African Journal of Biochemistry Research Vol. 4(9), pp. 220-224, September 2010
Available online at http://www.academicjournals.org/AJBR
ISSN 1996-0778 ©2010 Academic Journals

Full Length Research Paper

Alpha-amylase inhibitor of amadumbe (*Colocasia esculenta*): Isolation, purification and selectivity toward α- amylases from various sources

R. McEwan[1]*, R. P. Madivha[1], T. Djarova[1], O. A. Oyedeji[2] and A. R. Opoku[1]

[1]Department of Biochemistry and Microbiology, University of Zululand, P/B X1001, KwaDlangezwa 3886, South Africa.
[2]Department of Chemistry, University of Zululand, P/B X1001 KwaDlangezwa 3886, South Africa.

Accepted 25 June, 2010

Two proteins (A-1 and B-2) with α-amylase inhibitor activity were extracted and partially purified from *Colocasia esculenta* tubers through 80% ammonium sulphate fractionation, ion-exchange chromatography on DEAE-Sephacel and gel-chromatography on Sephadex G-100. The molecular weight of A-1 and B-2 were estimated to be about 17000 and 19000 daltons, respectively. The inhibitors inactivated α-amylases of animal origin, but had no effect on fungal amylase. Inhibitor A-1 also exhibited activity towards plant amylases, while inhibitor B-2 has no activity on plant amylases. Inhibitor A-1 was the most active against human salivary amylase at pH 6. Inhibitor A-1 was completely destroyed at temperatures above 50°C; while inhibitor B-2 was stable up to 70°C.

Key words: α-amylase inhibitor, amadumbe, *Colocasia esculenta*, diabetes, obesity.

INTRODUCTION

Colocasia esculenta is a tuber commonly known by several common names, such as Amadumbe (South Africa), Taro (Pacific islands) or Cocoyam (West Africa). It is a traditional food crop widely grown in the subtropical parts of South Africa. The tuber could be consumed as a good source of starch and the locally developed cultivars are commonly used as staple food in the KwaZulu-Natal (KZN) province of South Africa. Nutritional evaluation of the tubers showed storage of biologically active substances that have anti-nutritional properties (that is, they reduce the availability of nutrients to animals and humans). Amongst these substances, α-amylase inhibitors were present (McEwan, 2008).

Protein inhibitors of α-amylase occur widely in plants. The inhibitors are believed to make plants less palatable, even lethal to insects, thus contributing some selective advantage to the plants (Sasikiran et al., 2002). Amylase inhibitors are known as starch blockers because they prevent dietary starches from being digested and absorbed by the body. This could be useful for treating obesity and diabetes mellitus – a metabolic disorder characterized by chronic hyperglycaemia resulting from defects in insulin secretion (Ali et al., 2006). Plants play an important role in the treatment of diabetes, particularly in developing countries where most people have limited resources and do not have access to modern treatment. Because of the possible importance of these inhibitors in plant physiology and animal and human nutrition, extensive research has been conducted on their properties and biological effects (Garcia-Olmedo et al., 1987; Silano, 1987). In this study, we report the isolation and characterization of this specific inhibitor in *C. esculenta*.

*Corresponding author. E-mail: ronalda.mcewan@nmmu.ac.za Tel: +27 44 801 5018, +27 083 267 1317. Fax: +27 44 805 6622.

MATERIALS AND METHODS

Amadumbe (*C. esculenta*) tubers were obtained from the local market at Esikhawini, KZN, South Africa. The amylases (human salivary, type IX-A; porcine pancreatic, type I-A; sweet potato, barley, *Bacillus* species, type II-A; *Aspergillus oryzae*) and all other reagents used were obtained from Sigma Chemical Co., USA.

