PRIMARY METABOLITES OF CANDIDA TROPICALIS

THESIS SUBMITTED FOR
THE FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY

by

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Lastly, I would like to thank my parents whose constant encouragement enabled me to carry out this research work.

Atiya Abbasi
This is the first study on the characterisation of proteins and peptides in the cell free broth of *Candida tropicalis*. We are reporting the presence of a highly specific proteolytic enzyme cleaving at valine. This has been confirmed by digesting β-lactoglobulin and a number of synthetic peptides. The enzyme has a carbohydrate moiety and a molecular weight of 40,000 ± 7,000 as determined on SDS-polyacrylamide gel electrophoresis. Its optimum activity occurs at 37°C at a pH between 8-9. We have named it Valinease because of its selective cleavage.

The peptides separated on Dowex 50 have been tested against a number of pathogenic and non-pathogenic bacteria. At least four of the peptides have been found to be active against most of the bacterial strains. The amino acid composition and N-terminal residues of these peptides have been determined.
1 INTRODUCTION

1.1 GENERAL BACKGROUND

_Candida tropicalis_ belongs to the form family _Cryptococcaceae_. This family includes a number of false yeasts from the form genera of _Cryptococcus_, _Torulopsis_, and _Brettanomyces_ as well as yeast like fungi from the form genera of _Candida_ and _Trichosporon_. With other members of its genus _C. tropicalis_ produces Candidiasis, a well-recognised infection of the skin and mucous membranes and other parts of the body, in both man and animals.

Genus _Candida_ has been the subject to numerous studies to determine its chemical composition and biological activity. Some members have a high protein and vitamin content and have consequently been used as food\(^1\)\(^-\)\(^10\) especially by Germans during the World Wars.\(^11\) They have also been used, in the preparation of animal feeds.\(^12\)\(^-\)\(^15\) Enzymes isolated from various species have been utilised for different industrial processes, including conversion of sucrose to fructose.

The result of several experimental studies on mice are of great medical interest. Different polysaccharide fractions from various members of the genus _Candida_
have shown anti-cancerous activity against sarcoma-180, antiviral activity against tobacco virus and potato virus and induced interferon production effective against viral infection. Anticancerous activity has also been observed in a ribo-furanoside fraction, which incidentally is also effective in the treatment of gout.

Further work on these aspects is being done to exploit their use in the treatment of disease.

Many other metabolites of Candida have been found useful for agriculture and industry. A gibberlin-like substance synthesised by C. pulcherrima has been shown to increase growth rate and weight of pea when added to the soil. D-mannitol, $^{16}$ from various species of Candida, is used as additive in food and pharmaceuticals, and in the manufacture of synthetic resins, plasticizers and electrolytic dry condensers. Some lipids are used as anti-corrosives and anti-oxidants, $^{17}$ while others are effective as emulsifiers $^{18}$ for pesticides.
1.2 ENZYMES

In Candida, different families of enzymes have been isolated. Some of these such as the dehydrogenases, oxidases, decarboxylases, acid and alkaline proteases, isomerases and transferases are common to all. Others are fairly specific to particular species e.g. coagulase and plasmocoagulase in C. albicans.

1.2.1 COAGULASE AND PLASMOCOAGULASE

Elinov and Zaikina\(^{19}\) (1965) reported the isolation of Coagulase and Plasmocoagulase from C. albicans. Both these enzymes are glycoproteins. The chemical composition of Coagulase reported by Zaikina\(^{20}\) in 1967 comprised a carbohydrate moiety containing glucose (85%), mannose (13%) and xylose (2%) and a protein moiety having mainly Asp, Gly, Lou, Phe, Val, Ala, Thr, Glu, Arg, Pro, Cys and yCys. However, later studies by the same workers\(^{21}\) showed a slightly different composition viz. glucose (81%), mannose (16%) and a lack of xylose, Gly, Asp, and cysteine.

Both Coagulase and Plasmocoagulase cause splitting of prothrombin into thrombin or similar substances.\(^{22}\) Plasmocoagulase and the polysaccharide\(^{23}\) from C. albicans
have also been shown invitro to inhibit phagocytosis
and to suppress the seizure of microbial cells by leukocytes and the digestion of phagocytosed microbes. In vivo experiments with simultaneous injection of the bacteria showed only a partial inhibition of phagocytosis.

The polysaccharide part of Plasmocoagulase protects the active site of the enzyme from the action of inhibitors. This has a substrate specificity similar to leucine aminopeptidase from pig kidney. 24

1.2.2 ASPARAGINASE

Asparaginase has been obtained from different species. Sakamoto25 observed anti-tumour activity in the asparaginase from C. utilis. This anti-tumour activity was lower in the asparaginase from C. utilis as compared to that from E. coli. This might be due to the faster clearance of the enzyme from the blood.

1.2.3 CANDITOXIN

Iwata and Uchida26 reported the isolation of a toxic substance from C. albicans in 1967. They thought it was an acidic protein of high molecular weight. Later reports27 showed it to have a weak alkaline phosphatase
activity which was activated 40-fold by heat treatment at 55°C for 30 minutes. Its toxicity was completely destroyed by this treatment.

1.2.4 KININASES

Gerald and Ulrich²⁸ (1975) observed time-dependent inactivation of bradykinin by intact, washed cells, cell homogenized blastopore suspension from C. albicans and C. tropicalis. 1:10 dilution of the preparation was however found to be inactive. Enhanced kinin inhibition was observed on cell homogenization. Treatment with 1,10 phenanthroline, heat or acid inactivated the enzyme.

1.2.5 FRUCTOFURANOSIDASE

Negoro²⁹ (1973) reported the isolation of β-fructofuranosidase from C. kefyr. The enzyme possessed inulin and sucrose hydrolysing ability. It was found to be more potent in hydrolysing inulin as compared to the one obtained from C. utilis or S. cerevisiae.

1.2.6 MILK COAGULATING ENZYME

Yoshino³⁰ et al., observed milk coagulating activity in a proteolytic enzyme isolated from culture
broth of *C. lipolytica*. A similar effect was produced by a protease from *C. muscorum*.31

Ovacharov and co-workers32 in 1974 reported the isolation of a chymotrypsin-like protease from *C. guilliermondii*.

Most species of *Candida* have shown some proteolytic activity. However, there have been some contradictory reports regarding the presence of proteases in *C. albicans* and *C. tropicalis*.33,34
1.3 PROTEINS

A number of proteins and glycoproteins have been isolated from the genus *Candida*. Much work has been done on their chemical composition, biological activity and toxicity. Mankowski\(^35\) analysed the glycoprotein from *C. albicans* and found that it contained glucose and mannose in the ratio of 3:1 as the polysaccharide component and at least 15 amino acids with 0.5% glucosamine as the protein moiety. This glycoprotein was found to inhibit growth in Swiss mice and produced toxic effects in the new born rodents; it also stimulated the course of 3-methyl cholangthrone induced carcinogenesis and thus acted as a cocarcinogen.\(^36\)

Nosal\(^37\) (1974) isolated a water soluble glycoprotein from *C. albicans* which induced histamine release in mast cells from male rats with an almost linear dosage response curve. This effect was temperature-dependent with more than 60% release being observed at 45\(^\circ\) and no release at 0\(^\circ\)C. Later studies by the same author using light microscopy showed that release at higher temperatures was due to degranulation.\(^38\) Histamine release was observed only with the intact glycoprotein. The effect of this histamine
release was to decrease the number of fat cells in the peritoneal tissues by at least 10 times. Other effects observed were hypotension, broncho-constriction, increased vascular permeability and local edema.\textsuperscript{39} Chemical studies revealed the presence of mannose, glucose, glucosamine and 17 different amino acids. It has a molecular weight of 36,000. The Thr/Scr of the protein molecule is linked to mannose of the carbohydrate moiety.\textsuperscript{40}

El-Sokkary\textsuperscript{41} et.al., (1975) reported the isolation of a toxic protein from \textit{C. guilliermondii}. The toxin was found to be stable at 100°C. Purified toxin possessed a MLD of 4.9 and LD\textsubscript{50} of 3.18mg in mice.

Yamamoto and Iwata\textsuperscript{42} (1977) reported the presence of three glycoprotein toxins designated as Fr-1, Fr-I-2 and FR-II-2. All three fractions had a different magnitude of toxicity and a different stability against physical and chemical treatments. One of the toxins showed limulus amoebocyte lyzate (LAL) gelling activity. This activity manifested itself at high temperature, shortened the plasma coagulation time and had acute toxicity and lethality on intravenous administration in mice.\textsuperscript{43} This LAL-gelling activity was attributed to the presence of D-mannose, which was found to be the sole carbohydrate,
accounting for 86-92% of the glycoprotein and the protein moiety had a characteristic amino acid composition. The toxin was stable against various physical and chemical treatments, had good antigenicity and no hemolytic activity.

A purified mannan preparation was also found to exhibit LAL-gelling activity; however, lethality of this preparation was less as compared to the glycoprotein itself. 43

Sattarelli et al. 44 (1980) demonstrated the existence of an extracellular glycoprotein from C.albicans. The preparation was found to inhibit Sarcoma-180 in mice. Inhibition with 10-80% regression was observed with a single dose. Multiple doses caused 60-90% regression of all the tumours. This glycoprotein was also found to be immunologically active.

