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Research Article

Isolation, Characterization and Purification of α-Galactosidase from Peas

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Abstract: In India, the peas are cultivated on a large scale. The α-galactosidase enzyme has principle applications in food industries and in the medical field. The α-galactosidase enzyme was purified from dry peas (*Pisum sativum*). The purification steps include acetone precipitation and DEAE-cellulose column chromatography. The enzyme has maximum activity at 40°C and optimum pH=7.5. The molecular weight of the enzyme was determined by using SDS-PAGE analysis and found to be 110 kDa. The purified enzyme was immobilized by sodium alginate and it was observed that the immobilized enzyme showed an increase in thermal tolerance.

Keywords: α-galactosidase, Dry peas, DEAE Cellulose column chromatography, SDS-PAGE, DNSA, Immobilization.

1. Introduction

Enzymes are highly specialized proteins which act as biocatalyst and enhance the rate of reaction without itself taking part in the reaction. There is considerable interest in the enzymes that catalyse hydrolysis of glycosidic bonds, due to their extensive industrial, therapeutic and biochemical applications. α-galactosidase [α-D-galactosidase galactohydrolase E (3.2.1.22)] is an exoglycosidase that catalyses hydrolysis of terminal α-1, 6 linked galactosyl residue from a wide range of substrates including oligosaccharides of raffinose family sugars such as raffinose, stachyose, melibiose, verbascose and polysaccharides of galactomannans, locust bean and guar gum. Moreover, it also hydrolyses glycoconjugates, glycoproteins and glycosphingolipids. α-galactosidase from various sources has been purified by multi-step conventional purification procedure. α-galactosidase is widely distributed in nature among plants, animals and microorganisms. The enzyme α-galactosidase is ubiquitous in legume seeds and for the first time, it was reported from sweet almond (1). The enzyme was also found in the leaves of *Cucurbita* and endosperm of coconut (2). Very recently it has been isolated from tomato fruit (3), grape flesh (4) and cultured rice (5). In plants α-galactosidase is believed to be involved in a variety of biochemical processes; the most important in the hydrolysis of oligosaccharides such as raffinose and stachyose during the germination period, resulting in the generation of free sugars which may serve as a ready source for the growing plants.

Multiple forms of enzymes, particularly α-galactosidase have been frequently reported. In seeds, these may reflect differences in their distribution between the tissues. α-galactosidase has been purified from mature leaves of *Cucurbita* using pH and ammonium sulphate fractionation. Gel filtration produced three forms of enzyme designated as L1, L2, and L3. Similarly, α-galactosidase from guar shows three different forms. These may be either anionic or cationic in nature. The three different forms are α-galactosidase-A (Anionic = 28%), α-galactosidase-C1 (Cationic =12%), α-galactosidase-C2 (Cationic = 60%). These may be glycoproteins, but their carbohydrate content and composition may vary. Their amino acid composition varies with respect to glutamic acid, methionine, histidine and arginine content; also their kinetic properties vary (6). Different methods can be used in the purification of α-galactosidase enzyme.

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Some of them used commercially are Ammonium sulphate precipitation, acetone precipitation and DEAE Sepharose ion exchange chromatography or DEAE Cellulose column chromatography. Thus, the purity of the enzyme obtained can be determined by SDS-PAGE analysis.

The objective of this study was to develop a suitable method for isolation, purification and characterization of α-galactosidase enzyme from peas. The stability of the immobilized enzyme towards heat was also evaluated.

2. Materials and Methods

2.1 Material

Peas were purchased from local markets. The chemicals used were sodium phosphate buffer, citrate buffer, acetone, ethanol, mercuric chloride, DNSA, Biuret reagent and melibiose; these were purchased from Hi-media chemicals.

2.2 Methods

2.2.1 Germination of peas

One gram of peas was weighed. The peas were subjected for surface sterilization which was carried out in laminar air flow. The peas were first washed with tap water followed by washing with 70% alcohol for 2 minutes. Finally, the peas were treated with 0.1% mercuric chloride and again the peas were washed with sterile distilled water for 4-5 times to remove all the sterilants. Then peas were transferred to a conical flask containing wet sterile cotton bed and were allowed to germinate for a varying time period of 24 h, 48 h, 72 h, and 96 h (7). Germination was carried out in dry as well as fresh peas and the method of germination followed for both was same. The maturity of fresh peas was observed after 120 h.

