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Characterization of Amylase Inhibitor from the Seeds of *Mucuna utilis*

Rajiv Bharadwaj P[1], K S Chandrashekharaiah[2*]
[1](Department of Biochemistry, Mangalore University, Chikka Aluvara, Kodagu-571232, Karnataka, India)
[2](Department of Biochemistry, Mangalore University, Chikka Aluvara, Kodagu-571232, Karnataka, India)
**Corresponding author: K S Chandrashekharaiah*

Abstract: *Proteinaceous amylase inhibitors are present in plants which regulate the activity of amylases. An α-Amylase inhibitor was isolated and purified by conventional protein purification techniques (ammonium sulphate fractionation, Sephadex G-10, sephadex G-50 chromatography and HPLC) from Mucuna utilis seeds. Its molecular weight as determined by gel-permeation chromatography on Sephadex G-100 was found to be 15 kDa. The purified inhibitor was temperature stable and retained more than 75% activity at 65 °C. Inhibitor was found to have pH optima of 6.9. 100% Zone of inhibition was observed when the inhibitor was added on the plated organisms. The Mucuna utilis amylase inhibitor was found to inhibit the activity of human salivary α-amylase. Inhibitory activity of α-amylase inhibitor against mammalian amylases could suggest its potential in treatment of diabetes and cure of nutritional problems, which result in obesity.*

Keywords: *Amylase inhibitors, isolation, characterization, Mucuna utilis seeds*

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I. INTRODUCTION

Plant seeds are known to produce a large number of varieties of proteinaceous enzyme inhibitors which are important tools of nature for regulating the activity of enzymes. α-Amylases (α-1, 4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) catalyze the initial hydrolysis of starch into shorter oligosaccharides, which is an important step towards converting polysaccharides into single units that can be assimilated by the organism. Proteinaceous inhibitors of α-amylases are widespread in plants, notably in cereals, wheat, barley and legume beans. Research into α-amylase inhibitors has relevance to several fields in the area of human health including the diagnosis of disorder associated with amylases function as well as the carbohydrate metabolism associated disorders such as control of diabetes, obesity and hyperlipidaemia[(1,2,3)]. α-Amylase inhibitors are also of great interest as possible tools to control insect pests [(4,5,6)]. Comparatively less is known about the inhibitors of these important carbohydrate digesting enzymes and little is known on the structure and properties of legume α-amylase inhibitors. More interest is now being given to explore the properties of these legume proteins because of their wide occurrence in both human and cattle diets.

The genus *Mucuna* is a tropical underutilized legume belongs to the family fabaceae (leguminoceae) which includes many species of annual and perennial legumes. The plant is infamous for its extreme itchiness produced on contact, particularly with the young foliage and the seedpods. The plant is an annual, climbing shrub with long vines and roots, leaves and seeds of the plant are commonly used in the treatment of various medical conditions including impotence, snake bite etc., and *Mucuna* is extensively used as cover and to control weeds and pests in agriculture. Many varieties and accession of wild legume, *Mucuna*, are in great demand in food and pharmaceutical industries. Traditional use of *Mucuna* as food crop by farmers at field level gain popularity due to good yield [(7)]. The immature pods and leaves serve as vegetables, while seeds are condiment and main dish by ethnic group in Nigeria [(8)]. In view of the importance of amylase inhibitors and seeds of *Mucuna* being a potential source of amylase inhibitors, the present study was undertaken. In the present study, purification and partial characterization of amylase inhibitor was described.

II. MATERIALS AND METHODS

2.1. Materials: The seeds of *Mucuna utilis* were collected from Sheege, Arasikere (TQ), Hassan (DT), Karnataka. α-Amylase, Acrylamide, N, N, Methylene bisacrylamide, Bovine serum albumin, Sephadex G-10 and G-50 were obtained from Sigma chemical company, USA. All other chemicals used were of technical grade.

