



Culture and Identification of *Candida Albicans* from Vaginal Ulcer and Separation of Enolase on SDS-PAGE

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Abstract

Candida albicans were isolated from patients with clinical symptoms of vaginal ulcer. Culture test for vaginal swab and scrapings were conducted on Sabouraud's dextrose broth and Sabouraud's dextrose agar plate respectively. Hichrome candida agar culture was used for differential identification of *Candida*. Smears from vaginal scrapings were prepared for gram staining. The suspected strain of *Candida* was inoculated on corn meal agar medium for chlamyospore formation. The suspected strain of *Candida* was inoculated in human serum for germ tube formation. Carbohydrate assimilation and fermentation tests were also conducted. The selected *Candida* colony was inoculated in YEPD medium for subculture and the cultured organism was harvested. The organisms were homogenized, centrifuged and the supernatant was filtered. The filtrate was extracted in chloroform. The extract was centrifuged and the aqueous phase was dialyzed. The dialyzed crude enolase was subjected to SDS-PAGE. The Sabouraud's dextrose broth inoculated with vaginal swab showed turbid growth. The scraping from vagina showed typical smooth creamy white colonies with a characteristic yeast odour on Sabouraud's dextrose agar plate. On Hichrome candida agar the *Candida* growth appeared as glistening green colored. Gram stained smears from vaginal scraps showed appearance of fungus as yeast

budding. On corn meal agar the suspected *Candida* growth showed the formation of large, highly refractive, thick walled terminal chlamydospores. Germ tubes were seen as long tube like projections extending from the yeast cells on human serum inoculated with suspected strain of *Candida*. The carbohydrate assimilation tests were positive for dextrose, maltose, sucrose, galactose, xylose and trehalose, and negative for lactose, melibiose, ellobiose, inositol, raffinose and dulcitol. The carbohydrate fermentation tests showed positive for dextrose, maltose, galactose and trehalose, and negative for sucrose and lactose. SDS-PAGE for enolase from *C. albicans* showed a single polypeptide band of around 46 – 48 kDa.

Keywords: *Candida albicans*, Enolase, Vaginal-ulcer

1. Introduction

The yeast *Candida albicans* is commonly inhabits in oral and vaginal mucosa and gastrointestinal tract of human beings as one of the commensal organisms. It causes opportunistic infections in immuno-compromised patients, produces allergic reactions and rarely causes morbidity and mortality (Douglas, 1988). It also causes a variety of infections that range from mucosal candidiasis to life-threatening disseminated candidiasis (Dixon *et al.*, 1996; O'Dwyer *et al.*, 2007; Terrier *et al.*, 2007).

The diagnosis of its deep seated infections remains a great challenge. One of the major reasons for the increase in *Candida* infections is the development of its resistant strains to azole drugs, such as fluconazole used in the prophylaxis and treatment of candidiasis (Diaz-Guerra *et al.*, 1998; Shahid *et al.*, 2006). The identification of invasive or disseminated candidiasis is based on clinical symptoms that are diffuse and not easily differentiated from those manifested by other infectious agents. Therefore considerable interest in the identification and characterization of antigens would be useful in the diagnosis and treatment of *Candida* infections.

It has been reported that in *C. albicans* and *Candida tropicalis* the cytosolic enzyme, enolase, was identified as an immuno-dominant antigen (Walsh *et al.*, 199; Mitsutake *et al.*, 1994; Angiolella, 1996). *Candida* enolase is a plasminogen- and plasmin-binding protein and the interaction of *C. albicans* enolase with the plasminogen system may contribute to invasion of the tissue barrier (Jong, 2003). Antibody to this bona fide cell wall protein is considered to be more predictive and specific as the cytoplasmic antigen is exposed only during invasive infection. Patients with localized candidiasis express only weak positive antibody titre (Van Deventer *et al.*, 1993). In recent years *Candida* infection increases clinical awareness. Therefore, the present work was aimed to culture and identify *C. albicans* from patients with suspected invasive vaginal candidiasis, and separate enolase on SDS-PAGE to identify its molecular weight.

