JJBS
Jordan Journal of Biological Sciences

Volume 5, Number 4, December.2012
ISSN 1995-6673
Pages 321 - 330

Cytotoxicity of Collagenases and Elastases Purified from *Candida* Species on Some Carcinoma Cell Lines

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Received 4th June 2012; accepted 8th July 2012

Abstract

The purpose of this work was to assay for the cytotoxicity of collagenases and elastases purified from *Candida parapsilosis* and *Candida krusei* on some carcinoma cell lines. Four *Candida* species and *Saccharomyces cerevisiae* were tested for their collagenolytic and elastinolytic enzyme activities. *C. parapsilosis* and *C. krusei* were proved to be high producers for the two enzymes in the culture filtrates. The four enzymes (collagenases and elastases from *C. parapsilosis* and *C. krusei*) were purified to full homogeneity using $(NH_4)_2SO_4$ precipitation, anion exchange column chromatography by DEAE- cellulose and gel filtration using Sephadex G100. The molecular masses of the four enzymes were determined by SDS-PAGE in parallel with all purification steps. However, the molecular masses of the single band of the purified enzymes were apparently determined to be, 97.2 and 53.4 KDa for collagenase and elastase in *C. parapsilosis,* 66.4 KDa and 23.5 KDa for collagenase and elastase in *C. krusei.* The four enzymes were characterized by studying the effect of temperature, pH, protease inhibitors & substrate specificity. Cytotoxicity assay of the four enzymes either in crude or in purified state was done singly or in combination mixtures using three carcinoma cell lines. It was found that larynx carcinoma cell line (HEP2) was the only sensitive cancer cells to proteases treatments, while colon carcinoma cell line (HCT116) and breast carcinoma cell line (MCF7) were not affected by enzyme treatments. Combination mixture of the four enzymes recorded the most potent cytotoxic effect on larynx carcinoma cell line (HEP2) with minimum value of IC_{50} (3.1 Uml^{-1}).

Keywords: *C. parapsilosis*; *C. krusei*; Collagenase; Elastase; Proteases; Antitumor Activity.

1. Introduction

Proteolytic enzymes have a long history of use in cancer treatment. In 1906, John Beard, a Scottish embryologist, reported on the successful treatment of cancer using a pancreatic extract in his book *The Enzyme Treatment of Cancer and its Scientific Basis*. In 1999, Gonzalez and Isaacs evaluated the benefit of proteolytic enzymes in patients with pancreatic cancer treatment in large scale study and showed dramatic improvements in these patients. However, several excellent compilations of the history of cancer science and treatment (Shimkin, 1977; Weiss, 2000), John Beard's ideas and its clinical results concerning enzyme therapy of tumors went without mention. Recently pancreatic extracts have been used to treat chronic or acute pancreatic disease or cystic fibrosis (Mossner *et al*., 1992). These oral preparations contain variable amounts of protease and active pancreatic enzymes which are formulated to pass through the gastric environment before they are deposited in the intestine (Layer and Keller, 1999). Active trypsin, chymotripsin, collagenase, elastase and other proteases are components of the commercially available enzyme mixtures "Wobe-Mugose" and "Phlogenzyme" produced by Mucos Pharma GMBH-Germany. These products were tested for treatment of neoplastic, autoimmune deficiency or viral diseases (Tamhankar *et al*., 2001). Several mechanisms, including suppression of cytokine levels (Desser *et al*., 1997), and enhancement of leukocyte-mediated cytotoxicity (Zavadova *et al*., 1995) were suggested to be responsible for the adjuvant affectivity of these enzyme mixtures. In medicine, collagenases and elastases are used in transplantation of pancreatic islet cells to alleviate diabetic symptom (Barker, 1975) and in some cancer treatments (Jain, 2008). Microbial collagenase is unique because it can degrade both water insoluble collagen and water soluble denatured ones. It can attack almost all collagen types (Mookhtair *et al*., 1985).

In order to invade and metastasize, it is necessary for malignant tumor cells to cross the basement membrane and penetrate the connective tissue stroma. It has been postulated that this process involves tumor cell production and/or activation of proteolytic enzymes, particularly

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matrix metalloproteases (MMPs), which degrade the extracellular matrix (ECM) (Murphy *et al*., 1989; Stetler *et al*., 1993; Mignatti and Rifkin, 1993). One of the major effects of the enzyme mixture at cellular level is the inhibition of tumor cell migration. Once proteolytic enzymes absorbed, the body produces antiproteases which are critical to the mechanism of action of proteolytic enzymes. These antiproteases block invasiveness of tumor cells as well as prevent the formation of new blood vessels (angiogenesis) and inhibit metastasis and enhance immune response (Murray *et al*., 2002).

Candida species were found to produce collagenases and elastases responsible for degradation of dental and pulmonary collagen and elastin. It was thought that *Candida* species may utilize collagen and elastin for growth (Kaminishi *et al*., 1986). Collagenases were used to anchor signaling molecules to collagen containing tissues, presenting a great potential for targeted drug delivery of anti-arthritic and cytotoxic reagents (Matsushita *et al*., 1994).

The current study aims at signaling out enzymatic target for cancer treatment as a new strategy for clinical drugs. Purification, characterization and cytotoxicity assays were carried out on four proteolytic enzymes extracted from *C. parapsilosis* and *C. krusei.*

2. Materials and Methods

2.1. Microorganisms

Five microorganisms, *Candida parapsilosis* (ATCC 22019), *Candida Krusei* (ATCC 6258), *Candida tropicalis* (ATCC 750), *Candida albicans* (ATCC 26555), and *Saccharomyces cerevisiae* (ATCC 2180- 1A) were screened for their collagenolytic and elastinolytic activities. These fungi were kindly supplied from Micro-Analytical Center (Microbiology lab) in Faculty of Science, Cairo University.

