IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
November 2015 Vol.:2, Issue:1

Biochemical Analysis of Amylases During Germination of Buckwheat *(Fagopyrum esculentum)* Seeds: A Pharmaceutical Plant

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Submission: 25 October 2015
Accepted: 2 November 2015
Published: 15 November 2015

Keywords: Buckwheat, Diabetes II, PAGE, Starch, Alpha and Beta Amylase

ABSTRACT

The main objective of study was to investigate different biochemical properties of the germinated Buckwheat *(Fagopyrum esculentum)* seeds regarding amylases and starch metabolism in seeds which could be used as a promising functional food for health promotion, prevention and treatment of various human diseases, and could also be helpful in improving the development of active components for functional food products and pharmaceuticals for the drug discovery. Biochemical analysis for total amylase, alpha and beta amylase activities was measured by the standard method designed by Bernfeld (1955). Quantification of total proteins was done by performing Lowry's Method (1950)**.** PAGE was performed as described by Laemmli (1970). The seeds of buckwheat showed the high level of amylolytic activity during different stages of germination. The total amylase, alpha and beta amylase activity were expressed as units per ml. The amylase activity starts increasing and becomes a maximum of 96 hours and starts decreasing and becomes lower at 192 hours, suggesting that alpha amylase plays a important role in starch metabolism . The moisture content in the seed at 0 hours was only 10% but it was increased to 25% after 24 hours by imbibitions of water by seed. Germinated buckwheat can be used for the drug discovery and treatment of several diseases like diabetes, polycystic ovary syndrome, constipation, bowel upsets, obesity and others.

INTRODUCTION

Buckwheat (*Fagopyrum esculentum*) belongs to the Polygonaceae family, is usually considered a cereal in agriculture and food technology because of its usage and the cultivation techniques used. It is recognized as valuable foods which successfully replace rice or potatoes on the daily menu. Originating from Asia and introduced into Europe around the 15th century, the cultivation of buckwheat has spread to Canada, the United States of America and to certain areas of Africa and Latin America, mountains of Nepal and India grows at high altitudes above 3, 000 meters in Nepal, India and Bhutan. Buckwheat is ubiquitous almost everywhere, but grows mainly in the northern hemisphere [1]. Buckwheat has engrossed increasing attention from food scientists for its therapeutic effects over chronic diseases. Buckwheat grain is characterized by a high content of starch, protein with an advantageous amino acid composition, a low content of α-gliadin and a high content of dietary fibre [2]. Seeds of buckwheat are one of the best sources of high quality, easily digestible, glutenless food and rich in potassium, phosphorous, calcium, iron, zinc, vitamins B, E, and rutin [3]. Additionally, extracts from buckwheat flour show anti-mutagenic activity, provide protection from oxidative stresses, and have the potential to improve diabetes symptoms [4]. Healthiness of buckwheat has been endorsed to the content of several natural antioxidants including tocopherols, phenolic acids, and flavonoids [5]. Distinctively buckwheat can be used in the production of foods for people with celiac disease, for those subjects who suffer from gluten intolerance, typhoid and liver ailments. Buckwheat is the only pseudocereal that contains rutin and it is a beneficial source of this flavonoid. Other phenolic compounds and flavones such as hyperin, quercitrin, and quercetin have been detected and isolated from immature buckwheat seeds [6]. They acquire special medicinal properties such as antihypertensive and antihypercholesterolemic effects at nontoxic concentrations in humans [7]. Buckwheat protein shows high biological value due to a well-balanced amino acid pattern and is rich in lysine and arginine and has many unique physiological functions, such as curing chronic human diseases, decreasing blood cholesterin, inhibiting mammary cancer caused by 7,12-dimethylbenzene, restraining gallstone and so on [8]. It is supposed to improve health in various ways, notably reducing serum cholesterol, suppressing gallstones and tumors, and inhibiting the angiotensin I-converting enzyme [9]. Much progress has been made in improving the nutrition and healing effects of buckwheat as a functional food in the last few years. In humans, the consumption of buckwheat is associated with a lower prevalence of hyperglycemia and improved