2.2. Methods:

2.2.1. Preparation of acetone powder: The acetone powder (10 %) of soaked seeds of *Mucuna utilis* was prepared according to the method of Wetter [(9)]. Seeds were blended in a homogenizer with chilled

acetone and filtered. The cake obtained was dried at 37° C, powdered and stored at 4°C until further use.

2.2.2. Preparation of crude extract: A 10 % extracts of acetone powder was prepared as described by Chandrashekharaiah et al [10] using sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 1.5 hr at 4° C. The extract was then centrifuged at 10,000 rpm for 15 min at 4° C. The supernatant was collected and used for qualitative and quantitative analysis of proteins/peptides for amylase inhibitory activity.

2.2.3. Ammonium sulphate fractionation: Ammonium sulphate precipitation was performed as described by Chandrashekharaiah et al [10] . The crude extract was subjected to ammonium sulphate precipitation. Solid powdered ammonium sulphate was added slowly with constant stirring over magnetic stirrer for 30 min at 4°C to obtain 0 - 90% saturation. After the addition of ammonium sulphate salt, the stirring over magnetic stirrer at 4°Cwas continued for 01 hour. The solution was allowed to stand for 1hr at 4°C. The precipitated protein was recovered by centrifugation at 10,000 rpm for 30 min. The protein pellet was dissolved in small amount 0.025 M sodium phosphate buffer, pH. 7.0.

2.2.4. Sephadex G-10 and G-50 gel-filtration chromatography: Gel-filtration chromatography was performed as described by Chandrashekharaiah et al [10] . Sephadex G-10 and G-50 were allowed to swell in excess of distilled water, decanted and then equilibrated with 0.05 M sodium phosphate buffer, pH 7.0 separately. The Sephadex G-10 gel was packed into a column of size 1.0 cm X 110.0 cm under gravity. The column was equilibrated with two bed volumes of 0.05M sodium phosphate buffer, pH 7.0 at a flow rate of 10 ml/hr. The concentrated Ammonium sulphate fractionation was loaded on to the gel and the proteins were eluted with 0.05M sodium phosphate buffer, pH 7.0 and 2.0 ml fractions were collected. Quantitative analysis of proteins, amylase and protease inhibitor activity was done. The fractions containing amylase inhibitor activity were pooled and subjected to sephadex G-50 chromatography which was performed as described for Sephadex G-10 gel-filtration chromatography. Quantitative analysis of proteins, amylase inhibitor activity was done for all the fractions obtained. The peak fractions containing amylase inhibitor activity were subjected to purification by HPLC.

2.2.5. RP-HPLC: RP-HPLC is carried out on Reversed-phase octadecylsilica (C18) column using binary solvent system with binary gradient capability and a UV detector. Buffer A is 0.1% (v/v) TFA in water and Buffer B is 100% acetoinitrile containing 0.1% (v/v) TFA. Column Equilibration and Blank Run was carried out using Buffer A with a flow rate of 1 mL/min at 220 and 280 nm respectively. Once the stable line is obtained, the sephadex G-50 precipitated sample was injected and eluted the sample with a linear gradient from 0 to 100% buffer B for 30 min.

2.2.6. Protein estimation: Protein was estimated from the crude extract and fraction of sephadex G-10 and G-50 according to the method of Lowry et al [11] .