2.2. Culture Media

Candida species were grown by submerged fermentation on malt extract glucose medium (MEG). This was composed of 3 gl^{-1} malt extract, 10 gl^{-1} glucose and 15 gl^{-1} agar. After incubation for two days at 30 °C in shaking incubator at 200 rpm, colonies were isolated and sub-cultured in the same but liquid media, for extracellular enzyme production.

2.3. Production of Collagenase and Elastase in Culture Filtrate

Preparation of (MEG) liquid media containing 2 gl^{-1} collagen or elastin as an enzyme inducer was performed, 25ml of this medium were placed in 100 ml Erlenmeyer flasks and sterilized by autoclaving then inoculated with disc of 0.5 cm diameter taken from two days old stock culture of the test fungus and incubated at 30°C for 2 days in shaking incubator at 200 g

2.4. Assay of Collagenolytic and Elastinolytic Activities in Reaction Mixture

Collagenolytic or elastinolytic activities were assayed after the method of Kaminishi *et al*. (1986).The reaction mixture contained, 10 ug of collagen or elastin (Sigma), 1ml of 0.05 M tris-maleat buffer pH (8), 1ml culture filtrate (as source of crude collagenase and elastase), or 1 ml of purified enzymes (10 Uml^{-1}). After incubation for 24 hours at 30 °C, an equal volume of 20 % trichloroacetic acid was added to stop reaction. The tubes were centrifuged at 2000 g for 10 min to remove the residual collagen or elastin. The collagenase or elastase unit was defined as the amount of enzyme releasing 1μg peptide in 1ml reaction mixture in one hour.

Protein was estimated after Lowry *et al*. (Lowry *et al*., 1951).

2.5. Partial Purification By $(NH_4)_2$ SO_4 Precipitation

The pH of crude enzyme preparation was adjusted at 7.5 by adding Tris/HCL (Tunlid *et al*., 1994). The culture filtrate was kept in an ice salt bath and this was followed by adding slowly 3.4 M $(NH_4)_2$ SO_4 to reach final concentration of 80 %. The culture filtrate was then placed on a magnetic stirrer at 4 °C for 15 min. After sedimentation for at least one hour, the supernatant was discarded and the precipitate was dialyzed over night and signaled as partially purified enzyme.

2.6. Anion Exchange Chromatography using DEAE-Cellulose

The partially purified enzyme extract was applied to DEAE-cellulose column (Pharmacia). Buffers used were a-10 mM tris/HCL (PH 7.5); b-10 mM Tris/HCL (PH 7.5) with 0.5 M NaC1. The gradient was 0.5 % b for 5 minutes, 0.5 %-100 % b for 35 minutes and 100% b for 5 minutes. The flow rate was 1.0 ml min^{-1}. Fractions of 5 ml were collected and assayed for collagenase or elastase and protein content of *Candida parapsilosis* and *Candida krusei* in the reaction tubes.

2.7. Gel Filtration Chromatography using Sephadex G100

The active fractions of the major peak from anion exchange chromatography were further purified using gel filtration column chromatography by sephadex (G100). The column was equilibrated using Tris-HCl buffer (5mM, pH 7.0) at flow rate of 1.0ml min^{-1} before application of the active fractions. The pooled active fractions were applied into the column. All the collected fractions were assayed for collagenolytic and elastinolytic enzymes activity & protein concentration, using standard assay methods.

2.8. Determination of the Molecular Masses of the Purified Collagenases and Elastases in Candida Parapsilosis and Candida Krusei By SDS-PA

Sodium dodecy1-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was adopted. The Laemmli SDS-PAGE discontinuous system (Laemmli, 1970) with homogenous gel was used under reducing or non-reducing conditions. A mini-protean II electrophoresis unit (Bio-Rad) was used.

2.9. Characterization of the Four Purified Proteases

The effect of pH, temperature, protease inhibitors and substrate specificity were carried out on the four proteases.

2.10. Cytotoxicity Assays of Collagenase and Elastase in Candida Parapsilosis and Candida Krusei

Potential cytotoxic activity of collagenase and elastase in *Candida parapsilosis* and *Candida krusei* were assayed using the method of Skehan *et al*. (1990) as follows:

Three cell lines: Colon carcinoma (HCT116), breast carcinoma (MCF7) and larynx carcinoma (HEP2) were planted in 96-multi well plate (10^4 cell/well) for 24 h before treatment with the-collagenase and elastase to allow attachment of cells to the wall of the plate. Different concentrations of enzyme under test (0.0, 10, 20, 30, 40, 50 and 60 U/ml) were added to monolayer cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the enzymes for 48 h at 37°C and 5% CO2 atmosphere. After 48 h, cells were fixed, washed and stained with sulfo-rhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured with an ELISA reader. The relation between surviving cells and enzyme concentrations was plotted to get the survival curve of each tumor cell line.

2.11. Statistical Analysis

For all determinations, the (one way ANOVA) statistical analysis was carried out including the calculation of the mean, standard deviation, standard error and t-value at level $p<0.05$.

