ANTIFUNGAL SPECTRUM DETERMINATION OF THE K5 TYPE YEAST KILLER PROTEIN ON FUNGI CAUSING SPOILAGE IN CITRUS FRUITS

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ABSTRACT

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Some yeast strains under certain conditions secrete polypeptide toxins which are inhibitory to sensitive fungal cells into the medium. These yeast strains are termed as killer yeasts and their toxins are designated as killer proteins or killer toxins. Killer proteins are classified into 11 typical types (K1-K11). These toxins have different killing mechanisms on sensitive cells. Some of them hydrolyze major cell wall component, β-1,3- glucans. As mammalian cells lack cell walls research and development of novel highly selective antifungals are mostly focused on the agents which target the components of the fungal cell wall. K5 type killer protein was characterized in our labarotory previously. This protein is an exo β-1,3-glucanase which is stable at pH's and temperatures appropriate for its biocontrol usage. β-1,3-

glucan hydrolyzing activity of the K5 type killer protein highlighted the potential use of this protein as a selective antifungal agent. According to CLSI methodology, antifungal activity of the K5 type yeast killer protein was tested against 6 fungal strains causing postharvest spoilage in citrus fruits and found to be effective on *Botrytis cinerea, Penicillium digitatum, Penicillium italicum* whereas non effective on *Colletotrichum gloeosporoides, Phythophythora citrophthora, Alternaria citri*. The MIC values of the toxin for *B.cinerea, P.digitatum, P.italicum* were found to be 16 µg/ml while IC_{50} values of the toxin were 2.12, 3.31, 2.57 µg/ml respectively. The results showed that K5 type yeast killer protein would be used as a novel and selective agent against *B.cinerea, P.digitatum* and *P.italicum*.

Key words: Biocontrol agent, K5 type yeast killer toxin, plant pathogenic fungi, MIC, postharvest citrus diseases

ÖZ

K5 TİPİ ÖLDÜRÜCÜ MAYA PROTEİNİNİN TURUNÇGİL MEYVELERİNDE BOZULMAYA NEDEN OLAN MANTARLAR ÜZERİNDEKİ ANTİFUNGAL SPEKTRUMUNUN TAYİNİ

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Bazı maya suşları belirli şartlar altında ortama duyarlı mantar hücrelerini inhibe edici polipeptid toksinler salgılarlar. Bu tip mantarlara öldürücü mantarlar ve salgıladıkları toksinlere öldürücü proteinler veya öldürücü toksinler adı verilmiştir. Öldürücü proteinler 11 değişik tip altında sınıflandırılmışlardır (K1-K11). Bu toksinlerin hassas hücreler üzerindeki etki mekanizması farklılıklar gösterir. Bazıları hücre duvarındaki β-1,3-glucan yapısını hidrolize eder. Memeli hücreleri hücre duvarına sahip olmadığı için yeni ve seçiciliği yüksek antifungalların araştırma ve geliştirilmesi mantarların hücre duvarı bileşenlerini hedef teşkil eden ajanlar üzerinde yoğunlaşmaktadır. Laboratuvarımızda yaptığımız çalışmalarda *P.anomala* suşunun

öldürücü etkisinden sorumlu olan K5 tipi öldürücü toksinin biyokimyasal karakterizasyonu sonucunda bu toksik proteinin biyokontrol alanında kullanım için uygun sıcaklık ve pH şartlarında aktivitesini koruyan bir exo β-1,3-glukanaz olduğu bulunmuştur. K5 tipi öldürücü proteinin duyarlı hücrelerin, hücre duvarlarındaki β-1,3 glukan yapısını hidrolize etme özelliği, bu proteinin seçici bir antifungal ajan olarak kullanımının önemini öne çıkarmaktadır. K5 tipi öldürücü maya proteininin antifungal aktivitesi CLSI metoduna göre turunçgillerde hasat sonrası bozulmaya neden olan 6 mantar üzerinde denenmiştir. K5 tipi öldürücü maya proteininin bu mantarlardan *Botrytis cinerea, Penicillium digitatum* ve *Penicillium italicum italicum* üzerinde etkili olduğu fakat *Colletotrichum gloeosporoides, Phythophythora citrophthora, Alternaria citri* üzerinde inhibe edici etkisinin olmadığı bulunmuştur. *B.cinerea, P.digitatum, P.italicum* için toksinin MİK değeri 16 µg/ml, IK_{50} değerleri ise sırasıyla 2.12, 3.31, 2.57 µg/ml olarak bulunmuştur. Bu çalışma K5 tipi öldürücü maya proteininin *B.cinerea, P.digitatum ve P.italicum*' a karşı yeni ve seçici bir biyokontrol ajanı olarak kullanılabileceğini göstermiştir.

Anahtar kelimeler: Biyokontrol ajanı, K5 tipi maya öldürücü proteini, bitki patojen mantarları, MİK, hasatsonrası turunçgil hastalıkları

To My Family

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bp	Base pair
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DSMZ	German National Resource Centre for Biological Material
DTT	Dithiothreitol
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory Concentration required to obtain 50% growth inhibition
kb	Kilo base
kDa	Kilo dalton
K_m	The Michaelis constant, is the substrate concentration required to reach half-maximal velocity ($V_{max}/2$).
L dsRNA	Large size double stranded RNA
MIC	Minimum inhibition concentration required to obtain 100% growth inhibition
MIC_2	Minimum concentration required to inhibit the growth of 50% of the cells
MIC_0	Minimum concentration required to inhibit the growth of all the cells
MFC	Minimum fungicidal concentration
MWCO	Molecular Weight Cut-Off
M dsRNA	Medium size double stranded RNA
NCCLS	National Comitee for Clinical Laboratory Standarts
OD	Optical Density
NCYC	National Collection of Yeast Cultures
TEMED	N, N, N' N'- Tetramethylethylenediamine
%T	Ratio of the sum of the weights of the acrylamide monomer and the cross linker in the solution , expressed as %w/v
%C	The ratio of cross-linker to acrylamide monomer

CHAPTER I

INTRODUCTION

Fungi are extremely successfull organisms with the ability to invade and colonize all kinds of ecological niches [1]. Unfortunately, they are also proficient at colonizing and using plants, humans and animals as substrates [2]. Fungal infections are an important problem especially in plant pathology and also in medicine in the care of immunosuppressed patients by infectious diseases [3].

Fruits and vegetables are highly perishable products, especially during the postharvest phase in which considerable detriments occur due to microbiological diseases, disorders, transportation and senescence [4].

Fruit crops are particularly susceptible to microbial infections in the post harvest period due to their high nutrient and water content in addition to the loss of natural resistance that they had while attached to the tree [5].

Losses of fresh fruits and vegetables after harvest have higher economic importance since they are related to a ready –for-marketing commodity with a high added value [4]. Worldwide, postharvest losses have been estimated at 50 % and much of this is due to fungal infections [6]. Generally, most of the harvested commodities are resistant to fungal infections during their early postharvest phase. However, they become more susceptible to infections during ripening and senescence [7]. In developing countries, postharvest wastages are often severe

due to the lack of adequate handling and refrigerated controlled atmosphere storage facilities [6].

Especially, mould growth destroys large amounts of fruits and vegetables, both pre- and post-harvest and causes serious looses throughout the world [8]. Growth of moulds in food leads to reduced nutritional values and production of allergenic spores and hazardous mycotoxins [9].

1.1. Postharvest Diseases of Citrus Fruits

Citrus fruits are the world 's premiere fruit crops, grown in over 100 countries on six continents. Worldwide production of citrus reached nearly 100 million tonnes in 2002 exceeding that of watermelons, bananas and grapes [10].

The life of a citrus fruit after harvest is frequently terminated by decay. Decay caused by fungal growth on citrus fruits during storage is a continuing problem that results in economic loss [11]. Although several fungal species have been reported, *Penicillium digitatum, Penicillium italicum, Alternaria citri, Colletotrichum gloeosporoidesi, Phythophthora citrophthora , Botrytis cinerea* are the primary organisms involved [12, 13].

1.1.1. Green Mould

Green mould caused by *Penicilliumdigitatum* is a universal postharvest disease of citrus. It is identified by the mass of olivegreen spores produced on infected fruit (Figure 1.1.). The extensive spore production by this pathogen ensures its presence wherever fruit is handled, including the field, packinghouse, equipment, degreening and storage rooms, transit containers and in the marketplace.

Figure 1.1. Green mould in citrus caused by *Penicilliumdigitatum* [10]

Infection takes place only through wounds where nutrients are available to stimulate spore germination and fruit decay begins at these infected injury sites. The early infection area appears as a soft watery spot. As the lesion progresses, white mycelia develop and produce the green spores. Within a few days the entire fruit can be covered with green spores. These green spores are easily dispersed by any physical motion or air currents and soil surfaces of healthy fruits [10].

1.1.2. Blue Mould

Blue mould is caused by *Penicillium italicum* which develops on citrus fruit via injuries like green mould. It occurs in all citrus-producing regions of the world. Blue mould is recognised by the mass of blue spores produced in decayed fruit (Figure 1.2.). Initial lesions are similar to the lesions of green mould but the spores are blue in color and are surrounded by a narrow band of white mycelium encompassed by water-soaked rind [10].

P. italicum is able to grow slowly at low temperatures and causes decay after extended storage. The fungus is a prolific spore producer and airborne spores easily contaminate packinghouse equipment and facilities. The decay can spread rapidly in packed containers and masses of spores produced on one infected fruit can infect surfaces of adjacent healthy fruit. The infected fruit can be covered entirely by a mass of blue spores [14].

Figure 1.2. Blue mould caused by *Penicilliumitalicum* [10]

1.1.3. *Alternaria* Stem-end Rot (Black Rot)

Alternaria black rot, caused by *Alternaria citri*, occurs throughout all citrus growing regions. The disease occurs primarily as a stem-end rot on fruit stored for a long time. The pathogen, *Alternaria citri,* lives saprophytically in the orchard and produces airborne conidia that cause latent infections in the button or stylar end of the fruit (Figure 1.3.). In the orchard, infected fruits colour prematurely and develop a light brown to blackish discoloration of the rind at or near the stylar end [10].

Alternaria black rot can also be a problem for the processing industry by contaminating the juice. Mycelia of the fungus and bitter taste of infected fruits can be major sources of this contamination [14].

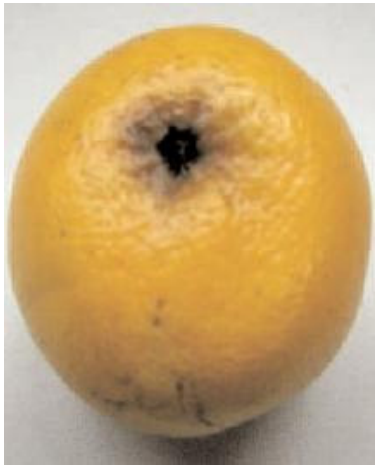


Figure 1.3. *Alternaria* Stem-end Rot caused by *Alternariacitri* in citrus fruits [10]

1.1.4. Antrachnose

Anthracnose is caused by the fungus *Colletotrichumgloeosporioides*. *Colletotrichum* is the most important and common fungal genus causing anthracnose. It has an extremely wide host range including vegetables, field and forage crops, fruit trees and ornamentals [10].

Fruit infections occur primarily from the localized dispersal of waterborne spores produced on the deadwood. Spores are disseminated to the fruit surfaces during the warm, rainy summer months [14].

Anthracnose lesions are initially silvery gray and leathery, being similar in firmness and elevation to adjacent healthy rind tissue. The infected rind becomes brown to grayish black and softens as the rot progresses (Figure 1.4.). Lesions vary in size and are irregular in shape. Pink spores may form on the lesion surface in humid environments [14].

Figure 1.4. Anthracnose lessions caused by *Colletotrichumgloeosporioides* [15]

1.1.5. Brown Rot

Two species of *Phytophthora*, *P.citrophthora* and *P.parasitica*, cause brown rot, but decay caused by *P.citrophthora* is usually more extensive because it produces spores more rapidly and abundantly on infected fruit and spreads more easily to healthy fruit in the upper part of the tree canopy [14].

The decay initially occurs as a light brown discoloration of the rind at any location on the fruit surface within 3-4 days of infection. Under humid storage conditions, delicate white mycelium forms on the lesion surface (Figure 1.5.). Decay will spread from infected to healthy fruit in packed cartons during transit and storage, particularly during conditions of export [15].

Figure 1.5. Brown rot in citrus caused by *P. citrophthora* [10]

1.1.6. Gray Mould (*Botrytis* Rot)

Gray mould, caused by the widespread fungus *Botrytis cinerea,* affects most vegetable and fruit crops, as well as citrus fruits and causes losses as great as 45%. Since the fungus is able to function at tempreatures just about freezing, it is also a problem on fruits and vegetables in cold storage and subsequent shipment. This pathogen can invade pre-harvest, during the flowering stage, and remain quiescent until conditions become favorable for its growth and development. It can also infect the fruit through wounds, through contact of senescing flower parts

with developing fruit, or through direct penetration of the skin if there is persistent fruit to fruit contact with a decayed berry, such as in a market basket [16].

Symptoms of *Botryti*s disease vary greatly depending on the host and the plant part attacked. Generalized symptoms include a gray to brown discoloration, water soaking, and a fuzzy whitish gray to tan mycelium and spores growing on the surface of affected areas. *Botrytis cinerea* also causes rind distortions especially in lemon (Figure 1.6.). In this case, symptoms typically consist of a series of conical or ridge-like outgrowths on the rind extending up to 4 mm from the fruit surface, usually associated with irregularly shaped, often stellate, corky scars at the tips. Fruit distortions have been noted on various other types of citrus [17].

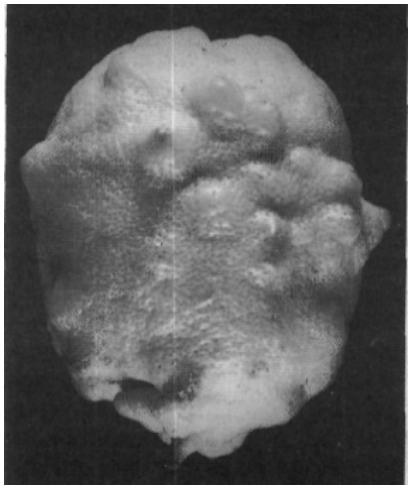


Figure 1.6. Typical symptoms of botrytis fruit distortion on a young lemon *(Citruslimon)* fruit [17]

Currently, synthetic fungicides such as imazalil and thiabendazole are the primary means of controlling postharvest disases of citrus fruits [11].

1.2. BiolologicalControl

Traditionally, effective control of fungal plant pathogens is achieved by application of synthetic fungicides [18]. However, the global trend is shifting towards reduced pesticide use in agriculture in general and in postharvest in particular. Pesticide residues on fruit and vegetables are a major concern to consumers and to the fruit and vegetable industry. Furthermore, the use of fungicides is more harmful in the post harvest period because of the short time between treatment and consumption [12]. With growing health and environmental concerns over pesticide disposal and the presence of chemical residues in the food chain, the development of fungicide-resistant strains of postharvest pathogens and the deregistration of some of the more effective fungicides has generated a growing interest in the development of safer alternatives to synthetic fungicides that are effective, economically feasible and pose no risk to human health and environment [6,12, 19, 4].

Over the past fifteen years, biological control has emerged as an effective strategy to combat major postharvest decay of fruits [20].

Biocontrol, a non-hazardous alternative to the use of chemical fungicides, involves the use of biological processes to reduce crop loss.

Currently, several promising biological control approaches that include antagonistic microorganisms, compounds of natural origin and induced resistance have been proposed as potential alternatives to synthetic fungicides for postharvest disease control. Among the proposed alternatives, development of antagonistic microorganisms has been the most studied one. The substantial progress has been made in this area [12] .

The postharvest phase is suited to the application of biological control methods [4]. The confined environment for the storage of harvested commodities provides a great opportunity for applying biocontrol agents since tempreature and humidity are stable and controllable [21]. Environmental conditions, such as temperature and relative humidity, can be managed to favor antagonist survival. Furthermore, biotic interference is minimal so antagonists encounter minimal competiton from indigenous microorganisms [20]. In addition, biocontrol agents are easy to apply, they act on a more concentrated plant parts. Mainly for these reasons, it is generally believed that biological control by means of microbial antagonists may have a greater potential for success when applied after harvest than before [22].

In recent years, research on the use of microbial biocontrol agents for the control of postharvest diseases of fruits has gained considerable attention and has moved from laboratory to commercial application [4].

Several antagonistic bacteria, yeasts and filamentous fungi have been isolated and shown to protect a variety of harvested commodities, including citrus fruits, against postharvest decay [12 , 4 , 16, 23].

The success of some of these microbial antagonists in laboratory and large scale studies has generated the interest of several agrochemical companies in the development and promotion of postharvest biological products for controlling rots on fruits and vegetables.

A number of microbial antagonists have been patented and evaluated for commercial use as a postharvest treatment. Currently, four antagonistic microorganisms _ two yeasts, *Candidaoleophila* and *Cryptococcus albidus*, and two strains of the bacterium *Pseudomonasyringae* _ are commercially available under the trade names Aspire, YieldPlus and Biosave-110, respectively [4, 6, 12, 13, 22].

Although the biocontrol activity of antagonistic microorganisms has been demostrated on a variety of commodities, the mode of action of the microbial biocontrol agents has not been fully elucidated [12].

In the case of bacterial antagonists, it has been suggested that their biocontrol activity may be associated with the production of antibiotics. The bacterial antagonist *Pseudomonas syringae,* which controls *Penicillium* moulds of citrus fruit and gray molds of pome fruit, produces an antibiotic, syringomycin, that inhibits the germination of *Penicilliumdigitatum* [24].

In recent years the use of antibiotic producing bacteria has been abandoned [22] in order to prevent the appearance of resistance in pathogen strains. In addition, for biological control to be effective, use of antagonists must be compatible with current handling and storage practices such as low temperature storage which could otherwise cause a reduction in the effectiveness of bacterial strains [23].

Since the antagonists are applied to consumable products, they must meet strict requirements for human safety. So, additional criteria for the efficiency of biocontrol agents may include pathogenicity of the antagonists to fruits and production of alergenic spores and mycotoxins [20].

Several important properties of yeasts make them useful for biocontrol purposes. For example yeasts do not produce allergenic spores or mycotoxins as many mycelial fungi do or antibiotics as possibly produced by bacterial antagonists. Yeasts generally have simple nutritional requirements and are able to colonize dry surfaces for long periods of time. They rapidly utilize available nutrients and can sustain many of the pesticides used in the postharvest environment. Yeasts can grow rapidly on cheap substrates in fermentors and are therefore easy to produce in large quantities. The suggested modes of action of biocontrol yeasts are not likely to constitute any hazard for the consumer [25]. Furthermore, yeast cells contain high amounts of vitamins, minerals and essential

aminoacids and several reports on the beneficial effect of yeast addition in both food and feed can be found in the literature [26, 27, 28].

1.3. Yeasts As Biocontrol Agents

Yeasts are unicellular fungi which are usually spherical or oval. They do not form filaments or a mycelium so the population of yeast cells remains as a collection of single cells [29].

Yeast cells mostly reproduce asexually by a process called budding (Figure 1.7.). A bud is formed on the outer surface of the parent cell as the nucleus divides. One nucleus migrates into the elongating bud. Cell wall material forms between the bud and the parent cell and the bud breaks away [31].