2.2.7. Amylase and amylase inhibitor activity: Quantitatively Amylase activity was determined by measuring liberated maltose using method of Bernfeld [12]. The amylase activity was defined as liberation of 1 µmole of maltose formed per min of maltose at pH 7.0 at 37°C. Amylase inhibitory activity was determined by measuring reduction in maltose liberated by salivary amylase using (DNS) dinitrosalisylic acid [13]. A typical Amylase assay mixture consists of 0.1 ml containing 100µg of amylase enzyme with 0.9 ml of phosphate buffer (pH 7.0) incubated for 10minutes at room temperature. For Amylase inhibitor assay 0.1 ml containing 100µg of amylase enzyme with 0.4 ml of phosphate buffer (pH 7.0) was incubated with 0.5ml of extracts/purified samples of *Mucuna utilis* for 10minutes at room temperature. The reaction was initiated by the addition of 1.0 mL of 1% soluble starch at 37°C. The reaction was stopped after 10 minutes by the addition of DNS (1.0 mL). Absorbance of the mixture was measured at 540 using UV-Visible spectrophotometer. The amylase inhibitory unit was defined as the number of amylase units inhibited under the assay conditions.

2.2.8. Polyacrylamide gel electrophoresis: An anionic disc gel electrophoresis was carried out essentially according to the method of Davis and Ornstein [14]. A discontinuous gel system consisting of 8% separating gel and 4% spacer gel was used. The electrophoresis was carried out in cold applying a current of 20 – 25 mA for 4 hours using tris – glycine (pH 8.3) as electrode buffer and bromophenol blue as marker dye. After the electrophoresis, the proteins were stained with coomassie brilliant blue R – 250 for 1 hour and destained using 7 % acetic acid.

2.2.9. Antibacterial activity by well diffusion method: Antibacterial activities of the processed samples were determined using agar well diffusion assay method with *E.coli, Pseudomonas aerogenosa, streptococcus sps* and Klebsella. A hundred ml nutrient broth culture of each bacterial organism was used to prepare bacterial lawns. Agar wells of 5mm diameter were prepared with the help of a sterilized cork borer. Five wells were prepared in the agar plates. The wells were labeled and were

loaded with 50µl of fraction, sterile water, and sample containing standard tetracycline. The plates were incubated at 37^0 c for 24 hours. The plates were examined for evidence of zones of inhibition.

III. RESULTS AND DISCUSSION

3.1. Purification: Crude extract of the soaked seeds of *Mucuna utilis* was subjected to ammonium sulphate fractionation (0 – 90%). The precipitated protein obtained was recovered by centrifugation at 10000 rpm at 4^0c. The precipitated protein was dissolved in small volume of 0.025M sodium phosphate buffer, pH 7.0 applied on to a Sephadex G-10 chromatography and eluted using same buffer. The fractions were analyzed both for proteins and amylase inhibitor activity. Four protein peaks were obtained (Fraction-I, II, III and IV) (Fig.1). The protein peak containing amylase inhibitor activity (Fraction-I) was pooled and concentrated using ammonium sulphate. The concentrated amylase inhibitor fractions were applied on to a Sephadex G-50 chromatography (Fig.2). Three protein peaks (Fraction-I, II and III) were obtained and the peak (Fraction-II) containing amylase inhibitor activity was further subjected to purification by HPLC.