glucose tolerance in people with diabetes [10]. The main phenolics of buckwheat extract was rutin, quercitrin, and quercetin. Rutin (quercetin-3-O-β-rutinoside) is the best-known glycoside derived from flavonol quercetin, which has relaxing effects on smooth muscles and is effective for preventing capillary apoplexy and retinal hemorrhage, reduce high blood pressure, and show antioxidant and lipid peroxidation activities. It also has a lipid-lowering activity by decreasing the absorption of dietary cholesterol as well as lowering plasma and hepatic cholesterol [11].

D-Chiro-inositol (DCI) is the main active nutritional ingredient in buckwheat. DCI is probably the main mediator of insulin metabolism by enhancing the action of insulin and decreasing blood pressure, plasma triglycerides, and glucose concentrations [12, 13]. Consequently, DCI has great potential to work as an adjunctive drug in the treatment of insulin resistance diseases such as type 2 diabetes and polycystic ovary syndrome [14]. D-Fagomine is an iminocyclitol first isolated from seeds of buckwheat. As D-fagomine and other iminosugars such as 1-deoxynojirimycin (DNJ) are intestinal glycosidase inhibitors, they are connected to a reduction in the risk of developing insulin resistance, becoming overweight and suffering from an excess of potentially pathogenic bacteria [15, 16]. Buckwheat was found to have high levels of angiotensin I-converting enzyme (ACE)-inhibitory activity. ACE converts angiotensin I to II, which is a pressor hormone in the reninangiotensin (RA) blood pressure control system [17]. High levels of vitamin E intake have been associated with a reduction in cardiovascular disease, lowering the risk of Alzheimer's disease and prostate cancer, improving the immune system, and delaying both age-related cataracts and age related macular degeneration which has been found predominant in buckwheat [18]. Active oxygen species were generated in the body cause damage to DNA and the lipid membrane structure of cells and play a role in the process of aging and the development of cancer. It is therefore important to control the amount of excess active oxygen in our body. Antioxidative components found in food have been shown to be effective as scavengers of active oxygen [19].

Another important phenomenon occurring in buckwheat is germination which makes the buckwheat more nutraceutical, pharmaceutical and medicinal. Germination is a complex process in which significant changes in the biochemical, nutritional and sensory characteristics occur due to the activation of dormant enzymes [20]. Starch is the most common reserve carbohydrate in seeds and an important commercial source is the endosperm of cereals. The amylose and

amylopectin in the native starch grain are first hydrolysed by a-amylase, which randomly breaks the α -1, 4 glycosidic links between the glucose residues. Starch degradation is aided by ß-amylase, which cleaves off successive disaccharide maltose units (glucose- glucose) from the non-reducing end of the large oligomers released by prior amylolytic attacks. Both α-amylase and β-amylase are present in seeds; β-amylase is present in an inactive form prior to germination, whereas α-amylase and proteases appear once germination has begun. As a result, the germinated seeds or sprouts are nutritionally superior to their original seeds with higher levels of nutrients, lower amounts of compounds that interfere with the absorption of nutrients and increased protein and starch digestibility [21]. Germinated buckwheat is an important raw material for food and functional food production, had better nutritional value than ungerminated buckwheat. The germinated seeds could help in the prevention and treatment of various human diseases, but could also be helpful in improving the development of active components for functional food products and pharmaceuticals. Therefore, this study has focussed on biochemical studies on amylases during germination of seed in buckwheat *(Fagopyrum esculentum)* to emphasize the values of buckwheat as medicinal, pharmaceutical and nutraceutical plant where the moisture content in the seeds, measurement of Alpha and Beta amylase activity from 0 to 192 hours, specific activity and purification of Alpha amylase by PAGE Technique has been estimated.

