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Extraction, partial purification and characterization of kiwifruit enzyme

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Abstract
Kiwifruit enzyme is a plant cysteine protease enzyme abundantly present in kiwifruit. Protease enzymes are multifunctional class of enzymes and henceforth can be used in different sectors of food industry such as for milk coagulation, for meat tenderization etc. So, present study was conducted for extraction, partial purification and characterization of kiwifruit enzyme from kiwifruit. Kiwifruit enzyme is belonging to same family of thiol proteases as papain and bromelain. The enzyme was estimated at various stages of fruit maturity followed by partial purification and characterization. The supernatant was precipitated with different concentration of ammonium sulphate i.e., from 10-90 %. Characterization of protease enzyme was done by using response surface methodology (RSM). Results showed that maximum enzyme activity was observed at immature stage of fruit and with 60% of ammonium sulphate concentration. The enzyme had an optimum temperature of 45°C, functioned best at pH 8.0. This enzyme can be exploited commercially in food industry.

Keywords: kiwifruit enzyme, protease, extraction, partial purification, characterization

Introduction
Kiwifruit (*Actinidia chinensis*) is native to Southern China (Ferguson, 1984) [5] and was originally called "Yang Tao" in china and "Chinese Gooseberry" in rest of world. Its cultivation were spread from China in the early 20th century, when Isabel Fraser introduced seed to New Zealand. In India, the plant was first grown in Bangalore, where it did not fruit, but the plants introduced in Shimla started bearing in 1969 (Dadlani *et al.,* 1971) [4]. Kiwifruit is very popular in human diet due to its pleasant taste and high content of vitamin C, minerals (potassium, phosphorus, iron) and low calorific value. Kiwifruits are good sources of folate, potassium and contain large amounts of vitamin E in the seeds. Moreover, kiwifruit is known to contain a highly active protease enzyme, actinidin (Kaur *et al*., 2010) [9]. The proteases are mainly obtained from microbial sources for industrial purposes. Though microbial proteases are the predominant source of industrial enzymes due to their broad biochemical diversity, rapid growth of microorganisms and limited space required for cell cultivation, it involves advanced technology in the field of biotechnology and microbiology (Rao *et al.,* 1998) [13]. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and renins. In the view of limited availability of livestock, high price of rennet, religious concern (e.g. Islam and Judaism), diet (vegetarian) or ban on recombinant calf rennet. In addition to these, proteolytic plant enzymes are superior to microbial derived enzymes as well as to animal derived enzymes mainly because of safety problems such as pathogenicity or other disadvantageous effects (Chen *et al.,* 2006) [3]. Henceforth the plant sources would be a possible alternative of microbial and animal proteases. Kiwifruit enzyme is a type of cysteine protease enzyme found in kiwifruits. This enzyme is part of papain-like peptidase C1 family (Baker *et al.,* 1980) [1] and consists of 220 amino acids with an apparent molecular weight about 23.0 kDa (Kamphuis *et al.,* 1985) [8]. It has wide substrate specificity, hydrolyzes most strongly the amide and ester bonds at the carboxyl side of a lysine residue and it is active at pH range 4-10 (Hussain *et al.,* 2003; Morimoto *et al.,* 2006) [7, 11]. Henceforth kiwifruit enzyme as a proteolytic enzyme can be used in food industry for milk coagulation (cheese preparation in dairy industry), for reducing cereal proteins in bakery industry and for meat tenderization.
So the present study was conducted for extraction, partial purification and characterization of kiwifruit enzyme.

Materials and methods

Procurement of Kiwifruits

Kiwifruits (*Actinidia* chinensis) were procured from Kiwifruit Orchad, Department of Fruit Science, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan Himachal Pradesh (INDIA). Kiwifruits were procured at three stages of fruit maturity i.e. one week before attaining the commercial harvest stage (TSS<6.5^{0}B), at the commercial harvest stage (TSS=6.5^{0}B) and after one week of commercial harvest stage (TSS<14^{0}B). After harvesting kiwifruits of uniform quality were carried to the laboratory for the study.

Extraction of enzyme

Crude enzyme

The kiwifruit tissues were homogenized in pestle-mortar with phosphate buffer (pH 8.0) under cool condition (0-5 ^{0}C) and extract was centrifuged at 10,000 rpm for 10 min in refrigerated centrifuge. The supernatant was labeled as "Crude enzyme".

Partial purification of enzyme

The procedure followed by Sadasivam and Manickam, 1998 [14] was employed for partial purification of enzyme extracted from kiwifruit. First partial purification of enzyme was done by using ammonium sulphate fractionation method as mentioned above. The crude extract of kiwifruit was precipitated by ammonium sulphate using different concentrations (0-90 per cent). Precipitation was carried out at 0-5 ^{0}C and the precipitate was recovered by centrifugation. The supernatant was discarded and the sediment from each concentration was dissolved in phosphate buffer solution (pH 8.0) and dialyzed over night against the same buffer. The dialyzed enzyme was used for further studies.