Oshino et al. 45 (1973) detected the presence of yeast hemoglobin in cells of C.mycoderma and C.robusta. Oxygen affinity of respiring and non-respiring cells was identical to the values observed for isolated yeast hemoglobin. Pretreatment of the cells with EtOOH resulted in
decomposition of the hemoglobin. The rate of cell multiplication, respiration behaviour of the redox states of the mitochondrial cytochromes, or cell multiplication was not affected by the presence or absence of hemoglobin.
1.4 POLYSACCHARIDES

Mackiewicz et al.\textsuperscript{46} (1959) studied the growth promoting action of \textit{C. albicans} on tubercule bacilli and attributed this action to the polysaccharide fraction. This accounted for approximately 10\% of cell weight and comprised mannose and glucose residue. It stimulated the growth of a streptomycin-dependent strain of mycobacterium in the absence of streptomycin.

Other polysaccharide fractions\textsuperscript{47} from various members of \textit{Candida} have been shown to possess antitumour and antiviral effects. High molecular weight polysaccharides from \textit{C. albicans}, \textit{C. guilliermondii}, \textit{C. pseudotropicalis} and \textit{C. utilis} showed anticarcinogenic activity. Kumano\textsuperscript{48} (1973) observed inhibition of carcinogenesis induced by 3-methyl cholanthrene with a mannan preparation from \textit{C. utilis}.

In an earlier study Oka et al.\textsuperscript{49} succeeded in isolating a mannan preparation from \textit{C. utilis}, which inhibited the growth of sarcoma-180 in mice. The preparation however was very toxic. Oka et al.\textsuperscript{50} later showed that lowering in toxicity without affecting anti-tumour
activity can be achieved by carbomethoxylation of the mannan preparation with \( \text{ClCH}_2\text{CO}_2\text{H} \) or \( \text{BrCH}_2\text{CO}_2\text{H} \). Loss of anti-tumour activity was however observed on partial hydrolysis or acetolysis of the mannan. This suggested that a large molecule is a pre-requisite to yeast polysaccharide anti-tumour activity.

Akensenov\(^{51}\) (1970) isolated a purified polysaccharide preparation from \( \text{C.albicans} \), \( \text{C.tropicalis} \) and \( \text{C.viswanthi} \) which induced the production of interferon in mouse fibroblast in culture and in mice in vivo, thus protecting them from viral infection. The interferonogenic activity may be due to \( \gamma \)-globulin containing a carbohydrate, or a carbohydrate-protein complex.\(^{52}\) Purified glucan, mannan and galactan preparation were found to be most active. Low metabolism of these substances might be responsible for the prolonged interferonogenic activity seen in animals.

Kovalenko\(^{53}\) (1971) obtained polysaccharide preparations from culture media and cells of \( \text{C.arborea} \) and \( \text{C.tropicalis} \) and found it to be virucidal against potato virus and tobacco mosaic virus. The preparation in a concentration of 0.001-0.1% produced 30-100% reduction
of infectivity. Trypsin, RNase and DNase had no effect on the antiviral activity of these substances. The inhibitory action was however partially destroyed by heating in 1N HCl or 1N NaOH. Complete inactivation was observed on heating with 2N H₂SO₄ or 2-4N HCl. Glucose and mannose were found to be the main polysaccharides by Moskovets et al. Glucose predominated in preparations from culture liquids, and mannose in preparations from cellular extracts. Eleven to fourteen different amino acids could be detected in the hydrolysates of these preparations.

Milchenko (1976) observed antiviral activity in the polysaccharide preparation from C. tropicalis which was found to be active against tobacco mosaic virus and also inhibited influenza A₂ virus in experiments on chick embryos causing 3.8 fold reduction in the titre of the virus hemagglutinins.

Alimova and Marysina studied the lipo-polysaccharide of C. tropicalis and found that it exhibited pyrogenic properties. Hydrolysis of the isolate afforded the pure polysaccharide containing glucose and mannose, with traces of ribose, galactose and xylose, 12 amino acids, and a lipid fraction containing 18 aliphatic acids of carbon chain ranging from 12 to 20. The pyrogenic activity was attributed to the lipid fraction.
1.5 LIPIDS

Different classes of lipids have been isolated from members of the genus Candida, notably the phosphosphingosides\textsuperscript{57} from C.intermedia and cerebrosides\textsuperscript{58} from C.utilis.

Lipids were found to be toxic for animals, and produced considerable local effect. Zalkina and Tsinzeiling\textsuperscript{59} therefore assumed that the lipid fraction might be responsible for the formation of granular tissue in Candidiasis, which is identical to that observed in tuberculosis.

Anti-tumorogenic activity was exhibited by an oil fraction\textsuperscript{60} from C.utilis. It was tested against Ehrlich ascitis carcinoma cells, and found to be effective.

Dykhovichnaya et al.\textsuperscript{61} (1977) studied the lipid composition of C.albicans. They found that different strains of this species had varying degrees of virulence, and increase in virulence was related to an increase in lecithin and lysolecithin content.
1.6 MISCELLANEOUS

Numerous other biological effects have been exhibited by various metabolites of members of the genus *Candida*.

Salvin\(^2\) showed the presence of a hemolysin in the yeast phase of *C. albicans*. This hemolysin was most active on hen and guinea pig erythrocytes, less active on horse, mouse and rat cells, and inactive on human cells in the concentrations used.

Ito et al.\(^3\) obtained hemipeptides on hydrolysis of cytochrome C from *C. krusei*. These were found to be useful in cerebral circulation problems.

Singh et al.\(^4\) (1972) demonstrated the existence of anti-bacterial components in extracts of *C. albicans* and *C. viswanthii*. In another study Barber et al.\(^5\) isolated polysaccharide protein conjugates from different species of *Candida* and found them to exhibit a wide range of antibiotic activity against both homologus and heterologus *Candida* species and against various enterobacteriaceae. Proteolytic digestion of the conjugate with papain resulted in loss of activity suggesting that the protein portion was responsible for the wide range of antibacterial activity.
Lingappa\textsuperscript{66} reported the isolation of autoantibiotics, phenethyl alcohol and tryptophol from \textit{C. albicans}. Nayaran et.al.\textsuperscript{67} also obtained phenethyl alcohol from \textit{C. guilliermondii} and \textit{C. tropicalis}.

Other compounds exhibiting antibiotic activity are tryptanthrin\textsuperscript{68} and its derivatives\textsuperscript{69} from \textit{C. lipolytica}, pulcherimic acid and pulcherimin\textsuperscript{70} from \textit{C. pulcherrima} and antiburastin\textsuperscript{71} from \textit{C. antipiricularis}.

Suzuki\textsuperscript{72} demonstrated the presence of 5-hydroxy L-tryptophan in extracts of \textit{C. tropicalis} which was useful as antineurotic agent.

Aseera\textsuperscript{73} observed an increase in growth rate and weight of pea and vicia plants by a gibberellin like substance synthesized by \textit{C. pulcherrima}.

Morimoto et.al.\textsuperscript{74} showed that \textit{C. utilis} is able to synthesize benzopyrene, an abundant environmental carcinogen. However others have shown that certain species of \textit{Candida} can absorb benzopyrene and have therefore been used to remove this from dairy and other food products.\textsuperscript{75}
Hsia\textsuperscript{76} has demonstrated that \textit{C. albicans} can synthesize benzylmethyl nitrosamine, an oesophageal carcinogen, by nitrosative oxidation of benzyl methyl amine.

Kupenov\textsuperscript{77} showed that members of the genus \textit{Candida} can effect the transformation of cardiac glycosides lanatoside A, lanatoside C and convallodore to digitoxin, digoxin and convallotoxin respectively.

Kupenov\textsuperscript{77a} (1978) observed that members of the genus \textit{Candida} can cause decomposition of terpene glycosides resulting in increase in essential oil content of rose (\textit{Rosa damascena}) flower distillate.

Anticarcinogenic activity has been claimed for 1-\textalpha-D-Ribofuranoside\textsuperscript{78} from \textit{C. tropicalis}. The riboside has also been found to be effective in the treatment of gout.

Canov et.al.\textsuperscript{79} reported increased levorin production on cultivating actinomyces levoris or streptomyces levoris in presence of \textit{C. tropicalis} or culture filtrate of \textit{C. tropicalis}. Enhancement of levorin production was attributed to a low molecular weight organic compound obtainable
on extraction with butanol followed by dialysis. Heat treatment and changes in pH in the range of 2-14 did not produce any undesirable effect.\textsuperscript{80}

Trnovec\textsuperscript{81} isolated a polysaccharide protein complex from cell wall of \textit{C.albicans} which produced a significant decrease in the ED\textsubscript{50} of the convulsion effect of pentylene tetrazole. Pretreatment of mice with methyl palmitate eliminated the effect. Carbon tetrachloride was also found to lower the threshold of pentylene tetrazole convulsions. Combined administration of carbon tetrachloride and polysaccharide protein complex however did not lead to a significant deepening of the effect. Absence of hepatotoxic effect was evident as the rate of elimination of sulphabromophthalein in blood remained unaltered.
1.7 BIOLOGICAL ASPECT

Some of the members of the genus *Candida* have been shown to be human pathogens producing candidiasis in various parts of the body. *Candida albicans* and *Candida tropicalis* are responsible for more than 80% of these cases. Conant et al. (1954) recognised the following types of candidiasis:

1. Cutaneous candidiasis
2. Candidiasis of mucous membrane
3. Bronchopulmonary candidiasis

1.7.1 CUTANEOUS CANDIDIASIS

The areas most commonly affected are the perianal and inguinal folds in the case of infants, and the interdigital areas, groins, gluteal crease, submammary folds, nail folds and axillae in the case of adults. These are the areas where warmth, moisture and maceration of the skin permits the organism to thrive. The affected perianal skin of infants exhibits red lacquer like sharply margined areas of erythema with loosely adherent scales at the periphery.
Systemic infection may superimpose cutaneous candidiasis or begin with chronic bronchitis, gastroenteritis, or meningitis. Involvement of the lungs often stimulates pulmonary tuberculosis.

