A MURINE MONOCLONAL ANTIBODY TO *PENICILLIUM ROQUEFORTI* TOXIN AND ITS APPLICATION FOR ENZYME-LINKED IMMUNOSORBENT ASSAY

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by
Joanna Yeu-Chin Ong

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A MURINE MONOCLONAL ANTIBODY TO PENICILLIUM ROQUEFORTI TOXIN AND ITS APPLICATION FOR ENZYME-LINKED IMMUNOSORBENT ASSAY

BY

JOANNA YEU-CHIN ONG

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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This Thesis Is Dedicated To My Dearest Parents,

Soh-Ching & Seng-Tee,

With Love

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Last, but not least, I wish to pay my tribute to Jesus Christ, the Saviour and Lord of my life, for His steadfast love and abundant blessings.

ABSTRACT

Penicillium roqueforti is used as a source of proteolytic and lipolytic enzymes in the production of blue-veined cheeses. However, many strains have the ability to secrete a secondary metabolite, *P. roqueforti* toxin (PRT). PRT is carcinogenic for rats, and impairs cell metabolism by inhibiting nucleic acid and protein syntheses. Recently, a radioimmunoassay which employs antiserum has been developed for the detection of PRT in cheese. This assay, however, requires special handling and disposal of radioactive material. In addition, it inherently suffers from limited availability of appropriate antisera. The purpose of this research was to develop a monoclonal antibody against PRT to be utilized in an enzyme-linked immunosorbent assay (ELISA).

A PRT-producing strain of *P. roqueforti* (ATCC no. 10110) was cultivated in a synthetic medium containing 2% yeast extract and 15% sucrose. Production of PRT was low (0.20 to 31 mg/L), and consequently a commercial PRT, which was further purified, was used for further research.

Purified PRT was conjugated to two structurally different protein carrier:bovine serum albumin (BSA), and egg-white ovalbumin (OV). Subsequently, the first conjugate was used for the immunization of BALB/c mice while the second for screening antisera and hybrids. Fusion of immune spleen and myeloma cells (P3X63-Ag8.653) was performed and the resulting hybridomas were screened for antibody

production using an indirect, non-competitive ELISA with the PRT-OV conjugate as a coating antigen. Performed fusion had a frequency of 1.2×10^{-5} and resulted in 20 hybridomas secreting antibodies against PRT. Of these, monoclonal antibodies (MAb, IgM) secreted by a 1H9 hybrid were highly reactive with PRT (100%), cross-reactive with PR-imine (70%) and exhibited no reactivity with PR-alcohol. These results indicate that the MAb-1H9 predominantly binds to PRT at other than its toxic site. MAbs were further propagated as ascitic fluid and used to develop a competitive ELISA. Several solvents were tested for compatibility with MAbs, and the results indicated that 10% acetonitrile in buffer caused no interference in ELISA and resulted in the best standard curve for PRT. The detection limit of the developed ELISA was 10 ng/assay. Comparison of the ELISA with the RP-HPLC method for the quantification of PRT in the range of 5 to 10^3 ng gave good correlation with r^2 of 0.9840.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HPLC	High-performance liquid chromatography
MAb	Monoclonal antibody
OV	Ovalbumin (egg-white)
PAb	Polyclonal antibody
PBS	Phosphate buffered saline
PDA	Potato dextrose agar
PEG	Polyethylene glycol
PRI	*Penicillium roqueforti* imine
PR-OH	*Penicillium roqueforti* alcohol
PRT	*Penicillium roqueforti* toxin
R_f	Relative mobility
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TLC	Thin-layer chromatography
UV	Ultraviolet
YES	Yeast extract with sucrose

I. INTRODUCTION

Mouldy food has been known to cause disease since the middle ages. Ergotism or "holy fire" is the earliest and best known form of mycotoxicosis (Davies and Diener, 1978). However, full scientific recognition was not given to the mycotoxin problem until it was discovered that the aflatoxins were responsible for the outbreak of the "Turkey x" disease causing the deaths of a large number of turkeys in England in 1960 (Blunden *et al.*, 1991). Since 1968, more than 100 species of fungi have been reported to produce over 300 mycotoxins, all having different characteristics with regard to their toxic effects on hosts (Polonelli *et al.*, 1984; Blunden *et al.*, 1991). Among them, some of the *Penicillium spp.* are known to produce toxic metabolites such as patulin, ochratoxin, sterigmatocystin, rubratoxin, penicillic acid, citrinin, roquefortine, and PR toxins (Davies and Diener, 1978).

Penicillium roqueforti toxin (PRT) is a secondary metabolite secreted by some strains of *P. roqueforti* (Wei *et al.*, 1973). This fungus was first isolated from mouldy grain and silage that was associated with the outbreak of bovine abortion and placental retention in cows (Still, 1973; Wei *et al.*, 1973). It has been reported that *P. roqueforti* can produce a number of mycotoxins including eremofortins, A, B, C, D (EA, EB, EC, ED), and PRT; the latter compound being the most toxic metabolite produced by *P. roqueforti* (Moule *et al.*, 1977a). The LD_{50} of PRT ranged from 5.8 to 11 mg/kg for mice and rats (Wei *et al.*, 1976). Furthermore, this compound impairs *in vitro* cell

metabolism by inhibiting nucleic acid and protein syntheses (Moule *et al.*, 1978; Moule *et al.*, 1980).

Penicillium roqueforti has been used in food manufacturing since 500 A.D. (Wei *et al.*, 1976). This mould is a source of proteolytic and lipolytic enzymes that are essential in the production of blue-veined cheeses such as, Roquefort, Danish Blue, Stilton, Gorgonzola, and others (Still, 1973; Frazier and Westhoff, 1976; Arnold *et al.*, 1978). Thus, discovering that *P. roqueforti* has the ability to secrete PRT has caused a concern to the dairy industries. Few analyses of cheeses for the presence of PRT have been attempted using thin layer (TLC) and high performance liquid (HPLC) chromatographies. None of these attempts, however, found PRT in cheeses, although low sensitivity and specificity of these methods may account for such results.

Recently, a radioimmunoassay (RIA) which employs polyclonal antiserum has been developed for the detection of PRT (Wei and Chu, 1988). In general, immunoassays have the potential to be very sensitive and highly specific tests. This, however, is dependent primarily upon the quality of antibodies employed. Although the proposed polyclonal antibody-based RIA is a sensitive and relatively fast procedure, it inherently suffers from limited availability of appropriate antisera. Moreover, this method requires special handling and disposal of radioactive material as well as the use of an expensive scintillator to detect a radiolabel.

The introduction of hybridoma technology by Kohler and Milstein (1975) has spurred the use of monoclonal antibodies (MAbs) in immunoassays due to their improved specificity, production of an unlimited supply, and the advantages of a world-wide assay standardization. This type of antibody is aproduct of hybrid cells formed

by fusion of antigen immunized B-lymphocytes, and myeloma cells. In addition, the introduction of an enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1971) has offered an elegant and safe replacement of RIA for the analysis of food products.

The overall purpose of this study was to develop a MAb against PRT that can be utilized in an ELISA for the detection of this toxin. The specific objectives of this research thesis are :

(1) To produce PRT from *P. roqueforti* culture.

(2) To develop and characterize MAb to PRT.

(3) To develop ELISA with the use of PRT-MAb for the detection of PRT.

II. LITERATURE REVIEW

1. Introduction

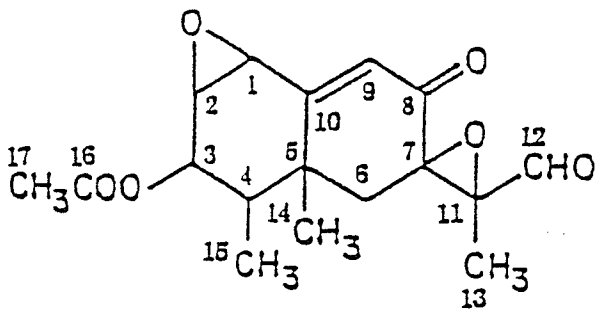
Penicillium roqueforti toxin is a toxic secondary metabolite produced by certain strains of *P. roqueforti.* During the last two decades, the toxicity, biosynthesis of PRT and the growth conditions of *P. roqueforti* were investigated by several groups of researchers (Wei *et al.*, 1975; Polonelli *et al.*, 1978). In addition, a number of semi-quantitative thin-layer chromatography methods and an immunoassay have been proposed for the determination of PRT in a variety of media.