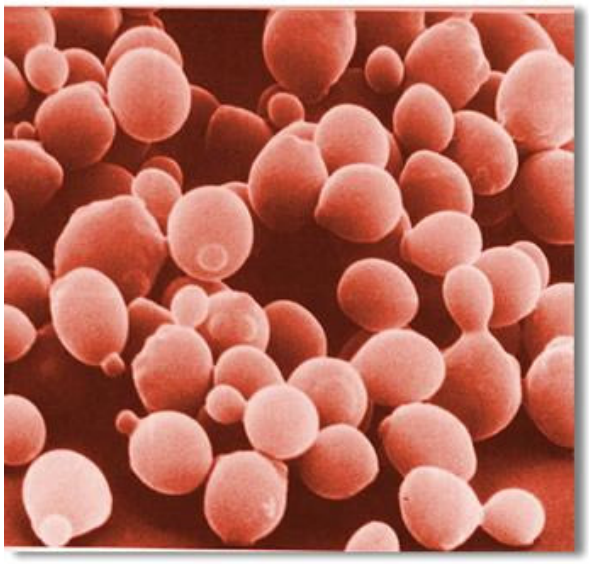


Figure 1.7. Yeasts multiplying by budding [30]

Yeasts can also reproduce sexually by a process called mating. Sexual spores (a and α) called ascospores which result from the fusion of the nuclei from two cells followed by meiosis. Life cycle of a yeast *S. cerevisiae* is shown in Figure 1.8. [32]. Sexual reproduction is much less common than asexual reproduction but does allow for genetic recombination [33].

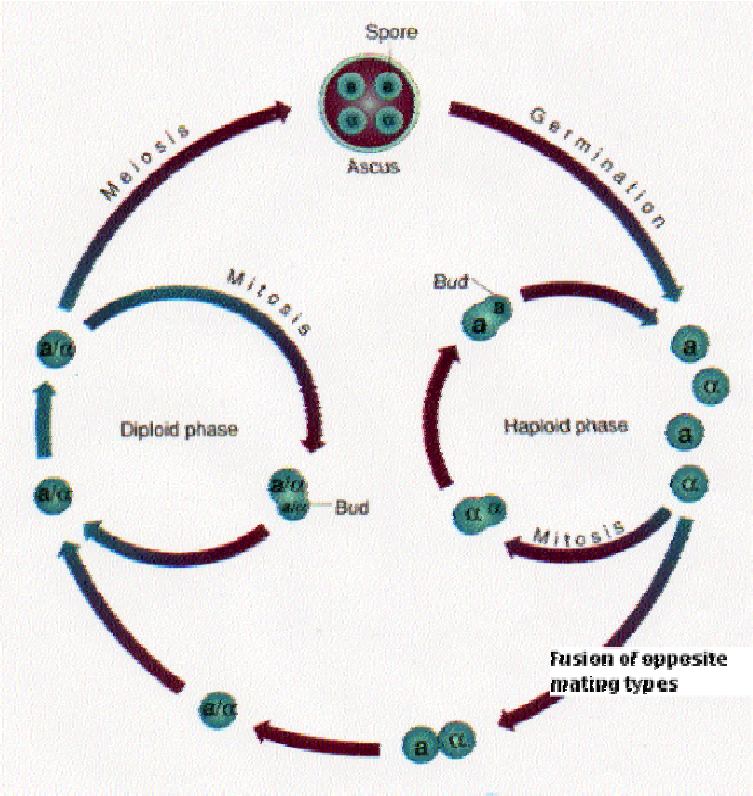


Figure 1.8 . Life cycle of *S. cerevisiae* [32]

Rapid growth, a budding pattern resulting in dispersed cells, the ease of mutant isolation, well-defined genetic system, and a highly versatile DNA transformation system are the properties making yeast suitable for biological

studies. Yeasts can be handled with few precautions since they are nonpathogenic [34].

Yeasts are the world's premier industrial microorganisms. They are cultured for the cells themselves, for cell components and for the end products that they produce during the alcoholic fermentation. Yeast cells are also used as sources of food, vitamins and growth factors [31]. Yeast is often taken as a vitamin supplement because it is fifteen percent protein and is a rich source of B vitamins, niacin, and folic acid.

The most well-known and commercially important yeasts are the related species and strains of *Saccharomyces cerevisiae*. These organisms have long been utilized to ferment the sugars from rice, wheat, barley, and corn to produce alcoholic beverages and in the baking industry to expand, or raise, dough. *Saccharomyces cerevisiae* is commonly used as baker's yeast and for some types of fermentation [31].

As a summary, yeast cells are used in industry for bread making, for food supplement, for animal feeds, for alcohol and glycerol production, for enzyme production such as invertase, galactosidase, for production of vitamins including vitamin B and D, for production of beer, whiskey, wine, brandy, vodka, rum also yeast are used extensively in environmental technologies such as bioremediation, waste utilization, crop protection, biosorption of metals [35, 36].

As an eukaryote that is easy to manipulate, the yeast *S .cerevisiae* is an attractive organism for the expression of heterologous genes and high level production of other molecules mainly for pharmaceutical use. Like bacteria, yeast can be cultivated in great quantitiy. In contrast to bacteria, yeast has a secretion-glycosylation system resembling the one found in animal cells and can carry out the posttranslational modifications often required for biological activity.

Yeast strains can easily be modified using molecular genetic techniques and their use in large scale industrial fermentations is well established. The yeast is necessarily the ideal host in any circumstance. The pharmaceutical products produced in yeast include hormones, growth factors, blood proteins, enzymes ,surface antigens of viruses and interferons. In addition to their wide exploitation in the production of foods, beverages and pharmaceuticals, yeasts also play significant role as model eucaryotic cells in furthering our knowledge in the biological and biomedical sciences [37, 38].

At present, yeasts appear to be promising biocontrol agents, providing alternatives to chemical fungicides in the postharvest storage of fruits and vegetables. There are several reports in the literature indicating the antagonistic activities of the yeast cultures against plant pathogens. Some of the yeast species reported with biocontrol activity are given in Table 1.1.

Table 1.1. Some of the yeast strains showing antagonistic activity against plant pathogens

Bicontrol Species	Target organism	Crop	References
Cryptococcus albidus	*Botrytis cinerea* *Penicilliumglabrum*	Bean tomato strawberry	39, 40, 41
Cryptococcus laurentii	*Penicilliumexpansum* *Botrytis cinerea*	apple	42, 43, 44, 45
Pichiaburtoni	*Penicilliumverrucosum*	seed	46
Pichia guillermondii	*Botrytis. cinerea* *Aspergillusflavus* *Penicillum digitatum*	Tomato, grape, soybean, grapefruit	47, 48, 49
Pichia membranifaciens	*Botrytis cinerea*	grapevine	50
Pichia anomala	*Penicilliumroqueforti* *B*otrytis cinerea *Aspergilluscandidus* *Penicilliumverrucosum*	Cereal grain, apple, grape,	51, 52, 53, 54, 55, 56
Metschnikowia pulcherrima	*Penicilliumglabrum* *Penicilliumexpansum* *Botrytis cinerea*	apple	41, 57, 58
Candida oleophila	*Penicilliumdigitatum* *Penicilliumitalicum* *Botrtis cinerea*	Citrus fruits, pome fruits	25, 59
Yarrowia lipolytica	*Botrytis cinerea*	apple	45
Rhodotorula glutinis	*Botrytis cinerea* *Penicilliumexpansum*	Bean, tomato, apple	42, 39
Debaromyces hansenii	*Penicilliumdigitatum*	Oranges, grapefruit	60, 61

Among the biocontrol yeast strains, *Pichiaanomala* has been reported to have a high level of reliable biocontrol activity against a variety of fungi in several different habitats [62] and is a potential candidate for biocontrol of moulds on grapevine and during postharvest storage of apples and airtight-stored grain [51-56]. *Pichia anomala* has also been shown to reduce ochratoxin A accumulation in co-culture with *Penicillium* verrucosum. Toxin production was inhibited at levels of *Pichiaanomala* lower than those inhiting growth [56].

A variety of mechanisms have been reported to contribute to the biocontrol of postharvest pathogens by introduced microorganisms. Commonly, the mechanism by which the biocontrol agent inhibits the target pathogen is poorly understood, as it is extremely difficult to construct experiments that can exclude all other possible mechanisms in the complex bicontrol environment [12, 20, 22].

Competition for nutrients has been suggested as the mode of action of several possible biocontrol organisms. This is in most cases a probable and reasonable explanation. However, competition for nutrients is diffucult to prove, as it is usually very hard to exclude all other mechanisms. In addition, no differences in sugar consumption were observed between yeasts with and without biocontrol activity, suggesting that additional factors are part of the inhibiting mechanisms [6, 20].

Biocontrol yeast *Pichia anomala* can reduce growth of *Penicillium roqueforti* both in vitro and in high moisture cereal grain in a test tube version of a malfunctioning airtight storage system [51, 52]. Competition for nutrients does not significantly contribute to the antifungal activity of *P.anomala* in the grain system. Morever, the finding that as many as 27 of the 38 species without biocontrol activity were able to grow to smilar levels as *P.anomala* in the minisilo system shows that the ability to grow, consume nutrients and compete for space on cereal grains is not enough to prevent the growth of *P.roqueforti*. Than, it has

been suggested that its antifungal activitiy is mainly caused by "killer protein" produced by biocontrol strain [51, 63-67]. There have been several reports indicating that killer protein production by biocontrol yeasts largelly contribute to their antagonistic activity (Table 1.2) .

Table 1.2. Summary of suggested mechanisms for antifungal effects of *P.anomala*

Target fungus	system	Suggested mode of action	references
Aspergillusflavus	Plate bioassay	--- ,killer toxin	63 , 64
Aspergillusfumigatus	Plate bioassay	--- ,killer toxin	63 , 64
Aspergillus nidulans	Plate bioassay	killer toxin	64
Aspergillus niger	Plate bioassay	killer toxin	51 , 64
Aspergillusparasiticus	Plate bioassay	killer toxin	64
Aurebasidiumpullulans	Plate bioassay	killer toxin	64
Candida albicans	Animals, plate bioassay	killer toxin	65 , 66
Candida glabrata	Animals, plate bioassay	killer toxin	65 , 66
Penicilliumcamembertii	Plate bioassay	killer toxin	64
Penicilliumnotatum	Plate bioassay	killer toxin	67
Pseudallescheria boydii	Plate bioassay	killer toxin	64 , 67
Phialophoraverrucosa	Plate bioassay	killer toxin	67
Rhizopusmicrosporus	Plate bioassay	killer toxin	67
Rhizopussolani	Plate bioassay	killer toxin	66
Scopulariopsisbrevicaulis	Plate bioassay	killer toxin	64 , 67
Sporothrix schenckii	Plate bioassay	killer toxin	64 , 67

*--- : No suggestion for possible antifungal mechanism was given in the reference

1.4. Yeast Killer Proteins : Killer Phenomenon In Yeast

The killing ability of microrganisms is not a new biological phenomenon .The first observation of antagonism in microorganisms was probably reported by Pasteur and Joubert [68]. They observed the inhibitory effect on *Bacillus anthracis* of bacteria isolated from urine.

The "killer" phenomenon in yeast cells was first discovered by Makower and Bevan who observed that certain strains of *Saccharomyces cerevisiae* could kill sensitive strains of the same species [69]. It was subsequently found that certain yeast strains termed " killer yeasts ", produce and excrete into the medium proteins or glycoproteins that are inhibitory to sensitive microbial cells. Thus, these proteins are designated as " killer proteins " or " killer toxins ". Killer yeast strains are immune to their own toxins but can be sensitive to the toxins of others [67 , 70-72].

The nature of the yeast killer phenomenon implies a potential role for competition, considering that yeast killer toxins may prevent antagonistic microorganisms from gaining access to resources that would provide a selective advantage during the early phases of microbial growth [67].

After the initial discovery of the killer phenomenon in *S. cerevisiae*, it soon became evident that killer strains are not restricted to the genus *Saccharomyces* but can also be found among many other yeast genera : up to now, toxin producing killer yeasts have been identified in *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces, Pichia, Ustilago, Torulopsis, Williopsis* and *Zygosaccharomyces*, indicating that the killer phenomenon is indeed widespread among yeasts [73-77]. Besides toxin secreting killer strains, a significant number of non-killer yeasts can be isolated that have lost their ability to produce various killer toxins but nevertheless retain immunity.

Killer toxins described to date are active against a variety of different yeasts often outside the genus and species of the producing strain [66]. Also growth inhibitory activity of killer toxins of certain yeast strains on gram positive pathogenic bacteria and plant pathogenic fungi has been shown [66 ,78].

These proteins are first classified into 10 different types (K1-K10) by Young and Yagiu [73]. A new killer protein producing strain (K11) was later added to this classification [79]. This classification is based on the killing spectra and immunity cross reactions of the strains. Killer strains and their proteins are shown in Table 1.3.

Table 1.3. Killer Yeasts and Their Protein Toxins [73].

Killer Protein Producing Strain	Classification
Saccharomyces cerevisiae	K1
Saccharomyces cerevisiae	K2
Saccharomyces capensis	K3
Candida glabrata	K4
Pichia anomala	K5
Kluyveromyces fragilis	K6
Candida valida	K7
Hansenula anomala	K8
Hansenula mrakii	K9
Kluyveromyces drosophilarum	K10
Candida glabrata	K11

1.4.1. Structure, Processing and Secretion of the Killer Proteins

Killer yeasts and their toxins have been used as model systems in fundamental research for studying the mechanisms of regulation of eukaryotic polypeptide processing, secretion and receptor binding. The killer system in yeast is thought to be closely parallel to the synthesis and action processes of mammalian hormones and neuropeptides [80].

Toxin secretion pathways are fully identified for K1 and K28 which are both secreted by *S. cerevisiae.* Although these two killer proteins have different amino acid compositions and mode of action; their synthesis, processing and secretion shows significant homologies. K1 and K28 toxins are both encoded by ds RNA viruses and consisting of two distinct disulfide bonded unglycosylated subunits, termed α and β. The α and β domains flank a segment called γ, which is not part of the mature toxin and assumed to be the immunity determinant. Killer toxins are initially translated as preprotoxin which undergoes post-translational modifications within the endoplasmic reticulum, and the golgi complex until it is finally secreted as mature α/β heterodimeric protein toxin [77].

Once synthesized, the preprotoxin enters the endoplasmic reticulum with the help of a highly hydrophobic signal peptide in the N-terminal region. The signal peptide is removed by a peptidase that probably cleaves to produce protoxin. In the endoplasmic reticulum, the γ domain is N-glycosylated and presumably folds into a form competent for translocation to the Golgi and for further processing .

A further modification proceeds in the golgi apparatus. During this stage the products of genes KEX1 and KEX2 are also apparently involved. KEX1 and KEX2 genes were shown to encode proteases necessary for processing both killer toxin and α-factor precursor proteins. The combined action of these two proteases yields mature toxin from protoxin. Kex2p, the gene product of KEX2, is an

endopeptidase and cleaves the pro-region, removes the intramolecular γ sequence. Kex1p, the product of KEX1, is a carboxypeptidase that removes the C terminal basic dipeptide exposed by Kex2p action. Figure 1.9. shows the preprotoxin processing in *S. cerevisiae* [76, 77]. Then mature toxin is transferred to a secretory vesicle and secreted out of the cell. Secretion process is possible with the products of SEC genes [77, 81 - 83].

At the same time, the killer cell is effectively protected against its own toxin. It is well known that the unprocessed toxin precursor is sufficient to confer protective immunity. However, the detailed mechanism of immunity is still largely unknown; it may be due to the binding of an immunity sequence to the toxin receptors of the producer cell. Thus the killer protein is inhibited from binding to the producer cell [77]. Secretion pathway of K28 is shown in Figure 1.9. and processing of K1 type yeast killer toxin is shown in Figure 1.10.

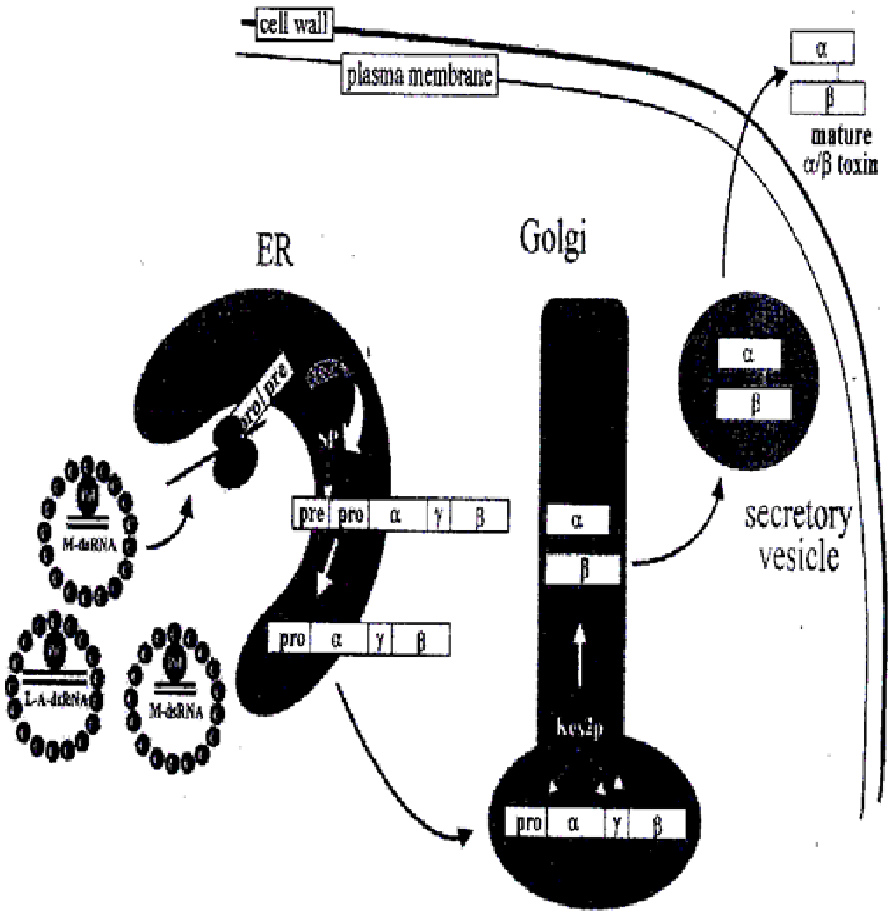


Figure 1.9. Structure and Processing of the Preprotoxin Encoded by M1 dsRNA [77].

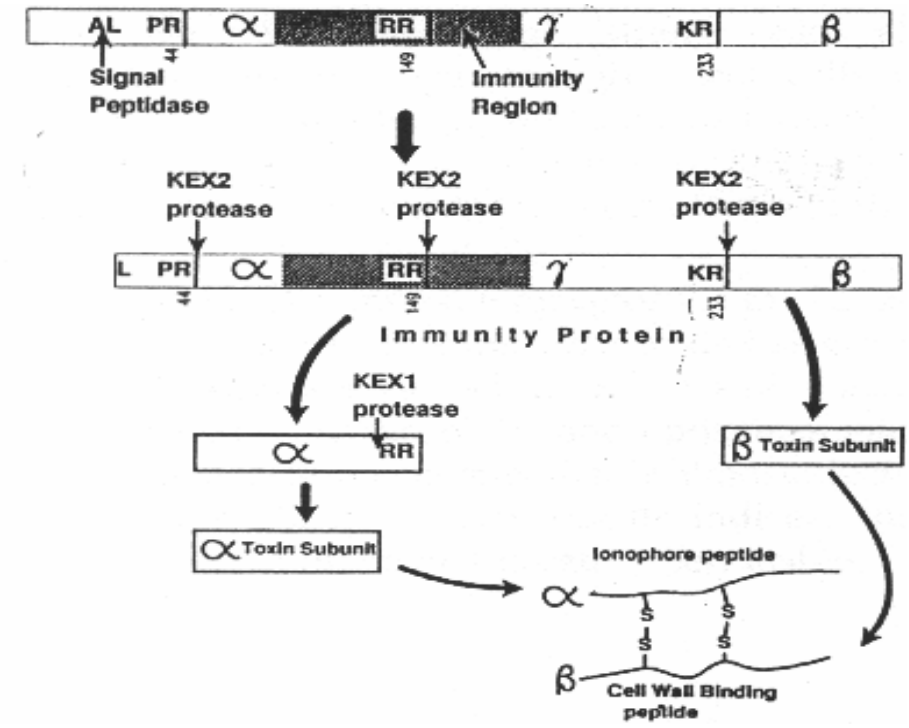


Figure 1.10. Secretory Pathway of Killer Toxin K28 in *S. cerevisiae* [77].