2. Materials and Methods

C. albicans strains were isolated from patients with clinical symptoms of vaginal ulcer and similar infections.

2.1 Collection and processing of vaginal samples

The suspected patients were used as subjects and the samples were collected and analyzed for *C. albicans*. The specimens were collected with meticulous care aseptically at the slit lamp.

2.2 Culture test from vaginal swabs

Sterile cotton swabs were prepared, gently smeared over the ulcers in the vaginal region and the swabs were immediately transferred to Sabouraud's Dextrose broth (pH, 5.6) containing dextrose (4 %), peptone (1 %) in double distilled water and incorporated with Chloramphenicol (5.0 mg). The culture tubes were incubated at 37⁰ C for 24 – 48 hr.

2.3 Culture test from vaginal scrapings

Using a heat sterilized Kimura's platinum spatula of Bard Parker blade, vaginal scrapings were collected and the scrap material was streaked onto Sabouraud's dextrose agar plates as a fine C-streak.

2.4 Preparation of Sabouraud's dextrose agar plates

The Sabouraud's Dextrose agar consists of Sabouraud's dextrose broth (pH, 5.6) and 1.5 % agar. This was autoclaved and cooled to 45⁰ C, and 5 ml of sterile defibrinated sheep blood was added to it aseptically. It was mixed thoroughly and dispensed into plates without air bubbles. The plates were incubated at 37⁰ C for 24 – 48 hr. The suspected isolates of *Candida* were sub cultured onto Sabouraud's agar plates.

2.5 Differential identification of candida

Hichrome Candida agar (pH, 6.5) containing 1.5 % peptic digest of animal tissues, 0.1 % dipotassium hydrogen phosphates, 1.2 % chromogenic mixture, 0.05 % Chloramphenicol and 1.5 % agar in double distilled water was used for the presumptive identification of clinically important *Candida* species. The scraps were directly streaked on Hichrome Candida agar and incubated at 37⁰ C for 48 hr.

2.6 Gram staining

Smears from the vaginal scrapings were prepared on slides cleaned with alcohol. They then heat-fixed; staining was done by flooding the smears with crystal violet solution for 1 min and then with Gram's iodine for 1 min. After washing, the smears were decolorized with 95 % ethanol and counter stained with aqueous basic fuchsin. The slide was subjected to observation of *Candida* morphology under oil immersion objective lens (100 x) of a Bright Field microscope.

2.7 Test for chlamyospore formation

The suspected *Candida* cultures were inoculated on corn meal agar medium (pH, 7) containing 4% corn meal powder, 1 % Tween 80 and 1.5 % agar in double distilled water. The plates were incubated at 37^o C for 48 - 72 hr.

2.8 Test for germ tube formation

The suspected *Candida* cultures were inoculated into 0.5 ml of human serum in a small tube and incubated at 37^o C for 2 hr. After desired period of incubation, a loop-full of culture was placed on a glass slide and overlaid with a cover-slip. The preparation was examined for germinating blastospores.

2.9 Carbohydrate assimilation tests

The mainstay of yeast identification to the species level is the carbohydrate assimilation test, which measures the ability of yeast to utilize a specific carbohydrate as the sole source of carbon in the presence of oxygen. Sugars used for assimilation tests include dextrose, maltose, sucrose, lactose, galactose, melibiose, cellobiose, inositol (a form of sugar, carbocyclic polyol, cyclohexanehexol), xylose, raffinose, trehalose and dulcitol (or galactitol, a sugar alcohol, the reduction product of galactose).

Candida cultures were suspended in saline (NaCl) and to which 1.5 ml of basal medium containing 67.8 % yeast nitrogen base was added. This was then added to 13.5 ml of molten, cooled agar containing 2 % agar powder, mixed well, poured into Petri dish and allowed to solidify. After the medium was set, 20 % sugar-soaked filter paper discs were placed on the medium. The plates were incubated at 25^o C for 10 – 24 hr and observed for the growth of *Candida*.

2.10 Carbohydrate fermentation tests

Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol. Production of gas rather than a pH shift is indicative of fermentation. Dextrose, maltose, sucrose, lactose, galactose and trehalose were used in the test.