MATERIALS AND METHODS

The present research work based study was conducted in the laboratory of Department of Biochemistry, College of Applied education and health sciences, Meerut, India regarding different biochemical activities and various changes on amylases during the germination of Buckwheat (*Fagopyrum esculentum* Moench) seeds.

The seeds of common Buckwheat (*Fagopyrum esculentum* Moench) were bought from the local market**.** Most of the chemicals used were of Analytical grade or Molecular Biology graded. Manufactured in India and procured through the local suppliers. Instruments manufactured by Indian companies were used and procured through local suppliers. Major equipments were refrigerated centrifuge (Eltech, Bombay, India); Oven (Yorko Ltd., India); Incubator (Yorko, Ltd., India); Electrophoretic unit (Genei, India); UV Spectrophotometer (Elico, India); Water bath (Elico, India); pH meter (Elico India); Projector (Zeistech India).

Glasswares used in the investigation were procured from Borosil India and Schott Duran, Germany. Plastic wares comprising of microcentrifuge tubes, falcon tubes, petridishes, microtips and tip boxes were procured from Tarson, India .Micropipette of different ranges were obtained from Eppendorf Germany. The consumable comprising of paraffin, tissue paper, cotton were procured from local chemical suppliers.

Germination of *Fagopyrum esculentum*

18 grams dry, healthy seeds of *Fagopyrum esculentum* out of which per 2 grams of seed were distributed in each petridishes and kept under laboratory conditions. The sample for the seed germination was drawn for every 24 hours interval from 0 to 192 hours. Each of 2 grams seed were washed with distilled water followed by soaking seeds in 0.1% $HgCl_2$ for 3 minutes for sterilization and then again seed were thoroughly washed under running tap water for 15 minutes to remove all the traces of sterilizing agent. The seeds were placed in sterilized petridishes containing moistened filter paper in dark for germination.

Isolation and extraction of enzyme

The seed coats of the sprouted seedlings were released with the help of sterilized forceps and also weight of the endosperm was taken before grinding. The endosperms were macerated in mortar and pestle with minute amount of distilled water. Then 5 ml of 50mM of phosphate buffer (pH 6.9) added for homogenization. The mixture was homogenized thoroughly and centrifuged at 8000 rpm for 15 minutes. The supernatant was taken with micropipette and was transferred to falcon tubes. Then after supernatant was taken for enzyme activity.

Moisture content:

For every 24 hours interval 1 gram of moistened endosperm were weighed and kept in the oven at 12^0C for 12 hours. Finally, moisture content was calculated by subtracting the final weight from the initial weight and the percentage of moisture was determined.

Biochemical Analysis:

Total amylase activity was measured by the method of Bernfeld (1955), wherein the reducing group liberated from starch was measured by reduction of 3, 5, dinitro salicylic acid [22]. Alpha

amylase activity was estimated in the same preparation after heating for 30 minutes at 70℃ for inactivating beta amylase. The value of beta amylase was calculated as a difference between total amylase and alpha amylase.

Quantification of Total Proteins:

Quantification of total proteins was done by performing Lowry's Method (1951) for enzyme extract and recording the Absorbance at 610nm with the help of colorimeter [23].

Purification of Amylase:

The protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) [24].

RESULTS AND DISCUSSION

The present paper reports the amylase activity in Buckwheat (*Fagopyrum esculentum*) seed during germination. Buckwheat seeds were taken for the study of amylase activity from 0 to 192 hours during germination as amylase was known to play a very important role in the germinating utilizing starch present in the seeds of many plants.