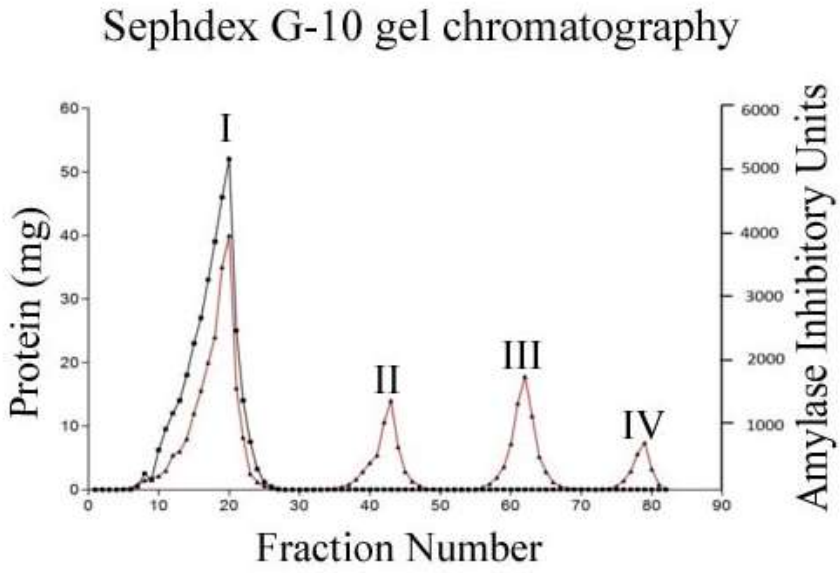

Fig.1. Elution of amylase inhibitor from soaked seeds of *Mucuna utilis* on Sephadex G-10.

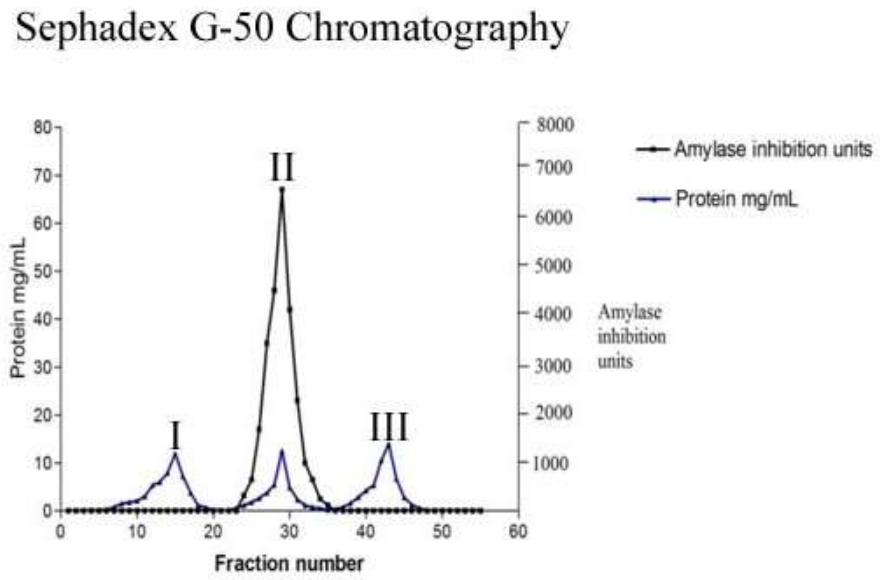

Fig.2. Elution of amylase inhibitor from soaked seeds of *Mucuna utilis* on Sephadex G-50.