2.2.2 Extraction of enzyme

The germinated peas were taken in pre-cooled mortal and piston and crushed using 25mM phosphate buffer (pH=7.2). The extract was cold centrifuged at 7000 rpm for 20 minutes and the supernatant obtained was collected and its volume was made 100mL by adding phosphate buffer. For 100mL of extract, 150mL of chilled acetone was added with continuous stirring and it was kept overnight at 4°C for precipitation. The next day centrifugation was carried out at 8000 rpm for 15 minutes and the pellet obtained was dissolved in 15mL sodium phosphate buffer. In dialysis, the pellet dissolved in buffer was taken in a dialysis bag which was closed at both ends and then tied to a glass rod. This glass rod along with the dialysis bag was suspended in a beaker containing the buffer solution and was kept in the freezer for overnight. During dialysis, the acetone present in the pellet gets diffused in the medium for maintaining equilibrium which is beneficial to get enzyme-free from acetone.

2.2.3 Protein estimation

Protein concentration of the enzyme was estimated according to Biuret method. This is done to determine the specific activity of enzyme (8).

2.2.4 Enzyme assay

The enzyme assay was carried out by using melibiose as the substrate. The melibiose was dissolved in 25mM phosphate buffer. The released sugar due to enzyme activity was estimated by DNSA method (9).

2.2.5 Characterization of enzyme

An extracellular α-galactosidase from germinated peas has been purified by cold acetone precipitation. The purified enzyme was used for the characterization such as the effect of pH & temperature on the enzyme activity (10).

2.2.6 Effect of pH on enzyme activity

Optimum pH of α-galactosidase from acetone precipitation was determined by assaying the enzyme activity over a pH range 3.0 to 10.0 using 25mM sodium phosphate buffer (pH=7.4), 25mM citrate-phosphate buffer, glycine NaOH buffer (pH= 9.0 to 10.0). The enzyme activity was determined by standard assay method i.e. DNSA method (9).

2.2.7 Effect of temperature on enzyme activity

The optimum temperature of α-galactosidase was determined by performing assay at varying temperatures of 20°C, 40°C, 50°C, 60°C, 70°C and 80°C. The enzyme activity was determined by performing DNSA method (9).

2.2.8 Ion exchange chromatography

The enzyme extracted from peas was loaded on Diethylaminoethyl cellulose (DEAE cellulose) column and fractions were eluted with NaCl gradient. The protein content and enzyme activity were studied.

2.2.9 Packaging of ion exchange column using DEAE cellulose

10g DEAE cellulose was weighed and dispersed in 100mL distilled water. This mixture was allowed to settle for 30 minutes to remove the supernatant. The resultant gel produced was washed with distilled water until pH=6. Then approximately 200mL of 0.5N NaOH was added to allow settling of the gel for 30 minutes; later on, alkali was discarded. Again gel was washed with distilled water until pH=7 and then 25mM phosphate buffer was added to the activated gel. The gel was filled up to ¾th of the height of the column with the help of the dropper and the column was kept packed for overnight. Next day the dialyzed enzyme was added to the column. After that, for every 7 mins, the fraction was collected as 5mL in each tube. Again buffer was added in the column to the buffer collected becomes up to 150mL. NaCl gradient was added (0.1 to 0.5) up to

the gel is immersed in it and then the next gradient was added. The fraction after addition of NaCl gradient was collected and its activity was determined. The fractions showing highest activity were used for further studies.

2.2.10 SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

The fraction showing the maximum enzyme activity was determined by SDS-PAGE by the method of Laemmli (11) for its purity. The bands were visualized by silver staining technique. The molecular weight of α-galactosidase was determined by comparison with standard molecular marker proteins.

2.2.11 Immobilization of α-galactosidase

The enzyme was immobilized using sodium alginate (5%) and calcium chloride (4%). About 15mL of enzyme was taken and added in 25mL of 5% sodium alginate to produce the beads which were dispensed in chilled $CaCl_2$. The beads; washed with sterile distilled water were kept in buffer for 24 h and its activity was determined after 4-5 days. The effect of temperature on immobilized enzyme was studied by using DNSA method (12).

3. Results and Discussion

3.1 Physicochemical analysis of pea powder

The α-galactosidase enzyme activity was higher for dried peas. Hence the physicochemical analysis was carried out for pea powder. The results obtained are given in Table 1. It was found from the table that, pea powder was rich in protein, total sugars and minerals. The predominating minerals like calcium and iron were also present in significant amount. Gopalan *et al.*, (1971) had reported similar results with respect to the nutritional components of dry peas (13).

Table 1. Chemical composition of pea powder.

