PURIFICATION AND CHARACTERIZATION OF PHOSPHOLIPASE FROM CABBAGE LEAF (*BRASSICA OLERACEA* L.)

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Abstract

Phospholipase (EC 3.1.4.4) is wide spread distribution in animals and plants. Phospholipase can occur naturally in cabbage leaves, peanut seeds, rat liver, human liver, snake liver, etc. Phospholipase enzyme catalyzed the hydrolysis of phospholipids to phosphatidic acid and the corresponding free base. Phospholipase activities are present in all organisms from bacteria to mammals. Phospholipase enzyme from cabbage leaves was with sodium chloride salt solution, successive ammonium sulphate (40 and 60%), finally crude phospholipase extract (250 mL) was obtained. Further purification was carried by using Sephadex G-200 gel filtration technique. The eluents (fraction numbers) were analyzed for protein content (280 nm) and phospholipase activity (558 nm). The fraction (32-42) showing the highest phospholipase activity were pooled and subsequent studies were done using this pooled solution. The protein contents of the enzyme was determined by using Biuret method. The wavelength of maximum absorption of copper-protein complex in Biuret method was found to be 550 nm. After purification, the specific activity, the relative purity of the enzyme, increased about (7) folds from crude to final purification step. In this research, the molecular weight of the purified phospholipase enzyme was determined by using SDS-PAGE Technique. The purity and homogenity of the phospholipase enzyme were confimed as a single band on gel electrophoresis chromatogram. In this research, effect of metal ions (Mn^{2+}, K^{+}, Na^{+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Hg^{2+}) on phospholipase activities were stud~~ied~~. The Ca^{2+} ion showed the highest activating effect on phospholipase activities. The Pb^{2+} and Hg^{2+} lead to the total denaturing of the enzyme proteins.

Keywords: Phospholipase, cabbage leaf, phosphatidyl choline, SDS-PAGE, phospholipids, Sephadex G-200 gel chromatograph~~y~~

Introduction

The aim of this work is to study isolation, purification and some enzymic characterization of phospholipase from cabbage leaf. The cabbage (*Brassica oleracea* Capitata Group) is a plant of the family *Brassicaceae or Cruciferae*. It is an herbaceous, biennial, and dicotyledonous flowering plant with leaves forming a characteristic compact cluster. Cabbages grown late in autumn and in the beginning of winter are called coleworts. Cabbage is a leaf vegetable. Cabbage has a long history of use both as a food and a medicine. Cabbage is an excellent source of vitamin C. It is also a very good source of fiber, manganese, folate, vitaminB6, potassium and omega-3 fatty acids. Cabbage is also a good source of thiamin (vitamin B1), riboflavin (vitamin B2), calcium, potassium, magnesium, vitamin A and protein. Cabbage also contains phytochemicals called indoles and sulforaphane, the breakdown products of compounds called glucosinolates (Ensminger, 1986). Cabbage leaves are used to treat acute inflammation. A paste of raw cabbage may be placed in a cabbage leaf and wrapped around the affected area to reduce discomfort. Some that it is effective for relieving painfully engorged breasts in breastfeeding women. Cabbage contains significant amounts of glutamine, amino acids, which has anti-inflammatory properties. It is a source of indol-3-carbin or I3C, a compound used as an adjuvent therapy for recurrent respiratory papillomatosis, a disease of the head and neck caused by human papilloma virus (usually type 6 and 11) that causes growths in the airway that can lead to death (Alison, 2006).

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Phospholipase

A phospholipase is an enzyme that converts phospholipids into fatty acids and other lipophilic substances. There are four major classes, termed A, B, C, D.

Phospholipase A_1**:** A fungal enzyme.

Phospholipase A_2**:** a) This enzyme removes the acyl group at C-2 for prostaglandin and leukotriene synthesis. This enzyme is also present in snake venom. Large amount of lysolecithin is produced after snake bite and acts as a potent haemolytic agent.

b) The enzyme lecithin cholesterol acyl transferase (LCAT) uses this acyl group for esterification of cholesterol.

c) The acyl group at C-2 is mostly unsaturated and prone to oxidation change.

