ARE GLYCOPROTEIN.

The glycoprotein which contain carbohydrate group attached covalently to the polypeptide chain represent a large group of wide distribution with considerable biological significance. Plant seed proteins contain in general 1-3% carbohydrate but there are exceptional cases in which the carbohydrate content may be about 10-12% (Lis and Sharon 1981). The percent by weight of carbohydrate group in different glycoprtoein may vary from less than 1% in ovalbumin to as 80% in the mucoprotein (Lehninger, 1981).

Many different types of monosaecharide derivatives have been found in glycoprotein. The linear or branched side chain of glycoprotein may contain from two to dozen of monosaccharide residues usually of two or more kinds. Some glycoproteins also contain oligosaceharides units. The sugar residues are generally mannose, galactose, lactose, xylose, glucose, raffinose, glucosamine etc. (Lis and Sharon 1981). Among them monnose and glucosamine are predominant.

AIM OF THE PRESENT STUDY

Rice bran, a by-product of milling paddy, is the most nutritious part of paddy rice, since it contains significant amount of protein, vitamin and minerals. It is also a good source of oil (14-22%) which can not be used for edible purposes as the oil becomes rancid on standing due to higher activity of enzyme (David *et al.,* 1965). Consequently, most of the oil of rice bran that extracted is used for soap manufacture (Barber *et al.,* 1980; Sayre *et al.,* 1982).

Rice bran oil has attracted much attention as a health food because it was found to lower serum cholesterol (Haumann, B.F., 1989). Rice bran oil contains oryzanol, tocopherol and sterol, which are thought to have biochemical activities (Shimizu. S., *et al.,* 1984).

Rice bran which is an industrially useful and economically important by-product of rice milling, has not been the subject of much use of human food with respect to its potentiality as a source of protein, mineral and vitamin like wheat and soybean. Proteins are widely distributed in nature. Till now, most of the proteins are purified from plant sources and the main sources of protein in plants are found in mature seeds. Researches on plant proteins are currently attracting much interest to the scientist because of their unique biological properties. Although hundred of proteins i.e. lectins are known but the structure of most proteins have not yet been fully elucidated. Further the physiological function (s) of plant proteins is still unknown. Rice bran may be considered to use as a supplementary sources of protein, since it contains about 10-16% protein.

The present investigation has therefore been undertaken to obtain comparative data of five new varieties of rice bran which were selected randomly. In this study the best variety rice bran, IRRI-28 have been selected for purification and characterization of proteins. Further the proteins was characterized with special emphasis on their agglutinating activity, molecular weight, subunit structure, sugar specificity etc.

The present thesis includes the following research works.

1) Investigation on the physico-chemical compositions of rice brans and their oils.

2) Preparation of bakery food product by supplementation with rice bran and detection of their quality.

3) Isolation, Purification and characterization of proteins from rice bran variety, IRRI-28.

4) Effect of physical and chemical agents on the stability of purified proteins.

CHAPTER-II

Investigation on physico-chemical characteristics and fatty acid composition of different varieties of rice brans and their oils

INTRODUCTION

Rice bran as it comes from the polisher has a fresh and some what sweet order. It is very nutritious cereal of rice industries since it contains lipid, protein, minerals and vitamins (Yokochi, K., 1974; Pauda. A.B., *et al.,* 1979). Rice bran also contains vitamin B_1 and vitamin A. It has also contained antisterility vitamin E (Gopala Krishnam, A.G., 1984). Rice bran is generally used as livestock feed in our country. It addition to its value as a feed stuff, rice bran constitutes a potential source of oil (15-25%) (Subramaniyam, V., 1971) with similar properties to that of edible oil. The fatty acids of rice bran lipid are the most important constituents from the stand point of food processing and nutrition. The fatty acid composition of rice bran oil was reported by several investigator (Nasjrullah *et al.,* 1989; Taira, H., *et al.,* 1980).

It has considerable calorific and nutritional values which provides a substantial quantity of vitamins, minerals and other nutrients in human diet. Rice bran, produced by current practices, is subject to a rapid deterioration after milling due to the higher activity of the fat hydrolytic enzymes (Loeb, J.R., *et al.,* 1952) which often causes a serious problem with its value as a stock feed (Enochina, R.V., *et al.,* 1981). In 1903, Enochina, R.V., *et al.,* (1981) first noticed the formation of free fatty acids in rice bran by the action of lipolytic enzyme. Further work was reported by West and Cruz (1933) which included that the formation of FFAS in rice bran is due to the action of enzyme in presence of moisture that acts as catalyst and accelerates the hydrolysis of the bran oil.

Many varieties of rice brans are available in our country but their nutritional and physico-chemical characteristics as well as essential minerals (basically calcium, phosphorus, iron and potassium), contents are not fully known individually. Therefore, in the present study five new varieties of rice bran have been selected for comparing physico-chemical characteristics and their oils contents.

COLLECTION OF RICE BRAN

Five varieties of rice brans (BR-5, BR-10, IRRI-28 BRRI-39 Kalijira) were collected from Janata Auto Rice and Flour Mills Ltd. Bogra; Noor-Habib Grain Industries Limited, Sopura, Rajshahi; North Bengal Auto Rice Mills, Naogan; Biswas Rice Mills, Natore, Rangpur and other auto rice mills of Dinajpur district. For the collection of fresh rice bran samples, the bran samples were taken from mill gates as they came fresh from the polishers and were immediately taken to the laboratory. The fresh bran samples were stored at room temperature

Rice bran usually contains coarse material like some fragments of rice kernel of polished rice. It may also have some broken rice hulls or other foreign matters. These coarse and foreign particles were separated from the bran samples by sieving (30 mesh) and stored in air tight polyethylene bags in a refrigerator.

MATERIALS AND METHODS

DETERMINATION OF MOISTURE CONTENTS OF RICE BRAN

Moisture content was determined by the standard method IUPAC (1977)

Materials

a) Porcelain Crucible
b) Electrical balance
c) Oven
d) Desiccator

Procedure

1-3 g of rice bran were weighed in a porcelain crucible (which was previously cleaned, heated to 100°C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six hours at 100°C. It was then cooled in a desiccator and weighed again.

Calculation

Percent of moisture content (g per 100 g of rice bran)

$$= \frac{\text{weight of moisture obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF ASH CONTENT OF RICE BRAN

Ash content of rice bran was determined following the method of AOAC (1980).

a) Porcelain crucible
b) Muffle furnace
c) Electrical balance (Mettler H-18)
d) Desiccator

Procedure

1-2 g of rice bran were weighed is a Porcelain Crucible (which was previously cleaned, heated to about 100°C, cooled and weighed). The crucible with its content was placed in a muffle furnace for about four hours at about 600°C. It was then cooled in a desiccator and weighed. To ensure completion of ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights were the same and the ash was almost white in colour.

Calculation

Percent of ash content (g per 100g of rice bran)

$$= \frac{\text{weight of the ash obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF TOTAL SOLUBLE SOLIDS

The total soluble solid (TSS) content of rice bran was directly determined from the percentage scale (0-90%) of Kyowa hand refractometer (Ranganna, S., 1986). A drop of rice bran solution was placed on the prism of refractometer and percent of TSS was obtained from direct reading.

DETERMINATION OF DRY MATTER

Dry matter Content was calculated from the data obtain for percentage of moisture content.

Calculation

Amount of dry matter in the rice bran (g per 100 g of rice bran)

$$= \frac{\text{weight of dry matter obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF CRUDE FIBRE

Crude fibre content of the different varieties of rice bran was determined by the following method (AOAC, 1980).

Reagents

a) Sulfuric acid (0.225 N)

b) Sodium hydroxide (1.25%)

c) Ethanol

d) Ether

Procedure

Three to Five gram of rice bran were taken in beaker (500ml) and 200 ml of boiling 0.225 N sulfuric acid was added to it. The mixture was boiled for 30 minutes, keeping the volume constant by the addition of water at frequent intervals (a glass rod was inserted in the beaker for smooth boiling). At the end of this period, the mixture was filtered through a Muslin cloth and the residue was washed with hot water, till free from acid. The material was then transferred into the same beaker and 200ml of boiling 1.25% sodium hydroxide was added. After boiling for 30 minutes (keeping the volume constant as before) the mixture was filtered through Muslin cloth. The residue was washed with hot water until free from alkali, followed by washing with some ethanol and ether. It was then transferred to a crucible, dried overnight at 80-100°C and weighed. The crucible was then heated in a muffle furnace at 600°C for 3 hours, cooled and weighed again. The difference in the weight represented the amount of crude fibre. The percentage of crude fibre (on dry basis) was calculated from the formula given below:

Calculation

Amount of crude fibre content in the rice bran (g per 100 g of rice bran)

$$= \frac{\text{Amount of crude fibre obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF TOTAL PROTEIN CONTENT OF RICE BRAN

Protein content of the different varieties of rice bran was determined by the method of Micro-kjeldhal (Jayaraman, J., 1981).

Reagents and Equipments

a) Solid potassium sulfate

b) Concentrated sulfuric acid

c) 5% $CuSO_4$, $5H_2O$ in distilled water

d) Concentrated sodium hydroxide solution (5N approx)

f) Few quartz chips

g) Boric acid solution containing bromocresol green (receiving fluid): 10 g of boric acid was dissolved in hot water (about 250 ml) and cooled. Then 1 ml of 0.1% bromocresol green in alcohol was added and diluted to 500 ml with distilled water.

h) Nitrogen determination apparatus according to Paranas-Wagner, made JENA Glass-all connections with interchangeable ground joints.

Procedure

A) Digestion: Concentrated H_2SO_4 (6-8ml), 1.0 g K_2SO_4, one to two drops of 5% $CuSO_4$ solution (catalyst) and some quartz chips were added (to avoid bumping) to 1- 2 g of rice bran in a kjeldhal flask. The mixture was heated till it had become light green (2-3 hours).

B) Collection of ammonia: The digestion was carried out in the steam distillation chamber of the nitrogen determination apparatus. The chamber is designated to act as a micro-kjeldhal flask and can be easily detached when needed. After completion of digestion the steam distillation chamber containing the digested mixture was fitted back to the nitrogen determination apparatus.

Boric acid solution (15 ml) in a small flask was placed so that the tip of the condenser outlet dipped below the surface of the boric acid solution. Sufficient amount of strong sodium hydroxide solution (approximately 30-40 ml) was added to digest in the chamber to neutralize the amount of acid present. Steam was generated from the steam generating flask and the sample in the chamber was steam distilled until 20 ml distillate was collected in the boric acid solution. The condenser outlet was then rinsed with little distilled water and the receiving flask was removed.

C) **Titremetric estimation of ammonia:** The ammonia in the boric acid solution was titrated with 0.01 N H_2SO_4 until the solution had been brought back to its original yellow green colour. The titration was repeated with a control containing only 15 ml of boric acid solution diluted to approximately the final volume of the titrated sample. The volume of acid required was noted.

The nitrogen content was calculated using the fromula given below.

1ml of 0.01N H_2SO_4≡ 140 µg of nitrogen in NH_3

Thus from the volume of standard H_2SO_4 used for titration, the amount of nitrogen in sample was calculated. The value multiplied by 6.25 give the approximate protein content of the sample tested.

Calculation

Percent of protein content in rice bran (g per 100 g of rice bran)

$$= \frac{\text{Amount of protein obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF WATER SOLUBLE PROTEIN

Water soluble protein content of rice bran was determined following the method of Lowry (Lowry, O.H., *et al.*, 1951) using BSA as the standard.

Reagents

a) 2% Na_2CO_3 solution in 0.1N NaOH

b) 0.5% copper sulfate in 1% sodium potassium tartrate

c) Folin-ciocalteaus reagent (FCR)

d) Standard protein solution: 10 mg/100 ml in distilled water

Procedure

Reagent a and b were mixed in the ratio of 50:1 and reagent-c was diluted with twice the volume of water.

The solution (1ml) was also taken in duplicate in different test tubes and 5ml of a:b mixture was added to each of the test tube. After 10 minutes, 0.5ml of FCR was added to each test tube, mixed well and kept for 30 minutes. Then spectrophotometric reading at 650 nm was recorded. The amount of soluble protein present in the rice bran was calculated by constructing a calibration curve using BSA as standard protein.

Calculation

Amount of soluble protein in the rice bran (g per 100g of rice bran)

$$= \frac{\text{Amount of soluble protein obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF POLYSACCHARIDE CONTENT OF RICE BRAN

The polysaccharide content of rice bran was determined by the Anthrone method (Jayaraman, J., 1981).

Reagent

a) Anthrone reagent (0.2% in Conc H_2SO_4)

b) Standard glucose solution (10 mg /100 ml distilled water)

c) 1 M HCl

Procedure

2-3 g of dry rice bran was homogenized with 10 ml of water. It was then filtered through double layer of Muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate, mainly the polysaccharide. After kept it over night in cold the precipitate was collected by centrifugation at 3000 g for 15 minutes. The precipitate was then dried over a steam bath. Then 40 ml of 1M hydrochloric acid was added to the dried precipitate and heated to about 70°C. It was then transferred to a volumetric flask and diluted to 100 ml with 1M HCl. Then 2 ml of diluted solution was taken in another 100ml volumetric flask and diluted further to 100 ml with 1M HCl.

Aliquot of 1ml of the extract of each variety was pipetted into different test tubes and 4 ml of anthrone reagent was added to the solution of each tube and mixed well. Glass marbles were placed on the top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes, then removed and cooled.

A reagent blank was prepared by taking 1ml of water and 4 ml of anthrone reagent in a test tube and treated as before. The absorbance of the blue-green solution was measured at 650 nm in a colorimeter. The amount of polysaccharide present in the rice bran was calculated from standard curve of glucose (Fig 1).

Calculation

The percentage of polysaccharide content (g per 100 g of rice bran)

$$= \frac{\text{Amount of polysaccharide obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF TOTAL SUGAR

Total sugar content of rice bran was determined colorimetrically by the anthrone method as describe in Laboratory Manual in Biochemistry (Jayaraman, J., 1981).

a) **Anthrone reagent:** 0.2 % in conc H_2SO_4

d) **Standard glucose solution:** 10 mg/100 ml

Extraction of sugar from rice bran: Extraction of sugar from rice bran was done by the following method as described by Loomis and shull (1937).

Five to six gram of rice bran was plunged into boiling ethyl alcohol and allowed to boil for 5-10 minutes (5 to 10 ml of alcohol was used for every g of rice bran). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of Muslim cloth and re-extracted the ground bran for three minutes in hot 80 percent alcohol, using 2 to 3 ml of alcohol for every gram of sample. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatman No-41 filter paper.

The volume of the extract was evaporated to about ¼ th the volume over a steam bath and cooled. This reduced volume of the extract was then transferred

to a 100 ml volumetric flask and made upto the mark with distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made upto the mark with distilled water (working standard).

Procedure

Aliquot of 1 ml of the bran extract from each varieties was pipetted into test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on top of each to prevent loss of water by evaporation. The test tubes were heated for 10 minutes in a boiling water bath and then cooled. A reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured by 650 nm in a colorimeter.

A standard curve of glucose was prepared by taking, 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard glucose solution in different test tubes containing 0.0, 10μg, 20μg, 40μg, 60μg, 80 μg and 100 μg of glucose respectively and made the volume upto 1.0 ml with distilled water. Then 4 ml of anthrone reagent was added to each test tube and mixed well. All these solutions were treated similarly as described above. The absorbance was measured at 650 nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

The amount of total sugar was calculated from the standard curve of glucose (Fig-1). Finally, the percentage of total sugar present in the rice bran was determined using the formula given below.

Calculation

Amount of total sugar in the rice bran (g per 100 g of rice bran)

$$= \frac{\text{Amount of total sugar obtained}}{\text{weight of rice bran}} \times 100$$

DETERMINATION OF REDUCING SUGAR

Reducing sugar content of rice bran was determined by dinitrosalicylic acid method (Miller, G.L., 1959).

Reagents

a) Dinitrosalicylic acid (DNS) reagent: Simultaneously 1 g of DNS, 200mg of crystalline phenol and 50 mg of sodium sulfite were placed in beaker and mixed with 100 ml of 1% NaOH solution by stirring. If it is needed to store then sodium sulfite must be added just before use.

b) 40% solution of Rochelle salt.

Procedure

Extraction of reducing sugar from rice bran: Reducing sugar was extracted from rice bran by the procedure as described earlier.

5 ml of the extract was pipetted into test tubes and 3 ml of DNS reagent was added to each of this solution and mixed well. The test tubes were heated for 5 minutes in a boiling water bath. After the colour has developed 1 ml of 40% Rochelle salt was added when the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3 ml of distilled water in a test tube and treated similarly. The absorbance of the solution was measured at 600 nm in a colorimeter. The amount of reducing sugar content in rice bran was calculated by constructing a calibration curve using glucose as standard.

Calculation

Amount of reducing sugar in rice bran (g per 100 g of rice bran)

$$= \frac{\text{Amount of reducing sugar obtained}}{\text{weight of rice bran}} \times 100$$

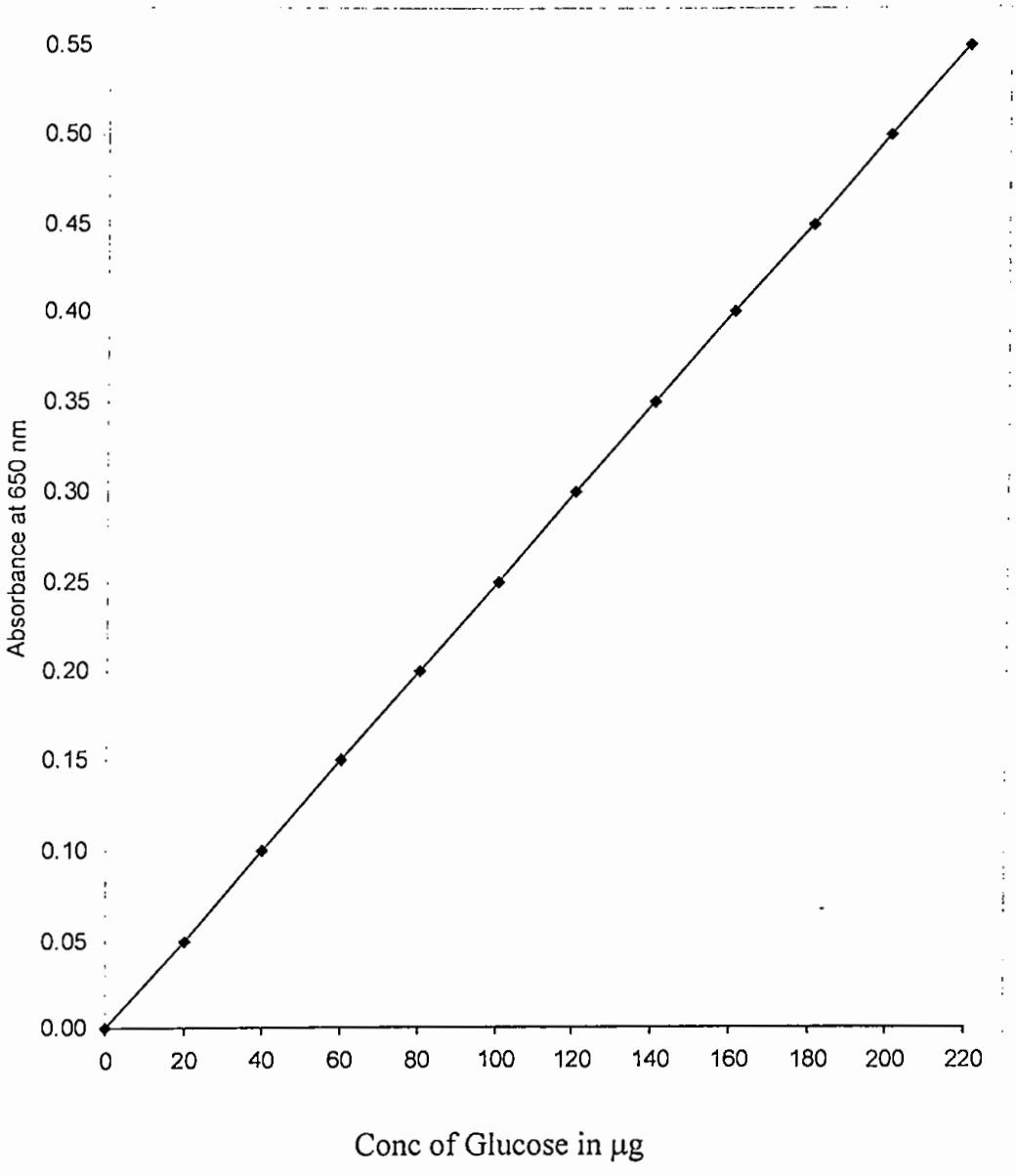

Fig-1: Standard curve of Glucose for estimation of sugar and polysaccharide.

DETERMINATION OF NON-REDUCING SUGAR

Non-reducing sugar was calculated from the formula as reported (Ranaganna, S. 1986).

DETERMINATION OF MINERALS CONTENT OF DIFFERENT VARIETIES OF RICE BRAN:

Rice bran was first converted to ash in order to determine minerals contents of rice bran. The organic and inorganic matter present in the ashes were determined by the following methods, the ash was digested with nitric acid and the minerals are released. Ca, Fe, Mn and Cu were determined by atomic absorption spectrophotometry, while K was determined by flame photometry, and P was determined by spectrophotometry.

Digestion

1.0 g of sun dried rice bran was weighed and taken into each of 38-nitrogen digestion tubes. The two remaining tubes were taken as blanks. 5 ml of 68% nitric acid was added to each of all 40 tubes. The content in each was mixed and left the tubes overnight. The tubes were placed in the digester and the tubes were covered with exhaust manifold. The temperature was set to 125°C. The digester was turned on and the digestion was continued for four hours after boiling started. After cooling the digestion mixture with distilled water was transferred to a 100 ml volumetric flask. The flask was made upto volume with water and mixed. Then it was filtered through a dry filter into a dry bottle, which could be closed with a screw cap. The filtrate was kept in the closed bottle. Ca, K, Fe, Mn, Cu and P were determined using specific filtrates.

Measurement of Calcium

Reagents

a) 68% nitric acid solution

b) 1: 100 diluted nitric acid solution

4 ml 68% nitric acid was transferred to a 2 liter volumetric flask, then the flask was made to volume with distilled water and mixed.

c) 3.25% La solution from $LaCl_3$

435 g of $LaCl_3.7H_2O$ weighed into a beaker, 100 ml. 5 M nitric acid was added to it. The total mixture was then transferred into a 5 liter volumetric flask and made upto the volume with distilled water. The mixing solution contained 3.25% La.

d) Calcium stock solution 1

2.502 g of $CaCO_3$ weighed into a 1000 ml volumetric flask. Some water and 15 ml 37% HCl were added. After complete dissolution of the $CaCO_3$, the volume was made upto the mark with distilled water. The mixing solution contained 1 g Ca per liter.

e) Calcium stock solution 2

100 ml calcium stock solution 1 was pipetted into a 500 ml volumetric flask. The flask was made to volume with distilled water and mixed. The solution contained 0.2 g Ca per liter.

f) Calcium standard solutions

00, 5.0, 10, 15 and 20 ml of calcium stock solution 2 were pipetted into a 500 ml volumetric flask. 20 ml $LaCl_3$ solution and 80 ml 1:100 diluted HNO_3 were added to each flask, and then made upto volume with distilled water. The mixing solution contained 00, 5.01, 10.02, 15.03 and 20.04 mg per liter Ca.

Procedure

20 ml diluted filtrate was transferred into a 50 ml volumetric flask using a pipette. 5 ml $LaCl_3$ solution was added, then made upto volume with water and mixed. The content of Ca was measured by Atomic Absorption Spectrometer (ASS). If the reading was higher than the highest standard solution, a larger dilution was made. In this case 1:100 diluted HNO_3 must be added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 5 ml.

Calculation

For Ca

$$\text{mg of Ca per kg rice bran} = \frac{a \times 25000}{b \times c}$$

Where, a = mg/L Ca measured on atomic absorption spectrometer

b = ml diluted filtrate transferred into the 50 volumetric flask for determination of Ca.

c = g of rice bran weighed into the digestion tube.

Additional dilution was made before transferred to the 50 ml volumetric flask and the result was multiplied with the dilution factors.

Measurement of Potassium

Reagents

a) 1: 100 diluted nitric acid solution

4 ml 68% nitric acid was transferred to a 2 liter volumetric flask, then the flask was made upto volume with distilled water and mixed.

b) Potassium stock solution 1

3.859 g of KCl weighed into a 1000 ml voilumetric flask and 500 ml water was added. After complete dissolution of the salt, the volume was made upto the mark with distilled water and mixed. The solution contained 200 mg K per liter.

c) Potassium stock solution 2

100 ml potassium stock solution 1 was pipetted into a 500 ml volumetric flaks and made upto volume with distilled water. The mixing solution contained 400 mg K per liter.

d) Potassium standard solutions

0.0, 5.0, 10, 15 and 20 ml potassium stock solution 2 were pipetted into a 500 ml volumetric flask. 40 ml 1:100 diluted HNO_3 were added to each flask, and made upto volume with distilled water. The mixing solution contained 0.00, 10.0, 20.0, 30.0 and 40.0 mg/l K.