3. Results

3.1. Screening the Collagenolytic and Elastinolytic Activities in Some Fungal Yeasts

Higher collagenolytic activities were exhibited by the tested *Candida* species than the elastinolytic activities, except in *Saccharomyces cerevisiae* where both enzyme activities were equal Table 1. *Candida parapsilosis* and *Candida krusei* recorded the highest collagenase activity (44 $Uml^{-1}$and 30 Uml^{-1}) and elastase activity (40 $Uml^{-1}$and 29 Uml^{-1}), respectively. Other tested fungi (*Candida tropicalis, C. albicans,* and *Saccharomyces cerevisiae)* attained weak collagenolytic and elastinolytic activities with minimum value (2.5 Uml^{-1}) in case of *Saccharomyces cerevisiae.* Consequently, the strongly active collagenolytic and elastinolytic yeasts, *Candida parapsilosis* and *Candida krusei* were chosen for completing this study.

Table 1. Assay of collagenolytic and elastinolytic activities of some yeast species.

Fungi	Collagenase activity (UmL^{-1})	Elastase activity (UmL^{-1})
C. albicans	6 ± .051	5.1 ± .032
C. krusei	30 ± .045	29 ± .050
C. parapsilosis	44 ± .036	40 ± .045
C. tropicalis	3.5 ± .046	4 ± .015
Saccharomyces cerevisiae	2.5 ± .045	2.5 ± .036

P> 0.05

3.2. Purification of Collagenase and Elastase from C. Parapsilosis and C. Krusei

Four enzymes were purified to full homogeneity using different purification steps namely, precipitation by $(NH_4)_2$ SO_4, anion exchange using DEAE cellulose column chromatography and gel filtration chromatography using sephadex G100.

3.3. Purification of C. parapsilosis collagenase

The first step of collagenase purification by precipitation with $(NH_4)_2$ SO_4 increased the specific activity from 0.52 U mg^{-1} in crude supernatant to 0.92 U mg^{-1} with purification fold of 1.76. Anion exchange column using DEAE cellulose resulted in two large peaks. Peak1 with enzyme activity 36 U ml^{-1}, specific activity 1.4 U mg^{-1} and purification fold of 2.69 of the crude enzyme Table 2. Peak 2 contained less active fractions than peak 1 and had 25 U ml^{-1}, 1.3 U mg^{-1} enzyme activity and specific activity with 2.5 purification fold. Further purification using gel filtration chromatography by sephadex G100 resulted in a single peak with maximum activity at fraction 15 Table 2. The enzyme activity was 44 U ml^{-1}, with specific activity of 2.9 U mg^{-1} and 5.5 purification fold of crude.

Table 2. Summary of purification steps of collagenase from *C. parapsilosis.*

Purification procedure		Collagenase activity (U ml^{-1})	Total protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification fold
Crude supernatant		48	92	0.52	1.0
$(NH_4)_2$ SO_4 precipitation		38	41	0.92	1.76
Anion exchange DEAE-cellulose chromatography	Peak1	36	26	1.4	2.69
	Peak2	25	19	1.3	2.5
Gel filtration chromatography sephadex G100		44	15	2.9	5.5

P >0.05

3.4. Purification Of C. Parapsilosis Elastase

The partially purified elastase resulting from $(NH_4)_2$ SO_4 precipitation recorded an increase in the specific activity of 0.76 U mg^{-1} compared to 0.61 in crude preparation. Anion exchange chromatography using DAEA cellulose resulted in two peaks (**Table 3**). Peak1 attained activity of 38 U ml^{-1} with specific activity of 1.3 U mg^{-1} and recovery 2.13 fold of crud value. Peak 2 resulted in enzyme activity of 32 U ml^{-1}, specific activity of 1.5 U mg^{-1} and purification fold of 2.54 of the crude enzyme. Gel filtration chromatography resulted in a single peak at which enzyme activity of 41 U ml^{-1}, specific activity 2.1 U mg^{-1} and purification fold of 3.4 were attained (**Table 3**).

Table 3. Summary of purification steps of elastase from *C. parapsilosis*

Purification procedure		Elastase activity (U ml^{-1})	Total protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification fold
Crude supernatant		43	71	0.61	1.0
$(NH_4)_2SO_4$ precipitation		33	43.5	0.76	1.24
Anion exchange DEAE–cellulose chromatography	Peak1	38	30	13	2.13
	Peak2	32	22	1.5	2.54
Gel filtration chromatography sephadex G100		41	20	2.1	3.4

3.5. Purification of collagenase from C. krusei

As the crude collagenase was precipitated by $(NH4)_2$ SO_4 up to 80 %, the specific activity increased to 0.61 Umg^{-1} compared to 0.55 Umg^{-1} in the crude enzyme (Table 4). Anion exchange chromatography resulted in two peaks, Peak 1 recorded collagenase activity of 36 Uml^{-1}, and specific activity of 1.44 Umg^{-1} with 2.6 purification fold of crude enzyme. Peak2 attained enzyme activity of 24 Uml^{-1}, specific activity of 1.33 Umg^{-1} with 2.4 purification fold. Gel filtration chromatography using sephadex G 100 resulted in appearance of a single peak with enzyme activity, specific activity and purification fold of 50 Uml^{-1}, 2.9 Umg^{-1} and 5.2 of the crude preparation, respectively.