Phospholipase B**:** Also acts at this site but its substrate is a lysophospholipid and not a phospholipid.

Phospholipase C**:** This is membrane bound enzyme. It is activated by certain hormones, generating into sitol-P_3 and diacylglycerol which modify activities of certain enzymes.

Phospholipase D**:** Basically, a plant enzyme is getting the attention of biotechnologists due to its very interesting applications (Dennis, 1991).

These enzymes are distinguished by the type of reaction they catalyze**:**

Phospholipase A

PL A_1- cleaves the SN-1 acyl chain

PL A_2- cleaves the SN-2 acyl chain

Phospholipase B- cleaves both SN-1 and SN-2 acyl chain, also known as lysophospholipase.

Phospholipase C- cleaves before the phosphate, releasing diacylglycerol and a phosphate – containing head group. Phospholipase C plays a central in signal transduction, releasing the second messenger Inositol triphosphate.

Phospholipase D-cleaves after the phosphate, releasing phosphatidic acid and an alcohol.

Occurrences of Phospholipase

Phospholipase D (PLD, phosphatidylcholine phosphatido-hydrolase, EC 3.1.4.4) has a very broad distribution in living organisms. It was first isolated in various kind of cabbage and has since been recognized in a number of plants including *ricinus*, castor beans, spinach leaves, soy beans and others (Madorey, 1997). Numerous bacterial sources are rich in PLD. The enzyme can be obtained in the culture broth of various *Streptomyces* (Ulbrich, 2003).

Phospholipase-catalyzed Reaction

Phospholipase enzyme exhibits two types of reaction are hydrolysis and transphosphatidylation. When this enzyme hydrolyzes phospholipids, such as phosphatidyl-choline (PC), it forms phosphatidic acid (PA) and choline (Liscovitch, 2000). Phosphatidic acid is an important second messenger in mammalian signal transduction pathways. Many PLDs, in the presence of an alcohol, are able to catalyze the exchange of the polar head group in addition to the hydrolysis product. Transphosphatidylation occurs with different degree of selectivity depending on the enzymatic source, the nature of the alcohol and its concentration (Yang, 1967).

Application of PLDs, PLs and PAs

Phospholipids are present in all living organisms. They are a major component of all biological membranes, along with glycolipids and cholesterol. Enzymes aimed at modifying phospholipids, namely, phospholipase, are consequently wide spreading nature. Phospholipase (A_1, A_2, C and D) are a complex and crucially important group of enzymes that hydrolyze phospholipids (PLs) releasing a variety of products, like for example lyso-phospholipids, free fatty acids (FFAs), diacylglycerols (DGs), choline phosphate and phosphatidates, depending on the site of hydrolysis. They play crucial roles in many biochemical processes related among others, digestion and inflammation (Shimizu *et al*, 2006). The use in industrial processes is increasing through the ability of optimizing the enzyme by protein engineering. Nowadays, phospholipase play key roles in bread making, egg yolk industry (emulsification for different applications) and refinement of vegetable oils (degumming). Phospholipids (PLs) are the constituents of all biological membranes. Due to their superior emulsification properties, phospholipids and their partial hydrolysis products, lysophospholipids, have numerous applications in food, cosmetics, pharmaceutical and other industries (Vance, 2002). Their properties depend on the fatty acid components and the polar component bound to the glycerol backbone. By changing the hydrophilic/lipohilic balance of phospholipids using lipase or phospholipases, it is possible to produce tailor made lecithin for specific applications (Cichowiez, 1993). Phosphatidic acid (PA) is a useful starting material for chemical synthesis of phospholipids. The chemical acylation of sn-glycrol-3-phosphate is the simplest way for preparing desaturated phosphatidic acid species. The most common methods for the preparation of phosphatidic acid containing unsaturated fatty acids are the extraction from wheat germ and the enzymatic degradation of phospholipids. This last reaction is catalyzed by phospholipase D (Hajdu, 2007).

