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Identification of a Proteinaceous Alpha Amylase Inhibitor from a Medicinal Herb *Oxalis corniculata* L. (Oxalidaceae)

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Abstract

Health management through traditional medicine is a promising approach worldwide as it represents a multifaceted approach to health care than conventional medicine. The heightened importance to validate the efficacy and standards of traditional herbal medicine is the thrust area of present day research. The present study is one such attempt to evaluate the alpha amylase inhibitory potential and identify the candidate inhibitor from *Oxalis corniculata* L, a potent indigenous medicinal plant. Sequential solvent extraction of the leaves of *O. corniculata* was performed in previous work and the aqueous extract, showing maximum inhibition against porcine pancreatic alpha amylase against starch as substrate (IC_{50} value 68.08+0.06), was selected for purification using ammonium sulphate precipitation. The pellet obtained at 40%-80% precipitation was further subjected to ion exchange chromatography on DEAE-cellulose and gel filtration chromatography using Sephadex G-100. The molecular weight of the proposed amylase inhibitor named AI-1 was estimated to be about 30 kda on SDS-PAGE. Temperature sensitivity and pH stability of the inhibitor was studied. The AI-1 protein was stable up to 400 C and was totally destroyed beyond 700 C and showed maximum activity at pH 6. Further characterization of the AI-1 protein and its sequence determination will help in discovering a novel proteinaceous alpha amylase inhibitor from *O. corniculata*. Research of this kind will help to usher the identification of active bioconstituents from plants with great medicinal activities.

Keywords: Alpha amylase inhibitor; *Oxalis corniculata*; Traditional medicine; SDS-PAGE; IC_{50}

Introduction

India is endowed with a rich plethora of medicinal plants and the use of these in the treatment of various ailments is in vogue since times immemorial. The growing awareness about the significance of thetraditional herbal medicine system among scientific and medical communities has changed the outlook of health care systems worldwide. Scientific evaluation of the curative phenomena characteristic of medicinal plants has been the key area of research [1]. The identification of bioactive constituents from plants has led to the development of new plant based drugs. Being able to use the innumerable medicinally potent plants bestowed by Nature, for development of drugs, will be a boon to the mankind [2,3]. More than 35,000 plant species are used for medicinal purposes worldwide. WHO has confirmed that above 80% of the rural population still use traditional herbal medicine to resolve health problems. Thus, the urge to develop efficient standards in herbal formulations and testing efficacy is the need of the hour [4].

The last decade has witnessed a rapid increase in diseases caused by improper lifestyle among mankind. These diseases pose a major threat as their treatment by conventional drugs is not possible either due to decreasing efficacy of synthetic drugs or their increasing contraindications [5]. Since the traditional medicine system works in accordance with mind-body complex and has a holistic approach towards disease treatment, phytotherapy or plant based medication is now the most sought to alleviate suffering and disease [6]. Diabetes mellitus is one such lifestyle disorder, a multifactorial disease which cannot be completely cured by any one medication [7,8]. A large number of medicinal plants with potential to curb diabetes in various ways are being studied widely as they contain a large number of bioconstituents that are effective against diabetes [9]. Alpha amylase inhibitors from plants possess the ability to lower post prandial hyperglycemia and can be used in supplementary treatment of diabetes. Hence there is a great scientific urge to extract them from plants and standardize them clinically [10,11]. An extensive progress has been made in the last decade in the research on the physicochemical properties, nutritional and physiological role of plant alpha amylase inhibitors [12]. While proteinaceous inhibitors have been isolated from cereals and legumes, [13,14] tubers and some leafy vegetables, [15,16] little is known about the existence of such proteins in small herbs and other medicinal plants. The present work is one such kind, to describe the extraction and purification of a proteinaceous alpha amylase inhibitory protein from a small herb *Oxalis corniculat.* L belonging to the family Oxalidaceae. The alpha amylase inhibitors characterized so far from various plants were grouped into six different classes based on their tertiary structure and ranging in molecular weight from 5-60 kda [17,18]. In this study we report the isolation of a 30 kda protein from the leaves of *O. corniculata* which was active against porcine pancreatic alpha amylase.