1.4.2.Genetic Basis of Killer System

There are different genetic factor for each killer system (Table 1.4.) [77]. They are are either cytoplasmically inherited encapsulated the dsRNA viruses that permanently resides in its host symbiotically, the linear cytoplasmic dsDNA plasmids and the chromosomal DNA [84, 85]. Table 1.4. shows genetic basis of some killer toxin producer yeast species.

Table 1.4. Genetic basis for killer phenotype expression in yeast [77].

Yeast	Genetic Basis	Toxin Gene
Saccharomyces cerevisiae	dsRNA virus	M1- , M2- , M28
Hansenulauvarum	dsRNA virus	M-dsRNA
Zygosaccharomycesbailii	dsRNA virus	M-dsRNA
Ustilago maydis	dsRNA virus	M-dsRNA
Kluyveromyces lactis	linear dsDNA plasmid	pGKl1
Kluyveromycesfragilis	Chromosomal	Not identified
Pichia acaice	linear dsDNA plasmid	pPac1
Pichiainositovora	linear dsDNA plasmid	pPin1
Pichiakluyveri	Chromosomal	Not identified
Pichiafarinosa	Chromosomal	SMK 1
Pichiaanomala	Chromosomal	Not identified
Williopsismrakii	Chromosomal	HMK
Williopsissaturnus	Chromosomal	Not identified
Schwanniomycesoccidentalis	Chromosomal	Not identified
Debaryomyces hansenii	Chromosomal	Not identified
Candidaglabrata	Chromosomal	Not identified

1.4.2.1.ds RNA Virus Based Yeast Killer Proteins

Double stranded RNA viruses were the first investigated agents responsible for the killing activity. In *S. cerevisiae*, the killer phenomenon is based on the presence of cytoplasmically inherited double-stranded RNA (dsRNA) viruses. These ds RNA's are found in the cytoplasm and they are surrounded by a protein coat and thus termed as yeast viruses [79].

Persistent infection of yeast cells with these viruses is symptomless, and in contrast to certain fungal viruses that are associated with adverse phenotypic effects (like La France disease in *Agaricus bisporus* or plaque formation in *Penicillium*), yeast dsRNA viruses have no (so far recognized) reported adverse effects for host cell.

In addition, yeast dsRNA viruses are considered non-infectious since no naturally occurring extra cellular route of transmission has been identified. They were therefore designated cryptic viruses or 'virus like particles' (VLPs) [79,86]. The genetic determinants of the killer trait shows non-mendelian inheritance. The particles are non-infective but can be transfered by sexual hybridization, protoplast fusion or cytoduction [76, 79]. In contrast to the horizontal transfer of most pathogenic plant and animal RNA viruses, yeast dsRNA viruses only spread vertically during mating and heterokaryon formation *in vivo* [87, 88].

The genome of dsRNA viruses constitutes 0.1% of the total nucleic acid in *S. cerevisiae* [79]. Genome of diploid *S. cerevisiae* cell is shown in Figure 1.11. [89].

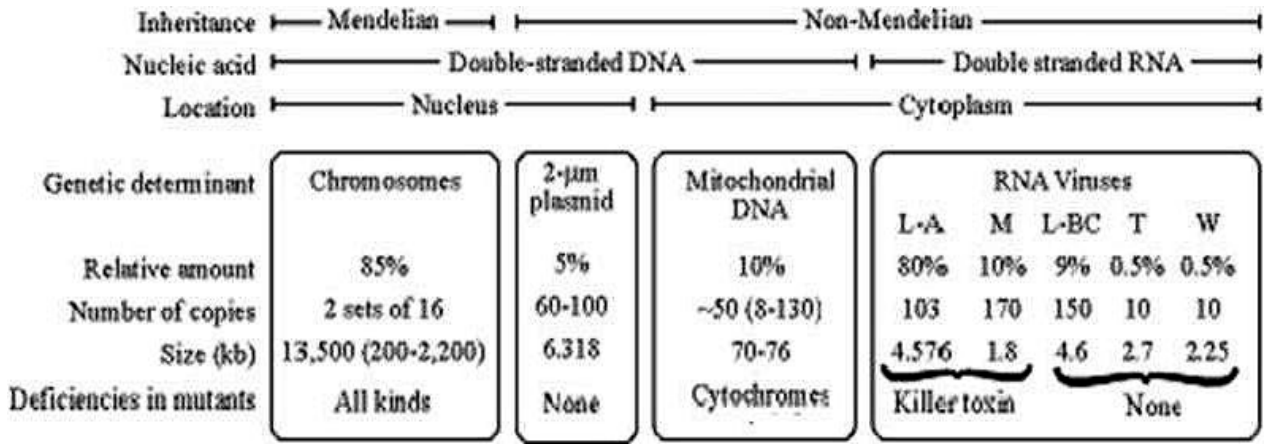


Figure 1.11. The Genome of a Diploid Cell of *S. cerevisiae*[17]

Strains of the yeast species harbor one or more nonhomologous species of double-stranded RNA (dsRNA), called L-A, L-BC, T, W and M(1-4). All of them show non-Mendelian inheritance [82 , 86].

M ds RNA virus like particles are responsible for the killer toxin production and immunity to it. M dsRNAs present in high copy number in the cell about 10-100 [73]. There are 3 types of M ds RNA viruses with different size ; M1, M2 and M3 which are responsible for the production of K1, K2 and K3 killer proteins respectively [82].

Most *Saccharomyces* strains, whether killer or not, have a 4.5 kB linear dsRNA called L dsRNA. L dsRNA encodes proteins for the viral capsid and for the synthesis and encapsidation of ssRNA replication intermediates. The maintenance, encapsidation and replication of M type dsRNA is mainly dependent on L type dsRNA. L dsRNA is present in a higher copy number (100-1000) in the cell and commonly closed in virus-like particles in almost all isolated strains of *S. cerevisiae* that usually do not contain M dsRNA and are thus sensitive to the killer protein.

L dsRNA comprises two unrelated families of RNA molecules called L-A family and L-BC family. L-A encodes the major coat protein that are used for encapsidation of both the M and L types of dsRNA molecules. L-BC dsRNAs have no apperent homology to L-A. It is not clear whether they have any functional relation to killer phenomena since some killer strains lack L-BC entirely.

In a series of *S. cerevisiae* strains other dsRNA types are also present which are designated as T, W and XL. They have no homology with each other or with L-A , L-BC or M dsRNAs. They are present in very low copy number in the cells. No functional relation has yet been established with the killer systems [79, 90].

The copy number of dsRNA viruses and their ability to replicate are controlled by both chromosomal and non chromosomal genes [79].

1.4.2.2. Linear ds DNA Encoded Yeast Killer Proteins

In some yeasts killer character is encoded by the linear ds DNA plasmids. These linear ds DNA plasmids have been identified for various yeast genera such as *Debaryomyces, Wingea, Kluyveromyces* and *Saccharomyces*.The best characterized example for ds DNA plasmids are pGKL1 and pGKL2 which are found in *Kluyveromyces lactis*. The killer protein of *K. lactis* is encoded by the pGKL1 plasmid. pGKL2 is thought to play a role in the replication of pGKL1 [91, 92, 34].

1.4.2.3.Chromosomally Encoded Yeast Killer Proteins

In some killer yeasts such as strains of *Williopsis, Pichia, Candida, Debaryomyces* and *Torulopsis* the killer character is not encoded by neither ds RNA viruses nor ds DNA plasmids. Thus it is suggested that genes responsible for killer character are located on a chromosome [93].

In one of the killer strains of *Saccharomyces cerevisiae*, the killer character was found to be encoded by two different genes which are mapped on chromosome V and chromosome IX [94].

1.4.3. Mode of Action of the Yeast Killer Proteins

Even though the killer toxins posses different modes of action, they do have one thing in common: all the secreted mature toxins can exert killer activity on susceptible cells by different mechanisms that require a specific initial binding to

a cell wall receptor. After the adsorption of toxin actual killing processes which vary with the toxin type occurs.

The biological action of the killer toxins is considered to consist of two step receptor-mediated process. Although actual cell killing mechanisms of these toxins show significant differences, they all use the yeast cell wall components as receptors. The first step involves a fast and energy-independent binding to a toxin receptor within the cell wall of a sensitive target cell. The second energy dependent step involves the toxin translocation to the cytoplasmic membrane and interaction with a secondary membrane receptor that leads to lethal effects [95, 96].

Yeast cell wall is mainly composed of mannoproteins, β- glucan backbone containing linear or branched β-1,3 and β-1,6,glucans and some chitin [97] (Figure 1.12.). It has been identified that these components serve as primary binding sites and cell wall receptors for the killer toxins [71, 98, 99].

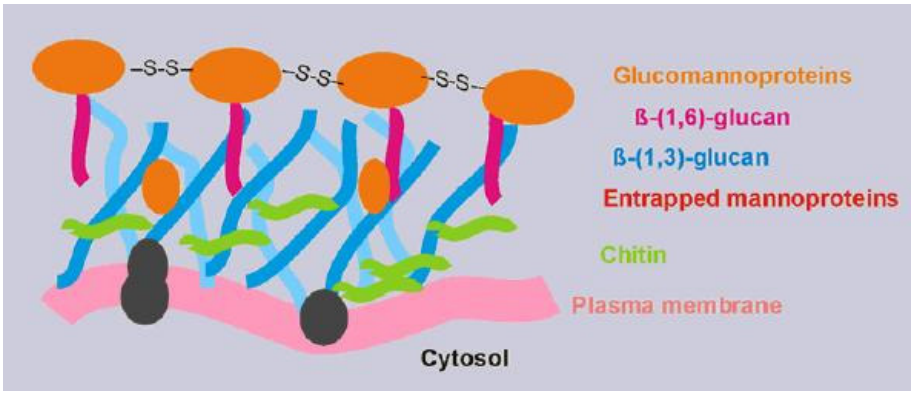


Figure 1.12. Constituents of a yeast cell wall [97]

Killer toxins of *Debaryomyces hansenii, Hanseniaspora uvarum, Pichia membranifaciens* and *Saccharomyces cerevisiae* (K1, K2) bind to β-1,6-glucan residues on the yeast cell wall as primary receptors whereas toxins of *Saccharomyces cerevisiae* (KT28), *Schwanniomyces occidentalis* and *Zygosaccharomyces bailii* bind to mannoproteins. Chitin residues has been described as cell wall receptor for *Pichia acaciae* and *Kluyveromyces lactis* killer toxins. After binding to the cell wall receptors actual cell killing step occurs. Killer toxins kill the sensitive cells by different mechanisms [100].

The mode of action of the killer toxin has been largely studied for the K1 and K2 killer system. It is assumed that β-subunit is involved in binding to the cell wall receptor. α component might be responsible for the lethal effect on the membrane.

Binding of K1 killer toxin to β-1,6-glucan of the cell wall is the initial step for the action of this toxin. It was demonstrated that damage of the cell by the killer protein caused by a changed permeabilty of the membrane and collapse of the proton gradient. This is followed by decrease of pH in the cell, inhibition of metabolic process, release of potassium ions and ATP to the medium and eventual death of the cell [72 ,76 , 77 , 101, 102].

Different from known killer toxins, the killer toxin from *S. cerevisiae* K28 binds mannoproteins of the cell wall. K28 causes cell cycle arrest, apparently in the G2 phase, and leading to non-seperation of mother and daughter cells.This toxin irreversibly blocks DNA synthesis [95].

Another mode of action of killer toxin is seen in *Kluyveromyces lactis* in which the toxin acts by arresting the sensitive cells at G1 phase of the cell cycle and leads to loss of viability [96].

The toxin from *Pichia kluyveri* causes ion-channel formation. These channels are relatively non-selective for common physiological cations and anions. The toxin-induced channels would cause a 'leak pathway' for major ions such as K^+ and H^+ and dissipate the normal ionic gradients across the plasma membrane. Finally, these toxin induced channels result in sudden cell death [103].

K5 type yeast killer toxin produced by *Pichiaanomala* inhibits the growth of sensitive microbial cells by hydrolyzing the major cell wall component β-1,3-glucan residues which results in cell burst [104]. Another mechanism that affects the cell wall of fungal cells is the inhibition of β-1,3-glucan synthase which is required for the synthesis of β-1,3-glucans. Killer protein of *Hansenula mrakii* causes pore formation by inhibiting the β-1,3-D-glucan synthesis occurring at a budding site which results in leakage of cell material and eventual cell death [77, 105].

1.4.4.Potential Uses of the Killer Yeast and Their Toxins

Several potential applications for the killer yeasts and their toxins have been studied and suggested. They have been used as model systems to study the mechanisms of regulation in eukaryotic polypeptide processing, secretion and toxin interaction with sensitive cells. Furthermore, the killer system in yeast provides useful models for the study of the control and expression of eukaryotic viruses. In recombinant DNA technology, killer plasmids from *S. cerevisiae* and *K. lactis* have the potential to serve as cloning vectors for the effective secretion of expressed polypeptides [106]. In addition, killer systems could have biotechnological applications both in food and fermentation industries and in medicine. They can be used as antifungal agents against both human and plant pathogens because of their wide killing spectrum on sensitive cells [84 , 99 , 100].

1.4.4.1.Heterologous Protein Expression

When expression of eukaryotic proteins is desired, bacterial systems often turn out to be ineffective hosts because of their limited capacity to perform multi-step post-translational modifications such as protein N-glycosylation, phosphorylation and acetylation. Therefore, unicellular eukaryotes such as the yeasts *S. cerevisiae, Pichia pastoris, Yarrowia lipolytica, Hansenula polymorpha, K.lactis* and *S.pombe* have become attractive hosts for the expression of heterologous proteins.

The killer trait can be used to produce large amounts of foreign proteins in yeast. Several heterologous genes, including genes coding biotechnologically important enzymes, have been expressed using killer plasmids as vectors.

Kluyveromyces lactis has recently become an attractive microbial host for the expression of foreign genes and protein secretion for several reasons, including (i) its food grade status, since *K.lactis* is present in various food products it is accepted as "GRAS" (generally recognized as safe) ; (ii) its excellent fermentation characteristics ; (iii) some major advantages of its linear plasmids (pGKL1 and pGKL2), when compared with circular plasmid such as their high copy number and extreme stability. Their cytoplasmic localization makes them independent of nuclear control in terms of replication and transcription ; (iv) its ability to secrete high molecular weight proteins [107 -110].

During the past decade, an increasing number of medically and pharmaceutically interesting secretory proteins (such as mouse K-amylase, human Antithrombin III or placental alkaline phosphatase) have been expressed as extracellular proteins by using homologous secretion signals derived from the plasmid-driven killer toxin of *K. lactis*. h-TPA (human tissue plasminogen activator) cDNA was fused with the leader sequence of *K. lactis* killer toxin in order to achieve high level expression of h-TPA in yeast [111].

Bacterial xylanase which is used in the paper manifacturing industry is expressed and secreted in *K. lactis* using a secretion signal derived from the pre- region of the *K. lactis* killer toxin[112 , 113].

Since the proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris* provides an excellent alternative to *E.coli* expression systems. A number of proteins have been produced using this system, including tetanus toxin fragment, human serum albumin and lysozyme [111, 113].

1.4.4.2.Medical Use

During the last two decades, human fungal infections have dramatically increased especially in immunocompromised patients due to intensive chemotheraphy, immunosuppressive drugs and HIV. Particularly patients infected with HIV-1 are under the extreme risk of mycoses [114].

There are two major problems in the treatment of fungal infections with the currently used antifungal agents such as amphotericin B, fluconazole, ketoconazole, itraconazole. These agents are not selective to the fungal cells and cause severe side affects on the host cells and these side affects are mainly related with liver problems [115]. Another problem is the resistance development to the antifungals especially in long term usages. Therefore development studies on novel antifungal drugs are focused on selective agents which target the components of yeast and fungal cell walls. These agents would not harm the host cell because mammalian cells do not have these components [67, 104].

Among the new antimicrobial molecules under investigation, specific yeast killer toxins represent promising candidates because they display wide spectra of activity. Since the cell wall components was shown to act as the primary binding site and cell wall receptor for the different yeast killer toxins,

antifungal research is currently focusing on the possible use of yeast killer toxins as novel antifungals [77, 95].

Killer toxins produced by *Hansenula, Pichia, Kluyveromyces* strains are demonstrated to have antifungal activity against pathogenic fungi that threat human, animal and plant hosts [65, 66, 116]. Especially *Pichia anomala* killer toxin has been investigated and shown to have antagonistic effect against a broad range of microorganisms. It is thought that *Pichia* toxins which shows relative high stability in comparison to toxins of other killer yeasts could be exploited in a therapeutic strategy [73, 75, 93, 117, 118].

In addition, killer yeasts have found applications in the biotyping of the pathogenic yeasts such as *Candida albicans* and *Cryptococcus* species by using their sensitivity or resistance to a wide range of killer toxins, especially those from *Saccharomyces, Pichia, Kluyveromyces* and *Candida*. The same methods are also approved applicable to classifying actinomycetes [119,120].

1.4.4.3.Fermentation industry

Contamination with killer-toxin producing yeast species is a potential problem in fermantation process. The killer phenomenon can be utilized for the protection of fermentation process against contaminating yeasts by the introduction of the killer trait into the sensitive starter strains. Immune industrial strains is constructed by using the methods of protoplast fusion or cytoduction In wine making, killer yeasts belonging to *S. cerevisiae* are currently used to initiate wine fermentation to improve the process of wine making and wine quality [71, 76,121,122].

1.4.4.4.FoodStorage

Fungal decay of fruits and vegetables in postharvest storage greatly limits their economic value. Many studies involving antagonistic microorganisms to control postharvest diseases of fruits and vegetables have been done. Among these antagonistic microorganisms, killer yeasts appear to be promising biocontrol agents providing alternatives to chemical fungicides in the postharvest storage of fruits and vegetables. With regard to the action of killer yeasts against plant pathogenic fungi, it is apparent that several yeast species display significant in vitro antifungal activity [39-61]. There are several reports in the literature indicating wide range intergeneric killing spectrum of *Pichiaanomala.* Therefore, *Pichia anomala* has been extensively studied and proposed as biocontrol yeast [63-67].

The ability of *P.anomala* to prevent mould growth has been validated in experiments using 0,2 m^3 pilot scale silos for airtight storage of 160 kg batches of moist wheat grain [123]. Strains of *P.anomala* can also prevent growth of the gray mold, *Botrytis cinerea* , on stored apples and on grape plants [25, 53, 54].

Based on the results of competition plate bioassays, killer strains of *P.anomala* markedly inhibited the growth of certain plant pathogenic fungi including *Phythophthora infestans , Botrytisfabae , Fusarium equiseti* [63-67].

Antagonistic activity of *P.anomala* on agar plates against the spoilage molds including *Penicillium species , Aspergillus flavus , Aspergillus fumigatus , Botrytis cinerea, Fusarium poae, Rhizopusstolonifer* was determined. *P.anomala* strongly reduced the growth and sporulation of most of the moulds tested on agar plates. The degree of inhibition was dose dependent [63].

The abilities of *P.anomala, Pichia guilliermondii* and *Saccharomyces cerevisiae* to inhibit the growth of the mold *Penicilliumroqueforti* in nonsterile

high-moisture wheat were compared by adding 10^3 *Penicilliumroqueforti* spores and different amounts of yeast cells per gram of wheat. *P.anomala* had the strongest antagonistic activity in wheat, with 10^5 and 10^6 CFU/g completely inhibiting the growth of *P. roqueforti* . *S. cerevisiae* inhibited mold growth only weakly at the highest inoculum level [63].