Table 4. Summary of purification steps of collagenase purified from *C. krusei.*

Purification procedure		Collagenase activity (Uml^{-1})	Total protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification fold
crude supernatant		44	80	0.55	1.0
$(NH_4)_2SO_4$ precipitation		32	52	0.61	1.11
Anion exchange DEAE-cellulose chromatography	Peak1	36	25	1.44	2.6
	Peak2	24	18	1.33	2.4
Gelfiltration chromatography sephadex G100		50	17	2.9	5.2

3.6. Purification of elastase from C. krusei

Ammonium precipitation lead to an increase in specific activity from 0.72 Umg^{-1} in crude preparation to 1.19 Umg^{-1} in the partially purified enzyme with purification fold of 1.63 of the crude (Table 5). Anion exchange chromatography using DEAE cellulose resulted in 2 peaks. The elastase activity at peak1 was 33 Uml^{-1}, while the specific activity and purification fold attained values of 1.57 Umg^{-1} and 2.18 of the crude preparation, respectively. Peak 2 recorded 21 Uml^{-1} elastase activity, 1.4 Umg^{-1} specific activity and 1.94 purification fold of the crude enzyme. Gel filtration chromatography resulted in a single peak with enzyme activity of 46 Uml^{-1}, specific activity of 4.2 Umg^{-1} and purification fold of 5.83 of the crude elastase (Table 5).

Table 5. Summary of Purification Steps of Elastase from *C. krusei.*

Purification procedure		Elastase activity (U ml^{-1})	Total protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification fold
Crude supernatant		47	65	0.72	1.0
$(NH_4)_2SO_4$ precipitation		37	31	1.19	1.63
Anion exchange DEA–Cellulose chromatography	Peak1	33	21	1.57	2.18
	Peak2	21	15	1.4	1.94
Gel filtration chromatography sephadex G100		46	11	4.2	5.83

3.7. Molecular Weight Study

The molecular masses of collagenases and elastases purified from C. parapsilosis and C. krusei were determined on each of add (a) crude preparations, (b) two peaks appeared after anion exchange chromatography on DEAE cellulose column and (c) single peak resulted from gel filtration

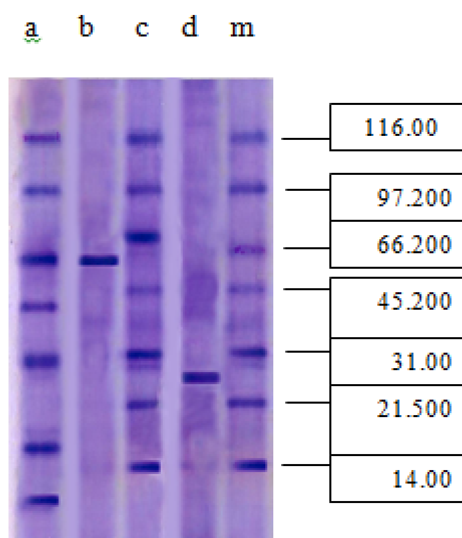

Figure (1a): The electrophoretic pattern of the purified collagenase and elastase from *Candida krusei* by gel filtration (Sephadex G-100) chromatography.
(a) crude elastase, (b) pure elastase (single bands after sephadex G100), (c) crude collagenase, (d) pure collagenase - (single bands after sephadex G100), (m) marker.

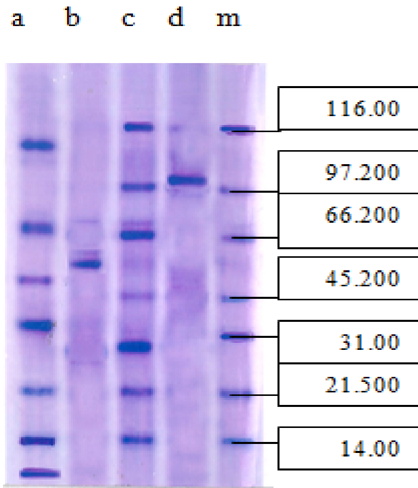

Figure (1b): The electrophoretic pattern of the purified collagenase and elastase from *Candida parapsilosis* by gel filtration (sephadex-G-100) chromatography.
a- crude elastase, b- pure elastase (single bands after sephadex G100), c- crude collagenase. d- pure collagenase -(single bands after sephadex G100), m – marker bands.

In crude enzymes, seven protein bands appeared with molecular masses ranged from 117.9 to 11.45 KDa. Anion exchange with DEAE cellulose resulted in appearance of two bands for each enzyme with molecular masses of approximately 96.5 & 45.5 KDa and 66.4 & 27.3 KDa for collagenase and elastase of *Candida parapsilosis* , while 73.9 & 35.4 kDa and 65.4 & 29.2 kDa for collagenase and elastase in *C. krusei*, respectively. The fully purified four enzymes which appeared as single band in gel filtration by sephadex G 100 had apparent molecular masses of 97.2 KDa for collagenase in *C. parapsilosis*, 53.4 KDa for elastase for *C. parapsilosis*, 66.4 KDa for collagenase in *C. krusei* and 23.5 KDa for elastase in *C. krusei*.

3.8. Characterization of the Four Purified Proteases

The effect of different pH values, temperatures, protease inhibitors & substrate specificity indicated that for collagenase, the optimum pH was 8 and the optimum temperature was 40 °C, while for elastase the optimum pH was 7 and the optimum temperature was 30 C. The four enzymes were metalloproteases inhibited by EDTA, EGTA and PMSF. The four enzymes were non specific proteases and could hydrolyze bovine albumin and gelatin in addition to collagen and elastin. They could not hydrolyze casein and fibrinogen (Table 6).

Table 6. Summary of characterization of the four protease enzymes.