2. Toxicity of PRT

Penicillium roqueforti isolated from toxic silage and grains was associated with cases of bovine abortion and placental retention (Wei *et al.*, 1973). This organism was later found to secrete several toxic metabolites, with PRT being the most toxic one (Moule *et al.*, 1977a). In addition, several strains of *P. roqueforti*, isolated from a number of blue cheeses, were also found to produce PRT (Polonelli *et al.*, 1984). In 1975, Wei *et al.* determined the chemical structure of PRT (Fig. 1), and more recently, the structures of other closely related compounds such as PR-imine, PR-alcohol and eremofortins A, B, C, and D were elucidated (Fig. 2).

Several research groups have investigated the toxicity of PRT on mice, rats and cultured cells. The earliest report by Wei *et al.* (1976) demonstrated that the

Figure 1. The structure of PR toxin.


O
O
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CHO
12
17
16
CH3COO
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2
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CH3
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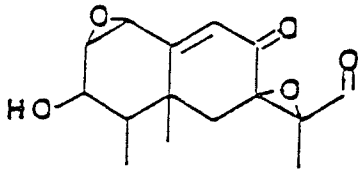
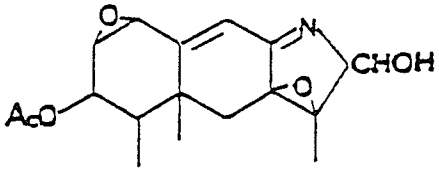
CH3
CH3
13

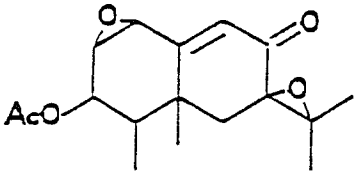
Figure 2. The structure of PR toxin related compounds.



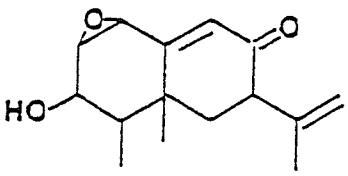
PR-alcohol



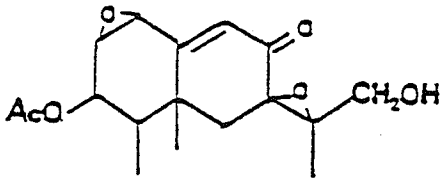
PR-imine



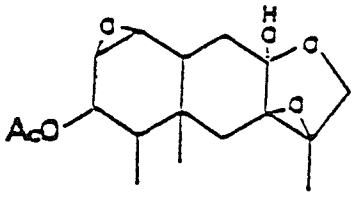
Eremofortin A



Eremofortin B



Eremofortin C



Eremofortin D

intraperitoneal LD_{50} values of PRT for weanling rats and mice were 11 and 5.8 mg/kg, respectively, while the oral LD_{50} value was 10 times greater for both animals. Mice, when fed with lethal doses of PRT, demonstrated in hypokinetics (Wei *et al.*, 1976; Arnold *et al.*, 1978; Polonelli *et al.*, 1978), followed by adipsia, anorexia (Arnold *et al.*, 1978) and breathing difficulties (Wei *et al.*, 1976; Arnold *et al.*, 1978; Polonelli *et al.*, 1978). Death of animals was shown to occur as early as two hours after PRT administration, although some mice survived up to a few days (Arnold *et al.*, 1978; Polonelli *et al.*, 1978).

Furthermore, PRT was found to inhibit nucleic acid and protein syntheses (Arnold *et al.*, 1978; Moule *et al.*, 1978; Chalmers *et al.*, 1981; Chang *et al.*, 1991). The mechanism of inhibition was investigated *in vitro* using rat-liver nuclei and polysomes (Moule *et al.*, 1976; Moule *et al.*, 1977a; Moule *et al.*, 1977b; Moule *et al.*, 1978). It has been reported that PRT impairs both, transcription carried out by nuclei, and translation promoted by polysomes. It is most likely that PRT inhibits both, initiation and elongation of the polynucleotide chain during the transcription process, while the translation process is affected *via* inhibition of the RNA polymerase. Results of recent studies have demonstrated that PRT induces DNA-protein cross-links in chromatin of isolated rat-liver (*in vitro*) and cultured cells (*in vivo*). In this process, the aldehyde group at position 12 on the PRT molecule (Fig. 1) is required for the induction of cross-linking in chromatin. Although the mechanism is not clear, it was suspected that methylene bridges between nucleic acid and protein are involved in this complex formation (Moule *et al.*, 1980). Studies of relationships between the chemical structure and the biological properties of PRT and related compounds indicated that

the aldehyde group at position 12 is directly implicated in the biological activity of the tested compounds (Moule *et al.*, 1977a).

It has been shown that mycotoxins such as EA, EB, and PR-imine which are related to PRT but lacking the aldehyde group in their structure, exhibit diminished toxicity (Arnold *et al.*, 1978). Thus, it is conceivable that the PRT toxic site is primarily composed of the aldehyde group. For instance, the two epoxide radicals (C_1-C_2 and C_7-C_{11}) do not play an important role since their presence in EA, EB and PR-imine does not implicate in biological activity (Moule *et al.*, 1977a). When comparing two equally toxic compounds, PRT and PR-OH, the acetylation of the eremophilane ring in position 3 did not change the toxic property.

Moreover, PRT exhibited inhibitory effects on DNA polymerases, α, β,and γ mitochondrial HCO_3^--ATPase activities, as well as mitochondrial respiration and oxidative phosphorylation in animal cells (Chang *et al.*, 1991). Furthermore, PRT was found to cause liver and kidney damage (Scott *et al.*, 1977), and to be mutagenic to *Salmonella typhimurium* (Hradec and Vesely, 1989), *Saccharomyces cerevisiae* as well as *Neurospora crassa* (Wei *et al.*, 1979). This toxin was also found to be carcinogenic to rats (Polonelli *et al.*, 1982) and recently its carcinogenicity was confirmed by Hradec and Vesely (1989) in the initiator tRNA acceptance assay.

3. Occurrence of PRT

Penicillium roqueforti toxin is a sesquiterpenoid compound, chemically known as 7 - acetoxy - 5,6 epoxy - 3,5,6,7,8,8a - hexahydro - 3',8,8a - trimethy - 3 - oxospiro[naphthalene -2(1H),2'- oxirane] - 3' - carboxaldehyde (Wei *et al.*, 1975). This