Procedure

10 ml diluted filtrate was transferred into a 50 ml volumetric flask using a pipette. The flask was made upto volume with water and mixed. The content of K was measured by flame photometer. When the reading was higher than the highest standard solution, a larger dilution was made, e.g. 5 ml filtrate was taken into a 50 ml. volumetric flask. In this case 1:100 diluted HNO_3 was added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 10 ml.

Calculation

For K

$$\text{mg of K per kg rice bran} = \frac{a \times 25000}{b \times c}$$

Where, a=mg/L K measured on a flame photometer.

b = ml diluted filtrate transferred into the 50 volumetric flask for determination of K

c = g of rice bran weighed into the digestion tube.

Additional dilution was made before transferred to the 50 ml volumetric flask and the result was multiplied with the dilution factors.

Measurement of phosphorus

Reagent:

a) 0.5 M $NaHCO_3$ solution

b) 0.3 M H_2SO_4 solution

c) 1:100 diluted nitric acid solution: 4 ml 68% nitric acid was transferred to a 2 liter volumetric flask, the flask was made upto volume with distilled water and mixed.

d) Ammonium molybdate-ascorbic acid solution: 4.0 g ascorbic acid was dissolved in 1 liter ammonium molybdate solution. This solution was not stable and used on the same day as it was prepared. Hence, a fresh solutions might be prepared daily.

e) Phosphorus stock solution 1: 0.4394 g KH_2PO_4 was weighed into a 1L volumetric flask and some water was added. When the salt was completely dissolved, this volume was made upto the mark with distilled water and mixed. The solution contained 100 mg P per liter.

f) Phosphorus stock solution 2: 10 ml phosphorus stock solution 1 was transferred into a 100 ml volumetric flask and made upto volume with distilled water and mixed. The solution contained 1.00 mg P per liter.

g) Phosphorus standard solutions: 5 ml 0.5 M $NaHCO_3$ and 5 ml 0.3 M H_2SO_4 were transferred into each of 50 ml volumetric flasks. During 30 minutes to complete effervescence mixed and shake frequently 0.00, 5.0, 10, 15, 20 ml phosphorus stock solution 2 was added to the flasks and

water to approximately 30 ml. 10 ml ammonium molybdate-ascorbic acid solution were added to each flask. After 15 minutes, the absorbance was measured on a spectrophotometer at 890 nm.

Procedure

10 ml diluted filtrate was transferred into a 50 ml volumetric flask. 25 ml distilled water was added and mixed. 10 ml ammonium molybdate-ascorbic acid solution was added made upto volume with water and mixed. After 15 minutes, the absorbance was measured on a spectrophotometer at 890 nm. When the absorbance was higher than that of the highest standard solution, the procedure was repeated using a smaller amount of filtrate. In this case 1:100 diluted HNO_3 was added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 5 ml. When the content of P was very high, it was diluted, the filtrate further before transfer to the 50 ml volumetric flask. The dilution was made with distilled water. After transferred of 5 ml diluted filtrate to the 50 ml volumetric flaks. 5 ml 1;100 HNO_3 diluted and water to approx. 30 ml were added. Then 10 ml ammonium molybdate-ascorbic acid solution was added and volume was made upto the mark with water and the absorbance was measured at 890 nm after 15 minutes.

Calculation

For phosphorus

$$\text{mg of P per kg rice bran} = \frac{a \times 2500}{b \times c}$$

Where, a = mg/L P measured on a spectrophotometer.

b = ml diluted filtrate transferred into the 50 volumetric flask for determination of P

c = g of rice bran weighed into the digestion tube.

Additional dilution was made before transferred to the 50 ml volumetric flask and the result was multiplied with the dilution factors.

Measurement of Iron, Manganese and Cupper

The content of these elements were measured by atomic absorption spectrometer (AAS) directly in the undiluted filtrate.

Reagents

a) Catalyst mixture. 1000 g potassium sulphate (K_2SO_4) and 100 g copper (11) sulphate ($CuSO_4$, $5H_2O$ in a mortar) was crushed and mixed.

b) 33% sodium hydroxide solution (NaOH): Sodium hydroxide solution was prepared by dissolving 1667 g NaOH with water in a 5 liter volumetric flask. After complete dissolution, the flask was filled to volume with water, and the content was mixed.

c) 0.05 M sodium hydroxide solution: 2.0 g of sodium hydroxide was dissolved with water in a one liter volumetric flask and made up to the volume with distilled water and mixed well.

d) Methyl red-methylene blue indicator solution; 0.667 g of methyl red was dissolved in a 500 ml 96% ethanol. 0.625 g of methylene blue was dissolved in a 500 ml 96% ethanol. The two solutions were mixed in equal volume.

e) Ammonium molybdate-potassium antimony tartrate solution: 24.0 g ammonium molybdate $\{(NH_4)_6\ Mo_7O_{24}.4H_2O\}$ was dissolved in 500 ml water in a beaker. In another beaker, 0.5816 g potassium antimony tartrate ($C_4H_4O_7KSb$) was dissolved in 100 ml water. Then it was transferred to a 5 liter volumetric flask.

Caution: The mixture became very hot, and it might be necessary to stop the addition of H_2SO_4 allowed cooling, and then contimixing addition of sulfuric

acid. After the addition of sulfuric acid has been completed, the solution was allowed to cool at room temperature. The solution of ammonium molybdate-potassium antimony tartrate was transferred to the flask, made to the volume with distilled water and mixed. The solution was stored in a dark bottle in a refrigerator.

Calculation

For Fe, Mn and Cu

$$\text{mg of Fe/Mn/Cu per kg rice bran} = \frac{d \times 100}{c}$$

Where, d= mg/L Fe, Mn or Cu measured on atomic absorption spectrometer or spectrophotometer.

c = g of rice bran weighed into the digestion tube.

If additional dilution was made before transferred to the 50 ml volumetric flask, the result was multiplied with the dilution factors.

Determination of total Sulphur

The organic matter was destructed and the sulphur was oxidized to sulfate by digestion with a mixture of nitric acid and perchloric acid. The sulfate was determined by precipitation as barium sulfate.

Reagents

a) 68% nitric acid
b) 70% perchloric acid solution
c) 0.05 M hydrochloric acid solution
d) 0.5 M barium chloride solution
e) 5% silver nitrate solution

Procedure

i) 1.0 g of different varieties of rice bran were taken into each of 38 nitrogen digestion tubes. The two remaining tubes were taken as blanks. 5 ml 68% nitric acid was added to each of all 40 tubes. The content in each was mixed and left the tubes overnight. The temperature was set to 80°C. The digester was turned on and the digestion was continued for 1.5 hours. After turned off the digester the tubes were cooled to room temperature.

ii) After complete cooling, 5 ml of 70% perchloric acid was added to each tube and mixed well. The temperature was set to 180°C, the digester was turned on and the digestion was continued for 3 hours. After 3 hours, the digester was turned off.

iii) After cooling, the digestion mixture was transferred to a 100 ml volumetric flask. The flask was made upto volume with distilled water and mixed. They were filtered on a dry filter into a dry bottle, which could be closed with a screw cap. The filtrate was kept in the closed bottle.

iv) 80 ml filtrate was transferred into a 600 ml beaker. Distilled water was added to the beaker to the volume of approx. 300 ml. 20 ml 1:1 hydrochloric acid was added, stirred and the solution was heated to boiling. While stirring, 20 ml of 0.5 M $BaCl_2$ solution was added dropwise, and the solution was boiled for 5 minutes.

v) The beaker was covered with a watch glass and it was placed on a boiling water bath for 1 hour. The water bath was turned off and the beaker leave on the water bath until the precipitate was settled and it was cooled to room temperature.

vi) The supernatant was decanted through Whatman No. 40 filter paper and the precipitate was transferred completely to the filter paper by using hot

distilled water. The precipitate tends to adhere to the beaker and it may be necessary to rub some precipitate off the inside of the beaker using a glass spatula with rubber sleeve. The precipitate was continued to wash on the filter with hot distilled water until chloride has been removed completely. The presence of chloride was checked by adding a few drops filtrate from the funnel to a few ml 0.5% silver nitrate solution.

vii) The filter is removed from the funnel, then it was folded carefully taking care that no precipitate was lost, and it was placed in a ceramic crucible which has been heated to 800°C, cooled and weighed on an analytical balance. The filter was placed in an oven at 105°C, and it left in the oven until it dry.

viii) The crucible was moved with dry filter to a muffle furnace adjusted to 800°C. after half an hour, the crucible was removed from the muffle furnace, it was cooled in a desiccator, and weighed the crucible with precipitate on an analytical balance. The heating was repeated in the muffle furnace. Cooling and weighing until weight became constant.

Calculation

$$\text{Percent of sulfur} = \frac{A \times 1374}{M \times W}$$

Where, A = Weight of $BaSO_4$ (g)

M = Amount of Solution transferred to the beaker for precipitation of $BaSO_4$, ml.

W = Weight of rice bran sample

$\% SO_3 = \%S \times 2.50$

DETERMINATION OF B-VITAMINS

Vitamin-B_1

Vitamin-B_1 content of rice bran was determined following the method of Anon (Anonymous, 1965).

Reagents

a) Potassium ferricyanide - 2%

b) Oxidizing reagent – 10 ml of 2% potassium ferricyanide was mixed with 3.5 N NaOH solution (90 ml). This solution might be used within 4 hours.

c) 0.2 N HCl

d) Alcohol

Standard thiamin hydrochloride solution

About 25 mg of thiamin hydrochloride was transferred in 1000 ml volumetric flask and it was dissolved in 300 ml of dilute alcohol solution. The pH was adjusted to 4.0 with dilute HCl and stored in a light resistant container.

Procedure

Five gram of fresh rice bran was homogenized with 0.2 N HCl. The mixture was heated on a steam water bath, then cooled and diluted. Standard thiamin HCl solution (5ml) was mixed rapidly with 3 ml of oxidizing reagent and 20 ml of isobutyl alcohol was added within 30 sec. Then mixed the mixture vigorously for 90 sec by shaking the tube manually. A blank was prepared only by substituting the oxidizing reagent with an equal volume of 3.5 N sodium hydroxide and proceeded in the same manner. Then 2 ml of dehydrate alcohol drawn off

and transferred into cuvettes, then measured the fluorescence. 5 ml of rice bran extract were pipetted in different test tubes and treated in the same manner as described above.

Calculation

The amount of mg of thiamin hydrochloride in each 5 ml of the bran extract was calculated from the formula (A-b)/(S-d), in which A and S were the average fluorometer reading of the portion of bran extract and standard preparation treated with oxidizing reagent respectively and b and d were the reading for the blanks of bran extract and standard preparation respectively.

mg percentage of vitamin-B_1 (mg per 100 g of fresh rich bran)

$$= \frac{\text{mg of vitamin} - B_1 \text{ obtained}}{\text{weight of rice bran}} \times 100$$

Vitamin-B_2 or (riboflavin)

Vitamin-B_2 content of rice bran was determined by the method of (Anonymous, 1965).

Reagents

a) 0.02 N acetic acid
b) 0.1 N H_2SO_4
c) 0.1 N NaOH
d) 0.1 N HCl
e) 4% potassium permanganate
f) Hydrogen peroxide.

Procedure

Standard preparation: 50mg riboflavin was mixed with 300 ml of 0.02 N acetic acid and the mixture was heated on a steam bath, with frequent agitation until the riboflavin was dissolved. Then the solution was cooled and made upto 500 ml with 0.02 N acetic acid. This solution was diluted appropriately with 0.02 N acetic acid to made final riboflavin concentration of 10 μg/ml.

Extraction of riboflavin from rice bran

Fresh rice bran (10 g) were homogenized well with 0.1 N H_2SO_4 (about 50 ml). The mixture was heated in a autoclave at 121-123°C for 30 minutes, then cooled it and filtered through double layer of muslin cloth. The filter was made upto 100 ml with distilled water. Then 25 ml of this solution was taken in a beaker and 25 ml of water was added to it. The mixture was agitated vigorously and adjusted the pH to 6.0 - 6.5 with 0.1 N NaOH. Immediately, 0.1 N HCl was added until no precipitation occurs. The extract was again filtered and pH of the extract was adjusted to 6.6 – 6.8 with 0.1 N NaOH.

Rice bran extract (10 ml) was taken in the test tube, 1 ml water and 1.0 ml of glacial acetic acid were added to it. The mixture was then mixed with 0.05 ml potassium permanganate solution, allowed to stand for two minutes and 0.5 ml of hydrogen peroxide solution was added, where upon the permanganate colour was destroyed within 10 seconds. The tube was shaken vigorously until excess oxygen expelled. Then 1 ml of standard solution was pipetted in a test tube and treated in the same manner as that described for bran extract. In a suitable fluorometer, the fluorescence o the tubes was measured. Then to each tubes 20 mg of sodium hydorsulfite were added, mixed well and measured the fluorescence within 5 seconds.

Calculation

The quantity in each of the rice bran extract was calculated by the formula, $0.0001 (I_U-I_B) (I_S-I_U)$ where.

I_U= Average reading for rice bran extract.

I_S= Average reading for standard preparation.

I_B= Average reading after mixing with sodium hydrosulfite.

mg percentage of vitamin B_2 content in rice bran (mg per 100 g of rice bran).

$$= \frac{\text{mg of vitamin } B_2 \text{ obtained}}{\text{weight of rice bran}} \times 100$$

IDENTIFICATION OF AMINO ACIDS IN RICE BRAN

Rice bran samples of the experimental varieties were sieved (30 mesh) and sun dried for 5 hours. Total amino acids of rice bran samples were determined by two dimensional paper chromatography following the method as described in Laboratory methods in Bio-chemistry (Jayaraman, J., 1985; Block and Bolling, 1951).

Preparation of extract for determination of total amino acids concentration

For extraction of protein, 5 g of fresh rice bran samples were homogenized well with petroleum spirit (40°C-60°C). After unifrom mixing, it was centrifuged at 1000 rpm and the supernatant being discarded. It was repeated three times. Final washing was done by acetone and the residue was air dried. This dried bran was homogenized with water and then kept overnight with occasional stirring. The sample was centrifuged at 8000 g for 15 minutes. The supernatant was taken and then mixed with ammonium sulfate upto 100% saturation.

The $(NH_4)_2$ SO_4 precipitate was again centrifuged at 8000 g for 15 minutes. The precipitate was dissolved in minimum volume of water and dialyzed against water for 24 hours at 4°C with 2-3 changes of water. The dialyzed solution was centrifuged again at 8000 g. The clear supernatant was used as a crude extract and stored at 4°C.

Hydrolysis of protein

Crude protein solution was hydrolyzed according to the method of Block and Bolling (1951). Protein solution was evaporated to dryness, dissolved in 6N HCl and heated at 110°C for 24 hours in a evacuated sealed tube. The hydrolyzate was again evaporated to dryness and the residue was redissolved in 1 ml of water. 0.1 ml of this solution was used for analysis of amino acid compositions by two-dimensional paper chromatography.

Two dimensional paper chromatography

This was carried out in large square sheets of Whatman No-1 filter paper (20″ × 20″). The standard amino acdis mixtures and a sample of hydolyzed protein was spotted. The paper was first developed in one direction with the solvent butanol: acetic acid: water (4:1:5), when solvent front had reached the top, the paper was removed and dried thoroughly in steam of warm air. The paper was then chromatographed in the second direction perpendicular to the first direction in a solvent containing phenol: water (80:20). For each direction, the solvent was run down for 10 to 12 hours duration. The paper after development were dried by air and sprayed with 0.2% ninhydrin solution and incubated at 100°C for a short period to develop the chromato-spots. The sports were identified by comparing the R_f values with those of the standard amino acids. The ninhydrin treated violet coloured sport of the amino acids were cut and eluted into 50% aqueous ethanol. The intensity of the colour was read at 570 nm. The concentration of the amino acids were determined by constructing a standard curve using glycine as a standard amino acid.

ANALYSIS OF RICE BRAN OIL

Extraction of oil from rice bran

Oil is the triglyceride portion of the bran which is extracted by suitable solvent under the operating condition. Continuous soxhlet extraction (South combe 1926) device was used for the extraction of oil.

Apparatus

a) Weighing balance.
b) Soxhlet apparatus.
c) Rotary evaporator.
d) Flask.

Chemical

1. n-Hexane, analytical reagent grade

Procedure

Fresh bran was sun dried for 6 hours to reduce the moisture content and then crushed well to almost powder form by a hand crusher. The powdered mass (100 g) was then placed in the thimble of the apparatus. The oil was then extracted with n-Hexane in the continuous soxhlet extraction apparatus. The extract was evaporated under reduced pressure (rotary evaporator) to obtain crude oil.

PURIFICATION OF CRUDE OIL

Materials and reagents:

1. Separatory funnel.
2. Diethyl ether.
3. Saturated sodium chloride solution.

Procedure

The oil obtained from rice bran by solvent extraction process was subjected to the following treatment for purification (Haque., M.E., 1975).

Rice bran oil (150 g) was taken in a separatory funnel mixed with water (100 ml), ether (200 ml) and saturated sodium chloride solution (25 ml). The content of the separatory funnel was shaken well and allowed to stand for sometimes when two distinct layers were separated. Discarding the aqueous layer, the organic layer was again mixed with 100 ml of distilled water and saturated solution from the aqueous layer and was subjected same as before once more. Finally, the ethereal extract was taken in a conical flask and dried over anhydrous sodium sulfate. The dried ethereal solution was then evaporated at 40°C and referred as pure oil.

CHARACTERIZATION OF RICE BRAN OIL

STUDY OF THE PHYSICAL CHARACTERISTICS OF OIL

Determination of specific gravity of the oil

The specific gravity (Sp. Gr) of the oil was determined by means of a specific gravity bottle using the formula (Hilditch, T.P., 1949).

Apparatus

a) Specific gravity bottle.
b) Water bath.
c) Electrical weighing balance.

Procedure

The specific gravity bottle was cleaned, dried and weighed. The bottle was filled with distilled water, which was boiled and cooled at room

temperature for avoiding the formation of bubbles. It was then immersed in a constant temperature water bath and heated for half an hour at 25°C.

The bottle was removed from the bath, wiped dry with tissue paper and allowed to stand for 15 minutes at room temperature. The bottle with its constant was then weighed again in Electrical balance.

The same procedure was followed by replacing water of the bottle with rice bran oil and finally determined specific gravity using the formula as given below.

Calculation

Specific gravity of rice bran oil (w/w)

$$\text{Specific gravity} = \frac{\text{weight of oil in bottle}}{\text{weight of distilled water in bottle}}$$

Determination of refractive index of the oil

Refractive index is the ratio of speed of light at definite wavelength in vacuum to its speed in media. The refractive index is determined (Hilditch, T.P., 1949)

Principle

Measurement by means of a suitable refractormeter.

Apparatus

i) Abbe refractormeter (standard model 60/70)

ii) Thermostat.

Procedure

The hinged prisms of refractormeter were opened and cleaned with little ether and tissue paper. Two or three drops of oil was placed on the face of prism. The prism was then closed and the apparatus left for 2 or 3 minutes to attain equilibrium temperature. The reading was directly measured by rotating the focusing telescope until line of total reflection passed through intersection of two hair lines fixed in the field of view.

Determination of smoke point, flash point and fire point of rice bran oil

The Smoke point, flash point and fire point were determined according to the Official Method of the American oil Chemists Society AOCS (1980).

Determination of pour point and solidification point of rice bran oil

The pour point and solidification point were determined according to the ASTM Standard Method (1952).

STUDY OF THE CHEMICAL CHARACTERISTICS OF OIL

Determination of Iodine value of oil.

Iodine value (I.V) was determined by Hanus method (Williams, 1966) using the formula.

$$\text{I.V.} = \frac{Sx(X-Y)\times 0.127\times 100}{W}$$

where

S= Strength of the sodium thiosulphate solution.

X= ml of thiosulphate required in the blank experiment.

Y= ml of thiosulphate required in the test experiment.

W= Weight of oil.

Principle

Addition to the test portion of an Iodine solution. After a time of reaction, determination of excess halogen by addition of potassium iodide aqueous solution and titration of liberated iodine with a standardized sodium thiosulphate solution.

Apparatus

i) Wide necked glass bottle with ground joint stopper.

ii) Burette/ pipette.

Reagents

i) Chloroform, analytical reagent grade

ii) Glacial acetic acid, free from ethanol

iii) Potassium iodide solution, 15%

iv) Bromine

v) Sodium thiosulphate solution, standardized before use.

vi) **Starch indicator:** Starch (1 g) was dissolved in 50 ml hot water and diluted to 100 ml with cold water.

vii) **Hanus solution:** Iodine (13 g) was dissolved in glacial acetic (12 ml) acid. It was warmed to dissolved iodine completely. The mixture was cooled at room temperature. Then enough bromine was added to double halogen content, usually about 3 ml was sufficient.

Procedure

The oil (4 g) was dissolved in 10 ml of chloroform in a dry glass stoppered bottle (500 ml). To the content 25 ml of Hanus solution was added and the mixture was allowed to stand in the dark for exactly 30 minutes with occasional shaking Potassium iodide solution (10 ml) was mixed to it and the

mixture was shaken well. Degassed water (100 ml) was added to the mixture and the content of bottle was titrated with sodium thiosulphate solution using starch solution as indicator.

A blank experiment was performed exactly in the same manner without oil.

Determination of saponification value and saponification equivalent

Saponification value (S.V) is the number of mg of KOH required to saponify 1 g of oil. The saponification value was determined (IUPAC, 1967) using the following formula

$$S.V = \frac{56.1 \times (A - B) \times \text{strength of acid used}}{W}$$

where

A= ml of acid required for blank experiment

B= ml of acid required for test experiment

W= weight of oil

Principle

Boiling of the test portion under reflux with ethanolic potassium hydroxide solution and titration of excess potassium hydroxide with hydrochloric acid in presence of an indicator.

Apparatus

i) Round bottom flask

ii) Reflux condenser

iii) Pipette and burette

iv) Water bath

Reagents

i) Alcoholic solution of potassium hydroxide (0.5 N) approximately.

ii) 0.5 N hydrochloric acid.

iii) Phenolphthalein solution (1% in alcohol)

Procedure

The oil (2-3 g) was taken in a conical flask and 25 ml alcoholic potassium hydroxide solution was added to it. The flask was then connected to reflux condenser, and heated on a boiling water bath so that the alcoholic solution boiled gently for 30 minutes. During this time the flask with its content was shaken occasionally to prevent agitation. A blank experiment (without oil) was performed simultaneously in same manner as described. After 30 minutes, both flasks were removed from water bath and their contents, while still hot were titrated with hydrochloric acid (0.5 N) using phenolphthalein as indicator.

Saponification equivalent (S.E) (Paech, K., *et al.,* 1955) was calculated from the saponification value using formula.

$$S.E = \frac{56100}{S.V.}$$

Determination of peroxide value of oil

Peroxide is milliequivalent of active oxygen per Kg of oil. Peroxide value of oil was determined (Jacobs, 1958) using the formula.

$$\text{Peroxide value} = \frac{(B-S)\times N}{W}\times 100$$

where

S= titration with sample

B= Blank titration

N= Exact normality of thiosulphate (0.01 N) solution

W= Mass of test portion

Principle

Treatment of the test portion, in acetic acid and in chloroform, by a solution of potassium iodide and titration of liberated iodine with standardized sodium thiosulphate solution.

Apparatus

i) Flask, glass stoppered
ii) Burette

Reagents

i) Acetic acid-chloroform solution.

ii) Potassium iodide, saturated aqueous solution, recently prepared, free from iodine and iodates.

iii) Sodium thiosulphate, accurately standardized aqueous solution, standardized just before use.

iv) Starch solution- 1 g starch was dissolved in 50 ml hot water and diluted to 100 ml with cold water.

Procedure

The oil (1.5-2.0 g) was taken in a 250 ml glass stoppered flask and 30 ml of acetic acid-chloroform solution was added to it. The oil was mixed well and 1 ml of saturated potassium iodide solution was added. The stopper was inserted quickly and the mixture was shaken for 1 minute. It was allowed to stand for 5 minutes away from light at a temperature of 15-25°C. About 75 ml of distilled water was added to mixture and liberated iodine was titrated with standardized thiosulphate solution by shaking vigorously, using starch solution as indicator.

A blank test was carried out simultaneously in same manner as described above.

Determination of acid value and % FFA (as oleic) of the oil

Determination of percentage of free fatty acid

The percentage of free fatty acids (% FFA) is measured (Augustus, *et al.*, 1933) using the following formula (as oleic)

$$\% \text{ FFA (as oleic)} = \frac{V \times S \times 28.2}{W}$$

where

V= Number of ml of alkali required to neutralize.

S= Strength of alkali.

W= Weight of oil.

Principle

Solution of oil to be analyzed in a mixture of ethanol and diethyl ether, followed by titration of free fatty acids with a solution of alkali.

Apparatus

i) Conical flask.
ii) Burette and pipette.

Reagents

i) Solvent mixture (1:1) of 95% (v/v) ethanol and diethyl ether, Neutralized exactly by means of KOH solution in presence of phenolphthalein indicator.
ii) Potassium hydroxide solution (0.1N) accurately standardized.
iii) Phenolphthalein indicator, in 1% ethanol.