Seed germination

Buckwheat seeds were germinated on filter paper in petriplates. Samples were drawn for amylase assay and other studies at 24 hours intervals from 0 to 192 hours as shown in fig. 1. Radicle protrusion through the structures surrounding the embryo is the usual event that terminates germination and marks the beginning of seedling growth. This occurs as a result of cell extension, which may or may not be accompanied by cell division. Synthesis of DNA occurs in the radicle cells soon after the start of imbibitions to repair damage to the macromolecule sustained during desiccation and rehydration, as well as synthesis of mitochondrial DNA. A second period of DNA synthesis occurs after radicle protrusion, along with an increase in tubulin, a microtubule component associated with cell division, although in some seeds these events may slightly precede cell elongation [25]. Emergence of the radicle by cell extension is a turgor driven process. The radicle cell walls must become more stretchable, and thus wall loosening could result from the activity of certain proteins, expansions, which disrupt the

hydrogen bonds linking cell wall polymers such as matrix polysaccharides and cellulose microfibrils. These proteins are present in germinating seeds, but their relationship to radicle extension remains to be determined. In some seeds, the embryo is surrounded by an endosperm or perisperm or a megagametophyte, which is sufficiently rigid to prevent extension of the radicle and completion of germination. However, a causal link between germination and enzymic weakening the cell walls of the surrounding structures remains to be established in most seeds [26, 27].

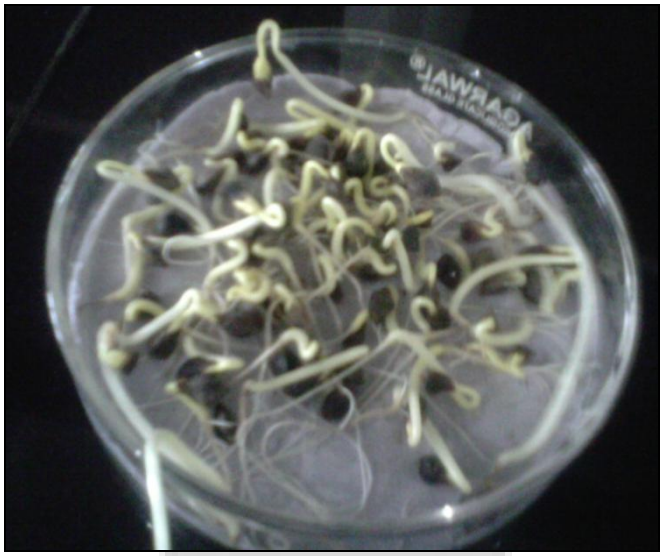

Fig. 1: Germination of Buckwheat seed

Moisture content

The moisture content of the seed plays a very important role in the germination of seed. Imbibed seeds rapidly resume metabolic activity. The essential cellular structures and enzymes for the commencement of metabolism are present within the dry seed, having survived the desiccation phase that completes seed maturation. One of the first activities to resume is respiration, which can be detected within minutes of the start of imbibitions. The glycolytic and oxidative pentose phosphate pathways recommence during Phase I, and Krebs' cycle enzymes become activated [28]. Dry seed tissues contain mitochondria and, although poorly differentiated following maturation drying, they contain sufficient Krebs' cycle enzymes and terminal oxidases to produce adequate amounts of ATP to support metabolism for several hours following imbibition [29]. When dry seeds take in water, a chain of metabolic events is initiated that results in the emergence of the radicle, thus completing germination. Thereafter, the major stored reserves

within the seed are mobilized, providing nutrients to support early seedling growth [30]. Uptake of water by a mature dry seed is triphasic. The initial influx (Phase I, imbibition) is a result of the very low water potential of the dry matrices of the seed (cell walls and storage components), which rapidly become hydrated, resulting in a plateau (Phase II). A further increase in water uptake occurs only after germination is completed, as the embryo grows into a seedling (Phase III). This kinetics of water uptake is influenced by the structure of the seed, in that water may not enter all parts equally, but may be directed preferentially towards the embryo or its radical [31]. The influx of water into the cells of dry seeds during Phase I cause temporary structural perturbations, particularly to membranes, that result in a rapid but temporary leakage of ions and low-molecular weight metabolites from the seed. In this study, the moisture content of buckwheat seeds was calculated when germination started. It was found that the moisture content in the seed at 0 hours was only 10% but it was increased to 25% after 24 hours by imbibitions of water by seed (Table-1). The moisture content of seed was found maximum at 192 hours i.e. 36%. The moisture content can be better understood using the table given below.