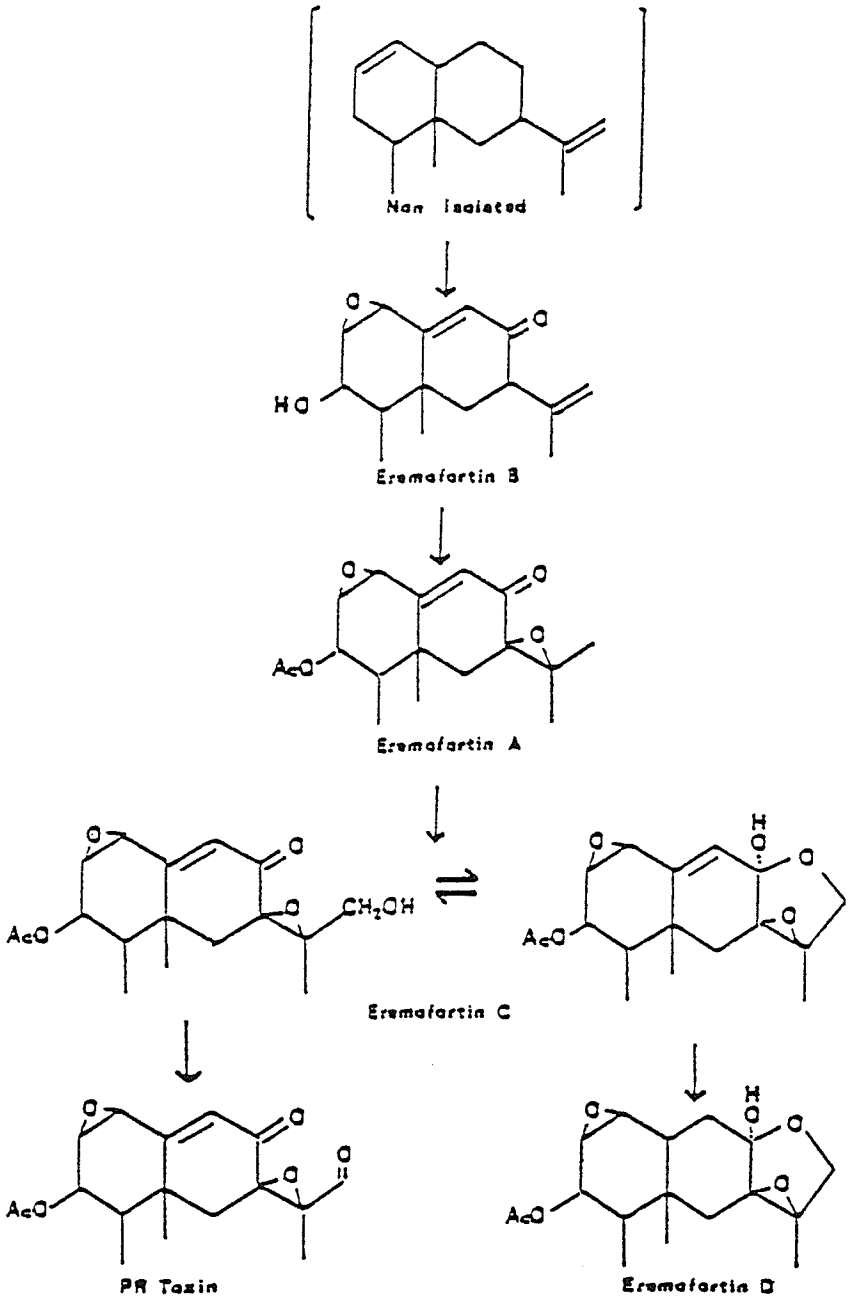
toxin resembles an eremophilane compound because of the similarities of the basic ring structure of these molecules. The evolvement of PRT, however, was believed to be from the sesquiterpenes (Moreau *et al.*, 1980).

The PRT closely related terpenoid compounds such as, eremofortin A, B, C, and D (EA, EB, EC and ED, respectively), were isolated from culture of the same *P. roqueforti* strains. The research by Moreau *et al.* (1980) was an attempt to study the syntheses of terpenoid compounds in order to understand the biochemical pathway used by *P. roqueforti* for PRT production. They hypothesized that PRT and its related mycotoxins are synthesized from their common carbon skeleton resembling the eremophilane ring structure *via* a cyclization of farnesyl pyrophosphate, followed by the rearrangement (Fig. 3).

Eremofortin C, which has a structure similar to PRT, was believed to be the precursor of PRT for two reasons. Firstly, the production of PRT by *P. roqueforti* was always associated with the production of EC in a way that an increase of PRT was accompanied by a decrease of EC (Moreau *et al.*, 1980). The second evidence was provided by Chang *et al.* (1985) who have identified and isolated an oxidase responsible for the oxidation of the hydroxyl group of EC and the subsequent conversion of EC to PRT. This enzyme always occurred in the time period that corresponded to the occurrence of PRT as proven by the time course studies (Chang *et al.*, 1985).

In addition, other eremofortin compounds such as EA, EB, and ED were identified to be related to PRT (Fig. 3). Although EA has a similar structure to PRT, it had failed to be proven as a direct precursor of PRT in the time course study. However, it was believed to be a precursor of EC. The latter compound was generated

Figure 3. Hypothetical biosynthetic pathway of *P. roqueforti* metabolites. The non-isolated skeleton is the hypothetical precursor for eremophilane-type sesquiterpenes (Moreau *et al.*, 1980)


Non Isolated
HO
Eremofortin B
AcO
Eremofortin A
AcO
CH2OH
Eremofortin C
AcO
PR Toxin
AcO
Eremofortin D

via a hydroxylation process of the aldehyde group of EA at position 12 (Moreau *et al.*, 1980).

Biosynthesis of PRT has been a subject of many investigations. Chalmers *et al.* (1981) studied the synthesis of PRT by growing *P. roqueforti* on a medium fortified with compounds thought to be potential PRT precursors. The results have shown that PRT arises from acetate *via* an isoprenoid biosynthetic pathway. In this pathway, mevalonate was formed from acetyl-CoA and its subsequent phosphorylation yielded isopentyl pyrophosphate. The assembly of isopentyl phosphates resulted in farnesyl pyrophosphate, which serves as the basic skeleton of PRT and other eremofortin compounds after the cyclization process (Chalmers *et al.*, 1981).

4. Production of PRT

Penicillium roqueforti strain that produces PRT, was isolated from mouldy grains and silage. The ambient conditions that enable the fungi to produce toxins were examined by growing them on synthetic media. Although many factors may affect the production of PRT during fermentation, some major factors that can be monitored in a laboratory environment were carefully assessed by several research groups. Those included strains of *P. roqueforti*, composition of fermentation media, temperature, pH, aerobicity of the culture, and the amount of light being provided during fermentation. The light factor was shown to be irrelevant to the production of PRT (Polonelli *et al.*, 1978; Chang *et al.*, 1991), while other factors which affect PRT production are discussed as follows.

4.1. Strains of fungi

Many strains of *P. roqueforti* were found to produce toxic secondary metabolites including eremofortin compounds and PRT. Therefore, a few research groups carried out experiments on different strains of *P. roqueforti*, and more than 15 *P. roqueforti* strains that are deposited in the American Tissue Culture Collection (ATCC) were found to produce PRT (Scott *et al.*, 1977; Wei and Liu, 1978; Chang *et al.*, 1991). In one experiment described by Scott *et al.* (1977), the ATCC strain no. 34906 was found to produce toxin at a maximum level of 770 mg/L of media over a period of 21 days and the level dropped immediately thereafter. Another experiment described by Wei and Liu (1978) revealed that the ATCC strain no. 6989 secreted PRT at a maximum amount of 300 mg/L, which occurred earlier at the 14th day of fermentation. Since different strains of *P. roqueforti* exhibit different behaviour in the production of a specific toxin, it is therefore very crucial to identify the time required for optimal PRT production, for each strain that is tested. The most common strain of *P. roqueforti* used for the production of PRT is the ATCC no. 10110. This strain was found to produce PRT at a maximum level of 250 mg/L between the 13 to 15th day of growth on medium with 2% yeast extract and 15% sucrose (Polonelli *et al.*, 1978; Wei and Liu, 1978).

Furthermore, the level of mycotoxin production is dependent on the morphology of the fungi used for fermentation. The size of inoculum and the density of spores may affect the fungal growth and consequently mycotoxin production (Shepherd and Carels, 1984). Sharma *et al.* (1980) reported that a small size of inoculum was associated with better mycelial branching and differentiation, which in

turn, resulted in a higher yield of aflatoxin production (Sharma *et al.*, 1980). The rationale behind this was that with a smaller spore population, the depletion rate of nutrients is slower which in turn stimulates better mycelial growth. According to the author, the diversified branching of mycelia would promote toxin production (Sharma *et al.*, 1980). On the other hand, the optimum density of inoculum is needed to stimulate better germination and branching of hyphae in later stages. Research has demonstrated the effect of different inoculum size to aflatoxin production. Apparently, smaller inoculum size (10^2-10^4) was better for the aflatoxin production than for the control (10^6) (Sharma *et al.*, 1980). Although studies on the effects of inoculum size on PRT production have not been carried out, a level of 10^6-10^8 spores/mL has been used as inoculum in the production of PRT (Chang *et al.*, 1991; Polonelli *et al.*, 1978; Wei *et al.*, 1973).

4.2. Nutrients for growth and PRT production

The composition of medium, particularly the nature and concentration of nutrients are the most important factors that may effect mycelial growth, sporulation and secondary metabolite production. It has been observed that mycotoxin production usually takes place when mycelial growth slows down due to the depletion of nutrients from the medium. The effect of the medium composition on PRT production was investigated by several researchers.

