Identification and Partial Purification of GAE in Cymbopogon Specieses

Anil Kumar
Department of Biotechnology Engineering and Technology,
Rama University, Uttar Pradesh, Kanpur, India.
anil.biotech@gmail.com

***Abstract*— Citronella and palmarosa leaf geranyl acetate esterase, GAE is identified and partially purified in vitro. The molecular weight of citronella GAE is determined with SDS-PAGE and with SG-150 column chromatography and it is 40 kDa. GAE has shown its optimum activity at pH 8.0, with sodium phosphate buffer. The GAE enzyme activity of citronella and palmarosa crude protein has 1.53 IU/mg and 1.85 IU/mg respectively. After the protein concentration/precipitation with ammonium sulphate (20-65%) the GAE enzyme activity has increased and it was reached to 2.52 and 3.25IU/mg respectively. A spectrophotometric enzyme assay for the GAE is developed at 405 nm maximum absorbance. GAE is showed its optimum enzyme activity at 35ºC with a time optimum for 20 min. This study has novelty and not mentioned yet in literature.**

Keywords*—GAE (Geranyl acetate esterase), Aromatic grasses, Esterase, Geraniol, Geranyl acetate.** ***(key words)

I. INTRODUCTION

Esterase is a set of hydrolytic enzymes that breaks ester bonds to alcohol and acid by addition of water and are universally present in different plants, fungi and animals. Different types of esterases such as pectin methyl esterase (PME) and acetylesterase (AE) have been identified in the organisms. Animal esterases are well studied and are catalytically and functionally classified into different groups while plant esterases have been viewed heedlessly and noted as biochemical markers for morphogenesis and genetic characterization of plants etc. Some volatile oil plants especially *Cymbopogon* species constitutively synthesize esterases to chop monoterpene esters, geranyl acetate, citronellyl acetate and linalyl which are the most common acyclic monoterpene esters. The plants synthesize esterases and some volatile mono to sesquiterpene to bear adverse conditions underneath certain ecological conditions such as herbivory, wounding, drought and flood.

The commercial use of esterase in plants is to the degradation of pectin during ripening of the fruit such as Pectin methylesterase is the most profuse esterase in the orange peel which hydrolyses the pectin to methanol and polygalacturonic acid during the ripening of fruit.

In *Cymbopogon* sp. geranyl acetate esterase, GAE (Shalit *et al.*, 2003) has been isolated which slowly hydrolyzes Geranyl acetate into geraniol during leaf development. The similar report of GAE is found in palamarosa (*Cymbopogon martinii*) inflorescence (Dubey and Luthra, 2001; Dubey *et al.*, 2003). In this report it has noticed that GAE plays a crucial role in the formation of geraniol during leaf development. In addition, to leaf development GAE also played different regulatory controls at organ, cellular, subcellular and enzyme levels for the controlling the balance level of geranyl acetate and geraniol.

In the present study the identification of esterase in the *Cymbopogon* sp. is reported herewith, since *Cymbopogon* sp. is well known aromatic grasses. A lot of secondary metabolites have been reported in theses grasses (Sangwan et al., 1989, 90, 91).

All the *Cymbopogons* grasses are well known for their high contents of essential oil, monoterpenes, phenolics etc. Theses active biomolecules are widely used in perfumery, cosmetics, soaps, detergents, sunscreen, confectionery and in synthesis of several vitamins (D. Ganjewala, 2008). Essential oil of these grasses was used as a mosquito replants since ancient times.

The entire plan of work and objectives of the present study is to identification and isolation and of esterase (especially, GAE) from the *Cymbopogon sps.* (Mainly in *Cymbopogon winterianus* and *Cymbopogon martini*).

