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Comparative Studies on Isolation and Characterization of Allinase from Garlic and Onion using PEGylation-A Novel Method†

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Allium plants including garlic (*Allium sativum*) and onion (*Allium cepa*) contain an enzyme allinase (E.C-4.4.1.4), which is an enzyme responsible for the production of thio sulphates, a promising therapeutically potential compound. PEG (polyethylene glycol) is used in this study for the first time, to precipitate a protein. Four fold purification was done for both garlic and onion. The protein yield of the garlic was greater than onion shoots. 6.2 mg protein was the yield at the step in garlic, whereas it was 0.6 mg in onion shoots. The total protein content in the garlic is 6.1 g in the 100 g of garlic where as it is 1.6 g in the onion shoots.

Keywords: *Allinase*, Garlic, Onion, Liquid chromatography, Polyethylene glycol.

INTRODUCTION

The garlic *(Allium sativum)* and the onion *(Allium cepa)* are the important plants used for culinary and medicinal purposes. The characteristic flavour of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.), occurs when the enzyme *alliinase* (EC 4.4.1.4) hydrolyzes the *S*-alk(en)yl-L-Cys sulfoxides (ACSOs) to form pyruvate, ammonia and odorous sulfur-containing volatile metablites. The sulfoxide substrates are located in the cytoplasm, but alliinase is present in the vacuole[1]. Rupturing makes these compounds to communicate with. The enzyme is a homo-dimeric glycoprotein from the fold-type I family of PLP-dependent enzymes.

Allin is the precursor which comes to a contact with *allinase* enzyme in the presence of co-factor pyridoxal phosphate which initially forms allicin, pyruvate and ammonia as products. Allicin is the bioactive compound present in the garlic. It possess antimicrobial, anticancer and cholesterol lowering property[2-4].

Allicin kills *Helicobacter pylori,* a kind of bacteria which is implicated in the cause of some stomach cancer and ulcers. National Cancer Institute is sponsoring a huge clinical trial on garlic's ability to prevent stomach cancer. In the Indian traditional cooking the garlic and onion are used as the basic ingredient in all types of food. It reduces the activity of many of the chemical reactions and also reduces the adverse effects of the chemical reaction.

Allicin is a very labile and volatile compound when exposed to air and the methods known today for its preparation are not satisfactory. The chemical synthesis involves many steps and is complicated, laborious, expensive and very inefficient. The enzymatic method seems to be more attractive, however *alliinase* is a so-called "suicidal enzyme" that is rapidly and irreversibly inactivated by its own reaction product, allicin. Therefore a few minutes incubation of *alliinase* with the substrate alliin or its product, allicin, leads to a biologically inactive enzyme after few cycles.

It is very important to isolate and characterize the *allinase* enzyme for the production of allicin and other associated bioactive compounds. In this study *allinase* enzyme is isolated from both garlic and onion and characterized.

EXPERIMENTAL

Sample preparation: Garlic bulbs and onion shoots were purchased from the local market and washed many times and same size of shoots and bulbs were sorted out. They were

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washed twice with water to remove soil. 100 g of shoots and bulbs were weighed and extracted with 100 mL of extraction buffer (Na-phosphate buffer 0.02 M pH 7.8, containing glycerol 10 %, pyridoxal phosphate 0.02 mM and 0.2 % 2-mercaptoethanol) and magnetically stirred for 30-45 min at 4 ºC[5]. The supernatant was collected and stored.

PEG precipitation: Concentrations varying from 25-55 % of PEG 6000 were used for both garlic and onion. They were stirred using magnetic stirrer under 4 ºC for 4 h and centrifuged for 10 min at 15000 rpm. The protein estimation was carried out using Lowry *et al.*[4] method with reference to the standard BSA.

Gel filtration chromatography: PEG precipitated sample was desalted using Akta Prime Plus with the buffer system Tris-Hcl buffer 0.02 mM pH-7.8. The matrix of the column is made up of Sephadex G-25. Column volume- 5 mL, void volume 1.5 mL, flow rate 5 mL/min and bead size 15- 70 µm. Every time the columns were washed with alcohol and water after a successful run. The optical density value and peak were noted for further reference. The protein content was estimated by Lowry's *et al.*[4].

Assay of *Allinase*: There is a simple and rapid spectrophotometric procedure for determination of allicin and *allinase* activity, based on the reaction between 2-nitro-5-thiobenzoate (NTB). The quantitative reaction of *alliinase* is assessed by the reaction at 412 nm due to the consumption of 2-nitro-5-thiobenzoate. 2-Nitro-5-thiobenzoate is not commercially available, it should be synthesized from DTNB (Ellman's reagent)[5,6].