Characterization	*C. parapsilosis*		*C. krusei*	
	Collagenase	Elastase	Collagenase	Elastase
Molecular mass of single band (KDa)	97.2	53.4	66.4	23.5
pH	8	7	8	7
Temperature (°C)	40	30	40	30
Protease inhibitors	Serine metalloprotease	Serine mettaloprotease	Serine mettaloprotease	Serine mettaloprotease
specificity	Non specific	Non specific	Non specific	Non specific

3.9. Cytotoxicity Assays of the Four Enzymes

In vitro cytotoxic activity of collagenases and elastases either singly or in combination, crude or purified from *Candida parapsilosis* and *Candida krusei* was assayed using different carcinoma cell lines of colon (HCT116), breast (MCF7) and larynx (HEP2).

3.9.1. Single treatment with crude enzymes

It was observed that single treatment with crude collagenase from *Candida parapsilosis* led to reduction in surviving cells of HEP2 carcinoma cell line with IC50 of 14.58 Uml^{-1} (Fig 2-A), while crude collagenase from *Candida krusei* exerted no cytotoxic activity on HEP2 carcinoma cell line and not achieved IC_{50} (Fig 2-C). Crude elastase preparations from both *Candida parapsilosis* and *Candida krusei* showed high cytotoxic activity on larynx carcinoma cell line (HEP2) with IC_{50} of 13 Uml^{-1} and 11.8 Uml^{-1}, respectively (Fig 2-B &D).

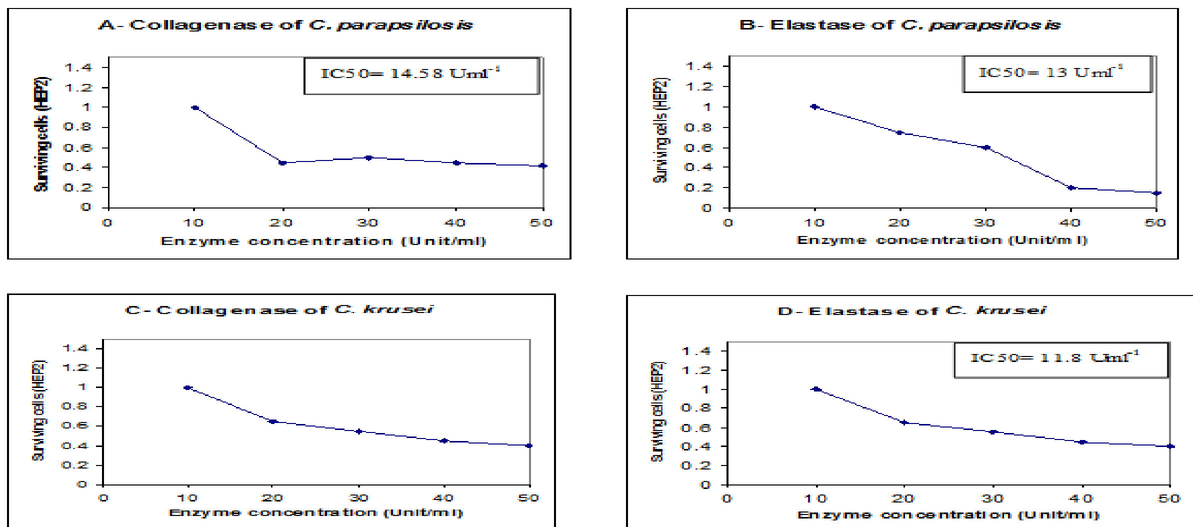

Figure 2. Single treatment with crude enzymes using HEP2

The four crude enzyme preparations had non-significant cytotoxic activity on breast carcinoma cell lines (MCF7) (Fig 3) and on colon carcinoma cell line (HCT116) (Fig 4) and no IC50 were detected in all treatments.

3.9.2. Single Treatment With Purified Enzyme

The single treatment with purified collagenase from *Candida krusei* exerted high cytotoxic activity on larynx carcinoma cell line (HEP2) with IC50 28.8 Uml^{-1} (Fig 5-C), while the purified collagenase from *Candida parapsilosis* was inactive and no IC50 was detected (Fig 5-A). Purified elastases from *Candida parapsilosis* and *Candida krusei* exerted significant cytotoxic activity on larynx carcinoma cell line (HEP2) with IC50 of 15.9 Uml^{-1} (Fig 4-B) and 13 Uml^{-1} (Fig 5-D), respectively.