Materials and Methods

In this research, cabbage sample was collected from local market. The mixture of light green inner leaves of cabbage (200 g) small pieces and 40% of sodium chloride solution were placed in a blender to blend this mixture. The solution mixture was filtered through a filter paper (first filtrate). The filtrate was taken and mixed with 50 g of sodium chloride. And then the solution mixture was filtered through a filter paper (second filtrate). A 72.68 g of ammonium sulphate was added into the 300 mL of filtrate to get 40% saturation. The solution was allowed to stand for 1hr and then filtration was carried out (third filtrate). The 250 mL of filtrate was fractionated by adding 32.5 g of solid ammonium sulphate to give 60 % saturation (fourth filtrate). The extract solution was placed in the refrigerator at 4·C. Phospholipase activity in the fourth filtrate enzyme solution fraction was determined by mixing 0.5mg/mL phosphatidyl choline, 0.3 mM sodium dodecyl sulphate, 0.1 mM phenol red and 40 mM calcium chloride in a test tube and incubating at room temperature for 10 min, and absorption spectra were recorded between 500 to 600 nm by UV-visible spectrophotometer (Hoppe, 1992). The maximum phospholipase activity was found at fourth filtrate. The wavelength of maximum absorption of copper-protein complex ~~was~~ determined by Biuret method was found at 550 nm. Crude phospholipase 2.0 mL was applied to a Sephadex G-200 gel filtration column previously equilibrated with the same medium. The flow rate was adjusted to 2 mL per 5 min. After collection of 2 mL fraction, the protein content of each tube was checked by measuring the absorbance at 280 nm wavelength using a UV-visible spectrophotometer. The enzyme in each test tube was also measured for phospholipase activity. The fraction which had the highest phospholipase activity was pooled. The pooled phospholipase fraction was measured for protein content by the Biuret method.

In this research, determination of molecular weight of the purified phospholipase was carried out by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis techniques in

European molecular biology Laboratory, Heidelberg, Germany. Before electrophoresis, purification of the crude phospholipase was carried out by Sephacryl S-200 Gel and Sepharose 6-B chromatographic technique. Activating and inhibiting effect of metal ions (Na^+, K^+, Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}) 1 x 10^{-2} M on phospholipase activities was determined by UV-visible spectrophotometric method.

Results and Discussion

Extraction of Phospholipase from Cabbage Leaf

Phospholipase was first identified in plants (Heller, 1978). Phospholipase is a widely distributed enzyme, occurring in various bacteria, fungi, animals and unrelated plants as cabbage, soy beans and maple trees, etc. (Tookey, 1956). The phospholipids of seeds disappear during germination, reappearing as water–soluble choline and other compounds, thus suggesting a general role for the enzyme in the utilization of reserve phospholipid. Plant phospholipase has the great potential for industrial applications such as food additives, industrial reagents and medical applications (Woodman, 2003). In this work, phospholipase enzyme was extracted from cabbage leaves by using the sodium chloride solution (40%) and two successive ammonium sulphate precipitations (40 and 60 %). Since ammonium sulphate has little effect on enzyme activity and in some cases stabilizes the enzyme, it is useful as the salt of choice in most isolation of enzyme (Seamen, 1963). Thus, it was used in the present isolation.

Phospholipase Activity of the Enzyme Solution

Phospholipase activity was determined spectrophotomatically using phosphatidyl choline (lecithin) as substrate. Figure 1 showed the reaction of phospholipase. Phospholipase enzyme catalyzed the hydrolysis of phospholipids to phosphatidic acid and the corresponding free base. In this research, the spectrophotometric assay was applied using phenol red indicator. The rate of the disappearance of the indicator was used to measure the rate of hydrolysis of the phosphatidyl choline substrate. Hydrolysis of lecithin can affect to decrease the pH due to the ionization of the new phosphate group, causing the indicator to change colour from red to yellow. Figure 2 showed absorption spectra of phenol red solutions under different concentrations. The wavelength of maximum absorption phenol red was found at 558 nm when 0.1 mL of phospholipase enzyme solution was added into the phenol red solution, spectra changed (after 10 min, 20 min, 30 min, 1 h and 2 h in different reaction time). Phospholipase activity was defined as changes of 0.001 absorbance unit at 558 nm per mL of enzyme solution per min of reaction time.