The moisture content was determined by the following formula:

$$\text{Moisture content} = \frac{\text{Fresh weight - dry weight X 100}}{\text{Fresh weight}}$$

Table 1: Moisture content in the germinating seeds of Buckwheat

Hours	Moisture content %
0	10%
24	25%
48	26%
72	27.5%
96	28.5%
120	30%
144	32%
168	33.5%
192	36%

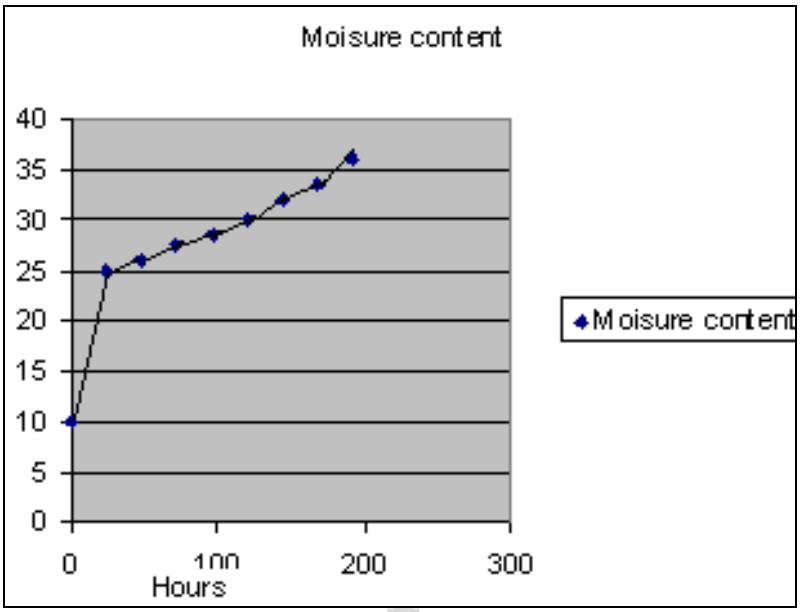

Fig. 2: Moisture content in Buckwheat seed

Amylase activity

The major mobilization of stored reserves in the storage organs commences after the completion of germination, and supports growth of the seedling until it becomes photosynthetically active (autotrophic). Seed storage organs contain substantial quantities of two or more of the major reserves, carbohydrates, oils and proteins, and the hydrolysis and utilization of these usually occurs concurrently. Starch is the most common reserve carbohydrate in seeds and an important commercial source is the endosperm of cereals. The amylose and amylopectin in the native starch grain are first hydrolysed by a-amylase, which randomly breaks the α (1,4) glycosidic links between the glucose (glucose) residues. Starch degradation is aided by b-amylase, which cleaves off successive disaccharide maltose units (Glucose-Glucose) from the nonreducing end of the large oligomers released by prior an amylolytic attack. These enzymes produce glucose and maltose from amylose and, in addition, highly branched short chains of glucose, called limit dextrins, from amylopectin. A debranching enzyme (limit dextrinase) releases the short chains by hydrolysing the -1, 6 branching points, which are further cleaved by the amylases. Maltose is converted to its constituent glucose units by maltase. This mode of starch breakdown is common in cereals, where the α -amylase, debranching enzyme and maltase are synthesized in, and released from, the embryo (scutellum) and surrounding aleurone layer into the nonliving storage cells of the starchy endosperm following germination; *ß*- amylase is already present in the latter

at grain maturity, and is activated when required. The resultant glucose is taken up into the growing embryo via the scutellum and is converted to sucrose for transport to, and utilization by, the growing regions of the developing seedling.