In spite of noteworthy antimicrobial activities of antagonistic microorganisms in laboratory scale, antogonists that show promise under laboratory conditions do not necessarily perform as well in a full scale trial [124, 63]. The short period between harvesting and placing fruit in storage, from less than a day to a few days, requires rapid antagonist action. Once fruit is placed in cold storage, metabolic rates of the host and associated microflora will decline depending on the temperature regime selected. The search for antagonists to control postharvest wound invading pathogens should be narrowed to rapid colonizers of the wound site that can still be metabolically active at low storage temperatures [20].

Furthermore, the way in which antagonist is introduced can affect both the original microbial flora and the antagonistic capacity of the introduced microorganism [48].

The biocontrol activity of microbial antagonists has been shown to increase with increasing levels of microbial antagonists and decreasing levels of the pathogen inoculum. Generally, microbial antagonists are most effective in controlling postharvest decay when applied at 10^8 CFU /ml. Often no control of decay was observed when antagonistic yeasts were applied at 10^5 CFU/ml. The nontarget microflora is also likely to be affected when an antagonist is introduced in such large numbers. The growth of an antagonist might change the environment , making it more suitable for other microorganisms [12, 63].

These limitations of antagonistic yeasts as biocontrol agents has generated an interest in yeast killer proteins which play major factor in antimicrobial

activities of biocontrol yeasts. Yeast killer proteins showing strong antimicrobial activity has been suggested as potential biocontrol agents [125].

1.5.K5 Type Yeast Killer Protein : Promising Antimicrobial Agent

According to the Young and Yagiu classification (1978), the K5 type yeast killer protein is produced by *Pichia anomala* NCYC 434 [73].

Pichia anomala is a fungi belonging to the phylum; *Ascomycetes*, class; *Hemiascomycetes*, order; *Saccharomycetales*, family; *Saccharomycetaceae*, genera; *Pichia* (*Hansenula*), species; *anomala* . *P. anomala* is present in various environments and have been isolated from fruit and plant material, cereal grain, maize silage, and from high sugar food products [126].

In several studies *P.anomala* toxins have been shown to have a wide-range intergeneric killing spectrum with relatively high stability in comparison to toxins of other killer yeasts [66 , 127 , 128]. Among *P. anomala*, especially the strain NCYC 434 has been extensively studied [129,130-133].

Production of K5 type yeast killer protein by *P. anomala* NCYC 434 cells is highly dependent on the composition of the medium. It is stimulated by the presence of β-glucan. Amount of the toxin increases in correlation with the amount of the β-glucan and the type of its linkage. Glucose is also required to maintain the highest level of K5 type toxin production [131].

K5 type yeast killer toxin was previously purified and characterized in our laboratory. It is a glycosylated protein with a molecular mass of 49 kDa and with a pI value of 3.7. Temperature and pH stability testing of the toxin shows that the optimum pH value for the K5 type yeast killer protein is 4.5 and about 70 % of the activity remains even at pH 2.5. However toxin is inactivated at pH values above

6.5 (Figure 1.13.). The optimum temperature for the toxin activity is 25 ºC. Toxin looses 10 % of its activity at 37 ºC and 50 % at 100 ºC (Figure 1.14.) [104].

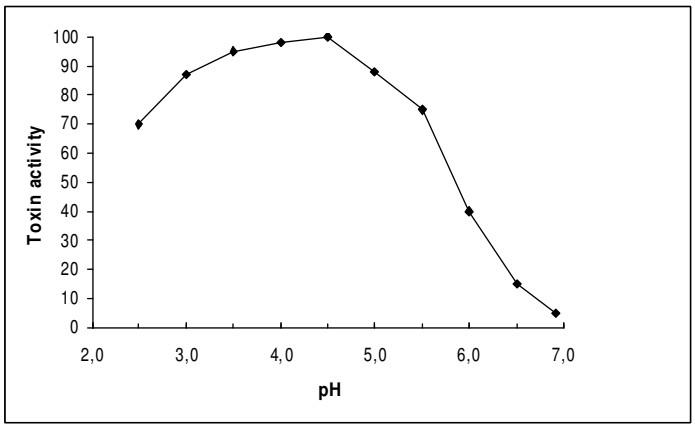


Figure 1.13. pH Stability of the K5 Type Yeast Killer Toxin [104].

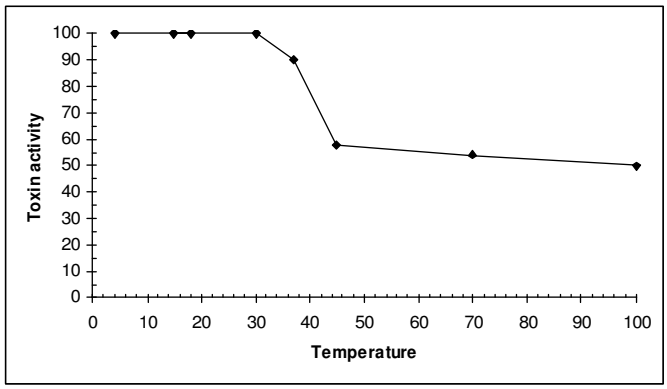


Figure 1.14. Temperature Stability of the K5 Type Yeast Killer Toxin [104].

Internal aminoacid sequences of the toxin shares 100 % homology with the exo-β-1,3-glucanase of *P.anomala* Strain K which is a glycoprotein of 45.7 kDa with a pI of 4.7 [104].

Mode of action of the K5 type yeast killer protein was also studied by İzgü et. al. K5 type yeast killer protein is mostly absorbed by laminarin which is mainly composed of β-1,3- glucans. This indicates that the toxin exerts its lethal affect by hydrolyzing β-1,3-glucan residues of the cell wall of sensitive fungal cells and causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure. Further studies on its mode of action shows that K5 type yeast killer protein exerts hydrolytic activity on the β-1,3- glucans in an exo like fashion [131].

Specific activity of the K5 type yeast killer toxin on laminarin is 120 U/mg and the Michaelis Menten constants K_m and V_{max} are 0.25 mg/ml and 370 µmol/min/mg respectively [131].

Affects of metal ions on the enzymic activity of the toxin was also studied. Activity is fully inhibited by Hg^{+2}, but increases with some other metal ions such as Ba^{+2}, Ni^{+2}, Cr^{+2}, Zn^{+2} ; most of all by Pb^{+2} [131].

High pH and thermo stability which are appropriate for both medical and biocontrol purposes, broad spectrum of antimicrobial activity of the K5 type yeast killer toxin, suggest its possible applications as an antimicrobial agent in food biocontrol, fermentation process and medicine [104].

Exo-β-1,3-glucanase activity of the K5 type yeast killer protein on sensitive cells highlighted the potential use of this protein as a highly selective antifungal agent since its hydrolyzing activity is specific to the fungal cell wall and does not affect the host cells.

To date, the growth inhibitory effect of the killer toxin was tested either by spotting *P.anomala* NCYC 434 cell suuspensions or the crude toxin preparations onto the plates seeded with the test strains [63-67]. Recently, we have determined antifungal spectrum of the pure K5 type yeast killer protein on human pathogenic fungal species including *Candida* species and dermathophytes, causing either systemic or superficial fungal infections and analysed its kinetics of cell killing.

K5 type yeast killer toxin was found to be affective against all of the tested 26 strains of the genus *Candida* within the MIC_0, MIC_2 and MFC ranges of 0.25-4, 0.5-8, 1-16 µg/ml respectively [132].

K5 type yeast killer toxin was found to be affective against all of the tested 9 strains of human pathogenic dermathophytes within the MIC_0 and MIC_2 ranges of 0.25-2, 1-8 µg/ml respectively [133].

It was shown that K5 type killer toxin exerted its cytotoxic effect within 2 hours and kills the entire population at time periods between 10-36 hours when tested at MFC.

Wide antifungal spectrum and selectivity of the toxin due to its strong exo-β-1,3-glucanase activity highlights the possible usage of the K5 type yeast killer protein as a novel antifungal agent in medicine.

In this study our aim was to determine the antifungal spectrum of the pure K5 type yeast killer protein on fungi causing spoilage in citrus fruits especially in postharvest period. Thereby, we would conduct a primary investigation about the potential usefullness of this novel yeast killer protein as a bicontrol agent against plant pathogens.

CHAPTER II

MATERIALS AND METHODS

2. 1. MATERIALS

2.1.1. Fungal Strains

The K5 type yeast killer protein producing strain, *Pichiaanomala* (NCYC 434), and killer toxin sensitive strain, *Saccharomyces cerevisiae* (NCYC 1006) were purchased from the National Collection of Yeast Cultures, Norwich, U.K. Plant pathogenic fungal strains used in antifungal susceptibility studies are given in Table 2.1.

Table 2.1. Plant Pathogenic Strains Used in Antifungal Susceptibility Studies.

STRAIN	STRAIN NUMBER\ SOURCE	ISOLATION
Penicilliumdigitatum	DSMZ 2776	lemon
Penicilliumitalicum	DSMZ2756	lemon
Botrytis cinerea *	DSMZ5145	*Vitis vinifera*
Alternaria citri	DSMZ62013	*Citrus sinensis*, necrotic spot on fruit
Colletotrichum gloeosporides	DSMZ62146	*Citrus sinensis*, necrotic spot on fruit
Phythophthora citrophthora	DSMZ62669	*Citrus sinensis*, rotting fruit

DSMZ: German National Resource Centre for Biological Material
* Resistant to benzimidazole- and dicarboximide-fungicides.

2.1.2. Culture Media

The K5 type yeast killer protein producing strain, *Pichiaanomala,* (NCYC 434) and killer toxin sensitive strain, *Saccharomyces cerevisiae* (NCYC 1006) were grown in YEPD medium at pH 5.5 consisting of 1% Bacto-yeast extract, 1% Bacto-peptone and 2% dextrose along with 2% Bacto-agar for the maintenance and routine growth. For the production of the K5 type yeast killer protein *P. anomala* cells were grown in YEPD medium buffered to pH 4.5 with phosphate-citrate buffer with the addition of 5% glycerol. YEPD medium with 2% Bacto-agar buffered to pH 4.5 with phosphate-citrate buffer was used for the determination of killer toxin activity.

Prior to antifungal susceptibility studies all pathogenic fungal cells were subcultured in potato dextrose agar (0,4% potato extract, 2% dextrose and 5% agar) plates to ensure purity and viability. Synthetic RPMI 1640 medium dissolved in pH 4.5 100 mM Na_2HPO_4- citric acid buffer was used for the broth microdilution antifungal susceptibility testing. Sabouraud medium consisting of 1% Bacto-peptone and 2% dextrose along with 2% Bacto-agar dissolved in pH 4.5 100 mM Na_2HPO_4- citric acid buffer was used for agar diffusion asssays in order to determine susceptibilities of pathogenic fungal strains to K5 type yeast killer protein.

2.1.3. Chemicals

The chemicals and the suppliers are listed in the Appendix A.

2.1.4. Buffers

Buffers and solutions used in the experiments are given in Appendix B.

2.2. METHODS

2.2.1. Sterilizations

The glass cultureware were sterilized on dry-cycle at 240 °C for two hours. The media for stock cultures and for routine growth of the yeast cells were sterilized at 121° C for 15 minutes on liquid cycle. RPMI 1640 medium was filtered through 0.45 μm and 0.22 μm (Sartorius, AG, Germany) cellulose acetate filters respectively for sterilization. Buffers used for the chromatographic purification steps were filtered through 0.45 μm cellulose acetate filters (Sartorius, AG, Germany) using filter device (Sartorius, AG, Germany) prior to sterilization on liquid cycle.

2.2.2. Maintenance of the Fungal Cultures

Freeze-dried cultures of *Pichiaanomala* (NCYC 434) and *Saccharomyces cerevisiae* (NCYC 1006) in glass ampoules were opened aseptically and 0.5 ml of YEPD medium was added to dissolve the dried culture using a sterile pasteur pipette. Dissolved yeast cells were plated onto petri dishes containing YEPD agar at pH 5.5 and incubated at 25 ºC until the colonies were formed [89].

Active cultures of the plant pathogenic strains in glass tubes were covered with adequate amount of sterile saline solution. Spores were scratched with the tip of a sterile pasteur pipette and 0.5 ml of the spore suspension was drawn under sterile conditions and plated onto PDA pH 5.5 agar plates and incubated at 25° C. All plate cultures were stored at 4 ° C for a maximum of 3 weeks and subcultured on to a new plate. Prior to antifungal susceptibility testing the strains were subcultivated to PDA plates to promote spore formation.

2.2.3. Production and Concentration of the K5 Type Yeast Killer Toxin

Production, concentration and purification of the K5 type yeast killer protein were done as described previously by İzgü and Altınbay [104]. *Pichia anomala* NCYC 434 cells were cultivated into 10 ml of YEPD pH 5.5 medium and incubated for 24 hours at 25 ^{o}C. One ml of cell suspension was further inoculated into 100 ml of the same medium. After the yeast cells were incubated at 25 ^{o}C for 24 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA), 10 ml of cell suspension was transferred to 1L of YEPD medium (containing 5% glycerol) buffered to pH 4.5 with phosphate-citrate buffer and incubated until stationary phase at 20 ^{o}C for 36 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). Centrifugation (KR 22i, Jouan, France) at 5000 rpm for 10 min. at 4^{o} C was applied to obtain the cell free culture medium and supernatant was filtered through 0.45µm and 0.2µm cellulose acetate membranes (Sartorius, AG, Germany) respectively for the sterilization of the medium.

Cell free culture medium containing the K5 type yeast killer protein was concentrated 100 fold by using 30 kDa molecular cut-off ultrafilters (Vivaspin VS2021, Sartorius, AG, Germany) at 4200 rpm, 4^{o}C (MR23i, Jouan, France) [104].

2.2.4.Purification of the Killer Toxin

Crude toxin obtained from the previous step was subjected to ion exchange and gel permeation chromatographies respectively by using a fully automated FPLC system (Biocad 700E Perseptive Biosystems, USA) including an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan). Detections were done with UV absorbance at 280 nm at 20 ^{o}C [104].

2.2.4.1. Anion Exchange Chromatography

Crude toxin was buffer exchanged to 30mM N-methylpiperazine-HCl (pH 4.8) by using 5 kDa molecular cut-off centrifugal filters (Vivaspin VS2021, Sartorius) at 4200 rpm, 4°C (MR23i, Jouan, France). Buffer exchange step was repeated for 3 times to maintain YEPD free samples. Samples were filtrated through 0.22µm disk filters (Sartorius, AG, Germany) prior to injection to the FPLC column.

750µL of the buffer-exchanged protein sample was put on an anion-exchange column (POROS HQ/M 4.6 mmD/100mmL, Perseptive Biosystems, USA) that is previously equilibrated with N-methylpiperazine-HCl (pH 4.8). The column was washed to 20 column volumes (CV) with the same buffer. Elution was done with a linear gradient of 0 to 500mM NaCl in N-methylpiperazine-HCl (pH 4.8) at a flow rate of 10 ml/min. Killer protein containing fractions (1600µL) that corresponds to 120mM NaCl were pooled. These eluted fractions were concentrated and buffer-exchanged to 100mM Na_2HPO_4- citric acid buffer, pH 4.5 by using 5 kDa molecular cut-off centrifugal filters (Vivaspin VS2021, Sartorius ,AG, Germany) at 4200 rpm, 4°C (MR23i, Jouan, France).

2.2.4.2. Gel-Permeation Chromatography

The concentrated and buffer exchanged sample obtained from the previous anion exchange step was then subjected to gel permeation chromatography using a TSK G2000 SW (7, 5 mmD/300mmL TosoHaas, Japan) column. Prior to injection of the sample, column was equilibrated with 100mM Na_2HPO_4- citric acid buffer, pH 4.5, containing 100mM Na_2SO_4 at a flow rate of 1 ml/min. The equilibration was performed until the base line was stable. Ninety µl of sample was injected into the column and elution was done with the same buffer at a flow rate of 1 ml/min. Killer toxin containing eluted fractions (1300µl) that corresponds to 8.5 ml were pooled. These active fractions are then concentrated

and buffer exchanged to the same buffer but the salt by using 5 kDa molecular cut-off ultrafilters (Vivaspin VS2021, Sartorius, AG, Germany). Thirty µl of the purified protein obtained from gel permeation chromatography was spotted on to YEPD (pH 4.5) agar plates seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells for the assessment of the killer toxin activity.

2.2.5. Determination of Killer Toxin Activity

Toxin activity at various stages of the study was tested according to Brown et al. [134] with an agar diffusion assay. Twenty five ml of molten YEPD agar (pH 4.5) was seeded with 1 ml of *Saccharomyces cerevisiae* (NCYC 1006) cells in sterile water at a density of 10^5 cells/ml and poured into petri dishes. Protein samples of 30µl were spotted onto petri dishes and incubated at 23 ^oC. The killer activity was determined by measuring the clear zone of growth inhibition of the seeded killer toxin sensitive strain after 48 hours of incubation. Killer toxin which gave a clear zone of 10 mm in diameter was defined as 1 arbitrary unit (AU).

2.2.6. Assessment of Protein Concentration

Protein concentration was measured by using protein-dye binding method of Bradford [135]. Bradford reagent used for the determination of protein concentration was prepared as follows. A hundered mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95 % ethanol then mixed with 100 ml of 85 % (w/v) phosphoric acid. Finally the solution was completed to a total volume of 1 L with distilled water and filtered through Whatman #1 paper.

Bovine serum albumin (Fraction V) dissolved in 100 mM Na_2HPO_4- citric acid buffer at the concentration of 0,5 mg / ml was used as the standart protein solution. From this stock solution different concentrations of bovine serum albumin was prepared in 100 mM Na_2HPO_4- citric acid buffer in a total volume of

800 µl and mixed with 200 µl filtered Bradford reagent to yield different concentrations of bovine serum albumin ranging from 2.5 to 25 µg/ml as shown in Table 2.2. Then a blank sample was prepared by mixing 800 µl 100 mM Na_2HPO_4- citric acid buffer and 200 µl Bradford reagent. 10 µl protein sample was also diluted in 790µl of the same buffer and mixed with 200 µl Bradford reagent. Then 100 µl from each standard solutions, protein sample and blank sample were pipetted into individual wells of 96-well microplate (Nunclon 167008; Nunc, Denmark)**.** Absorbances were immediately measured at 590 nm by using UV visible spectrophotometer (Spectramax 190, Molecular Devices, USA). Then the concentration of the protein in the sample was calculated using a standard curve of absorbance versus protein amount.

Table 2.2. Volume of Solutions Used in Constructing BSA Standard Curve.

Solution	Buffer(ml)	Stock BSA solution at the concentration of 500 µg / ml (µl)	Amount of BSA (µg)
1	795	5	2.5
2	790	10	5
3	780	20	10
4	770	30	15
5	760	40	20
6	750	50	25

2.2.7. SDS and Non-denatured SDS Polyacrylamide Gel Electrophoresis

Molecular weight determination of the pure toxin was done with a 5-20% linear gradient SDS polyacrylamide gel in a discontinuous buffer system as described by Laemmli [136], using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA). The 5-20% linear gradient gel was prepared with a gradient maker (Hoefer, USA). The density of the gel was 20% at the bottom and decreased to 5 % towards the top of the gel.

In order to ensure purity of the toxin, concentrated protein obtained from the gel permeation step was subjected to electrophoressis on a 15% linear, 0.75 mm thick native polyacrylamide gel in a discontinuous buffer system using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA). 2-β-mercaptoethanol was left out the standard Laemmli protocol for non-denaturing conditions.

Gradient (5%-20%) or 15% separating gel was prepared and poured into the electrophoresis unit and covered with saturated n-butanol to avoid contact of the gel with air and left for polymerization for 1 hour. When the polymerization was completed n-butanol was washed with water and stacking buffer respectively before the stacking gel was poured. Stacking gel was also left for polymerization for 1 hour. Separating and stacking gel components are given in Table 2.3 and 2.4.

