Production of Protease from a Strain of *Candida albicans*: Flour as Growth Substrate

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Abstract: Proteases are produced industrially and have a wide application. In the present investigation, *Candida albicans* inoculated into flour grew at 37°C with expression of proteolytic activity within ten days. The induced protease was partially purified by ammonium sulphate precipitation followed by dialysis. The partially purified protease had optimum activity at 30^{0}C and at pH 7.0. The alkaline protease was able to degrade casein with optimum activity expressed at 8mg/ml concentration. The enzyme was stimulated by zinc chloride and potassium chloride but inhibited by ethylene diamine tetraacetic acid suggesting a metalloprotease.

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Introduction

Proteases are hydrolytic enzymes involved the degradation of proteins with cleavage of peptide bonds and formation of small peptides and amino acids (Lehninger, 1982). They are produced by fungi including species of moulds (Voet and Voet, 1995). Proteases are affected by physiological parameters and are optimally active under specific conditions (Voet *et al*., 2013).

In the present investigation, a strain of *Candida albicans* isolated from a fifteen-year-old girl was grown on flour medium. Induced protease was partially purified. Certain physiological parameters affecting the enzyme activity were studied.

Materials and Methods

2.1 Source and Identification of Isolate

The strain of *Candida albicans* used in this research was obtained from the Department of Medical Microbiology and Parasitology, University College Hospital, Ibadan, Nigeria. It was from a fifteen-year-old girl with vaginal candidiasis and identified in the Department. The isolate was cultured on potato dextrose agar.

2.2 Culture Conditions and Inoculation

The isolate of *Candida aibicans* was subcultured from potato dextrose agar plates in to malt yeast extract broth medium. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of inoculated yeast extract broth were incubated at 37°C. One hundred and sixty eight-hr-old culture of isolate was used in this investigation.

2.3 Inoculation of Flour Medium

Flour was bought from a retailer of Flour Eagle Company, Ibadan, Nigeria at Challenge, Ibadan. It was brought to the laboratory, weighed in 100 ml distilled water (1% w/v) in conical flasks and sterilized at 15 Ib/in^{2} for 20 min. in an autoclave. The flour medium was inoculated with 1 ml of 10^{-2} dilution of isolate. Controls were uninoculated flour medium. Experimental and control flasks were incubated without shaking at 37^{0}C (Olutiola and Nwaogwugwu, 1982).

On a daily basis, the contents of each flask were filtered using glass fibre filter paper (Whatman GF/A). Filtrate was assayed for protease activity using the modified casein digestion method of Kunitz (1946). The crude protease obtained on day of optimum activity was precipitated with ammonium sulphate within the limits of 40 - 90% saturation and thereafter dialysed overnight with several changes of 0.02 M citrate phosphate buffer pH 6.0 containing 5mM sodium azide.

2.4 Enzyme Assay

2.4.1 Protease

Protease activity was assayed by a modification of the casein digestion method of Kunitz (1946). A 1% (w/v) casein (sigma) solution was prepared in 0.02M citrate phosphate buffer (pH 6.0) and heat denatured at 100°C for 15 minutes in a water bath. This was used as substrate.

One ml of the enzyme preparation was added to one ml of casein solution. Both were thoroughly mixed and incubated in water bath at 35°C for 1 hr.

The reaction was terminated by adding 3ml of cold 10% (w/v) trichloroacetic acid (TCA). Controls which initially contained only one ml of the substrate were incubated with the experimental tubes at 35°C for 1 hr. 3 ml cold 10% TCA was added to each control tube followed by 1 ml of enzyme. Both experimental and control tubes were left to stand at 0°C for 30 min to allow complete precipitation of unhydrolysed protein. The supernatant was carefully filtered using filter paper (Whatman No. 1). Optical density readings of filtrates were taken at 280nm. One unit of protease activity was expressed as the amount of enzyme in 2 ml of reaction mixture which caused an 0.1 increase in absorbance at 280nm per hr under assay conditions.