The 5 ml of carbohydrate (pH, 7.4) containing 1 % peptone, 1 % sugar, 0.3 % beef extract and 0.5 % NaCl, 0.2 % Bromothymol blue in distilled water medium was dispensed in sterilized Durham tube and 0.2 ml of saline suspension of the test organism was added and incubated at 37^o C for 10 days.

2.11 Subculture and harvesting of yeast cells

The selected organism was inoculated in yeast-extract-peptone-dextrose (YEPD) culture medium (pH, 7) containing 1 % yeast extract, 2 % peptone and 2 % dextrose, and incubated at 30^o C for 24 hr in a mechanical shaker at 150 rpm. A 10 ml starter culture provided the basis for 500 ml *Candida* culture to be grown under similar conditions in a 2 l Erlenmeyer flask.

The *C. albicans* culture was harvested from the 2 l Erlenmeyer flask by centrifugation at 10,000 rpm for 15 min at 4^o C. The cells were washed with distilled water and again collected by centrifugation. The supernatant was discarded and the cell pellets were re-suspended in protein-extraction buffer (pH, 6.5) containing 0.2 % Bis-Tris. The yeast cells were centrifuged as described above then re-suspended and pooled for further processing.

2.12 Cell fractionation

The organisms were suspended in a breaking buffer (pH, 6.8) containing 62.5 mM Tris-HCl buffer, 15 % glycerol, 1mM dithiotheritol and 20 mg of phenyl methyl sulfonyl flouride and broken by mechanical disruption in a Braun's homogenizer for 2 min with intermittent cooling in the presence of glass beads 0.45 mm in dia. After disruption of the cells, the glass beads and unbroken cells were removed by centrifugation at 500 x g. The homogenate was then centrifuged at 6000 x g for 20 min and the pellet was collected. The supernatant containing the soluble protein fraction was recovered and passed through a 0.45 µm filter membrane. The filtrates were then extracted with an equal volume of chilled chloroform. Following centrifugation at 4^o C for 15 min at 2000 x g, the upper aqueous phase was aspirated and transferred to a dialysis tube. The crude protein fraction was dialyzed in column binding buffer (20 mM Bis-Tris, pH 6.5) for 24 hr. The dialysed crude enolase was subjected to Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS – PAGE).

2.13 SDS - PAGE

SDS – PAGE was performed following the method of Laemmli (1970). Equal amount of sample loading buffer was added to the enolase samples (20 µg protein). The samples were boiled for 1 min and loaded on 12% SDS – PAGE. Protein standard of known molecular weight was also run parallel. The wells and tanks were filled with electrophoresis running buffer. The protein was allowed to isolate at 50 V DC in the stacking region and 100 V DC in the separating gel for about 3 hrs. The gel was stained overnight in Coomassie Brilliant Blue solution. The stained gel was de-stained in methanol – acetic acid solution until the gel background turns clear for visualizing the polypeptide band.

3. Results and Discussion

Candida is a dimorphic organism, meaning it can exist in two shapes and forms simultaneously. One form is a yeast-like state that is a non-invasive, sugar fermenting organism. The other is a fungal form that produces very long root-like structures, called rhizoids that can penetrate the mucosa and is invasive.

Healthy immune system prevents this yeast from becoming an infectious fungus. It is when our bodies lose their proper immune protection or the intestinal pH is altered unfavourably, that the organism can change from the yeast form to the fungal form. When this happens, the now-parasitic fungal form penetrates the gastrointestinal mucosa and breaks down the boundary between the intestinal tract and the rest of the circulation in our bodies. This allows partially digested dietary proteins to travel into the blood stream, where they exert a powerful antigenic (antibody-stimulating) assault on the immune system. It has been officially estimated that about 80% of the population may have candidiasis that is out of control.

The immune system is attacked by *Candida* as a result of the prolonged use of antibiotics, taking steroids or oral contraceptives on a regular basis, or due to high sugar diet. It is also known that *Candida* increases its numbers during periods of stress and lowered immune states.