Preparation of 2-nitro-5-thiobenzoic acid: 2-Nitro-5-thiobenzoate was prepared according to Degani and Patchornik *et al.*[6]. 2-Mercaptoethanol (5 mL) was added to the solution of DTNB (1 g) in 50 mL of 0.5 M Tris-HCl buffer, pH 8. After 5 min, the solution was acidified to pH 1.5 by the addition of 6 N HCl and kept overnight at 4 ºC. Orange colour crystals were formed and filtered washed with dilute HCl.

***Allinase* assay:** The assay of *allinase* activity is based on the reaction of allicin produced during the enzymatic reaction and 2-nitro-5-thiobenzoate. The standard reaction mixture contained NTB (0.001 M) in 50 mM Na-phosphate, pH 6.5 containing EDTA (1 mM), pyridoxal phosphate (2 × 10 M) and 2.5- 10 units *allinase* and alliin 10 mM in total volume of 1 mL, enzymatic activity at 23 ºC started by adding the alliin and the initial rate was monitored spectrophotometrically by recording the decrease in absorbance at 412 nm.

RESULTS AND DISCUSSION

***Allinase* assay:** Lowrys *et al.*[4] method was used to find out the protein concentration in each and every step of purification. Calibration chart was drawn and the slope was calculated and represented in Fig. 1. Desalting, process is used to remove the salts in the precipitated sample. AKTA prime plus is used for the desalting process along with HiTrap Desalting column matrixes with sephadex G-25, cross linked dextran. Desalting graph showed two peaks in Blue colour which indicated the UV of the sample elution Shown in Fig. 2. The sample was collected in a fresh, clean tube and the peak elutes was stored at 10 ºC. This sample was later injected into the

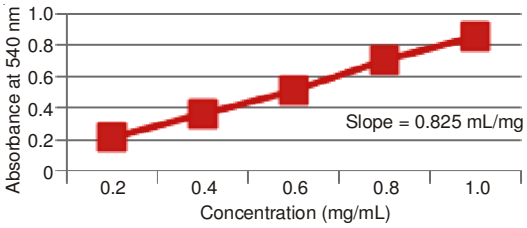

Fig. 1. Lowry's calibration chart

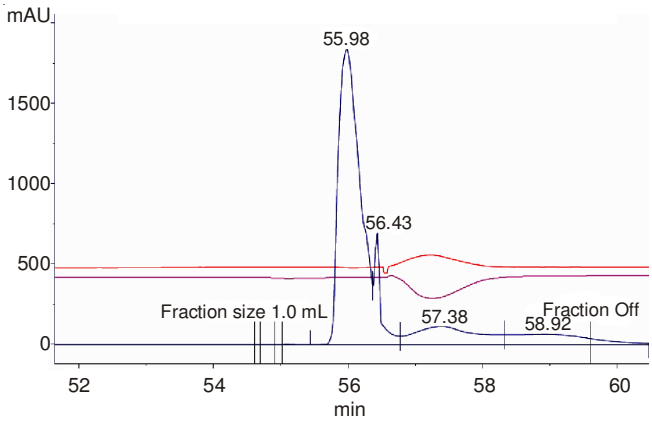

Fig. 2. UV absorption spectrum of sample purified using HiTrap column chromatography

liquid chromatography in an AKTA prime plus sephadex column. The results obtained were presented in Fig. 3. There was a peak at 36 min with 1938 mAU which was collected and stored at 4 ºC for further use. Table-1 gives the total protein yield of garlic and onion using fourfold purification steps. This involved precipitation, desalting and liquid chromatographic analysis. Results were tabulated in Table-1. The results exhibited that garlic possessed greater protein yield than the onion. The resultant solution from liquid chromatography was analyzed using BRUKER NMR. DMSO was used as solvent. The proton near the carboxylic group showed a triplet signal around 4.30 ppm and there were a triplet signal around 3.15 ppm for the proton near NH group. There might be triplets of triplet peak for the remaining protons which were slightly merged and messed up Fig. 4. *Allinase* assay was done according to Miron *et al.*[7,8]. The spectrophotometric results were diagrammatically represented in Fig. 5. The results showed 5 % PEG garlic at 0.5 h and showed high peak *allinase* activity.