Characterization of enzyme

The effect of pH, temperature and incubation time on enzyme activity was studied by using the RSM (Response Surface Methodology). As per the design treatments chosen were shown in table-1

Table 1: Range of values for the RSM

Variables	-1	0	+1
pH	4	8	12
Temperature (^{0}C)	30	45	60
Incubation Time (min)	10	20	30

The different combinations were made as per expert RSM design version 7.0(Stat Ease, Inc, Minneapolis, USA). The details are given in Table-2.

Table 2: Experimental plan of characterization of enzyme as per the design

Treatments	Factor 1 A:pH	Factor 2 B:Temp (^{0}C)	Factor 3 C: Time of incubation (min)
T_1	8.00	45.00	20.00
T_2	4.00	30.00	10.00
T_3	1.27	45.00	20.00
T_4	8.00	45.00	20.00
T_5	8.00	45.00	20.00
T_6	12.00	60.00	30.00
T_7	8.00	45.00	20.00
T_8	8.00	45.00	36.82
T_9	12.00	30.00	10.00
T_{10}	8.00	70.23	20.00
T_{11}	4.00	30.00	30.00
T_{12}	12.00	60.00	10.00
T_{13}	8.00	45.00	20.00
T_{14}	4.00	60.00	30.00
T_{15}	4.00	60.00	10.00
T_{16}	14.73	45.00	20.00
T_{17}	8.00	45.00	20.00
T_{18}	12.00	30.00	30.00
T_{19}	8.00	19.77	20.00
T_{20}	8.00	45.00	3.18

Enzyme assay

The procedure followed by Thimmaiah (2006) [15] was employed for assessment of enzyme assay with slight modifications. For this, one ml of reaction mixture containing 1 per cent casein in 1 ml of 0.05 M phosphate buffer, pH 8.0 and 1 ml of enzyme was incubated at 45°C for 20 min. After 2hr, the reaction was stopped by adding 2ml of cold 10 per cent trichloroacetic acid (TCA) and for blank, immediately after incubation reaction was stopped with 10 per cent TCA. After 2 hour, the culture filtrate was centrifuged at 10,000 rpm for 10 min in refrigerated high speed research centrifuge (Model TC 4100 F/RC 4100 F) to remove the precipitate and absorbance of the supernatant was read spectrophotometrically at 660 nm. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine under standard assay conditions.

Estimation of soluble protein in kiwifruit

Protein concentration in kiwifruit was determined using procedure followed by Lowry (1998) [10].

Result and discussion

The present study was conducted for the extraction, partial purification and characterization of protease enzyme from kiwifruit.

Extraction of kiwifruit enzyme at various stages of fruit maturity

This was done in order to estimate the right stage of enzyme extraction. Three different stages of fruit maturity i.e. immature stage (7 days before attaining the commercial harvest date (TSS<6.5^{0}B)), mature (commercial harvest date (TSS=6.5^{0}B)) and ripened stage (8-10 days after commercial harvest date (TSS<14^{0}B)) was taken. The data in Table 3 represents the protein content and enzyme activity of kiwifruit at various stages of fruit maturity as protein content indirectly represents the enzyme. The highest protein content and enzyme activity (0.42±0.20 mg/gm and 200.32±0.20 µg/gm) was observed at immature stage of fruit followed by mature (0.28±0.10 mg/gm and 131.50±0.20 µg/gm) and then ripened (0.25±0.20 mg/gm and 130.25±0.20 µg/gm) stage respectively. It is shown from the experiment that in kiwifruit maximum enzyme activity was shown at immature stage. Similar findings were reported by Whitaker (1958) [16] that the enzyme activity of *Ficus carica* fruit was highest when they were unripened green.

Table 3: Protein content and enzyme activity of kiwifruit

(Mean±SE)		
Stage	**Protein (mg/gm)**	**Enzyme activity (µg/gm)**
Immature (TSS<6.5 ^{0}B)	0.42 ± 0.20	200.32 ± 0.20
Mature (TSS=6.5 ^{0}B)	0.28 ± 0.10	131.50 ± 0.20
Ripened (TSS<14 ^{0}B)	0.25 ± 0.20	130.25 ± 0.20

Partial purification of kiwifruit enzyme

In the Table 4, purification profile of kiwifruit enzyme was presented. With ammonium sulphate precipitation, the fractionation was carried out in different ranges i.e. 20-90 per cent. Each fraction was assayed for its protease activity. Highest enzyme activity, yield and purification fold was found with 40-60 per cent concentration of ammonium sulphate, i.e. 86 per cent protease enzyme yield of 1.65 purification fold and 0.86 /mg of protein specific activity were found in kiwifruit. However Chaiwut *et al.* (2007) [2] and Otani *et al.* (1991) [12] reported the highest activity of protease enzyme extracted from fig, precipitated with 50-70 per cent ammonium sulphate.