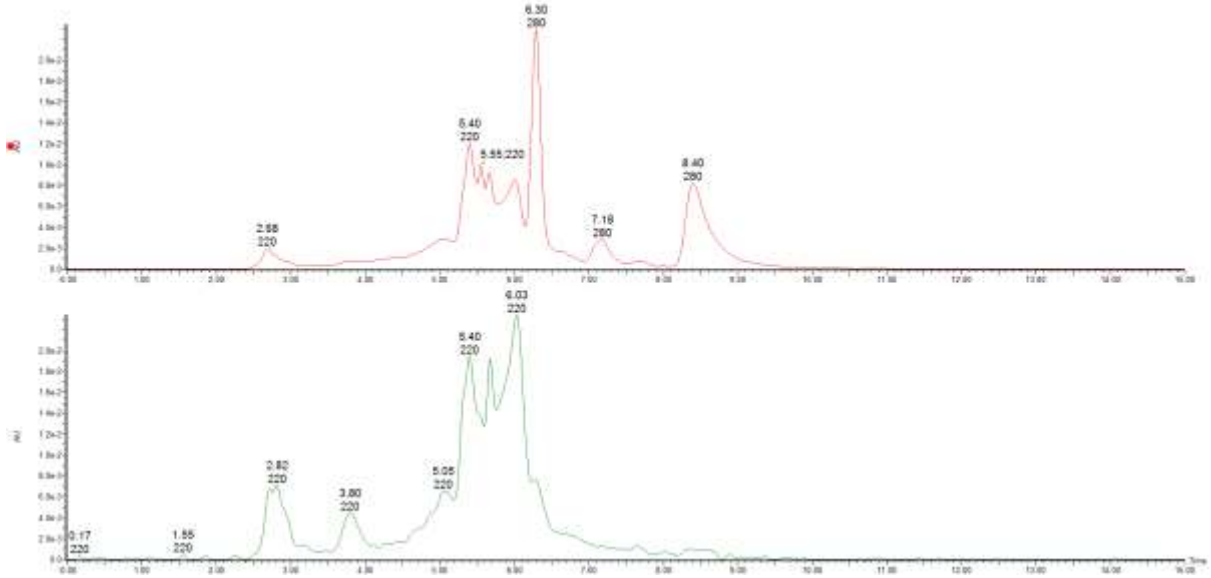

Fig.3. Elution profile of Sephadex G-50 amylase inhibitor fraction on RP- HPLC.

The *Mucuna utilis* amylase inhibitor was purified to 36.68 fold with a recovery of 52.61% and purified inhibitor showed a specific inhibitor activity of 61.18. An α-amylase inhibitor was purified employing conventional protein purification techniques such as ammonium sulphate precipitation, ethanol fractionation, chromatographic separation on Sephadex and reversed phase-high profile liquid chromatography [15, 16, 17, 18].

3.2. Molecular weight: The molecular weight of the purified amylase inhibitor from the seeds of *Mucuna utilis* as determined by gel-permeation chromatography on Sephadex G-100 was found to be 15 kDa. Alpha amylase inhibitor composed of three subunits with a molecular weight of 15,488, 18,620 and 26,302 daltons has been reported from bean cultivars [18]. A heat labile alpha amylase inhibitor was isolated from white kidney beans composed of three subunits α, β, and γ with molecular weights of 7800, 14000 and 22000, respectively [19]. A similar heat labile amylase inhibitor composed of three different subunits was reported from white kidney bean [20]. A glycoprotein alpha amylase inhibitor was purified from *Phaseolus vulgaris* and its molecular weight was found to be 45000 having subunit molecular weights of 14000 and 30,000 daltons. No trypsin inhibitor activity was found in the purified amylase inhibitor during present studies. When tested for the presence of carbohydrates by Molisch and Anthrone's tests, negative results were obtained. However the amylase inhibitor from Phaseolus bean cultivars was reported to be a glycoprotein [21, 22]. In present studies, the inhibition was found to increase up to 85% with the increase in concentration of purified α-amylase inhibitor in the assay, however, at higher concentrations the degree of inhibition was constant. There was a linear increase in inhibition up to 70–75% with the increase in concentration of purified wheat α-amylase inhibitor [23]. Kutty and Pattabiraman[24] found that inhibition was linear up to 80% with increasing levels of sorghum inhibitor concentration and this inhibitory pattern deviated from linearity at higher concentrations.

In the present studies the maximum activity for the purified α-amylase inhibitor from *Mucuna utilis* was observed at 25 °C– 45 °C. Similar results were observed in case of bean seeds [25] and for rye α-amylase inhibitor [26]. The purified amylase inhibitor from *Mucuna utilis* was inhibited the human salivary amylase. The α-amylase inhibitor purified from *P. vulgaris* (KR-9) was found to be effective on human salivary α-amylase. Similarly, purified inhibitor from *P.vulgaris* and wheat, *A. aspera* and rye [26, 27, 28, 29] was found to be effective against human salivary amylase. However, protienaceous alpha amylase inhibitors from chick pea, kidney bean, maize, and millet seeds did not inhibit human saliva α-amylase activity [30].