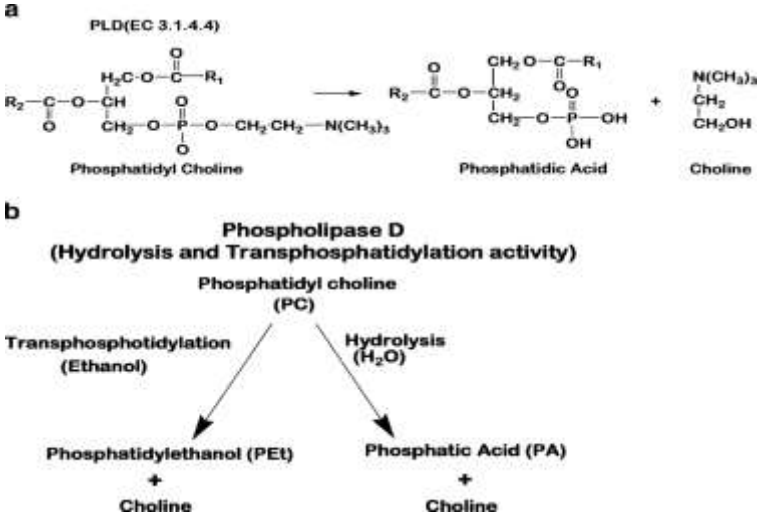

Figure 1 Reaction of phospholipase

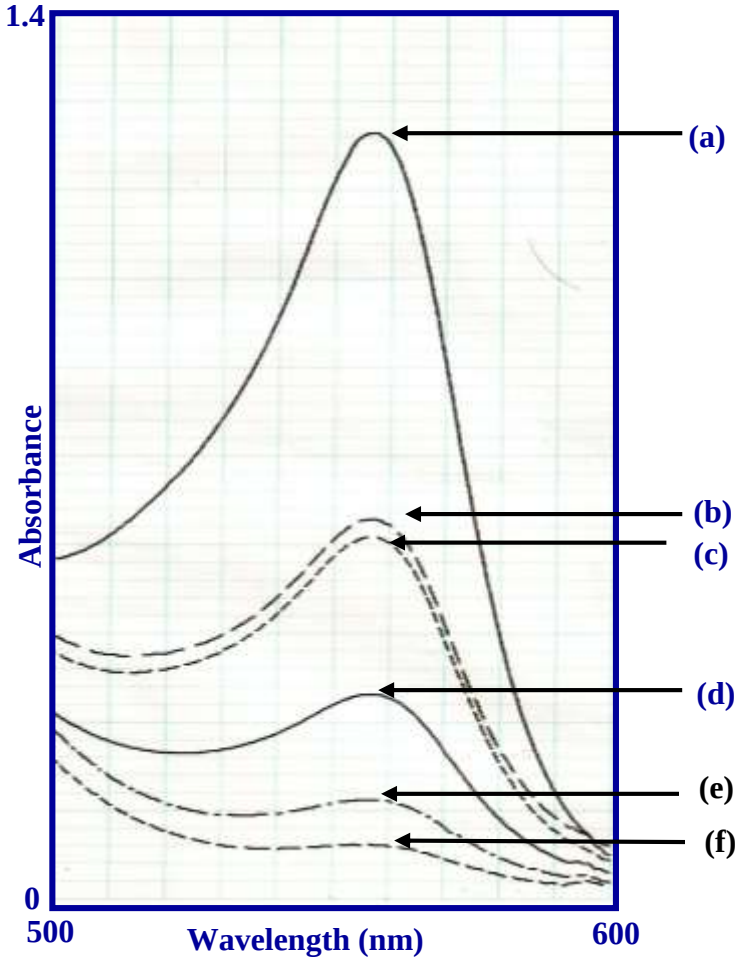

Figure 2 Absorption spectra of phenol red solution
(a) before adding enzyme solution
(b) after adding enzyme solution of crude extract (0.1) mL (reaction time 10 min)
(c) after adding enzyme solution of crude extract (0.1) mL (reaction time 20 min)
(d) after adding enzyme solution of crude extract (0.1) mL (reaction time 30 min)
(e) after adding enzyme solution of crude extract (0.1) mL (reaction time 1 h)
(f) after adding enzyme solution of crude extract (0.1) mL (reaction time 2 h)