Oxalis corniculata L is a small herbaceous plant of the family Oxalidaceae, indigenous to tropical and subtropical regions of the

world. In Indian traditional medicine, the plant is widely used as anti-inflammatory, antibacterial, antiviral, anti-implantation and abortifacient, diuretic, and digestive agent [19,20]. The plant is well known for its medicinal value as a good appetizer and as a remover of anemia, dyspepsia, cancer, dementia, convulsion, and piles [21]. There is extensive study on the phytochemical analysis of the plant which showed the presence of flavonoids, tannins, phytosterols, glycosides, fatty acids, sterols, and amino acids [22]. Many of its therapeutic uses have been validated in vivo and in-vitro but no studies have been reported so far on the alpha amylase inhibitory potential of this plant. The preliminary evaluative studies have been conducted to analyze the alpha amylase inhibitory potential of the leaf extracts of this plant against porcine pancreatic alpha amylase using starch as substrate [23]. Further research was carried out to extract and purify the proposed alpha amylase inhibitor from the leaves of *O. corniculata* using ammonium sulphate precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography. The molecular weight of the proteinaceous inhibitor was characterized by SDS-PAGE. Temperature sensitivity and pH stability of the inhibitor was also studied.

Materials and Methods

Plant material

The plant *Oxalis corniculata* L., has been identified by the taxonomist in the Department of Botany, Osmania University, Hyderabad and has been documented with a voucher specimen number UCW-019, at the Department of Botany, Osmania University College for Women, Hyderabad. Freshly collected leaves of *Oxalis corniculata* were washed and shade dried for 2 days. The dried leaves were powdered in a grinder and subjected to sequential solvent extraction in the previous work [24].

Chemicals and reagents

Porcine pancreatic alpha amylase, Acarbose, DEAE-cellulose were obtained from Sigma. Ammonium sulphate, NaCl, and other chemicals were obtained from Himedia.

Alpha amylase inhibitory assay

The dried leaf powder was subjected to cold water extraction for 24 hrs. The extract was filtered using a muslin cloth and the obtained crude extract was studied for its alpha amylase inhibitory assay against porcine pancreatic alpha amylase using starch as substrate according to the method described by Miller [25] as mentioned previously.

Protein precipitation

The crude extract analyzed for alpha amylase inhibition was subjected to 40-80% ammonium sulphate precipitation. Protein pellets obtained after centrifugation (12000x g, 15 min) were redissolved in a minimum amount of phosphate buffer (0.02M, pH 6.9, with 0.3M NaCl). The ammonium sulphate extract thus obtained was dialysed extensively against the buffer for 48 hrs (with the buffer being changed every 6-8 hrs) and then analyzed for amylase inhibitory activity as mentioned above [26].

Ion exchange chromatography

The ammonium sulphate extract was further purified through anion exchange chromatography (10×1.5 cm DEAE cellulose) equilibrated with 0.02M phosphate buffer. The elution of the column was done with a linear NaCl gradient of 0.2-0.5 M at the flow rate of 30 ml/h). The 5 ml fractions collected were then monitored at A280 nm. Individual peaks were pooled and then analysed for their amylase inhibitory activity.

Gel filtration chromatography

The above peaks with AI activity were then allowed to flow through a column of Sephadex G-100 (1.5×100 cm) equilibrated with 0.02M Tris-HCl buffer pH 8.0 with a flow rate 20 ml/h. 3 ml fractions were collected and the active fractions showing maximum absorption at 280 nm were pooled and stored at 4°C.

Gel electrophoresis

The purified inhibitor was subjected to SDS-PAGE according to the method of Laemmli [27]. The running and stacking gel concentration was 12% and 5% of polyacrylamide respectively. 20 µl of laemmli solution added to 20 µl samples and 35 µl of the mixture was injected to the running gel [28]. Medium molecular weight proteins (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lactalbumin) were used as standard for calculating the molecular weight of separated protein. Electrophoresis was performed on a mini-protein cell apparatus (Bio-Rad Laboratories) in 0.02M Tris-glycine buffer pH 8·6. The run was 3hr at a current of 20 mA.The protein bands were stained with Coomassie brilliant blue R-250. De-staining was done by washing with 10% acetic acid.