II. REVIEWS AND LITERATURES

In immature palmarosa (*Cymbopogon martinii*, var. *motia*) inflorescence with unopened spikelets it has been noted that proportion of geranyl acetate in the oil decreased significantly with a corresponding increase of geraniol, during inflorescence development. An esterase (GAE) activity has been reported in the transformation of geranyl acetate to geraniol, with the use of gas chromatographic technique. The geranyl acetate esterase (GAE), has found active in the alkaline pH optimum at pH 8.5. The time linearity catalysis of GAE has noted for 6 hours. The GAE enzymatic preparation and isolation and its physiological activities has mentioned in this report (V. S. Dubey and R. Luthra., 2001).
In the lemongrass (*Cymbopogon flexuosus*, mutant cv. GRL-1) leaves it has been reported that the composition of geraniol and geranyl acetate in the essential oil varied throughout its development (D. Ganjewala and R. Luthra, 2009). The amount of geranyl acetate and geraniol in the essential oil has been mentioned at day 10 after leaf appearance. The magnitude of

geranyl acetate and geraniol had just about 59% and 33% correspondingly. Nevertheless, the level of geranyl acetate has noted down from roughly 59 to 3% while the level of geraniol grown up from something like 33 to 91% throughout the leaf growth period from 10 to 50 days. Still, the turn down in the level of geranyl acetate was most marked in the early days (10 to 30) stage of leaf development. The fashion of changes in the proportion of geranyl acetate and geraniol has been evidently showed the role of an esterase that has been concerned in the conversion of geranyl acetate to geraniol during leaf development. In this report it has been mentioned about the isolation GAE esterase from leaves of different. It has reported that GAE activity obviously changed during the leaf development. It had been a clear linked with the monoterpene (geranyl acetate and geraniol) ratio during leaf development. GAE had been an optimum pH at 8.5 (sodium phosphate buffer) and temperature at 30 ºC (D. Ganjewala and R. Luthra, 2009).

The essential oil isolated from the *C. martini* has been identified by the use of x-ray diffraction of its 1:1 solvate with deuteriochloroform (Bottini et al. 1987). Only immature palmarosa (*C. martini*) Wats. Inflorescence with raw spikelets has been accumulated essential oil significantly. Geraniol and geranyl acetate together proportion about 90% of palmarosa oil. The proportion of geranyl acetate in the oil decreased significantly with a corresponding increase of geraniol during inflorescence development. An esterase activity has been implicated in the conversion of geranyl acetate to geraniol, was found from

the immature inflorescence via a gas chromatography method. The enzyme, named as geranyl acetate esterase (GAE), had found was active in the alkaline pH range with the optimum at pH 8.5. The catalysis of geranyl acetate had been found linearly far up to 6 hours, and after 24 hours of incubation, 75% of the geranyl acetate incubated had been hydrolyzed in this report (Dubey and Luthra 2001).

III. MATERIALS AND METHODS:

The chapter deals with wide metabolic application of esterase have impelled to study the in selected aromatic plant citronella and palmarosa. It is proposed to carry out the identification and isolation of esterase for description of their activity. Since some of the technical elements were specific to plant resource of citronella and palmarosa to the identification of esterase, the materials and methods for their investigation are presented in the following deferent sections.

A. Plant Material

Citronella and palmarosa plants were grown at the experimental farm of CIMAP, Lucknow (India) following standard agronomic practices. Young and mature leaves were harvested for the identification and isolation of the esterase enzyme.

B. Chemicals

All biochemicals and reagents like chromogenic substrates (geranyl acetate), buffers components etc. were purchased from Sigma Chemicals Co. USA and Fluka India Pvt.Ltd. etc. Other specifically used chemicals/biochemicals, tools and techniques were find their description at required place. All the buffers and reagents were prepared in triple distilled water made in Quartz-condenser distillation unit (M/s Bhanu Scientific Co., India).

C. Optimization of enzyme isolation and assay

To separate optimal extraction of esterase from citronella and palmarosa leaf, the enzyme was isolated using extraction buffers of different pH- 8.0 mM Sodium phosphate of 100 mM contained 10 mM β-mercaptoethanol, 5 mM thiourea and 2 mM EDTA. The homogenate was centrifuged at 10,000 x g for 30 min at 4 °C. The enzyme activity was assayed in the collected supernatant and the total protein content was estimated to calculate specific activity of the enzyme. An enzyme assays for esterase was carried out under optimum conditions with respect to time and temperature.

D. Enzyme extraction

All the steps of enzyme extraction and purification were carried out 0-4 °C unless specified otherwise. Leaf tissue (18 g) (Citronella and palmarosa was immediately surface rinsed with distilled water, de-moistened with a tissue paper and made powder with liquid nitrogen in a pestle-mortar.