Protein samples were subjected to acetone precipitation and resuspended in 125 mM Tris-Cl pH 6.8. They were heated at 100 °C for 5 min in equal volume of sample buffer (20% (v/v) glycerol, 4% (v/w) SDS, 0.02% (v/w) bromophenol blue pH 6.8) for the non-denaturing SDS PAGE and 10% 2-β-mercaptoethanol was added to the sample buffer for SDS PAGE. The samples were loaded onto the gel after the polymerization of the stacking gel was completed.

Electrophoresis was done at 15 mA/0.75 mm gel (Power supply PP4000, Biometra, Germany) at 15 ^{o}C using a circulating water bath (Heto Holten, Denmark).

The gels were visualized by silver staining. For the molecular weight determination the SDS gel was scanned using a GT9500 Color Image Scanner (Epson, Japan) and the data were processed with Gelworks 1D software (UVP Products, UK).

Table 2.3. Separating Gel Mixtures.

	5% Gel	15% Gel	20% Gel
Acrylamide-bisacrylamide (30:0.8)	3.34 ml	12.5 ml	13.2 ml
4X Seperating Gel Buffer (1.5M Tris-Cl , pH:8.8)	5 ml	7.5 ml	5 ml
10 % SDS	0.2 ml	0.3 ml	0.2 ml
ddH_2O	11.4 ml	9.6 ml	-
Sucrose	-	-	3 gr
10 % Ammonium persulfate*	66µl	150 µl	66µl
TEMED*	6.6µl	10 µl	6.6µl

*Ammonium Persulfate and TEMED were added after deaeration

Table 2.4. Stacking Gel Mixture (4%T).

Acrylamide-bisacrylamide(30:0.8)	1.33 ml
4X Stacking Gel Buffer (0.5M Tris-Cl , pH:6.8)	2.5 ml
10 % SDS	0.1 ml
ddH_2O	6 ml
10 % Ammonium persulfate*	50 µl
TEMED*	5 µl

*Ammonium Persulfate and TEMED were added after deaeration.

2.2.8. Protein Detection in Gels: Silver Staining

Protein bands were visualized by silver staining method. After the gel was removed, it was placed in Destain 1 solution (100 ml) and incubated for 30 minutes with gentle shaking. Then the solution was replaced with Destain 2 solution (100 ml). After 30 minutes Destain 2 solution was discarded and replaced with 100 ml of cross-linking solution. Gel was washed with several changes of water over 2 hours. After the final wash DTT solution was added and incubated for 30 minutes with slow shaking. DTT solution was replaced with 100 ml of Silver Nitrate Solution and again shaken slowly for 30 minutes. Gel was washed with distilled water and developing solution respectively and 100 ml of fresh developing solution was added. Staining was occurred within 5-10 minutes. When the desired staining level was reached, development was stopped by replacing the development solution with the destain 2 solution [137].

2.2.9. Antifungal Susceptibility Testings

Pathogenic fungal strains (6 *in toto*) causing postharvest decays in citrus fruits were tested for their susceptibility to K5 type yeast killer protein both *in vitro* and *in vivo*.

In order to use in these antifungal susceptibility testings inoculum suspensions composed of spores were prepared as follows. The isolates were subcultured on PDA plates and incubated at 25° C. Inoculum suspensions were prepared from fresh , mature (1 to 2 week old) cultures. In some cases an extended incubation was required for proper sporulation of the isolates such as *Alternaria citri, Phythophthora citrophthora*. To obtain spore suspensions, cultures were flooded with sterile distilled water containing %0.05 Tween 80 (Merck). Spores were gently scraped from fungal clonies with a sterile bacterial loop. Resulting spore suspension was transferred into a sterile tube. After heavy particles were allowed to settle down for 5 to 10 minutes, the upper homogenous suspension is transferred to a sterile tube and filtered through a filter with a pore diameter of 11 µm (Millipore NY1104700 , Madrid , Spain). This step removes hypha and yields a suspension composed of spores. Resulting suspension is vortexed (Heidolph, Germany) for 15 seconds. Then the concentration of spore suspension was determined by microscopic enumeration with a cell-counting haemocytometer (Neubauer Improved Chamber, Brand, Germany). Appropriate dilutions were performed in order to get the desired concentration required in the assays.Adjusted inoculum concentrations were controlled by plating spore suspensions on PDA plates. The colonies were counted as soon as possible after the observation of the visble growth.

For the optimum activity of K5 type yeast killer protein during testings, all the mediums used in the testings of antifungal activity of K5 type yeast killer protein were dissolved in 100 mM Na_2HPO_4- citric acid buffer adjusted to pH 4.5.

2.2.9.1. *In vitro* Antifungal Susceptibility Assays

Antifungal activity of the K5 type yeast killer protein on fungi causing spoilage in citrus fruits was determined by agar diffusion assays performed on SDA plates (consisting of 1% Bacto-peptone and 2 % dextrose along with 2% Bacto-agar) dissolved in pH 4.5 100mM Na_2HPO_4- citric acid buffer.

Minimum inhibition concentrations (MICs) of the K5 type yeast killer protein for plant pathogenic fungal strains were determined by the broth microdilution method acording to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) -formerly NCCLS- in document M-38-A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi) [138] using 96 well microtitre plates. Growth inhibitory effect of K5-type yeast killer protein on fungal strains was also evaluated quantitatively by measuring the increase in OD at 492 nm [139-141].

2.2.9.1.1.Agar Diffusion Assay

The spore suspensions of the pathogenic fungal strains were prepared as described previously and adjusted to the concentration of 2 X 10^4 spores/ml by microscopic enumeration with a cell counting haemocytometer (Neubauer Improved Chamber, Brand, Germany).

One ml of the spore suspension of each strain at the desired concentration was inoculated into the twenty ml of molten SDA plates consisting of 1% Bacto-peptone, 2% dextrose, 2% Bacto-agar dissolved in pH 4.5 100mM Na_2HPO_4- citric acid buffer and poured into petri dishes.

Protein sample of 50 µl was spotted on these SDA plates for each fungi. SDA plates on which 50 µl of pH 4.5 100mM Na_2HPO_4- citric acid buffer was

spotted served as growth control plates. After 5 days of incubation at 25° C, the antifungal activity was determined by observing the zone of growth inhibition of the seeded pathogenic fungal strain as compared with the growth control plates . Experiments were replicated three times.

2.2.9.1.2.Broth Microdilution Assay

Determination of the minimum inhibition concentration of the K5 type yeast killer protein on fungal strains was performed according to CLSI (Clinical and Laboratory Standarts Institute-formerly NCCLS-) M38-A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi) [138] methodology. Minimum Inhibition Concentration (MIC) assays were done in 96-well round-bottomed microtitre plates (Nunclon 167008; Nunc, Denmark) using a two-fold dilution series of K5 type yeast killer protein ranging from 32 to 0,5 µg/ml which were prepared in 100 mM Na_2HPO_4-citric acid buffer adjusted to pH 4,5. A completely synthetic medium, RPMI 1640 (Sigma Aldrich , 095K8310), was prepared at twice its final concentration (2x RPMI1640) and buffered to pH 4.5 with the same buffer for these susceptibility testings as the test medium.

Fungal inoculum suspensions consisting of spores were prepared for each fungi as indicated previously. For the adjustment of the inoculum concentrations haemocytometer counting was used instead of the spectrophotometric adjustment at 530 nm which was recommended as the inoculum preparation method in antifungal susceptibility testing of filamentous fungi by the reference method M38-A. Since the use of spectrophotometric adjustment for inoculum preparation requires separate standardization for each species, to avoid variability due to the size and colour of the spores, haemocytometer counting was preferred to yield a reliable and appropriate inoculum size. The inoculum suspensions were counted with a haemocytometer and adjusted to the concentration of 4×10^5 to 50×10^5 spores / ml. Then, the suspensions were diluted 50 fold with 2 fold concentrated

RPMI 1640 medium to obtain two fold test inoculum (0.8 x 10^4 -10 x 10^4 spores/ml).

Each microdilution well containing 100 µL of the corresponding two-fold protein dilution ranging from 32 to 0,5 µg /ml was inoculated with 100 µL of the spore suspension prepared in two fold concentared RPMI 1640 medium. This step diluted the medium (1xRPMI 1640) and protein concentrations (16 to 0.25 µg/ml) and also inoculum densities ($0.4x10^4$ to $5x10^4$ spores/ml) to the desired test concentrations.

Each row on the microtitre plate also included both protein free growth control wells (containing 100 µl spore suspension in RPMI 1640 medium and 100 µl 100 mM Na_2HPO_4-citric acid buffer at pH 4.5) and sterility control wells (consisting of 100 µl 100 mM Na_2HPO_4-citric acid buffer at pH 4.5 and 100 µl spore suspension free RPMI 1640 medium). The microdilution trays were incubated at 25 ° C for 48 hours.

The growth in each well was compared with the growth control well both by visually and spectrophotometrically**.** The growth was determined quantitatively by measuring the absorbance at 492 nm in a fully automatic microtitre plate reader (Spectramax 190, Molecular Devices, USA) at 12 hours intervals for 48 hours [139-142]**.**

Readings at 492 nm were taken at the start of the experiment as the background absorbance. Net fungal growth was calculated by substracting these absorbance values at the beginning of the experiment from the absorbance values at each 12 hours interval. Absorbance readings from the final time interval were used to calculate the percent inhibition of the growth. For all the microtiter plates, absorbance values of the growth control wells were averaged.

The average absorbance value obtained for the growth control wells was individually divided into the average absorbance values obtained for the dilution series of the K5 type yeast killer protein. Percent inhibition of each protein concentration on fungal growth was calculated using the following equation : (OD of K5 type yeast killer protein containing well _ background OD) \ (OD of the growth control well _ background OD of growth control well) X 100. Then each value obtained from this equation was subtracted from 100 to acquire the percent growth inhibition for each concentrations of the K5-type yeast killer protein[141].

By using percent growth inhibition as x values and the protein concentration as y values, linear regression analysis was performed to obtain the (Minimum Inhibitory Concentration) MIC and (Inhibitory Concentration) IC_{50} values [141].

MIC was defined as the lowest concentration of the K5 type yeast killer protein where wells were optically clear (without any visible growth/turbidity) or 100% reduction in the growth which was determined by using an automatic microtitre plate reader at 492 nm. IC_{50} was defined as the the concentration of the K5-type yeast killer protein required to obtain 50% growth inhibition [139-142].

In all of the experiments, three replicates were prepared for each treatment and the experiments were replicated at least twice for each fungal strain.

Fungal growth was routinely double checked by an inverted microscope (Nicon, Melville, NY) to confirm absorbance data. At different times, microscopic observation of the growth in wells containing different concentration of the K5 type yeast killer protein were done and compared to the growth control

wells.Differences were photographed with a Kodak camera attached to the photoport of the microscope.

2.2.9.2. *In vivo* Antifungal Susceptibility Assays

In vivo antifungal activitiy of the K5 type yeast killer protein was tested on strains found to be susceptible to protein *in vitro* testings. Experiments were carried out on freshly harvested organic lemon fruits (*C*itrus limon) which were not treated with any antifungal agent [140-142].

Firstly, fruits were surface sterilized by submerging for 5 minutes in a 10% commercial bleach solution and subsequently washing three times distilled water. After air drying fruits were treated with 70% ethanol. Fruits were again airdried and wounded by making punctures three sides around the equator of the fruit with a edge of the sterile tip.

Equal amounts of spore suspensions (at the concentration of 10^4 spores / ml for *Penicllium sp.* [141] and 10^5 spores/ml for *Botrytis cinerea* [140,142]) and protein solutions (at the concentration corresponding to 2 x MIC value) were mixed in a sterile ependorphe tube. For the growth control groups spore suspensions were mixed with equal amount of 100 mM Na_2HPO_4-citric acid buffer adjusted to pH 4.5 instead of the K5 type yeast killer protein. From these solutions , 10 µl of sample was applied onto each wound. For each treatment three replicas (3 fruits per replica , 3 wounds per fruit) were applied. Experiment was repeated three times.

Fruits were maintained at 20 ° C with a high humidity and symptoms were scored daily [140,142].

CHAPTER III

RESULTS

3.1. Production and Concentration of the K5 Type Yeast Killer Protein

Since the production of the killer toxins are highly dependent on the pH of the cultivating medium and incubation temperature [144] *P. anomala* cells were grown in YEPD pH 4.5 medium with the addition of 5% glycerol as toxin stabilizer at 20 ^{o}C to maintain the highest degree of killing activity [104].

3.2. Purification of the K5 Type Yeast Killer Protein

P. anomala cells were removed from the culture liquid by centrifugation and cell free culture medium was concentrated 100 fold by ultrafiltration. Crude toxin obtained from ultrafiltration step was buffer exchanged prior to anion exchange chromatography. K5 type yeast killer protein was purified by anion exchange chromatography using POROS HQ/M column for the optimal resolution (Figure 3.1). Toxin was eluted in the fraction (indicated by arrow) corresponding to 120 mM of NaCl and the resulting elute contained 280 fold purified toxin. Killer protein obtained from anion exchange chromatography was concentrated and buffer exchanged then put on a gel permeation column TSK G 2000SW (Figure 3.2). Active fraction was eluted at 8.5 ml (indicated by arrow). Final purification of 400 fold was achieved by this step.

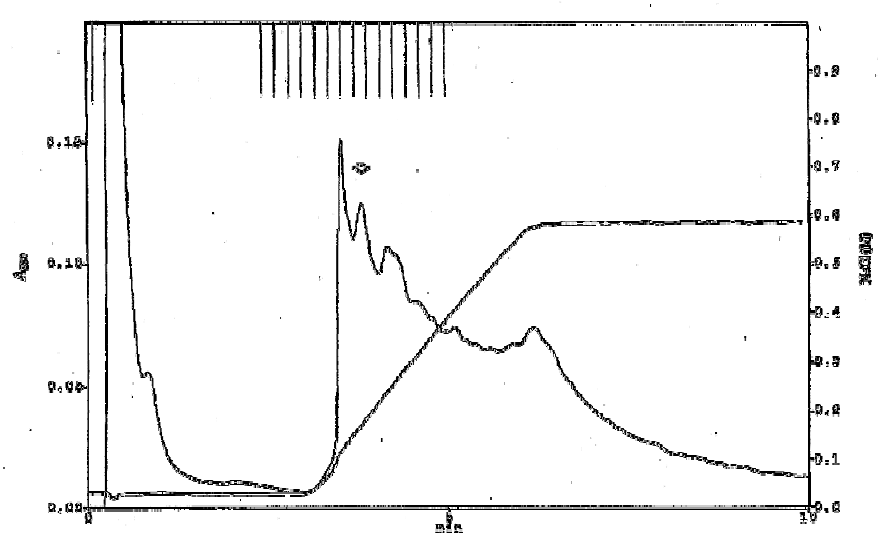


Figure 3.1. Elution Profile of the K5 Type Toxin on a POROS HQ/M Column. Column size: 4,6 mmD/100mmL; Sample: 750 µl; Starting buffer 30 mM N-methyl piperazine- HCl pH:4,8; Gradient: 0-500 mM NaCl in the starting buffer in 20 CV; Flow rate: 10 ml/min; Detection: UV 280 nm.; Fraction volume: 1600 µl. Fraction indicated by arrow contains the K5 type killer toxin corresponding to 120 mM NaCl.

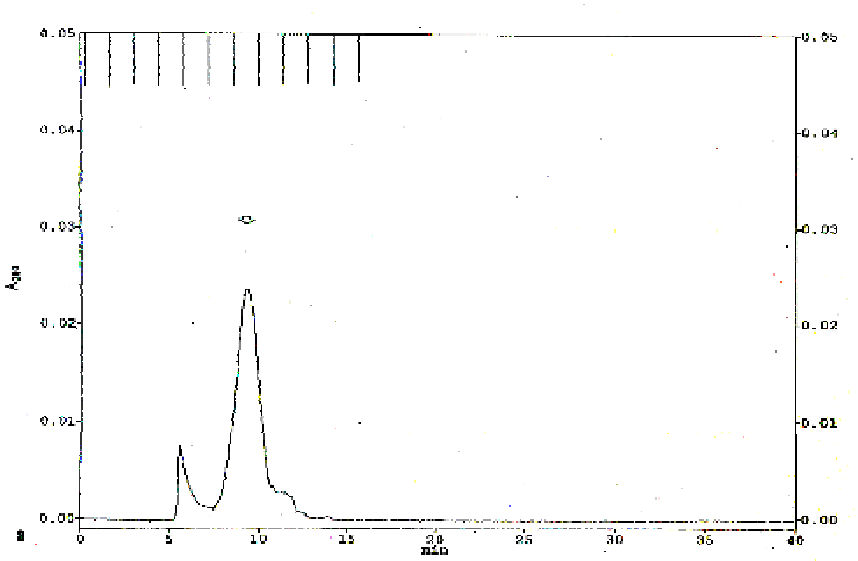


Figure 3.2. Elution Profile of the K5 Type Toxin on a TSK G2000SW Column Column size:7.5 mmD / 300 mmL; Sample: 40 µl; Elution buffer: 0.1M Na_2HPO4 pH 4.5 + 0.1 M Na_2SO4 ; flow rate 1 ml/min; detection 280 nm UV Fraction volume 1300 µl. Fraction containing killer protein is eluted at 8,5 ml and indicated by arrow.

Killer toxin containing fractions obtained from gel permeation chromatography were concentrated and buffer exchanged to 100 mM Na_2HPO_4-citric acid buffer, pH 4.5.

3.3. Determination of Killer Toxin Activity

Killing activity of the K5 type yeast killer protein at different stages of this study was tested with agar diffusion assay. Thirty µl of the protein was spotted onto YEPD pH 4.5 agar seeded with killer toxin sensitive *S. cerevisiae* (NCYC 1006) cells both prior to and after the purification step. After 24 hours of incubation a clear growth inhibition zone of 22 mm (Figure 3.3) and 15 mm (Figure 3.4.) each corresponding to 2.2 and 1.5 AU was observed respectively.

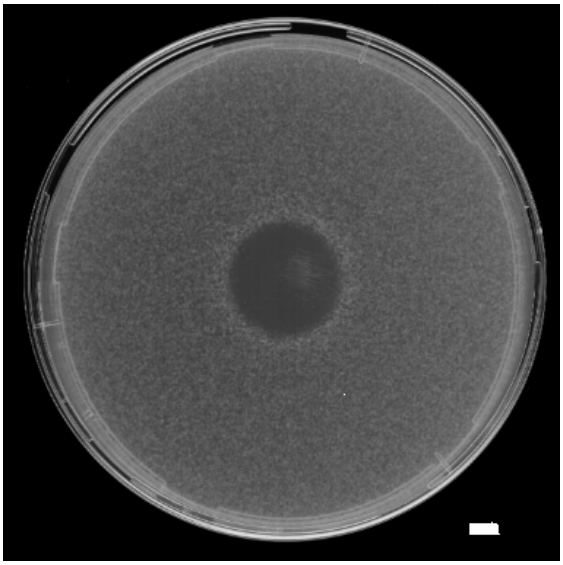


Figure 3.3. Killer Activity of the Concentrated Crude K5 Type Toxin Determined by Agar Diffusion Assay. Bar scale represents 5 mm

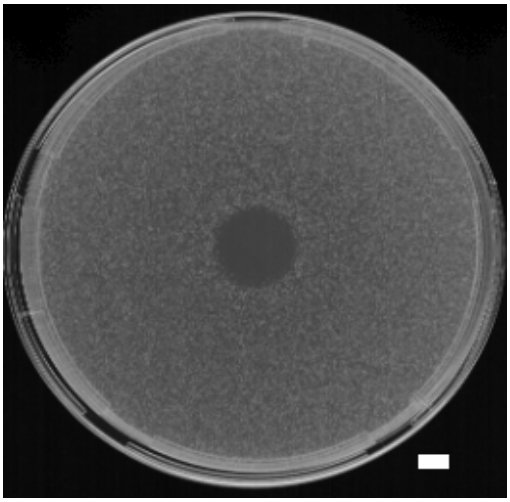


Figure 3.4. Killer Activity of the Purified K5 Type Toxin Determined by Agar Diffusion Assay. Bar scale represents 5 mm

3.4. Assesment of Protein Concentration

Protein concentration was determined as 32 µg/ml by Bradford assay using a standard curve drawn with bovine serum albumin solutions at different concentrations (Figure 3.5.).