Procedure

The oil (1.0-1.5 g) was taken in a conical flask. The oil was dissolved in the neutralized solvent mixture of ethanol and diethyl ether (1:1, v/v), 2-3 drops of phenolphthalein solution was added and shaken well. The mixture was titrated, by shaking with standardized potassium hydroxide solution to the end point of indicator.

Determination of acid value

It is the number of mg of KOH required to neutralize free fatty acid present in one gram of oil. This is used for determining the rancidity due to free fatty acids.

Regents

Same as used in the determination of % FFA.

Procedure

Acid value (A.V) was determined using the formula (Devive *et al.*, 1961).

$$A.V = \frac{V \times S \times 56.1}{W}$$

where

V= Number of ml of alkali, needed for titration
S= Strength of alkali.
W= Weight of oil.

The procedure was same as described earlier for % FFA.

Determination of quantity of unsaponifiable matter

The unsaponifiable matter is a fraction of fat or oil that remain insoluble after saponification of fat sample by alkali. The unsaponifiable matter includes the sterols, higher alcohols, pigments and hydrocarbons.

Principle

Saponification of oil by an alcoholic alkaline solution, dilution and then extraction of unsaponifiable matter.

Apparatus

i) Round bottom flask (ground joint)
ii) Reflux condenser.
iii) Desiccator
iv) Separatory funnel
v) Water bath
vi) Oven, regulated at 150°C ± 2°C

Regents

i) Diethyl ether, Analytical reagent grade
ii) Acetone, Analytical reagent grade
iii) Alcoholic solution of potassium hydroxide (0.5 N)
iv) Phenolphthalein, 1% solution in ethanol

The amount of unsaponifiable matter present in oil was determined using the method as described (Williams, 1966).

Procedure

The oil (4 g) was taken in a round bottom flask and 1N alcoholic potassium hydroxide (50 ml) was added to it. The mixture was then refluxed for 45 minutes on a water bath at 70°C to 80°C with occasional swirling. The solution was transferred to a separatory funnel and rinsed with 100 ml water. The solution while still perceptibly warm, extracted four times with ether using 50 ml each time. Each extraction was done by vigorous shaking in the separatory funnel and allowing the two layers to be separated. The aqueous alcoholic layer at the bottom of separating funnel was run off and the ethereal solution from top was poured into another separating funnel containing 20 ml water. The total ether extracts in the separating funnel was washed thrice using 20 ml water each time.

The ethereal layer was then washed three times with 20 ml aqueous potassium hydroxide solution. Each alkali wash was followed by a wash with 20 ml water. After the aqueous alkali wash, the solution was washed with sufficient amount of water, till the washing did not show alkaline reaction to phenolphthalein solution.

The ether extract was then transferred into a weighed flask and ether was evaporated. The residue was dried to constant weight at 80°C. Drying was assisted by adding 2-3 ml of acetone to the extract when nearly all ether was evaporated. The flask with its contents was then weighed. The quantity of unsaponifiable matter present in 100 g of oil was calculated from the following formula.

$$\text{Unsaponifiable matter} = \frac{\text{Wt of unsaponifiable matter}}{\text{Wt, of oil taken}} \times 100$$

Glyceride composition of rice bran oil

The determination of mono-, di- and triglyceride in oil were carried out by column chromatography (Gofur, *et al.,* 1985) on Silica gel (70-120 mesh, E Merck, Darmstade) free from ether soluble substances. The silica was made free from moisture by heating at 120°C for 2 hours and cooled to room temperature in a desiccator. The gel was then hydrated with 5% water (w/w) and allowed to stand for atleast 2 hours with shaking occasionally to ensure complete water distribution. One gram of oil was dissolved in 15 ml of chloroform and applied to the prepared column (15 mm dia and 250 mm long). The triglyceride fraction was eluted with 200ml of benzene, while diglyceride fraction with 200 ml of a mixture of benzene and diethylether (9:1 v/v) and monoglyceride fraction with 200 ml of diethylether. The flow rate was adjusted to 2 ml of effluent per minutes. FFA, present in lipid, was eluted with diglyceride fraction.

The elution of each fraction was monitored by microslide. Thin layer chromatography (TLC) was performed to ensure uniformity of separation of each class of glyceride during silicic acid chromatography and the eluted solvent were collected in a weighed flask. The fractions thus obtain were evaporated in rotary vacuum evaporator and were dried under reduced pressure before being weighed. The purity of glyceride classes was further checked by TLC using silica gel bounded plaster, ascending development with n-hexane-diethyl ether 80:20 (v/v) and visualization with chromic-sulphuric acid at 180°C. The glyceride classes were identified by R_f comparison with standard references.

Separation of saturated and unsaturated fatty acids from different varieties of rice bran oil

Separation of saturated and unsaturated fatty acid were carried out by lead ether method (Das, R.K., 1989). The oil (50g) was saponified with alcoholic caustic soda to obtain soap solution. A slight excess of lead acetate solution was added to the soap solution to form lead salts of fatty acids which were then separated. Ether was added to the mixture of lead salts the whole mixture was boiled and then cooled at 0°C for 24 hours. The precipitated lead salts of saturated fatty acids so formed were separated from the solution of lead salts of unsaturated fatty acids by filtration. The lead salts of unsaturated fatty acids were obtained by removing ether from the ethereal solution. Each group of lead salts was suspended in water and treated with sufficient hydrochloric acid to form fatty acids and lead chlorides. The mixture was then extracted with ether to obtain ethereal solution of each group. On evaporating ether, the fatty acids were obtained in separated group. Finally masses of saturated and unsaturated fatty acids were obtained by weighing them separately.

Separation of lipid classes by column and thin layer chromatography

Fractionation of lipids was done by silicic and column chromatography (Ali, M.A. *et al.,* 1985) with elution rate of 0.5-1.0 ml/ minute. The silicic acid (E.Merck Darmstadt, W.Germany, 80-120 mesh) was washed with water and methanol to remove fines and impurities. It was then activated at 120°C overnight and again for 1 hour immediately before the column was prepared. For each column, 25 g silicic acid was washed with 250 ml of chlorofrom/methanol (7:1 v/v), 125 ml chlorofrom/methanol (15:1 v/v) and 160 ml chlorofrom. A slurry of 25 g of silicic acid in chlorofrom was poured into the column (2.5 cm). The column was then washed with 150 ml of diethyl ether and 350 ml of 4% diethylether in petroleum ether (b.p 60-80°C). 300 mg of total lipids were dissolved in 10 ml eluting solvent and quantitatively transferred to the column. The solvent systems used was similar to those described (Paech, K., *et al.,* 1955). In a typical fractionation 60ml of 4% diethyl ether in petroleum ether was used to elute the hydrocarbons and then 700 ml of the same solvent system removed triglycerides. Free sterols were eluted with 300 ml of chloroform and partial glycosides with an additional 350 ml of chloroform. The polar lipids in glycolipid and phospholipids were eluted with 700 ml of acetone and 400 ml of methanol respectively. The elution was controlled with a flow rate of 2.0-2.5 ml/min.

The elution of each fraction was monitored by microslide thin layer chromatography to ensure uniformity of separation of each lipid class during silicic acid chromatography and the eluted solvents were collected in flask of known weight. The fraction thus obtained were evaporated in a rotary vacuum evaporator and were dried under reduced pressure before being weighed again. The purity of lipid classes was further checked by TLC on 20 × 20cm plates coated with a layer (0.5 mm) of silica gel. The lipid classes were identified by R_f comparison with standard references.

PREPARATION OF FATTY ACID MIXTURE FROM RICE BRAN OIL

Fatty acid mixture was prepared from oil, following the procedure as reported earlier (Clark. J.M., 1964).

Reagents

a) Oil
b) Diethyl ether
c) Hydrochloric acid (5 N approximately)
d) Concentrated hydrochloric acid
e) Alcoholic potassium hydroxide (5.6%)

Procedure

The oil (15 g) was taken in a round bottom flask (250 ml) and alcoholic potassium hydroxide solution (100 ml) was added to it. The mixture was then refluxed 45 minutes on a water bath untill it became clear. The reaction mixture was allowed to cooled and neutralized with hydrochloric acid (5 N). Alcohol was then removed from the neutralized solution by evaporation over a steam bath. Water (25 ml) was then added to alcohol free solution and the pH of the solution was adjusted to 1-2 by adding concentrated hydrochloric acid dropwise. The acidified aqueous mixture was then extracted with 20 ml of ether in a separatory funnel and the extraction was repeated for three times. The combined ether extract was washed twice with 15 ml of water in order to remove any adhering hydrochloric acid. Ether was then removed from the extract to give fatty acid mixture.

PREPARATION OF METHYL ESTER FROM FATTY ACID MIXTURE

Methyl esters were prepared from fatty acid mixture, using the procedure (Golbar Hossain, 1973).

Reagents

a) Fatty acid mixture obtained from oil.
b) 0.25 M methanolic solution of sulphuric acid.
c) Diethyl ether.
d) Dilute sodium carbonate solution.

Procedure

The fatty acid mixture obtained from oil was used for preparation of methyl ester mixture. The fatty acid mixture was taken in a round bottom flask (250 ml) and methanolic solution of sulphuric acid (0.25 M, 5 ml per gram of acid) was added and refluxed for about 2 hours until the solution became clear. The reaction mixture was then dissolved in ether (25 ml) in a separatory funnel and was washed with dilute sodium carbonate solution until the effervescence ceased. It was then washed twice with 10 ml of water and dried over anhydrous sodium sulphate. Ether was finally removed to give the methyl ester mixture.

GAS LIQUID CHROMATOGRAPHIC EXAMINATION OF THE MIXTURE OF FATTY ACID METHYL ESTER OBTAINED FROM RICE BRAN OIL

Analysis of fatty acid composition of the rice bran oil was carried out with a PYE-UNICAM 4500 U gas chromatograph fitted with a flame-ionization detector, following the procedure (Kansella *et al.*, 1977).

Column: Internal diameter 2mm, length 1.5 meter

Packing material: 10% DEGS (Diethylene glycol succinate) coated with solid material Gas chrom p, 100-120 mesh.

Recorder: For qualitative and quantitative evaluation of results a PM 8251, single pen recorder (Phillips) was used.

Sample: Dilute solution of methyl ester mixture of oil sample in chloroform (5 μg/100 μl) was used.

Standard samples: Methyl ester of Myristic, palmitic, oleic, stearic, linoleic, linolenic and arachidic acid (Sigma chemical Co. Ltd. USA) (5μg/ 100μl).

Procedure

a) **Preparation of chromatograph:** One μl of all standard methyl ester mixture was injected to injection port. The separation was affected on column at 130-230°C, 4°C per minute and the temperature of injection port and detector were maintained at 240°C and 250°C, respectively. An standard chromatograph was obtained after 40 minutes of sample injection. Chromatograph for lipid sample was also prepared by similar procedure described above.

b) **Identification of fatty acid:** The fatty acids present in oil samples were identified by comparing the relative retention time and peak position of the sample chromatograph with those of the standard.

c) **Quantification of fatty acids:** Amount of fatty acids present in a oil sample are proportional to the areas under the peaks. So area under the peak representing each fatty acid was calculated and summed. Then area under each peak (or amount of each fatty acid) was divided by total area under all peaks (total amount of fatty acids) to obtain proportion of each fatty acid. The area under each peak can be obtained by an integrator or by using the following formula:

$$\text{Area} = \frac{1}{2}(H \times W)$$

Where

H= Height of a peak measured from the base line of the chromatograph.

W= Width of the peak at half the height of the peak.

RESULTS AND DISCUSSION

The data on the milling paddy rice in some automatic rice mills of Bangladesh are given in Table-3. The quantity and quality of finished products of paddy rice depends upon the method of milling, and the condition of rice before milling. The average moisture content of paddy rice samples was 10.54% while the average moisture content of rice bran was 10.39%. After sieving the bran samples through a 30 mesh sieve, the percentage of the fine bran which passed through the sieve and also the percentage of the coarse material which retained by the sieve were determined. The average percentage of the fine bran was 91.89 and that of the coarse bran was 8.09

Table-3: Data on experimental rice brans obtained from automatic rice mills.

Quality and Quantity of mill products	Variety of paddy Rice					Average
	BR-5	BR-10	IRRI-28	BRRI-39	Kalijira	
Polished Rice %	65.20	64.79	66.33	65.37	64.29	65.20
Rice bran, % (non sieved)	6.25	6.14	6.90	6.75	6.85	6.42
Rice hull, %	28.55	27.04	28.43	27.88	28.66	28.38
Polished rice (Finished product) Rice grain, %	98.18	98.75	98.85	98.10	97.95	>97.95
Broken hull, %	0.71	0.66	0.37	0.48	1.10	<1.5
Rice bran, %	0.91	0.56	0.72	1.22	0.95	<1.5
Rice bran (Sieved 30 mesh)						
Fine bran, %	91.18	92.53	92.54	90.92	92.36	91.89
Coarse bran, %	8.82	7.47	7.46	9.08	7.64	8.09
Paddy rice moisture, %	10.06	10.40	10.01	11.02	11.06	10.54
Rice bran moisture, %	10.17	10.90	9.98	10.41	10.50	10.39

The polished rice contained more than 97% of rice grains, while in broken hall, rice bran were found to be present less than 1.5%.

The yield of polished rice was found to be more or less very similar. As shown in Table-3, the amount of milled rice bran in different varieties was found to be between 6.14 to 6.9%.

MOISTURE, ASH, TOTAL SOLUBLE SOLID AND CRUDE FIBRE CONTENTS OF DIFFERENT VARIETIES OF RICE BRAN

Moisture: As shown in the Table-4, the moisture content of different varieties of rice bran were varied from 9.98 to 10.90%. BR-10 variety contained the highest percentage of moisture (10.90%) whereas BR-5 variety contained the lowest amount of moisture (10.17%). The present results are very similar to that reported for the different varieties of Japanese rice bran(9.34-11.87%) by Tci-Hidaka (1939).

Ash: The ash content of different varieties of rice bran were tabulated in Table-4, The amount of ash content of different varieties of rice bran were found to be ranged between 9.45% to 11.12%. BR-10 variety contained the highest amount (11.12%) while IRRI-28 variety contained the lowest amount of ash (9.45%). The present results are very similar to that reported for the different varieties of Bangladeshi rice bran (10.09-11.01%) by Maksud Ali M. *et al.* (1998).

Table-4: Moisture, ash, total soluble solid and crude fibre contents of different varieties of rice bran.

Name of variety	Moisture (g%)	Ash (g%)	Total soluble Solute (g%)	Crude fibre (g%)
BR-5	10.17 ± 0.021	10.96 ± 0.025	11.25 ± 0.030	10.35 ± 0.016
BR-10	10.90 ± 0.019	11.12 ± 0.020	10.06 ± 0.020	10.15 ± 0.013
IRRI-28	9.98 ± 0.020	9.45 ± 0.038	10.39 ± 0.025	9.35 ± 0.016
BRRI-39	10.41 ± 0.016	10.25 ± 0.019	10.17 ± 0.020	9.45 ± 0.012
Kalijira	10.50 ± 0.015	10.06 ± 0.30	10.18 ± 0.30	9.18 ± 0.016

Total soluble solid (TSS): The analytical data for TSS content in different varieties of rice bran are shown in Table-4. It appears that TSS content in the bran was ranged from 10.06% to 11.25%. BR-5 variety contained the highest amount of TSS (11.25%) whereas BR-10 variety contained the lowest amount of TSS (10.06%).

Crude fibre: Crude fibre is the substances which are insoluble upon boiling in 0.225% H_2SO_4 and in 1.25% NaOH (A.A.C.C, 1962). The main components are cellulose and lignin, and it has pronounced effect on digestion and absorption process of nutrients. As given in the Table-4, the crude fibre content of different varieties of rice bran was found to be in the range of 9.18% to 10.35%. Among the varieties tested the BR-5 variety contained the higher amount of crude fibre followed by BR-10 and so on in decreasing order. The present results are very similar to that reported for different varieties of Bangladeshi rice bran (9.13-9.81%) by Mokhlesur Rahman, M. *et al.* (1999).

TOTAL PROTEIN, WATER SOLUBLE PROTEIN AND DRY MATTER CONTENTS OF DIFFERENT VARIETIES OF RICE BRAN:

Total Protein: Protein plays important roles in all the biological processes and its functions are: (a) exzymatic catalysis, (b) transport and storage, (c) co-ordination motion, (d) generation and transmission of nerve impulses, (e) control of growth and differentiation, and (f) as regulators. The amount of protein in different varieties of rice bran are shown in Table-5. As found, the protein content in different varieties of rice bran were ranged from 12.45% to 14.06%. Of the varieties examined IRRI-28 variety contained the highest and BR-10 variety contained the lowest amount of protein. David *et al.* (1964) reported that the protein content of raw rice bran varied from 5.7-15.5%, while Krishnamuti and Sree Ramle (1982) suggested that the protein content of bran varied depending upon the variety Finding of present results suggested that rice bran might be used as a potential source of protein.

Water soluble protein: As shown in Table-5, the water soluble protein content of different varieties of rice bran were found to be ranged between 3.20% to 4.71%. IRRI-28 variety contained the highest amount while BRRI-39 variety contained the lowest amount of water soluble protein.

Table-5: Total protein, water soluble protein and dry matter contents of different varieties of rice bran.

Name of variety	Total protein (g %)	Water soluble protein (g %)	Dry matter (g %)
BR-5	13.33 ± 0.011	3.98 ± 0.012	89.83 ± 0.030
BR-10	12.45 ± 0.016	3.25 ± 0.013	89.10 ± 0.031
IRRI-28	14.06 ± 0.020	4.71 ± 0.018	90.02 ± 0.030
BRRI-39	12.71 ± 0.016	3.20 ± 0.020	89.59 ± 0.030
Kalijira	12.81 ± 0.020	3.26 ± 0.011	89.50 ± 0.041

Dry matter: The dry matter content of rice bran was ranged from 89.10%-90.02% indicating no such significant variation in dry matter contents among the varieties.

POLYSACCHARIDE, TOTAL SUGAR, REDUCING SUGAR AND NON-REDUCING SUGAR CONTENTS OF DIFFERENT VARIETIES OF RICE BRAN

The analytical data of polysaccharide, total sugar, reducing sugar and non-reducing sugar contents of different varieties of rice bran are given in Table-6.

The amount of polysaccharide present in different varieties of rice bran samples were varied from 10.95%-12.86%. As shown in the Table-6, the kalijira variety contained the highest amount (12.86%) whereas IRRI-28 variety contained the lowest (10.95%) amount of polysaccharide.

As presented in Table-6, the total sugar content of different varieties of rice bran were ranged between 4.35% to 5.04%. Among the variety examined Kalijira contained the highest amount (5.04%) while BR-10 variety contained the lowest amount of total sugar (4.35%).

Table-6: Polysaccharide, total sugar, reducing sugar and non-reducing sugar contents of different varieties of rice bran.

Name of variety	Polysaccharide (g %)	Total sugar (g %)	Reducing sugar (g %)	Non-reducing sugar (g %)
BR-5	12.25 ± 0.015	4.96 ± 0.012	1.45 ± 0.011	3.51 ± 0.014
BR-10	11.35 ± 0.011	4.35 ± 0.011	1.25 ± 0.011	3.10 ± 0.021
IRRI-28	10.95 ± 0.019	4.85 ± 0.013	1.36 ± 0.014	3.49 ± 0.026
BRRI-39	11.06 ± 0.020	4.73 ± 0.015	1.15 ± 0.015	3.58 ± 0.025
Kalijira	12.86 ± 0.021	5.04 ± 0.012	1.08 ± 0.012	3.96 ± 0.030

As given in Table-6, both reducing and non-reducing contents were found to be varied between 1.08-1.45% and 3.10-3.96% respectively. Of the varieties examined BR-5 contained the highest and Kalijira variety contained the lowest amount of reducing sugar while non-reducing sugar content was found to be highest in Kalijira and the lowest in BR-10 variety.

Minerals content of different varieties of rice bran

Table-7: Minerals (K, Ca, Mg, Na, P, S, Fe, Zn and Mn) contents in different varieties of rice bran.

Minerals	Varieties of rice bran				
	BR-5	BR-10	IRRI-28	BRRI-39	Kalijira
K(%)	0.780	0.630	0.840	0.960	0.800
Ca(%)	0.140	0.340	0.180	0.260	0.280
Mg(%)	0.413	0.312	0.210	0.396	0.400
Na(%)	0.168	0.154	0.197	0.203	0.163
P(%)	0.200	0.219	0.316	0.301	0.271
S(%)	0.146	0.138	0.106	0.099	0.140
Fe(%)	0.075	0.098	0.164	0.152	0.175
Zn(%)	0.0062	0.094	0.0167	0.104	0.084
Mn(%)	0.036	0.081	0.059	0.092	0.160

Minerals content of five different varieties of rice bran were presented in Table-7. Nine minerals such as potassium, Calcium, Magnesium, Sodium, Phosphorus, sulphur, Iron, Zinc and manganese were found to be present in all the varieties of rice bran. BR-5 variety contained the highest amount of magnesium (0.413%) and sulphur (0.146%) while BR-10 variety contained the highest amount of calcium (0.340%). Again IRRI-28 variety contained the highest amount of potassium (0.840%), Phosphorus (0.316%) and Zinc (0.167%). The highest value of sodium (0.203%) was observed in BRRI-39

variety whereas Kalijira variety contained the highest amount of Iron (0.175%) and manganese (0.160%).

VITAMIN CONTENTS OF DIFFERENT VARIETIES OF RICE BRAN

Vitamin-B_1 (Thiamine)

Thiamine in tissues mostly in the form of thiamine pyrophosphate, known as co-carboxylase. TPP Serves as co-enzyme in carbohydrate metabolism. Beside the metabolic role, thiamine has a specific role in neurophysiology depending on its co-enzyme function. Thiamine is essential for growth, normal appetite, digestion and healthy nerves.

Table-8, shows the analytical values of vitamin B_1 content of the different varieties of rice bran. As shown in the Table, vitamin-B_1 content was ranged from 2.98-3.81 mg%. IRRI-28 variety rice bran contained the maximum percentage of vitamin-B_1 followed by Kalijira and so on decreasing order.

Table-8: Vitamin B_1 and B_2 contents of different varieties of rice bran.

Name of Variety	Vitamin B_1 (mg/100 g bran)	Vitamin B_2 (mg/100 g bran)
BR-5	3.04 ± 0.015	0.85 ± 0.021
BR-10	3.25 ± 0.020	0.76 ± 0.032
IRRI-28	3.81 ± 0.036	0.86 ± 0.034
BRRI-29	2.98 ± 0.013	0.61 ± 0.022
Kalijira	3.45 ± 0.026	0.95 ± 0.036

Riboflavin (Vitamin B_2)

Vitamin B_2 is a crystalline pigment, the principal growth-promoting factor of the vitamin B-complex. It functions as flavoprotein in tissue

respiration. Riboflavin may be used as medicine, animal feed supplement, enriched flours, dietary supplement etc. It functions as a co-enzyme for many flavine enzymes.

Table-8, shows the analytical values of vitamin B_2 content of the different varieties of rice bran. As described in the Table, vitamin B_2 content was ranged between 0.61-0.95 mg%. Of the varieties examined IRRI-28 variety contained the highest amount while BRRI-39 variety contained the lowest amount of vitamin-B_2.

Table-9. Some amino acid compositions determined by paper chromatography of different varieties of rice bran

(percentage in fresh rice bran)

Amino acid	Rice bran varieties				
	BR-5	BR-10	IRRI-28	BRRI-39	Kalijira
Alanine	0.896	0.730	0.874	0.703	0.569
Arginine	0.436	0.519	0.642	0.529	0.538
Aspartic acid	0.998	0.845	0.976	0.842	0.735
Glutamic acid	1.301	0.976	1.425	0.897	0.784
Glycine	0.610	0.740	0.415	0.437	0.533
Histidine	0.205	0.316	0.419	0.217	0.253
Leucine	0.412	0.521	0.317	0.254	0.316
Lysine	0.438	0.243	0.419	0.295	0.327
Phenylalanine	0.614	0.539	0.678	0.413	0.476
Proline	0.531	0.610	0.705	0.546	0.739
Serine	0.406	0.541	0.627	0.639	0.343
Tyrosine	0.714	0.459	0.913	0.615	0.516

Some amino acids contents of five different varieties of rice bran were presented in Table-9. Twelve amino acids such as alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine, proline, serine and tyrosine were detected by paper chromatography in all the varieties of rice bran.

As shown in the Table, BR-5 variety contained the highest amount of alanine (0.896%) aspartic acid (0.998%) and lysine (0.438%). Again BR-10 Variety contained the highest amount of glycine (0.740%) and leucine (0.521%). The highest value of arginine (0.642), glutamic acid (1.425%), histidine (0.419%) and phenylalanine (0.678%), were obtained in variety IRRI-28 whereas BRRI-39 variety contained the highest amount of serine (0.639%). and Kalijira variety (0.739%) contained the highest amount of proline

ANALYSIS OF RICE BRAN OIL

Physical characteristics of rice bran oil.