GAE was isolated and assayed according to the modified method as described by Dubey and Luthra (2001). GAE was extracted by means of 100 mM sodium phosphate (sodium phosphate) buffer (pH 8.0) containing of 50 mM sodium metabisulphite, 10 mM each of 2-mercaptoethanol and ascorbic acid, 0.25 M sucrose and 1 mM EDTA. Leaf samples (18 g) were ground by mixing of insoluble PVPP (50% w/w) in extraction buffer (1:1 w/v). The homogenate was filtered through four layers of muslin cloth and the filtrate was centrifuged at 10,000 x g (30 min). The clear supernatant (10 ml) was collected. Collected supernatant (crude enzyme) was used for enzyme assayed and enzyme activity.

The supernatant collected was treated with purified amberlite X-AD-4 resin (50% of the tissue weight) for 5 min at 4 °C to remove the endogenous phenolics.

E. Enzyme assay

The esterase activity was assayed by the modified method of Sharma et al. (2008) using $NADH_2$. The assay mixture in a total volume of 1 ml contained 2.5 mM NADH2 (50ul), 100 mM sodium-phosphate buffer (pH 8.0) and the enzyme preparation. The reaction was started by the addition of enzyme and the reaction run at 35 °C for 20 min following which the reaction was stopped by the addition of 750 µl of 1.0 M carbonates and the rate of formation of product was detected in terms of increase in absorbance at 405 nm in a UV-visible spectrophotometer (Perkin Elmer). The control, that enclosed all the assay mixture components but wherein the reaction was blocked at time zero, was run parallel to set the background absorbance to zero. One unit of enzyme

activity was defined as that catalyzing the formation of one µ mole of geraniol formed per minute, under the defined conditions.

F. Protein estimation

Protein estimation was done by the method of Lowry et al. (1951) using bovine serum albumin as reference standard (0.1mg/ml).

IV. Results and Discussions

This section of the dissertation mentions with the results obtained and their discussions during the present study to gather objectives of the study. The results and discussions section are discussed below.

Identification of esterase activity was done in citronella and palmarosa simultaneously with replicates (five no). Zero time control and substrate negative were put parallel with experimental one while the experiment.

In the following table 4.1, GAE enzyme activity of citronella and plamarosa leaf is recorded.

TABLE I. Estimation of GAE enzyme activity (IU/mg) in crude leaf extract of citronella and palmarosa.

	Esterase activity in citronella crude enzyme			Esterase activity in palmarosa crude enzyme		
Step	**Protein (mg/ml)**	**Activity (IU/ml)**	**Specific activity (IU/mg)**	**Protein (mg/ml)**	**Activity (IU/ml)**	**Specific activity (IU/mg)**
1.	**0.67**	**0.93**	**1.39**	**0.53**	**0.97**	**1.83**
2.	**0.58**	**0.85**	**1.47**	**0.49**	**0.91**	**1.85**
3.	**0.51**	**0.78**	**1.53**	**0.50**	**0.83**	**1.66**
4.	**0.71**	**0.83**	**1.17**	**0.47**	**0.87**	**1.85**
5.	**0.57**	**0.79**	**1.38**	**0.54**	**0.97**	**1.79**

A. Partition of GAE esterase protein with ammonium sulphate

Crude protein from citronella and palmarosa was partionated with the 20-65% of ammonium sulphate addition. Precipitated protein was desalted with SG-20 column chromatography. The esterase activity in the active fractions was increased than crude protein samples. SG-20 passed GAE enzyme was showed greater enzyme activity than the crude protein might be due to concentrated of protein. The obtained GAE enzyme activity is mentioned in the following table 4.2.

TABLE II. Estimation of GAE enzyme activity (IU/mg) in SG-20 passed enzyme of citronella and palmarosa.