Infection of the nails is characterised by painful swelling at the nail beds and resembles pyogenic infection.

1.7.2 CANDIDIASIS OF MUCOUS MEMBRANES

Thrush infection of buccal mucosa exhibits eruptions of quite a different character. Papillae are atrophied with bright red, glazed, and smooth surface having vesicles or erosions sometimes occurring on edges.

1.7.3 BRONCHOPULMONARY CANDIDIASIS

Mild bronchopulmonary candidiasis is accompanied by persistent cough and sputum containing cell debris and yeast-like cells. More extensive pulmonary candidiasis resembles miliary tuberculosis with cough, fever, dyspnea, chest pain, hemoptysis and night sweat. Signs of pleural thickening and consolidation may be evident.
1.8 PATHOGENESIS BY CANDIDA TROPICALIS

*Candida tropicalis* has been frequently isolated from human sites described earlier. It has also produced vaginal candidiasis. In pregnant patients failure to treat the infection before delivery may lead to candidal infection in the neonate.  

1.8.1 EYE CANDIDIASIS

Endogenous *Candida* endophthalmitous was first reported by Miale in 1943 and the first known clinical diagnosis and successful treatment reported by Lauria and Dineen in 1960's.

More recently in 1980 endogenous *Candida* endophthalmitous has been reported in infants by Palmer.

A case of endogenous tumorous hemorrhagic mycotic iritis caused by *C. tropicalis* and *C. zeylanoides* has been reported by Krudyz and Witoli.

1.8.2 CARDIAC CANDIDIASIS

The involvement of fungi in causing endovascular infections is quite rare, however cases of endocarditis and cerebral embolism have been reported by
Kunstadter et al.\textsuperscript{91} viz \textit{C. albicans}, \textit{C. tropicalis}, \textit{C. parakrusei} and \textit{C. guilliermondii}.

Cardiac candidiasis was characterised by myocardial microabscesses. In cases of infective endocarditis, the mitral valve was most frequently involved.\textsuperscript{92}

1.8.3 \textbf{CEREBRAL CANDIDIASIS}

Postmortem examination of human cerebral candidiasis carried out by Parker et al.\textsuperscript{93} (1980) showed cancer in 21\% cases. All individuals had proven or gram negative sepsis. \textit{Candida} species were identified outside the brain in nearly every patient and included the kidneys (90\%) heart (80\%) and other organs.

1.8.4 \textbf{TEETH CANDIDIASIS}

Presence of yeast was also demonstrated frequently in caries teeth, and as many as 1/3 of the chronically inflamed tonsils had a mycological genesis mostly by \textit{Candida}.\textsuperscript{94}

1.8.5 \textbf{OROPHARYNGEAL AND OESOPHAGEAL CANDIDIASIS}

Cases of chronic oropharyngo-oesophageal candidiasis have been reported.\textsuperscript{95} Chronic oropharyngo-oesophageal candidiasis results in impressive oropharyngeal lesions and stricturing of mid oesophagus.
1.8.6 **DISSEMINATED CANDIDIASIS**

Incidence of disseminated candidiasis is more frequent with acute leukaemia. Gastro-intestinal candidiasis with hepatic (75%) and splenic (94%) involvement being common in such patients. 96

1.8.7 **CANDIDIASIS IN BURNED PATIENTS**

Candidiasis has been seen to occur as a pre-terminal phenomenon in burned patients. It coincided with a general collapse of host defense and homeostatic mechanism. It rarely responded to treatment and had a high mortality. 97

1.8.8 **CANDIDIASIS IN ANIMALS**

*Candida tropicalis* is also known to cause disease of a similar nature in animals like mice, cows and goats. Studies carried out on the pathogenicity of *Candida albicans* and *Candida tropicalis* show that most strains of *C. tropicalis* possess a considerable degree of virulence for mice. 98
1.9 EXPERIMENTALLY INDUCED CANDIDIASIS IN ANIMAL

Hurley and Winner\textsuperscript{99} studied the pathogenicity of \textit{C. tropicalis} by intravenous administration in mice and observed that all the inoculated animals showed focal mycotic lesions throughout the heart, brain and kidneys. Severe pyelonephritis was observed in some cases. The animals died in 1-10 days.

Experimental endogenous \textit{Candida} endophthalmitous in rabbits showed preretinal spheroid opacities, the course of these lesions being compatible with that described in humans. Conditions such as this can lead to the destruction of eye and has caused corneal perforations in fatal cases of \textit{Candida} septicemia of which \textit{C. tropicalis} is an established causative agent, being second in frequency to \textit{C. albicans}.\textsuperscript{100}

Experimentally induced mammitis in sheep with this yeast like fungus produced irreversible sclerosis, whereas inoculation by intragalactophoric route caused predominant necrotic granulomatous lesions.\textsuperscript{101}
1.10 OBJECTIVES OF PRESENT STUDY

The cell free broth of *Candida tropicalis* has not been studied for its chemical constituents or biological activity. This is the first effort in this direction. We have undertaken to study the protein and peptide contents; to determine proteolytic action of proteins/protein conjugates and any antimicrobial activity that might be present in peptides.
2 EXPERIMENTAL

2.1 COMPOSITION OF CULTURE MEDIA

*Candida tropicalis* culture CBS 94 was obtained from Central Bureau Voor Schimmel Cultures, Yeast Division, Baarn Holland and grown initial on following media:

1) Basal Synthetic Medium

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<td>MnSO₄</td>
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<td>FeSO₄·7H₂O</td>
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<td>MgSO₄·7H₂O</td>
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</tr>
<tr>
<td>Biotin</td>
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<tr>
<td>Thiamine</td>
<td>100 mg.</td>
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<td>H₂O</td>
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2) Asparagine Medium

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<tr>
<td>KH₂PO₄</td>
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<tr>
<td>Thiamine</td>
<td>5 mg.</td>
</tr>
<tr>
<td>H₂O</td>
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3) Sabourauds Medium

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<td>Glucose</td>
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<td>Peptone</td>
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b) Sabourauds Sucrose

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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>10 g.</td>
</tr>
<tr>
<td>Peptone</td>
<td>20 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 l</td>
</tr>
</tbody>
</table>

c) Sabourauds Starch

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>20 g.</td>
</tr>
<tr>
<td>Starch</td>
<td>10 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 l</td>
</tr>
</tbody>
</table>
iv) Czapex (Dox) Medium

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>2 g.</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1 g.</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.01 g.</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g.</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 ℓ</td>
</tr>
</tbody>
</table>

v) Potato Dextrose Medium

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200 g.</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 ℓ</td>
</tr>
</tbody>
</table>

These media with certain variations were also tried for the growth of this fungus.

Certain solid growth media were also tested. These include following:

i) Sabourauds Glucose Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>20 g.</td>
</tr>
<tr>
<td>Glucose</td>
<td>40 g.</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 ℓ</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

ii) Potato-Dextrose Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>20 g.</td>
</tr>
<tr>
<td>Potato</td>
<td>200 g.</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 ℓ</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
</tr>
</tbody>
</table>
iii) Potato agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200 g.</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g.</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 l</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Growth of the culture was very slow in most of the test media. Sabouraud's Glucose broth was however found to be best. Fig.-1 shows the growth of different media. The effect of pH was studied in Sabouraud's medium and maximum growth was found to be at pH 6.5 (Fig.-2).

The studies carried out on the metabolites and reported here are on Sabouraud's-Glucose broth and Sabouraud's-Glucose agar (Solid Media).

2.2 PREPARATION OF CULTURE MEDIA

The culture media was prepared by dissolving the components in distilled water. The pH of the solution was adjusted using 1M NaOH. The pH adjusted culture media was sterilized at 15 lbs/sq. inch. (121°C-temperature), for 10 minutes. The sterilized culture media was kept for 10 hours after sterilisation to establish sterility, and then inoculated with the culture.
Fig. -1 Growth pattern of *Candida tropicalis* on different media.
A=Sabourauds
B=Asparagine
C=Čapex (Dox)
D=Basal
Fig. - 2 The curve showing effect of pH on growth of \textit{Cand. tropicalis} in sabouraud's medium.
2.3 GROWTH OF THE CULTURE

The culture was grown in 100 ml flasks, 1/3 filled with the media and a slant of the culture introduced under aseptic conditions to prevent the culture media from contamination. The culture was grown under shaking conditions at 37°C for 6 days.

