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Original article

Immobilization and Kinetic Studies of Bromelain: A Plant Cysteine Protease From Pineapple (*Ananas Comosus*) Plant Parts

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ABSTRACT

Bromelain is a cysteine protease enzyme present in the plants. In this study we isolated the enzyme from pineapple plant parts like leaves, green fruit, ripe fruits and immobilized it by using sodium alginate. We observed the effects of different activators and inhibitors, kinetic properties and different concentrations of substrate and estimated enzyme activity on free and immobilized bromelain from pineapple plant parts with hemoglobin as substrate. The protease enzyme showed an effective temperature of 40-60^{o}C and optimal temperature of 50-60^{0}C, effective p^{H} of 4.0-8.0 and optimal p^{H} is 4.5-5.5. These all properties of enzymes will be useful in keeping enzyme in an active state in various industrial and medical preparations.

KEYWORDS: *Ananas Comosus*, Bromelain, Calcium alginate, Hemoglobin, Immobilization

INTRODUCTION

Proteases constitute the major share in the enzyme industry because of their wide range of clinical and practical applications. These enzymes collectively accounts for 40% of all enzyme sales [1]. Proteases have been found in both monocotyledonous plants like pineapple, cereals and dicotyledonous plants like papaya, milk weed and Euphorbia [2] . Bromelain is a protease enzyme and its isolation was first recorded by the Venezuelan chemist Vicente Marcano in 1891 from the fruit of pineapple [3] . In 1892, Chittenden, assisted by Joslin and Meara, investigated fully and named as a 'bromelian'. It was first introduced as a therapeutic supplement in 1957. Research on bromelain was first conducted in Hawaii but more recently has it has been conducted in countries in Asia, Europe, and Latin America. This enzymes has valuable

therapeutic applications in inflammatory[4], arthritis[5], indigestion, hay fever, ulcers, wound de bridement, prevention of pulmonary edema [6] and in folk medicine. According to literature immobilization has generally been accepted as a complementary method to the existing analytical tools. Immobilized enzymes offer a tremendous scope as they can be separated easily from the solvent system and reused over a long period [7]. The greatest advantage of immobilized enzymes is that they have high stability as compared with the native enzymes . In the present study bromelain from different parts of pineapple was entrapped in the calcium alginate beads and the kinetic properties of the free and immobilized bromelain were studied.

MATERIALS AND METHODS:

Collection of plant material:

Fresh pineapple plant parts were collected from local area (Kulasekhram, Kanyakumari) market. All required chemicals were purchased from (Hi-Media Labs Ltd. Mumbai).

Isolation of bromelain:

Plant parts were washed thoroughly under tap water. Juice of leaves, green fruits and ripe fruits were separately obtained by mechanical grinding and filtering [8]. From the juice bromelain was isolated according to the method suggested by Murachi [9]. The juice was allowed to cool and one volume of acetone was added. The precipitate obtained was discarded. Further precipitation of enzyme with two volumes of cold acetone was done and centrifuged at 30,000 rpm for 5 min. The precipitate was then collected and dried. [10]. The dried product was mixed with sodium citrate buffer and centrifuged at same rpm. The supernatant was then collected and used for the study .

Immobilization of bromelain:

Sodium alginate was used as the immobilizing agent for the bead preparation. Various concentrations of aqueous sterilized sodium alginate solution ranging between 1% to 6% was prepared to find out an optimum concentration for stable bead preparation [11]. The sample was suspended in cooled alginate and the sample-alginate suspension was transferred into a disposable plastic syringe. Then the alginate-sample mixture was extruded drop by drop into a gently stirred 0.2M calcium chloride solution and hardened in this solution for 30 min [12]. The resulting calcium alginate gel beds were thoroughly washed with sterile saline water and used.

Estimation of bromelain activity using various substrates

The estimation involved digestion of denatured hemoglobin by dilute bromelain under standard conditions after which the undigested protein was precipitated with TCA and the precipitate was removed by filtration [13]. The bromelain activity was measured by Folins Phenol reagent [14].

Effect of activators and inhibitors on bromelain activity:

The enzyme was incubated with various thiol specific inhibitors (Potassium ferricyanide, Potassium permanganate, Hydrogen peroxide)[15] and activators (Cysteine, Sodium cyanide, hydrogen sulphide) for one hour at room temperature to observe inhibitory and activator effects on enzyme[16] .