Parameters	Values (%)
Moisture content	10.47
Protein content	16.27
Fat content	1
Total Sugars	14.2
Reducing Sugars	1.7
Ash content	2.47
Crude fiber	1.11
Calcium	1.002
Phosphorous	0.15
Iron	0.33

Each value is an average of three determinations

3.2 Extraction of the enzyme and study of its activity

Initially, the effect of solvents on the germination of peas was noted for which different types of solvents were used in the experiment. The results obtained for germination of both dry and fresh peas are given in Table 2. It was found that germination took place only in the presence of tap water which may be due to the presence of minerals in the tap water as they help for germination.

Table 2. Effects of solvents on germination of peas.

Types of Solvents	Germination	
	Fresh peas	Dry peas
Tap water (pH=7.5)	-	+
Distilled water (pH=6.5)	-	-
Phosphate buffer (pH=7.2)	-	-

Each value is an average of three determinations

The mature peas contain large amount of raffinose family sugars such as melibiose, verbascose & stachyose. During the germination period, these sugars are hydrolyzed by the α-galactosidase enzyme and provide carbohydrate source to the developing embryo. So, the activity of enzymes extracted during germination was measured at different germinating periods. The activity measured in respective periods (in h) (Table 3).

Table 3. α-galactosidase activity during germination for dry and fresh peas.

Time/h	Enzyme activity/(U/mL)	
	Dry peas	Fresh peas
0	0	0
24	0	0
48	12	0
72	26	0
96	15	12
120	15	16
144	15	12
168	15	12

The maximum enzyme activity of dry peas and fresh peas were observed after 72 and 120 h respectively. The maximum activity in both peas is shown in Fig. 1.

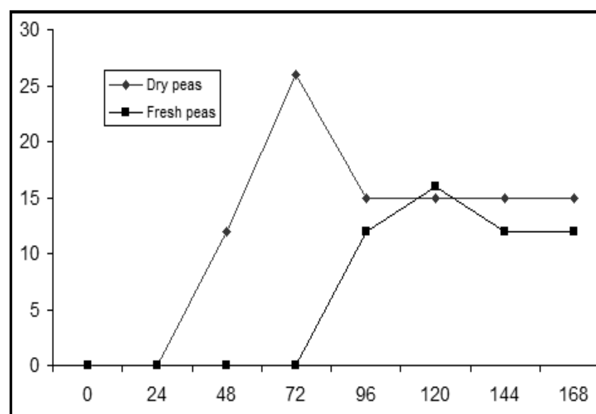

Fig. 1. α-galactosidase production during germination.

Thus, Fig. 1 revealed that the α-galactosidase production was 2.17 times higher in case of dry peas germinated in tap water as compared to fresh peas. Hence, the hydration of peas during germination

possibly induces α-galactosidase activity leading to raffinose oligosaccharides breakdown, releasing reducing sugars to increase the α-galactosidase activity.

3.3 Purification of α-galactosidase by DEAE Cellulose Column Chromatography

The α-galactosidase was extracted from the dry peas after 72 h of germination period which was partially purified by the cold acetone precipitation method. The purification of the partially purified enzyme was carried out by DEAE-cellulose ion exchange column chromatography. Here, the number of protein fractions eluted from the extracted sample was observed. The enzyme was eluted in the fractions of 0.2–0.3M NaCl concentration. The activity of fractions collected is shown in Table 4. The protein content and enzyme activity profile is shown in Fig. 2.

Table 4. Protein content and enzyme activity of purified α-galactosidase.

Fraction No.	Protein Content (Absorbance at 530nm)	Enzyme activity/(U/mL)
1	0.1	0
2	0.15	0
3	0.12	0
4	0.11	0
5	0.13	0
6	1.4	0
7	0.85	0
8	0.56	0
9	0.85	0
10	1.2	0
11	1.3	0
12	1.2	0
13	1.0	9
14	1.2	15
15	2.0	20
16	1.59	15
17	1.55	12
18	1.4	10
19	1.0	9
20	0.79	5
21	0.66	2
22	0.56	0
23	1.2	0
24	1.5	0
25	0.9	0
26	0.64	0
27	0.32	0
28	0.24	0
29	0.25	0
30	0.16	0
31	0.1	0
32	0.09	0
33	0.07	0
34	0.07	0
35	0.11	0
36	0.09	0
37	0.09	0
38	0.065	0
39	0.065	0
40	0.05	0
41	0.045	0
42	0.03	0
43	0.04	0
44	0.04	0
45	0.18	0
46	0.04	0
47	0.04	0
48	0.02	0
49	0.02	0
50	0.02	0