The synthetic medium composed of 2% yeast extract and 15% sucrose was used by Scott *et al.* (1977) to stimulate the PRT production by five different *P. roqueforti* strains. All five strains grew well and secreted PRT in the range of 44-530 mg/L. In

contrast, media supplemented with different sugars such as, mannitol and glucose (Ohmomo *et al.*, 1975) or other compounds such as succinic acid (Ohmomo *et al.*, 1977) and L-tryptophan (Scott *et al.*, 1977) did not support PRT production. Furthermore, attempts to replace sucrose with lactose, and sodium lactate comfirmed that sucrose is the best carbon source for PRT production, indicated by a toxin level of 500 mg/L (Medina *et al.*, 1985). Polonelli *et al.* (1978) also investigate the use of 2% yeast extract with different level of sucrose ranging from 5 to 35%. The results demonstrated that optimum PRT production occurred at 15% of sucrose.

More recently, Chang *et al.* (1991) showed that the production of PRT and EC was better when *P. roqueforti* was grown on a solid medium composed of cereals rather than legumes. Mould grown on corn and oat produced by far, the greatest amount of toxin. Moreover, it was reported that the addition of corn extract to a liquid medium composed of yeast extract and sucrose enhanced PRT production. When the medium was supplemented with 20% corn extract, the toxin level increased two- to three-fold over the level obtained when the medium contained only 2% yeast and 15% sucrose (Wei and Liu, 1978).

4.3. Temperature and pH

The temperature and pH were also found to affect fungal growth and consequently PRT production. Polonelli *et al.* (1978), reported the effect of temperature ranging from 4 to 37°C on the toxin production. The maximum amount of PRT production occurred over a broad range from 20 to 30°C. It is, therefore, evident that temperature is not a crucial factor with regards to PRT production.

However, Chang *et al.* (1991) showed that *P. roqueforti* strain ATCC no. 48936 produced the highest toxin level at 24° C and therefore, this temperature is the most commonly used in the production of PRT (Wei *et al.*, 1973; Scott *et al.*, 1976; Moreau *et al.*, 1980).

The effect of pH on the PRT production was studied by Polonelli *et al.* (1978) and Chang *et al.* (1991). Maximum toxin level occurred at pH 5.5 (Polonelli *et al.*, 1978) and a substantial decrease in the toxin level was observed when the pH was changed towards either alkalinity or acidity. In contrast, Chang *et al.* (1991) observed two pH optima at 4.0 and 4.5 for the production of PRT by different *P. roqueforti* strains. The discrepancies may be attributed to the differences between strains and the composition of the medium, since different media were used.

4.4. Effects of aeration

The growth of fungi occurs under aerobic and microaerobic conditions. However, PRT is produced only under aerobic conditions (Polonelli *et al.*, 1978). Conversely, the increased in aeration provided by a shaken culture was found to have little or no effect on toxin production (Polonelli *et al.*, 1978; Chang *et al.*, 1991). The reasons for this contradiction are still unknown, however, the effect aeration on the primary metabolism, which latter affects the secondary metabolism of fungi may account for this result.

In the primary metabolism, the two major glycolytic pathways, the pentose phosphate (PP) and the Embden-Meyerhof-Parnas (EMP) pathways are regulated by the oxygen level (Garraway and Evans, 1984). The PP pathway is favoured by the

relatively high aerobicity provided by the shaken culture, while the EMP is triggered by the lowering of the oxygen level. It is the EMP that provides toxin precursors such as, pyruvate and other primary metabolites, thus in static cultures, the EMP pathways predominates (Garraway and Evans, 1984). The accumulation of primary metabolites may eventually trigger the enzymes responsible for secondary metabolites where toxins are produced (Garraway and Evans, 1984).

Moreover, the high oxygen level provided by agitation would facilitate the conversion of any EC to PRT by catalyzing the oxidase, despite the fact that a lower amount of EC is usually present in the shaken than in the static culture (Chang *et al.*, 1991).

5. Detection methods for PR toxin

5.1. Chromatographic techniques

It is not uncommon that mould may concomitantly secrete several mycotoxins during their growth. In addition, these secondary metabolites may occur in very low quantities. Therefore, it is imperative that the isolation, detection and quantification of mycotoxins be performed by means of sensitive, high resolution chromatographic procedures. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) have been routinely used for the determination of mycotoxins. Of these techniques, the least convenient is GC because very often, samples have to be transformed into volatile derivatives such as, trimethylsilyl ether or methyl oximes (Gorst-Allman and Steyn, 1984).

5.1.1. Thin layer chromatography. This technique has been applied extensively in the detection of mycotoxins such as, aflatoxin B_1, B_2, G_1, and G_2, ochratoxin A, citrinin, patulin, penicillic acid, and sterigmatocystin (Scott *et al.,* 1979). The detection is usually carried out using a normal phase silica gel TLC, and different solvent systems. The systems include the chloroform based solvents such as, methanol/chloroform and acetone/chloroform as well as non-chloroform based solvents: benzene/acetate, ethyl acetate/methanol/ammonia, formic acid/ethyl ether, benzene/methanol/acetate and ethyl acetate/acetone/HCl. The developed plates are visualized either in visible or ultraviolet light, since some mycotoxins may not be visible under the ordinary light.

A number of qualitative and semi-quantitative TLC methods have been used to detect PRT. The common solvent system used for PRT analysis include methanol/ chloroform (Wei *et al.*, 1973; Lafont *et al.*, 1976); chloroform/methanol/ammonium hydroxide (Scott and Kennedy, 1976, Scott *et al.*, 1977); or toluene/ethyl acetate/ formic acid (Scott and Kanhere, 1979; Scott *et al.*, 1977). After the plates have been developed, they are examined either by exposure to ultra-violet light or by spraying concentrated sulphuric acid, and charred with high heat. PRT which would fluoresce under UV light, is characterized by a dark blue spot when exposed to short-wave followed by long-wave UV light (Wei *et al.*, 1973; Scott and Kanhere, 1979), and became visible as a yellow spot in visible light thereafter (Wei *et al.*, 1973). In contrast, when TLC plates are sprayed with concentrated HCl and charred, PRT appears as yellowish-brown coloured spot under visible light (Wei *et al.*, 1973). Comparing the colour intensity of the toxin with known amount of standard PRT, it is possible to semi-

quantify the amount of PRT present in cheese (Scott and Kanhere, 1978). The method, however, relies on a visual comparison of unknown samples with standard PRT and yielded inaccurate results. Furthermore, since TLC is a simple separation technique, it has been frequently employed as a clean up step prior a more sensitive chromatographic method such as HPLC.

5.1.2. High performance liquid chromatography. This method has several advantages over other chromatographic techniques such as high sensitivity and accuracy. In addition, HPLC analyses can be performed usually within a maximum of 30 minutes due to the high pressure exerted by pumps that are employed in the system. Furthermore, samples which are thermally unstable or too polar to be analyzed by GC can be used, without further derivatization, for HPLC analyses. For the determination of mycotoxins, which display different UV maxima, HPLC offers the advantage of having variable wavelength UV detectors.

Recently, a normal-phase HPLC technique has been used to study the occurrence of different metabolites such as, EA, EB, EC, as well as PRT during fermentation. Since these metabolites have UV absorption maxima in the region of 250 nm, a 254 nm filter was chosen for the detection of these compounds (Moreau *et al.*, 1979; Moreau *et al.*, 1980; Chang *et al.*, 1991). Solvents employed in the proposed HPLC system include n-hexane and tetrahydrofuran (75:25, v/v), and chloroform. The detection limit of 10 ng of PRT was obtained using this method.

Danielli *et al.* (1980) reported a micro HPLC system for the determination of PRT in culture broth. This system composed of a reversed-phase μC_{18}-Bondapack

column, and the technique utilized a mixture of water-acetonitrile as a solvent. The detection limit was 0.2 ng for PRT, which indicated great improvement as compared to the aforementioned normal phase HPLC system. A reversed-phase HPLC system for PRT detection was also briefly mentioned by Gorst-Allman and Steyn (1984). Analysis of PRT in cheese, however, was not reported until recently, when Siemens and Zawistowski (1991) developed a reversed-phase HPLC system for the determination of PRT in cheese. The system consisted of a μC_{18}-Bondapack column and UV detector with a 254 nm filter. The solvent system consisted of a mixture of methanol and water in ratios of 70:30 (v/v) and 65:35 (v/v). The detection limit was as low as 3 ng, which is more sensitive than the detection limit of the normal phase HPLC, but not as sensitive as the micro-HPLC.