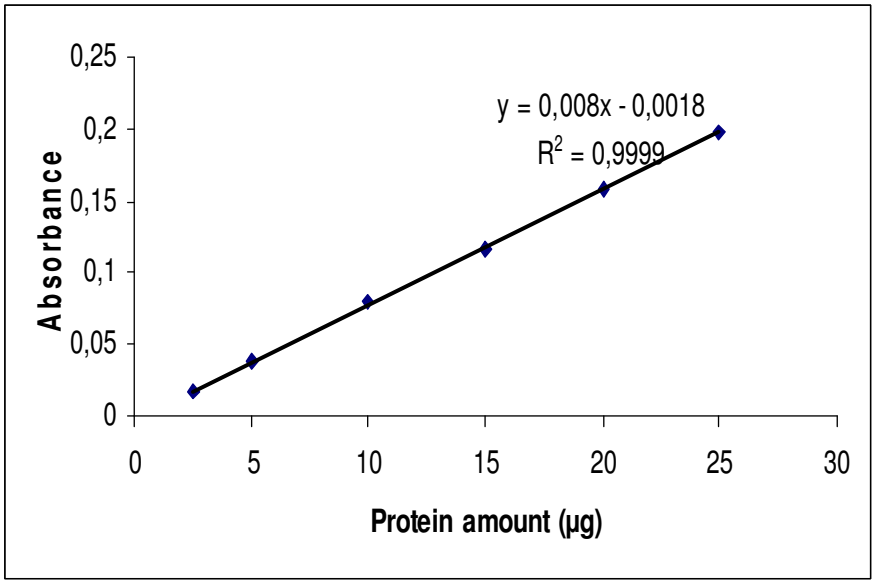


Figure 3.5. Standard Curve of Protein Amount versus Absorbance. Bovine serum albumin solutions were used as standards. 10 µl of the purified K5 type yeast killer protein gave an UV absorbance of 0,00076 at 590 nm which corresponds to 0,32 µg protein amount.

3.5. SDS and Non-denatured SDS Polyacrylamide Gel Electrophoresis

Purified killer protein was electrophoresed on a 15% linear SDS-PAGE gel in a discontinuous buffer system under non-denaturing conditions to check its purity. Observation of single protein band on the silver stained gel indicates the absence of any contamination. Molecular weight of the K5 type yeast killer

protein was determined with a 5%-20% linear gradient SDS-PAGE gel and was found to be 49 kDa (Figure 3.6.).

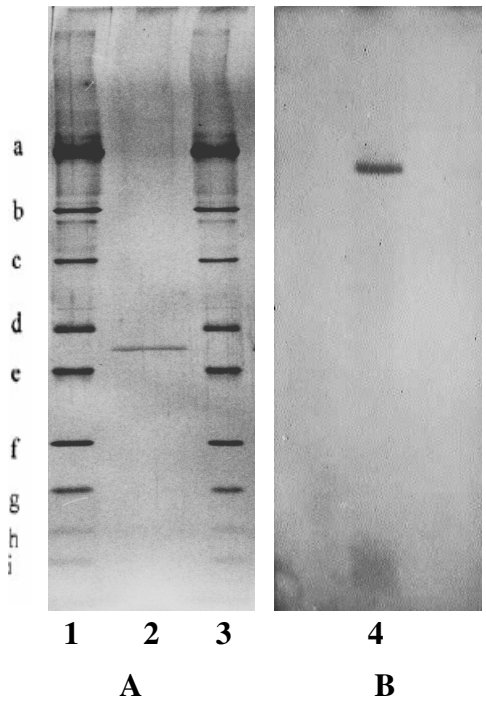


Figure 3.6. Gradient SDS-PAGE (**A**) and Non-denatured SDS-PAGE (**B**) Profiles of the K5 Type Yeast Killer Protein. Lanes 2-4 are K5 type yeast killer protein. Lanes 1-3 are molecular mass markers.(Da)

a) α_2-macroglobulin(170,000), b) β-galactosidase(116,353), c) fructose-6-phosphate kinase (85,204), d) glutamate dehydrogenase (55,562), e) aldolase (39,212), f) triose phosphate isomerase (26,626), g) trypsin-inhibitor(20,100), h) lysozyme (14,307), i) aprotinin(6,500)

3.6. Antifungal Susceptibility Studies

The antifungal activity of the pure K5 type yeast killer protein on plant pathogenic fungal isolates was tested *in vitro* by agar diffusion and broth microdillution testings and also *in vivo* on lemon fruits.

3.6.1. In Vitro Antifungal Susceptibility Assays

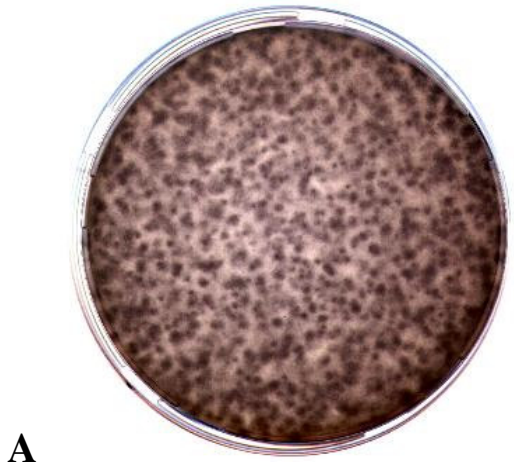
3.6.1.1.Agar Diffusion Assay

The susceptibilities of the pathogenic fungal strains to purified K5 type yeast killer protein was tested with agar diffusion assay.

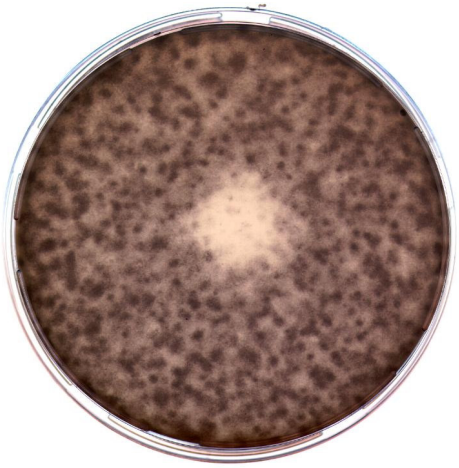
50 µl of the pure protein was spotted onto SDA pH 4,5 plates seeded with spore suspension of each fungal strain. These plates and growth control plates on which 50 µl of 100 mM Na_2HPO_4-citric acid buffer, pH 4.5 spotted were incubated at 25 ° C.

After 5 days of incubation, growth inhibition zone of 15, 12, 10 mm was observed in SDA plates seeded with spore suspension of *Botrytis cinerea* (Figure3.7), *Penicillium digitatum,Penicilliumitalicum* respectively .

No difference was observed between the growth control plates and purified protein spotted plates for *Alternaria citri, Colletotrichum gloeosporoides, Phythophthoracitrophthora*. There was no growth inhibition zone on the plates on which 50 µl of pure K5 type yeast killer protein was spotted (Figure 3.8.).

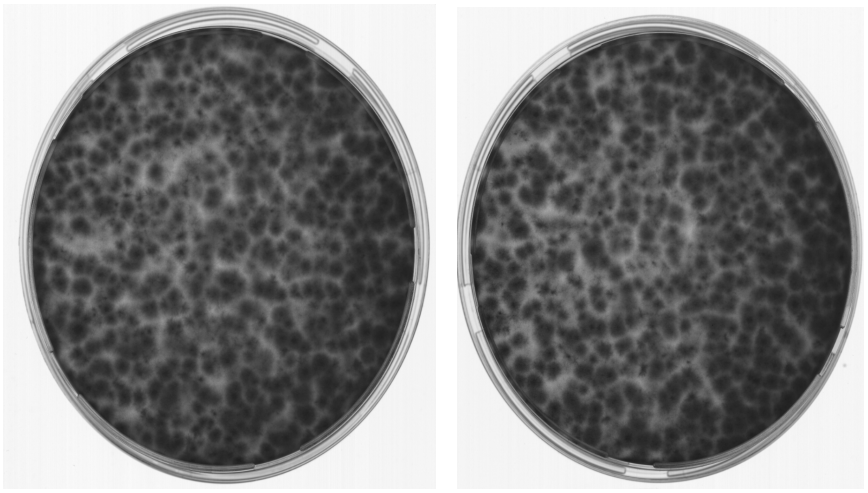


A



B

Figure 3.7.Determination of the Antifungal Activity of K5-type Yeast Killer Protein on SDA Plates Seeded with the Spore Suspension of *Botrytis cinerea*. A)Growth control plates on which 50 µl of 100 mM Na_2HPO_4-citric acid buffer was spotted. B) Growth inhibition zone observed in SDA plates on which 50 µl of pure K5 type yeast killer protein was spotted



A B

Figure 3.8. Determination of the Antifungal Activity of K5-type Yeast Killer Protein on SDA Plates Seeded with the Spore Suspension of *A. citri.*

A) Growth control plate on which 50 µl of 100 mM Na_2HPO_4-citric acid buffer was spotted.

B)SDA plates on which 50 µl of pure K5 type yeast killer protein was spotted

3.6.1.2.Broth Microdilution Assays

Susceptibility of pathogenic isolates to purified K5 type yeast killer toxin in different concentrations ranging from 0.25 to 16µg/ml was tested according to CLSI (Clinical and Laboratory Standards Institude) M38-A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi) methodology.

MICs were determined both visually and spectrophotometrically by comparing the growth in each protein containing well with the growth control wells. MIC was defined as the lowest concentration of the K5 type yeast killer protein where wells were optically clear (without any visible growth/turbidity) or 100% reduction in the growth which was determined by using an automatic microtitre plate reader at 492 nm (Spectramax 190, Molecular Devices, USA).

Three of the tested strains including *Alternaria citri, Colletotrichum gloeosporoides, Phythophthora citrophthora* were determined to be not susceptible to the K5 type yeast killer protein.For these isolates there was no difference between protein containing wells and growth control wells both visually and spectrophotometrically.

Other strains including *Penicillium digitatum, Penicillum italicum, Botrytis cinerea* were found to be susceptible to the toxin. After 48 hours of incubation at 25°C, first wells of the microplate corresponding to the protein concentration of 16 µg/ml were visually clear whereas growth control wells were highly clouded (Figure 3.9.,3.10.).

Figure 3.9. The Susceptibilities of *Penicillium sp.*to the K5 Type Yeast Killer Toxin on a Microtitre Plate.

The colours of the protein containing wells ranged between dark to light brown due to the colur of the protein.

Arrows indicate the MICs (visually clear wells).

Protein concentrations; a) 0.25 µg/ml b) 0.5 µg/ml c) 1 µg/ml d) 2 µg/ml e) 4 µg/ml f) 8 µg/ml g) 16 µg/ml

1) *Penicillium italicum* MIC: 16 µg/ml

2) *Penicilliumdigitatum* MIC: 16µg/ml

A) Sterility control B)Growth control.

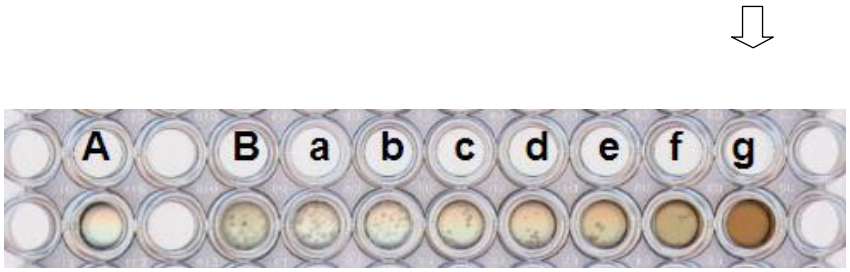


Figure 3.10. The Susceptibility of *Botrtis cinerea* to the K5 Type Yeast Killer Toxin on a Microtitre Plate.

Arrow indicates the MIC (visually clear well)value corresponding to 16µg/ml.

The colours of the protein containing wells ranged between dark to light brown due to the colur of the protein.

Protein concentrations; a) 0.25 µg/ml b) 0.5 µg/ml c) 1 µg/ml d) 2 µg/ml e) 4 µg/ml f) 8 µg/ml g) 16 µg/ml

A) Sterility control B) Growth control

In order to quantify the growth inhibition also a microtiter plate assay was set up to test the effect of the protein on the growth of fungi by measuring the increase in OD at 492 nm over time. Quantitative comparison of the OD_{492} values of the growth control wells with the K5-type killer protein containing wells allowed the calculation of protein concentration required to obtain 100% and 50% inhibition of fungal growth. By using percent inhibition as y values and the protein concentration as x values, linear regression analysis was performed to obtain the MIC and IC_{50} values for the strains *Penicilliumdigitatum, Penicilliumitalicum, Botrytis cinerea* as displayed in Figure 3.11.

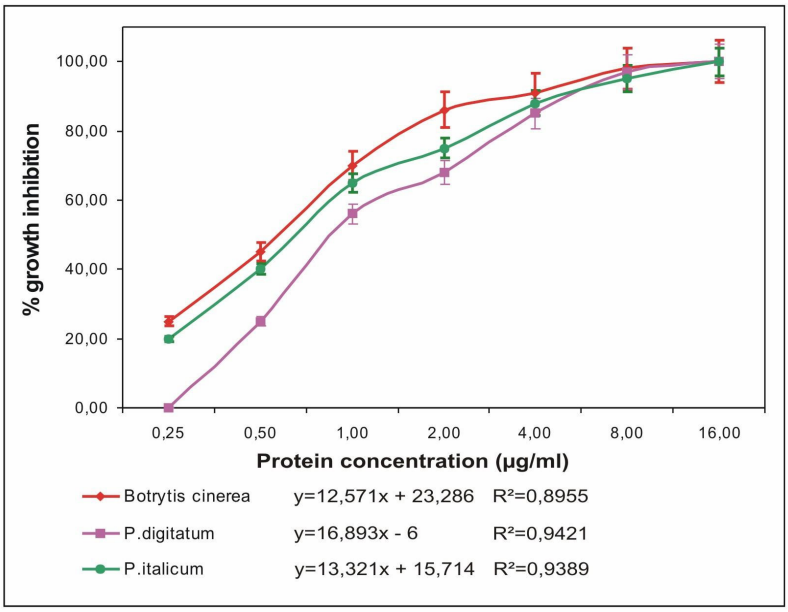


Figure 3.11. *In vitro* Growth Inhibitory Activity of the K5 Type Yeast Killer Protein on *Botrytis cinerea, Penicilliumdigitatum, Penicilliumitalicum*

Data are shown as the mean percentage ± SD of *in vitro* growth inhibition at each K5 type yeast killer protein concentration with respect to growth control wells after 48 hours of incubation.

The MIC and IC_{50} values were expressed as the mean of three replicates ± the standart deviation of three independent experiments and presented in Table 3.1.

Table 3.1. Susceptibility ($IC_{50,}$ MIC Values) of Tested Strains to the K5 Type Yeast Killer Protein

STRAIN	**STRAIN NUMBER\ SOURCE**	**IC_{50} µg/ml**	**MIC µg/ml**
Botrytis cinerea	DSMZ 5145	2,12	16
Penicillium digitatum	DSMZ 2776	3,31	16
Penicillium italicum	DSMZ 2756	2,57	16
Colletotrichum gloeosporoides	DSMZ 62146	*NI	› 16
Alternaria citri	DSMZ 62013	*NI	›16
Phythophthoracitrophthora	DSMZ 62669	*NI	›16

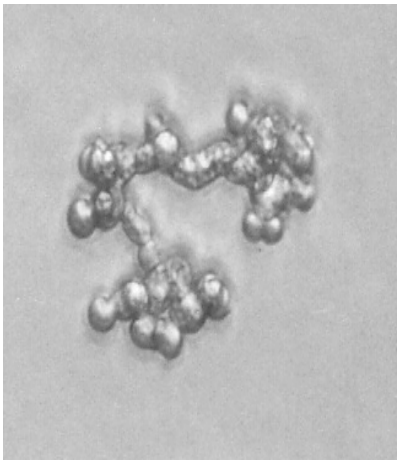
*NI : Not inhibitory

Fungal growth inhibition mediated by K5-type yeast killer protein was also analyzed microscopically. At different times microscopic observations of the wells containing different concentrations of K5-type yeast killer proteins were done and compared with the growth control wells. After 18 hours of incubation, efficient inhibition of spore germination was observed in the wells containing K5-type yeast killer protein at the concentration of 16µg/ml corresponding to MIC value.

Microscopic examinations of the spores incubated in the presence of the K5-type yeast killer protein at the concentrations corresponding to the values lower than the MIC value revealed significant reduction in germination,

abnormalities in the germination process and germ tube elongation of protein-treated spores. K5-type yeast killer protein treatment lead to slower germ tube elongation, hypher-branching of mycelium and morphological changes such as leakage of cytoplasm and cell swelling as shown in Figure 3.12., 3.13.,3.14.

A



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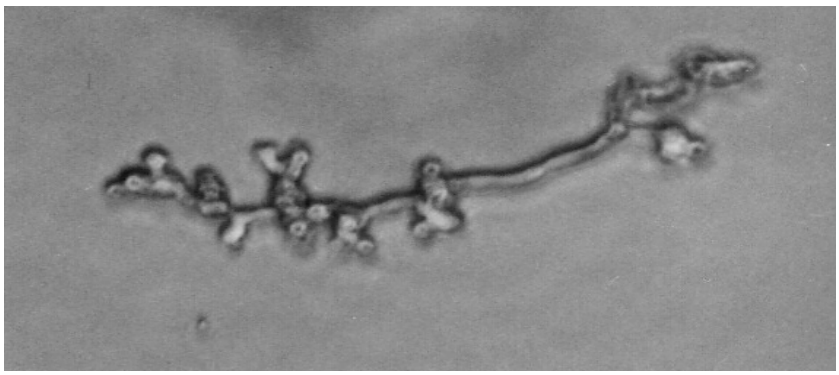

Figure 3.12.Reduction of *Botrytis cinerea* Germ Tube Elongation and Induction of Morphological Changes by K5-type Yeast Killer Protein.

K5-type yeast killer protein at the concentration of 4µg/ml lead to

A) reduction of germ tube elongation .

B) Spores not treated with protein had well extended germ tube.

Photographs were taken after 24 hours of incubation.