Phospholipase Activity, Protein Content and Specific Activity of the Enzyme Solutions

In this research, Sephadex G-200 gel chromatographic purification was carried out. The phospholipase activity was determined by using the spectrophotometric method and protein content of the enzyme solution was determined by using the Biuret method at final purification step. The phospholipase activity was found to be 2.265 EU per gram of cabbage leaf and specific activity was 271.37 μmol min^{-1} mg of protein. Table 1 showed phospholipase activity, protein contents and specific activity of the enzyme solution at different purification steps. Table 2 showed variation of protein absorbance at 280 nm and phospholipase activity with fractions numbers in Sephadex G-200 chromatography. Figure 3 showed the purification of crude phospholipase enzyme by G-200 gel filtration chromatography.

Table 1 Phospholipase Activities, Protein Contents and Specific activities of Enzyme Solutions at Different Purification Steps

No	Purification Steps	Protein Content (mg/mL)	Phospholipase Activity (μmol min^{-1} mL^{-1})	Total Phospholipase Activity (μmol min^{-1} mL^{-1})	Specific Activity (μmol min^{-1} mg^{-1})	Protein Recovery (%)	Degree of Purity (fold)
1	Crude extract	4.257	370	55500	39.93	100	1
2	After purification with 40% ammonium sulphate	2.877	439	52680	152.59	94.92	3.61
3	After purification with 60% ammonium sulphate	2.760	564	50760	204.35	91.46	4.84
4	After passing the Sephadex G-200 column (32-42)	1.404	381	3733.8	271.37	6.73	6.80

Table 2 Variation of Protein Absorbance at 280 nm and Calculated Phospholipase Activity with Fractions Numbers in Sephadex G- 200 Gel Chromatography

Fraction Number	Protein Absorbance at 280 nm	Phospholipase Activity (μmol min^{-1} mL^{-1})
1	0.005	0
3	0.007	0
5	0.008	0
7	0.016	0
9	0.046	0
11	0.072	0
13	0.082	0
15	0.126	1
17	0.487	5
19	0.616	10
21	0.516	103
23	0.273	105
25	0.358	110
27	0.323	118
29	0.355	145
31	0.303	175
33	0.277	204
35	0.308	227
37	0.210	253
39	0.261	222
41	0.334	95

Fraction Number	Protein Absorbance at 280 nm	Phospholipase Activity (μmol min^{-1} mL^{-1})
43	0.233	101
45	0.240	97
47	0.249	98
49	0.235	89
51	0.205	23
53	0.185	0
55	0.087	0
57	0.085	0
59	0.080	0
61	0.007	0