After maximum growth of the fungus the culture broth was separated under aseptic conditions from the cells by centrifugation. The cells were washed twice with sterilized distilled water and stored in refrigerator till required.

2.4 ISOLATION OF COMPONENTS FROM BROTH

The cell free cultured broth was concentrated on a rotary evaporator to a smaller volume. Isolation of metabolites was carried out using:

i) Direct precipitation method

ii) Solvent extraction method

In direct precipitation method ethanol was employed for the separation of proteins and peptides, whereas the solvent extraction method was adopted for extraction of smaller organic compounds. Flow Chart-I
shows the fractionation of cell free cultured broth by solvent extraction procedure.

```
Cell-Free Culture  Broth (Concentrated)
     /             |
     |              |
     |              |
Benzene extract  Aq. Layer  CH₃Cl.
     |              |
     |              |
Aq. Layer  CHCl₃  ext.(B)
     /              |
     |              |
EtOAc  EtoAc ext.(C)  Aq. Layer  EtoH
     |              |
     |              |
EtOH ext.(D)  ppt
```

2.5 GEL FILTRATION CHROMATOGRAPHY

The crude proteins/peptides were subjected to gel filtration chromatography an important technique in biochemical separation. Dextran gel i.e., Sephadex G-100 was used for this purpose. Sephadex is an anhydro-glucose polymer cross linked to epichlorohydrin. The separation is based on molecular size discrimination, and it often produces a high degree of resolution.
Sephadex G-100 was swollen in 0.2M acetic acid for 72 hours. The gel was deaerated and packed in a desired column filled with 0.2M acetic acid up to one third of its height. The column was allowed to pack under the influence of gravity. After packing the column was run in 0.2M acetic acid at a flow rate of 20ml/hour. Column void volume was determined with Dextran blue.

2.6 ELECTROPHORESIS OF THE PROTEIN

Polyacrylamide Gel Electrophoresis: Protein/peptides sample were analyzed by electrophoresis on poly-acrylamide gel. The following solutions were used:

Solution A: 36.6 g. Tris, 48ml 1N HCl and 0.46ml N,N,N',N'-
tetramethylene diamine (E. Merck, Darmstadt) made to 100ml with distilled water pH 8.0

Solution B: 30.0 g. Acrylamide and 0.8 g. N,N'-methylene bis acrylamide (Serva Feinbiochemica, Heidelberg) dissolved to make 100ml with distilled water.

Solution C: 11.0 g. Ammonium peroxodisulfate dissolved to 50ml with distilled water (freshly prepared).
TABLE-I

MIXING RATIO FOR GELS WITH 2.6% CROSS-LINKING

<table>
<thead>
<tr>
<th>GEL SOLUTION</th>
<th>5%</th>
<th>7.5%</th>
<th>10%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sol.A</td>
<td>1.6</td>
<td>2.1</td>
<td>3.25</td>
<td>4.8</td>
</tr>
<tr>
<td>Sol.B</td>
<td>3.2</td>
<td>1.8</td>
<td>6.5</td>
<td>9.74</td>
</tr>
<tr>
<td>Sol.C</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>H₂O</td>
<td>13.2</td>
<td>10.8</td>
<td>8.25</td>
<td>3.4</td>
</tr>
<tr>
<td>Total volume</td>
<td>20ml</td>
<td>20ml</td>
<td>20ml</td>
<td>20ml</td>
</tr>
</tbody>
</table>

Gel tubes (14x0.6 cm) were filled with this solution to a height of 12 cms and left for polymerization for approximately 30 minutes. A small amount of water was added carefully to the top of the gel to produce a smooth surface.

Sample preparation: 1mg of the protein sample was dissolved in the upper electrode buffer and the solution denatured at 100°C for 10 minutes.

Sample application: The tubes were first layered carefully with a saturated solution of sucrose and then with a known quantity of the sample, containing bromophenol blue as the tracking dye. Finally the gel tubes were filled with the upper electrode buffer.
Electrode Buffers:

1) Upper electrode buffer-Tris 5.16g, Glycine 3.48g, pH 8.8 made upto 1 litre with water.

2) Lower electrode buffer-Tris 14.5g, adjusted to pH 8.0 with HCl and made to 1 litre with water.

Fixing Solution:

1) 7% acetic acid-8 hours for staining with Amidoblack.

2) 20% aqueous sulfosalicylic acid-18 hours at room temperature for staining with Comassie Brilliant blue R-250.

Staining Solution:

1) 2% Amido black 10-B in 7% acetic acid.

2) 2% Comassie Brilliant Blue R-250 in 25% methanol and 10% acetic acid in water.

Destaining Solution:

1) 7% acetic acid in the case of Amido black 10-B.

2) 25% methanol, 10% acetic acid in water for Comassie Brilliant Blue R-250.

**SDS Polyacrylamide Gel Electrophoresis:** Molecular weight was determined by SDS-gel electrophoresis. Electrode buffers contained 0.1% SDS. Gels also contained 0.1% SDS.
Sample preparation: For SDS-gel electrophoresis the sample was dissolved in electrode buffer and 50μl of a 50% solution of mercaptoethanol added. The sample was heated for 10 minutes in a boiling water bath. Electrophoresis apparatus used was from ATTO Corporation, Tokyo, Japan.

2.7 AFFINITY CHROMATOGRAPHY

Enzyme purification was carried out with amino hexyl Sepharose-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) linked to pepstatin.

105 Sepharose is a bead-formed agarose gel which displays virtually all the features required of a successful matrix for immobilizing biologically active molecules. The hydroxyl groups on the sugar residues can be easily derivatized for covalent attachment of a ligand. Sepharose 4B has the exclusion limit of MW 20x 10^6, and makes the interior of the matrix available for ligand attachment ensuring good binding capacities. It exhibits extremely low non-specific adsorption.

AH-Sepharose 4B was swollen for 15 minutes and washed with 0.5M NaCl to remove all additives. It was treated with pepstatin and the product finally washed several times with 0.2M sodium citrate buffer pH 6.0 containing 1M NaCl. The treated resin was packed in a column (10 x 0.7cms) and was used for the purification of the enzyme.
2.8 ENZYME ASSAYS

Protease enzyme assay was carried out using \textsuperscript{106}\textsuperscript{Kunitz}'s method. The procedure involves treatment of enzyme with casein in 0.1 M Tris-HCl pH 8.0. The solution is incubated at 37° C for 20 minutes. The reaction was stopped by the addition of 6 ml of 0.3 M TCA followed by centrifugation at 5000 rpm. The supernatant was left for 30 minutes and the optical density was recorded at 280 nm against blank.

Activity units were calculated as follows:

\[ 1 \text{ activity unit} = 0.001\Delta \text{ absorption units} \]

Solution used: Casein dissolved in 0.1 M Tris-HCl pH 8.00, Enzyme dissolved in 0.1 M Tris-HCl pH 8.00, 0.3 M TCA.

2.9 OXIDATION WITH PERFORMIC ACID

Protein samples were oxidized with performic acid\textsuperscript{107} prior to hydrolysis. The performic acid was prepared by

\textsuperscript{*Trichloroacetic acid}
mixing formic acid and hydrogen peroxide in the ratio of (9:1) (v/v) and left in a refrigeration at 0°C for 4 hrs. The mixture of protein and performic acid was maintained at 0°C overnight, water and excess performic acid from the sample was then removed by desiccation over KOH.

2.10 SEPARATION OF PEPTIDES ON DOWEX-50x2

Ion-exchange chromatographic process was adopted for the separation of Candida tropicalis peptides. Dowex-50x2 (200 mesh) was selected for this purpose. The resin was first treated with 2M-NaOH for 45 minutes and then washed to neutral with deionized distilled water. The resin was then treated with 2M HCl for 45 minutes, washed with deionized distilled water through suction until neutral. The resin was equilibrated with 0.02M Pyridine acetate buffer pH 2.8 degassed and packed in a jacketed column maintained at a constant temperature of 55°C at a flow rate of 30ml/hour. Peptides sample dissolved in Pyridine acetate buffer pH 2.0 was applied on top of the column and development was carried out with Pyridine acetate buffer-linear pH gradient.

The buffer vessel used were either step-wise gradient forming or continuous with linear gradient formation. Linear gradient apparatus was with two way connector having on either side a cylindrical vessel.
The buffer flow rate was maintained with a 12000 pump (LKB/Varioperpex, peristaltic pump) at a constant flow rate of 30ml/hour.

Pyridine GR (E.Merck, Darmstadt) used for buffer preparation was distilled twice over ninhydrin.

Linear gradient buffers were found to produce better separation in this case.