IV. CONCLUSION

The α-amylase inhibitor was purified from the seeds of under-utilized legume, *Mucuna utilis.* The purified inhibitor existed as a monomer with molecular weight of 15 kDa. Purified amylase inhibitor was found to inhibit the activity of human salivary α-amylase. Inhibitory activity of α-amylase inhibitor against mammalian amylases could suggest its potential in treatment of diabetes and cure of nutritional problems, which result in obesity.

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REFERENCES

[1]. M D O' Donnell, O. FitzGerald and K. F. McGeeney, Differential serum amylase determination by use of an inhibitor and design of a routine procedure. Clinical Chemistry, 23, 1977, 560-566.

[2]. E Gilles Turcotte, Linda Nadeau, Jean-Claude Forest, Pierre Douville, Pierre Leclerc, Jean Bergeron, Bernard Fruteau de Laclos, A new rapid immunoinhibition pancreatic amylase assay: Diagnostic value for pancreatitis. Clinical Biochemistry, 27(2), 1994, 133 – 139.

[3]. H Bischoff, H.J. Ahr, D. Schmidt and J. Stoltefuss, Acarbose—ein neues Wirkprinzip in der Diabetestherapie Nachrichten aus Chemie, Technik und Laboratorium, 42, 1994, 1109–112.

[4]. M J Chrispeels, M.F. Grossi-de-Sa, T.J.V. Higgins, Genetic engineering with α-amylase inhibitors seeds resistant to bruchids. Seed Science Research, 8, 1998, 257-263.

[5]. A M R Gatehouse, Gatehouse, J.A. Identifying proteins with insecticidal activity: Use of encoding genes to produce insect-resistant transgenic crops. Pesticide Science, 52, 1998, 165-175.

[6]. A Valencia, A.E. Bustillo, G.E. Ossa, M.J. Chrispeels, Alpha-amylases of the coffee berry borer (Hypothenemus hampei) and their inhibition by two plant amylase inhibitors. Insect Biochemistry and Molecular Biolo gy, 30, 2000., 207-213.

[7]. R Gilbert, *Mucuna pruriens* in Malawi: A promising legume with a troubled history. In: Food and Feed from *Mucuna*: Current Uses and the Way Forward (Editors, Flores B M, Eilittä M, Myhrman R, Carew L B and Carsky R J), Workshop, CIDICCO, CIEPCA and World

[8]. Hunger Research Center, Tegucigalpa, Honduras (April 26-29, 2000), 2002; 48-59.

[9]. K O Adebowale, and O. S. Lawal, Functional properties and retrogradation behavior of native and chemically modified starch of *Mucuna* bean (*Mucuna pruriens*). Journal of Science of Food and Agriculture, 83, 2003b, 1541-1546.

[10]. L R Wetter, Some properties of lipase present in germinating rapeseeds. Journal of American Oil Chemists Society, 34, 195, 66–69.

[11]. K S Chandrashekharaiah, N. Ramachandra swamy K.R. Siddalinga Murthy, Carboxylesterases from the seeds of an underutilized legume,

[12]. *Mucuna pruriens*: Isolation, purification and characterization. Phytochemistry, 72(18), 2011, 2267-2274.

[13]. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193 (1), 1951, 265–75.

[14]. P Bernfeld, Alpha Amylases, α and β, Methods in Enzymology, 1(1), 1955, 149–158.

[15]. K. Fossum, J.R. Whitaker, Simple method for detecting amylase inhibitors in biological materials, Journal of Nutrition, 104, 1974, 930- 936.

[16]. B J Davis, L. Ornstein, Disc Electrophoresis–2, Method and Application to Human Serum Protiens. Annals of the New York Academy of Sciences, 121 (2), 1964, 404-427.

[17]. M F Ho, J.F. Whitaker, Purification and partial characterization of white kidney bean α-amylase inhibitor from two experimental cultivars. Journal Food Biochem.istry, 17, 1993, 15–33.

[18]. E Kokiladevi, A. Manickam, B.Thayumanavan, Characterization of alpha-amylase inhibitor in Vigna sublobata., Botanical Bulletin- Academia Sinica Taipei, 46, 2005, 189–196.