Fungal infection of vagina is sometimes called a thrush. Candidiasis is also known as yeast infection is a common fungal infection that occurs when there is overgrowth of the fungus called *Candida* which is always present in the body in small amounts. However, when an imbalance occurs, such as when the normal acidity of the vagina changes or when hormonal balance changes, *Candida* can multiply. When this happens, symptoms of candidiasis appear. This may include depression, dry, itchy, flaky skin, anxiety, recurring irritability or mood swings, heartburn, indigestion, lethargy, food and environmental allergies, joint soreness, chest pain or other skin problems, recurring cystitis/vaginal infections, premenstrual tension and menstrual problems. Immune suppression, AIDS, diabetes and thyroid disorder may also cause candidiasis.

Vaginal thrush was characterized by typical white lesions developed on the epithelial surfaces of vulva, vagina and cervix. In the present study, the Sabouraud's dextrose broth inoculated with swab from the lesions of vagina showed turbid growth after incubation. On Sabouraud's dextrose agar, the scrapings from lesions of vagina showed typical smooth creamy white colonies with a characteristic yeast odour. Scrapings from the lesions of vagina were examined in the Gram stained smear. The fungus appeared as budding yeast cells and pseudomycelium was present in most cases (Fig. 1). On Hichrome candida agar, the scrapings from vagina produced glistening green color colonies (Fig.2). The suspected stains of *C. albicans* isolates were grown on the corn meal agar. It showed the formation of large, highly refractive and thick walled terminal chlamydo spores (Fig. 3). When the suspected *C. albicans* was inoculated with human serum the formation of germ tubes was seen as long tube like projections extending from the yeast cells. There was no constriction at the point of attachment to the yeast cells. The germ tubes were formed within two hours of incubation in *C. albicans* (Fig. 4).

The carbohydrate assimilation tests for *C. albicans* are presented in table 1. It shows that the test was positive for dextrose, maltose, sucrose, galactose, xylose and trehalose. At the same time, the tests were negative for lactose, melibiose, cellobiose, inositol, raffinose and dulcitol. Similarly, the carbohydrate fermentation tests for *C. albicans* are presented in table 2. The tests for fermentation of dextrose, maltose, galactose and trehalose were positive but negative for fermentation of sucrose and lactose.

The SDS-PAGE profile of the crude sample prepared from *C. albicans* for enolase showed several polypeptide bands from 205 kDa to 29 kDa. Among these, the prominently resolved single polypeptide band of around 48 kDa was considered as enolase (EC: 4.2.1.11) (Fig. 5). This confirmation was based on the reports in the literature (Strockbine *et al.*, 1984; Walsh *et al.*, 1988, 1991; Savolainen *et al.*, 1990; Sundstrom and Aliaga, 1992; Mason *et al.*, 1993; Ito *et al.*, 1995; Chang *et al.*, 2002). The enolase from the pathogenic yeast *C. albicans* has been identified as an immunodominant antigen in disseminated infection and in allergic disease (Mitsutake *et al.*, 1994). The *Candida* species such as *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. lusitaniae* are increasingly being identified as significantly causing infections in humans (Hazen and Hazen, 1992).

The cell wall of *Candida* is a dynamic and multilayered structure located external to the plasma membrane. The cell wall of *C. albicans* is composed of different types of carbohydrates (80 – 90 %): (i) Mannan or polymers of mannose

covalently associated with proteins to form glycoproteins, also referred to as mannoproteins, (ii) α -glucans that are branched polymers of glucose containing α -1,3 and α -1,6 linkages, and (iii) chitin, which is an un-branched homopolymer of N-acetyl-D-glucosamine (Glc-NAC) containing α -1,4 bonds (Gomez *et al.*, 1996). The other constituents are proteins (6 - 25 %) and lipids (1 - 7 %) which are present as minor wall constituents (Shepherd, 1987). Yeast cells and germ tubes are similar in their cell wall composition, although the relative amounts of α -glucans, chitin and mannan may vary with morphology. Although glucans are present in greater abundance than mannan in *C. albicans*, they are immunologically less active (Poulain *et al.*, 1985).

