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Proteases of germinating of green gram (Vigna radiata) and black chana (Cicer arietinum) : purification and characterization of an alkaline proteases

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Abstract: The crude protease was extracted from germinated seeds of *Vigna radiata* and *Cicer arietinum*. Protease was purified from these germinated seeds. Protein content was estimated by using Lowry method. Qualitative analysis was done where casein hydrolysis was observed by forming clear zones. Quantitative analysis was carried out by using casein as substrate. Effects of temperature and pH was also examined in which optimum pH was 8 while optimum temperature was 50℃. Partial purification of germinated seeds was executed by ammonium sulphate precipitation with high osmotic resistance.

Introduction

Enzymes are renowned as biocatalysts that form horde of chemical reactions and are commercialized as food, detergent diagnostics and pharmaceutical. Enzymes are productive as well as conservationist. They are characterized by specificity, catalytic activity and aptitude at moderate temperatures and pH (Nadeem, 2009). Enzymes from microbes utilize in baking, alcohol production, brewing, cheese making, etc.(Beg *et al*., 2003).

The primmest enzyme used in today's world is proteases. These are the group of enzymes that catalyzes the peptide bonds. These are also depicted as peptidase. Proteases are present in biomass and microbes. (Boller, 1986). Industrial proteolytic enzymes are applicable inprocessed food, bread manufacturing, meattenderization, brewing, leather and textile industry (Kaneda *et al*., 1997). The taxonomy of proteases based on their ability to degrade N- or C- terminal peptide bond i.e exopeptidase and endopeptidase (Al- sherhi and Mostafa, 2004).

Germinated seeds has reduced the starch content which make it facile assimilate. Green gram and black chana are tremendous source of roughage, protein, vitamins, minerals and significant proportion of bioactive compounds. Their medic able in treatment of numerous diseases such as cancer, gastrointestinal tract related issues, cardiovascular diseases, diabetes, etc. they are good for detoxification and consist of immunomodulatory properties (Hou *et al*.,2019, Wallace *et al*.,2016).

Materials and method

2.1 Collection of plant materials

Green gram and black chana seeds were decontaminated with 0.1% mercuric chloride solution for 5 minutes and removed by various changes of distilled water. Than the seeds were sprouted on piece of watered cotton cloth for 3 days. After propagation they were stored for further use.

2.2 Extract preparation

With the help of motor and pestle 10gram of sprouted seeds were grounded in 15ml of Tris-HCl(10mM) (Akhtaruzzama*et al.*,2014). Centrifuged the extract at 4℃ for 15 minutes at 10,000 rpm and supernatant was utilized to evaluate the protease activities.

2.3 Protein estimation by Lowry's method

BSA was used as standard protein for estimation. Pipette out 0.1 ml of extract added distilled water to make final volume 1 ml. added 4 ml of alkaline copper sulfate and allowed to stand for 10 min and add on 0.5 ml of Folin' reagent. Incubated the mixture for 30 min. at 37℃. Observed the reading at 660 nm (Lowry *et al*,1951).

2.4 Protease activity

2.4.1 Qualitative analysis by using agar plate techniques

Protease activity was analyzed on agar plate using the recognized method of (Galil, 1992).0.65% w/v of casein powder was dissolved in agar (1%w/v) which was prepared in Tris-HCl buffer (10mM;pH8). After solidification, with the help of gel puncture wells of 9mm diameter were made and a particular amount of crude extract of protein (50µg) was poured into the wells. Then incubated them for 24 hr at 37℃. Furthermore, the solution of Coomassie Brilliant Blue (0.1%w/v) was overwhelmed on the surface and after 3 hr incubation the dye was drained off and surface was washed by the destaining solution consist of methanol: acetic acid : water (4:1:5). Transparent zone formed which is known as hydrolytic zone.

2.4.2 Quantitative estimation

Casein was used as a substrate for proteases as described by (Hema and Shiny, 2012). Reaction mixture composed of 5 ml of 0.65% casein in 10mM Tris-HCl buffer (pH 8) and 1 ml of enzyme solution. At 37℃ 10 min. incubation was given and with the addition of 5 ml of 10% trichloroacetic acid (TCA) the reaction was ceased. Later on a 30 min. incubation was provided at 37℃. The un-reacted casein was precipitated by centrifugation at 10,000 rpm for 15min. the 2ml of supernatant was dissolved in 5ml of 0.5 M $Na_2CO_3$and at 660 nm absorbance was measured against a blank reagent.

2.5 Determination of optimum pH

Optimum pH of protease was studied in the pH range 5 to 9. The reaction mixture comprised 1ml of enzyme preparation and 1ml of casein in Tris-HCl buffer of appropriate pH protease activity was assayed using the standard method described above 2.4.2.

2.6 Determination of optimum temperature

Optimum temperature of protease was determined over the range 30- 60°C by adding 1ml of enzyme preparation to 1ml of casein in Tris-HCl and incubated at respective temperatures. Protease activity was assayed using the method described above 2.4.2.

2.7 Partial purification of protease

2.7.1 Ammonium sulphate precipitation

Enzyme solution was taken and mixed with ammonium sulphate to get 70% saturation and incubated at 4° C for one hour. Centrifuged at 5000 rpm for 25 minutes and the pellet was dissolved in Tris-HCl buffer(pH 8 and 0.1M) and determined the enzyme activity and protein content (Ahmed *et al.*,2009).