Protein extraction

Tubers with no physical signs of infection were washed, peeled and

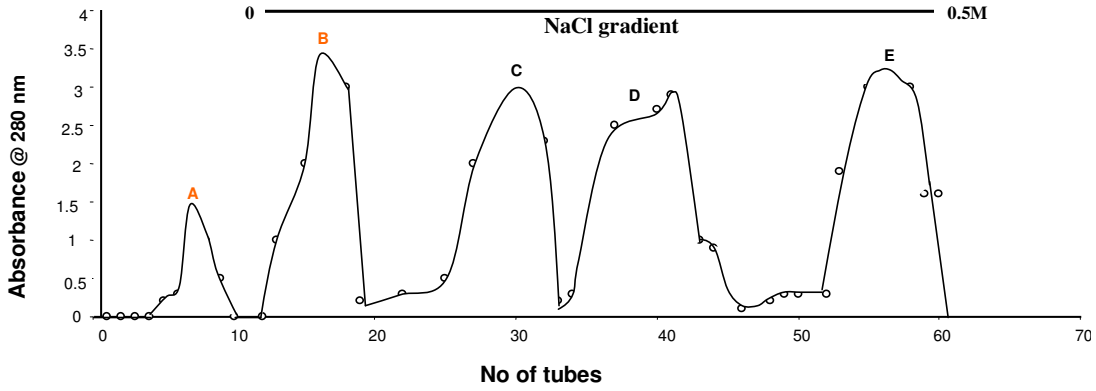

Figure 1. Ion exchange chromatography.

cut into small (2 × 3 cm) pieces and dried at 40°C for 24 h. The dried material was milled (68 mesh) and the flour were defatted with hexane and air-dried. The defatted flour were added (1:5 w/v) to distilled water (containing 1% polyvinylpolypyrrolidone), stirred for 2 h, and filtered. The residue was re-extracted and the combined filtrate was centrifuged at 12 000 × *g* for 20 min. The supernatant (designated as the crude extract) was subjected to 80% $(NH_4)_2SO_4$ saturation and left overnight at 4°C. Protein pellets obtained after centrifugation (12 000 × *g,* 20 min) were re -dissolved in minimum volume of phosphate buffer (0.02 M, pH 6.9, containing 0.3 M NaCl), dialyzed extensively (48 h, with 6 h change of buffer) against the buffer (designated the ammonium sulfate extract) and analyzed for amylase inhibitor (AI) activity.

Ion-exchange chromatography

The ammonium sulfate extract was further purified through ion-exchange chromatography (6 × 1.1 cm DEAE-Sephacel equilibrated with 0.02 M phosphate buffer, the column was eluted with a linear NaCl gradient of 0.2 - 0.5 M at the flow rate of 20 ml/h; 5 ml fractions were collected). The absorbance of the effluent was monitored at 280 nm. Individual peaks were pooled and analyzed for protein and AI activity.

Two peaks (A and B, Figure 1) with AI activity were then separately chromatographed on a Sephadex G-100 column (35 × 1.1 cm, equilibrated with the phosphate buffer and eluted with same buffer at a flow rate of 15 ml/h; 5 ml fractions were collected and the absorbance at 280 nm was determined). Pooled fractions were analyzed for protein and AI activity. Fractions (A-1 and B-2, Figure 2) with AI activities were collected, dialyzed extensively (48 h, with 6 h change of 0.02M phosphate buffer), freeze-dried and dissolved in deionized water.

Enzyme inhibitor assay

Amylase and amylase inhibitor activity assays were based on Bernfeld's method for amylase assay (Bernfeld, 1955). Amylase inhibitor extracts were mixed with amylase and incubated for 30 min at 37°C. The reaction was started by adding extract-enzyme mixture to test tubes containing buffered starch solution (2 mg starch in 20 mM phosphate buffer of pH 6.9 containing 0.4 mM NaCl) and was incubated for 20 min. This reaction was terminated by adding 3,5-dinitrosalicyclic acid (DNS) reagent to the assay mixture. The assay tubes were kept in a boiling water bath for 5 min, cooled under tap water and the colour formed by maltose oxidation was measured at 530 nm. Controls without inhibitor were run simultaneously. One amylase unit is defined as the amount of enzyme that will liberate 1 µmol of maltose from starch under the assay conditions (pH 6.9, 37°C, 5 min). Inhibitory activity is expressed as the percentage of inhibited enzyme activity out of the total enzyme activity used in the assay.

