Protease From a Strain of *Candida albicans* Grown in a Defined Medium

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Abstract: A defined medium with galactose as carbon source and sodium nitrate as nitrogen source was inoculated with *Candida albicans*. Incubation was at 37°C. Protease activity was expressed by the isolate within a period of six days. Optimum activity was expressed on the fifth day. The protease was precipitated at 90% saturation and dialysed with buffer. The partially purified enzyme was stimulated by salts of Zn^{2+} and Na^{+} but inhibited by $HgCl_2$ and EDTA. [Adejuwon AO, Haruna AA, Arojojoye OA, Akintobi OA. **Protease From a Strain of *Candida albicans* Grown in a Defined Medium.** *Rep Opinion* 2013;5(6):27-31]. (ISSN: 1553-9873). http://www.sciencepub.net/report. 6

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1. Introduction

Proteases are hydrolytic enzymes and are classified based on: the type of reaction catalyzed; their chemical nature and catalytic site (Voet and Voet, 1995; Stryer, 1995). They can initiate protein degradation in reactions, setting amino acids free (Kimball, 1978). They catalyze cleavage of peptide bonds (Lehninger, 1982). Peptide cleaving enzymes can be divided into endopeptidases and exopeptidases according to their specific mode of action (Voet *et al.*, 2013). Microorganisms have broad biochemical diversity with a susceptibility to genetic manipulation (Nester *et al.*, 2004; Stryer, 1995). Microbial proteases are affected by chemical and physical factors and can be obtained by molecular genetic manipulation (Bond, 1989; Devlin, 2010).

This current investigation was designed to characterize a protease from a strain of *Candida albicans*.

2. Materials and Methods

2.1 Source and Identification of Isolate

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

2.2 Culture Conditions and Inocula

The isolate was subcultured from potato dextrose agar plates into malt yeast extract broth medium. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of inoculated broth were incubated at 37°C. One hundred and sixty eight-hr-old culture of isolate was used in this investigation. Based on the method of Olutiola and Ayres (1973), culture was grown in a defined medium of the following composition: Na_2NO_3 (9.9g), $MgSO_4.7H_20$ (0.1g), KH_2PO_4 (0.5g), $CaSO_4$ (0.1g), $MnSO_4$ (1mg), $CuSO_4$ (1mg), $ZnSO_4$ (1mg), biotin (0.005mg), thiamine (0.005mg) and $FeSO_4.7H_20$ (1mg), galactose (10 g) (Sigma) in 1 litre of distilled water. One hundred millilitre of growth medium in conical flasks (250 ml) was inoculated with 1 ml of 10^{-2} dilution of isolate. Controls were uninoculated growth medium. Experimental and control flasks were incubated without shaking at 37^0C (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 enzyme obtained on day of optimum activity was precipitated with ammonium sulphate at 90% saturation and thereafter dialysed overnight with several changes of 0.02 M citrate phosphate buffer pH 6.0 containing 5mM sodium azide.

2.3 Enzyme Assay

2.3.1 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^{o}C for 15 minutes in a water bath. This was used as substrate.

One millilitre of the enzyme preparation was added to 1 ml of casein solution. Both were thoroughly mixed and incubated in water bath at 35^{o}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^{o}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^{o}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 defined as the amount of enzyme in 2ml of reaction mixture able cause an 0.1 increase in absorbance at 280nm in 1hr under assay conditions.

2.4 Characterization of Enzyme

The partially purified enzyme was diluted with 0.02 M citrate phosphate buffer pH 6.0 and used in characterization.

2.4.1 Effect of temperature

The reaction mixture, 1 ml substrate (1% denatured casein in 0.02 M citrate phosphate buffer pH 6.0) and 1 ml of enzyme was incubated at different temperature (20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C) 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.

2.4.2 Effect of pH

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

2.4.3 Effect of substrate concentration

Casein of different concentrations (1, 2, 4, 5 and 6 mg respectively) was prepared in 0.02 M citrate phosphate buffer pH 6.0. The reaction mixture was 1 ml substrate and 1 ml enzyme. Incubation was at 35^{o}C for 1 hr. The reaction was terminated by adding 3 ml of cold trichloroacetic acid (TCA). Protease activity was thereafter determined.

2.4.4 Effect of some salts and chemicals

Different concentrations of salts ($ZnCl_2$ and NaCl) 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 5, 10 and 15mM respectively. They were used as substrate in the enzyme assay.

Different concentrations (2, 5 and 10 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^{o}C for 1 hr. Protease activity was determined thereafter.

3. Results

In this study, a defined growth medium was innoculated with *Candida albicans*. Incubation was at 37^{o}C. There was growth over a period of six days with production of the enzyme protease. Expressed protease activity increased gradually within twenty-four hours of incubation reaching an optimum on the fifth day after which there was a decline. Protease activity was expressed as 4000 units at twenty-four hour and 12800 units on that fifth day (Table 1).