Table 4: Purification profile of kiwifruit enzyme

Observations					
Purification step	**Protein (mg/gm)**	**Enzyme activity (µg/gm)**	**Specific activity (mg/protein)**	**Purification fold**	**% Yield**
Crude enzyme	0.42	220.00	0.52	1	100
Ammonium sulphate precipitation (40-60%)	0.22	190.00	0.86	1.65	86

Characterization of kiwifruit enzyme

The results obtained from response surface design for enzyme activity are presented in figure 1. From figure it was clearly shown that with increase in pH (8.0), temperature (45^{0}C) and time of incubation (20 min) up to a certain limit significant increase in enzyme activity was observed up to a certain level. After that there was decrease in enzyme activity with further increase in value of independent factors. Maximum enzyme activity of 342.00 µg/gm was observed at 8.0 pH and at 45^{0}C temperature.

Table 5: Experimental results for CCD of RSM for characterization of kiwifruit enzyme

Run	pH	Temp (^{0}C)	Time of incubation (min)	Enzyme activity (µg/gm)
1	8.00	45.00	20.00	342.00
2	4.00	30.00	10.00	200.00
3	1.27	45.00	20.00	146.00
4	8.00	45.00	20.00	342.00
5	8.00	45.00	20.00	342.00
6	12.00	60.00	30.00	163.00
7	8.00	45.00	20.00	342.00
8	8.00	45.00	36.82	136.00
9	12.00	30.00	10.00	171.00
10	8.00	70.23	20.00	141.00
11	4.00	30.00	30.00	200.00
12	12.00	60.00	10.00	177.00
13	8.00	45.00	20.00	342.00
14	4.00	60.00	30.00	283.00
15	4.00	60.00	10.00	121.00
16	14.73	45.00	20.00	187.00
17	8.00	45.00	20.00	342.00
18	12.00	30.00	30.00	226.00
19	8.00	19.77	20.00	330.00
20	8.00	45.00	3.18	220.00

The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. So that the response (enzyme activity) could be expressed in terms of the following regression equation:

Enzyme activity = 340.20 + 2.36A + 19.89B + 76.19C - 96.15A2 - 1.15 B2 + 13.88C2 - 24.25AB - 9.58AC + 6.08BC

Analysis of variance gave the enzyme activity as a function of the initial values of parameters. The coefficient of determination (R^2) was calculated as 0.5882 indicating that the statistical model can explain 58.82% of variability in the response. Adequate precision measures signal to noise ratio. For enzyme activity, an adequate precision of 4.815 was recorded (A ratio greater than 4 is desirable), which indicates an adequate signal. The analysis of both pH and temperature dependence and stability of kiwifruit enzyme (Figures 2a, b, c) suggests that the enzyme might be fully compatible with conditions used in cheese manufacture as well as with rennet action. Furthermore, the decrease in the enzyme activity observed after 20 min of incubation and that ensures a lowering of the proteolysis rate during the cheese making process, thus limiting the amount of short peptides that might be responsible for the enhancement of bitter taste. However, Hullikere *et al.* (2014) [6] observed the protease enzyme of papaya with maximum activity at 40 ^{0}C temperature and 7 pH, whereas Otani *et al.* (1991) [12] characterized the proteolytic enzyme of fig and found optimum temperature and pH 65 ^{0}C and 7.5 respectively and further reported that ficin retained more than 90 per cent of its original activity after a period of 1 hr incubation at 55 ^{0}C.

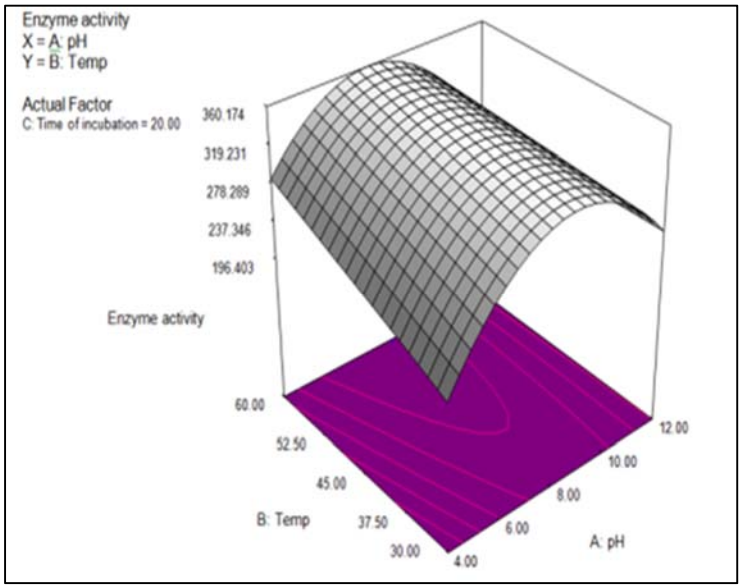

Fig 1: Effect of pH and temperature on enzyme activity

Conclusion

Kiwifruit enzyme has many applications in food due to its proteolytic nature such as used as an alternative of rennet in cheese production and for reducing fermentation time in buns production and for tenderization of spent hen chicken in addition to traditional plant proteases such as papain, ficin and bromelain. Henceforth higher temperature and pH stability of kiwifruit enzyme show its potential for industrial applications.

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