2.11 AMINO ACID ANALYSIS

The Amino acid analyzer systems involve cation exchange resins of definite specification based on the pioneering work by Moore and Stein,\textsuperscript{109} Spackman, Stein and Moore,\textsuperscript{110} Hamilton and Anderson\textsuperscript{111} and Peterson and Sober.\textsuperscript{112}

\textbf{Amino Acid Analyzer LC 6001} (Biotronic, West Germany) is a fully automatic analyzer with automatic sample injector and a computing integrator. The system involves photometric detection with ninhydrin. The photometer is attached to a recorder which in turn is attached to the computing integrator. The analyzer can be represented schematically (Flow Chart-2). Separation is achieved as follows:
### TABLE - 2

<table>
<thead>
<tr>
<th>Value</th>
<th>Buffer A</th>
<th>Buffer B</th>
<th>Buffer C</th>
<th>Buffer D</th>
<th>Regeneration Solution</th>
<th>Sample Diluting Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.50</td>
<td>4.25</td>
<td>5.35</td>
<td>10.20</td>
<td>-</td>
<td>2.20</td>
</tr>
<tr>
<td>Mn-Concentration</td>
<td>0.12 n</td>
<td>0.18 n</td>
<td>0.18 n</td>
<td>0.2 n</td>
<td>0.2 n</td>
<td>0.12 n</td>
</tr>
<tr>
<td>Ce-Concentration</td>
<td>0.08 m</td>
<td>0.06 m</td>
<td>0.06 m</td>
<td>0.033</td>
<td>-</td>
<td>0.08 m</td>
</tr>
<tr>
<td>Lm hydroxide</td>
<td>9.6 g</td>
<td>14.4 g</td>
<td>14.4 g</td>
<td>-</td>
<td>16 g</td>
<td>4.8 g</td>
</tr>
<tr>
<td>Lm acetic acid</td>
<td>33.6 g</td>
<td>25.2 g</td>
<td>25.2 g</td>
<td>-</td>
<td>-</td>
<td>16.8 g</td>
</tr>
<tr>
<td>Sodium chloride 37%</td>
<td>6.0 ml</td>
<td>14 ml</td>
<td>5 ml</td>
<td>-</td>
<td>8 ml</td>
<td></td>
</tr>
<tr>
<td>2-Methylcellulose</td>
<td>200.0 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>30% solution</td>
<td>4 ml</td>
<td>4 ml</td>
<td>4 ml</td>
<td>4 ml</td>
<td>4 ml</td>
<td>-</td>
</tr>
<tr>
<td>2-Methylakuromanol</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
</tr>
<tr>
<td>Ethanol 25% in H₂O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20 ml</td>
</tr>
<tr>
<td>Lm formic acid</td>
<td>2 g</td>
<td>2 g</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lm citrate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19.6 g</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lm tetraborate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>38.1 g</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### COLUMN DATA FOR THE STANDARD HYDROLYZATE PROGRAM

- **Column**: 0.4 x 25 cm
- **Buffer Flow**: 20 ml/h
- **Reagent Flow**: 20 ml/h
- **Temperature T₁**: 47.5°C
- **Temperature T₂**: 61.5°C
1. Buffers stored in glass vessels are connected to a buffer selection valve driven both automatically and manually.

2. Buffer from the buffer selection valve enters the bubble trap which removes any air or gas present.

3. The buffer then enters the pump. The pump is connected to the dividing block, which in turn is connected to the buffer pressure gauge and pre-wash column. The pre-wash column serves to remove ammonia from the buffer.

4. Samples are injected in the sample injector.

5. The buffer stream coming from the pre-wash column carries the sample into the column. Development is carried out with buffer gradients. The composition of buffer is given in Table-2. Sample eluting from the column enters the mixing block, where it receives ninhydrin and enters into the reaction bath.

Detection: The ninhydrin is stored in cool dark coloured bottle with an atmosphere of nitrogen and connected to two equilibrating vessels. The ninhydrin coming from the vessel enters a bubble trap being connected to the pump. From the pump the ninhydrin enters the ninhydrin dividing block. The dividing block is connected to the pressure gauge and a back pressure valve. The back pressure valve serves to ensure a constant flow and safe cut off of the reagent when the pump is stopped. The reagent enters the mixing block where it comes in contact with the eluate. The mixture then enters the reaction bath. The following reaction takes place.
The exact mechanism for the reaction of proline (an imino acid) with ninhydrin is not yet known. Still the yellow product obtained has been studied by Grassman and van Haastrecht, who found the mol. formula is $C_{22}H_{13}NO_4$.

$$2 \text{C}_2\text{H}_6\text{O}_4 + \text{C}_5\text{H}_9\text{O}_2\text{N} \rightarrow \text{C}_{22}\text{H}_{13}\text{O}_4\text{N} + 2\text{H}_2\text{O} + \text{CO}_2$$

The reaction products flow through the photometer and the absorbance value for each component, is detected at 570nm and 410nm and recorded.
Preparation of Ninhydrin Reagent: 20g. of ninhydrin and 1g. of stannous chloride were dissolved in 750ml of methyl cellulose in presence of nitrogen stream. 250ml of 4 M sodium acetate buffer pH 5.5 is then added and N₂ passed for 20 minutes at a pressure of 0.1 bar to remove oxygen.

Sample Preparation: A known amount of the protein/peptide sample was dissolved in 6 N HCl and sealed in vacuo. The sealed tube was heated for 24, 48, 72 or 96 hrs. at 110°C. The tube was then cooled to room temperature and HCl from the hydrolysed sample was removed on a rotary evaporator. The dried content was then dissolved in 0.2 M sodium citrate buffer pH 2.2.

2.12 Paper Chromatography of Peptides and Amino Acids

Paper chromatography of the hydrolysate of each fraction was used as one of the tools for identifying the different fractions.

Ascending-Unidimensional Chromatography: Whatman No. 1 (7"x7") was taken and a line drawn 1.5 cms away from either side of the paper. The sample was applied as a line (0.5cms) and developed with Butanol:Pyridine:water
or Butanol:Acetic acid:water (1:1:2.2) or as described. The paper was then dried and sprayed with Cu-ninhydrin or Sr-ninhydrin in the case of amino acids and aniline phthalate in the case of carbohydrates.

Ascending-Two Dimensional: Whatman paper (7"x7") was taken and spotted as described for unidimensional chromatography. The paper developed with first solvent system of Butanol:Pyridine:water (1:1:1), dried, developed in the second solvent system of Butanol:Ethanol:water (4:1:2.8), again dried and turned to 90° and then developed with third solvent system of Phenol:water (80:20). The paper was dried and sprayed with Sr-ninhydrin.

Descending-Unidimensional: Descending paper chromatography was carried out on (36x45cms) sheets. Samples were spotted 4.5 cms away from the base and then the paper developed for 20, 36 or 72 hours. The paper dried and sprayed with their respective reagents viz. ninhydrin for amino acids and aniline phthalate for carbohydrates.
2.13 TESTING OF ANTIMICROBIAL ACTIVITY

Source of Bacterial cultures: Clinical isolates of bacteria were obtained from Abbasi Shaheed Hospital, Karachi (Pakistan) and Department of Microbiology, University of Karachi (Pakistan). These cultures were tested for their purity and strain in each batch of the experiment. The pure cultures were stabbed in soft agar and stored in a refrigerator until required.


Preparation of Bacterial Culture: The culture of bacteria grown overnight at 37°C, were used for testing the antibacterial activity of different fractions of
Candida tropicalis separated on Sephadex G-100 and Dowex 50x2 columns. The assay was carried out by Overlayagar and Seed plate method.

**Overlay Agar Preparation:** The method due to Ames et.al. was employed the plates were formed with two different percentages of agar i) the thick hard layer or underlay made by oxoid nutrient medium containing sterilized 1.5% agar (Difco) poured in molten form to pre-sterilized petri-plates. The plates were then left for hardening on a smooth surface. Following the hardening of agar the plates were incubated at 37°C to keep out swabbing and for sterility testing. The soft agar overlay medium was prepared with 0.6% agar, pH adjusted to 7 and an aliquot of 2.5ml was taken in test tubes, sterilized and stored in a refrigerator till required. The soft agar tubes were melted in duplicate corresponding to the number of available pure cultures and kept in a water bath at 45°C. The pure cultures were added to soft agar in duplicate mixed well and poured on to the sterile hard agar plates and left for half an hour. The method employed for testing the Candida tropicalis fractions were Drop, Disc or Well. In Well method experiment a positive control, Kanamycin 12.5mg/ml was used and results are reported as zone of inhibition with respect to standard control.
Seed Plate Method: In this technique oxidid nutrient medium containing 1.4% agar adjusted to pH 7 was distributed in 40ml quantity in screw capped bottles and sterilized. The bacterial culture was then added aseptically to the agar medium at 45°C, mixed well and poured immediately in duplicate to the presterilized petriplates and left for hardening on smooth surface. Following the hardening of agar, wells were cut and the C. tropicalis fractions were placed in these wells. The plates incubated at 37°C and results recorded after 48 hours.

2.14 N-TERMINAL ASSAY BY DANSYLATION

N-terminal assay was carried out according to Gray and Hartley. The reaction involves coupling of the terminal amino group with 1-dimethyl amino naphthalene-5-sulphonylchloride (DNS-Cl) to give the dansylated derivative having a yellowish green fluorescence.
The protein/peptides sample was dissolved in 0.2M NaHCO₃ solution at pH 8.5. 1% solution of dansyl chloride in acetone added, pH checked and the whole solution incubated at 37°C for 4 hours with occasional shaking and then left overnight. The solution was centrifuged to remove excess NaHCO₃. The supernatent was dried in vacuo and dissolved in 6M HCl, the tubes sealed in vacuo and heated at 110°C for 20 hours. HCl from the hydrolysed samples was removed under vacuum and extraction was carried out with ethylacetate. The ethyl acetate extract was analysed by thin layer chromatography on silica gel.