The oil content of different varieties of rice bran were extracted with soxhlet apparatus using n-hexane as a extracting solvent and it was found to be ranged between 14.95% to 16.16% as shown in Table-10. Kalijira variety contained the highest amount (16.16%) while IRRI-28 variety contained the lowest amount of oil (14.95%) (Table-10)

The oil contents of all the varieties under investigator are very similar within range so far reported by different workers Murti and Dollear (1948) found that the rice bran contained 11.9%-16.9% oil. Hussain, M.G. *et. al.*, (1995) reported that rice bran grown in the climatic condition of Bangladesh contained 12.24% oil while. Subramaniyam, V. (1971) and Desikachar H.S.R. (1977) reported an increase in oil content in the bran due to boiling.

Specific Gravity (Sp. Gr.)

The specific gravity of fats or oil does not vary as general rule to an extent which makes this properly useful in discriminating between one fat and another. The specific gravity of particularly all fats or oils lies between 0.900 to 0.950 (Hilditch, P. 1949). As shown in the Table-10, the specific gravity of the rice bran oil of presently examined varieties were varied from 0.924 to 0.966 at 25°C.

BR-5 variety gave the maximum value while BRRI-39 variety gave the minimum value. The rest of the variety gave the value in between. The values obtained in the present studies are slightly higher to that reported for rice bran oil (0.918-0.922) by Maksud Ali M. *et al.* (1998).

Table-10: Oil content and physical characteristics such as specific gravity and refractive index of different varieties of rice bran oil.

Name of variety	Amount of oil (%)	Specific gravity	Refractive Index
BR-5	15.38 ± 0.011	0.966 ± 0.014	1.563 ± 0.010
BR-10	15.08 ± 0.012	0.925 ± 0.010	1.569 ± 0.013
IRRI-28	14.95 ± 0.010	0.927 ± 0.012	1.564 ± 0.015
BRRI-39	15.11 ± 0.013	0.924 ± 0.013	1.568 ± 0.013
Kalijira	16.16 ± 0.009	0.929 ± 0.009	1.561 ± 0.019

Refractive Index (R.I)

The refractive power of fats or oils varies somewhat widely and is chiefly governed by the proportion and degree of unsaturated matter present.

It was found from the Table-10, that the R.I of the oils of different varieties of rice bran varied from 1.561 to 1.569. As shown in the Table, BR-10 variety rice bran gave the maximum value followed by BRRI-39.

For comparison, it may be mentioned that the refractive index of Brassica oil, Linseed oil, Sesame oil, Sunflower oil and Olive oil are 1.470, 1.475, 1.446 and 1.466 respectively (Rezaul Karim 1992). The present values of this investigation is quite similar to that of the reported values for other plant oils.

Table-11: Smoke point, flash point, fire point, pour point and solidification point of different varieties of rice bran oil.

Constants	Rice bran oil				
	BR-5	BR-10	IRRI-28	BRRI-39	Kalijira
Smoke point (°C)	206	211	214	209	217
Flash point (°C)	331	335	337	334	340
Fire point (°C)	351	359	354	348	357
Pour point (°C)	-6.52	-6.43	-6.65	-6.39	-6.42
Solidification point (°C)	-8.49	-8.57	-8.80	-9.04	-9.18

As shown in the Table-11, the smoke point of the different varieties of rice bran oils were found to be ranged from 206°C-217°C. Kalijira variety gave the maximum value where as BR-5 variety gave the minimum value. It may be also concluded from the present data that flash point, fire point, pour point and solidification point of different varieties of rice bran oils were more or less very similar.

CHEMICAL CHARACTERISTICS OF RICE BRAN OIL

Saponifiction Value

As shown in the Table-12, the Saponification value of different varieties of rice bran oils were found to be ranged between 181.40-189.00. The IRRI-28 variety gave the maximum value followed by Kalijira and so on in slightly decreasing order. This result is found to be very similar to that reported for rice bran oil by Maksud Ali M. *et al.* (1998).

Iodine Value

As shown in the Table-12, the iodine value of different varieties of rice bran oil was ranged between 100.18 to 102.99. The IRRI-28 variety gave the maximum value (102.99) followed by BRRI-39 (101.04), BR-5 (101.00), BR-10 (100.85) and Kalijira (100.1 8). In the literature (Jacobs M.B. 1958) the iodine value is found within the range of 120-141. Some vegetable oil namely olive oil, linseed oil, sunflower oil and cotton seed oil have the iodine values of 80-85, 175-200, 125-141 and 102-114 respectively (Kishan Lal 1990). The present data showed quite similar data to that of the reported values.

Peroxide value

As described in the Table-12, the peroxide value of different varieties of rice bran oil was ranged between 1.08 to 1.71. The Kalijira variety contained the highest while IRRI-28 variety contained the lowest peroxide value. Rice bran oil contained natural antioxidants for this reason rice bran oil is very resistant to oxidative deterioration. Experimental values are within the range of reference value for rice bran oil by Maksud Ali M. *et al.* (1998).

Table-12: Chemical characteristics of different varieties of rice bran oil.

Constituents	Rice bran oil				
	BR-5	BR-10	IRIR-28	BRRI-39	Kalijira
Saponification value	185.00	183.00	189.00	181.40	186.18
Iodine value	101.00	100.85	102.99	101.04	100.18
Peroxide value (m.eq O_2/kg lipid)	1.650	1.415	1.080	1.560	1.710
Acid value	3.95	3.42	3.10	3.15	3.86
Free fatty acid % (as oleic)	2.29	2.36	2.09	2.15	2.54
Unsaponifiable matter %	3.29	3.09	3.46	3.39	3.28

Fresh rice bran oil was used to determined the values.

Percentage of free fatty acids (% of FFAS)

Acid value (A.V) is measure of the hydrolysis that has occurred in fat. In the present investigation the acid value and the % FFA of different varieties of rice bran were estimated in normal laboratory conditions and presented in Table-12. It has been found from the table that the acid value of different varieties of rice bran oil was varied from 3.10 to 3.95 and % FFA from 2.09 to 2.54. The BR-5 variety have the highest acid value while the IRRI-28 variety have the lowest acid value. Again Kalijira variety also contained highest % FFA while the IRRI-28 variety have the lowest % FFA

It has been found that the free fatty acid of the oil does not exceed beyond the values needed to be consumed by the human system if the oil should be extracted immediately from the fresh bran of the mill. So it can be concluded from the results that the present varieties of rice bran oils under investigation might be suitable for edible purposes, if extracted under fresh condition.

Unsaponifiable matter

The unsaponifiable matter is a fraction of fat or oil that remains insoluble after Saponification of the sample by alkali.

The unsaponifiable matter present in the different varieties of rice bran oil varied from 3.09% to 3.46%. Of the varieties examined IRRI-28 contained the highest amount and BR-10 variety contained the lowest amount of unsaponifiable matter. Although the unsaponifiable matter of crude rice bran oil is rather high due to the presence of wax, but the edibility of rice bran oil is comparable to other oils including groundnut, soybean and rapseed oil (Hussain M.G. *et. al.,* 1995). Moreover the rice bran oil has better keeping quality due to the presence of natural antioxidants.

Table-13: Effect of storage with respect to days on peroxide value at room temperature.

(Figures in the margin indicate the peroxide values)

No of days oil store	Rice bran oil				
	BR-5	BR-10	IRIR-28	BRRI-39	Kalijira
0	1.650	1.415	1.080	1.560	1.710
15	1.731	1.430	1.251	1.620	1.736
30	1.738	1.435	1.316	1.630	1.784
45	1.742	1.440	1.340	1.641	1.788
60	1.800	1.445	1.352	1.650	1.792
75	1.805	1.461	1.375	1.667	1.799
90	1.871	1.500	1.401	1.673	1.801
120	1.915	1.601	1.420	1.721	1.815
150	1.946	1.681	1.485	1.758	1.886

The storage effect on oxidative deterioration of the different varieties of rice bran oil was studied at room temperature for 5 months (Table-13). From the results it is seen that after 5 months of storage, the peroxide values, of the BR-5, BR-10, IRRI-28, BRRI-39 and Kalijira varieties were increased from 1.650 to 1.946, 1.415 to 1.681, 1.080 to 1.485, 1.560 to 1.758 and 1.710 to 1.886 respectively. The values obtained in the present studies are silghtly higher then that reported for rice bran oil by Maksud Ali M. *et al.* (1998).

Glyceride compositions of different varieties of rice bran oil (wt %).

The oils obtained from rice bran of different varieties were separated into mono, di-and triglyceride fraction by means of column chromatography and the results are presented in Table-14. From the table it was found that triglyceride in all the varieties accounted for over 90% of the total weight of the oil. The oils of IRRI-28, variety contained 93.08%, 1.75% and 1.54% of

triglyceride, dighyceride and monoglyceride respectively. On the other hand the lowest amount of triglyceride, diglyceride and monoglyceride were found in kalijira, kalijira and BR-5 respectively.

Table-14: Glyceride composition of oils extracted from different varieties of rice bran.

Name of variety	Glyceride			
	Monoglyceride	Diglyceride	Triglyceride	Non-Glyceride (unsap+FFA)
BR-5	1.45 ± 0.020	2.18 ± 0.010	92.36 ± 0.020	4.01 ± 0.010
BR-10	1.61 ± 0.011	2.30 ± 0.013	91.84 ± 0.018	4.25 ± 0.011
IRRI-28	1.54 ± 0.013	1.75 ± 0.011	93.08 ± 0.016	3.63 ± 0.010
BRRI-39	1.69 ± 0.015	1.75 ± 0.010	92.67 ± 0.011	3.89 ± 0.008
Kalijira	1.49 ± 0.015	1.51 ± 0.009	91.76 ± 0.015	5.24 ± 0.009

Saturated and unsaturated fatty acids composition from different varieties of rice bran oil are presented in Table-15, The amount of saturated fatty acid present in rice bran oil was varied from 24.85 to 28.30%. As shown in the Table, the BR-5 variety contained the highest percentage of saturated fatty acid (28.30%) followed by Kalijira (27.10%), BR-10 (26.85%) BRRI-39 (25.26%) and IRRI-28 (24.85%) Further the different varieties of rice bran oils contained 71.06 to 74.95% unsaturated fatty acid. Of the varieties examined IRRI-28 variety contained the highest amount while BR-5 variety contained the lowest amount of unsaturated fatty acids.

Table-15: Saturated and unsaturated fatty acids composition of different varieties of rice bran oil.

Name of the variety	Saturated fatty acid (%)	Unsaturated fatty acid (%)
BR-5	28.30 ± 0.011	71.06 ± 0.020
BR-10	26.85 ± 0.010	72.31 ± 0.018
IRRI-28	24.85 ± 0.009	74.95 ± 0.015
BRRI-39	25.26 ± 0.011	72.46 ± 0.019
Kalijira	29.10 ± 0.009	71.90 ± 0.012

Compositions of total lipid were further fractionated into lipid classes by silicic acid coloumn chromatography and the results are presented in Table-16. It was found that rice bran oils contained mainly triglyceride which was ranged from 91.76 to 93.08%. Of the varieties examined IRRI-28 variety contained the highest amount of triglyceride while Kalijjra variety contained the lowest amount. In addition to triglyceride the rice bran oils also contained 3.81 to 6.11% partial glyceride, 0.71% to 0.85% glycolipid, 0.59 to 0.99% phospholipid, 0.21% to 0.40% free sterols and 0.18% to 0.40% hydrocarbon.

Table-16: Weight percentage of different classes of lipids present in rice bran oil.

Constituents	Name of variety				
	BR-5	BR-10	IRRI-28	BRRI-39	Kalijira
Triglyceride	92.36	91.84	93.08	92.67	91.76
Hydrocarbons	0.18	0.35	0.25	0.38	0.40
Free sterols	0.21	0.40	0.31	0.29	0.36
Glycolipid	0.75	0.71	0.84	0.84	0.85
Phospholipid	0.65	0.59	0.99	0.89	0.92
Partial glyceride	4.85	6.11	4.52	3.81	4.52

GAS LIQUID CHROMATOGRAPHIC (GLC) EXAMINATION OF THE METHYL ESTER MIXTURE FROM THE RICE BRAN OIL

Fatty acid analysis of the oil from the different varieties of rice bran by GLC are carried out after making their corresponding methyl esters. Among the rice bran oils examined, IRRI-28 variety contained the highest amount of oleic acid (43.15% followed by Kalijira (42.01%), BR-5 (41.08%), BRRI-39 (40.18%) and BR-10 (40.16%) (Table-17). It was also seen from the Table that linoleic and arachidic acid contents were found to be varied from 28.45-31.15 and 1.01%-2.17% respectively.

Besides, these unsaturated fatty acids rice bran oils also contained trace amounts of lenolenic acid (0.8-1.92%). In addition rice bran oil also contained saturated fatty acid such as myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and stearic acid ($C_{18:0}$), and their contents were found to be around (0.38-0.45%), (19.08-20.86%) and (3.13-4.21%) respectively. The GLC analysis data indicated that all the five varieties of rice bran oil contained higher amount of unsaturated fatty acid (70.33-75.38%). Similar results are also demonstrated for the rice bran oil by Bernardini, E. (1973).

From the present data it might be suggested that all the five varieties of rice bran oil are suitable for edible purposes as they contained significant amount of unsaturated fatty acid.

Table-17: Fatty acids composition of the different varieties of rice bran oils.

Name of the variety	Fatty acid percentage							
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	Others
BR-5	0.45	19.08	4.15	41.08	28.45	0.80	2.17	0.75
BR-10	0.42	19.86	4.01	40.16	30.84	1.92	2.11	0.68
IRRI-28	0.39	20.17	3.40	43.15	31.09	1.14	1.59	1.14
BRRI-39	0.52	20.86	3.13	40.18	31.15	1.25	1.01	0.90
Kalijira	0.38	20.26	4.21	42.01	30.18	1.26	1.20	0.50

Conclusion

Many varieties of rice brans are available in our country. Among them, the important widely cultivated varieties are BR-5, BR-10, IRRI-28, BRRI-39 and Kalijira. These varieties are also distinguished by their principal physical characteristics such as length, width and thickness of the grain, the colour of the hull and tips, flavour and other properties. Bangladesh rice mills products consist principally of polished rice, hull and bran. In the country, the paddy rice when milled, yielded an average of 6.42% of rice bran.

Rice bran of different varieties in general contain-moisture (9.98-10.90%), ash (9.45-11.12%) crude fibre (9.18-10.35%). protein (12.45-14.06%) polysaccharide (10.95-12.86%). and total sugar contain (4.35-5.04%). The minerals estimated, in BR-5, BR-10, IRRI-28, BRRI-39 and Kalijira varieties contained the highest amount of magnesium (0.413%), calcium (0.340%) Potassium (0.840%), sodium (0.203%) and iron (0.175%) respectively. Vitamin B_1 and Vitamin B_2 contents were found to be highest in IRRI-28 and Kalijira variety respectively.

Twelve amino acids were found in all the five varieties of rice brans in the total form. Among the variety, IRRI-28 contained relatively higher amount of total amino acid. The physical constant of different varieties of rice bran oils varies (14.95-16.16%), specific gravity and refractive index varies from (0.924-9.66) and (1.561-1.59). The important chemical characteristics of the oils studied were saponification value (181.40-189.00), Iodine value (100.18-102.99), peroxide value (1.080-1.710), acid value (3.10-3.95%) and unsaponifiable matter (3.09-3.46%). Kalijira variety contained the highest amount of %. FFA (2.54) and IRRI-28 variety contained the lowest amount (2.09) of % FFA.

Five varieties of rice bran oils were analyzed for their glyceride and fatty acid composition. More than 90% triglyceride in the experimental varieties. The saturated fatty acids present in the oil samples were mainly palmitic acid (19.08%-20.86%) and stearic acid (3.13%-42%). The unsaturated fatty acids were mainly oleic (40.16-43.15%), linoleic acid (28.45-31.15%). Rice bran oils of different varieties content high percentage of unsaturation which can be used as edible purposes.

CHAPTER-III

A Comparative analysis on the quality characteristics of prepared bread supplementation with rice bran and locally available market brand bread

INTRODUCTION

Rice bran contain fatty acids which is important constituents for food processing and nutrition, but this potential by-product is not utilized for human consumption. It offers a potential source for exploitation as human food. In Bangladesh, the diet of masses consists largely of white rice and is more or less deficient in fat, protein, minerals and vitamins. In our country, beriberi is very common and fatal disease among the poorer classes who live on a diet which is deficient of vitamin B_1. Rice bran is a rich source of antineuritic vitamin B_1. The yield of paddy rice in Bangladesh accounts to over 28 million tones, thus the yield of rice bran obtained as a by-product accounts to 1.95 million tones (6.42%). Despite being a one of major producer of this valuable nutritious by-product but the country till now use other raw materials for production of food materials such as bread. Keeping this view in mind, in this study, we have been trying to produce quality breads by utilizing different percentage of rice brans, wheat flour and other ingredients.

Materials and Methods

Five different varieties of rice brans (BR-5, BR-10, IRRI-28, BRRI-39 and Kalijira) were used in this study. The results are tabulated by taking triplicate data for each of the parameters.

Preparation of bread using different varieties of rice bran

Wheat flour (85-95%) and (5-15%) of properly sieved fresh rice bran flour were, mixed with required amount of water, vegetable fat, sugar, yeast, milk powder, salt etc. The whole mass was kept undisturbed for 3-4 hours. Then it was needed for proper mixing. Weighed amount of kneaded mass

(properly mixed mass) was taken in a dice of desired size. The dice along with the mass was baked in the baking oven for the production of final product (bread).

ANALYSIS OF THE PHYSIO-CHEMICAL CHARACTERISTICS OF PREPARED BREAD.

Determination of moisture contents of prepared rice bran bread

Moisture content was determined following the standard method (IUPAC 1977).

Determination of ash and fibre content of prepared rice bran bread

Ash and fibre content of prepared rice bran breads were determined following the method of AOCS (1980)

Extraction of oil from prepared rice bran bread

Fat is the triglyceride portion of the bran bread and biscuit, which is extracted by suitable solvent under the operating condition using continuous soxhlet extraction device A.A.C.C (1962).

Determination of protein content of prepared rice bran bread

Protein content of prepared rice bran bread was determined by the method of Micro-kjeldhal Jayaraman, J., (1981), as described in chapter-II.

Determination of total sugar content of prepared rice bran bread

Total sugar content of rice bran bread was determined colorimetrically by the Anthrone method Jayaraman, J., (1981), as described in chapter-II.

Solubility of proteins in aqueous solution and different solvents

The solubility of protein of rice bran breads was followed as reported by method Ghyasuddin, *et. al.*, (1970). The sample (5g) was mixed separately in a container containing 200ml distilled water. The mixture was stirred for 20 minutes at room temperature and then centrifuged at 2000g for 10 minutes. The clear extract was filtered and the residue was extracted with two portion of 50 ml distilled water for 20 minutes. These extracts were combined and the total protein content was determined using the method of Lowry *et. al.*, (1951). The residue left removal of water soluble materials specially protein was extracted in the same way using three portions of 2% NaCl solution. After this extraction the residue was extracted for 20 minutes with 80% alcohol at 70°C. Finally the residue was suspended in three successive portions of 0.25% NaOH solution and stirred for 20 minutes. The above protein content of extracted solution in each of the solvent was determined by the Lowry method.

Determination of saponification value, acid value and iodine value of prepared rice bran bread

Saponification value, acid value and iodine value of prepared rice bran breads were determined according to the methods as described earlier in Chapter II.

Results and Discussion

The chemical composition of the prepared rice bran bread and the market brand breads were tabulated in Table-18 and 19. From the table it was seen that the moisture content of the prepared breads were varied from (26.02-28.35%) while that of the locally market made bread contained (30.14-32.95%) of moisture. So bread available in local market contained less solid materials than the prepared bread.

Table-18: Analysis of the nutrient compositions of breads, produced by supplementation of different varieties of rice brans (g/ 100g).

Wheat flouer and bran ratio used (w/w)		Moisture	Ash	Fibre	Fat	Protein	carbohydrate	Total sugar	Reducing sugar
BR-5	95% + 5%	28.06±0.015	2.56±0.006	1.66±0.022	7.01±0.006	6.64±0.002	44.04±0.040	6.40±0.005	0.56±0.003
	90% + 10%	27.15±0.016	2.64±0.008	1.88±0.019	7.34±0.009	6.73±0.006	44.49±0.030	6.21±0.008	0.48±0.007
	85% + 15%	27.05±0.017	2.72±0.010	1.96±0.010	7.51±0.013	6.82±0.009	44.81±0.020	6.08±0.012	0.63±0.009
BR-10	95% + 5%	28.35±0.009	2.67±0.012	1.59±0.024	7.49±0.018	6.70±0.023	45.21±0.015	6.59±0.007	0.61±0.016
	90% + 10%	27.45±0.010	2.77±0.015	1.64±0.026	7.72±0.023	6.81±0.020	45.35±0.020	6.48±0.003	0.73±0.019
	85% + 15%	27.09±0.012	2.83±0.017	1.70±0.031	7.81±0.013	6.88±0.017	45.84±0.010	6.35±0.005	0.80±0.021
IRRI-28	95% + 5%	27.28±0.007	2.30±0.005	1.54±0.006	7.86±0.035	7.09±0.0.21	45.63±0.050	6.04±0.002	0.69±0.025
	90% + 10%	27.03±0.009	2.41±0.009	1.61±0.008	7.91±0.030	7.18±0.009	46.01±0.040	5.85±0.007	0.75±0.005
	85% + 15%	26.45±0.013	2.49±0.012	1.76±0.012	8.04±0.028	7.25±0.005	46.27±0.020	5.60±0.009	0.84±0.003
BRRI-39	95% + 5%	28.12±0.017	2.45±0.006	1.78±0.011	6.95±0.025	6.84±0.012	44.39±0.030	6.51±0.008	0.55±0.007
	90% + 10%	27.07±0.019	2.61±0.008	1.80±0.015	7.01±0.023	6.88±0.015	45.18±0.020	6.40±0.004	0.69±0.012
	85% + 15%	26.95±0.021	2.70±0.010	1.85±0.020	7.25±0..029	6.93±0.008	45.38.0.015	6.12±0.012	0.73±0.017
Kalijira	95% + 5%	26.42±0.005	2.17±0.016	1.96±0.006	7.12±0.030	7.03±0.025	43.18±0.035	6.80±0.016	0.64±0.019
	90% + 10%	26.11±0.007	2.31±0.018	1.90±0.009	7.41±0.020	7.17±0.020	44.25±0.030	6.71±0.012	0.69±0.011
	85% + 15%	26.02±0.009	2.40±0.021	1.95±0.013	7.59±0.022	7.20±0.016	44.49±0.25	6.54±0.009	0.78±0.009

Table-19: Analysis of the nutrient compositions of different breads available in local market (g /100 g).

Name of the company	Moisture	Ash	Fibre	Fat	Protein	Carbohydrate	Total sugar	Reducing sugar
Kundo Bread & Co, Rajshahi	31.14±0.005	2.70±0.017	2.04±0.006	8.12±0.008	6.21±0.009	41.42±0.003	7.13±0.011	0.78±0.004
Moshen Bread & Co, Rajshahi	32.06±0.009	2.81±0.013	2.61±0.009	7.91±0.012	5.49±0.019	40.75±0.006	8.25±0.019	0.95±0.009
Nowhata Bread & Co, Rajshahi	32.95±0.012	2.93±0.009	2.32±0.014	8.04±0.015	6.00±0.017	39.98±0.017	8.61±0.022	0.75±0.011

The ash and fibre content of the prepared rice bran breads were found to be varied bwtween 2.17-2.83% and 1.54-1.96% while that of locally available maket brand breads were found to be between 2.70-2.93% and 2.04-2.61% respectively. It is important to note that the locally available market brand breads contained slightly higher percentage of ash and fiber than the prepared rice bran breads.

It can also be observed from comparison of the major nutrient contents of prepared rice bran and locally available bread that the prepared rice bran breads contained higher percentage of carbohydrate and protein while the locally available bread in the market contained slightly higher amount of oil. So on the nutrition point of view, prepared rice bran bread might be considered superior than the locally available bread in the market.

Table-20: Solubility of protein prepared rice bran bread in different solvents (g /100g).