Each value is an average of three determinations

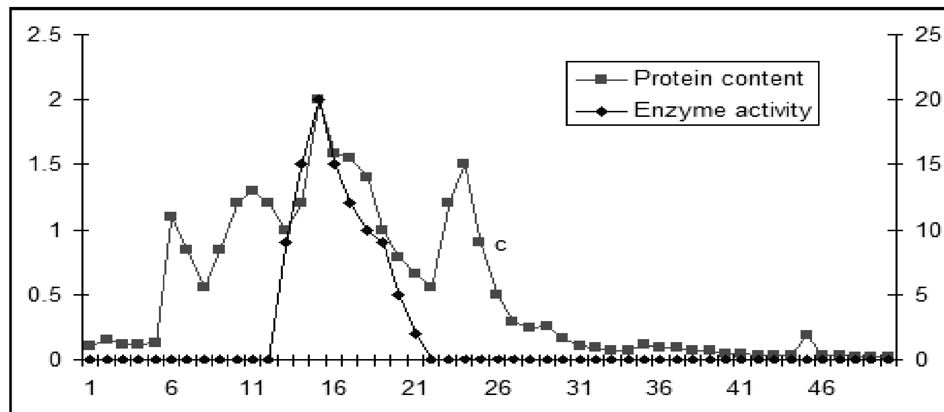

Fig. 2. Elution profile of α-galactosidase purified by DEAE-cellulose chromatography.

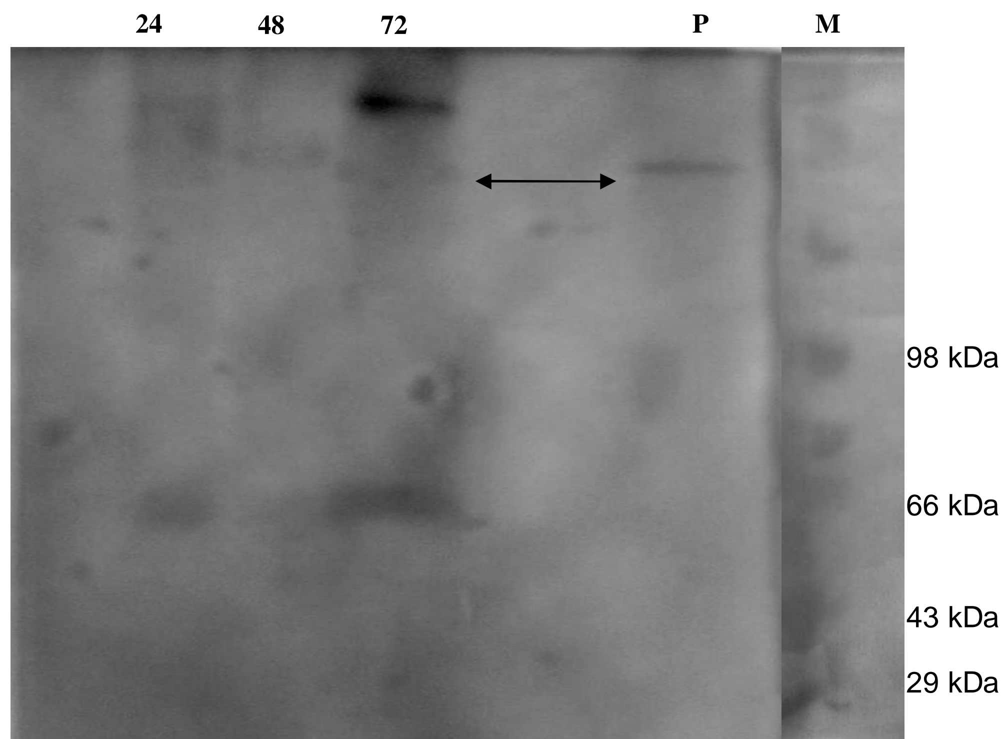

Fig. 3. SDS-PAGE analysis of purified and crude α-galactosidase.

The single peak from Fig. 3 confirmed the presence of α-galactosidase enzyme. Thus, the highest peak shows maximum protein content. It is clear from Table 4 and Fig. 3 that the maximum protein content was observed at 15th fraction and corresponding maximum enzyme activity was 20U/mL.

3.4 Purification of α-galactosidase by SDS-PAGE analysis

The SDS-PAGE of purified fractions was done to determine the purity of the enzyme. The separation of bands can be seen in the Fig. 3.

The enzyme which was of high purity (i.e. Purified by a single step of DEAE-cellulose chromatography) along with the crude sample collected at 24, 48 and 72 h of germination were analyzed by SDS-PAGE. The purified enzyme showed only one band with 110 kDa molecular weight. The crude enzyme extracted at 72 h of germination showed a similar band at the same alignment along with many other bands whereas, crude enzyme extracted at 24 and 48 h germination showed many bands which did not show any alignment with single band of purified enzyme. The α-galactosidase enzyme produced by the dry pea was monomer, which hydrolyzes the raffinose family sugars.