	Esterase activity in citronella in partial purified (SG-20 passed) enzyme			Esterase activity in palmarosa in partial purified (SG-20 passed) enzyme		
Step	**Protein (mg/ml)**	**Activity (IU/ml)**	**Specific activity (IU/mg)**	**Protein (mg/ml)**	**Activity (IU/ml)**	**Specific activity (IU/mg)**
1.	**0.34**	**0.83**	**2.44**	**0.29**	**0.82**	**2.82**
2.	**0.35**	**0.86**	**2.45**	**0.32**	**0.95**	**2.96**
3.	**0.42**	**1.06**	**2.52**	**0.40**	**1.30**	**3.25**
4.	**0.44**	**0.97**	**2.20**	**0.50**	**1.03**	**2.06**
5.	**0.49**	**0.87**	**1.77**	**0.52**	**0.91**	**1.75**

The molecular weight of GAE was determined with SG-150 column chromatography with standard protein markers (BSA, oval albumin and Ribonuclease A). The obtained molecular weight of GAE was 40 kDa. The native molecular weight determination of GAE was done in citronella but it could not be done in the case of palmarosa..

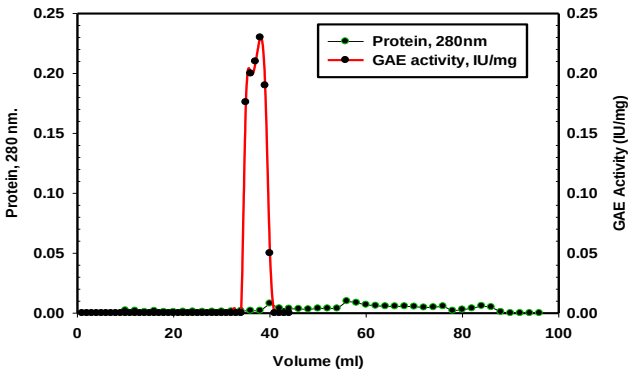

Fig. 4.1, SG-150 chromatograph of GAE protein and enzyme in citronella leaf.

The following chromatogram (4.1) was obtained from the gel filtration chromatography (SG-150) of GAE in citronella

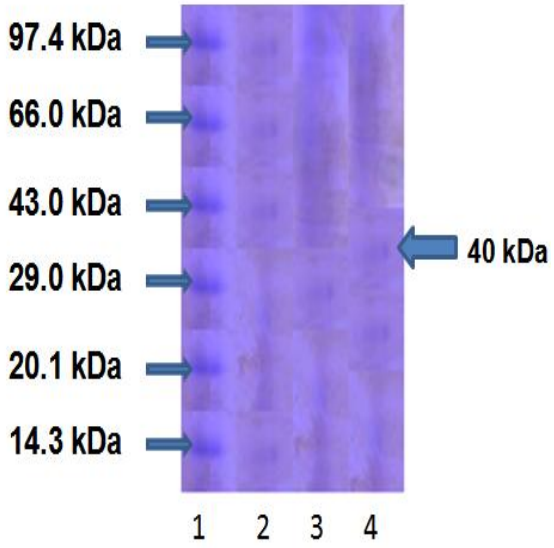

Fig. 4.2, Coomassie stained SDS-PAGE gel the citronella and palmarosa GAE enzyme preparation in crude protein. Lanes: 1, standard molecular weight marker mix; 2, crude enzyme of citronella; 3, crude enzyme preparation of palmarosa; 4, Partial purified GAE enzyme of citronella.

In overall conclusion, this study provides basic biochemical analysis of a vegetative esterase from aromatic plants (*Cymbopogon winterianus* and *Cymbopogon martinii*). GAE governs the proportation of several acyclic monoterpenol such as citronellol and geraniol nerol etc in plants. To obtain a defined image and role of the esterase in aromatic grasses, and its regulation, additional studies in intensity is required.

V. Summary and Conclusions

Geranyl acetate (ester) breaking esterase (GAE) activity is noticed in the leaf extracts of the *Cymbopogon winterianus* and *Cymbopogon martinii*. The esterase enzyme was partially purified and separated on SDS-PAGE showing several bands on gel. GAE is showed its highest enzyme activity in the palmarosa leaf than citronella. The molecular weight of GAE in citronella was found 40 kDa.The activity pattern of GAE evidently suggests its participation in the monoterpene composition on developmental and growth phages of the leaf. Identification and partial purification of GAE in citronella and palmarosa is a novel finding not reported in literature yet. Further investigation, purification and characterization of GAE are requires deep study to known the exact role of GAE in citronella and plamarosa.

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