Although HPLC can be used to detect PRT with great sensitivity and reproducibility, there are a few drawbacks associated with this method. The method requires extensive sample clean-up prior to analysis and it is time consuming, since only one sample can be analyzed at one time. Furthermore, instruments as well as solvents are expensive and analysis can be only performed by specially trained personnel.

5.2. Immunochemical Techniques

Recently, immunochemical techniques for the detection of mycotoxins have gained popularity due to their simplicity and the lack of the disadvantages of HPLC methods. Immunoassays were initially developed for clinical diagnoses in the medical field. The first immunoassay was reported by Yalow and Berson (1960) for the

detection of insulin. Since then, immunoassays have been modified for different applications in medical sciences (Clifford, 1985). All immunoassay techniques are based on the reversible, non-covalent binding of antigen and antibody, using a labelled form of one or the other to quantify the system.

5.2.1. Preparation of immunogen. Essential elements of any immunoassay are antibodies. Antibodies are raised in animals such as mice, rats, rabbits, goats etc. *via* multiple immunizations with an appropriate immunogen against which antibodies are being produced. In order to be immunogenic, compounds have to be foreign to the animal used for injections, chemically complex and be of high molecular weight. Proteins and carbohydrates are considered to be good immunogens. In contrast, chemically complex compounds such as mycotoxins, which have molecular weight below 1,000 are not immunogenic, although they may be able to bind to antibodies. These compounds, which are known as haptens, may gain immunogenicity when conjugated to larger macromolecules, usually proteins. When conjugated to a protein carrier, a hapten can induce an immune response leading to the production of antibodies.

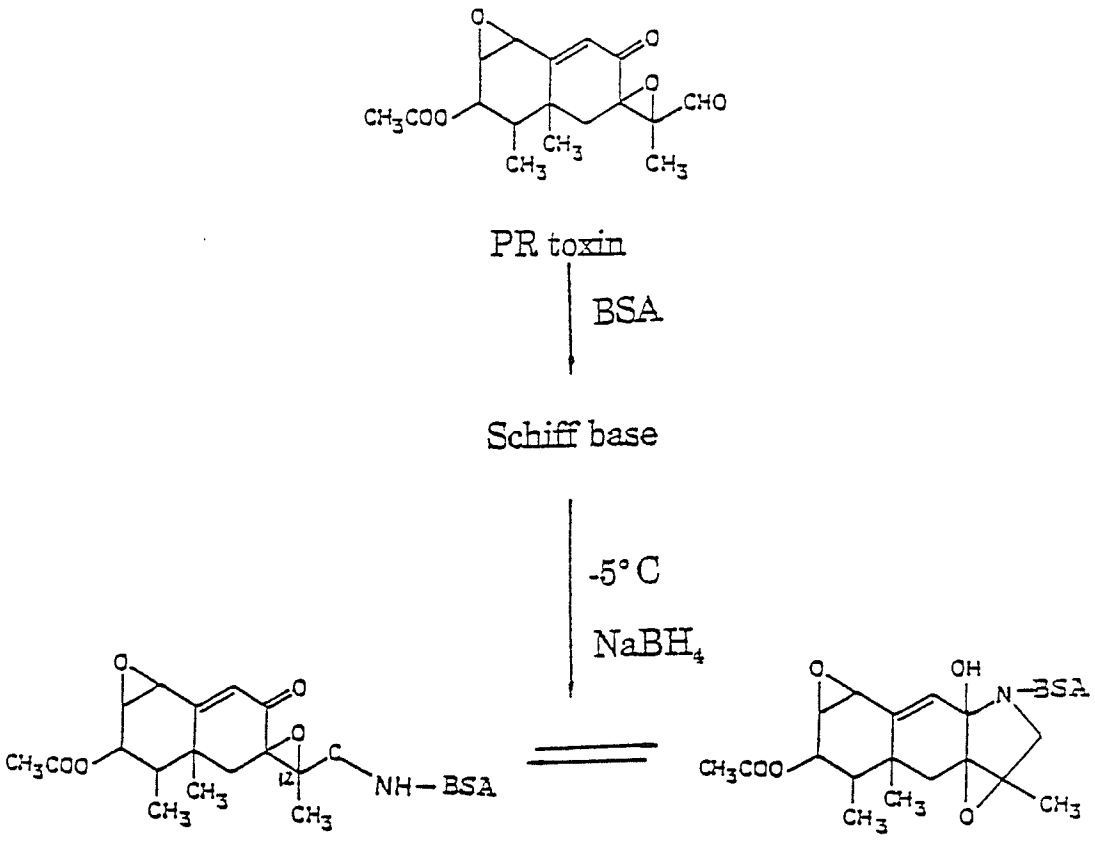
The choice of a carrier for the conjugation is very important because it is the part of the molecule that is responsible for stimulating the immunogenic response (Benjamini and Leskowitz, 1988). Globulin fractions, serum albumins of various species, haemocyanin, thyroglobulin, fibrinogen and ovalbumin, and even certain polypeptides are used as protein carriers (Erlanger, 1980). The carrier should be soluble to yield a soluble conjugate. Although solubility of the conjugate is not desired

for triggering immunogenic responses, it is important for further applications of the conjugate in ELISA techniques. It has been reported that, conjugates consist of bovine serum albumin (BSA) are more soluble than those that contain proteins such as globulin or ovalbumin (Erlanger, 1980). Therefore, BSA is a preferred carrier for toxin conjugation to serve as a soluble antigen. In addition, when BSA is conjugated to haptens, the resulting conjugate becomes more immunogenic than those that are composed of a synthetic polypeptide carrier.

Another important feature of a carrier is its ability to bind a high number of haptens. This varies with the nature of the hapten as well as the availability of carrier functional groups. The readily available functional groups in BSA include the ϵ-amino group of lysine residues, the α-amino group, and the hydroxyl group of tyrosine, the sulfhydryl group of cysteine residues, and the imidazole group of histidine residues (Erlanger, 1980). Furthermore, a successful conjugation depends also on the nature of the reactive groups present on the haptens.

Mycotoxins are commonly conjugated to BSA using either the carbodiimide reaction, the mixed anhydride method or the reductive alkylation technique (Erlanger, 1980). Penicillium roqueforti toxin, which contains several functional groups including an aldehyde, an acetoxy, α, β-unsaturated ketones, three methyl groups and two stable epoxides (Wei *et al.*, 1975; Wei and Chu, 1988), can be conjugated to BSA using the reductive alkylation method (Fig. 4). In this conjugation process, the amino group of BSA forms a covalent bond with the aldehyde group of PRT *via* a Schiff base formation (Shaw *et al.*, 1984). Shaw *et al.* (1984) has reported that the highly reactive aldehyde group of PRT actually formed a Schiff base with the ϵ-NH_2 group of the

Figure 4. Conjugation of PRT to BSA by the reductive alkylation method (Wei and Chu, 1988).


PR toxin
BSA
Schiff base
-5° C
NaBH4
NH—BSA
N—BSA
OH

lysine residue, while the α, β-unsaturated ketone in position 8, could bind with the sulfhydryl group of cysteine. Since each BSA molecule has 62 lysine residues and only 1 cysteine residue, it is most likely that PRT would bind BSA *via* lysine residues forming a Schiff base. Although Shaw and coworkers (1984) did not find any evidence that PRT binds any other amino acids, they did not exclude the possible involvement of either hydrogen bonding or hydrophobic interactions in the reaction between the toxin and BSA. However, this hypothesis would need further investigation to confirm its reliability.

Shaw *et al.* (1984) have also hypothesized that the efficient conjugation process could only be achieved when the concentration of both BSA and PRT are optimized. When concentration of BSA is too high, the free functional groups of the amino acid residues in the albumin molecules may become unavailable for ligand binding due to conformational changes imposed by aggregation. On the other hand, when a PRT level reached a saturation point, the binding between PRT and BSA would not increase with any further increase of the PRT amount.

The PRT-BSA conjugate is purified prior to immunization usually by dialysis (Wei and Chu, 1988). Next, the prepared conjugate is injected into an animal to stimulate the production of antibodies. Mice, rats, hamsters, guinea pigs, rabbits, and goats have been used as laboratory animals. However, the choice of animals is dependent on the kind and the amount of antibodies one choose to produce. Wei and Chu (1988) have produced polyclonal antibodies (PAbs) against PRT by immunizing rabbits with PRT-BSA conjugate. The PAbs were then used to develop an immunoassay for the detection of PRT.