Gel chromatography on Sephadex G-100

Molecular weight was determined by gel chromatography on Sephadex G-100, under the conditions described by Sigma. Cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa) were used as molecular weight markers. Proteins were determined with the Sigma kit.

pH and temperature stability of inhibitors

The inhibitors (70 µg) were separately mixed with 20 µmol of phosphate buffer (pH 6) and incubated at the various temperatures (20 - 100°C) for 30 min. Aliquots were withdrawn and assayed for residual inhibitory activity against human salivary amylase as already described.

Inhibitor A1 (70 µg) was incubated with 20 µmol of each of HCl (pH 1, 2), citrate buffer (pH 3, 4), acetate buffer (pH 5), phosphate buffer (pH 6, 7, 8), glycine-NaOH buffer (pH 9, 10) in a volume of 0.2 ml at 25°C for 30 min. Aliquots were assayed for inhibitory activity against human salivary amylase as described. pH stability of inhibitor B2 was not determined.

RESULTS

Two proteins (A and B-1) with α-amylase inhibitory activities were purified from Amadumbe tubers by ion-exchange chromatography on a DEAE-Sephacel column. The elution pattern of the ion-exchange chromatography is shown in Figure 1. Only two (A and B) of the five protein peaks showed α-amylase inhibitory activity.

The inhibitor peaks (Figure 1) from the DEAE-Sephacel chromatography were subsequently separated by gel filtration. Two (A1 and B2) of these proteins had inhibitory activity against α-amylase (Figure 2).

AI-A and AI-B were purified (3.89- and 4.42-fold, respectively from the crude extract with a yield of approximately

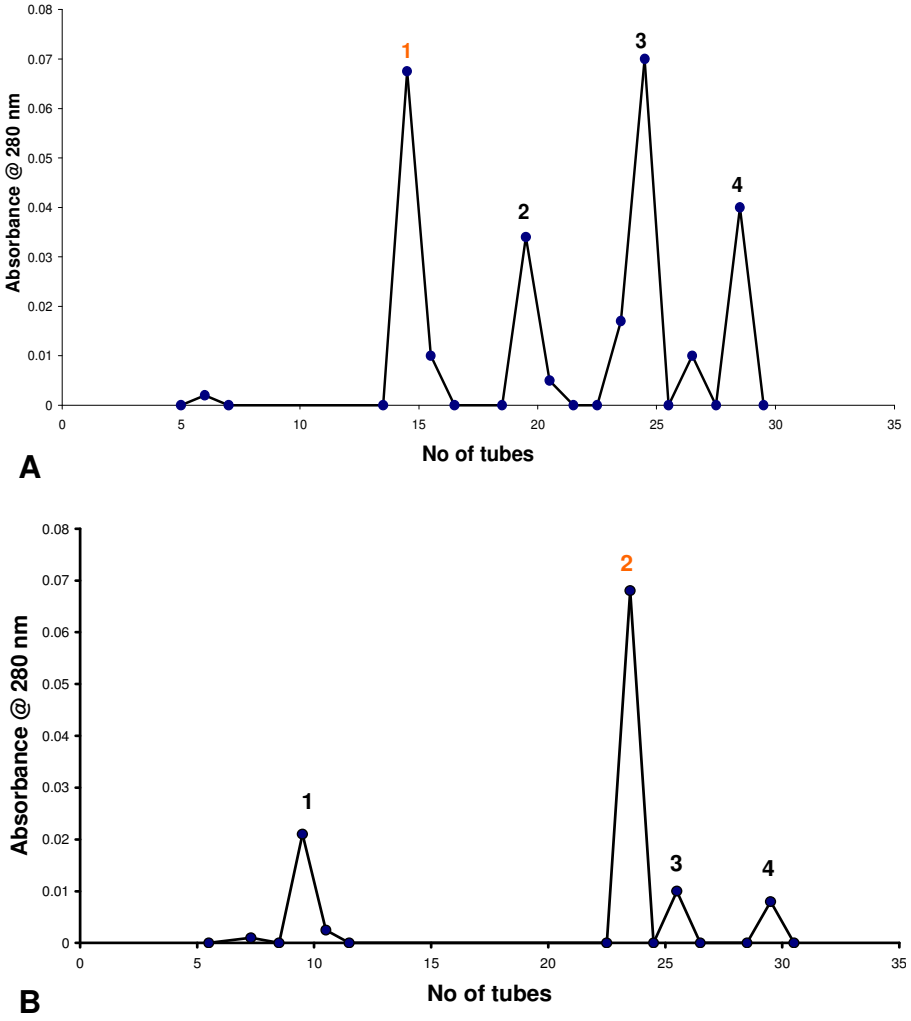

Figure 2. Gel chromatography for sample A and B.