Kinetics of free and immobilized bromelain:

Effect of p^H on bromelain was investigated by digesting 5ml portions of the protein substrate buffered at various p^H ranging from 4.5-9.5. The amount of digestion was measured [17]. The effect of temperature on bromelain was studied at various temperature ranging from 40 to 65^0C of the reaction mixture[18]. The effect of time on the activity of bromelain was studied at various incubation times from 0-30min of the reaction mixture[19]. The enzyme concentration and substrate concentration effect on bromelain activity was studied at different concentrations [20].

RESULTS

The bromelain was immobilized on calcium alginate beds. We obtained best quality beads with 4% alginate solution. Bromelain activity was more towards hemoglobin (leaf, green fruit

, ripe fruit) compared with other substrates. (Table 1). Cysteine has maximum activator activity (2.64, 3.10, 3.29) and potassium ferricyanide has maximum inhibitory activity (0.82, 0.10, 0.52) on bromelain (Table 2&3). Free bromelain from leaf p^H 5.5, green fruit p^H 7.5, ripe fruit p^H 6.5 has maximum activity but Immobilized bromelain green and ripe fruit have maximum activity p^H 7.5 but leaf p^H 6.5 (Table 4). Free and immobilized bromelain have optimum activity temperature 50^0C (Table 5). The activity of free and immobilized bromelain was maximum at 15min (Table 6). Depending on the enzyme concentration the free and immobilized bromelain activity also increased (Table 7). Free and Immobilized bromelain activity increased till substrate concentration reached 0.4 in the leaf, green fruit and ripe fruit (Table 8).

Table 1: *Bromelain activity towards various substrates*

Substrate	Bromelain activity (milli moles of tyrosine per min)		
	Leaf	Green fruit	Ripe fruit
Casein	2.16	3.19	3.28
Ovalbumin	1.86	4.38	6.10
Peptone	1.23	4.02	6.06
Haemoglobin	4.52	4.85	6.19
Gelatin	3.08	3.67	4.06

Table 2: *Effect of activators on bromelain activity*

Reagents	Concentration	Bromelain activity (milli moles of tyrosine per min)		
		Leaf	Green fruit	Ripe fruit
Crude sample	-	2.0 (100%)	2.5 (100%)	2.6 (100%)
Cysteine	0.03M	2.64 (32%)	3.10 (24%)	3.29 (26.5%)
Sodium cyanide	0.1M	2.60 (30%)	3.02 (20.8%)	3.26 (25.3%)
Hydrogen sulphide	0.1M	2.14 (7%)	2.06 (17.6%)	2.52 (3.07%)

Table 3: *Effect of inhibitors on bromelain activity*

Reagents	Concentration	Bromelain activity (milli moles of tyrosine per min)		
		Leaf	Green fruit	Ripe fruit
Potassium Ferricyanide	0.01M	0.82 (59%)	0.10 (96%)	0.52 (80%)
Potassium Permanganate	0.001M	0.11 (94.5%)	0.03 (98.8%)	0.09 (96.5%)
Hydrogen Peroxide	0.003M	0.10 (95%)	0.02 (99.2%)	0.06 (97.6%)

Table 4: *Effect of p^H on free and immobilized bromelain activity*

p^H	Free bromelain activity in (millimoles of tyrosine per min)			Immobilized bromelain activity in (millimoles of tyrosine per min)		
	Leaf	**Green fruit**	**Ripe fruit**	**Leaf**	**Green fruit**	**Ripe fruit**
4.5	1.86	0.50	1.30	1.66	1.30	1.56
5.5	2.63	0.83	2.68	2.20	2.27	2.43
6.5	1.79	1.33	3.11	2.69	2.91	2.93
7.5	1.56	1.87	2.20	2.67	3.70	4.62
8.5	1.37	1.32	1.78	2.55	3.25	3.90
9.5	1.36	0.77	1.32	2.50	2.48	3.19

Table 5: *Effect of temperature on free and Immobilized bromelain activity*

Temperature in ^{0}C	Free bromelain activity in (millimoles of tyrosine per min)			Immobilized bromelain activity in (millimoles of tyrosine per min)		
	Leaf	**Green fruit**	**Ripe fruit**	**Leaf**	**Green fruit**	**Ripe fruit**
40	3.12	2.05	1.99	1.71	1.73	2.89
45	3.21	2.48	4.17	2.69	2.79	3.69
50	6.26	3.33	5.59	3.84	2.94	3.81
55	4.25	3.67	3.78	4.98	4.62	5.58
60	3.64	3.36	3.41	2.91	3.91	4.95
65	2.98	2.05	2.49	2.13	2.88	3.72