2.5 Characterization of Enzyme

The partially purified enzyme was diluted with 0.02 M citrate phosphate buffer pH 6.0. and used to characterize the enzyme.

2.5.1 Effect of temperature

The reaction mixture was 1 ml of substrate (1% denatured casein in 0.02 M citrate phosphate buffer pH 6.0) and 0.5 ml of enzyme. The substrate was allowed to stabilize at each of different temperature (20°C, 25°C, 30°C, 40°C and 50°C) before adding enzyme. Incubation at each temperature was for 1 hr. The reaction was terminated with 3 ml cold 10% trichloro acetic acid (TCA). Protease activity was determined as described under enzyme assay.

2.5.2 Effect of pH

Casein was prepared in 0.02 M citrate phosphate buffer of different pH values (pH 3.0 ó 9.0) and employed as substrate in the protease assay. The reactants (1 ml substrate and 1 ml enzyme) were incubated at 35°C for 1 hr. Protease activity was determined as described under enzyme assay. The reaction was terminated with 3 ml cold 10% (w/v) trichloro acetic acid (TCA). Protease activity was determined as described under enzyme assay.

2.5.3 Effect of substrate concentration

Casein of different concentrations (2, 4, 6, 8 and 10 mg respectively) were prepared in 0.02 M citrate phosphate buffer pH 6.0. The reaction mixture was 1 ml substrate and 1 ml enzyme. After incubation at 35°C for 1 hr, the reaction was terminated by adding 3 ml of cold trichloroacetic acid (TCA). Protease activity was thereafter determined.

2.5.4 Effect of some salts and chemical

Different concentrations of each salt ($ZnCl_2$ and KCl) were prepared in casein (1% denatured casein in 0.02 M citrate phosphate buffer pH 6.0) such that the final concentration of each cation was 2, 10, 15 and 20mM respectively. They were employed as substrate in the enzyme assay.

Different concentrations (2, 4 and 6 mM) of ethylene diamine tetraacetic acid (EDTA) were prepared in casein (1% denatured casein in 0.02 M citrate phosphate buffer pH 6.0). These were substrate preparations. Reaction mixtures were incubated at 35°C for 1 hr. Protease activity was determined as described.

3. Results

In this research, flour was innoculated with *Candida albicans*. Incubation was at 37°C. There was growth over a period of ten days with induction of protease activity. Activity increased gradually within twenty-four hours of incubation reaching an optimum on the sixth day after which there was a gradual decline. Protease activity was expressed as 3800 units at twenty-four hour and 13800 units on that sixth day (Table 1).

Protease produced by *Candida albicans* was influenced by temperature. At 20°C incubation, activity was 7300 units. As temperature of the medium increased, protease activty increased. Optimum activity was expressed at 30°C and was 12000 units (Table 2).

pH of the reaction medium affected protease produced by *Candida albicans.* As the pH of the medium increase from 3.0, activity increased from 3800 units. Activity was optimum at pH 7.0 and expressed as 13400 units. As the pH further increased, protease activity gradually declined. An optimum activity expressed at pH 7.0 indicates that the protease is an alkaline protease (Table 3).

As substrate (casein) concentration of reaction medium increased from 2 mg/ml to 10 mg/ml protease induced from *Candida albicans* increased from 9600 units. Optimum activity was expressed as 13000 units at 8 mg/ml casein concentration. Protease activity declined thereafter (Table 4).

Zinc chloride and potassium chloride incorporated into reaction medium stimulated protease activity produced by *Candida albicans*. As the concentration of each salt increased from 2 mM to 20 mM, preotease activity gradually increased with no decline. Activity was expressed as 15200 units with 20mM concentration of zinc chloride and 15000 units with 20mM concentration of potassium chloride (Tables 5 and 6).