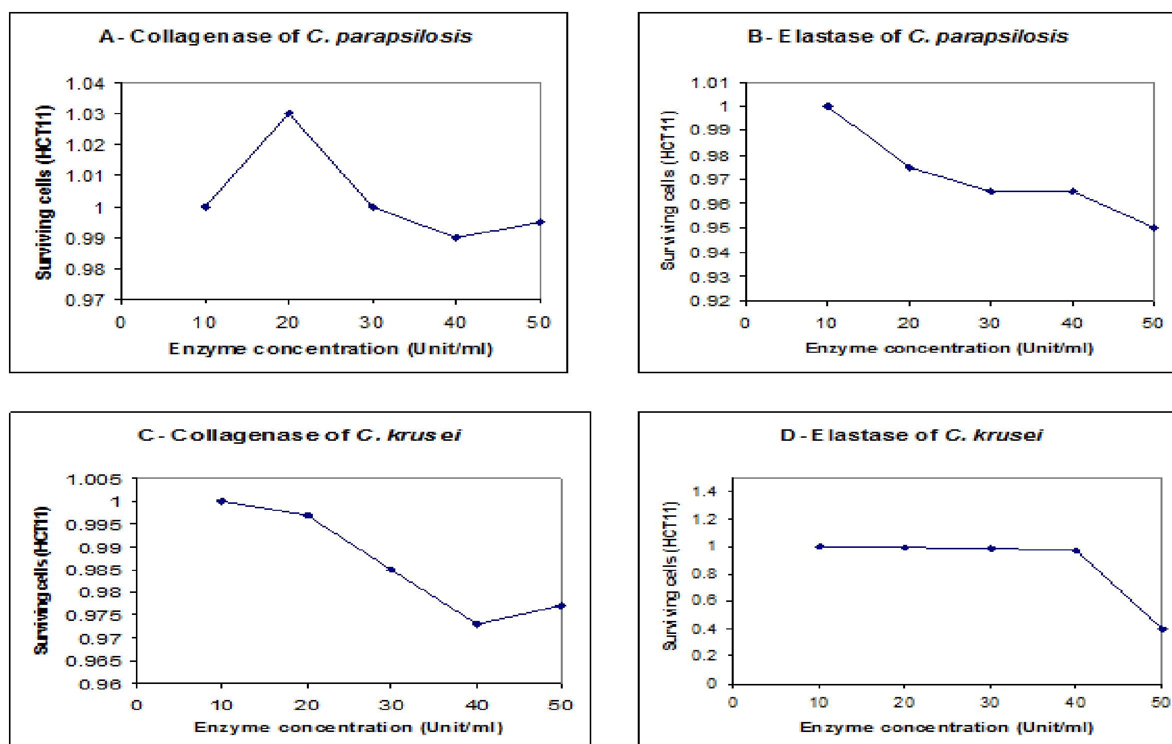

Figure 3. Single treatment with crude enzymes using HCT11

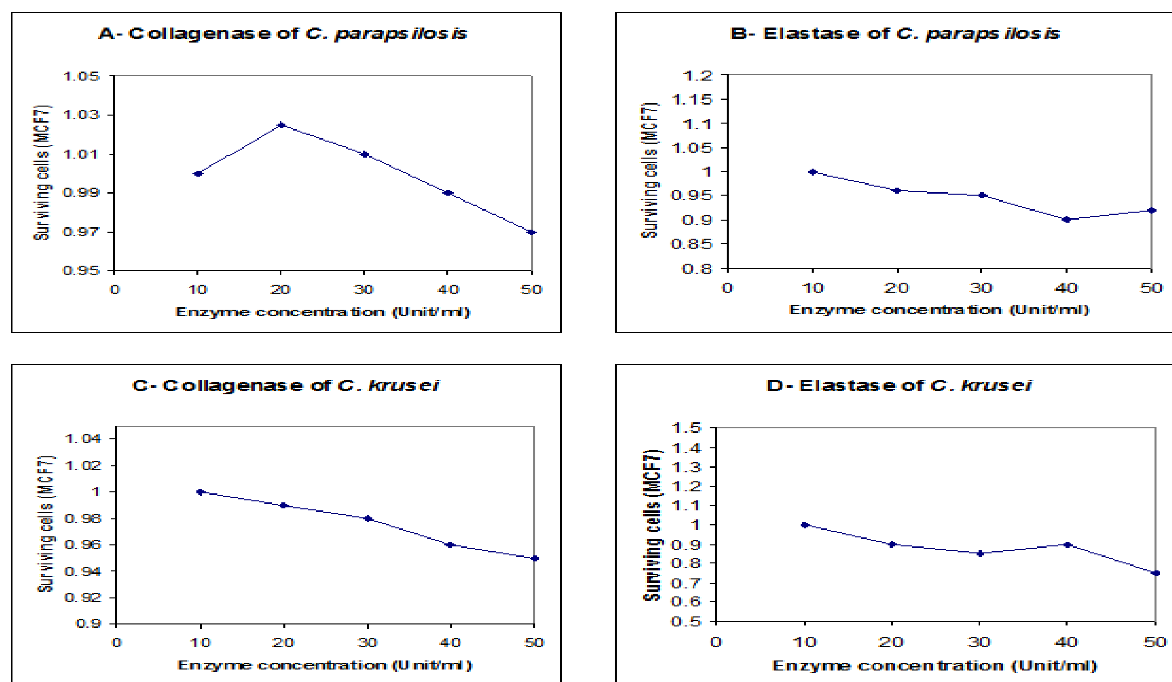

Figure 4. Single treatment with crude enzymes using MCF7

3.9.3. Combined Treatment with Purified Enzyme Mixtures

Combined mixture containing purified collagenase and elastase from *Candida parapsilosis* exerted high cytotoxic activity on larynx carcinoma cell lines (HEP2) with IC50 12.8 Uml^{-1} (Fig 6-A), while the same mixture of purified enzymes but from *Candida krusei* had no cytotoxic activity on larynx carcinoma and not achieved IC50 Fig 5-B.