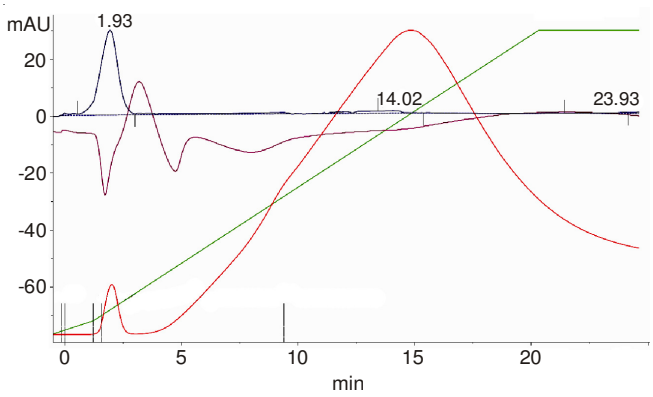

Fig. 3. Liquid chromatography elution profile through Sephadex G-25

TABLE-1
TOTAL PROTEIN YIELD

S.No.	Steps	Protein		Units		Specific activity		Recovery		Purification	
		Garlic	Onion	Garlic	Onion	Garlic	Onion	Garlic	Onion	Garlic	Onion
1	Raw sample	6.2 g	1.6 g	365	123	0.14	0.11	100	98	1.0	1
2	PEG precipitation	2.2 g	0.9 g	91	47	0.11	0.9	25	25	0.8	0.8
3	Desalting	1.9 g	0.8 g	81	28	0.11	0.9	20	22	0.8	0.6
4	Liquid chromatography	1.6 mg	0.6 mg	53	13	6.9	1.1	14	9	4.9	2.2

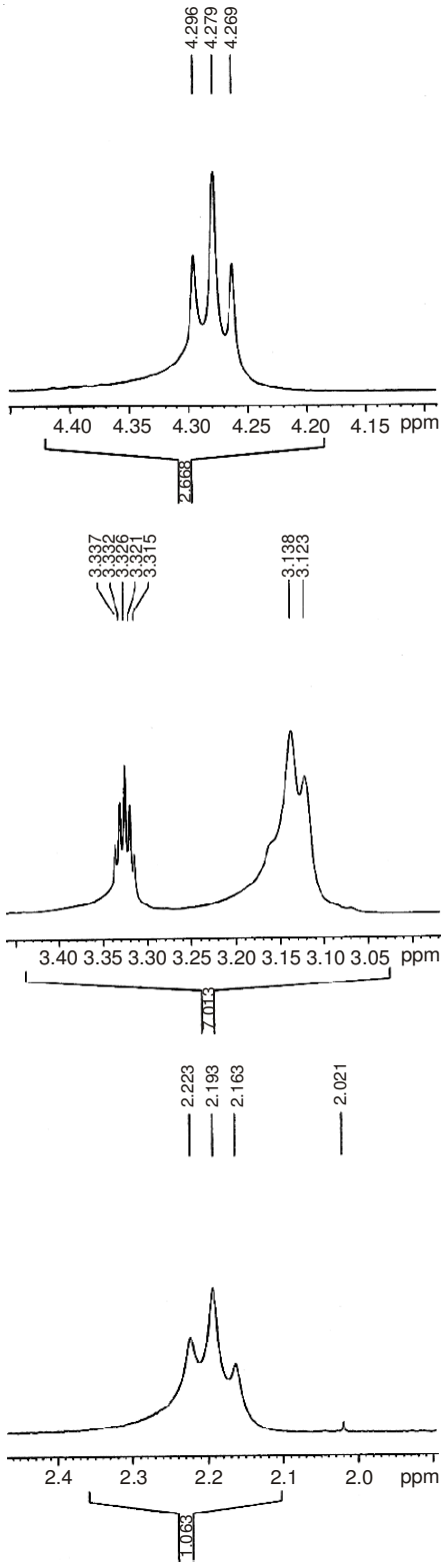

Fig. 4. Alliin NMR spectra

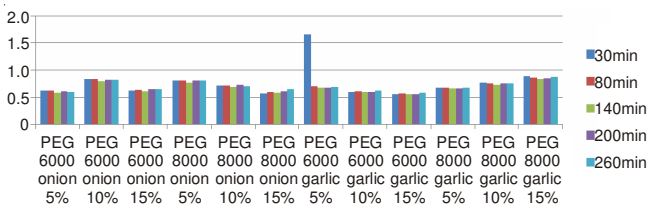

Fig. 5. Allinase assay

Conclusion

Since there are many secondary metabolites in allium plants, the isolation of a single metabolite is a challenging task, leading to the isolation of *allinase* catalysing reaction that can help significantly to isolate a single desired metabolite. Method discussed in this paper can help researchers in getting a greater yield of enzyme and a product for a better drug design and development.

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