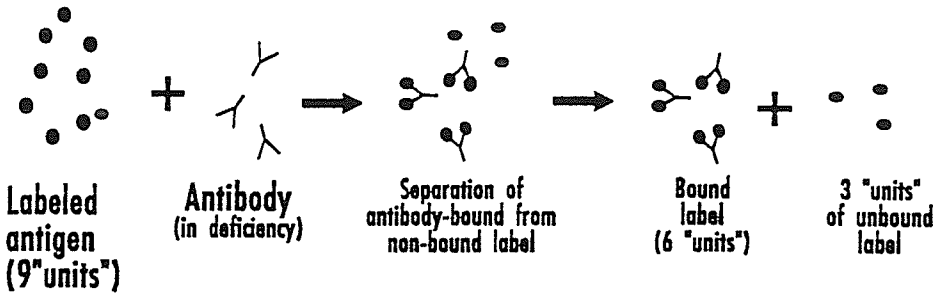
5.2.2. Radioimmunoassays. The first food application of an immunoassay was introduced by Porath (1968) for the detection of specific proteins in food extracts. The method, referred to as radioimmunoassay (RIA) had a detection limit of 1 pg protein/ml of extract. The high sensitivity was possible *via* the determination of extremely low concentrations of a radioisotope-labelled antigen or antibody.

The principle of RIA is illustrated in Figure 5. When a known amount of radioactively labelled antigen reacts with a limited amount of antibody, the antibody/labelled antigen complexes are formed. After the separation of these complexes from the free antigens, the radioactivity bound to the complexes is determined (Fig. 5a). Next, labelled antigen is pre-mixed with unlabelled antigen (Fig. 5b). The unlabelled antigen competes with the labelled antigen for the antibody and as a result, less label is bound to antibody than in the absence of unlabelled antigen. The more unlabelled antigen present in the reaction mixture, the smaller the ratio of antibody-bound, radiolabelled antigen to free, radiolabelled antigen. This ratio can be plotted as a function of the concentration of the unlabelled antigen used for competition (Fig. 5c). Consequently, an unknown concentration of antigen in a sample can be determined by mixing a sample with predetermined amounts of labelled antigen and antibody, and measuring the ratio of bound/free radioactivity. This is then compared with data on the standard curve (Fig. 5c).

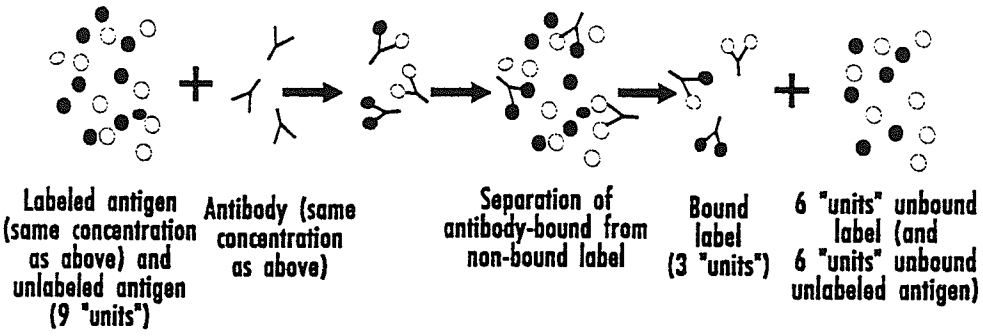
Radioimmunoassay has been used in the food area for the detection of low molecular weight molecules such as, drugs and mycotoxins (Newsome, 1986). For example, RIA has been used for the detection of aflatoxin B_1 (Chu and Ueno, 1977),

Figure 5. Radioimmunoassay. (a) Amount of labeled antigen bound to antibody after incubation of constant amounts of antibody and labeled antigen. (b) Competition of non-labeled and labeled antigens for antibody. (c) The standard curve showing the inhibition of binding of labeled antigen to antibodies by non-labeled antigen. (Benjamini and Leskowitz, 1988).

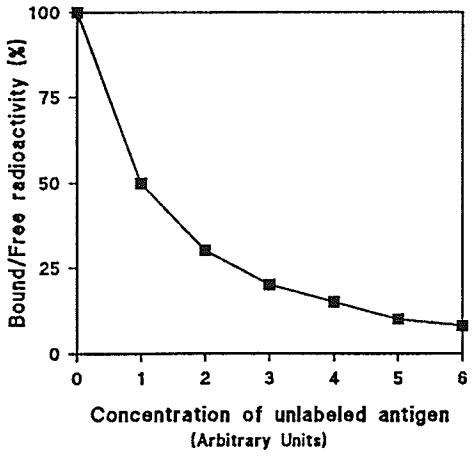
(a)


Labeled antigen (9"units")
Antibody (in deficiency)
Separation of antibody-bound from non-bound label
Bound label (6 "units")
3 "units" of unbound label

(b)


Labeled antigen (same concentration as above) and unlabeled antigen (9 "units")
Antibody (same concentration as above)
Separation of antibody-bound from non-bound label
Bound label (3 "units")
6 "units" unbound label (and 6 "units" unbound unlabeled antigen)

(c)


Bound/Free radioactivity (%)
100
75
50
25
0
0
1
2
3
4
5
6
Concentration of unlabeled antigen
(Arbitrary Units)

ochratoxins (Chu *et al.*, 1976), and T-2 toxin (Chu *et al.*, 1979).

Wei and Chu (1988) developed a PAb-based RIA to determine PRT in blue cheeses. The method has a detection limit of 1-2 ng of toxin per assay. Cross-reactivity studies showed that PAbs employed in the developed RIA had broad specificity. They reacted with PR-imine (100%), PRT (71%), tetrahydro-PRT (50%), EC (30%), AC-EC (10%) and ED (1%). All measurements were made by utilizing tritiated PRT as a marker.

Although most RIAs exhibit high sensitivity, they have not been accepted by most food scientists for several reasons. These include the difficulties associated with handling and disposal of radioactive materials, the expensive scintillators, and most importantly, the concern of potential health-hazardous radioactivity in close proximity with food products.

5.2.3. Enzyme immunoassays. An alternative to RIA technique came with the introduction of an enzyme label. The first reports of food enzyme immunoassay (EIA) are attributed to Engvall and Perlmann (1971), where the detection of parasites in pigs initiated the second generation of food immunoassays. These assays gained popularity in food applications because a hazardous radioactive label was replaced by a harmless enzyme (Clifford, 1985). The most commonly used EIA today is an enzyme-linked immunosorbent assay (ELISA). The principle of ELISA is based on the concept of 'reagent excess', where the solid phase reagents are used for separation of a free label from a bound label, which also facilitates the removal of excess reagents after each step. There are several types of ELISAs. A typical non-

competitive, indirect ELISA is shown in Figure 6. Briefly, an excess of antigen is attached to a solid surface such as the inside wells of the ELISA plate. Unbound substances are removed by washing and the plate is incubated with a sample containing antibodies. Only antibodies reactive with the immobilized antigen are captured on the plate. After removing the excess of antibodies by washing, the plate is incubated with enzyme-labelled anti-immunoglobulins (second antibodies). The second antibodies bind to the first antibodies, labelling the whole complex. When substrate is added, it is converted to a coloured product *via* an enzymatic reaction. The intensity of the developed colour can be read in a spectrophotometer and is directly proportional to the amount of tested antibodies or antigens.

Enzyme-linked immunosorbent assays have been used in the analyses of a variety of mycotoxins such as, aflatoxins, ochratoxins and T-2 toxin where high sensitivity and minimal sample preparation are needed. The sensitivity of ELISA for aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1), ochratoxin (OA) and T-2 toxin (T-2) is presented in Table 1 (Chu, 1984). These results indicate the sensitivity of enzyme immunoassays when polyclonal antibodies were employed.