Protease activity by *Candida albicans* was affected by temperature. At 20^{o}C incubation, activity was 5200 units. As temperature of the medium increased, protease activty increased. Optimum activity was expressed at 35^{o}C and was 12500 units. There after there was decline in activity (Table 2).

pH of the reaction medium influenced protease produced by *Candida albicans.* As the pH of the medium increase from 3.0, activity increased from 4500 units. Activity was optimum at pH 7.0 and expressed as 12800 units. With further increase in pH, protease activity gradually declined. Optimum protease activity expressed at pH 7.0 indicates an alkaline protease (Table 3).

As substrate concentration of the medium increased from 1 mg/ml to 6 mg/ml protease activity from *Candida albicans* increased from 9800 units. Optimum activity was expressed as 12800 units at 6 mg/ml casein concentration (Table 4).

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

Day	Protease activity (Units) x 10^2
1	40
2	62
3	89
4	106
5	128
6	104

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

Temperature (^oC)	Protease activity (Units) x 10^2
20	52
25	78
30	96
35	125
40	116
45	105

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

pH	Protease activity (Units) x 10^2
3.0	45
4.0	68
5.0	93
6.0	116
7.0	128
8.0	121

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

Casein concentration (mg/ml)	Protease activity (Units) x 10^2
1	98
2	106
4	118
5	125
6	128

Zinc chloride and sodium chloride incorporated into reaction medium enhanced protease activity produced by *Candida albicans*. As the concentration of each salt increased from 5mM to 15mM, preotease activity gradually increased. Activity was expressed as 16200 units with 15mM concentration of zinc chloride and 14200 units with 15mM concentration of sodium chloride (Tables 5 and 6).

Ethylene diamine tetraacetic acid (EDTA) and mercuric chloride inhibited protease produced by *Candida albicans*. As the concentrations of EDTA and mercuric chloride increased from 2mM to 10mM protease activity decreased gradually. At 10mM concentration of EDTA, there was approximately 34 percent loss in activity (Table 7). At 10mM concentration of mercuric chloride, there was approximately 66 percent loss in activity (Table 8).

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

$ZnCl_2$ concentration (mM)	Protease activity (Units) x 10^2
0	125
5	138
10	145
15	162

Table 6: Effect of sodium chloride on protease produced by *Candida albicans* in inoculated defined medium

NaCl concentration (mM)	Protease activity (Units) x 10^2
0	128
5	132
10	138
15	142

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

EDTA concentration (mM)	Protease activity (Units) x 10^2
0	126
2	96
5	84
10	62

Table 8: Effect of mercuric chloride ($HgCl_2$) on protease produced by *Candida albicans* in inoculated defined medium

$HgCl_2$ concentration (mM)	Protease activity (Units) x 10^2
0	123
2	104
5	98
10	42

4. Discussion

Candida albicans grew in a defined medium with the enzyme protease produced by the fungus during growth at 37°C. *Candida albicans* which causes candidiasis in man is able to grow at body temperature of 37°C (Nester *et al.*, 2004). Fungi are capable of growth under specific conditions with the elaboration of enzymes such as proteases (Nester *et al.*, 2004; Adejuwon and Olutiola, 2003). According to Adejuwon and Olutiola (2005), *Fusarium oxysporum* is able to grow on tomato fruits and produce a protease with molecular weight estimate of 11200 Daltons. The Human Immunodeficiency Virus (HIV) is able to replicate with involvement of its genetic products which includes a structural protease (Alimonti *et al.*, 2003).

Temperature affected protease activity expressed by *Candida albicans.* Optimum protease activity was expressed at 35°C. Enzymes are optimally active at specific temperatues (Lehninger, 1982). Olutiola and Nwaogwugwu (1982) reported from their studies, a protease from *Aspergillus aculeatus* with optimum activity at 37°C. However, according to Chakraborty and Srinivasan (1992), *Pseudomonas species* produced an extracellular thermostable protease which expressed optimum activity at 30°C.

The pH of reaction medium affected protease from *Candida albicans.* Optimum activity was expressed at pH 7.0. This indicates an alkaline protease nature. The catalytic activity of an enzyme is affected by the hydrogen ion concentration of its reaction medium and the nature of amino acid found on its catalytic site (Voet *et al.*, 2013). The alkalinity of the vagina will support such protease activity expressed by *Candida albicans* during vaginal trush in candidiasis probably enhancing pathogenicity of the fungus in such infection.

Protease from *Candida albicans* was 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; Nester *et al*., 2004).

The protease from *Candida albicans* was stimulated by salts of zinc ion and sodium ion but inhibited by ethylene diamine tetracaetic acid and mercuric chloride. Metalloenzymes are inhibited by chelating agents such as ethylene diamine tetraacetic acid (Dixon and Webb, 1971). The protease produced by our strain of *Candida albicans* used in this research might actually be a metalloprotease.

In conclusion, *Candida albicans*, inspite of the harm it causes man, can be used industrially in the synthetic production of proteases which are useful in the production of detergents and leather processing.

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