[19]. V K Hivrale, N.P. Chougule, A.P. Giri, P.J. Chhabdaa, M.S. Kacholea, Biochemical characterisation of α- amylase inhibitors from Achyranthes aspera and their interactions with digestive amylases of coleopteran and lepidopteran insects. Journal of Science of Food and Agriculture, 91, 2011, 1773–1780.

[20]. Mridu Gupta, Pratima Sharma, and Amarjit K. Nath, Purification of a novel α-amylase inhibitor from local Himalayan bean (Phaseolus vulgaris) seeds with activity towards bruchid pests and human salivary amylase., Journal of Food Science and Technology, 51(7), 2014, 1286–1293.

[21]. H Yamaguchi, Isolation and characterization of the subunits of a heat labile a-amylase inhibitor from Phaseolus vulgaris white kidney bean. Bioscience Biotechnology Biochemistry, 57, 1993, 297-302.

[22]. S Wato, K.. Kamei, T. Arakawa, J. Philo, J. Wen, S.Hara, H. Yamaguchi, A chimera-like a- amylase inhibitor sug- gesting the evolution of Phaseolus vulgaris a-amylase inhibitor, Journal of Biochemistry, 128, 2000, 139- 144.

[23]. S Sawada, Y. Takeda, M. Kanamori, M.Tashiro, Purification and characterization of alpha-amylase inhibitors from the seeds of three cultivars of the genus Phaseolus, Journal of Japanese Food Science and Tech.nology, 48, 200, 182–188.

[24]. M Y Yang, X.Q. Zhang, Y. Ma, J. Shen, J.R. Song, H.L. Zhu, . Purification and partial characterization of a glycoprotein alpha-amylase from white kidney bean, Journal of Food Biochemistry, 32, 2008, 72–84.

[25]. A V M Kutty, T.N. Pattabiraman, Isolation and characterization of an amylase inhibitor from sorghum seeds specific for human enzymes, Journal of Agricultural Food Chemistry, 34, 1986, 552–557.

[26]. J M Frels, J.H. Rupnow, Characterization of two α-amylase inhibitors from black bean (Phaseolus vulgaris). Journal of Food Science, 50, 1985, 72–78.
[27]. J R Power, J.R. Whitaker, Purification and some physical and chemical properties of red kidney bean (Phaseolus vulgaris) α-amylase inhibitor, Journal of Food Biochemistry, 1, 1977, 217–238.
[28]. Granum PE. Purification and characterization of α-amylase inhibitor from rye flour. Journal of Food Biochemistry, 2, 1978, 103–107.
[29]. H Yoshikawa, M. Kotaru, C. Tanaka, T. Ikeuchi, M. Kwabata, Characterization of kintoki bean (Phaseolus vulgaris) α-amylase
[30]. inhibitor: inhibitory activities against human salivary and porcine pancreatic α-amylases and activity changes by proteolytic digestion, Journal of Nutritional Science and Vitaminology, 45, 1999, 797–802.
[31]. R Heidari, S. Zareae, M. Heidarizadeh, Extraction, purification and inhibitory effect of α-amylase inhibitor from wheat (Triticum aestivum var. Zarrin). Pakistan Journal of Nutrition, 4, 2005, 101–105.
[32]. J Iulek, O.L. Franco, M. Silva, C.T. Slivinski, J.C. Bloch, D.J. Rigden, M.F. Grossi-desa, Purification, biochemical characterization and
[33]. partial primary structure of a new α-amylase inhibitor from Secale cereale (rye), International Journal of Biochemistry and Cell Biology, 32, 2000, 1195–1204.
[34]. N Khan, In vitro effects of protienaceous alpha amylase inhibitors on red flour Bettle, Tribolium castaneum, Science Research Reports, 1, 2011, 101–104.

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