The enzyme enolase was reported to be associated with glucan in the inner layers of the cell wall as well as in culture supernatants. Enolase seems to be the most immunodominant antigen in humans. In mice, enolase stimulates both humoral and cellular immune responses. The glycolytic enzyme, enolase catalyzes the reversible dehydration of 2-phospho-D-glycerate to high-energy phosphoenolpyruvate (Matthews *et al.*, 1984). Characterization of antigenic components in cytoplasmic extracts of *C. albicans* that were recognized in sera from patients with disseminated candidiasis has been reported (Strockbine *et al.*, 1984). Further, they reported that these patients had circulating antibodies directed against a 48 KDa protein antigen purified by anion-exchange chromatography from the *Candida* extract. Individuals colonized with *C. albicans* without evidence of candidiasis did not have detectable levels of antibodies to this antigen, which was subsequently identified as enolase (Franklyn *et al.*, 1990). Circulating anti enolase antibodies may have potential value for the diagnosis of candidiasis (Mitsutake *et al.*, 1994).

Antigenemia with the 48-KDa antigen has been reported in disseminated candidiasis in the absence of fungemia and correlated with deep tissue infection (Walsh *et al.*, 1988). *C. albicans* enolase antigenemia is a marker for deep tissue invasion even in the absence of fungemia (Walsh *et al.*, 1991). Some major protein allergens of *C. albicans* have been identified by immunoblotting with anti-human IgE antibodies. For instance, in a study, serum samples from asthmatic patients reacted with fractions containing a 46 KDa protein (Walsh *et al.*, 1991). The enzyme enolase from *Saccharomyces cerevisiae* and *C. albicans* acts as a major allergen and such allergen detected was believed to be enolase, a 46 KDa protein (Savolainen *et al.*, 1990; Ito *et al.*, 1995; Nittner-Marszalska *et al.*, 2001; Kustrzeba-Wojcicka and Golczak, 2000; Chang *et al.*, 2002). In *S. cerevisiae* the glycolytic enzyme, enolase has been localized as a 47 kDa protein (Pardo *et al.*, 2000; Kim and Park, 2004).

The humoral and cellular immune responses to enolase were evident in germ-free mice colonized with *C. albicans*. The lymphocytes from intravenously challenged mice responded to enolase as an immunodominant humoral antigen (Del Prete, 1992). Although the cytoplasmic location of enolase appeared to be established, enolase was detected by radioimmuno precipitation in cell wall extracts obtained after digestion of the wall α -glucan network with α -glucanase, zymolyase (Stec and Lebioda, 1990; Sundstrom and Aliaga, 1992; Angiolella *et al.*, 1996). The release of enolase may be important in defining the selective stimulation of the host antifungal response during infection. It has recently been reported that anti-enolase antibodies may have an immunoprotective effect in mice. Cilofungin, a lipopeptide antibiotic affecting α -1, 3-D-glucan synthesis inhibited the incorporation of a 46 to 48 KDa glucan-associated protein, a cell wall associated form of enolase (Mason *et al.*, 1993).

To summarize, *C. albicans* were isolated from patients with invasive candidiasis and characterized. Suspected *C. albicans* strains were selectively isolated on Hichrome Candida Agar and identified. The organism was grown on YEPD medium and harvested. The cell pellets were suspended in protein-extraction buffer. The cells were again suspended in the breaking buffer and broken by mechanical disruption with glass beads. The homogenate was centrifuged and the supernatant containing the soluble protein fraction was passed through the membrane filter. The filtrate was subjected to extraction with chloroform. The dialysed crude enolase cytoplasmic extract was subjected to SDS - PAGE along with standard markers and the *C. albicans* enolase was confirmed as a single polypeptide band of around 48 KDa.

Further, to investigate the immunological cross reactivity of enolase with appropriate antibodies and other biochemical studies, purification of enolase is required. Such type of investigation is very much needed to study the pathology of *C. albicans*. Immunological relevance and interspecies relationship of the *Candida* enolase antigens can also be investigated.

Acknowledgement

Mr. C. Sureshkumar, M.Sc., M.Phil., Microbiologist, AR Labs and Research Foundation, Salem, Tamilnadu, India is gratefully acknowledged for providing technical support.