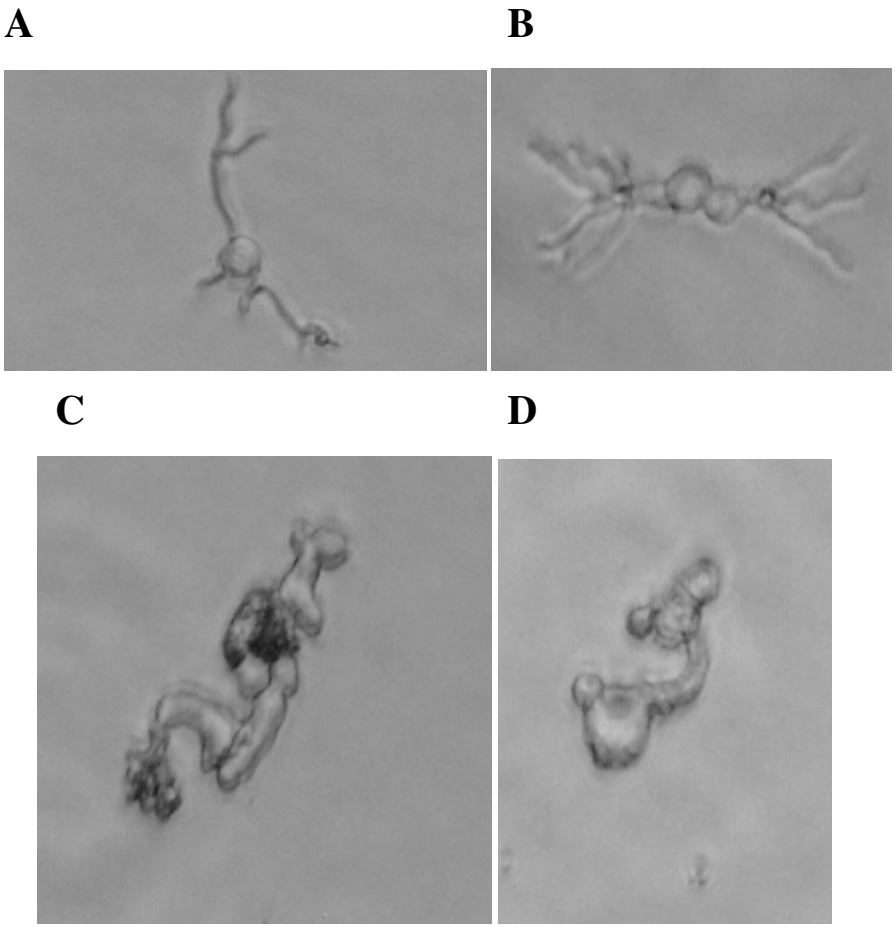

Figure 3.13.Growth Anomalies in Spores of *Penicilliumitalicum* when Incubated in the Presence of K5 Type Yeast Killer Protein.

A-B) In the absence of the K5-type yeast killer protein spores germinated normally and had thin and long germ tube.
K5-type yeast killer protein at the concentration of 4 µg/ml C) inhibited germ tube elongation and caused D) cell swelling.
Photographs were taken after 24 hours of incubation.

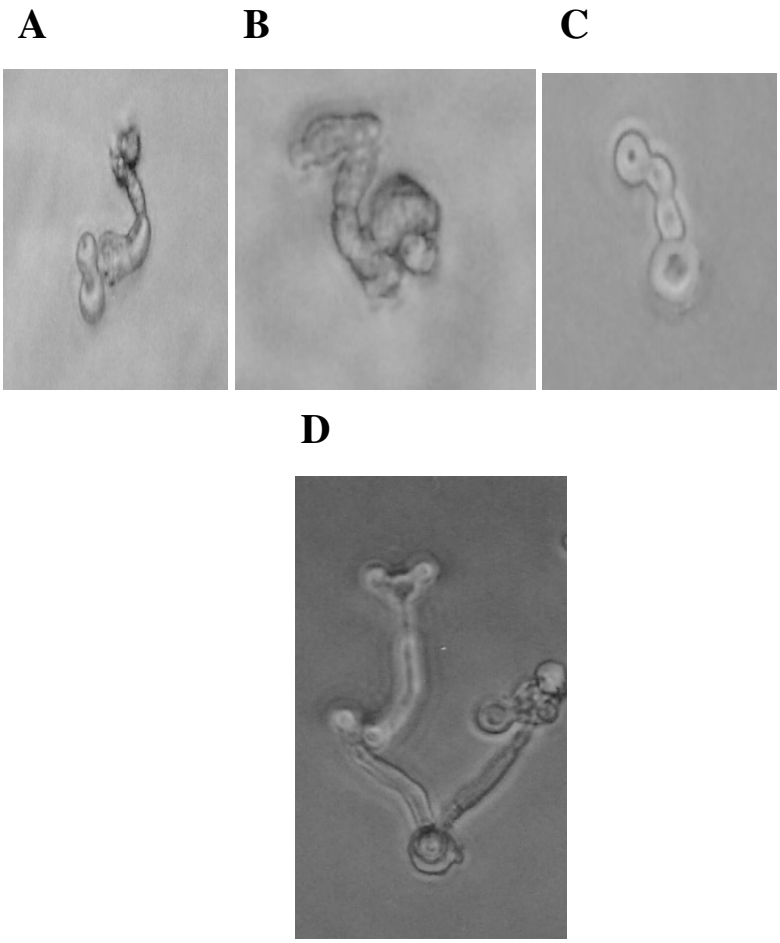

Figure 3.14.Effect of K5-type Yeast Killer Protein on Germ Tube Elongation from Germinated Conidia of *P.digitatum*.

A-C) In the presence of the K5 type yeast killer protein at the concentration of 4µg /ml, few spores with short, swollen germ tubes were observed.

D) In the absence of the K5-type yeast killer protein, many spores with well-extended, long germ tube were observed.

Photographs were taken after 24 hours of incubation.

Short hyphae were frequently observed in the wells containing K5-type yeast killer protein at the concentrations below the MIC. If longer, the hyphae showed a marked alteration in their morphology, with constricted regions either in the apical or central regions (Figure3.15.).

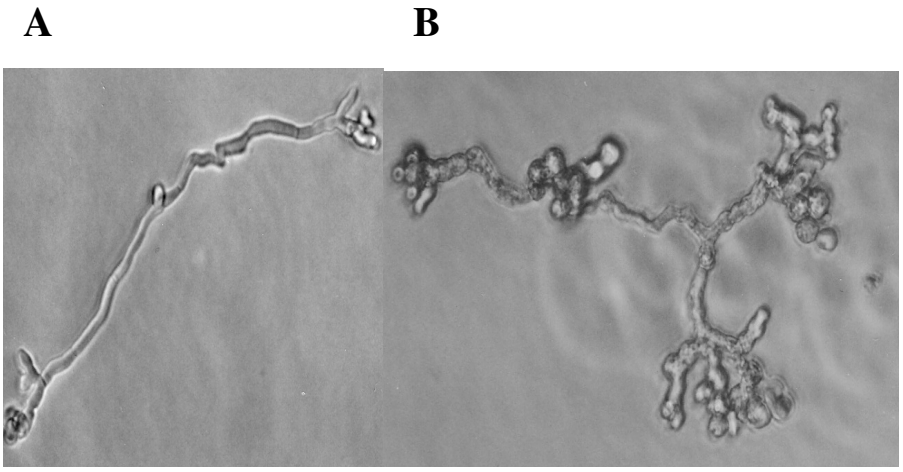

Figure3.15.Effect of K5-type Yeast Killer Protein on Hyphal Growth of *Botrytis cinerea*.

A) In the absence of K5-type yeast killer protein,well extended, long hyphae with smooth surface were observed.

B) In the presence of K5 type yeast killer protein (at the concentration of *4* µg/ ml) highly branched hypha with constricted regions and rough surface were observed.

Photographs were taken after 36 hours of incubation.

Examination of the growth of *Botrytis cinerea, Penicillium digitatum, Penicillium italicum* in the presence of K5-type yeast killer protein at the concentrations that partially inhibit growth revealed hyperbranching of germinated spores, compared with thin, elongated, well-extended mycelial growth in controls. The hypha grown for approximately 3 days exhibited numerous, condensed, shorter thicker cells giving them a bead-like appearance Figure 3.16, 3.17,3.18.

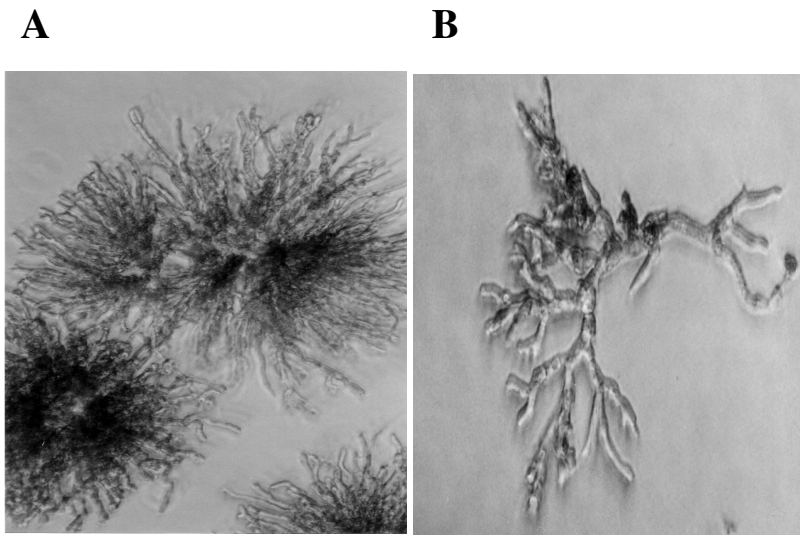

Figure 3.16.Effect of K5-type Yeast Killer Protein on Mycelial Growth of *Botrytis cinerea.*

A) Thin, elongated hyphae forming the well-extended mycelium were observed in the absence of K5-type yeast killer protein

.B)K 5-type yeast killer protein at the concentration of 4 µg /ml inhibited mycelial growth and caused highly branched, shorter, thicker hyphae.

Photographs were taken after 48 hours of incubation.

A **B**

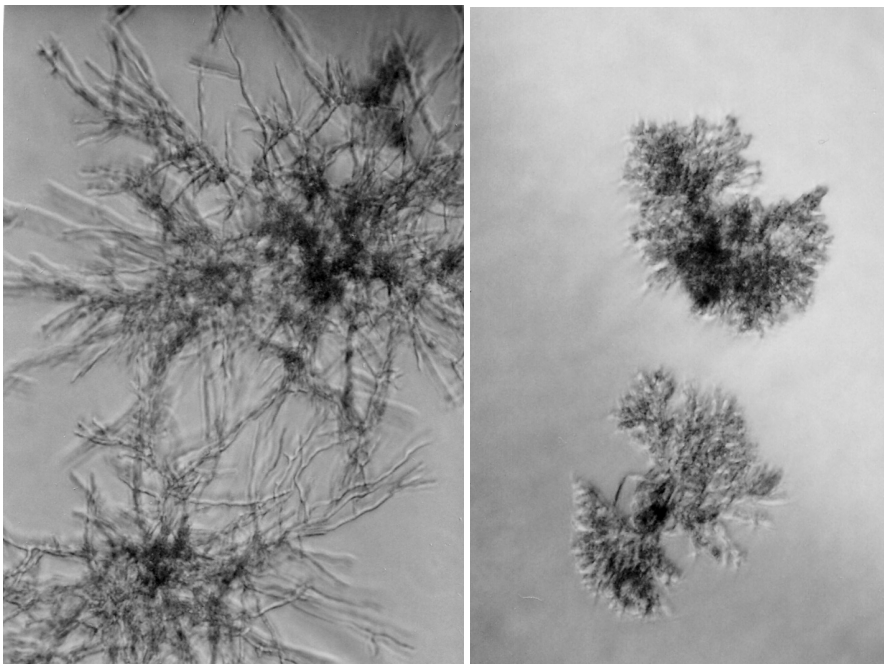

Figure 3.17. Effect of K5-type Yeast Killer Protein on Mycelial Growth of *P.italicum.*

A)Long, thin hyphae forming the well extended mycelial growth in the absence of K5-type yeast killer protein

B) K5-type yeast killer protein at the concentration of 4µg/ml caused to shorter,thicker hyphae and reduction in mycelial growth.

Photographs were taken after 48 hours of incubation.

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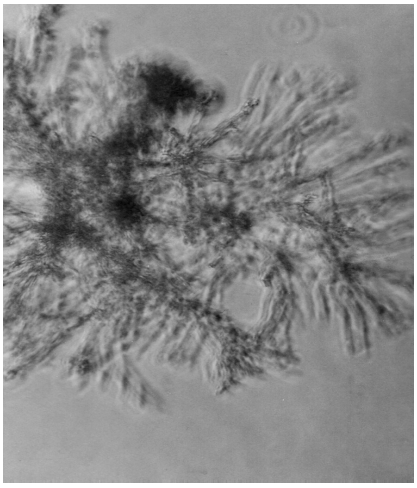
A



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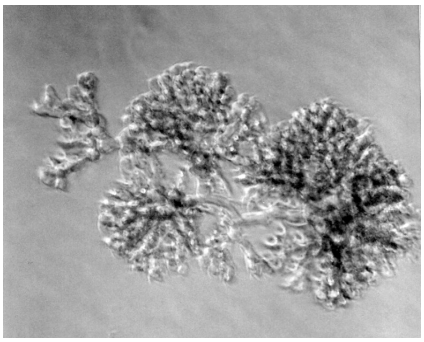

Figure 3.18. Effect of K5-type yeast killer protein on mycelial growth of *P.digitatum.*

A) Well extended mycelial growth was observed in the absence of K5 type yeast killer protein

B) In the presence of K5 type yeast killer protein at the concentration of 4µg/ ml, hypha exhibited numerous, condensed, shorter, thicker cells giving them a bead-like appearance.

Photographs were taken after 48 hours of incubation.

3.6.2.*In vivo* Antifungal Susceptibility Assays

Antifungal activitiy of the K5 type yeast killer protein against *Botrytis cinerea, Penicillium digitatum, Penicillium italicum* was tested on lemon fruits. For each fungal strain mentioned above, fruit inoculations were conducted to determine whether the observed *in vitro* antifungal activity of K5-type yeast killer protein correlated with an inhibition of fruit rot caused by fungal infections.

Lemon fruits inoculated with the spore suspensions (at the concentration of 10^4 spores/ml for *Penicllium sp.* and 10^5 spores/ml for *Botrytis cinerea*) mixed with equal amounts of 100 mM Na_2HPO_4-citric acid buffer served as growth control group. Lemon fruits inoculated with the same spore suspensions mixed with K5- type yeast killer protein (at the concentration of 32 µg/ml) were used to evaluate the potency of K5-type yeast killer protein in inhibiting diseasae progression.

Tissue maceration appeared in all the fruits at the end of second day after inoculation. By the end of fourth day, all wounds on fruits in growth control group were infected whereas no disease symptom was observed on fruits inoculated with spore suspensions mixed with K5-type yeast killer protein (Figure 3.19., 3.20., 3.21.).

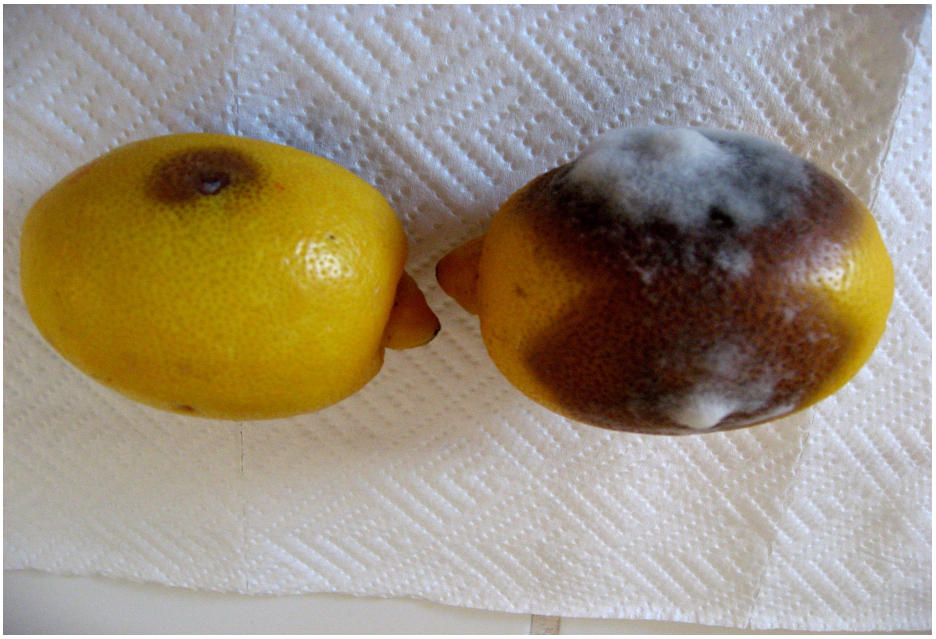

Figure 3.19. Antifungal Activity of the K5 -type Yeast Killer Protein against *Botrytis cinerea* on Lemon Fruits.

Fruit on the left inoculated with the spore suspension mixed with equal amounts of K5- type yeast killer protein (at the concentration of 32 µg /ml) had no sign of infection.

Fruit on the right, inoculated with the spore suspension mixed with equal amounts of 100 mM Na_2HPO_4-citric acid buffer had disease symptomps including large size, well-sporulated lesions.

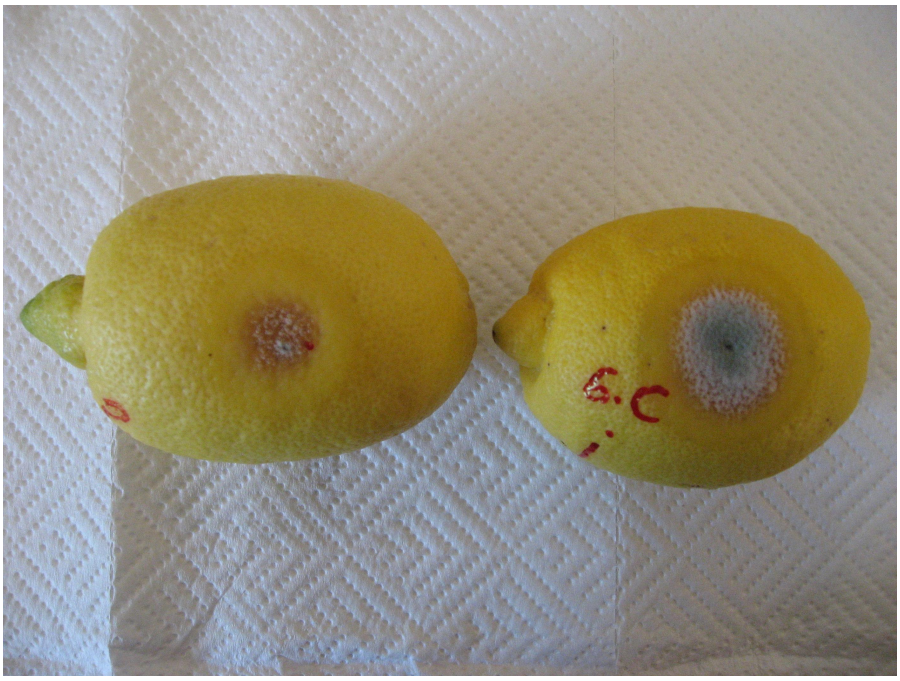

Figure 3.20. Antifungal Activity of the K5-type Yeast Killer Protein Against *P.italicum* on Lemon Fruits.

K5- type yeast killer protein (at the concentration of 32 µg /ml) inoculated with equal amounts of spore suspension inhibited fungal growth and sporulation on the fruit (on the left).

Fruit on the right inoculated with the spore suspension mixed with equal amounts of 100 mM Na_2HPO_4-citric acid buffer had disease symptomps with large size lesions.

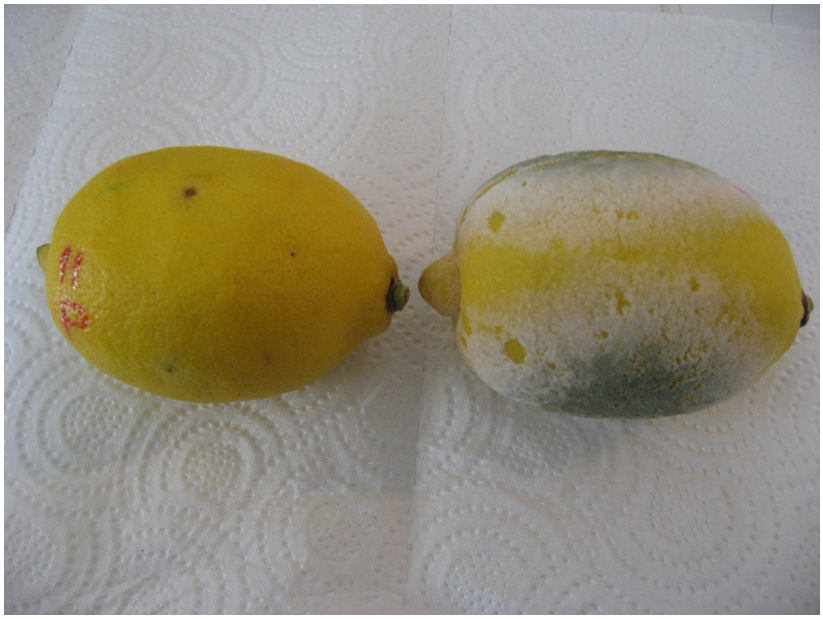

Figure 3.21. Antifungal Activity of the K5-type Yeast Killer Protein Against *P.digitatum* on Lemon Fruits.