Silica gel plates (Merck, precoated) were spotted with the sample along with standard DNS-amino acids and developed with Benzene: Pyridine: Acetic acid (40:10:1). Fluorescent spots were located and identified under a UV lamp at 254 nm.

2.15 CARBOHYDRATE ANALYZER*

Carbohydrate analyzer based on the work of Voelter and Bauer was employed for the detection of mono/disaccharides.

The analyzer consists of a buffer compartment, an anion-exchange resin column, the detecting unit and recorder.

*Biotronic GmbH Frankfurt, West Germany.
The resin is a copolymer of divinyl benzene
cross linked polystyrene substituted with quaternary amine
functional group. It is stable to high temperatures and
alkaline pH. The composition of buffers are shown in
Table-3.

Preparation of Cu-bicinchoninat reagent:

1. 207g potassium carbonate dissolved in
750ml of deionized water.

2. 2.5g potassium carbonate, 1.8g Aspartic acid
and 0.5g copper sulphate dissolved in 50ml
deionized water.

3. 1g sodium bi-cinchoninat dissolved in 50ml
deionized water.

4. Final volume made to 1 litre with deionized
water.

The reagent is stable in this form for few
weeks and requires no cooling or oxygen free atmosphere.

The reagent and the eluate are mixed in a 1:1
ratio. The reaction takes place in a 30 meter long teflon
tube having an inner diameter of 0.5mm imersed in a boiling
water bath. The intensity of coloured complex formed is
determined at 570nm.
Preparation of Samples: Samples for carbohydrate analysis were hydrolysed in 2M H₂SO₄ acid or 4M HCl in a sealed tube at 100°C for 8 hours. The hydrolysed samples were evaporated to dryness and dissolved in starting borate buffer pH 8.0.

Analysis of Amino Sugar: Amino sugars were analysed on cation exchange resin (BTC 2710) using 0.15M sodium citrate buffer pH 5.49 containing 0.45M NaCl. Detection was carried out with Qu-bi-cinchoninat.
### Table 3

<table>
<thead>
<tr>
<th>Buffer</th>
<th>0.1M</th>
<th>0.2M</th>
<th>0.3M</th>
<th>0.4M</th>
<th>0.5M</th>
<th>Buffer</th>
<th>Regeneration Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molarity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boric acid</td>
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Column Resin: DA-X₈-11
Red height: 18.5cm
Column Temperature 60°C
Flow rate of buffer: 40ml/hr
Flow rate of reagent: 40ml/hr
3. RESULTS

3.1 ISOLATION OF PROTEINS AND PEPTIDES

Proteins/peptides present as the extra cellular components in the broth were precipitated with 70% ethanol. The concentrated broth was treated with ethanol and left in a refrigerator overnight. The filtrate was then drawn off and kept separately. The precipitates were studied for their protein/peptides pattern using polyacrylamide gel-electrophoresis and column chromatography on Sephadex and ion-exchange resins.

3.2 ELECTROPHORESIS

Electrophoresis of the ethanol precipitated sample containing protein/peptides was carried out using 5%, 10% and 15% polyacrylamide gels (25x0.8cms). Tris-glycine pH 8.8 and Tris-HCl pH 8.0 were used as the upper and lower electrode buffers respectively. A constant current of 10mA per tube was applied. Figure-3 shows the electrophoretic pattern of crude protein precipitate on charge and molecular weight basis. Electrophoresis showed several distinctly visible bands.
Fig. 3 Disc electrophoresis of *Candida tropicalis* crude proteins on polyacrylamide gels (5-15%, 25x0.8 cms); current 10 mA/tube; running time 3.5 hours; (bands observed visually).

a) With SDS
b) Without SDS
Fig. 4 Elution profile of *Candida tropicalis* crude proteins on Sephadex G-25 (90x2.5cm); eluted with 0.2M Acetic acid; fraction volume 3ml; Abs at 254 nm.
Fig. 5 Separation profile of *Candida tropicalis* prote on Sephadex G-200 (50x1cm); elution with 0.2M Acetic acid; fraction volume 1ml; absorbance a 254nm.
3.3 SEPARATION OF PROTEINS/PEPTIDES ON SEPHADEX

500mg of the precipitated protein/peptides sample was dissolved in 0.5ml of 0.2M \( \text{CH}_3\text{CO}_2\text{H} \) and subjected to a Sephadex G-100 (90x2.5 cms) column. Elution was carried out with 0.2M \( \text{CH}_3\text{CO}_2\text{H} \) and collected as 3ml fractions on an LKB fraction collector ULTRO RAC 7000. The UV spectrum was recorded using 206 and 254nm filters. Figure-4 shows the elution pattern of different proteins on Sephadex G-100, and Fig.5 on Sephadex G-200.

Two peaks CTExS\(_1\) and CTExS\(_2\) appeared within the first void volume. Another set of eleven unresolved proteins/peptides peaks (CTExS\(_3\)-CTExS\(_{13}\)) appeared near the end of second void volume and were thus pooled together and labelled as CTEx-P. The pooled fractions were evaporated on a rotary evaporator. Traces of acetic acid were removed with repeated distilled water re-evaporation. The samples were then taken in deionized distilled water for further studies.

3.4 PURIFICATION OF THE ENZYME

An affinity column was employed with pepstatin linked to aminohexyl-sepharose-4B. The sample was adsorbed to the column (10x0.7cms) in 0.2M citrate buffer, pH 6.0
Fig. - 6  Disc electrophoresis of CTEpS: gel polyacrylamide 7.5%; 12x0.6 cms); current 4 mA/tube; running time 3 hours; staining agent 0.2% Coomassie Brilliant blue R-250.

a) With SDS
b) Without SDS
and 1M NaCl and was desorbed with 0.1M Tris-HCl pH 8.3 and 1M NaCl.

3.5 ELECTROPHORESIS OF CTEpS₁

The protein sample CTEpS₁ was subjected to SDS polyacrylamide disc gel electrophoresis (5-15%) using Tris-glycine pH 8.8 as the upper electrode buffer and Tris HCl pH 8.0 as the lower electrode buffer. A constant current of 5mA per tube was applied. The tubes were run for 3 hours. After electrophoresis the tubes were fixed in 20% Sulphosalicylic acid for 18 hours, these were stained with 0.2% comassie Brilliant Blue R-250 in 25% methanol and 10% acetic acid for 18 hours. Destaining was carried out in 25% methanol in 10% acetic acid solution. Standard proteins were run under similar conditions and results are represented in Figure-6. The molecular weight was found to be 40,000 ± 7,000.

3.6 AMINO ACID COMPOSITION OF CTEpS₁

A known quantity of the oxidised protein sample was hydrolysed with 6M HCl in a sealed tube for 24 hours at 110°C. HCl from the sample was removed in vacuo and the dried content was taken in sodium citrate buffer pH 2.2. The analysis was carried out on Biotronic Automatic Amino Acid Analyzer LC 6001. Table-4 shows the amino acid composition.
TABLE-4

AMINO ACID COMPOSITION OF CTEP浓缩

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<th>AMINO ACID</th>
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TABLE-5

CARBOHYDRATE COMPOSITION OF CTEP浓缩

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<td>Glucosamine</td>
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<td>Galactosamine</td>
<td>traces</td>
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</table>
3.7 N-TERMINAL ASSAY OF CTEps₁

The protein sample CTEps₁ was dissolved in 200μl 0.2M NaHCO₃ and to it was added 200μl of a 0.5% solution of Dansyl chloride in acetone. The pH checked and the solution kept overnight at 37°C. Excess Dansyl chloride was then extracted with ethylacetate and the sample hydrolysed with 6M HCl. After hydrolysis HCl was removed under vacuum. The dried material was extracted with 1:1 solution of ethylacetate and water. Thin layer chromatography of it was carried out in Benzene:Pyridine:Acetic acid (40:10:1). System. A mixture of known Dansyl amino acids was also run as standard. The spot/identified to be DNS-Glycine (Rf, 0.38).

3.8 CARBOHYDRATE CONTENTS OF CTEps₁

Paper Chromatography: The protein sample CTEps₁ was assayed for its carbohydrate contents by Molisch test and found to contain a carbohydrate moiety. A sample of it was therefore analysed for its sugar contents by paper chromatography. The sample hydrolysed with 4M HCl for 8 hours was subjected to paper chromatography in various solvent systems. Three sugars having Rf Gal x 100: 69, 65 and 38 were found to be present in Butanol: Ethanol: Water (4:1:2.2).
Carbohydrate Analyzer: A sample of CTEps₁ (1mg) was hydrolysed with 4M HCl in a sealed tube for 8 hours. After hydrolysis the sample was dried in vacuum and taken in potassium borate buffer pH 8.0 and the sugar composition was determined quantitatively on Biotronic Autoanalyzer LC 8001. The results are shown in Table 5.