Wheat flour and bran ratio used (w/w)		Total protein %	water soluble protein %	water in soluble protein % in different solvents			Total (water soluble + water in soluble)	% of protein denatured
				2% NaCl	0.25% NaOH	80% Alcohol		
BR-5	95% + 5%	6.40	0.81	0.98	1.02	0.21	3.02	47
	90% + 10%	6.21	0.96	1.00	1.14	0.30	3.40	55
	85% + 15%	6.08	0.99	1.06	1.26	0.41	3.72	61
BR-10	95% + 5%	6.59	0.75	1.09	1.31	0.31	3.46	52
	90% + 10%	6.48	0.80	1.21	1.42	0.42	3.85	59
	85% + 15%	6.35	0.83	1.30	1.56	0.50	4.19	66
IRRI-28	95% + 5%	6.80	0.71	0.61	0.89	0.21	2.42	35
	90% + 10%	6.71	0.83	0.74	0.96	0.31	2.84	42
	85% + 15%	6.54	0.62	0.89	1.10	0.46	3.07	47
BRRI-39	95% + 5%	6.51	0.70	1.10	1.12	0.08	3.00	46
	90% + 10%	6.40	0.75	1.24	1.36	0.12	3.47	54
	85% + 15%	6.12	0.84	1.38	1.44	0.15	3.81	62
Kalijira	95% + 5%	6.04	0.63	0.94	0.99	0.12	2.68	47
	90% + 10%	5.85	0.72	0.99	1.24	0.19	3.14	54
	85% + 15%	5.60	0.89	1.11	1.36	0.28	3.64	65

Table-21: **Solubility of protein market made breads in different solvents (g /100 g).**

Name of the company	Total protein %	water soluble protein %	water in soluble protein % in different solvents			Total (water soluble + water in soluble)	% of protein denatured
			2% NaCl	80% Alcohol	0.25% NaOH		
Kundo Bread & Co, Rajshahi	4.51	1.15	0.86	0.25	1.86	4.12	91
Moshen Bread & Co, Rajshahi	3.65	0.98	1.08	0.31	1.54	3.91	84
Nowhata Bread & Co, Rajshahi	4.51	0.65	0.79	0.41	1.75	3.60	79

The solubility of protein present in prepared bread and market bread is also used as a criteria for considering as the quality characteristics. The results indicated that the protein contents of the ingredients were drastically denatured due to baking at relatively high temperature and made the protein insoluble in aqueous solution as well as in mild to harsh solvents. As shown in the Table 20 and 21, the percentage of denaturation of prepared bran breads were varied from 35% to 66% while that of the market brand breads were varied from 79% to 91%. So, the protein in market brand breads were more denatured as compared to that of prepared breads.

Table-22: Quality of fat present in prepared rice bran bread.

Varieties of bran used		Saponification value	Acid value	Iodine value
BR-5	95% + 5%	159.00	1.26	92.14
	90% + 10%	155.00	1.35	93.00
	85% + 15%	150.18	1.40	93.91
BR-10	95% + 5%	166.00	1.16	94.00
	90% + 10%	161.17	1.42	94.90
	85% + 15%	156.00	1.45	96.00
IRRI-28	95% + 5%	160.00	1.31	95.00
	90% + 10%	155.17	1.43	97.00
	85% + 15%	150.00	1.49	99.15
BRRI-39	95% + 5%	166.00	1.21	93.00
	90% + 10%	160.12	1.29	95.13
	85% + 15%	155.27	1.35	97.00
Kalijira	95% + 5%	169.00	1.32	98.00
	90% + 10%	165.00	1.40	99.13
	85% + 15%	163.00	1.51	100.15

Table-23: Quality of fat present in market made different types of breads.

Name of the company	Saponification value	Acid value	Iodine value
Kundo Bread & Co, Rajshahi	142.16	1.63	71.00
Moshen Bread & Co, Rajshahi	1.38.00	1.71	63.06
Nowhata Bread & Co, Rajshahi	149.15	1.86	57.40

Some quality characteristics of oil such as saponification value, acid value, iodine value etc. of rice bran made breads and market available breads were compared and the results are tabulated in table 22 and 23. It was observed that the saponification as well as iodine value of rice bran made breads were much higher than that of locally available market made breads. It is also interesting to note that the acid values of market made breads were higher than that of the rice bran made breads. From this data it may also conclude that the quality of rice bran made breads are better than locally available market breads.

Further, the higher iodine value of prepared bran bread indicated that the prepared bran bread might be better than the locally available market brand breads for health.

CHAPTER-IV

Purification and characterization of rice bran protein

INTRODUCTION

Animal kingdom is the main sources of proteins but plants kingdom also possesses a lot of proteins and most of them are glycoproteins in nature. Proteins (glycoproteins) are isolated from various sources such as plant, bacteria fungi etc. Many authors have described the isolation, purification, chemical and biological activities of protein (lectins) from different plants sources. Rice bran contained about 14% protein but still now no detailed work has done been on the nature of protein present in it. Keeping this view in mind, this study describes the purification, characterization and structure function analysis of three rice bran proteins.

Collection of Rice Bran

IRRI-28 variety of rice bran, collected from Noor-Habib Grain Industries Ltd. Sopura, Rajshahi is used for purifie of proteins.

METHODS

Preparation of Fat-Free Meals

In order to purify protein from rice bran in biologically active from unless otherwise indicated all the operations were performed at 4°C. First the dried rice bran was taken in a mortar and pounded uniformly into fine powder. To these powder pre-cooled petroleum ether (40-60°C) was added and homogenized uniformly with a homogenizer. The desired temperature was maintained by putting ice in the outer chamber of the homogenizer. The oily extract was then kept in a beaker at 4°C for an hour with occasional stirring. The homogenate was then filtered though a clean muslin cloth. The process was repeated again by adding more pre-cooled petroleum ether in order to make the homogenate quite fat-free. The filtrate was then further clarified by centrifugation at 8×10^3 g for 12 minutes. The residue obtained after centrifugation were collected, air dried at room temperature and stored it in the refrigerator for experimental purposes.

Selection of suitable extracting solvents

Proteins from fat free rice bran meals were extracted under identical conditions using six different extracting solvents. After extraction, the total concentration of the protein was determined by measuring the absorbance at 280 nm. The suitable extracting solvent was selected from their-ratio of absorbance at 280 nm and 260 nm as reported by Clark and Switzer (1977).

Table 24. Preparation of crude protein extract from rice brans in different extracting solvents.

Extracting media	Amount of rice bran (g)	O.D at 280 nm	O.D at 260 nm	Ratio of O.D. 280 & 260
1% CH_3COOH	1.0	0.570	0.779	0.731
Tris-HCl Buffer pH-6.9	1.0	1.225	1.455	0.841
10mM phosphate buffer pH-6.6	1.0	1.366	1.586	0.861
Distilled water	1.0	1.348	1.510	0.892
Distilled water containing 0.15 M NaCl pH-5.9	1.0	1.014	1.096	0.925

From the above table, the highest ratio of absorbance (280/260 nm) was found in distilled water containing 0.15 M NaCl Solution, pH-5.9 therefore this solution was selected as suitable extracting solvent.

Preparation of crude protein extract

The fat-free dry powder was mixed uniformly with precooled extracting solvent 0.15 M NaCl in H_2O, pH 5.9 in a beaker and kept overnight at 4°C with occasional stirring. The suspension was filtered through a muslin cloth in the cold room. Then the filtrate was collected and clarified further by centrifugation at 8×10^3 g, 4°C for 20 minutes. The clear supernatant was collected and adjusted to 100% saturation by adding solid ammonium sulfate. The ammonium sulfate precipitate was then centrifuged again at 8×10^3 g, 4°C for 20 minutes. The precipitate was collected,

dissolved in minimum volume of pre-cooled deionized water and dialyzed against cold distilled water for 12 hours with three changes and against 10 mM Tris-HCl buffer, pH 8.0 for 24 fours at 4°C. After centrifugation the clear supernatant was used as crude protein extract.

Purification of protein from crude extract

Purification of proteins by gel filtration

i) **Activation of gel G-50 powder:** Sephadex G-50 powder was suspended in 10% acetic acid containing 1M sodium chloride (1 mole of NaCl was dissolved in one liter of 10% acetic acid) in a beaker and left it to swell for overnight. It was stirred by glass rod after short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality.

ii) **Packing of the column:** This is very critical and important step in all types of column chromatographic experiment. If the column is not packed properly, accurate results can never be expected. Because a poorly packed column gives rise to uneven flow rates. The gel suspension was taken in a filtering flask and deaerated by vacuum pump; otherwise it would affect the flow rate of the column after packing. The gel suspension was adjusted so that it was a fairly thick slurry, but not thick enough to retain bubbles. The column as mounted on a stable laboratory stands and its narrow end was fitted with an outlet tube. It was ensured that there was no air bubble in the dead space of the bed support. This was easily achieved by filling approximately $1/4^{th}$ of the column, including the outlet tube with distilled water. When dead space was properly filled, the outlet tube was closed with pinch cork and the gel suspension from a gel reservoir was added gently to the column. In order to avoid trapping of any bubble, this was performed by pouring the gel in inner wall of the column. In this way, a column of desired length was packed uniformly with the gel suspension.

iii) **equilibration of the column:** After completion of the column packing it was equilibrated with the eluant buffer (10 mM Tris-HCl buffer, pH 8.0). The buffer was continued to run through the column until the pH of the eluate became same as the pH of the eluant buffer.

iv) **Application of sample:** Before loading of the sample, the outlet tube of the column was opened and the eluant buffer from the top of the gel bed was allowed to diffuse into the gel. The crude extract (3-5 ml) was then loaded on the top of bed. After diffusion of the sample, about 1 ml of eluant buffer was poured on the top of the gel bed and was allowed to diffuse. Then an additional amount of buffer was added, so that the space about 3-4 cm above the gel bed was filled with eluant. The buffer was then allowed to flow continuously through the column at a flow rate of about 25 ml per hour and 3 ml fractions of the eluate were collected by an automatic fraction collector. Absorbance of each fraction was measured at 280 nm. The protein concentration of each fraction was also measured by the method of Lowry *et. al.*, (1951).

Purification of proteins by DEAE-cellulose column chromatography

A. Procedure

i) **Activation of DEAE-cellulose powder:** The DEAE-cellulose powder was suspended in 0.5 M HCl solution in a beaker and left it to swell for few hours. During swelling it was stirred gently at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water several times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.5 M NaOH and left for few hours. It was again washed with distilled water to neutralized its pH.

ii) **Packing of the column:** The activated DEAE-cellulose suspension was taken in a filtering flask and deaerated by vacuum pump. A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

iii) **Equilibration of the column:** After packing, the column was equilibrated with 10 mM Tris-HCl buffer, pH 8.4.

iv) **Preparation and application of sample:** The fraction one (major fraction) obtained by gel filtration was dialyzed against distilled H_2O for 12 hours and against 10 mM Tris-HCl buffer, pH 8.4 for 12 hours at 4°C. The dialyzed sample was loaded onto DEAE column at 4°C. The proteins were eluted from the column with the same buffer containing NaCl by linear and stepwise elution.

Test of purity

Sodium dodecyl sulfate polyacrylamide slab gel electrophorieis (SDS-PAGE) Method

The purity of separated rice bran proteins obtained from DEAE-cellulose column was tested by SDS-PAGE method.

Principle: Polyacrylamide gel electrophoresis method is commonly used for checking the purity of proteins and of their molecular weight determination. Sodium dodecyl sulfate (SDS) is an anionic detergent which binds to most proteins in amounts roughly proportional to moleculr weight of the protein, about one molecule of SDS for every two molecules of amino acid residues. The bound SDS contributes large net negative charge, rendering the intrinsic charge of the proteins insignificant. In addition, native conformation of the protein is altered when SDS is bound and most proteins assume similar shape and thus similar ratio change to mass. Slab gel electrophoresis in presence of SDS therefore separated proteins almost exclusively on the basis of mass, with smaller polypeptides migrating more rapidly. Protein-SDS complexes will therefore all move towards the anode during electrophoresis and their movement is inversely proportional to the log 10 of their molecular weights by using standard proteins of known molecular weights. The molecular weight was determined using 10% SDS-PAGE according to the method of Laemmli (1970)

Reagent and solutions

i) Preparation of 30% acrylamide gel solution: 14.5 g acrylamide and 0.5 g N, N-methylene-bis-acrylamide were dissolved in 35 ml of DDW (Deionized distilled water) in a 50 ml volumetric flask and the final volume of was made upto mark by adding DDW. Then the solution was filtered and stored in a dark bottle at room temperature.

ii) Preparation of 1.5 M Tris-HCl buffer, pH 8.4: 18.15 g of Tris base (MW 121.088) was dissolved in 90 ml of DDW in a conical flaks and mixed well. The pH of the solution was adjusted to 8.4 by adding concentrated HCl and the final volume was made upto 100 ml with DDW.

iii) Preparation of 10% SDS (Sodium dodesyl sulphate) solution: 10% SDS solution was prepared by dissolving 5 g of SDS in about 40 ml of DDW. After adjusting the pH to 8.0 with conc. HCl the final volume was made upto 100 ml DDW.

iv) Preparation of 10% APS (ammonium sulphate) solution: 10% APS solution was prepared by dissolving 0.5 g of APS in about 4 ml of DDW. The final volume was made upto 5 ml with DDW. Then the solution was stored in eppendrof tubes (500 µl in each tube) at 20°C.

v) **TEMED:** The commercially available preparation TEMED of Sigma Chemical Co., U.S.A. was used without any modification.

Preparation of marker sample: 1.5 ml of buffer solution was taken in vial and 2 to 10 µgm of each marker protein is added to it and mixed well for complete solubilization. Before the application of this mixture to the gel it should be incubated in a boiling water bath for one minute. Then 5 to 10 µl solution was applied respectively to the well. The following proteins are used as markers-

Proteins	Approximately molecular weight
Lysozyme	14,000
Trypsin inhibitor	20,000
Albumin (Egg white)	45,000
Albumin (BSA)	67,000
β-galactosidase	1,16,000

Procedure for SDS-PAGE: Clean and dry plates (10 cm × 7 cm) were assembled with spacer (0.5 cm thick) and were held together on a gel casting stand. The assembly was carefully checked for leakage.

Preparation of separating gel: The separating gel was prepared by mixing the following components.

Components	Separating gel 7.5%	Separating gel 10%
Deionized water	8.4 ml	7.55 ml
40% acrylamide	2.55 ml	3.40 ml
1.5M Tris-HCl buffer, pH 8.8	3.75 ml	3.75 ml
10% SDS	0.15 ml	0.15 ml
10% APS	0.15 ml	0.15 ml
TEMED	0.15 ml	0.15 ml

The freshly prepared separating gel was poured between the glass plates carefully to avoid the inclusion of air bubbles. The gel poured leaving one cm gap between the bottom of the gel and the upper level of the stacking gel. The upper level of the separating gel was upto-overlaid level. Then it was kept undisturbed for 30 minutes.

Preparation of stacking gel: The stacking gel was prepared by mixing the following components.

Components	Stacking gel
Deionized water	6.2 ml
40% acryamaide	1.10 ml
0.5 m Tris-HCl buffer, pH 6.8	2.50 ml
10% SDS	0.10 ml
10% APS	0.10 ml
TEMED	0.10 ml

Before pouring the stacking gel, DDW was soaked off. After pouring of the gel, a 10 well comb was inserted carefully into the gel. Meanwhile, the loading samples were prepared by mixing the sample and the sample buffer in the ratio of 2:1 and was boiled in a water bath for about 2 minutes. It was then cooled down to the room temperature and ready for use. The comb was gently removed form the stacking gel after 30 minutes. The portion that did not polymerize, was removed by soaking cautiously with filter paper. The samples along with the standard marker proteins were loaded in the well carefully avoiding cross contamination. The samples were then overlaid with electrophoresis buffer. A constant voltage (20 volts) was firstly applied for 30 minutes still the stacking gel dye cross the stacking gel. Then 100 volts was applied for one hour. When the gel front reached the bottom of the gel, the electricity supply was disconnected and the gel was removed from the glass plates very carefully and kept immersed in freshly prepared staining solution for 2 to 3 hours on constant shaking in a shaker. It was then immersed in freshly prepared destaining solution until the gel become transparent.

CHARACTERIZATION OF PURIFIED RICE BRAN PROTEINS

MOLECULAR WEIGHT DETERMINATION

By gel filtration

The molecular weight of the purified protein was estimated from the data of gel filtration on Sephadex G-150 (0.75×100cm) with Lysozyme (14000), trypsin inhibitor (20,000), egg albumin (45,000), bovine serum albumin (67,000) and β-galactosidase (116000) as reference proteins following the procedures as described by Andrews (1965).

By sodium dodecyl sulfate polyacrylamide slab gel electrophoresis

The molecular weights of the purified protein was determined by the method as Laemmli (1970) which was described above.

HEMAGGLUTIONATION STUDIES

A. Materials

(i) Phosphate buffer saline (PBS), pH-7.2.
(ii) 4 % Rat red blood cells (RBC) in PBS
(iii) Protein solution

B. Procedure

Just before experiment, blood from albino rat was collected in centrifuged tube containing equal amount of pre-cooled 5 mM phosphate buffer saline pH 7.2. The blood sample was immediately centrifuged at 3×10^3 g for 3 minutes. The supernatant was discarded and the cells were washed similarly for three times with the above buffer. Finally a 4% suspension (W/V) of RBC was prepared and the hemagglutination was performed in siliconized test tubes (0.5 × 4 cm) as follows:

0.2 ml of 4% RBS was mixed with 0.2 ml of protein solution in PBS and mixed well by gentle stirring. The mixture was incubated at 30°C for an hour. A control containing 0.2 ml of PBS, pH 7.2, instead of protein solution and 0.2 ml cell suspension were used as reference. After 1 hour incubation, the sedimented erythroytes were gently mixed with the supernatant and one drop of this suspension was examined under microscope. Results were recorded as 3^+, 2^+, 1^+ (Read, W.P., (1981).

The agglutinating activity was expressed as the titre, the reciprocal of the greatest dilution at which visible agglutination could be detected. The specific activity was expressed as titre / mg of protein.

Hemagglutination inhibition studies

A. Materials

(i) 5 mM phosphate buffer saline, pH-7.2.

(ii) Different sugar solutions. D-glucose, D-Galactose, D-mannose etc.

B. Procedure

The hemagglutionation-inhibition test was performed in the presence of different sugars as described below. Protein solutions (0.1 ml) containing minimum concentration of protein needed for visible agglutination were added to 0.1 ml of sugar solutions of various concentrations and mixed gently, and then 0.2 ml of 4% RBC in PBS was mixed and incubated at 30°C for an hour. Reactions were compared with a positive control (0.1 ml protein + 0.1 ml buffer + 0.2 ml 4% RBC) and a negative control (0.2 ml PBS + 0.2 ml 4% RBC) as reported by Atkinson *et al.,* (1980).

Test for glycoprotein and estimation of sugar

Phenol-sulfuric acid

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric microdetermiantion of sugars and their methyl derivatives, oligosaccharide and polysaccharides as descrived by Dubois *et al.* (1956). This method was also employed for detecting the presence of sugar in protein.

A. Materials

(i) 5% phenol (in water)

(ii) Conc. sulfuric acid

(iii) Protein solution

B. Procedure

The protein solution (0.1 ml, 0.085-0.095 mg/ml) was taken in a test tube and made upto 2 ml by distilled water. Then 1 ml of 5% phenol was added to it and finally 5 ml of conc. H_2SO_4 was added rapidly. To obtain good mixing the stream of acid being directed against the liquid surface rather than against the side of the test tube. The tube was allowed to stand for 10 minutes. After mixing by shaking it was kept in the dark at 25 to 30°C for 20 minutes and then the absorbance of the solution was measured at 490 nm. The conc. of sugar was then estimated from a standard curve which was constructed by using glucose as standard sugar (Fig.-2). From this calibration curve the concentration of sugar in the purified proteins was calculated.

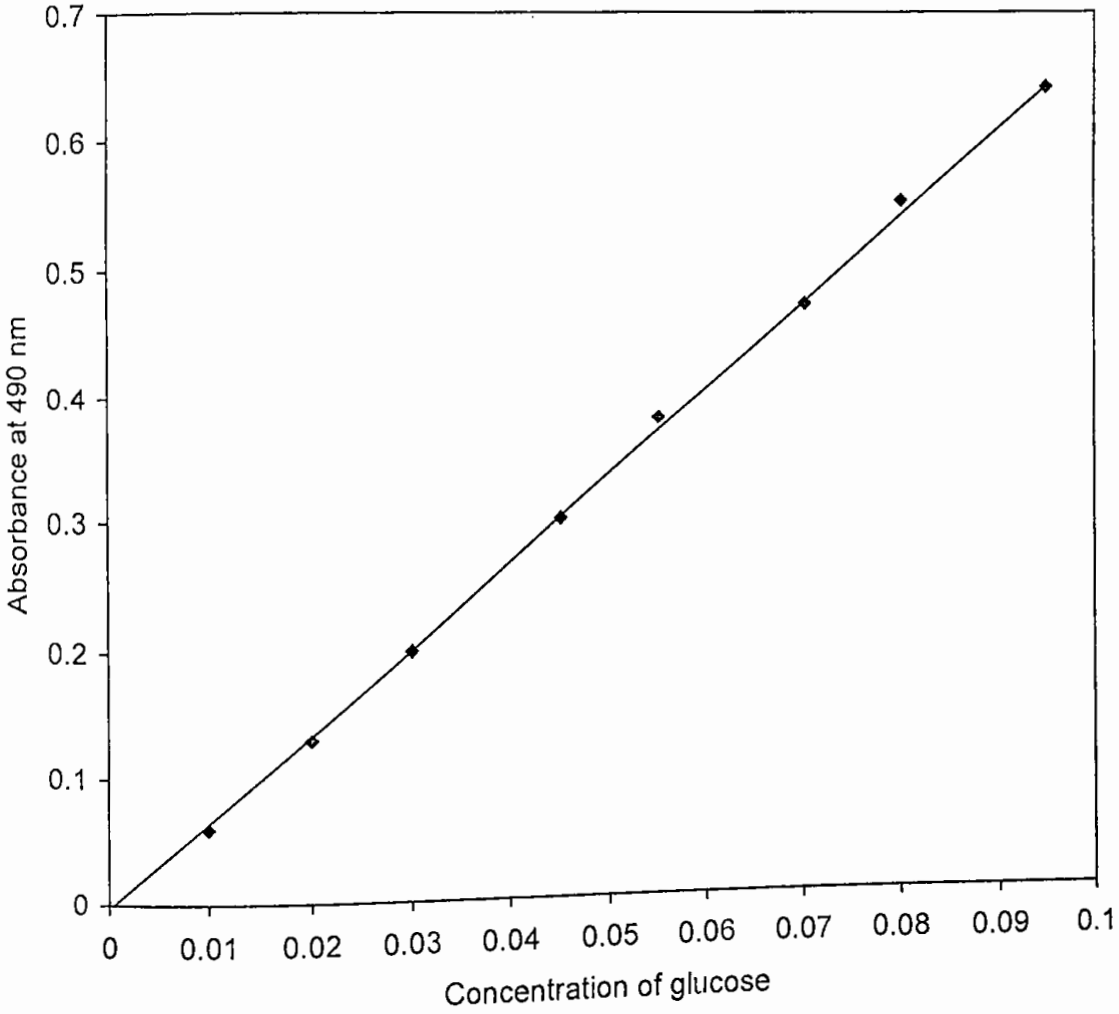

Fig. 2: Standard curve for estimation of sugar present in glycoprotein.

Determination of Optical Density (O.D at 280 nm) VS Protein Concentration Relation by the Folin-Lowry Method (Lowry *et al.* 1951)

A. Materials

(i) Alkaline sodium carbonate solution (20 g / litre Na_2CO_3 in 0.1 mol/litre NaOH)

(ii) Copper sulphate and Solution potassium tartrate solution (5 g/litre $CuSO_4.5H_2O$ in 10 g/litre Na-K tartarate). Freshly prepared,

(iii) Alkaline solution: Mixture of solution (i) & (ii) in the preparation of 50:1, respectively.

(iv) Folin-Ciocalteau's reagent (Diluted with equal volume of H_2O, Just before use).

(v) Standard protein (bovine serum albumin 1 mg/ml in dist. H_2O) solution.

B. Method

From standard protein solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml were taken in different test tubes and made the volume upto 1 ml by distilled water. Then 3.5 ml of the alkaline solution was added to the protein solution in different test tubes and mixed thoroughly. It was allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-Ciocalteau's reagent was added rapidly with immediate mixing and left for 30 minutes. The dark blue color formed was measured at 650 nm against the appropriate blank. By applying the same procedure described above the absorbance of purified protein solution was measured and the concentration of protein was determined by constructing a standard curve, using BSA as standard (Fig.-3).