from the crude extract, with a yield of approximately 18 and 15%, by the procedures shown in Table 1. The proteins had specific activity of 11.29 and 12.83 U/mg, respectively.

The purified AI-A1 and AI-B2 fractions showed a molecular weight of approximately 17 and 19 kDa, respectively, as estimated by Sephadex G-100 gel filtration (Figure 3) and the use of standard proteins (cytochrome C, carbonic anhydrase, alcohol dehydrogenase, and β-amylase).

The results obtained from the studies on the effect of temperature, pH, and the inhibitory activity against various amylases are presented in Table 2. The two AI-A1 and B2 showed no inhibition to the fungal amylase and AI-B2 showed no inhibition to plant amylases from barley and sweet potato. However, both inhibitors did show activity against amylase from human saliva and porcine pancreas. The two proteins displayed temperature optima for activity at 40°C and a complete loss of activity at 80°C. The purified proteins showed optimum activity at pH 4.0 and 6.0 with a rapid decline as the pH increase above 7.5.

DISCUSSION

While proteinaceous inhibitors of α-amylase from cereals (Roy and Gupta, 2000; Heidari et al., 2005; Muralikrishna and Nirmala, 2005) and legumes (Giri and Kachole, 1998; Melo et al., 1999) have been well characterized, little is known of these inhibitors in starchy tubers. The present work describes the purification and characterization of the two α-amylase inhibitors present in an ammonium sulphate extract of *C. esculenta.* The little available information on α-amylase inhibitors present in *C. antiquorum* (Sharma and Pattabiraman, 1980), taro (Seltzer and Strumeyer, 1990) and sweet potato (Rekha et al., 1999) seemed to indicate that most tubers had two proteins that showed inhibiting activity. The molecular weight of these proteins ranged between 11 and 25 kDa. Temperature is

Table 1. Purification of amylase inhibitors from Amadumbe.

Fraction	Total protein (mg)	Inhibitor (units)	Specific activity (units/mg)	Yield (%)	Purification
Crude extract	20.9	62	2.9	100	
Ammonium sulphate	18.5	56	3.02	88.5	1.04
DEAE Sephacel					
Peak A	7.2	54	7.5	33.44	2.59
Peak B	5.9	50.5	8.56	28.22	2.93
Sephadex G-100					
Peak A1	3.7	41.8	11.29	17.70	3.89
Peak B1	5.6	39.8	12.83	14.83	4.42

The inhibitor units were calculated using human salivary amylase.

Table 2. Kinetic studies of *C. esculenta* α-amylase inhibitors.

α-Amylase inhibitor activity								
Action of inhibitors on different amylases			**Action of inhibitors on different temperature**			**Action of inhibitors using different pH with human salivary amylase**		
Source of amylases	**% Inhibition**		**Temperature (°C)**	**% Inhibition**		**pH**	**% Inhibition**	
	A1	**B2**		**A1**	**B2**		**A1**	
Human saliva	62	56	20	15	35.7	1	0	nd
Barley	56	0	30	0	17.8	2	0	nd
Sweet potato	4.7	0	40	13	28.5	3	0	nd
Bacillus species	10.2	23	70	0	2.07	4	17.2	nd
Aspergillus species	0	0	80	0	0	5	14.2	nd
Porcine pancreas	28.5	48.5	100	0	0	6	33.8	nd
						7	7.3	nd
						8	1.8	nd
						9	0	nd
						10	0	nd

nd – not determined.