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Table 1. Carbohydrate assimilation tests for *C. albicans*

Species	Substance	Detection
<i>C. albicans</i>	Dextrose	+ ve
	Maltose	+ ve
	Sucrose	+ ve
	Lactose	- ve
	Galactose	+ ve
	Melibiose	- ve
	Cellobiose	- ve
	Inositol	- ve
	Xylose	+ ve
	Reffinose	- ve
	Trehalose	+ ve
	Dulcitol	- ve

Table 2. Carbohydrate fermentation tests for *C. albicans*

Species	Substance	Detection
<i>C. albicans</i>	Dextrose	+ ve
	Maltose	+ ve
	Sucrose	- ve
	Lactose	- ve
	Galactose	+ ve
	Trehalose	+ ve

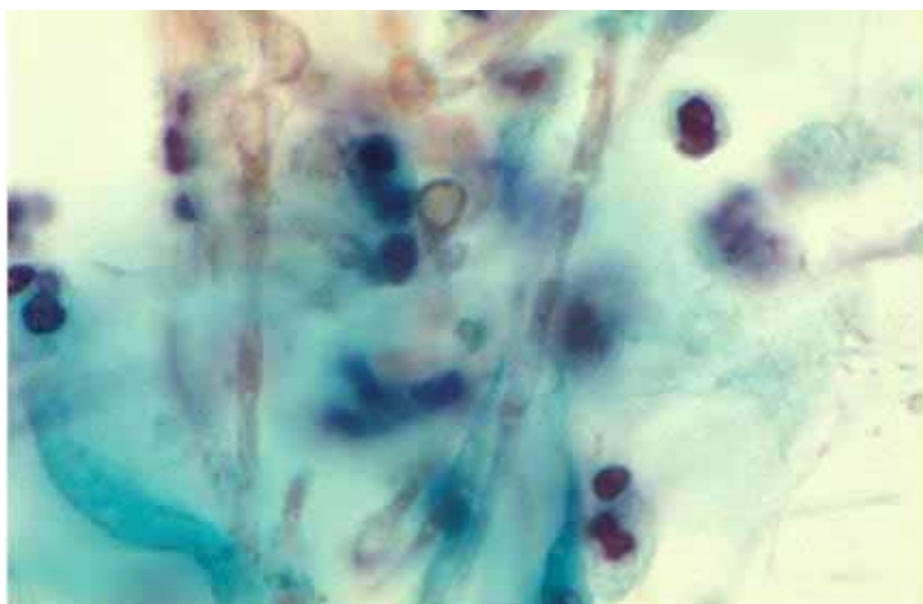


Figure 1. Budding yeast cells with pseudohyphae of *C. albicans*



Figure 2. Glistening green colour colonies produced by *C. albicans* on Hithrome canaida agar