Table 1: Protease activity from *Candida albicans* in inoculated flour with days of incubation

Day	Protease activity (Units) x 10^2
1	38
2	42
3	67
4	95
5	126
6	138
7	120
8	108
9	95
10	82

Table 2: Effect of temperature on protease produced by *Candida albicans* in inoculated flour

Temperature (^oC)	Protease activity (Units) x 10^2
20	74
25	82
30	120
40	108
50	68

Table 3: Effect of pH on protease produced by *Candida albicans* in inoculated flour

pH	Protease activity (Units) x 10^2
3.0	38
4.0	65
5.0	92
6.0	129
7.0	134
8.0	121
9.0	96

Table 4: Effect of substrate (casein) concentration on protease produced by *Candida albicans* in inoculated flour

Casein concentration (mg/ml)	Protease activity (Units) x 10^2
2	96
4	120
6	132
8	130
10	128

Ethylene diamine tetraacetic acid (EDTA) inhibited protease produced by *Candida albicans.* As the concentration of EDTA increased from 2mM to 6mM protease activity decreased gradually. At 6mM concentration, there was approximately 41 percent loss in activity (Table 7).

Table 5: Effect of zinc chloride on protease produced by *Candida albicans* in inoculated flour

$ZnCl_2$ concentration (mM)	Protease activity (Units) x 10^2
0	132
2	139
10	148
15	150
20	152

Table 6: Effect of potassium chloride on protease produced by *Candida albicans* in inoculated flour

KCl concentration (mM)	Protease activity (Units) x 10^2
0	139
2	140
10	146
15	148
20	150

Table 7: Effect of ethylene diamine tetraacetic acid (EDTA) on protease produced by *Candida albicans* in inoculated flour

EDTA concentration (mM)	Protease activity (Units) x 10^2
0	134
2	120
4	108
6	80

4. Discussion

Candida albicans grew in a medium containing flour as substrate. The enzyme protease was produced by the fungus during growth. According to Adejuwon and Olutiola (2003), a species of *Fusarium* was able to produce protease during its infection of tomato fruits. Also according to Adejuwon and Olutiola (2005), *Fusarium oxysporum* is able cause extensive degradation of tomato (*Lycopersicon esculentum* Mill.) fruits with production of an alkaline protease.

Temperature had an effect on protease activity expressed by *Candida albicans.* Optimum activity was expressed at 30°C. Similarly, optimum activity at 30°C was reported on protease from *Aspergillus aculeatus* associated with the black tongue disease (Olutiola and Nwaogwugwu, 1982). Earlier reports of Olutiola (1976) also revealed a similar temperature for optimum growth of *Aspergillus flavus.*

The hydrogen ion concentration (pH) of medium affected protease from *Candida albicans.* Optimum activity was expressed at pH 7.0. This indicates that the protease is an alkaline protease. Olutiola and Nwaogwugwu (1982) reoprted that

Aspergillus aculeatus is able to produce a protease with optimum activity expressed at pH 7.0. However, protease from a strain of *Fusarium oxysporum* seem to express optimum activity at pH 6.0 (Adejuwon and Olutiola, 2005). The genetic constitution of the isolates actually accounts for this obsevrved variability (Tortora *et al*., 2004).

Protease from *Candida albicans* is able to degrade casein with optimum activity expressed at 6mg/ml concentration. Enzymes are biological catalysts capable degradation of substrate with activity optimum at particular values (Lehninger, 1982).

The protease from *Candida albicans* was stimulated by zinc chloride and potassium choride but inhibited by ethylene diamine tetracaetic acid. According to Dixon and Webb (1971), metalloenzymes are affected by ethylene diamine tetraacetic acid (EDTA) which chelates the enzyme with possible removal of ions of the prosthetic group. Bond (1989) reported that zinc could actually be found in the active sites of certain metalloproteases and are involved in catalysis.

Conclusively, *Candida albicans* is capable of producing a protease in contaminated flour. During industrial production of enzymes, the possible use of *Candida albicans* and flour can be explored for the possible production of an alkaline protease. The usesfulness and benefits of this isolate may on the long run outweigh its diseaese implication.

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