Table 6: *Effect of time on free and Immobilized bromelain activity*

Time (min)	Free bromelain activity in (millimoles of tyrosine per min)			Immobilized bromelain activity in (millimoles of tyrosine per min)		
	Leaf	**Green fruit**	**Ripe fruit**	**Leaf**	**Green fruit**	**Ripe fruit**
5	2.02	2.79	3.54	1.99	2.80	3.09
10	3.53	3.10	3.95	2.36	2.85	3.95
15	4.26	4.28	5.32	3.88	4.29	5.69
20	4.26	4.56	5.44	3.88	4.88	5.69
25	4.26	4.56	5.44	3.88	4.88	5.69
30	4.26	4.56	5.44	3.88	4.88	5.69

Table 7: *Effect of enzyme concentration on free and Immobilized bromelain activity*

Enzyme concentration (mg)	Free bromelain activity in (millimoles of tyrosine per min)			Immobilized bromelain activity in (millimoles of tyrosine per min)		
	Leaf	Green fruit	Ripe fruit	Leaf	Green fruit	Ripe fruit
0.5	0.88	1.09	1.29	1.26	1.60	1.67
1.0	1.84	2.05	1.70	2.35	2.17	1.69
1.5	3.09	2.57	2.68	2.69	2.66	2.96
2.0	3.79	3.95	4.31	4.60	4.21	4.47
2.5	5.32	5.55	5.61	5.68	5.67	5.69

Table 8: *Effect of substrate concentration on free and Immobilized bromelain activity*

Substrate concentration (µm)	Free bromelain activity in (millimoles of tyrosine per min)			Immobilized bromelain activity in (millimoles of tyrosine per min)		
	Leaf	Green fruit	Ripe fruit	Leaf	Green fruit	Ripe fruit
0.1	2.37	2.12	3.49	1.75	2.15	3.41
0.2	3.59	2.93	3.49	3.67	4.08	3.79
0.3	4.18	4.85	4.57	5.06	5.44	3.99
0.4	4.26	4.85	5.44	5.73	5.44	5.34
0.5	4.26	4.85	5.44	5.73	5.44	5.34
0.6	4.26	4.85	5.44	5.73	5.44	5.34

DISCUSSION

Bromelain has wide range of medical and industrial uses. It is available on the name of 'Ananase'. This protease enzyme is used as medicine in the treatment of different diseases in the medical field. Bromelain which was first introduced in medical research in 1957, may work by blocking some pro-inflammatory pathways when applied topically and it can be of use in reducing inflammation after surgery. This anti-inflammatory activity is believed to be due to inhibition of neutrophil migration to sites of acute inflammation. As a potential anti-inflammatory agent, it may be of use in treating arthritis it has never been confirmed in human studies for this use. The Natural Medicines Comprehensive Database suggests that bromelain, can used in conjunction with trypsin and rutin and it is as effective as some of the prescription analgesics in the management of osteoarthritis. Bromelain may also be used in a variety of other conditions like hay fever, ulcerative colitis, removal of dead and damaged tissue after debridement in burns, preventing the collection of fluid in the lung pulmonary edema, relaxing muscles, stimulating muscle contractions, slowing clotting of blood, improving the absorption of antibiotics, preventing cancer, shortening delivery time, and helping the body to get rid of fat. However it is not scientifically confirmed and not approved by regulatory authorities like the Food and Drug Administration of USA. These proteolytic enzymes can be used for systemic enzyme therapy in the treatment of plasmacytoma, breast, and colorectal cancer patients. Studies in

mice with experimental colitis, the enzyme was observed to decrease the colonic inflammation. It also showed reduction in the number of cancerous lesions in the colon in the colorectal cancer models. This enzyme can be used in the meat industry for tenderizing. If the enzyme is allowed to act for too long, the meat may become too "mushy" for many consumers' preferences.. This study will give a fundamental idea about the optimum activity of bromelain under different conditions. From this background we conclude that improvement in the use of bromelain in medical as well as other industrial purposes can be obtained by maintaining optimum temperature, time, P^H along with use enzyme activators. There is also an increased requirement of more human studies to expand the use of bromelain enzyme to include treatment of various other diseases.

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