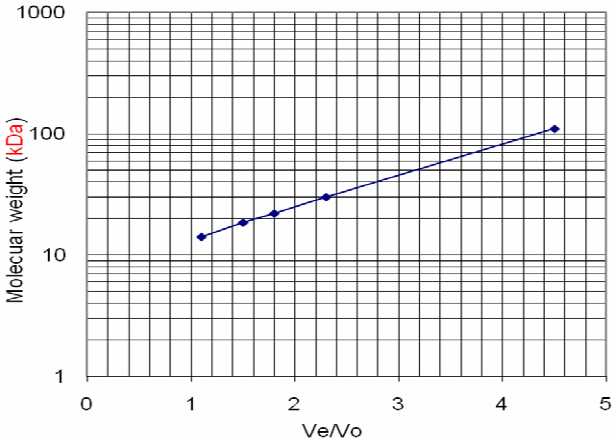

Figure 3. Standard (Mw of standards in Daltons) log molecular weight graph using gel filtration. Standards cytochrome C (12 400) carbonic anhydrase (29 000) alcohol dehydrogenase (150 000) β-amylase (200 000).

one of the most important parameters that affect the rate of enzyme hydrolysis. The optimum temperature displayed for both the *C. esculenta* inhibitors are similar to those reported for the navy-bean amylase inhibitor (Hoover and Sosulski, 1984) and for the α-amylase inhibitor from *Pachyrhizus erosus* tuber (Noman et al., 2006). We can also conclude that alpha-amylase inhibitors are fairly heat-stable (Prathibha et al., 1995). The residual activity in processed Amadumbe could be attributed to this property (McEwan, 2008).

α-Amylase inhibitors show strict target enzyme specificity and recognize only one out of several closely related isoenzymes or enzymes from different species (Weselake et al., 1983; Franco et al., 2000). In most cases the mechanism of inhibition occurs through the direct blockage of the active centre at several sub sites of the enzyme (Payan, 2004). Literature (Sharma and Pattabiraman, 1980, 1982; Ida et al., 1994) indicates that many AI present in tubers are active against mammalian amylases, but exhibit no activity on plant amylases. Alpha-amylase inhibitors present in Amadumbe also had no effect on the *Aspergillus* amylase. Amadumbe grow in moist, humid conditions and fungal infections are prevalent. It is possible that fungi have become resistant to the action of these α-amylase inhibitors. Sharma and Pattabiraman (1982) have reported similar results for *Dioscorea alata*.

Bifunctional properties have been demonstrated by a number of inhibitors and have therefore, received a particular attention as the appealing candidates for pest-control (Maskos et al., 1996). α-Amylase inhibitors found in wheat (Franco et al., 2000), barley (Richardson, 1991) and Indian finger millet (Campos and Richardson, 1983) have been shown to efficiently inhibit α-amylases from different insect sources. It is possible that the two AI present in Amadumbe may complement each other, with defense against a wider spectrum of intruders.

However, as reported, increases in urbanization and the associated changes in life-style, incidences of obesity and diabetes are on the increase. Inhibitory activity of plant amylases against mammalian amylases could cause a marked decrease in the availability of digested starch (Ali et al., 2006). This could suggest a potential in the prevention and treatment of diabetes and nutritional problems, which result in obesity. Based on the results of this study, the two α-amylase inhibitors of *C. esculenta* may have similar potential in the prevention and therapy of obesity and diabetes. Additional studies are needed to further investigate this possibility.

REFERENCES

Ali H, Houghton PJ, Soumyanath A (2006). α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. J. Ethnopharmacol., 107: 449-455.

Bernfeld P (1955). Amylases α and β. In: Colowick, S.P. and Kaplan, N.O. (eds), Methods in Enzymology, Academic Press, New York, (1): 149-158.

Campos FAP, Richardson M (1983). The complete amino acid sequence of bifunctional α-amylase/trypsin inhibitor from seeds of ragi (Indian finger millet, *Eleusine coracana* Gaertneri). FEBS Lett., 152: 300-304.

Franco OL, Rigden DL, Melo FR, Bloch C, Silva CP, Grossi-de-Sá MF (2000). Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. Eur. J. Biochem., 267: 2166-2173.

Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Royo J, Carbonero P (1987). Plant proteinaceous inhibitors of proteinases and alpha-amylases. In: Miflin, B. (Ed.), Oxford University Press, Oxford, Oxford Surveys Plant Mole. Cell Biol., 4: 275-334.