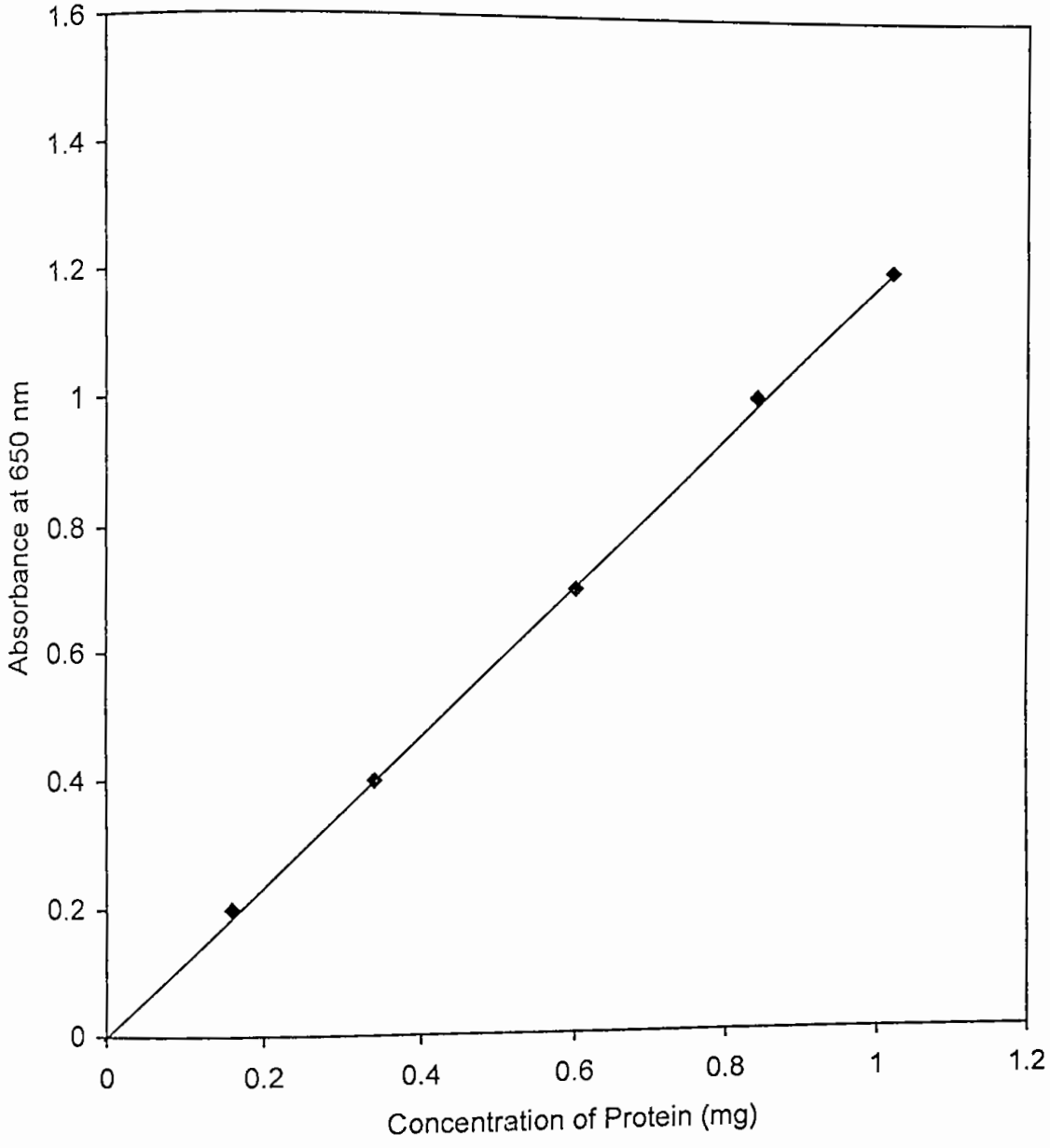

Fig. 3: Standard curve for estimation of concentration of proteins.

Determination of purified rice bran proteins concentration by drying process

Materials

a) Small test tube
b) Oven
c) Electric balance
d) Desiccator
e) Protein solutions

Procedure

The optical density (O.D) of purified protein was measured at 280 nm and one ml was taken in a small test tube (previously weighed). The protein solution in the test tube was dried up completely by heating at 100°C in a water bath under vacuum. When the solution was evaporated, removed the test tube from water bath and placed in a desiccator and allowed to cool. After cooing, the test tubes with dried proteins were weighed again and the amount of proteins were calculated.

Brine shrimp lethality: A rapid general bioassay for cytotoxic effect: (Mayer *et al.*, 1982)

Brine shrimp lethality bioassay test is a recent development in the bioassay for bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological toxic in high doses. Here in vivo, lethality of simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive products.

There is a positive correlation between brine shrimp toxicity and cytotoxicity. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activities of natural products.

Materials

1) *Artemia salina* Leach (brine shrimp eggs)
2) NaCl (Sea water)
3) Pipettes (5 ml & 1 ml)
4) Micro pipette (10-100 µl adjustable)
5) Two drum vials
6) Magnifying glass
7) Smalll tank with perforated dividing drum to grow shrimp, including cover and lamp to attract shrimp.

Procedures

A) **Preparation of sea water:** 38 gms of NaCl was weighed, dissolved in one liter of distilled water and then filtered off.

B) **Hatching of brine shrimp eggs:** Sea water was taken in small tank and shrimp eggs were added to one side of the divided tank which was covered. The shrimps were allowed for two days to hatch and matured as nauplii. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforations in the drum. These nauplii were taken for bioassay.

C) **Preparation of sample solution:** Protein solution (1 mg/ml) in Tris-HCl buffer, pH 8.4 was dialyzed separately against distilled water for 3 hours at 4°C.

D) **Application of test solution and nauplii in the vials:** At room temperature 2, 5, 10, 20, 30 & 40 µl of the protein solutions were taken in different vials and made upto 5ml with the sea water to each vial containing 10 brine shrimp nauplii, so, the concentrations of sample in the vials were 0.4, 1, 2, 4, 6 and 8 µg/ml respectively. Three vials were used for each concentration and control was used containing 10 nauplii in 5 ml of sea water.

E) **Counting of nauplii:** After 24 Hours incubation the vials were observed and the number of survivors in each vial were counted using magnifying glass and noted. From this data, the percentage of mortality of the nauplii was calculated at each concentration.

IDENTIFICATION OF REDUCING SUGAR PRESENT IN THE PURIFIED RICE BRAN PROTEINS BY THIN LAYER CHROMATOGRAPHY (TLC)

Ascending one dimensional thin layer chromatography was perfomed in an attempt to identify the sugar components present in the hydrolyzed protein solution.

A. Materials

i) Standard sugars

D-Glucose, D-galactose, D-arabinose, D-mannose and D-ribose (0.01 mg/ml) were used as standard sugars for the experiment.

ii) **Preparation of protein sample:** The protein sample (1 ml) of having concentration (A_{280} = 0.4-0.5) was evaporated by heating to dryness under vacuum pump. Then 2 ml of 4N HCl was added to it and after deaeration, the tube was sealed by heating. It was then heated at 110°C for four hours. The hydrolyzate was evaporated to dryness and dissolved in a few drops of distilled water and used for analysis.

iii) **Stationary phase:** Activated silica Gel-G.

iv) **Mobile phase:** Isopropanol; Acetic acid: Water (3:1:1)

v) **Spary reagent:** (Aniline-Phthalate) (Partridge 1949; Parchke 1965)

Phthalic acid	4.0 g
Aniline	2.5 ml
Water saturated butanol	250 ml

B) Procedure

i) **Preparation of plates:** The plates were prepared and activated by applying conventional procedure.

Plate size:	(20×20) cm
Thickness:	0.5 mm
Activation:	110°C for 1 hour

ii) **Chamber saturation:** Glass chamber with airtight lid was used for development of the plates. The chamber was saturated with the vapors of the selected solvent system in the usual manner.

iii) **Resolution of compounds:** A small spot of the standard sugar solution was applied at 2 cm above the lower edge of the activated silica gel-G plate and about 4 cm apart by means of a capillary tube. The spot diameter was kept below 0.5 cm. A spot of the hydrolyzed protein solution was also applied on the side of the plate as for standard. A straight line was cut 2 cm below the upper edge of the plate and it was the indication for the solvent front. Then the spotted plate was placed gently in the chromatographic tank containing the selected solvent system. When the developing mobile phase reached the marked point, the plate was taken out and dried in air.

iv) **Detection of Compounds:** Aniline-phthalic acid mixture was sprayed on the plate. The plate was heated at 110°C for 10 minutes and location of spots were marked.

v) **Calculation of R_f values:** The R_f values were determined by using the following formula:

$$R_f = \frac{\text{distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

RESULTS

PURIFICATION OF RICE BRAN PROTEIN

Gel filtration

The 100% ammonium sulfate saturated crude protein extract after dialysis against distilled water and against 10 mM Tris-HCl buffer, pH 8.0 was applied to a Sephadex G-50 column at 4°C which was previously equilibrated with the same buffer. As shown Fig. 4, the components of the crude protein extract were eluted as one sharp major peak F-1, and another broad minor peak, F-2. After analysis it was found that only F-1 fraction possessed the biological activity. The F-1 fraction as indicated by solid line was then pooled, precipitated by 100% $(NH_4)_2$ SO_4, fractionation and dialyzed against 10 mM Tris-HCl buffer pH 8.0 for further purification by ion exchange chromatography. It may be mentioned that the minor fraction F-2 was not used for further study as it contained mostly protein of low molecular weight as well as this fraction did not contined any detectable biological activity.

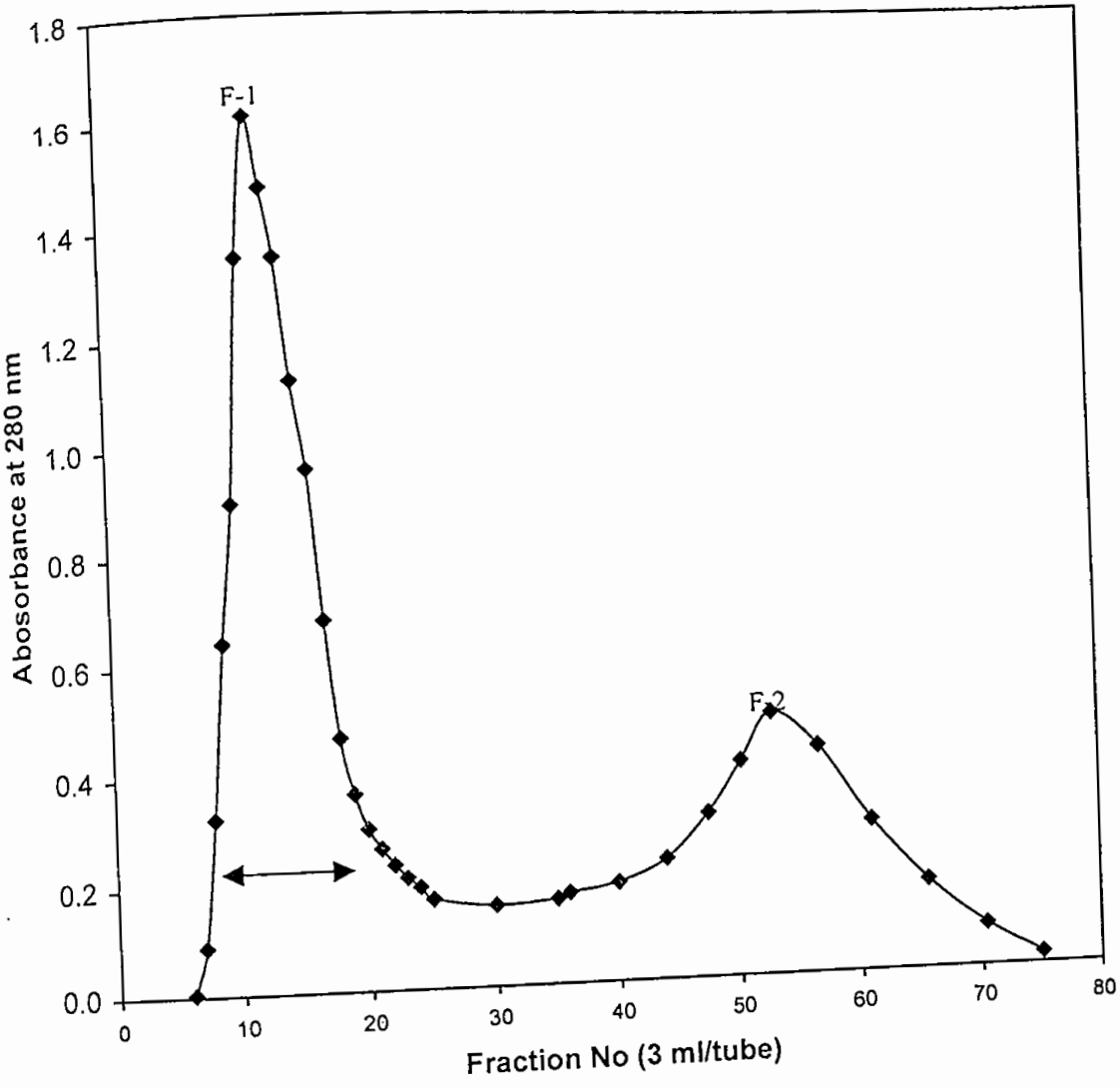

Fig. 4: Gel filtration of 100% $(NH_4)_2SO_4$ saturated crude protein extract (Rice bran IRRI-28 Variety) on Sephadex G-50. The crude extract (95 mg) was applied to the column (3.0 × 120 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH-8.0 at 4°C and developed with the same buffer, Flow rate; 25 ml/hour.

DEAE-Cellulose chromatography of the active F-1 fraction

The ammonium sulfate precipitate of F-1 fraction as obtained by centrifugation at 8000g was dissolved in minimum volume of distilled water, dialyzed against distilled water for 12 hours and against 10 mM Tris-HCl buffer, pH 8.4 at 4°C for 12 hours with three changes of the buffer. After centrifugation, the clear supernatants was applied to a DEAE-cellulose column at 4°C previously equilibrated with the same buffer and eluted by a linear gradient of NaCl from 0 to 0.4 M in the buffer. As shown in Fig. 5. the components in the fraction were eluted from the column in a single but broad peak, indicating that the fraction might be contained more than one component. In order to separate these components, the elution was carried out stepwise with increasing concentrations of NaCl in the same buffer under identical conditions.

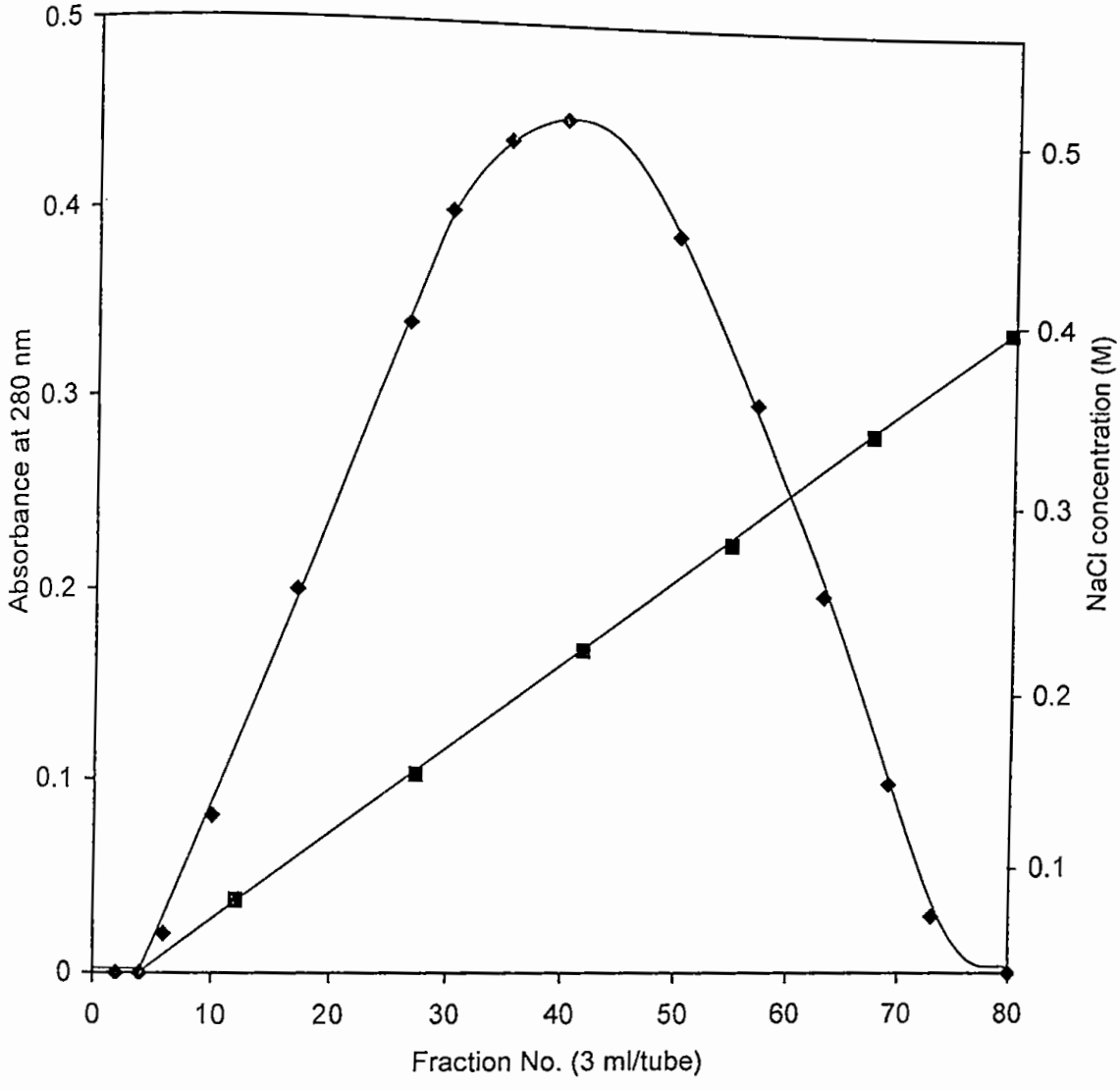

Fig. 5: Ion exchange chromatography of F-1 fraction on DEAE-cellulose. F-1 fraction (65 mg) obtained by gel filtration, was applied to the column (2.0 × 24 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH-8.4 at 4°C and eluted by a linear gradient of NaCl (0 to 0.4 M) in the same buffer Flow rate; 45 ml/hour.

As shown in Fig. 6, most of the components of F-1 fraction was bound to the column and the components were eluted from the column in different distinct peaks. Of these peaks, the fraction F-1d, and F-1c are major while F-1a, F-1b and F-1e are minor peaks. Again the factions F-1a, F1b, F-1c, F-1d

and F-1e were eluted from the column by the buffer containing 0.02, 0.1, 0.4, 0.6 and 0.8M NaCl respectively. Among the peaks F-1b, f-1c and f-1d were found to be contained biological activities while F-1a and f-1e possessed no such detectable biological activities. So, only the purities of F-1b, F-1c and F-1d were detected by SDS-PAGE on slab gel.

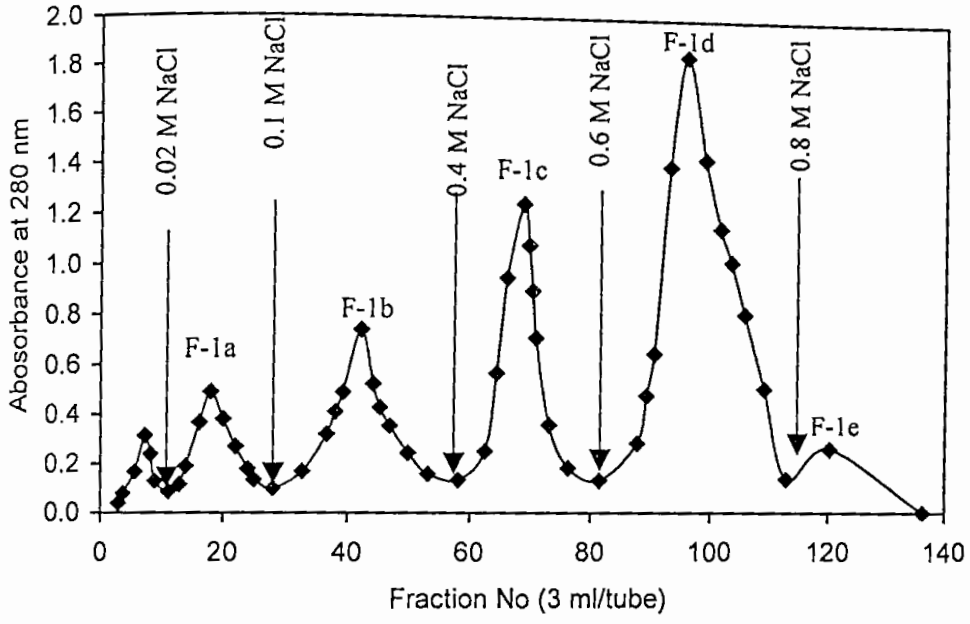

Fig 6. Ion exchange chromatography of F-1 fraction on DEAE-cellulose. F-1(45 mg) fraction obtained by gel filtration was applied to the column (2.0×24 cm) prewashed with 10 mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by stepwise increases of NaCl concentrations in the same buffer. Flow rate: 45 m/ hour.

SDS-PAGE

SDS polyacrylamide slab gel electrophoresis of the different protein fractions obtained from the DEAE cellulose chromatography i.e., F-1b, F-1c and F-1d were performed on 10% gel at room temperature using 10 mM. Tris-HCl buffer pH-8.4 and the photographic representation of the electrophoretic patterns are shown in Fig. 7.

From the results it might be concluded that all the fractions, F-1b, F-1c and F-1d contained pure protein as they gave single band on the gel.

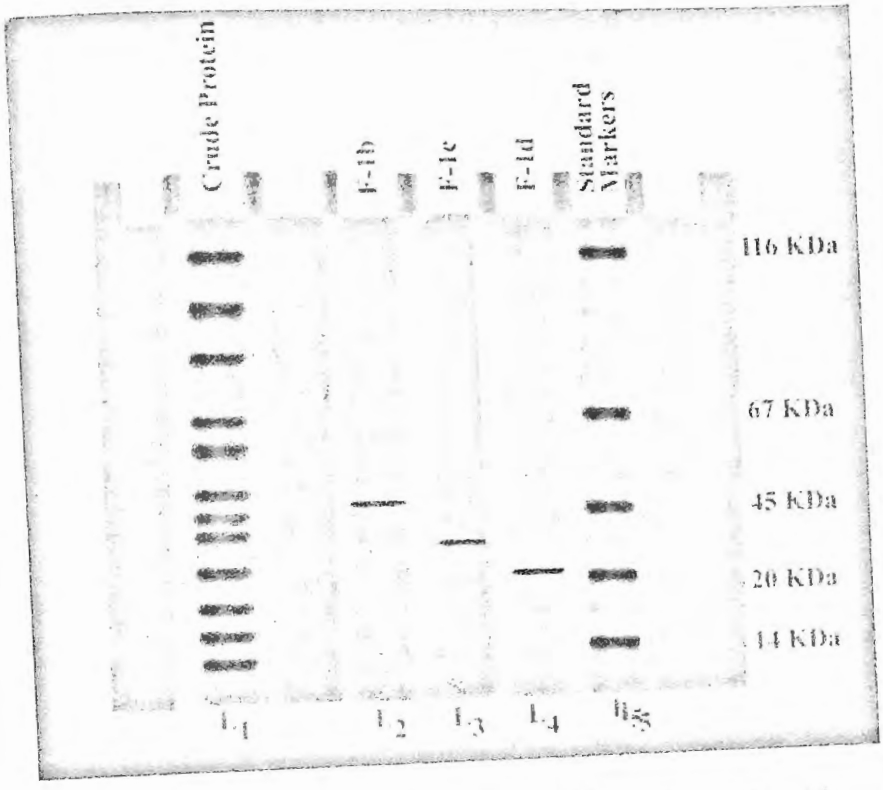

Fig.7: Photographic representation of SDS-polyacrylamide slab gel electrophoretic patterns of the crude and purified protein on 10% gel.

L_1 = Crude protein
L_2 = F-1b,
L_3 = F-1c,
L_4 = F-1d,
L_5= standard markers

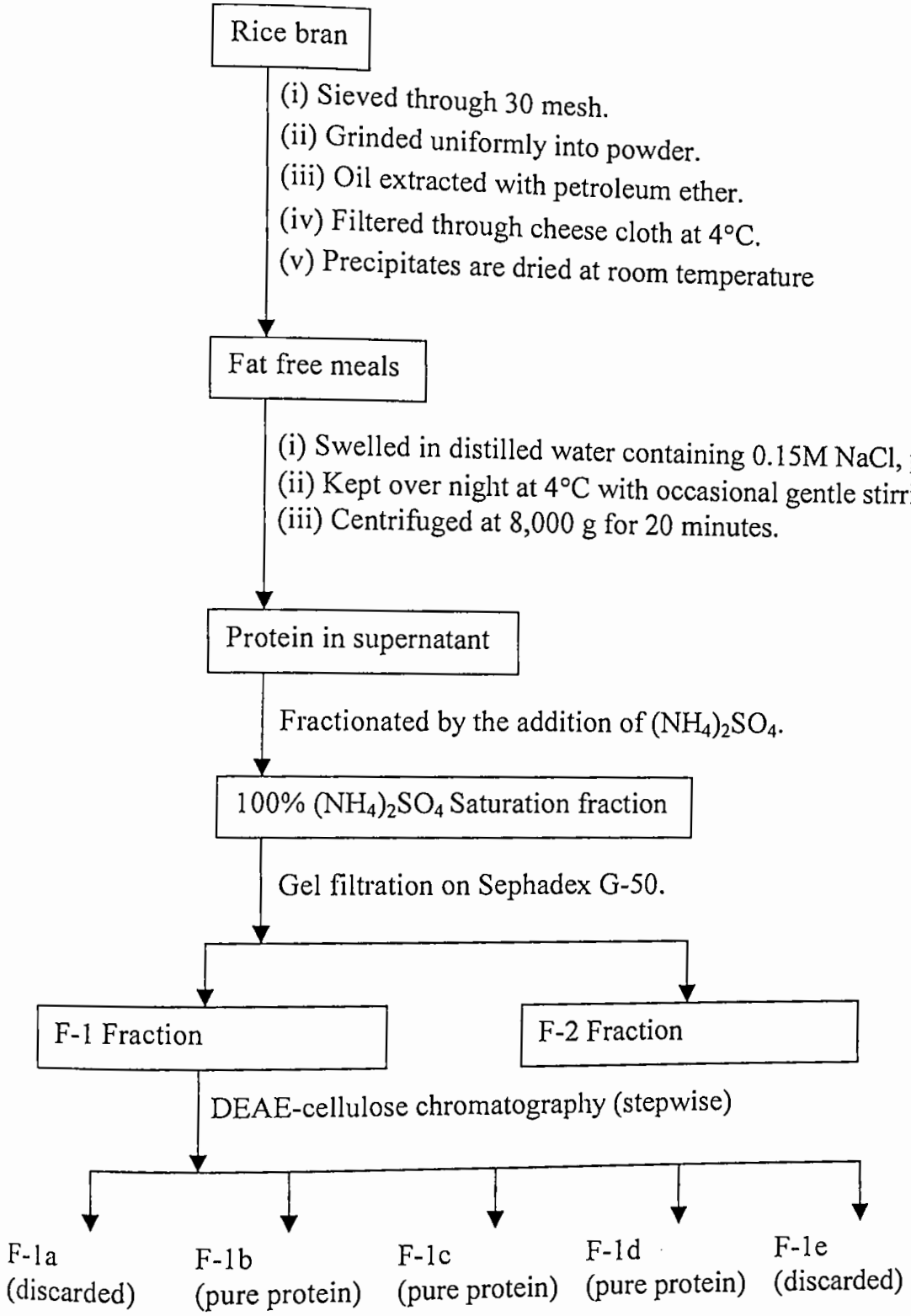

Fig. 8: Schematic representation of protein purification steps.