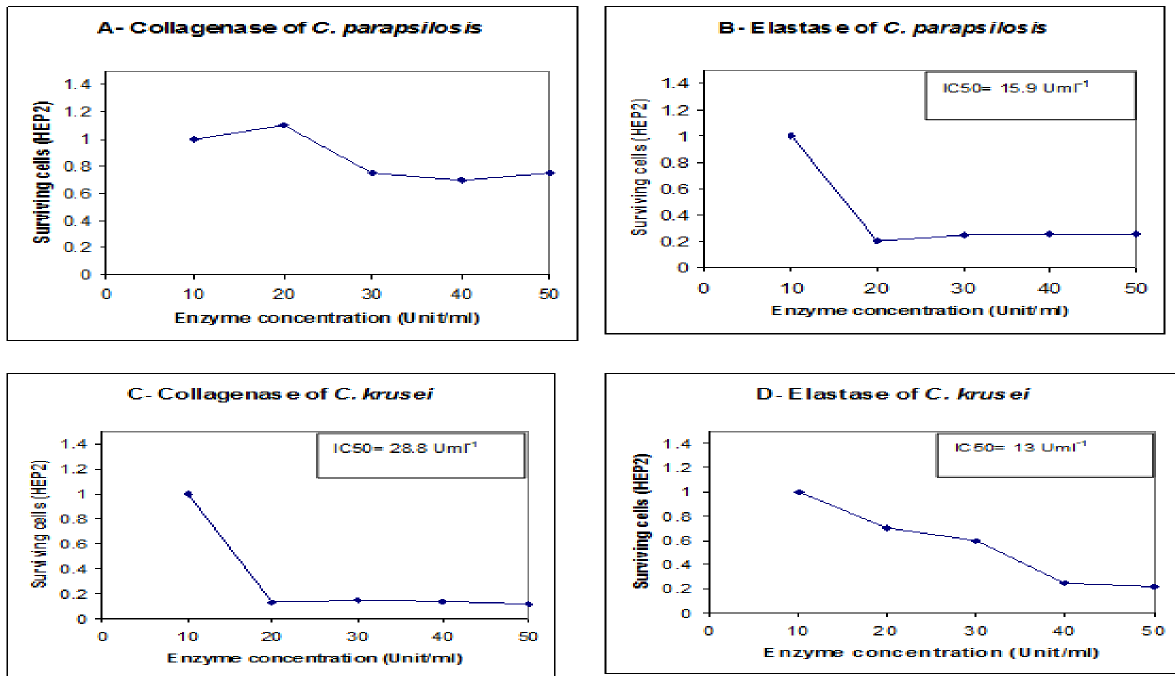

Figure 5. Single treatment with purified enzyme using HEP2

Combined mixture containing the four purified enzyme preparations from the two *Candida* species exerted the highest cytotoxic activity with very low IC50 of 3.1 Uml^{-1} Fig 6-C. It is worthy noting that the cytotoxic activity of all tested enzyme preparations was concentration dependent.

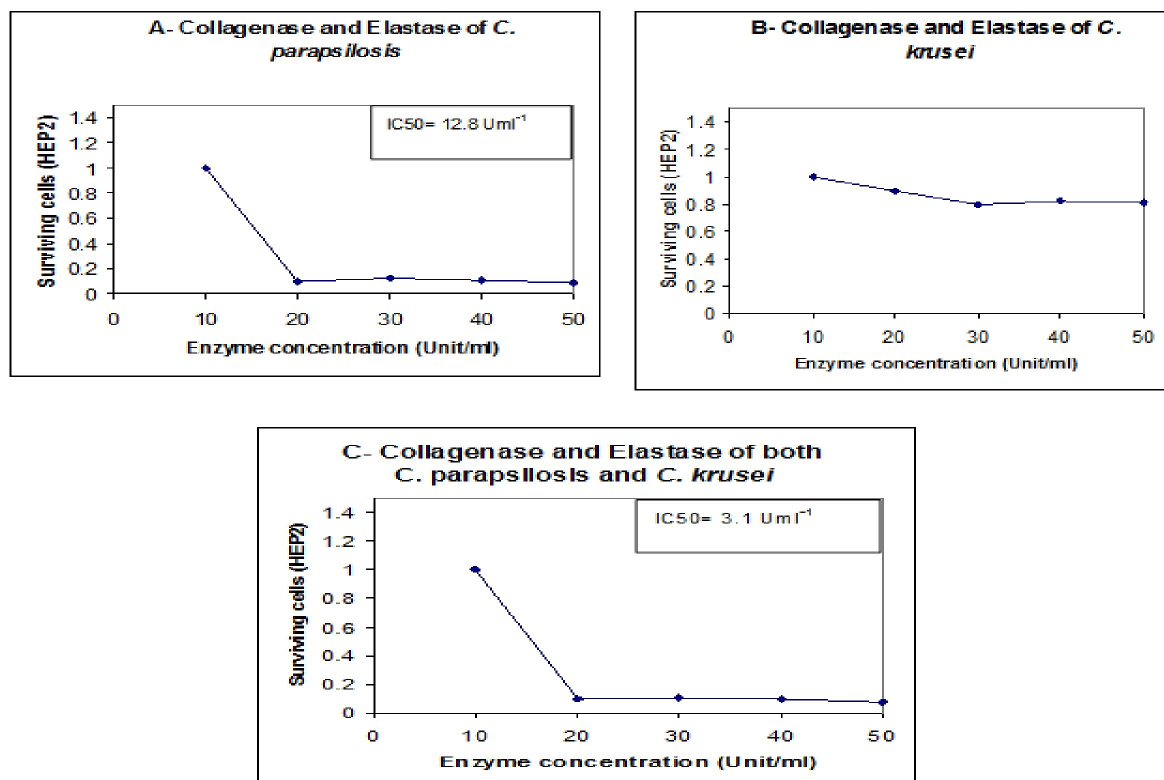

Figure 6. Combined treatment with purified enzyme mixtures using HEP2

4. Discussion

Several workers isolated and purified different proteases including keratinases, collagenases and elastases from different dermatophytes (Yu *et al*., 1968, 1971; Takiuchi and Higuchi, 1977; Takiuchi *et al*., 1982; Sanyal *et al*., 1985). In the present study, five fungal yeasts were tested for their collagenolytic and elastinalytic activities. *C. parapsilosis* and *C. krusei* proved to have the strongest collagenolytic and elastinolytic activities, however, *C. albicans*, *C. tropicalis* and *Saccharomyces cerevisiae* showed weak activities in both enzymes. *Candida* species secreted collagenolytic enzyme to utilize collagen in medium containing collagen as sole nitrogen source (Kaminishi *et al*., 1986). A number of potentially virulence – related proteases such as elastases and collagenases have been documented in both pathogenic and non pathogenic fungi (Sanyal *et al*., 1985). S*treptomyces* strain 3B constitutively secreted collagenolytic enzymes during the post exponential growth phase (Petrova and Derekova, 2006).