K5- type yeast killer protein (at the concentration of 32 µg /ml) inoculated with equal amounts of spore suspension inhibited fungal growth and sporulation on the fruit (on the left).

Fruit on the right inoculated with the spore suspension mixed with equal amounts of 100 mM Na_2HPO_4-citric acid buffer was infected.

CHAPTER IV

DISCUSSION

The battle against postharvest decays of fruits and vegetables has been fought for decades but has not yet been won. About 70 % of the major postharvest diseases are caused by fungi with an economic cost of billions of dollars a year. Application of the synthethic fungicides is the usual practice in the fight against plant pathogens. However postharvest use of fungicides has been increasingly curtailed by the development of pathogen resistance to many key fungicides, lack of replacement fungicides and public perception that fungicides are harmful to human health and the environment. This negative perception has promoted governmental policies restricting use of fungicides [20]. Thus, alternative methods to control postharvest diseases are urgently needed.

Currently, several promising biological control approaches that include antagonistic microorganisms, compounds of natural origin have been shown to be potential alternatives to synthetic fungicides for postharvest disease control. Among the proposed alternatives, biological control through antagonistic microorganisms has been the most studied one [12].

Although the various antagonistic microorganisms have been shown to reduce postharvest diseases, each alternative comes with limitations that can affect its commercial potential. Most antagonistic microorganisms provide only a protectant affect that diminishes with ripening and has no curative activity.

Antagonist are most effective when applied prior to pathogen inoculation, a prerequisite that is diffucult to meet under commercial conditions. More importantly, under commercial conditions none of the biological control approaches has been shown to offer consistent disease control comparable to that obtained with synthethic fungicides. All of these drawbacks in biological control by means of antagonistic microorganisms has generated great interest on natural antifungal compounds.

Recently, considerable attention has been placed on yeast killer proteins as potential antifungal compounds that could substitute for the current use of synthethic fungicides. Among the killer yeasts *Pichia anomala* has been widely studied. There are several reports in the literature indicating wide range intergeneric killing spectrum of *Pichia anomala*. Therefore, *Pichia anomala* has been proposed as biocontrol yeast due to its strong antagonistic activity against a wide range of plant pathogenic fungi. In several studies, *P.anomala* toxins that play major factor in antagonistic activity have been shown to have a wide-range intergeneric killing spectrum with relatively high stability in comparison to toxins of other killer yeasts [66 , 127 , 128].

K5-type yeast killer toxin secreted by *P. anomala* NCYC 434 was suggested as a potential antifungal agent [144-148]. High pH and thermo stability which are appropriate for both medical and biocontrol purposes, broad spectrum of antimicrobial activity of the K5 type yeast killer toxin, suggest its possible applications as an antimicrobial agent in biocontrol, and medicine [104].

Exo-β-1,3-glucanase activity of the K5 type yeast killer protein on sensitive cells highlighted the potential use of this protein as a highly selective antifungal agent since its hydrolyzing activity is specific to the fungal cell wall and does not affect the mamalian cells.

The fungal cell wall protects the organism against a hostile environment and relays signals for invasion and infection of hosts. Fungi have a significant

internal turgor pressure so that even slight perturbation of the cell wall can result in cell lysis [99].

To date, the growth inhibitory effect of the killer toxin was tested either by spotting *P.anomala* NCYC 434 cell suspensions or the crude toxin preparations onto the plates seeded with the test strains [63-67]. Purification and characterization of the K5-type yeast killer protein performed in our laboratory [104] made it possible to determine the actual killing spectrum and cell killing patterns of this toxin on test strains. Recently, we have determined antifungal spectrum of the pure K5 type yeast killer protein on human pathogenic fungal species including *Candida* species, dermathophytes and analysed its kinetics of cell killing [132].

K5 type yeast killer toxin was found to be affective against all of the tested 26 strains of the genus *Candida* within the MIC_0 (Minimum Inhibition Concentration required for 50% growth inhibition), MIC_2 (Minimum Inhibition Concentration required for 100% growth inhibition) and MFC (Minumum Fungicidal Concentration) ranges of 0.25-4, 0.5-8, 1-16 µg/ml respectively .

K5 type yeast killer toxin was found to be effective against all of the tested 9 strains of human pathogenic dermathophytes within the MIC_0 and MIC_2 ranges of 0.25-2, 1-8 µg/ml respectively [133].

Wide antifungal spectrum and selectivity of the toxin due to its strong exo-β-1,3-glucanase activity highlights the possible usage of the K5 type yeast killer protein as a novel antifungal agent in medicine against human pathogenic fungi.

In this study we have determined antifungal spectrum of the pure K5-type yeast killer protein on fungi causing spoilage in citrus fruits. Among the tested strains, *B.cinerea, P.digitatum,P.italicum* were found to be susceptible to the K5-type yeast killer protein whereas other tested strains including *Colletotrichum*

gloeosporoides, Alternaria citri, Phythophthora citrophthora found to be not susceptible. Minimum inhibition concentrations (MICs) of the K5-type yeast killer protein against the growth of *B.cinerea, P.digitatum,P.italicum* were 16 µg/ml. MIC values of the K5-type yeast killer protein for *Colletotrichum gloeosporoides,Alternaria citri,Phythophthora citrophthora* were higher than 16 µg/ml.

K5-type yeast killer protein exerts its lethal affect by hydrolyzing β-1,3-glucan residues of the cell wall of sensitive fungal cells and causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure. Thus, susceptibility of the tested strains to the K5-type yeast killer protein varied due to different cell wall compositions and different polysaccharide content of the cell wall. Distinct structures present in the spores of the tested fungi and the different content of the cell wall might allow the survival of these fungi from hydrolytic activity of the K5-type yeast killer protein.

Conidia of *C.gloeosporoides* posses a fibrillar 'spore coat' as well as a cell wall. The spore coat was identified to be composed of densely packed fibres arranged perpendicular to cell wall by transmission electron microscopy[149]. The conidia are produced in a acervuli embedded in a water-soluble mucilage composed of high molecular mass glycoproteins. This structure is suggested to function in protection conidia from toxic plant metabolites[150]. Also, it might serve to protect the conidia from hydrolytic effect of K5-type yeast killer protein.

Other strain found to be not susceptible to the K5-type yeast killer protein is *Alternaria citri.* The spores of *Alternaria citri* are multi-celled and pigmented and they are produced in chains or branching chains. The spores have a distinctive appearance that makes them easy to recognize. They are tapered towards the apex, rough-walled and have septate in two directions, both transverse and longtitudinal [151]. This complex structure might prevent K5-type yeast killer protein from damaging the cell wall of the conidia. (Figure 4.1).

Figure 4.1. Rough-walled, multicelled, large conidia of *Alternaria citri* [151]

Acording to the results obtained in this study, K5-type yeast killer protein was not effective on the *Phythophthora citrophthora*. This may be originate from cell wall composition of the strain, since the cell wall components was shown to act as the primary binding site for yeast killer proteins. The cell wall of *P. citrophthora* is mainly composed of cellulose [152]. Cellulose monomers (β-glucose) are linked together through β-1,4-glycosidic bond. Thus, K5-type yeast killer protein could not exert exo-β-1,3-glucanase activity that is responsible for its fungicidal effect.

Another significant sense of these results is the confirmation of the fact that K5-type yeast killer protein exerts its lethal effect on sensitive microbial cells through its strong exo-β-1,3-glucanase activity.

IC_{50} values of K5-type yeast killer protein for *B.cinerea, P.digitatum, P.italicum* were 2.12, 3.31, 2.57 respectively. K5-type yeast killer protein completely inhibited spore germination of these strains at the MIC value corresponding to 16 µg/ml.

K5 type yeast killer protein partially inhibited the growth of fungi at the concentrations below the MIC value. Microscopic observations revealed morphological anormalities in germ tube elongation and hyphal growth of the fungi treated with K5-type yeast killer protein at the concentrations below the MIC value. K5-type yeast killer protein disturbed the rigidity of the hypha and caused leakage of cytoplasm.

This destructive mode of action of the K5-type yeast killer protein on hypha is crucial in terms of plant pathogenesis. For successful development of the disease, a crucial step is the penetration of pathogenic fungi into plant cell wall. For this purpose, rigid, undamaged, modified hypha serve as infection structure. Also, turgor pressure constitutes essential component of fungal penetration ability of the hypha[153]. Thus, loss of rigidity and turgor pressure of the hypha caused by K5-type yeast killer protein leads to abnormally growing hyphae that are unable to cause infections.

One of the most noteworthy aspects of this study is that the concentrations of K5-type yeast killer protein required for complete inhibition of infection on fruits are equal to the MIC values determined in *in vitro*. Using an infection assays on fruits, we have demonstrated that K5-type yeast killer protein at the concentrations corresponding to *in vitro* MIC value prevented fruit decay caused by the inoculated fungal strain. The results of the *in vivo* susceptibility assays also indicated that K5-type yeast killer protein remained biologically active in the lemon fruit. So we have shown that K5-type yeast killer protein has growth inhibitory effect on *B.cinerea, P.digitatum* and *P.italicum* not only in vitro but also in vivo by the absence of disease symptoms on citrus fruits.

The fungus *Botrytis cinerea* has a wide host range (over 200 plant species) and infects fruits (both in the field and after harvest), flowers and vegetative tissues. It is an economically important pathogen of a wide range of economic plants. Several groups of fungicides are used to fight *B.cinerea* and resistance to some of them have appeared in natural populations [154]. On the other hand, the two major postharvest diseases of citrus are green and blue moulds, caused by *Penicillium digitatum* and *Penicillium italicum* respectively. These wound-obligate pathogens have a relatively short disease cycle and can produce 1-2 billion conidia that are efficiently dispersed via air currents on a single fruit [155].

In packinghouses, citrus fruits are treated with imazalil and thiabendazole to control *Penicillium* decay. These fungicides are used ina manner that is highly conducive to the selection and proliferation of resistant biotypes of *Penicillium digitatum* and *Penicillium italicum.* The fruit surface is completely covered by the fungicides and the residue is persistent for the life of the fruit. Many packinghouses process fruit the year around, resulting in continous selection pressure on the pathogen population. The common practice of repacking after storage to remove diseased fruit results in efficient aerial dispersal of conidia of fungicide-resistant biotypes to recently harvested fruit being processed in the same packinghouse[156].

o- Phenylphenol and thiabendazole have been used routinely on citrus fruits over the past 3 decades, resulting in a serious problem of resistance to these two fungicides by the late 1970s [157]. Imazalil was enthusiastically adopted instead of thiabendazole which was largely ineffective due to the large population of thiabendazole-resistant *Penicillium spp.* Imazalil resistant biotypes of *P.digitatum* were reported in 1987, just 5 years after the introduction of imazalil as a commercial treatment in packinghouses. The wide distribution of biotypes of *P.digitatum* and *P.italicum* resistant to thiabendazole, imazalil and o-phenylphenol has been recently documented [157,158].

Microorganisms gain resistance to conventional antifungals especially in long term usages and these resistances spread rapidly among other microorganisms and cause problems in the fight against pathogenic fungi.

Therefore, there is a critical need for new antifungal agents which are fungicidal, have a broad spectrum of activity and pose no risk to human health and environment.This study demonstrates that K5-type yeast killler protein might be a potent alternative to synthethic fungicides used against *B.cinerea, P.digitatum* and *P.italicum* , as evidenced by the *in vitro* and *in vivo* assays indicating the growth inhibitory effect of K5-type yeast killler protein on these fungi. Considering that benzimidazole and dicarboximide resistant strain of *B.cinerea* tested in this study was susceptible to K5-type yeast killer protein, possible use of this protein against resistant strains of *B.cinerea* comes into prominence.

Development studies on novel antifungal drugs are focused on selective agents which target the components of yeast and fungal cell walls. These agents would not harm the host cell because mammalian cells do not have these components [67,104]. K5 type yeast killer toxin exhibits its cytotoxic effect by hydrolyzing β-1,3- glucans of the cell wall of sensitive fungal cells and causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure. [104]. β 1,3 glucan hydrolyzing activity of the K5 type killer protein on sensitive cells highlighted the potential use of this protein as a highly selective antifungal agent.

Activity of the yeast killer proteins are depended on pH and temperature of the environment which limits their use in relevant fields. K5 type yeast killer toxin has a wide range pH (2.5-5.5) and temperature (4-37 ^{o}C) stability and looses only 50% of its activity at 100 ^{o}C [104]. These characteristics along with its high affinity to β-1,3- glucans are enormously increasing the application area of this protein and especially highlight the use of this toxin as a novel antifungal agent.

The use of the K5 type yeast killer protein as a biocontrol agent against *B.cinerea, P.digitatum* and *P.italicum* will be feasible with appropriate formulation studies upon the antifungal activity determination of the toxin in this study.

Future researches should be focused on the development of a suitable formulation which enhances the stability and efficacy of the K5-type yeast killer protein for long term storage, determination of the optimum application techniques, investigations on the timing of applications with regard to storage conditions, monitoring the effectiveness of the K5-type yeast killer protein on different crops and in different storage conditions.

CHAPTER V

CONCLUSION

1. K5 type yeast killer toxin was found to be effective against *B.cinerea, P.digitatum* and *P.italicum* that cause postharvest diseases in a variety of fruits. MIC of the K5 type yeast killer toxin for these fungi was 16 µg/ml. *Colletotrichumgloeosporoides, Alternariacitri, Phythophthoracitrophthora* were found to be not susceptible to K5 type yeast killer protein due to their different cell wall compositions protecting them from exo-β-1,3-glucanase activity of the K5-type yeast killer protein.

2. Inhibitory Concentrations of the K5 type yeast killer protein required for 50% growth inhibition (IC_{50}) were 2.12, 3.31, 2.57 µg/ml for *B.cinerea, P.digitatum* and *P.italicum* respectively.

3. The microscopic observations of the wells containing K5-type yeast killer protein at the concentration of 16µg/ml (corresponding to MIC value) showed that K5-type yeast killer protein efficiently inhibited spore germination with respect to growth control wells which were protein free. Microscopic examinations of the spores incubated in the presence of the K5-type yeast killer protein at the concentrations corresponding to the values lower than the MIC value revealed significant reduction in germination and abnormalities in the germination process and germ tube elongation of protein-treated spores. K5-type yeast killer protein treatment lead to short and thick germ tubes, cell swelling,

slower germ tube elongation, hypher-branching of mycelium and damage in hyphae that results in loss of rigidity and leakage of cytoplasm.

4. Results obtained from *in vivo* antifungal susceptibility testings showed that K5 type killer toxin also effective against *B.cinerea, P.digitatum* and *P.italicum* also *in vivo* as compatible with *in vitro* antifungal susceptibility results. Concentrations of K5-type yeast killer protein required for complete inhibition of infection on fruits are equal to the MIC values determined in *in vitro* antifungal susceptibility testings..

4. Wide antifungal spectrum and selectivity of the toxin due to its strong exo-β-1,3-glucanase activity, its high stability in wide range pH (2.5-5.5) and temperature (4-37 ^{o}C) highlights the possible use of the K5 type yeast killer protein against pathogenic fungi as a novel antifungal.

5. K5 type yeast killer protein can be used against *B.cinerea, P.digitatum* and *P.italicum* as biocontrol agent with appropriate formulations.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Acetic Acid (Merck, Germany)

Aceton (Merck, Germany)

Acrylamide (Boehringer Mannheim, Germany)

Ammoniumpersulphate (Pharmacia Biotech, Sweden)

Bacto-agar (Difco, USA)

Bacto-peptone (Difco, USA)

Bis-acrylamide (Boehringer-Mannheim, Germany)

Bovine Serum Albumine Fraction V (Boehringer-Mannheim, Germany)

Bromophenol Blue (Sigma, USA)

Butanol (Merck, Germany)

β-mercaptoethanol (Sigma, USA)

Citric Acid (Merck, Germany)

Coomassie Brilliant Blue R-250 (ICN, USA)

Coomassie Brilliant Blue G-250 (ICN, USA)

Dithiothreitol (DTT) (Boehringer Mannheim, Germany)

D-Glucose (Merck, Germany)

Di-sodium Hydrogen Phosphate (Merck, Germany)

Di-potassium Hydrogen Phosphate (Merck, Germany)

Ethanol (Merck, Germany)

Formaldehyde (Riedel-de Haen, Germany)

Glutaraldehyde (Fluka, Switzerland)

Glycerol (Merck, Germany)

Hydrochloric Acid (Merck, Germany)

Malt extract (Difco, USA)

Methanol (Merck, Germany)

PDA (Merck, Germany)

Phosphoric acid (Merck, Germany)

Potassium Dihydrogen Phosphate (Merck, Germany)

RPMI 1640 (Sigma, USA)

Silver Nitrate (Merck, Germany)

Sodium Carbonate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium Dodecyl Sulfate (Merck, Germany)

Sodium Hydroxide (Merck, Germany)

Sodium Sulfate (Merck, Germany)

TEMED (Pharmacia Biotech, Sweden)

Tween 80 (Merck, Germany)

Trichloroacetic Acid (Merck, Germany)

Tris (Merck, Germany)

Yeast extract (Difco, USA)

APPENDIX B

BUFFERS AND SOLUTIONS

Table A.1.Buffers and Solutions

Buffers / Solutions	Composition
1.SDS-PAGE Monomer Solution	30.8% T , 2.7% C_{bis}
4X Running Gel Buffer	1.5 M Tris-Cl , pH 8.8
4X Stacking Gel Buffer	0.5 M Tris-Cl , pH 6.8
SDS	10%
Initiator	10% Ammonium Persulfate
2X Treatment Buffer	0.125 M Tris-Cl , 4% SDS , 20% Glycerol , 10% β-mercaptoethanol , 0.020% Bromophenol blue , pH 6.8
Tank Buffer	0.025 M Tris , 0.192 M Glycine , 0.1% SDS , pH 8.3.
2.SILVER STAIN Destain Solution I	40% Methanol , 7% Acetic Acid
Destain Solution II	5% Methanol , 7% Acetic Acid
Cross-linking Solution	10% Glutaraldehyde
(DTT) Solution	5 µg/ml
Silver Nitrate Solution	0.1% w/v
Sodium Carbonate	3% w/v
Developing Solution	3% sodium carbonate, 0.019% formaldehyde

Table A.2. Components of RPMI 1640 Medium

RPMI 1640 COMPONENTS	**Conc. (mg/L)**
INORGANIC SALTS:	
Calcium nitrate (Ca(NO3)2 4H2O)	100.00
Potassium chloride (KCl)	400.00
Magnesium sulfate (MgSO4)	48.84
Magnesium sulfate (MgSO4 7H20)	100.00
Sodium chloride (NaCl)	6000.00
Sodium Phosphate (Na2HPO4)	800.00
OTHER COMPONENTS:	
Glucose	2000.00
Glutathione Reduced	1.00
Phenol red	5.00
AMINO ACIDS:	
L-Arginine	200.00
L-Asparagine	50.00
L-Aspartic Acid	20.00
L-Cystine dihydrochloride	65.00
L-Glutamic Acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine hydrochloride	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine disodium, dihydrate	29.00
L-Valine	20.00
VITAMINS:	
Biotin	0.2
D-Ca Pantothenate	0.25
Choline Chloride	3.00
Folic Acid	1.00
i-Inositol	35.00
Niacinamide	1.00
p-Aminobenzoic Acid (PABA)	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20