A brief scheme of the overall purification of rice bran proteins is shown in Fig-8. The extent of purification, recovery and yield at each steps are summarized in Table-25. As give in the Table, the specific activity of the different protein fractions were increased at each subsequent purification step. It can be shown from the table that more than 94% of protein was lost during the complete purification procedure. Although the total yield in biological activities of proteins are found to be about 39% but the purification folds, as a whole were increased more than 20 folds. The decrease in yield might be due to the lengthy purification procedures which may cause denaturation of some proteins.

Table-25: Summary of purification data of rice brans proteins using IRRI-28 variety.

Fraction		Total protein (mg)	Total Hemaggluti-nation activity (titre)	Specific activity (titre/mg)	Yield (%)	Purificati on (fold)
Crude extract		560	1720	3.07	100	1.00
100% $(NH_4)_2SO_4$ Saturated		195	860	4.41	50	1.43
After gel filtration		80	730	9.12	42.44	2.97
DEAE-Cellulose fractions	F-1b	12.00	340	28.33	19.76	9.22
	F-1c	10.60	210	19.81	12.20	6.45
	F-1d	8.5	120	14.11	6.97	4.59

Hemagglutionation activity (titre)= Reciprocal of highest dilution showing visible Hemagglutionation.

$$\text{Yield} = \frac{\text{Observed Hemagglutionation activity}}{\text{Initial Hemagglutionation activity}} \times 100$$

$$\text{Fold} = \frac{\text{Observed Specific activity}}{\text{Initial specific activity}}$$

CHARACTERIZATION OF PURIFIED RICE BRAN PROTEIN

Molecular weight determination

I) By gel filtration

The molecular weight of the proteins were determined by gel filtration on Sephadex G-150. The standard curve was constructed by plotting the log of molecular weight of the proteins against elution volume using Lysozyme, Trypsin inhibitor, Egg-albumin, Bovine serum albumin and β-Galactosidase as reference proteins. From the curve the calculated molecular weight of F-1b (designated as rice bran proteins-1, i.c. RBP-1), F-1c (designated as rice bran proteins-2, i.c. RBP-2) and F-1d (designated as rice bran proteins-3, i.c. RBP-3) were found to be 47 KDa, 35 KDa and 28 KDa respectively (Fig-9)

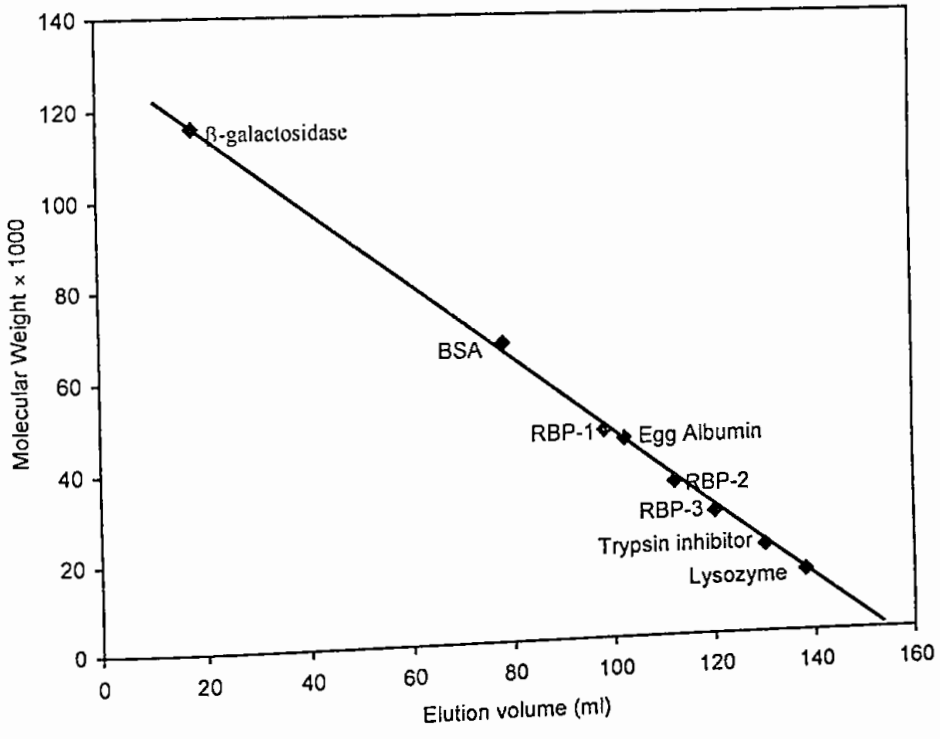

Fig-9: Standard curve for the determination of molecular weight of proteins by gel filtration.

Column : 1.8 × 90 cm
Buffer : 10 mM Tris-HCl, pH 8.4
Flow rate : 24 ml/hour

Determination of Molecular weight and subunit structure of rice bran proteins by SDS-PAGE slab gel electrophoresis

The molecular weight and the subunit structure of the purified rice bran proteins was also determined by SDS-PAGE on slab gel electrophoresis using Lysozyme, Trypsin inhibitor, Egg Albumin Bovin Serum Albumin and β-D glactodisdase as reference proteins. A standard curved was constructed by plotting $\log_{10}$ of molecular weight versus the relative mobility of proteins in the gel after SDS-PAGE. From the graph the molecular weight of purified proteins were estimated to be 46 KDa, 33 KDa and 26 KDa for RBP-1, RBP-2, RBP-3, respectively (Fig. 7 and 11). Further all the three proteins gave single slightly broad band on the gel when SDS-PAGE was performed in the presence of 0.1% SDS and 1% β-mertcaptoethanol and the molecular weight of the three proteins purified from rice bran were calculated to be 24-25KDa, 17-18KDa and 13-15KDa for RBP-1, RBP-2 and RBP-3 (Fig. 10 and 12) respectively. From these data it may suggeste that all the three rice bran proteins contained two subunits which are held together by S-S bonds. Advanced sophisticated modern techniques such as N-terminal sequences should be applied to confirm finally the subunit composition of each of the purified protein.

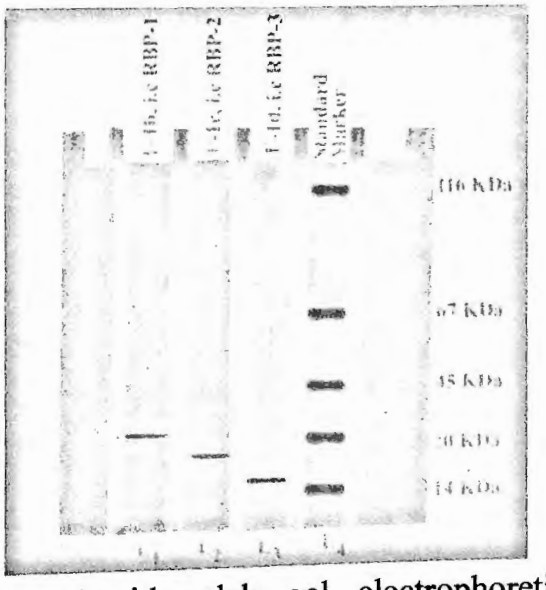

Fig. 10: SDS polyacrylamide slab gel electrophoretic pattern for the determination of Molecular weight of subunit structure of F-1b, F-1c and F-1d in the presence of 0.1% SDS and 1% β-mercaptoethanol.

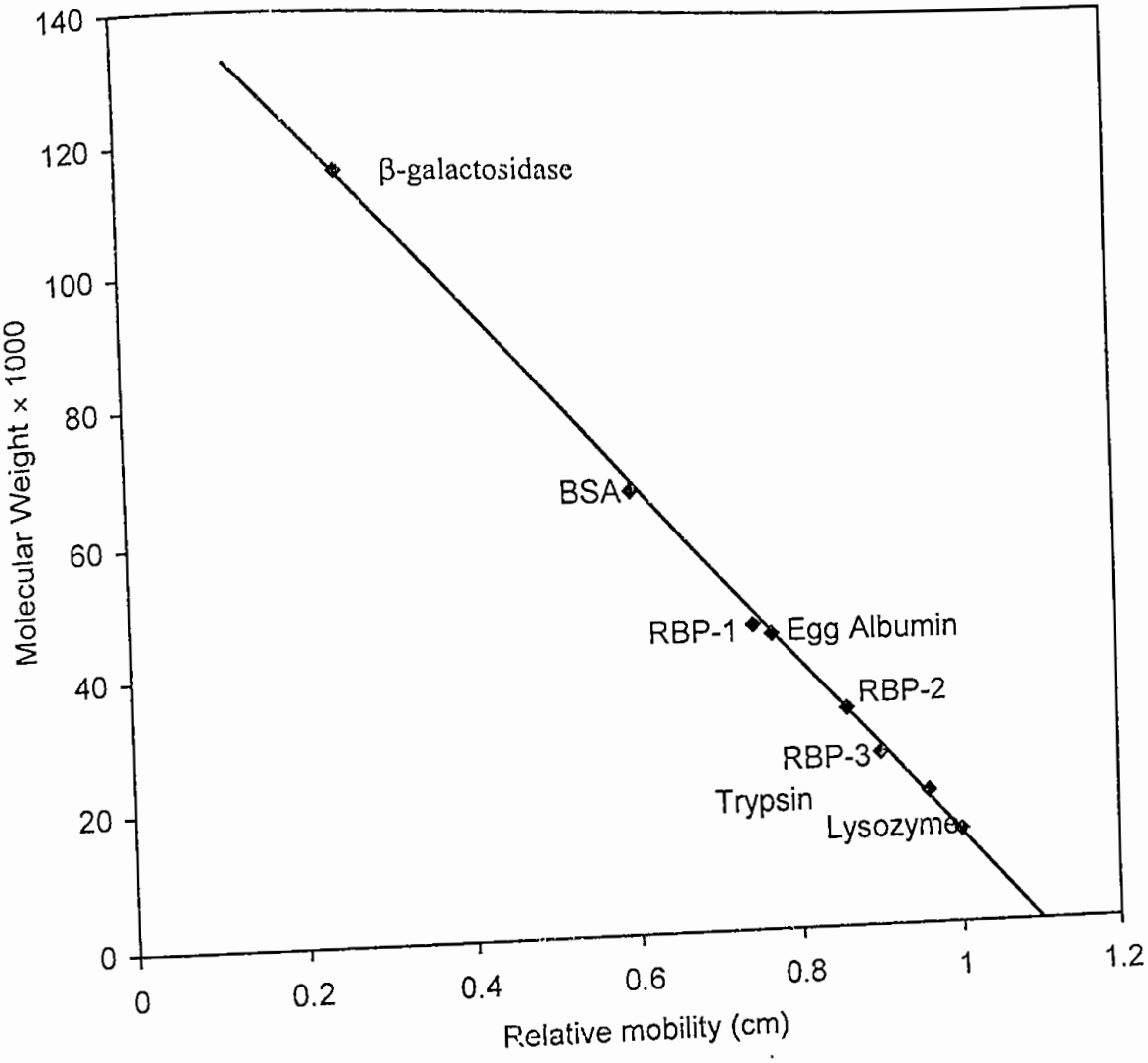

Fig-11: Standard curve for the determination of molecular weight of proteins by SDS polyacrylamide slab gel electrophoresis.

Buffer : 10 mM Tris-HCl, pH-8.7

Column size : 2.5 ×100 cm.

Flow rate : 25 ml/hour

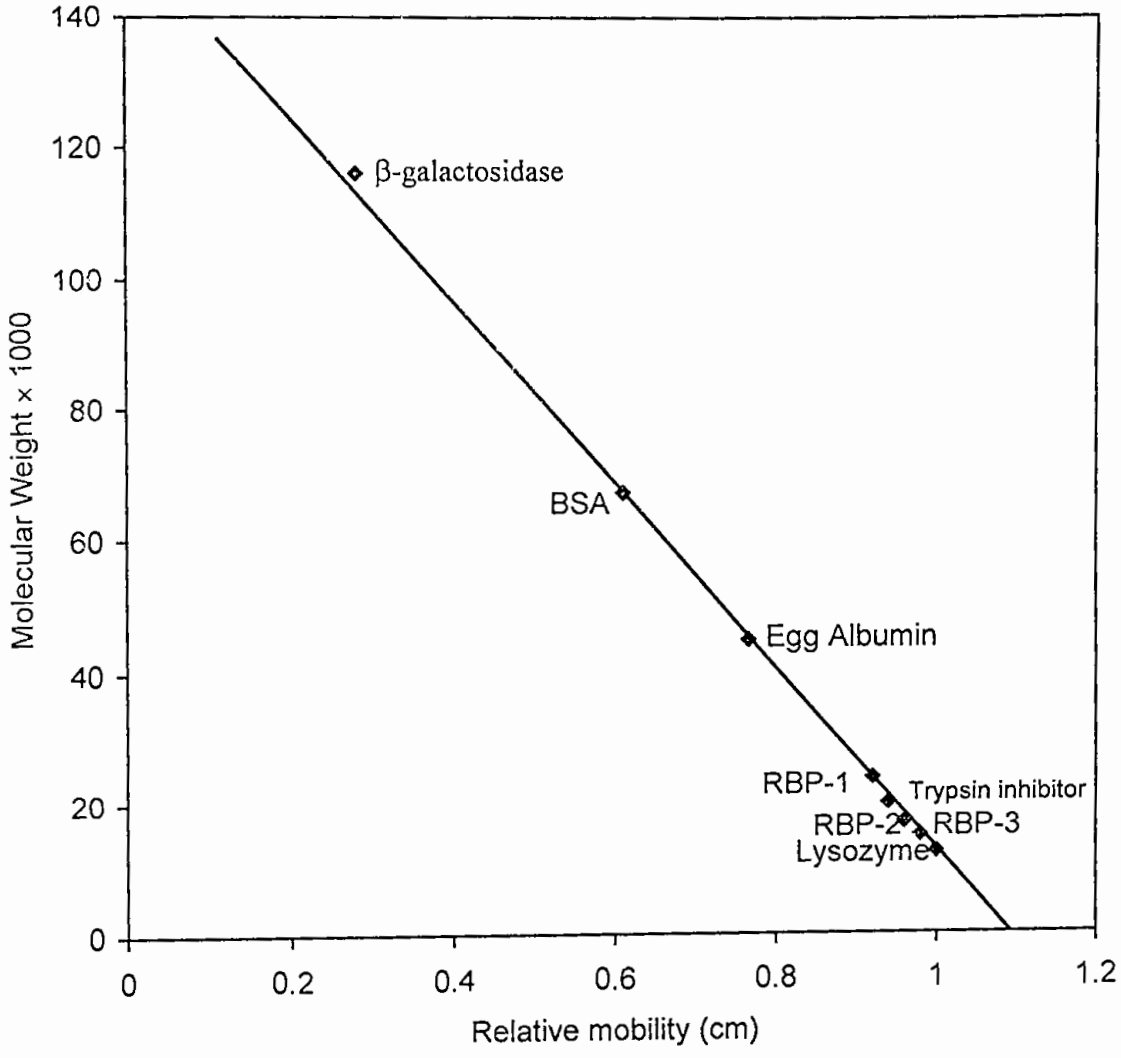

Fig. 12: Standard curve for determination of Molecular weight of subunit structure of the purified proteins by SDS-PAGE.

Hemagglutinating Activities of the proteins

All the purified proteins agglutinated specifically albino rat red blood cells and the hemaglutination potency of rat red blood cells by the proteins were shown by photographic representation in (Fig-13,14, 15) as given in Table-26. The minimum protein concentration at 280 nm needed for visible agglutination were found to be 8.2μg, 15.4μg and 26.0μg/ml for RBP-1, RBP-2, RBP-3 respectively.

Table. 26: Hemagglutination activities of three purified rice bran proteins with 4% red blood cell from albino rat.

Protein sample	Absorbance at 280 nm	Concentration (mg/ml)	Degree of hemagglutination
RBP-1	0.05	0.041	3^+
	0.04	0.032	2^+
	0.03	0.024	1^+
	0.01	0.0082	±
RBP-2	0.07	0.0534	3^+
	0.06	0.0462	2^+
	0.04	0.0308	1^+
	0.02	0.0154	±
RBP-3	0.06	0.0558	3^+
	0.05	0.0465	2^+
	0.034	0.0319	1^+
	0.0028	0.0260	±

3^+ Indicate complete aggregation of all most all cells.

2^+ Indicate lesser degree of agglutination where smaller number of cells remained free.

1^+ Indicate all the cells were present in small aggregation of varying sizes.

± Indicate major cell were present in small aggregates.

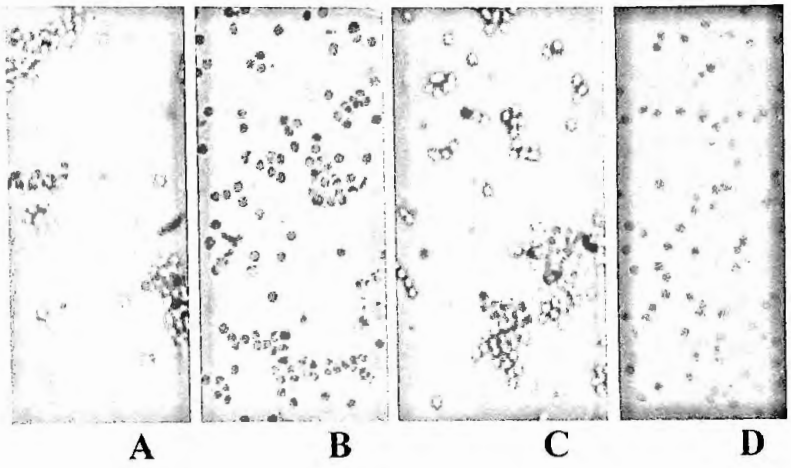

Fig. 13: Agglutination of albino rat red blood cells by RBP-1
A=3^+; B=2^+; C=1^+ and D=control

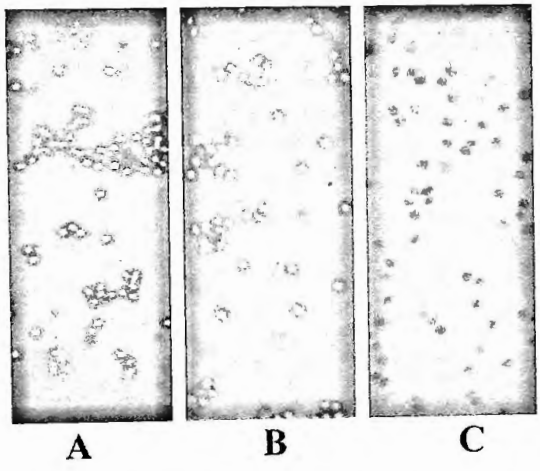

Fig. 14: Agglutination of albino rat red blood cells by RBP-2
A=3^+; B=2^+ and C=1^+

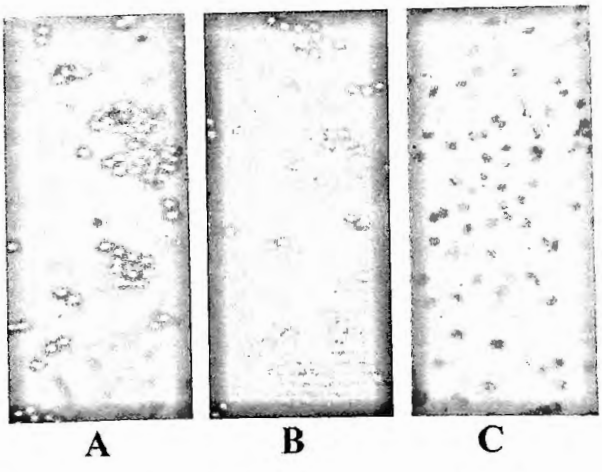

Fig. 15: Agglutination of albino rat red blood cells by RBP-3
A=3^+; B=2^+ and C=1^+

HEMAGGLUTINATION-INHIBITION STUDIES:

Hemagglutination inhibition of rat red blood cells by RBP-1, RBP-2 and RBP-3 were perfomed in prsence of different sugars and their derivatives in various concentration. The results of the hemagglutination inhibition test of the rice bran proteins with sugars are presented in Table-27. It was evident from the results that the agglutination of rice bran protein (RBP-1) was inhibited by D-glucose and D-glucosamine whereas that of RBP-2 and RBP-3 were inhibited by D-glucose, N-acetyl-D-glucosamine and D-glucosamine-HCl.

Table. 27: Hemagglutination-inhibition assay of rice bran proteins by different sugar and their derivatives.

Proteins	Sugar	Conc. (mM)	Inhibition
RBP-1	D-glucose	40	I
	D-mannose	110	NI
	D-galactose	110	NI
	N-acetyl-D-glucosamine	110	NI
	Methyl-α-D-galactopyranoside	110	NI
	Methyl-β-D-galactopyranoside	110	NI
	D-glucosamine-HCl	35	I
RBP-2	D-glucose	50	I
	D-mannose	110	NI
	D-galactose	110	NI
	N-acetyl-D-glucosamine	40	I
	Methyl-α-D-galactopyranoside	110	NI
	Methyl-β-D-galactopyranoside	110	NI
	D-glucosamine-HCl	40	I
RBP-3	D-glucose	35	I
	D-mannose	110	NI
	D-galactose	110	NI
	N-acetyl-D-glucosamine	35	I
	Methyl-α-D-galactopyranoside	110	NI
	Methyl-β-D-galactopyranoside	110	NI
	D-glucosamine-HCl	30	I

NI= No Inhibition, I=Inhibition

ULTRAVIOLET ADSORPTION SPECTRA OF THE PURIFIED PROTEINS

The purified protein RBP-1, RBP-2 and RBP-3 in aqueous solution gave absorption maxima around 277, 280 and 275 nm and minima around 240, 245 and 250 nm respectively (Fig. 16).

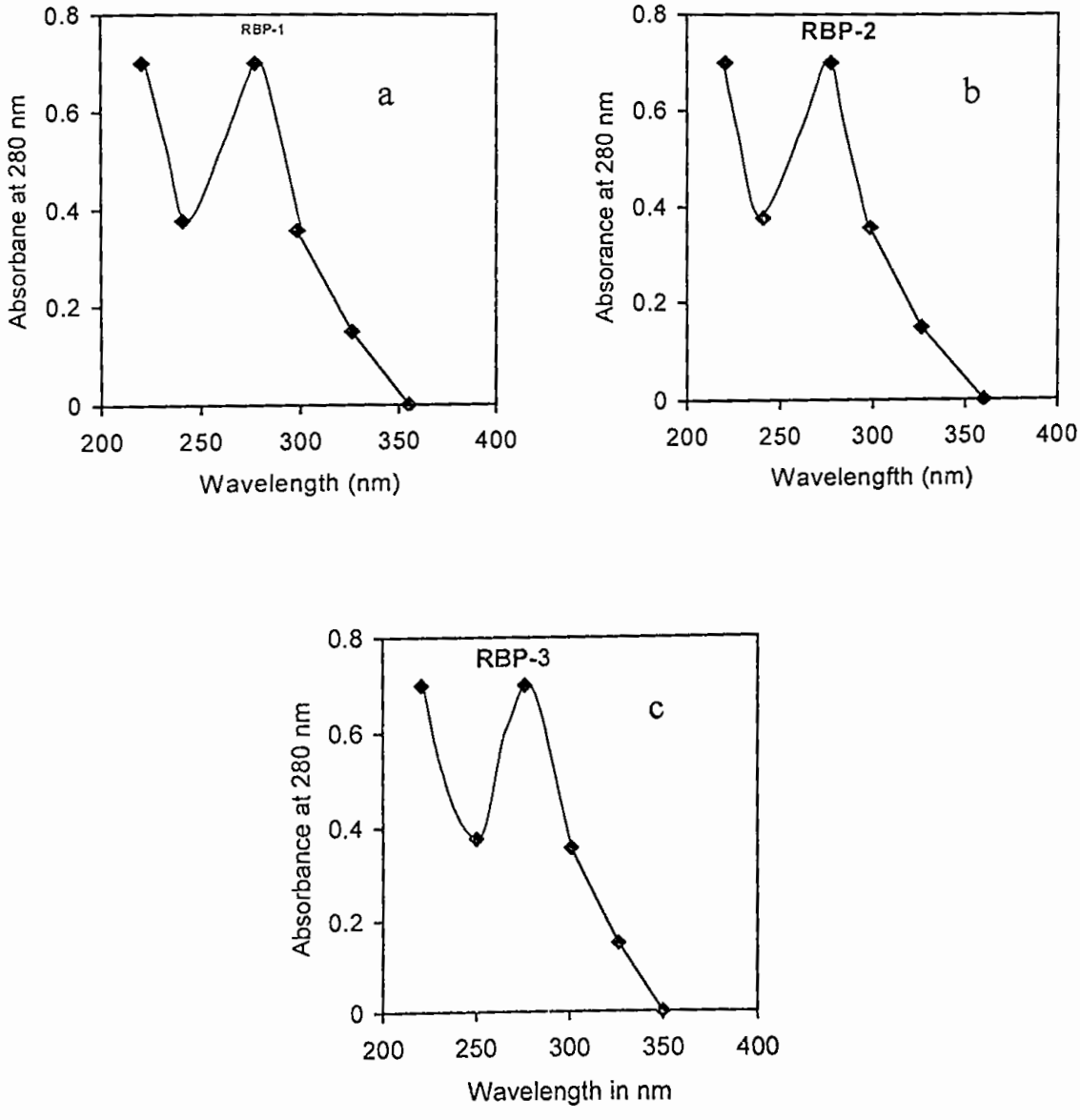

Fig. 16: Ultraviolet absorption spectra of a) RBP-1, b) RBP-2 and c) RBP-3.