Temperature and pH stability of the inhibitor

To understand the temperature and pH sensitivity of the purified inhibitor, 0.5 ml was pre-incubated with 1 ml (50 mM) of each of the buffers HCl-KCl (pH1, 2) citrate buffer (pH3, 4), acetate buffer (pH5), phosphate buffer (pH6, 7, 8), glycine-NaOH (pH9, 10) at 25°C for 30 min. The residual inhibitory activity against porcine pancreatic amylase was studied as described earlier and plotted on a graph. The inhibitor dissolved in 0.5 ml phosphate buffer was incubated at various temperatures ranging from (20-100°C) for 30 min. The aliquots were then assayed for amylase inhibitory activity as mentioned above.

Results

An alpha amylase inhibitory protein AI-1 was purified from the leaves of Oxalis corniculata, by ion exchange chromatography on DEAE cellulose and gel filtration chromatography on Sephadex G-100. The elution pattern of ion exchange chromatography as in Figure 1 shows one major peak and two minor peaks. The major peak shows an alpha amylase inhibitory activity against porcine pancreatic alpha amylase with a specific activity of 2U/mg of protein and 8.16% of purification folds.

The major peak from the DEAE cellulose chromatography was then separated on a gel column using Sephadex G-100. The resulting peak from gel chromatography column as shown in Figure 2 also presents one major peak with specific activity of 3.1 U/mg protein and 13 fold purification as shown in Table 1.

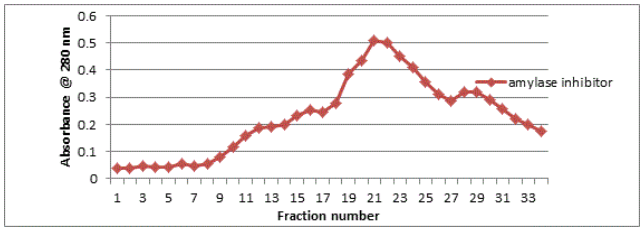

Figure 1: Ion exchange chromatography of *O. corniculata* inhibitor on deae- cellulose column

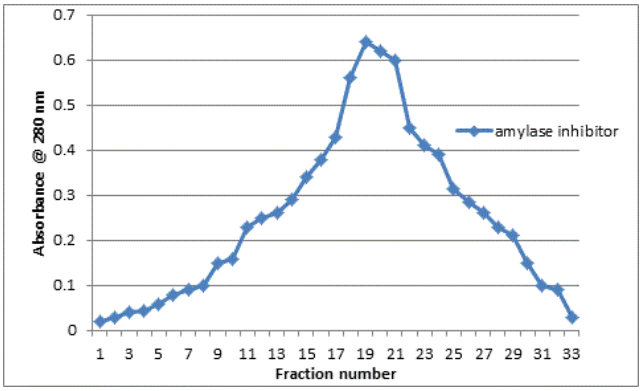

Figure 2: Gel filtration chromatography of *O. corniculata* amylase inhibitor on sephadex G-100 column.

Fraction	Volume (ml)	Activity (Units/ml)	Protein Concentration (mg/ml)	Specific Activity (U/Mg)	Total activity (U * ml)	Fold purification	% yield
Crude extract	40	63	27	2.3	2520	1.00	100
Ammonium sulphate	30	59	19	3.10	1770	1.4	74.1
DEAE cellulose fraction	15	57	11.2	5.08	855	2.07	61
Gel filtration	12	38	5.3	7.16	456	3.11	53.3

Table 1: Purification Table of *O.corniculata* alpha amylase inhibitor

The purified AI-1 showed a molecular weight of 30 kda approximately as estimated using SDS-PAGE by comparison with the standard protein marker as shown in Figure 3.