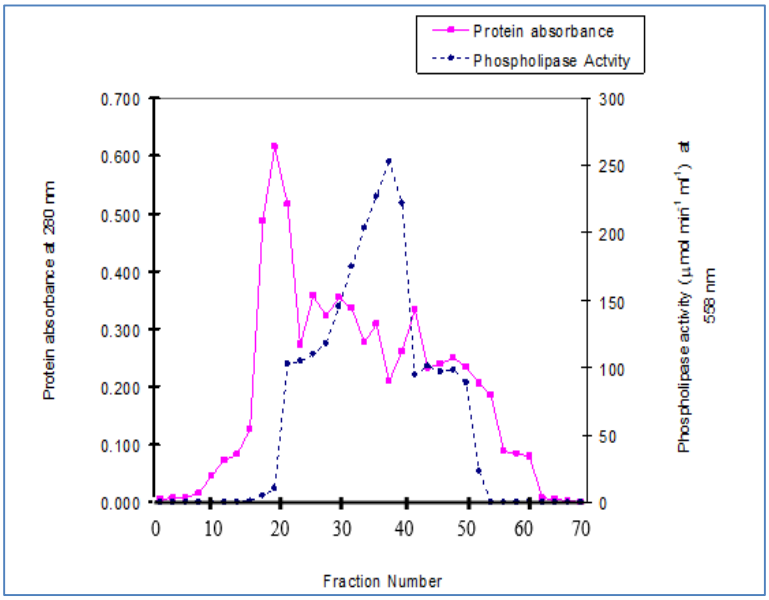

Figure 3 Purification of crude phospholipase enzyme by gel filtration chromatography

Molecular Weight of Purified Phospholipase Enzyme

The molecular weight of a protein may be determined by a variety of methods (Murray, 1993). The most accurate molecular weights are those obtained from sequence studies, a procedure hardly suitable in routine studies. Usually, molecular weight is obtained by methods involving the analytical ultracentrifuge and other methods; gel filtration, osmotic pressure, light scattering, electron microscopy, etc., have been employed (Scopes, 1994). Molecular weights are best determined when both the band of interest and the standards appear as sharp, narrow bands so that there is no mistake as to where to measure the migration distance. Maximal resolution is obtained with low protein loads. In this study, protein from the pharmacia high molecular weight (HMW) calibration kit: thyroglobulin (667,500), ferritin (439,000), urease trimer (230,000), β-glactosidase (139,000) and fructose-6-phosphate kinase (66,500) were used for molecular weight determination by SDS-PAGE (Table 3, Figure 4). The homogeneity of the purified phospholipase was confirmed by (SDS-PAGE). The use of polyacrylamide gel electrophoresis for determining protein molecular weights has become a routine laboratory technique (Pharmacia Inc, 1987). The molecular weight of a protein under investigation is determined by comparing its electrophoretic mobility with that of protein standards of known molecular weights. The principal limitation of the method, as related to protein work, appears to be its relatively low molecular weight exclusion characteristics. The

purified phospholipase enzyme showed a single band on SDS-PAGE where the molecular weight of purified phospholipase was located near the standard protein (mol.wt. 230,000). The molecular weight of purified phospholipase was measured according to the method of Halim and Smith (Smith, 1975). An estimated molecular weight of purified phospholipase from cabbage leaves sample was found as 158,489 Daltons from the log of known HMW marker proteins vs R_f values from SDS-PAGE (Figure 5).

Table 3 Relationship between Log of Molecular Weight of Standard Marker Proteins and Relative Mobility (R_f) Value Obtained from SDS – PAGE

No	Standard HMW marker proteins	MW (Daltons)	Log of MW	R_f
1	Thyroglobulin	667,500	5.8244	0.04
2	Ferritin	439,000	5.6425	0.13
3	Urease trimer	230,000	5.3617	0.33
4	β-Glactosidase	139,000	5.1430	0.44
5	Fructose-6-phosphate kinase	66,500	4.8228	0.63

The R_f of phospholipase was found to be 0.39 so that the molecular weight was determined to be 158, 489 Daltons (Figure 5).