Polyclonal antisera, however, have several disadvantages. To produce specific antibodies, it is necessary to use a highly purified antigen of interest for immunization of animals, although most antigens are multivalent and multideterminant having many kinds of epitopes. In this respect, antibodies present in serum are usually a heterogenous mixture of immunoglobulins resulting in high level of cross-reactivity. Thus, polyclonal antibodies are characterized by a low specificity towards the antigen of interest. Furthermore, the supply of polyclonal antibodies is limited and dependent

Figure 6. A schematic representation of an indirect, non-competitive enzyme-linked immunosorbent assay (Benjamini and Leskowitz, 1988).

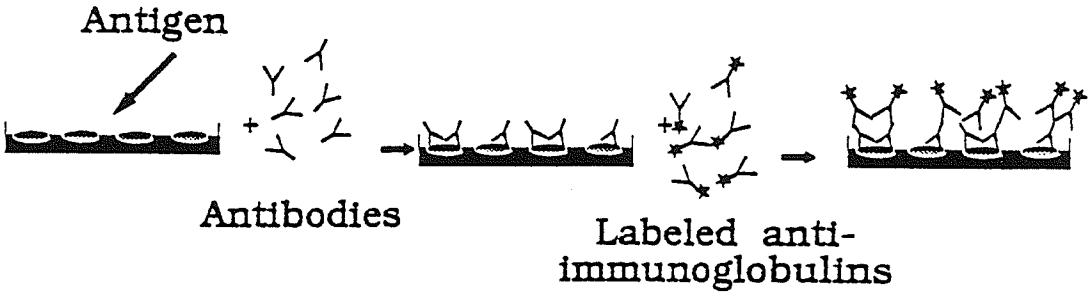

Antigen
Antibodies
Labeled anti-
immunoglobulins

TABLE 1. Sensitivity of ELISA for mycotoxins.

Mycotoxins	Standard range (pg)	Detection limits (μg/kg)	Samples tested[a]	Ref.
AFB_1	25-1,000	3.0	C(70),P(72),W(62)	El-Nakib *et al.*, 1981
		5.8	C(73),P(97),W(81)	
AFM_1	25-1,000	0.25	M(120-130)	Pestka *et al.*, 1983
		0.50	M(96-120)	
OA	25-500	1-2	W(85)	Lee and Chu, 1984
T-2	2.5-200	2.5	W(96)	Pestka *et al.*, 1981
		5.0	C(109),W(83)	

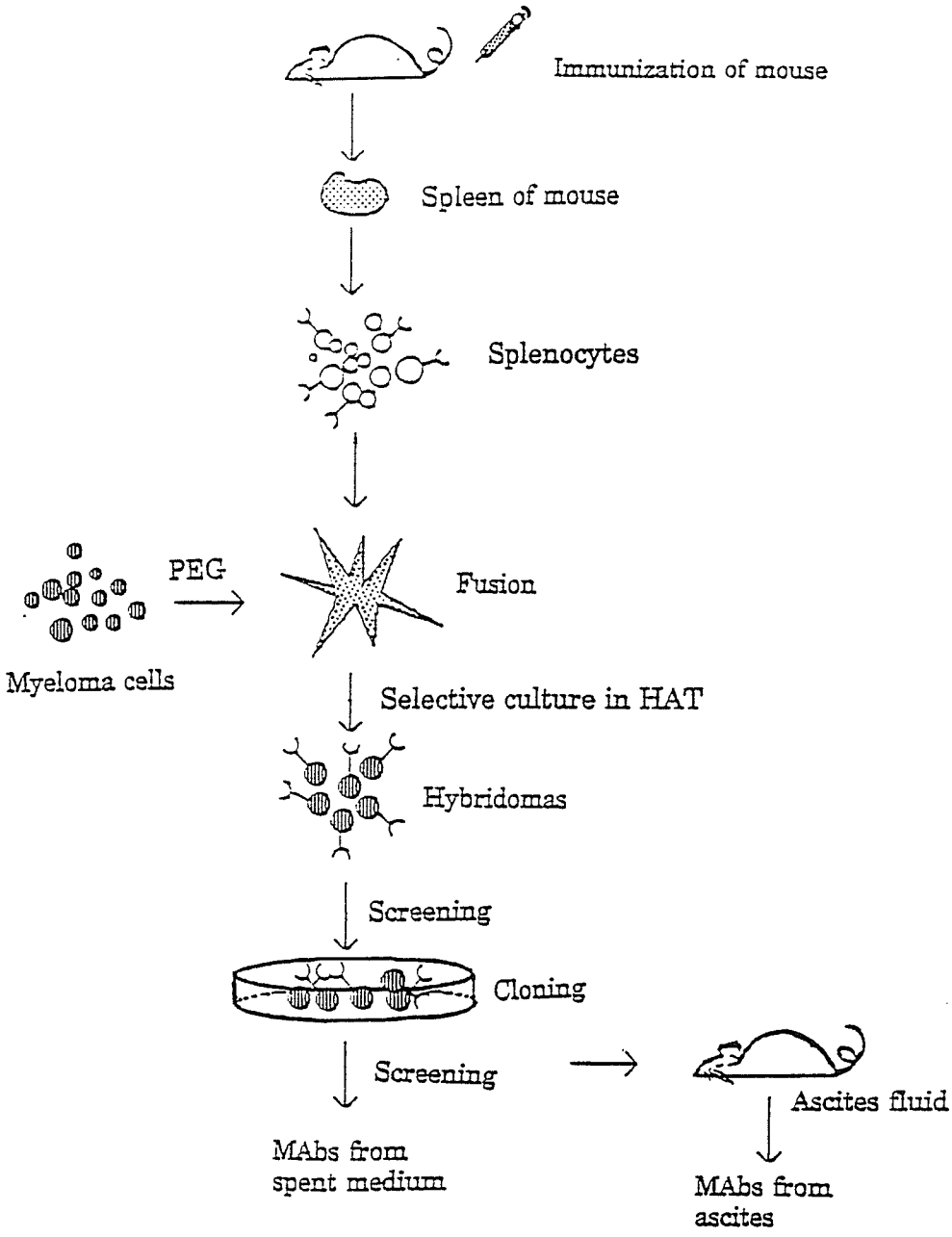
[a] Abbreviations used: C-corn; M-milk; P-peanut; S-serum; U-urine; W-wheat. The values in parentheses indicated % of recovery.

on the size of animal used for the immunization. The common problem associated with polyclonal antibodies is a natural batch variation, since the same combination of specific antibodies is impossible to reproduce in a new animal.

5.2.4. Monoclonal antibody production. To overcome the problems associated with PAbs, Kohler and Milstein (1975), who pioneered the work on the production of highly specific monoclonal antibodies, introduced hybridoma technology. This technology involved the fusion of immunized antibody-secreting B-lymphocytes with myeloma cells in order to produce immortal hybridomas which have the ability to secret antibodies of interest. The basic principles of monoclonal antibody production are shown in Figure 7. There are five basic steps which include immunization, fusion of cells, culturing of cells in selective medium, the screening of cells against antigen and finally, cloning in order to isolate a single hybridoma cell that secretes antibodies against the antigen of interest.

In this protocol, a mouse is immunized several times (3-4) with the antigen of interest until an appropriate titre of antibodies in serum is obtained. Different kind of adjuvants (eg. Freund's adjuvant) are used in conjunction with the antigen in order to enhance immunogenicity of the injected substances. Freund's adjuvants have been the most widely used adjuvants for generating good antibody responses. The mechanism of the action of adjuvants is obscure and complex. It includes slow, prolonged release of antigen in a highly aggregated form, together with pharmacologically active substances such as muramyl dipeptide from the mycobacteria which stimulates synthesis of antibodies by B-lymphocytes (Goding, 1980). After

Figure 7. Monoclonal antibody production.


Immunization of mouse
Spleen of mouse
Splenocytes
PEG
Fusion
Myeloma cells
Selective culture in HAT
Hybridomas
Screening
Cloning
Screening
Ascites fluid
MAbs from spent medium
MAbs from ascites

immunization, the mouse is sacrificed, the spleen is removed, and spleen cells (B-lymphocytes) are isolated from the tissue and prepared for fusion with myeloma cells. The spleen is considered as one of the best sources of concentrated immunized B-lymphocytes.