3.9. ENZYME ACTIVITY OF CTEps₁ ACCORDING TO KUNITZ

The enzyme activity was determined according to Kunitz. The reaction was carried out at pH 8.0 using Casein (10mg) in 0.1M Tris HCl buffer as substrate at 37°C. The Casein solution was first denatured at 100°C for 10 minutes, the solution was cooled down to room temperature. 50μg of CTEps₁ was then added to it and the mixture incubated at 37°C. The reaction was stopped after 30 minutes by the addition of Greel of 0.3M Trichloro acetic acid (TCA), the solution left for another 30 minutes for complete precipitation. The precipitates were removed by centrifugation at 5000 rpm for 10 minutes. The optical density was recorded at 280nm against a blank solution. The activity units were calculated according to Kunitz. The enzyme was found to possess an activity of 1040U. The protease activity with this method was reported for Ile, Leu and Val., suggesting that it is cleaving at one/all of these amino acids.
Fig. 7. The curve showing the effect of pH on enzyme activity.
Substrate Casein in 0.1M Tris-HCl
Temperature 37°C
3.10 EFFECT OF pH ON ENZYME ACTIVITY

The enzyme activity was determined both at acidic as well as alkaline pH however the activity was detectable only in the alkaline region. 2ml of the Casein solution (5mg/ml) in 0.1M Tris-HCl buffer was taken at pH 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0, separately denatured at 100°C for 10 minutes brought to room temperature and the pH of each was adjusted. 20µl (15mg/ml) of the enzyme solution was added to each of the tubes containing buffer of different pH. The tubes were incubated at 37°C for 2 hours with occasional shaking. The reaction was stopped by adding 8ml of 0.3M TCA. The samples were left for 30 minutes and centrifuged at 5000rpm for 10 minutes. The absorbance of the supernatent solution was determined at 280nm in presence of a blank solution. Figure 7 shows the effect of pH on enzyme activity. The optimum pH was found to be in the range of 8.5-9.0.

3.11 EFFECT OF TEMPERATURE

In order to determine the temperature range for this enzyme; experiments were carried out having Casein as a substrate, 5mg Casein dissolved in 1ml of 0.1M Tris HCl buffer adjusted to pH 8.5 was denatured by keeping at 100°C for 10 min. brought to room temperature as above,
Fig -8. The curve showing the effect of temperature on enzyme activity.
Substrate casein in 0.1M Tris-HCl pH 8.5
50μl (15mg/ml) of the enzyme solution was added to the samples and incubated at 25°, 30°, 35°, 40°, 45°, 50°, and 60°C for 1 hour. The reaction was stopped by addition of 6ml 0.3M TCA, left for 30 minutes and centrifuged. The supernatant was taken off and absorbance determined at 280nm using a blank solution prepared under similar conditions. Effect of temperature is shown in Figure 8. No activity was detectable above 40°C and below 25°C.

3.12 SPLITTING OF β-LACTOGLOBULIN

β-Lactoglobulin (10mg) was dissolved in 10ml deionized water. The solution was denatured as before its pH adjusted to 8.5 using 0.5M NaOH, CTEPS₁ (1mg) was added and the solution kept at 37°C. The pH was maintained for 6 hours by adding the base if required at an interval of 10 minutes and then left overnight. Fig. 9 shows the amount of sodium hydroxide utilized. The digested material was vacuum dried and peptide mapping was carried out using electrophoresis in one direction and paper chromatography in the other. This showed the development of nine bands with Sr-ninhydrin solution, corresponding to Val/Ile residues in β-lactoglobulin. The sequence of β-lactoglobulin is shown in Table 6.
Fig. 3: Amount of sodium hydroxide utilised to maintain the pH of β-lact during digestion with CTBPS.
TABLE - 6
AMINO ACID SEQUENCE OF $\beta$-LACTOglobulin

Leu-Ile-Val₁ - Thr-Gln-Thr-Met-Lys-Gly-Leu-Asp-Ile-Gln₂
      - Lys-Val₃ - Ala-Gly-Thr-Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-
      - Asp-Ile-Ser-Leu-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg-Val₄ - Tyr₅
      - Val₆ - Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-
      - Leu-Gln-Lys-Trp-Glu-Asn₆₇ - Glu-Cys-Ala-Gln-Lys-Lys-Ile-Ile₈
      - Ala-Glu-Lys-Thr-Lys-Ile-Pro-Ala-Val₉ - Phe-Lys-Leu-Asp-Ala-
      - Ile-Asn-Glu-Asn-Lys-Val₁₀ - Leu-Val₁₁-Leu-Asp-Thr-Asp-Tyr-Lys-Lys-
      - Tyr-Leu-Leu-Phe-Cys-Met-Glu-Asn-Ser-Ala-Glu-Pro-Glu-Gln-Ser-Leu₁₂
      - Val₁₃ - Cys-Gln-Cys-Leu-Val₁₄ - Arg-Thr-Pro-Glu-Val₁₅ - Asp-Asp-Glu-Ala-Leu₁₆
      - Glu-Lys-Phe-Asp-Lys-Ala-Leu-Lys-Ala-Leu-Pro-Met-His-Ile-Arg₁₇
      - Leu-Ser-Phe-Asp-Pro-Thr-Leu-Gln-Glu-Glu-Gln-Cys-His-Ile₁₈.
3.13 SPLITTING OF SYNTHETIC PEPTIDES

Initial studies showed that the enzyme probably cleaves at Valine / Isoleucine residues.

Synthetic peptides containing Valine/Isoleucine as a residue were therefore employed to confirm the site of cleavage. 5ug each of a number of synthetic peptides were dissolved in 50ul of 0.2M NaHCO₃, pH 8.2 and to it was added 5ul of CTEps followed by incubation at 37°C for varying time periods. The digested peptides were subjected to paper chromatography using a modified procedure. The chromatogram showed the cleavage at valine and confirmed its specificity. The following peptides* were used.

1) L-Valyl-L-Methionine  9) L-Valyl-L-Glycine
2) L-Valyl-L-Serine    10) L-Valyl-L-Proline
3) L-Valyl-L-Phenyl alanine  11) L-Valyl-L-Leucyl-L-Serine
4) L-Valyl-L-Valine    12) L-Alanyl-L-Valyl-L-Leucine
5) L-Valyl-L-Aspartic acid  13) L-Isoleucyl-L-Glutamyl-L-Thr.
6) L-Valyl-L-Alanine    14) L-Isoleucyl-L-Isoleucyl-L-Asp.

*Synthetic peptides were received through the courtesy of Professor Dr. Wolfgang Voelter, Tuebingen University, Tuebingen, West Germany.
Fig. 10 Elution pattern of peptides on Dowex 50 (45x1.0 cms) eluted with 0.02M pyridine acetate buffer linear pH gradient 2.8-3.2; 3.2-4.0; 4-5.0; 5.0-6.0.

Fraction volume 5ml; flowrate 30ml; detection with ninhydrin.
3.14 SEPARATION OF CTEp-F ON DOWEX 50 x 2

The pooled and concentrated peptide fraction CTEp-F was subjected to a column of Dowex 50 x 2 (45x10 cm) and eluted with pyridine acetate buffer 0.02M using a linear gradient of 2.8 + 3.2; followed by 3.2 + 4.0, 4.0 + 5.0 and finally 5.0 + 6.0. The temperature of the column was maintained at 55°C. Fractions of 5.0ml were collected. 400μl was drawn off from alternate fractions and 600μl HCl (10M) was added to it. The tubes sealed and kept at 110°C for 16 hours. The hydrolysed samples were neutralised with NaOH to pH 5.5 and treated with 1ml of 1% ninhydrin solution in methyl-cellusolve at 100°C for 45 minutes. The optical density was read at 570nm using Spectronic 21 (Bausch and Lomb). Fig.10 shows the elution pattern of peptides from Dowex 50. The fractions were pooled and dried in vacuo. The samples were taken in deionised distilled water stored at 4°C.

3.15 AMINO ACID ANALYSIS OF PEPTIDES

A known quantity was taken from each of the separated peptide fractions and hydrolysed under vacuum in a sealed tube as described earlier. The hydrolysed samples were vacuum dried; taken in sodium citrate buffer
### TABLE - 7

**AMINO ACID COMPOSITION OF PEPTIDES (µ MOLES / 100 µ MOLES)**

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**Additional Information:**

- Val, Ser, Tyr, Glu
- Amino acid composition
- Peptide retention volume
pH 2.2 and subjected to analysis on Biotronic Amino acid analyzer LC 6001. The amino acid composition is shown in Table 7.

3.16 N-TERMINAL ASSAY OF PEPTIDES

Peptide samples were dissolved in 0.2M NaHCO₃ and an equal amount of 0.25% Dansyl chloride in acetone was added. The pH checked and adjusted to 8.9. The solution was kept in an oven at 37°C for about 16 hours. Excess Dansyl chloride removed, and the samples hydrolysed for 8 hours at 110°C using 6M HCl. Hydrolysed samples were then vacuum dried and extracted with ethyl acetate:water (1:1). Thin layer chromatography was carried out in Benzene:Pyridine:Acetic acid (40:10:1). Fluorescent DNS-amino acid were observed under uv light 254nm and N-terminal amino acids were identified are shown in Table 7.