During the process of germination the total amylase activity & alpha and beta amylase activity was also determined separately. The sample was taken from the germinating seeds from petriplates firstly at 0 hours and then after every 24 hours interval till 192 hours. At 0 hour a low amylase activity was found. Amylase activity gradually increased from 24 hours to 96 hours and was seen maximum at 96 hours. After 96 hours the total amylase activity started decreasing and became very low at 192 hours. Such trend in the amylase activity was found because starch was the only source of energy for the germinating seeds. So, amylases start degrading starch present in seed embryo after 24 hours and this activity become maximum at 96 hours. But after 96 hours the seedling starts emerging out and the concentration of starch in the seed embryo starts decreasing so the amylases activity was degraded. Starch is the major storage component of buckwheat grains. It is accumulated in the endosperm as an energetic material necessary for the plant growth. In the whole grain of buckwheat, starch content varies from 59% to 70% of the dry mass, demonstrating fluctuations under variable climatic and cultivation conditions [32]. Amylose content of buckwheat starch granules fluctuates between 15% and 52% and its degree of polymerisation varies from 12 to 45 glucose. Buckwheat starch granules are spherical, oval and polygonal in shape with noticeable flat areas due to compact packing in the endosperm, the granule size distribution ranges from 2 to 6 μm [33].

In this study, the alpha amylase activity was also studied separately and it was observed that the alpha amylase was the main contributor in the total amylase activity in the Buckwheat seed. It was found that the alpha amylase activity initially started at 24 hours and become maximum at 96 hours and then show the decrease in activity as the days passes and then become very low at 192 hours. Same trend is found for the beta amylase activity but its activity was very low in the comparison to the alpha amylase activity. At 24 hours beta amylase activity increases and after 24 hours beta amylase activity decreases at 2^{nd} day and again increases at 3^{rd} day and same as 4^{th} day and then decreases till 192 hours. The total amylase, alpha and beta amylase activity were expressed as units per ml.

Table 2: Total amylase, Alpha amylase, and Beta amylase activity in units per 30 minutes

Hours	Total amylase units/ micromoles/30 minutes	Alpha amylase units/ micromoles/ 30 minutes	Beta amylase units/micromoles/ 30 minutes
0	2.42	1.50	0.90
24	2.90	1.60	1.30
48	3.82	2.72	1.10
72	4.26	2.86	1.40
96	4.80	3.40	1.40
120	4.42	3.22	1.20
144	3.74	2.44	1.30
168	3.05	1.95	1.10
192	2.72	1.92	0.80