CONCENTRATION OF PROTEIN

The absorbance of 1.0 at 280 nm for RBP-1, RBP-2 and RBP-3 were found to be equal to 0.82, 0.77 and 0.93 mg of proteins, respectively, by the Lowry methods but drying methods gave slightly higher values than the Lowry method.

Table-28: Optical density (O.D) and protein concentration relationship of the proteins.

Proteins	O.D. of proteins at 280 nm	Amount of proteins by Lowry method (mg)	Amount of protein obtained by drying method (mg)
RBP-1	1.0	0.82	0.87
RBP-2	1.0	0.77	0.83
RBP-3	1.0	0.93	0.97

Glycoprotein Test and sugar content

The purified proteins RBP-1, RBP-2 and RBP-3 gave yellow orange colour in the presence of phenol-sulfuric acid, indicating that the proteins contained sugar. i.e. the proteins are glycoprotein. The percentage of neutral sugar present in the glycoproteins, RBP-1, RBP-2 and RBP-3 were found to be 3.01% 3.81 and 2.16% respectively.

BRINE SHRIMP LETHALITY BIO-ASSAY

In the brine shrimp lethality bio-assay the purified three proteins RBP-1, RBP-2 and RBP-3 showed positive results indicating that the proteins are cytotoxic in nature (Table-29).

The mortality rate of brine shrimp nauplii was found to be increased with the increase of concentration of the sample and a plot of log of concentration vs. percent mortality gave an almost linear correlation (Fig 17). From the graph, the LC_{50} (concentration at which 50% mortality of the brine

shrimp nauplii occurred) was determined by extrapolation and the LC_{50} values of RBP-1 RBP-2 and RBP-3 were calculated to be 9.12, 6.91 and 11.22 µg/ml respectively.

Table-29: Result of brine shrimp lethality bio-assay of the RBP-1, RBP-2 and RBP-3.

Test Sample	Conc. of sample (µg/ml)	Log conc. (log C)	No of Shrimp taken (each vial)	No. of survial (average)	% of mortality	LC_{50} (µg/ml)
Control	0.00	0	10	10	00	
RBP-1	1.15	0.06	10	9	10	9.12
	2.3	0.36	10	8	20	
	4.6	0.66	10	6	40	
	9.2	0.96	10	5	50	
	18.4	1.26	10	3	70	
	36.8	1.56	10	2	80	
RBP-2	1.15	0.06	10	9	10	6.91
	2.3	0.36	10	7	30	
	4.6	0.66	10	6	40	
	9.2	0.96	10	3	70	
	18.4	1.26	10	2	80	
	36.8	1.56	10	1	90	
RBP-3	1.15	0.06	10	9	10	11.22
	2.3	0.36	10	8	20	
	4.6	0.66	10	7	30	
	9.2	0.96	10	4	60	
	18.4	1.26	10	3	70	
	36.8	1.56	10	2	80	

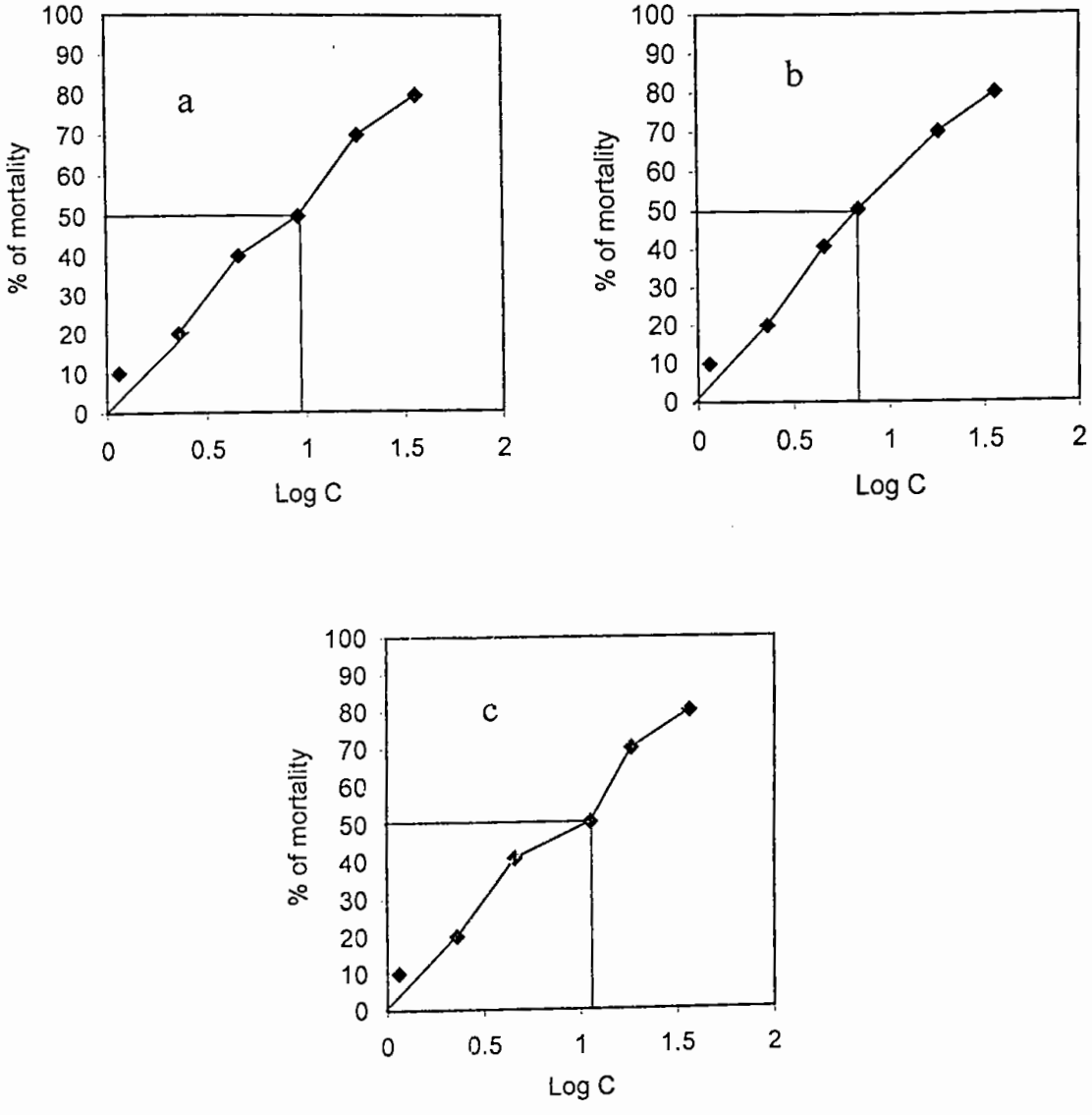

Fig. 17: Determination of LC_{50} of the proteins using brine shrimp eggs a) RBP-1, b) RBP-2 and c) RBP-3.

IDENTIFICATION OF REDUCING SUGAR PRESENT IN THE PURIFIED PROTEIN BY THIN LAYER CHROMATIOGRAPHY.

The reducing sugar composition of the purified proteins was determined by one dimensional thin layer chromatography. It was detected that the proteins RBP-1, RBP-2 and BRP-3 contained D-galactose/arabinose, D-mannose and D-glucose respectively.

Table-30: Data of the values obtained by TLC examination of the sugars from hydrolyzed protein samples (RBP-1, RBP-2 and RBP-3) and standard sugars.

Standard sugars & protein sampled	Distance traveled by the solvent system	Distance traveled by the standard sugar & protein	R_f values
Sugar			
D-glucose	11.6	8.20	0.70
D-galactose	11.6	6.50	0.56
D-mannose	11.6	7.75	0.66
D-arabinose	11.6	8.15	0.69
D-ribose	11.6	9.80	0.84
Protein:			
RBP-1	11.6	6.45	0.55
RBP-2	11.6	7.60	0.65
RBP-3	11.6	8.20	0.70

DISCUSSION

Although only one lectin was reported to be isolated and purified so far from different varieties of rice bran by some research laboratory (Shen et all (1983) purified a rice bran lectin of M.W 23.000, M Tsuda (1979) also purified a lectin from rice bran of M.W 44.000) but S. Rashel Kabir (2001) first time reported in our laboratory that rice bran from Amon variety contained two lectins which are dimer in nature. In this thesis, it has been found that rice bran variety of IRRI-28 contained at least three proteins which are catagorized by further evidences as lectins. All the three proteins are glycoprotein in nature as they gave yellow orange colour in the presence of phenol sulfuric acid. The presence of sugar in the proteins was also confirmed by TLC examination. The proteins, RBP-1, RBP-2 and RBP-3 are so designated as RBL-1. RBL-2 and RBL-3 respectively. From the estimation of percentage of neutral sugar (2-4%) it might be suggested the three lectins purified in this study has been quite different from the lectins so far reported from different laboratories as well as from our laboratory. The rice lectin purified by Shen *et. al.,* (1984) contained only 0.8% carbohydrate while Takahashi *et al.* (1971) reported that rice seed hemagglutinine contained abnormally higher amount of carbohydrate (26.8%). On the other hand. Tsuda (1979) detected neither neutral nor amino sugars in rice bran lectin but S. Rashel Kabir *et al.,* (2001) found that rice bran lectins contained 4.0-5.4% neutral sugar.

From the comparison of molecular weight it may conclude that the presently purified lectins are quite different from those purified in other laboratories. In the present study the molecular weight of the lectins RBL-1, BRL-2 and RBL-3 were estimated to be 46-47 KDa, 33-35 KDa and 26-28 KDa respectively. All these lectins are dimer in nature and their subunits are held together by disulfide bonds, Similar subunit structure of rice bran lectins was also reported by S. Rashel Kabir *et. al.,* (2001) which were purified from rice brans of Amon variety. It is interesting to note that Shen *et. al.,* (1984)

reported that under denaturing condition in the presence as well as in the absence of β-mercaptoethanol, the rice bran lectin gave three bands on the gel with molecular weight of 11,300, 13,700 and 19,000 while Tsuda (1979) reported the molecular weight of rice bran lectin to be 44,000 by SDS-PAGE but by gel filtration on Sepharose 6B in 6M guanidine-HCl it was estimated to be 19,000 which was dissociated further into two non-identical subunits of molecular weight-11,000 and 8,200 if the lectin was reduced with gel filtration. On the other hand Takahashi *et. al.,* (1971) purified a rice seed hemagglutain of monomer with molecular weight of 10,000.

The agglutinating activities of rice bran lectins so far purified from different rice bran varieties were inhibited mainly by N-acetyl-D-glucosamine as well as also by D-glucose and D-glucosamine (S. Rashel Kabir, 2001).

In the present study it was also found that the agglutinating activities of all the three lectins are inhibited by D-glucose and D-glucosamine-HCl while the activities of RBL-2 and RBL-3 were also inhibited by N-acetyl D-glucosamine.

The lectins, purified in this study are cytoxic in nature as they affect significantly the mortality rate of brine shrimp. Similar results were also reported for rice bran lectins by S. Rashel Kabir (2001). Further rice bran lectin was reported to be mitogenic against mouse splenic and human peripheral lymphoeytes (Tsuda, 1979 and Takahashi *et. al.,* 1971). From the comparison of the results, it could be suggested that RBP-2 is more toxic as compared to that of RBP-1 and RBP-3.

In conclusion the proteins purified from rice brans of variety IRRI-28 might be considered as lectins as they showed the following properties such as agglutinating activities, toxicity and also contained neutral sugar. Again, the purified lectins RBL-1, RBL-2 and RBL-3, besides being specific for rat red blood cell agglutination, can also be added as an additional members of the list of glucose and glucose containing sugars such as N-acetyl D-glucosamine and D-glucosamine binding lectins.

CHAPTER-V

Effect of physical and chemical treatments on the biological activities of rice bran lectins

INTRODUCTION

The tertiary and quaternary structure of a protein is governed by its primary structure and its environment. The organized native structure (conformation) of a protein is known to be affected from the effect of external environmental changes such as temperature, acidity, urea or other denaturing solution and a number of other chemicals.

In structural studies of proteins it is often necessary to established conditions for reversible denaturation. The choice of denaturating condition depends on the stability of the protein of interest. Among the techniques used for reversible denaturation are lowering of the pH (Itano *et al.* 1985), freezing and thawing in concentrated salt (Market, C.L. 1963), and adding denaturants such as urea and Guanidine-HCl (Chilson *et al.* 1965).

The glycoproteins i.e namely RBL-1, RBL-2 and RBL-3 were purified form rice bran, specifically agglutinate rat red blood cells and might be useful to perform many other biological activities.

In this chapter these glycoproteins were subjected to various physical and chemical treatments, and their effects on the hemagglutinating activities were observed. The study is expected to provide important information regarding some of the physico-chemical properties such as pH stability, thermal stability, the stability of the protein towards denaturing agents etc. The results might also be helpful in understanding the relationship between structure and function.

MATERIALS AND METHODS

Acetic acid and urea were the products of British Drug House (BDH), Poole, England. Guanidine-HCl was the products of Bio-Red Laboratories, Richmond, California, USA. All other reagents used were of analytical grade.

HEMAGGLUTINATING ACTIVITY

The hemagglutinating activity was performed by using 4% albino rat red blood cells according to the method of Lin *et al,* (1981) as described earlier in Chapter-4.

DETERMINATION OF pH STABILITY

The pH stability of RBL-1, RBL-2 and RBL-3 were examined by incubating the sample solution (0.25-0.35% for each) in 50 mM respective buffers possessing pH ranges from 2.0-10.2 for 10 hr at 30°C. The hemagglutinating activity retained was determined after dialysis against 5 mM phosphate buffer saline, pH 7.2 for 12 hr at 4°C.

DETERMINATION OF HEAT STABILITY

The purified proteins (0.3-0.5%) in 5 mM phosphate buffer saline, pH 7.2 were heated at various temperature ranges for 1hr in a temperature controlled water bath. Then the relative hemagglutinating activity of the protein was determined after cooling the heated sample solutions in an ice bath and diluting with the same buffer.

TREATMENT WITH ACETIC ACID

The purified rice bran proteins (0.85 mg/ml), were added with acetic acid at different concentration. After an incubation period of 1 hr at 4°C, the sample solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hr at 4°C and the hemagglutinating activity was determined.

TREATMENT WITH UREA

The purified protein solutions (0.85 mg/ml in 10 mM Tris-HCl buffer, pH 8.4), were added with solid urea to the concentrations of 0.2, 0.4, 0.8, 1.0, 2.0, 3.0, 5.0, 7.0 and 8.0 M. After incubation at 15°C for 12 hr, the solutions were dialyzed

against 5 mM phosphate buffer saline, pH 7.2 for 12 hr at 4°C to remove urea and the hemagglutinating activity was determined.

TREATMENT WITH GUANIDINE-HCL

The purified rice bran protein solutions (0.85 mg/ml in 10 mM Tris-HCl buffer, pH 8.2), were added with solid Guanidine-HCl to the concentrations of 0.4, 0.8, 1.5, 2.0, 4.0, 6.0 and 6.5 M. After incubation at 20°C for 12 hr, the solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hr at 4°C to remove the reagent and the hemagglutinating activity was determined.

TREATMENT WITH VARIOUS SALTS

The metallic salts of different concentrations were added to the protein solution (0.85 mg/ml in 10 mM Tris-HCl buffer, pH 7.2), and incubated for 30 minutes at room temperature. The hemagglutinating activity was assayed as described before. In this experimental procedure deionized water was used.

RESULTS

EFFECT OF pH

The effect of pH on the hemagglutinating activities of RBL-1, RBL-2 and RBL-3 are summarized in Table-31. The results demonstrated that the biological activity of RBL-1, RBL-2 and RBL-3 were markedly influenced by the pH changes. The hemagglutinating activities of the three purified rice bran lectins were found to be maximum in the neutral pH. The activities were found to be abolished sequentially in both in the acidic as well as in the basic pH-regions. More than 50% activities of the lectins were destroyed at pH-5.0 and at pH-9.0, and the lectins lost almost complete activities at pH-2.0 and at pH 10.2.

Table 31: Hemagglutinating activities of the lectins at different pH values.

pH (Buffer composition)	Relative hemagglutinating activity (%)		
	RBL-1	RBL-2	RBL-3
2.0 (KCl-HCl)	8	7	5
2.5 (AcONa-HCl)	15	18	20
4.0 (AcONa-CH_3COOH)	25	20	26
5.0 (NaH_2PO_4-Na_2HPO_4)	30	35	40
6.0 (NaH_2PO_4-Na_2HPO_4)	50	55	50
6.5 (NaH_2PO_4-Na_2HPO_4)	80	70	75
7.2 (NaH_2PO_4-Na_2HPO_4)	100	100	100
8.0 (NaH_2PO_4-Na_2HPO_4)	84	85	80
9.0 (NaH_2PO_4-Na_2HPO_4)	40	35	30
10.2 (NaH_2PO_4-Na_2HPO_4)	20	15	10

EFFECT OF TEMPERATURE

In Table-32, the hemagglutinating activities of RBL-1, RBL-2 and RBL-3 were present with respect to the changes of temperature. In the study the optimum temperature for maximum hemagglutinating activities of the lectins were calculated between 30°C to 40°C. Again, the hemagglutinating activities of RBL-1, RBL-2 and

RBL-3 were abolished rapidly with further rise of temperature and at 80°C the activities of all the lectins were found to be completely destroyed.

Table 32: Heat stability of purified lectins.

Temperature (°C)	Relative hemagglutinating activity (%)		
	RBL-1	RBL-2	RBL-3
20	100	95	100
30	100	100	100
40	100	100	100
50	65	55	65
60	20	25	30
70	5	10	10
80	00	00	00

EFFECT OF ACETIC ACID

The effect of acetic acid on the hemagglutinating activities of RBL-1 RBL-2 and RBL-3 are given in Table-33. It was found that the hemagglutinating activities was found to be almost 100% even after treatment with 0.6% acetic acid solution, but then the activity was gradually decreased with the increase in acetic acid concentration and in presence of 10% acetic acid solution about 75-80% activities of the lectins were destroyed.

Table 33: Effect of acetic acid on the hemagglutinating activities of purified rice bran lectins.

Concentration of acetic acid (%)	Relative hemagglutinating activity (%)		
	RBL-1	RBL-2	RBL-3
0.0	100	100	100
0.2	100	100	100
0.6	100	100	100
1.00	80	85	90
3.00	70	75	65
6.00	50	55	45
10.00	20	15	20
15.00	00	00	00

EFFECT OF UREA

As shown in Table-34, the hemagglutinating activities of RBL-1 RBL-2 and RBL-3 were decreased rapidly after treatment with increasing concentration of urea and all the lectins lost their activities completely after treatment. with 8M urea.

Table 34: Effect of Urea on the hemagglutinating activities of purified rice bran lectins.

Concentration of Urea (molar)	Relative hemagglutinating activity (%)		
	RBL-1	RBL-2	RBL-3
0.0	100	100	100
0.2	100	100	100
0.4	100	100	100
0.8	100	100	100
1.00	100	100	100
2.00	85	75	70
3.00	60	55	50
5.00	30	35	40
7.00	15	20	10
8.00	00	00	00

EFFECT OF GUANIDINE HYDROCHLORIDE

As presented in Table-35, the hemagglutinating activities of RBL-1, RBL-2 and RBL-3 were also decreased markedly after treatment with guanidine hydrochloride and the present data indicated that the lectins retained about 40-50% activities even after treatment with 1.5M Guanidine-HCl. The activities of the lectins were decreased further with rise in guanidine-HCl concentration and all the lectins lost their activities completely after treatment with 6.0M guanidine-hydrochloride.

Table 35: Effect of Guanidine-HCl on the hemagglutinating activities of purified rice bran lectins.

Concentration of guanidine-HCl (molar)	Relative hemagglutinating activity (%)		
	RBL-1	RBL-2	RBL-3
0.0	100	100	100
0.4	90	85	80
0.8	80	75	70
1.5	45	50	40
2.00	25	30	25
4.00	10	12	10
6.00	00	00	00

EFFECT OF METALLIC SALTS

The effect of various metal ions and salts concentration on the hemagglutinating activities of RBL-1, RBL-2 and RBL-3 were showed in Table-36. From the table, it is evident that the presence of EDTA (100 mM) abolished completely the hemagglutinating activities of RBL-1, RBL-2 and RBL-3 while the presence of Ca^{2+} and Cu^{2+} increased the activities significantly. On the other hand the metallic salts such as Na^{+} and K^{+} had profound inhibitory effect of the activities of the proteins but the metallic salt Mg^{2+} had no such effect.

Table 36: Effect of EDTA and various metal salts on the hemagglutinating activities of purified rice bran lectins.

Salt added	Concentration (nM)	Relative hemagglutinating activity (%)		
		RBL-1	RBL-2	RBL-3
None	---	100	100	100
EDTA	50	5	5	0
	100	0	0	0
$MgCl_2$	50	100	100	90
	100	100	100	80
Na_2SO_4	50	70	50	60
	100	60	40	45
KCl	50	40	35	50
	100	35	30	50
$NaNO_3$	50	70	50	60
	100	60	40	55
$CaCl_2$	50	120	105	100
	100	130	110	105
$CuCl_2$	50	110	115	120
	100	125	130	135

DISCUSSION

The aim of this study is to determine the stabilities of RBL-1, RBL-2 and RBL-3 by using physical and chemical means. The results clearly demonstrated that the activities of the lectins are more sensitive in acidic pH region than alkaline pH region and the maximum activities were found at the neutral pH. The lost in activities at the extreme pH values, both of acidic or basic region might be due to ionization of the groups located at or near the saccharide binding site(s) or due to disorganization of the structures of the proteins at these extreme pH values.

The present data also indicated that the activities of all the three lectins are depended on temperature and they gave optimum activities around 30-40°C. The

decrease in activities above these temperature ranges might be due to destruction of the native structure or denaturation of the proteins.

It was also found the activities of the protein were affected with increases in concentration of chemicals such as acetic acid and denaturants such as urea and guanidine HCl. The decrease in activities of the proteins in the presence of acetic acid might be evaluated with the destruction of activities in extreme acidic pH-values, indicating that the conformation of the binding site(s) of the lectins are changed abruptly at higher acetic acid concentration. Although all the lectins are found to be sensitive to both the denaturing agents but Guanidine HCl is considered to be slightly more sensitive than urea.

The activities of the lectins were also carried out in the presence of various metallic salts as well as EDTA. As shown in table-36. the activities of all the purified rice bran lectins were abolished completely after treatment with EDTA. It may also mentioned that the activities of the proteins were enhanced significantly in the presence of some metallic ion such as Ca^{2+} and Cu^{2+}. From the results it could be concluded that the metal ions which are essential for activities of lectins, might be released from the proteins after treatment with EDTA. The inhibitory effect of EDTA on hemagglutinating activity have also been observed on lectin of other sources such as TM (Tora-mame) lectin, a lectin from *phaseolus valgaris seeds* (Itoh, *et al.*, 1980).

Many lectins have been reported to be metalloproteins (I.J. Goldstein 1986 and C.E. Hayes, 1978) and a part of the metals is necessary for activities such as hemagglutination (Takahashi *et al.*, 1971; Paulova *et al.*, 1971; Tunis M., 1965; Alford, R.H. 1970), polysaccharide precipitation (Paulova *et al.*, 1971) and lymphocyte transformation (Takahashi *et al.*, 1971 & Alford R.H. 1970) In rice bran lectins metal ion like Ca^{2+}/Cu^{2+} may be present in low concentration and an amount of these metals might have been removed from the protein molecules during the lengthy purification procedures.

In conclusion, it may conclude that the rice bran proteins are of metalloprotein types and their activities are greatly depended on pH and temperature.

CHAPTER-VI

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