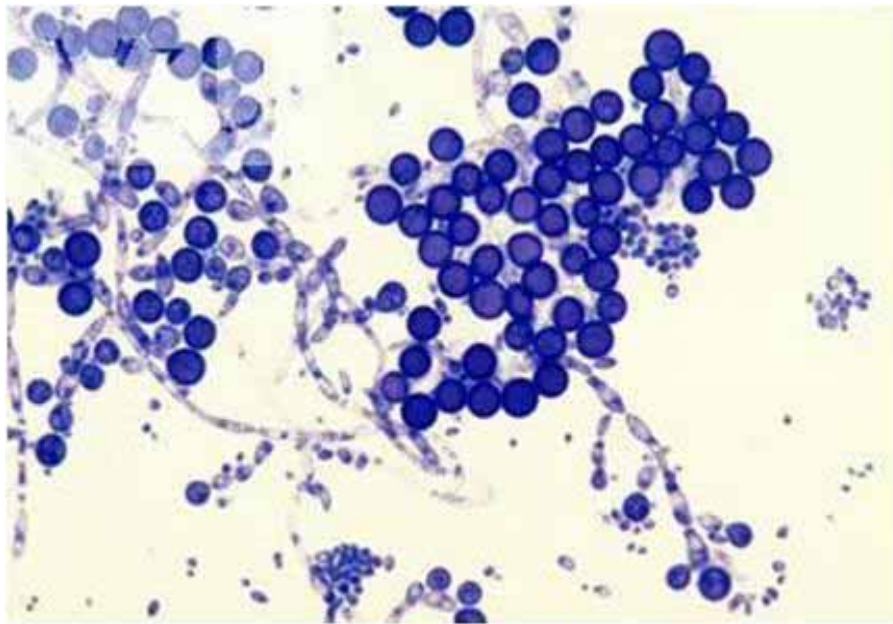


Figure 3. *C. albicans* with thick walled terminal chlamydospores



Figure 4. *C. albicans* with germ tubes

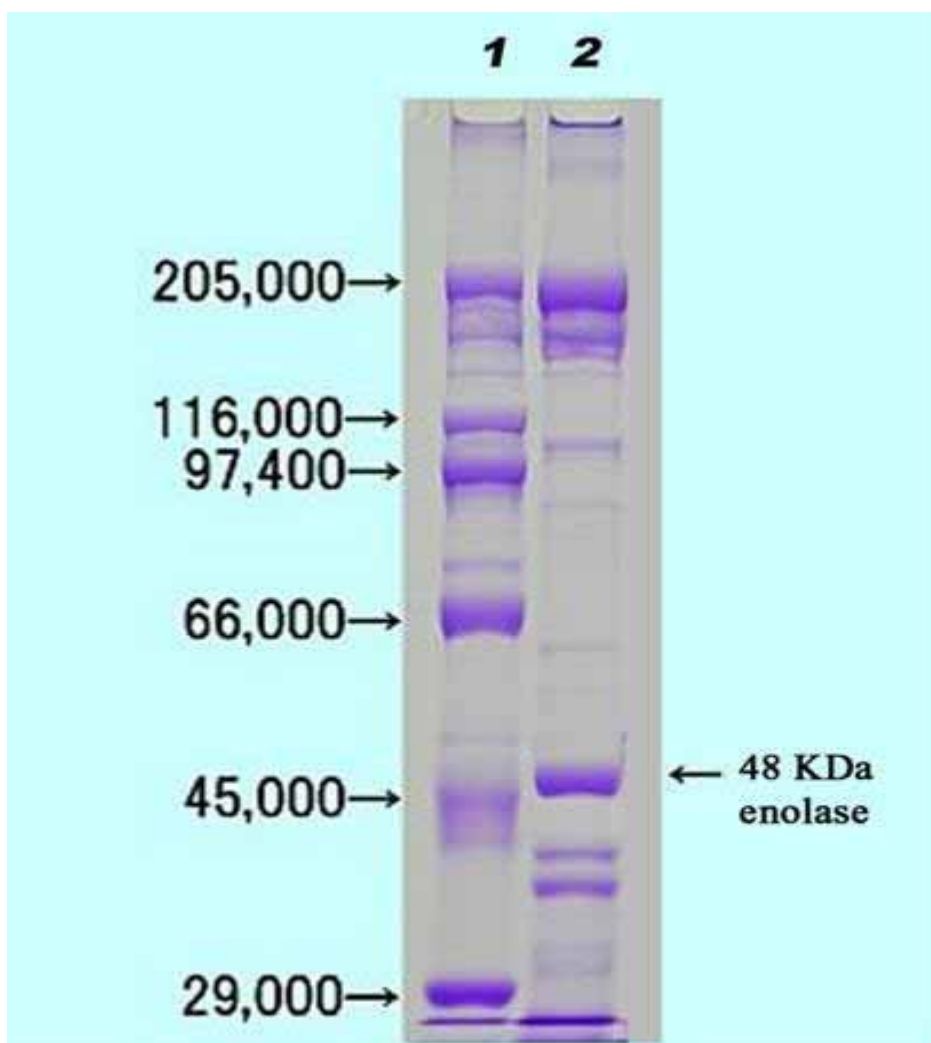


Figure 5. SDS-PAGE profile of crude enolase from *C. albicans* (1, standard protein markers; 2, crude enolase sample)