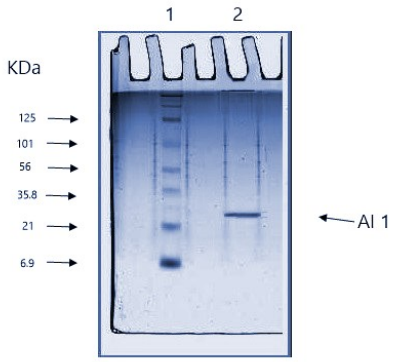

Figure 3: SDS- GEL picture of the purified AI 1 from *O. corniculata*

The results obtained from the studies on the effect of temperature and pH on the inhibitory activity as shown in Table 2 and 3 indicates that the AI-1 inhibitory protein has temperature optima at 40°C and is active up to 60°C. It shows a total loss of activity at 80°C as seen in Figure 4. The purified protein presents optimum activity at pH 6.0 and declining activity beyond pH 8 as seen in Figure 5.

Temparature (°C)	% Inhibition
20	48.5
30	67
40	83
50	76
60	75
70	20
80	09
100	0

Table 2: Effect of temperature on inhibitory activity

Discussion

Proteinaceous inhibitors have so far been identified only in few plant species like cereals, legumes, tubers and some leafy vegetables [29]. Identification and characterization of more of these inhibitors from medicinally important plants adds much value to the contemporary alternative and traditional medicine systems that are being relied upon by a large population for the treatment of life style diseases [30].

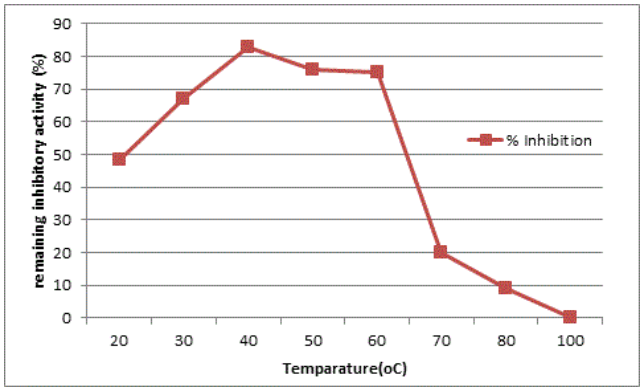

Figure 4: Effect of temperature on inhibitory activity

pH	% Inhibition
1	0
2	0
3	0
4	65.3
5	75
6	82.6
7	09
8	0
9	0
10	0

Table 3: Effect of pH on the inhibitory activity

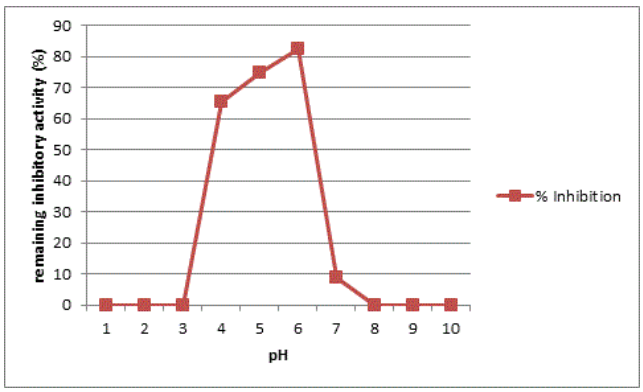

Figure 5: Effect of pH on inhibitory activity

The inhibitor AI-1 isolated from *O. corniculata*, shows a molecular weight of 30kda on SDS-gel electrophoresis. The inhibitor has an optimum temperature range of 40-60°C and is fully inactive at higher temperatures. The inhibitor is effective in the pH range of 4-6 and inactive in alkaline pH. There is extensive evidence that these kinds of alpha amylase inhibitors, obtained from plants, are a good source for controlling postprandial hyperglycemia, a major problem in type-II diabetes [31,32]. The present work is a contribution towards the identification of a novel alpha amylase inhibitor from a small medicinal herb *O. corniculata*. Further structural elucidation needs to be done on the inhibitor identified in this study in order to relate it to a specific class of amylase inhibitors identified so far from different plants [33]. Phytotherapy has gained great importance in the treatment of various lifestyle disorders like diabetes, obesity, heart diseases etc. [34,35]. Complete structural elucidation of this inhibitor might prove important in developing a natural plant based medication for the treatment of type-II diabetes.

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