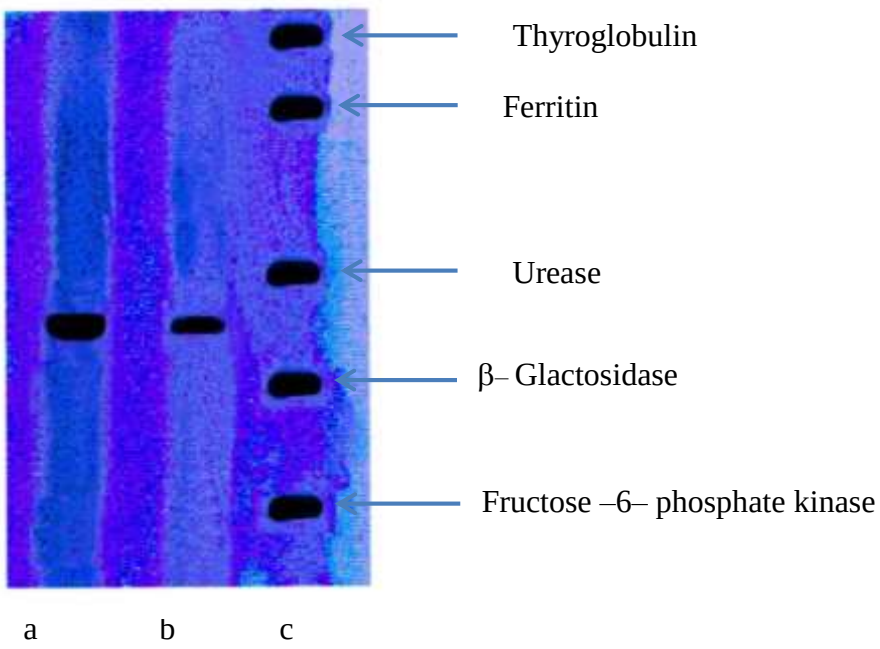

Figure 4 Photograph of sodium dodecyl sulphate polyacrylamide gel electrophoresis
a. Purified phospholipase fraction obtained from Sephadex G-200
b. Purfied phospholipase fraction obtained after successive purifications with Sephacryl S–200 and Sepharose 6–B
c. High molecular weight marker proteins

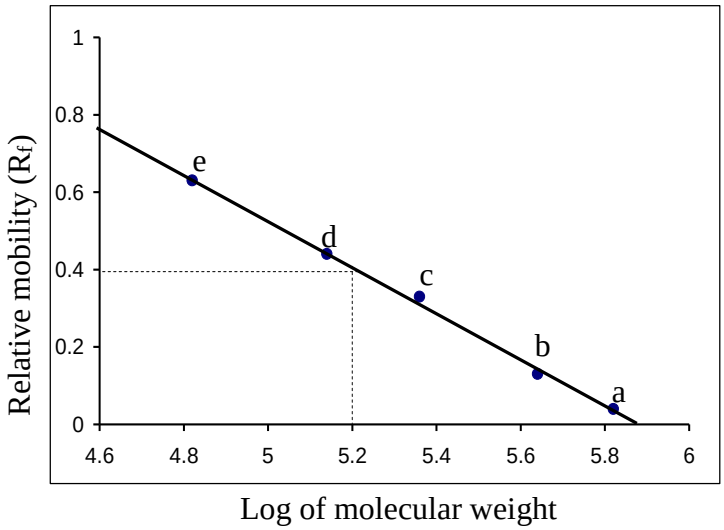

Figure 5 Plot of log of molecular weight of high molecular weight (HMW) marker proteins as a function of relative mobility (R_f) obtained from SDS – PAGE
(a) Thyroglobulin
(b) Ferritin
(c) Urease trimer
(d) β– Glactosidase
(e) Fructose – 6 – phosphate kinase

Effect of Some Metals Ions on Phospholipase Activity

In this research, effect of some metal ions (Mn^{2+}, K^{+}, Na^{+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, $Pb^{2+}$and Hg^{2+}) on phospholipase activity was studied. The metal ions; Mn^{2+}, K^{+}, Na^{+}, and Ca^{2+} were found to be activators (Table 4, Figure 7). Among them, Ca^{2+} showed highest degree of activation (20.64%). In general, salt solutions of alkali and alkaline earth metal ions can stabilize the native structure of enzyme protein in solution. Therefore, phospholipase activity increased in the presence of alkali and alkaline earth metal ions. A Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, and Hg^{2+} ions exhibited as inhibitors. In the presence of 1 x 10^{-2} M Pb^{2+} and Hg^{2+} ions, phospholipase activity became zero due to the denaturing of enzyme protein by these metal ions. Heavy metals can bind to the –SH, -NH_2, and –OH groups in the protein structure of the enzyme. As a result, deformation occurred in native structure of enzyme protein and the phospholipase activity decreased.