Giri AP, Kachole MS (1998). Amylase inhibitors of pigeonpea (*Cajanus cajan*) seeds. Phytochemistry. 47: 197-202.

Heidari R, Zareae S, Heidarizadeh M (2005). Extraction, purification, and inhibitory effect of alpha-amylase inhibitor from wheat (*Triticum aestivum* Var. *Zarrin*). Pakistan J. Nutr., 4: 101-105.

Hoover R, Sosulski F (1984). Characteristics and concentrations of α-amylase inhibitor in *Phaseolus vulgaris* biotypes. Starch/Starke. 36: 246-250.

Ida EI, Finardi-Filho F, Lajolo FM (1994). Purification and partial characterization of two proteinaceous α-amylase inhibitors from triticale. J. Food Biochem., 18: 83-102.

Maskos K, Huber-Wunderlich M, Gloskshuber R (1996). RBI, a one-domain α-amylase/trypsin inhibitor with completely independent binding sites. FEBS Lett., 397: 11-16.

McEwan R (2008). Anti-Nutritional content of *Colocasia esculenta* (Amadumbe) a traditional crop food in Kwazulu-Natal, pp 59-74. PhD thesis, University of Zululand, Empangeni, South Africa.

Melo FR, Sales MP, Pereira LS, Bloch C, Franco OL, Ary MB (1999). Alfa-Amylase inhibitors from cowpea seeds. Protein and Peptide Lett., 6: 385-390.

Muralikrishna G, Nirmala M (2005). Cereal α-amylases – an overview. Carbohydr. Polym., 60: 163-173.

Noman ASM, Hoque MA, Sen PK, Karim MR (2006). Purification and some properties of α-amylase from post-harvest *Pachyrhizus erosus* L. tuber. Food Chem., 99: 444-449.

Payan F (2004). Structural basis for the inhibition of mammalian and insect α-amylases by protein inhibitors. Biochem et Biophys., 1696: 171-180.

Prathibha S, Bala N, Leelama S (1995). Enzyme inhibitors in tuber crops and their thermal stability. Plant Foods Hum. Nutr., 48: 247-257.

Rekha MR, Padmaja G, Easwari Amma CS, Sheela MN (1999). Genotype differences in the α-amylase inhibitor activity in sweet potato and yam tubers. J. Root Crops, 25: 95-101.

Richardson M (1991). Seed storage proteins: the enzyme inhibitors In: Rogers, L.J. (Ed). Methods in Plant Biochemistry, vol. 5, Academic Press, New York, pp 259-305.

Roy I, Gupta MN (2000). Purification of a 'double-headed' inhibitor of alpha-amylase / proteinase K from wheat germ by expanded bed chromatography. Bioseparation, 9: 239-45.

Sasikiran K, Rekha MR, Padmaja G (2002). Proteinase and alpha-amylase inhibitors of sweet potato: Changes during growth phase, sprouting, and wound induced alterations. Bot. Bull. Acad. Sinica, 43: 291-298.

Seltzer RD, Strumeyer DH (1990). Purification and characterization of Esculentamin, A proteinaceous alpha-amylase inhibitor from the taro root, *Colocasia esculenta*. J. Food Biochem., 14: 199-217.

Sharma KK, Pattabiraman TN (1980). Natural plant enzyme inhibitors. Isolation and characterization of two α-amylase inhibitors from *Colocasia antiquarum* tubers. J. Sci. Food Agric., 31: 981-991.

Sharma KK, Pattabiraman TN (1982). Natural plant enzyme inhibitors. Purification and properties of an amylase inhibitor from yam (*Dioscorea alata*). J. Sci. Food Agric., 33: 255-262.

Silano V (1987). Alpha-amylase inhibitors. In: Kruger JE, Lineback D, Stauffer CE (Eds.), Enzymes and their roles in Cereal Technology, American Association of Cereal Chemists, St. Paul, MN., pp 141-199.

Weselake RJ, MacGregor AW, Hill RD (1983). An endogenous α-amylase inhibitor in barley kernel. Plant Physiol., 72: 809-812.