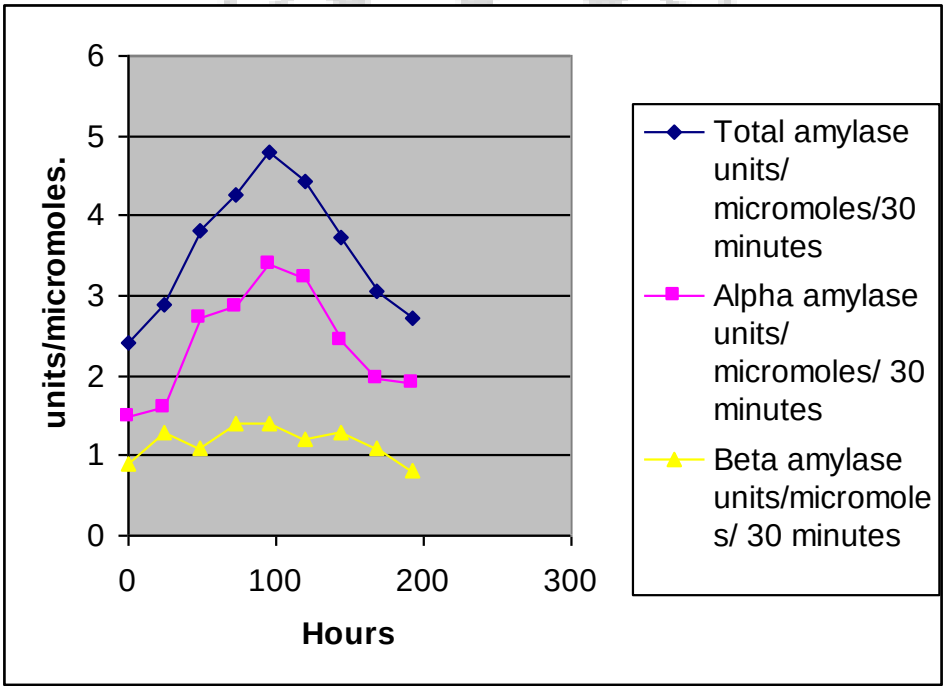

Fig. 3: Amylases activity (units/micromoles/30 minutes)

Table 3: Specific activity of Total, Alpha and Beta amylases in germinating Buckwheat seeds from 0 to 192 hours

Hours	Total amylase units/mg/30 minutes	Alpha amylase units/mg/30 minutes	Beta amylase units/mg/30 minutes
0	2.104	1.304	0.782
24	2.521	1.391	1.130
48	3.820	2.720	1.100
72	6.656	4.468	2.187
96	6.000	4.250	1.750
120	6.138	4.472	1.666
144	4.986	3.253	1.733
168	3.351	2.142	1.208
192	3.400	2.400	1.000

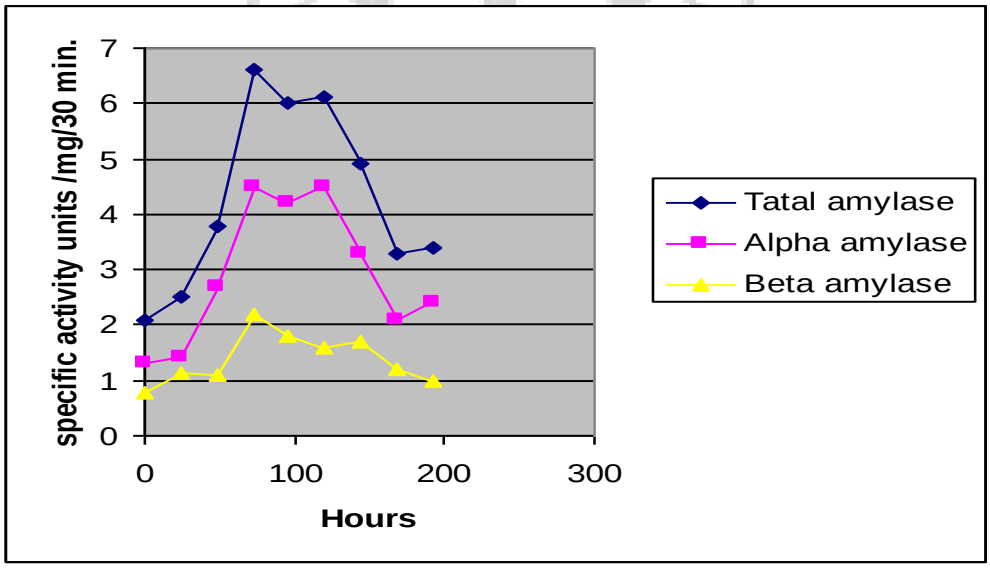

Fig 4: Specific activity of Total, Alpha and Beta amylases in germinating Buckwheat seeds from 0 to 192 hours

Isozymic Analysis: Amylases was also purified by the process of Polyacrylamide Gel Electrophoresis. The bands of amylase were observed in the fig. 5. The bands of Amylase were clear prominent and the bands of Amylase were found maximum at the stage of 72 hrs. A total of 7 alternative protein bands were seen .