The molecular mass studies clearly indicated that the molecular masses of collagenases are higher than that of elastases in both *Candida* species. Furthermore, the molecular masses of both enzymes in *C. parapsilosis* are higher than those in *C. krusei*. The dependence of enzyme molecular mass on the producing microorganism was reported by Jain (2008) in *Streptomyces exofoliatus* collagenase with molecular mass of 14.5 KDa, Hamdy (2008) in *Rhizoctonia solani* collagenase with molecular mass of 66 KDa, Rhodes *et al.* (1990) in *Aspergillus flavus* elastase with molecular mass 23 KDa and Petrova and Derekova (2006) in *Streptomyces* strain collagenase I & II with molecular masses of 116 & 97 KDa.

In our present study, the optimum pH and temperature were 8 and 40 C for collagenases while they were 7 and 30 ^{o}C for elastases. The four enzymes were metalloproteases and non specific.

The single treatments of the four crude enzyme preparations exerted cytotoxic activity against larynx carcinoma cell lines (HEP_2) only, while colon and breast carcinoma were non responsive to enzyme treatments. The single treatments of the four pure enzymes against HEP_2 carcinoma cell lines resulted in less cytotoxic effect with higher values of IC50 than that recorded in case of crude enzyme treatments. Furthermore, elastases exerted stronger cytotoxic activity with lower values of IC50 than collagenases treatment. Mixture of pure collagenase and elastase from *C. parapsilosis* and mixture of pure collagenase and elastase from *C. kruse*i revealed different results. While the first mixture increased the cytotoxic activity against larynx carcinoma cell lines (HEP2), the second mixture reduced cytotoxic activity and not achieved IC50 compared to single preparation.

Treatment with combination mixture containing the four purified enzymes from *C. parapsilosis and C. kreusi* resulted in highly significant cytotoxic activity against the larynx carcinoma cell lines (HEP_2) with minimum IC50 ($3.1 Uml^{-1}$). It could be concluded that mixture of collagenases and elastases from more than one microorganism acted synergistically and exerted stronger cytotoxic activity than enzymes from one microorganism.

Several mechanisms were reported about the role of proteases in cancer therapy. Proteolytic enzyme therapy is an effective alternative cancer treatment by reversing the tumor from malignant to benign, inducing apoptosis in tumor cells, breaking down the tumor by eliminating the toxins and cancer cells, strengthening the body and immune system and reversing the internal environment from anaerobic, acidic pH (favorable to cancer cells) to a healthy, aerobic slightly alkaline condition (Aoyama and Chen, 1990). Proteolysis enzyme treatment to patients of multiple myelnoma increases the response rate and prolongs survival in patients. It has been hypothesized that proteolytic enzymes help break the protective coating produced by cancer cells, thereby giving immune system a better chance to attack the tumor cells (Warrior, 2010). Heavy dosing of proteoloytic enzymes supplement has succeeded with even terminal cancer patients. These enzymes digest complete rip up cancer cell wall, leaving the cancer cells vulnerable to the immune system (Bell, 2011). The major components of extracellular matrix in cancer cells are collagen (type 1-V) proteoglycans, elastin, laminin and fibronectin. The interactions of various proteases have been identified to degrade these barriers to affect cancer cells.

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The treatment of cultured tumor cells with proteases mixture causes complete arrest of directional movement of metastatic cells. Conversely, the same treatment of normal cells results in enhanced motility and an acceleration closure of gap created the cell monolayer (Novak and Trnka, 2005). Protease treated tumor cells contain a disrupted action cytoskeleton and exhibit a loss of front to back polarity (Novak and Trnka, 2005). It is hypothesized that provision of proenzymes rather than the enzymes was of crucial importance to the clinical effectiveness in human tumor. Proteases mixtures are components of commercially available drugs named ((Wobe Mucos-E)) and plogenozyme (Moco Pharma Gmbh, Geretsried, Germany). When protease mixture is provided orally, it resulted in measurable relief from cancer disease and cause significant extension of survival (Dale *et al*., 2001; Billigmann, 1995). Different modes of action concerning proteases treatment of cancer cells have been reported: including i) suppression of cytokine levels, (Desser *et al*., 1997). ii) enhancement of polymorphonuclear leukocyte mediated cytotoxicity (Zavadova *et al*., 1995) iii) reduction of the side effect of radiation therapy (Gujral *et al*., 2001). The activation of trypsinogen proenzymes may occur within the tumor environment by auto activation

(Colombatti *et al*., 1993; Ohta *et al*., 1998). The cytotoxic enzymes treatment include matrix metalloprteases and serine proteases especially collagenases and elastases (Woolley, 1993).

5. Conclusion

Combination mixture of the four proteases (collagenases & elastases) recorded the most potent cytotoxic activity against larynx carcinoma cell line (HEP2) with minimum value of IC_{50} (3.1 Uml^{-1}).

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