3.5 Effect of temperature on enzyme activity of purified α-galactosidase enzyme

The purified enzyme was immobilized using sodium alginate method for further study to determine

the effect of varying temperature on the enzyme activity by performing DNSA method. The effect of temperature was noticed for partially purified and immobilized form of the enzyme. The enzyme activities observed for partially purified and immobilized enzyme is given in Table 5. The immobilized enzyme showed higher resistance towards heat up to 60°C compared to the controlled enzyme. The partially purified enzyme showed the activity between 20–50°C, whereas the optimum activity was observed at 40°C. The immobilized enzyme showed the activity between 20–60°C. The result observed is shown in Fig. 4.

Table 5. Effect of temperature on purified and immobilized α-galactosidase activity.

Temperature/(°C)	Enzyme activity/(U/mL)	
	Control	Immobilized
10	0	0
20	10	11
30	26	30
40	30	35
50	10	25
60	0	15
70	0	0
80	0	0

Each value is an average of three determinations

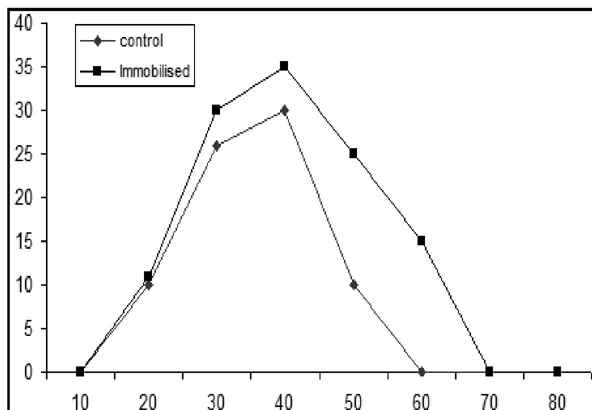

Fig. 4. Effect of temperature on purified and immobilized α-galactosidase activity.

Table 6. Effect of pH on partially purified α-galactosidase activity.

pH	Enzyme activity/(U/mL)
3	0
3.5	0
4	0
4.5	9
5	13
5.5	13
6	18
6.5	26
7	31
7.5	36
8	32
8.5	18
9	6

Each value is an average of three determinations

3.6 Effect of pH on partially purified enzyme activity

The effect of pH on purified enzyme activity was studied by performing DNSA method. The enzyme activity at different pH is shown in Table 6 and the activity at optimum pH is shown in Fig. 5. It was seen that the enzyme was stable in between pH 4.5 – 9.0 but the optimum activity was at pH=7.5.

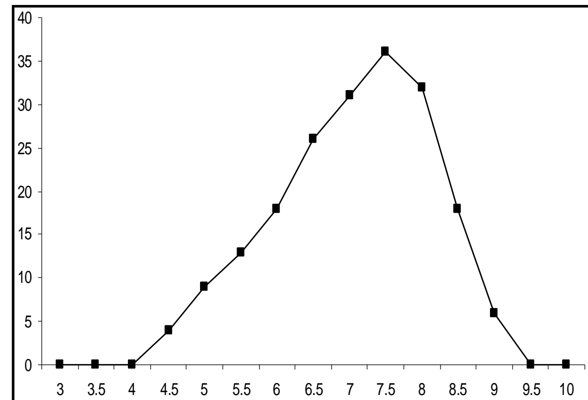

Fig. 5. Effect of pH on enzyme activity of partially purified enzyme.

4. Conclusion

α-galactosidase can be produced in higher amount of dry peas than fresh peas. It was produced during germination i.e. it is an inducible enzyme. As reported in several cases, the biosynthesis of α-galactosidase could be induced in the presence of sugars viz: raffinose, melibiose, stachyose and verbascose. The maximum α-galactosidase production was after 72 hours. The α-galactosidase produced can be purified by DEAE-Cellulose ion exchange column chromatography and the purity was determined by SDS-PAGE analysis. The optimum temperature was 40°C and stability towards heat was increased by sodium alginate immobilization technique. This is a very important characteristic of enzyme to make it applicable in processing industries. The purified enzyme showed maximum activity at pH=7.5. Further, α-galactosidase extracted from dried peas can be used in many food products containing raffinose family oligosaccharides as a digestive aid. This enzyme can also be used for animal feed and medicinal purposes.

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