Results

The extraction of proteases from plants has been elevated due to their medicinal properties and industrial applications. In this study extraction of protease was aimed from germinated seeds of green gram (*Vignaradiate)* and black chana (*Cicerarietinum).*

3.1 Estimation of protein by lowry method

The protein was estimated on the basis of standard plot. The protein concentration of green gram and black chana was 1.454 mg/ml and 2.00 mg/ml respectively.

3.2 Enzyme activity

3.2.1 Qualitative analysis by using agar plate technique

The agar plate proteases exhibited a zone of clearance surrounding the enzyme growth. Crude extract of green gram as well as black chana was loaded. When zone of clearance was measured in green gram showed higher protease activity due to bigger zone of clearance i.e 24mm while in black chana it was measured to be 19mm.

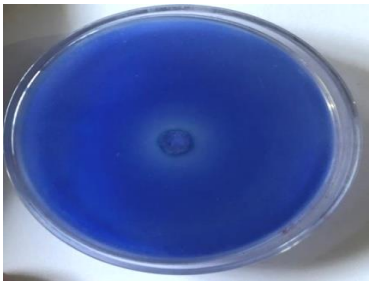

Figure 3.1: Zone of casein hydrolysis produced on inoculation of crude extract of green gram.

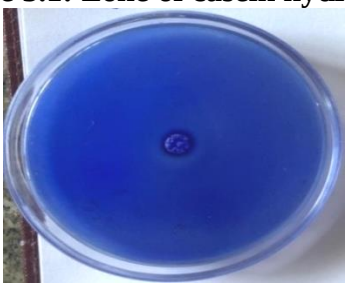

Figure 3.2: Zone of casein hydrolysis produced on inoculation of crude extract of black chana.

3.2.2 Quantitative analysis of proteases

Quantitative analysis was estimated by using standard plot by using different concentrations of tyrosine. In case of crude extract of germinated green gram tyrosine released was found to be 305.8 µg/ml and in case of black chana it was found to be 237.5µg/ml. Tyrosin conc. is directly corresponds to higher protease activity i.e more casein is hydrolyzed.

3.3 Protease characterization

3.3.1Optimization of temperature

The protease activity in both the cases of germinated green gram and black chana was first increased and then decreased with increasing temperature, with enzyme showing optimum temperature of 50℃.

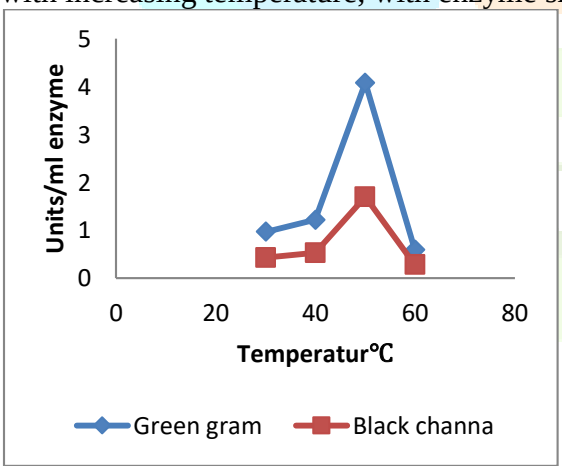

Figure 3.3: Optimum temperature of protease

3.3.2 Optimization of pH

The protease activity in both the cases of germinated green gram and black chana was first increased and then decreased with increasing pH, the enzyme shows optimal pH of 8 respectively.

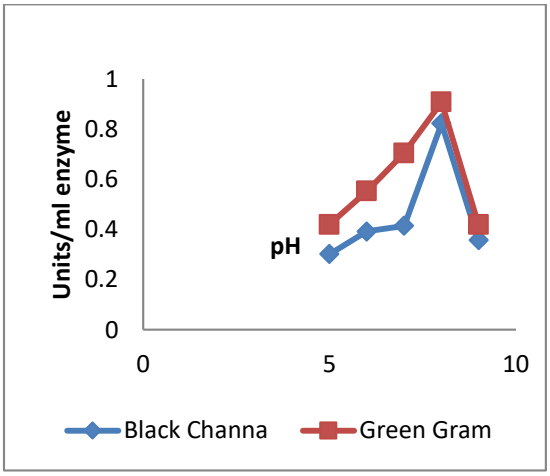

Figure 3.4: Optimum pH of proteases

3.4 Purification of protease

3.4.1 Partial purification by Ammonium precipitation

Germinated seeds of green gram and black chana were used for the extraction of protease. Protease from crude extract was purified by precipitation in a high osmotic strength ammonium sulphate salt solution. The result of the purification of protease are sum up in table 3.1 and 3.2.

Table 3.1: Purification summary of germinated green gram

Steps of purification	Protein conc.(mg/ml)	Enzyme (Units/ml)	Specific activity (U/mg)
Crude	2	0.721	0.0164
Ammonium salt precipitation	0.366	0.732	0.0912

Table 3.2: Purification summary of germinated black chana

Steps of purification	Protein conc. (mg/ml)	Enzyme (Units/ml)	Specific activity(U/mg)
Crude	1.454	0.929	0.0294
Ammonium salt precipitation	0.176	0.748	0.194

Discussion

Proteases isolated from soybean and green gram seeds show maximum activity at 50℃while optimum pH of other proteases extracted from leguminous seeds was 7.5 Akhtaruzzaman*etal*.,2012. In case of *Moringaoleifera* optimum pH was reported 8 Banik et al.,2018. The specific activity of green gram seeds was 0.004578U/ mg and black gram seeds was 0.003536 U/ mg Akhtaruzzaman*et al*.,2012 so, our results are consistent to previous ones.

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