Fig. 5. Native PAGE for Isozymic analysis of Amylase in Buckwheat. Lanes (7 bands) from left to right- 1.

CONCLUSION

The present study was conducted on the amylases in Buckwheat (*Fagopyrum esculentum*) seeds during germination biochemical studies on amylases was conducted. This study emphasizes buckwheat seed germination and the embryo was taken for the preparation of crude extract. The crude extract was then centrifuged and the supernatant was used for enzyme assays through DNS method and using Lowry method for protein estimation.

The moisture content of the seeds was also calculated and it was found low at 0 hours i.e.10% and maximum at 92 hours i.e. 36%. The total amylase activity in germinating seeds was also calculated. It was observed that amylase activity was low at 0 hours and starts increasing and become maximum at 96 hours and starts decreasing again and become low at 192 hours. Alpha and Beta amylase activity was also calculated separately.

Specific activity of total amylase, alpha amylase and beta amylase was also calculated. The specific activity of total amylase, alpha amylase and beta amylase was found highest at 72 hours, 120 hours and 72 hours. Amylase was also purified by the process of Polyacrylamide Gel

Electrophoresis. The bands of amylase were observed in the fig. 5. The bands of Amylase were clear prominent and the bands of Amylase were found maximum at the stage of 72 hrs.

For the past decades, there has been an increasing interest in the investigation of different extract obtained from plants for nutritional and therapeutic purposes. Buckwheat *(Fagopyrum esculentum)* is a rich source of protein, minerals, lipids, β-glucan, avenanthramides, indole alkaloid, flavonoids, triterpenoid, saponins, lipids and sterols. It exerted many pharmacological effects including antioxidant, anti-inflammatory, dermatological, immunomodulatory, antidiabetic, gastrointestinal, hypolipidemic, neurological, cardiovascular and many other biological activities. The treatment with buckwheat increased insulin activity and improved sensitivity for normalizing blood glucose level and reduce glucose production by the liver [32]. The glycaemic and insulinaemic response to buckwheat were tested in a small-scale clinical study [34].

On the other hand the amylases present in the seed of buckwheat play a very important role in the germination of seed as these help in utilization of starch present in the endosperm of buckwheat seed [26]. Enzymes being the most important products obtained for human needs have stimulated renewed interest in the exploration of industrially relevant enzymes from several natural sources including plants, animals and microorganism. Amylases are important enzymes employed in the starch processing industries for the hydrolysis of starch into simple sugars by the breakdown of 1, 4-D glucosidic linkages between adjacent glucose units there by hydrolyzing single glucose units from the non-reducing ends of amylase and amylopectin in a stepwise manner. This enzyme is extensively used in starch liquefaction, paper industries, food, pharmaceutical and sugar industries [27, 28]. Germinated buckwheat is an important raw material for food and functional food production, had better nutritional value than ungerminated buckwheat. As a result, the germinated seeds or sprouts are nutritionally superior to their original seeds with higher levels of nutrients, lower amounts of compounds that interfere with the absorption of nutrients and increased protein and starch digestibility. In their study, the researchers found that the improvement of flavonoids led to significant enhancement of the antioxidant activities of germinated buckwheat. Germinated buckwheat had better nutritional value and antioxidant activities than ungerminated buckwheat and it represented an excellent natural source of flavonoids and phenolic compounds, especially rutin and C-glycosyl flavones

[21]. It is important to promote ancient nutritive food crop, buckwheat for various health reasons. The germinated seeds could help in the prevention and treatment of various human diseases, but could also be helpful in improving the development of active components for functional food products and pharmaceuticals.

ACKNOWLEDGEMENT

Facilities provided by Department of Biochemistry, College of Applied Education and Health sciences, Meerut, India are highly acknowledged. The authors are also thankful to faculty members of Biochemistry department for their consistent support, cooperation and creating a good research environment for the completion of this research.

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