Table 4 Relationship between Metal Ions and Degree of Activation (+)/Inhibition (-)

No	Metal ions	Δ A at 558nm	Phospholipase activity (10^2 μmol $min^{-1}mL^{-1}$)	Degree of activation /inhibition
1	Without	0.281	2.81	-
2	Mn^{2+}	0.293	2.93	+4.27
3	K^+	0.304	3.04	+8.19
4	Na^+	0.336	3.36	+19.85
5	Ca^{2+}	0.339	3.39	+20.64
6	Co^{2+}	0.172	1.72	-38.79
7	Zn^{2+}	0.164	1.64	-41.64
8	Cu^{2+}	0.072	0.72	-74.38
9	Pb^{2+}	0	0	-100
10	Hg^{2+}	0	0	-100

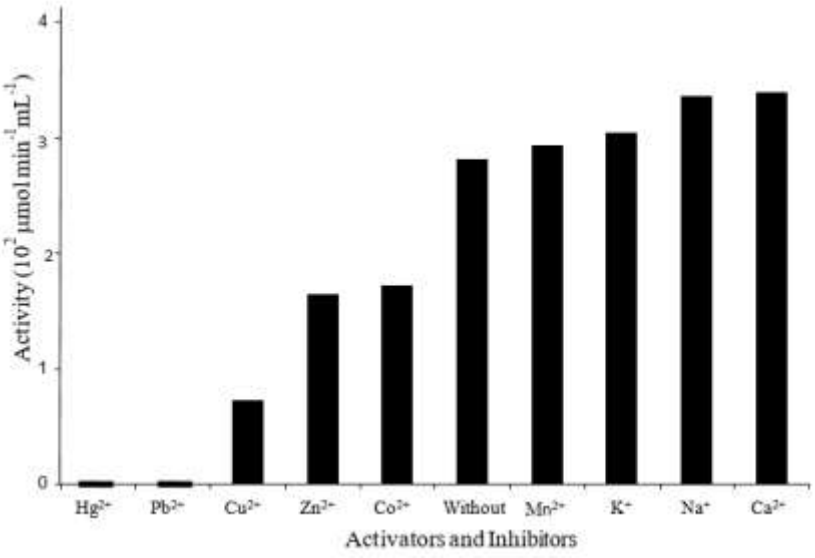

Figure 7 Phospholipase activities in the presence of various inhibitors and activators (1 x 10^{-2} M)

Conclusions

In this research, crude phospholipase enzyme was extracted from cabbage leaf and crude phospholipase extract solution (250 mL) was obtained from 200 g of cabbage leaf. The phospholipase activity in crude extract was to be 2.265 EU per gram of cabbage leaf. After Sephadex G-200 gel chromatographic separation, the specific activity (the relative purity of the enzyme) increased about (7) folds from crude to final purification step. The specific activity was found to be 271.37 EU per mg of enzyme protein at final purification step. The purity of the enzyme was confirmed by SDS-PAGE as a single band. The molecular weight of the purified enzyme as determined by SDS-PAGE technique was 158,489 Dalton. The Mn^{2+}, K^+, Na^+ and Ca^{2+} showed the activating effect on phospholipase activity, whereas Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Hg^{2+} exhibited the inhibitory effect on phospholipase activity. The Ca^{2+} ion ($1x10^{-2}$ M) showed the highest activating effect (20.6%) on phospholipase activity. The Pb^{2+} and Hg^{2+} ($1x10^{-2}$ M) lead to the total denaturing of the enzyme protein and therefore phospholipase activity became zero. Degrees of activation were found to be 4.27%, 8.19%, 19.58% and 20.64% for 0.01 M of Mn^{2+}, K^+, Na^+ and Ca^{2+} ions, respectively. The inhibitory effect of Zn^{2+} ion on phospholipase activity was found to be

non-competitive type (enzyme denature) and degrees of inhibition were found to be 38.79%, 41.64%,74.38%, 100% and 100% for 0.01M of Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Hg^{2+} ions, respectively.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education, Ministry of Education, Myanmar, for provision of opportunity to do this research and also to the Myanmar Academy of Arts and Science for allowing to present this paper.

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