3.17 ANTIMICROBIAL ACTIVITY

The separation on Sophadex G-100 chromatography was not well defined peaks as such component were not separated. However, the top of the peak was taken for preliminary antibacterial activity test.
TABLE - 8

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<tr>
<td>Nisseria cattarhalis</td>
<td>+++</td>
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<tr>
<td>Proteus mirabilis</td>
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<td>Proteus morgani</td>
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<td>Proteus vulgaris</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
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<tr>
<td>Salmonella typhi</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Salmonella typhi A</td>
<td>+++^4</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Salmonella typhi B</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Salmonella typhi LT1</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Sarcina lutea</td>
<td>+++</td>
<td>+</td>
<td>++</td>
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<td>Shigella sonni</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Zone of inhibition upto 10mm.
2. "  "  "  "  "  "  15mm.
3. "  "  "  "  "  "  25mm.
4. "  "  "  "  "  "  35mm.
5. No zone of inhibition = -

Peptides D3, D6 and D8 did not show any inhibitory action.
The fractions obtained from chromatography on Dowex 50 were subjected to antimicrobial testing. Table 8 shows the activity of 9 peptide(s) against 24 common bacterial strains. At least four fractions were found to be active against 10-13 bacterial strains. These tests were routinely performed using standard procedures as described in Section 2.13. In all cases Kanamycin 12.5mg/ml was used as the standard antibiotic.
4 DISCUSSION

4.1 CHEMICAL ASPECT

FRACTION CTEpS₁ (VALINEASE)

There is no report in the literature on the chemical analysis of the culture filtrate of *Candida tropicalis* or *albicans*, although there are numerous studies on the cellular extracts. These have revealed the presence of biologically active metabolites of high molecular weights, mainly belonging to carbohydrates, proteins, conjugated proteins and peptides. There are some low molecular weight fractions but their chemical identification has not been established.

Our investigation has been aimed at studying the proteins and peptides in the culture filtrate of the *Candida tropicalis*. The study on the cell free broth of this culture has revealed two high molecular weight protein peaks (CTEpS₁ and 2) and a number of peptides of varying chain lengths.

The protein CTEpS₁ has been studied further and it has been observed that it is a conjugated protein with a carbohydrate moiety attached to it. Its early elution on Sephadex G-200 column (Fig. 5) suggests high
molecular weight, but this could be deceptive as glyco- and sialoglyco- proteins do not separate strictly according to their molecular weights on cross linked dextran gels. In fact a recent study shows that the actual molecular weight of sialoglyco-/glycoproteins could be 8-9 times less than the molecular weight obtained on cross linked dextran gels. Estimates on SDS- Polyacrylamide gel-electrophoresis using different gel-percentages and standard protein markers have shown fraction CTEpS₁ to have a molecular weight of 40,000 ± 7000. Even this may be slightly high as in glycoproteins the carbohydrate moiety exhibits little affinity for sodium dodecyl sulphate, while the protein moiety binds only nominally with it. This results in overall decreased binding ratio of glycoproteins with SDS and hence there is decreased electrophoretic mobility.

Protein CTEpS₁ has been found to possess proteolytic activity as determined by Kunitz method. The activity is specific, involving leucine, isoleucine and valine and is of the order of 10-10U. The proteolytic activity of CTEpS₁ was tested with β-lactoglobulin. This globulin has 22 Leu, 10 Ile and 9 Val. After digestion with CTEpS₁ only 9 ninhydrin-positive spots were observed.
This suggests that cleavage is occurring either at Val or at Ile and either one or two cleaved peptides respectively are not resolved in peptide mapping, or certain preceding or following amino acids restrict the digestion.

In order to ascertain the cleavage site a number of synthetic peptides were digested with CTEpS₁. Some of these peptides had Ile and others Val residue. A dipeptide Val-Val was also studied. The results of all these digestions show that the splitting occurs at Val residue and that any preceding or following amino acid does not prevent its activity. The probable reason for 9 spots in the digested β-lactoglobulin peptide mapping is that the peptide leu-Ile-Val (1), and leu-val (7) are overlapping. The enzyme is therefore highly specific for cleaving at valine and has been designated as Valinease. The optimum temperature has been found to be 35-40°C, and the optimum pH between 8.5-9.0 suggesting that it is an alkaline protease. The temperature and pH is the same as that of other site-specific enzymes namely trypsin, chymotrypsin and staphlococcal protease (Table 9).
TABLE - 9

OPTIMUM TEMPERATURE & pH OF SOME ALKALINE PROTEASES

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>TRYPsin</th>
<th>CHYMOTRYPSIN</th>
<th>STAPHYLOCOCCAL CTEPS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.0</td>
<td>7.8-8.0</td>
<td>7.8</td>
</tr>
<tr>
<td>Temp.</td>
<td>37°C</td>
<td>37°C</td>
<td>37°C</td>
</tr>
</tbody>
</table>

The amino acid composition of the enzyme Valinecase shows 29% Gly, 9% Ala, 8% His, 8% Glu, 7% Cys, 5% Arg, 5% Asp, 5% Pro (Table 1) accounting for 76% of the total amino acids. This also suggests that it should have a number of S-S bonds in its secondary structure and is predominated by hydrogen bondings as 38% of its composition comprises of Gly and Ala. The amino acid composition of Valinecase is compared with trypsin and chymotrypsin in Table 10.

The N-terminal has been found to be Gly.

Glucose forms a major part of this carbohydrate moiety. This is rather unusual as the glycoproteins rarely contain glucose.
TABLE 10

AMINO ACID COMPOSITION OF TRYPsin, CHYMOTRYPSIN AND VALINEAS (RESIDUES/1000)

<table>
<thead>
<tr>
<th></th>
<th>Cow. Trypsin</th>
<th>Cocconase</th>
<th>Streptomyces griseus</th>
<th>Bovine chymotrypsin</th>
<th>Valinease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>62.78</td>
<td>60.18</td>
<td>31.87</td>
<td>54.32</td>
<td>33.2</td>
</tr>
<tr>
<td>His</td>
<td>13.45</td>
<td>18.5</td>
<td>4.9</td>
<td>7.81</td>
<td>83.6</td>
</tr>
<tr>
<td>Arg</td>
<td>8.96</td>
<td>27.77</td>
<td>41.19</td>
<td>16.46</td>
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<tr>
<td>Asp</td>
<td>98.65</td>
<td>120.37</td>
<td>88.76</td>
<td>89.71</td>
<td>53.0</td>
</tr>
<tr>
<td>Thr</td>
<td>44.84</td>
<td>74.07</td>
<td>80.43</td>
<td>94.65</td>
<td>28.0</td>
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<tr>
<td>Ser</td>
<td>147.44</td>
<td>106.48</td>
<td>69.64</td>
<td>123.86</td>
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<tr>
<td>Glu</td>
<td>52.78</td>
<td>69.44</td>
<td>85.33</td>
<td>58.43</td>
<td>81.5</td>
</tr>
<tr>
<td>Pro</td>
<td>40.25</td>
<td>41.66</td>
<td>39.23</td>
<td>35.8</td>
<td>53.1</td>
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<tr>
<td>Gly</td>
<td>12.1</td>
<td>101.85</td>
<td>139.28</td>
<td>95.88</td>
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<tr>
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<td>74.07</td>
<td>98.08</td>
<td>89.3</td>
<td>90.6</td>
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<tr>
<td>Leu</td>
<td>76.29</td>
<td>92.59</td>
<td>87.29</td>
<td>92.18</td>
<td>34.0</td>
</tr>
<tr>
<td>Ser</td>
<td>8.96</td>
<td>4.62</td>
<td>13.24</td>
<td>7.81</td>
<td>19.2*</td>
</tr>
<tr>
<td>Glu</td>
<td>67.26</td>
<td>55.5</td>
<td>39.23</td>
<td>40.74</td>
<td>17.8</td>
</tr>
<tr>
<td>Pro</td>
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<td>55.5</td>
<td>53.94</td>
<td>77.36</td>
<td>31.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>44.84</td>
<td>41.66</td>
<td>40.21</td>
<td>16.87</td>
<td>9.3</td>
</tr>
<tr>
<td>Phe</td>
<td>13.45</td>
<td>23.14</td>
<td>27.95</td>
<td>26.74</td>
<td>19.1</td>
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<tr>
<td>Tyr</td>
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<td>13.88</td>
<td>ND</td>
<td>28.80</td>
<td>ND</td>
</tr>
<tr>
<td>yCys</td>
<td>51.81</td>
<td>18.5</td>
<td>29.91</td>
<td>41.15</td>
<td>66*</td>
</tr>
</tbody>
</table>

ND = Not determined

* = Determined after performic acid oxidation.
Peptides from Dowex 50 chromatography have been tested against 24 bacterial strains. Clear zones of inhibition have been seen after 24-48 hrs. incubation at 37°C. Peptides D₁, D₂ and D₇ are most active producing zones of inhibition against 19, 15 and 17 strains respectively (Table 8). Increase in the initial dose serves to increase the lethality. Peptide fraction D₁ has been found to be more active than Kanamycin against an isolate of E.Coli. In case of Proteus morgani and Staphlococcus doubling of the original dose serves to produce zones of inhibition.

Peptides D₅ and D₉ have been found to be active against 10-12 bacterial strains.

All peptides have been found to be inhibitory for Nisseria Cattarhalis and Corynebacterium hoffmannii.
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