Subsequently, a fusion is performed. The purpose of the fusion is to produce hybrids which will contain genes inherited from immunized spleen cells coding for the antibody against the antigen of interest and genes (from myeloma cells) conferring ability to multiply indefinitely. Cell fusion is a rare event and the rationale behind it is still poorly understood (Goding, 1982) It is made possible by the addition of fusogen such as Sendai virus. This was later replaced by a high molecular weight polyethylene glycol (PEG), since it permitted higher fusion efficiencies with minimal toxicity for cells. Wojcieszyn *et al.* (1983) have attempted to explain the rationale behind the fusion mediated by PEG. They proposed that, cell fusion may occur as a result of a series of interrelated events. First, the adjacent plasma membrane of two cells are brought to close contact by the ability of PEG to bind with water that surrounds the cells. Next, a molecular rearrangement of the two membranes occurs due to the destabilization of the normal bilayer structure of the membranes, and finally, the previously, separated cytoplasmic compartments join to become one. Polyethylene glycol alone, however, does not appear to be a complete fusogen. According to Honda *et al.* (1981), the antioxidants and polymerization agents that are added to commercial PEG may also be responsible for the fusion activity.

Although fusion of cells could be facilitated by chemical agents, the fusion events are poorly controlled. Other non-hybrids tumour cells, which still exist in a

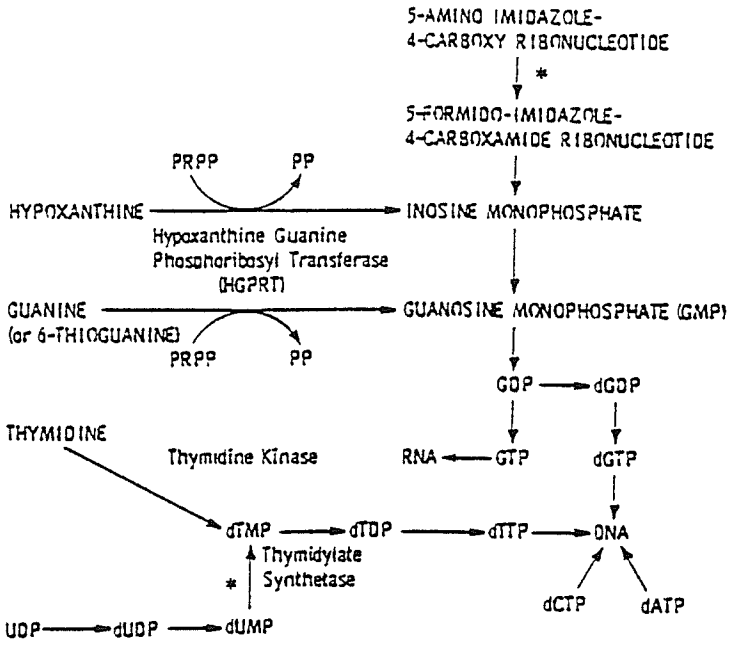
great number may overgrow the hybrids. In order to isolate the hybrids from two cell types, a selective culture technique is needed.

A selective medium containing hypoxanthine, aminopterin and thymidine (HAT), is added to the cell cultures after fusion has taken place. This selective medium blocks the main biosynthetic pathway for guanosine in the presence of a folic acid antagonist, aminopterin. An alternative "salvage" pathway of cell metabolism can be used where pre-formed nucleotides are utilized in the presence of thymine and hypoxanthine *via* a reaction catalysed by the enzymes, thymidine kinase (TK) and hypoxanthine phosphoribosyl transferase (HPRT) (Fig. 8). The mutant myeloma cells do not contain these enzymes and since their main biosynthetic pathway for the synthesis of purine and pyrimidine has been blocked, they cease growing on the HAT selective medium. Although both splenocytes and the resultant hybridomas possess these enzymes, the unfused splenocytes die naturally in culture, leaving only the hybrids, which are able to survive and reproduce (Goding, 1983).

Each hybrid cell is able to produced only one type of antibody that is specific for a single determinant. When this cell is isolated and further reproduced into one cell line, it produces a homogenous antibody, known as monoclonal antibody.

The process of isolation of this one cell from the remain is called a cloning process. Although there are several methods of cloning, the most commonly used method is a limiting dilution procedure. In the cloning process, culture cells are diluted successively and set up in culture such that the wells contain only one clone. The progeny of this cell can be isolated and then grown on as an individual clone. Each established clone is screened against the antigen in order to identify positive

Figure 8. The metabolic pathways of nucleic acid synthesis of hybrid selection in HAT medium containing hypoxanthine, aminopterin (*) and thymidine (Goding, 1983).


5-AMINO IMIDAZOLE-
4-CARBOXY RIBONUCLEOTIDE
*
5-FORMIDO-IMIDAZOLE-
4-CARBOXAMIDE RIBONUCLEOTIDE
PRPP
PP
HYPOXANTHINE
INOSINE MONOPHOSPHATE
Hypoxanthine Guanine
Phosphoribosyl Transferase
(HGPRT)
GUANINE
(or 6-THIOGUANINE)
GUANOSINE MONOPHOSPHATE (GMP)
PRPP
PP
GDP
dGDP
THYMIDINE
Thymidine Kinase
RNA
GTP
dGTP
dTMP
dTDP
dTTP
DNA
Thymidylate
Synthetase
*
dCTP
dATP
UDP
dUDP
dUMP

wells that contain cells producing the antibody of interest. Further cloning may be necessary to ensure that the cell clone secretes only one type of monoclonal antibody.

For the continual supply of antibodies, hybrids can be produced *in vitro* by cultivating cells in tissue culture or fermenter, or *in vivo* as ascitic fluid in the abdominal cavity of an animal, which is usually the strain of mouse used for antibody production. The latter method is inexpensive and antibody concentration is higher than that in tissue culture (Goding, 1980). However, there is an ethical concern about using animals while the propagation of antibodies can be performed *in vitro*.

Monoclonal antibodies have been produced against several mycotoxins such as, aflatoxins, trichothecenes, ochratoxins and zearalenone. These MAbs have been used to develop ELISA tests for detection of respective mycotoxins. Candlish *et al.* (1988) developed MAbs-based ELISAs for the detection of aflatoxin B_1, T-2 toxin, and ochratoxin. Recently, Ramakrishna *et al.* (1990) improved the sensitivity of the test for the determination of the aforementioned toxins in barley. The competitive ELISA detected 0.1 ng/ml of aflatoxin B_1, 10 ng/ml of T-2 toxin and 1 ng/ml ochratoxin. However, the development of MAbs to PRT have not been reported. Such MAbs used in ELISA could offer many advantages lacking in the developed PAb-based RIA.

III. MATERIALS AND METHODS

1. Materials

1.1. Chemicals, cells and organisms

Potato dextrose agar (PDA) and yeast extract were purchased from Difco Laboratories, Detroit, MI. *Penicillium roqueforti* (ATCC no. 10110), mouse myeloma cells P3X63-Ag.8.653 were purchased from American Type Culture Collection (ATCC), Rockville, MD. Penicillic acid, patulin, aflatoxin B_1, B_2, G_1, G_2, pyridine, bovine serum albumin, Freund's complete and incomplete adjuvants, Tween 20 and 80, polyethylene glycol 4000, *p*-nitrophenyl phosphate, trypan blue were purchased from Sigma Chemical Co., St. Louis, MO. Ochratoxin A was provided by the Animal Science Department, University of Manitoba, Winnipeg, MB. Egg-white ovalbumin was purchased from STL Laboratories Inc., Winnipeg, MB. while 2, 6, 10, 14 - tetramethylpentadecane (Pristane) was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Hypoxanthine, aminopterin, thymidine and RPMI-1640 tissue culture medium were purchased from Flow Laboratories Inc., McLean, VA. Fetal calf serum was purchased from Bocknek Laboratories, Rexdale, ON. Goat anti-mouse IgG-alkaline phosphatase, acrylamide, N, N'-methylene - bis - acrylamide, N, N, N', N'-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue R-250, low molecular weight protein standards for SDS-PAGE, antibody isotyping kit, protein determination assay kit were purchased from BIO-RAD Laboratories, Richmond, CA. Sodium

borohydride (98%) and diethanolamine were purchased from Mallinckrodt Inc., Pointe-Claire, Quebec. All other chemicals and organic solvents were of analytical and HPLC grades, respectively.

1.2. *Penicillium roqueforti* toxins - standards

Crystalline PRT was obtained from Sigma Chemical Co., St. Louis, MO, USA and was further purified as described previously (Siemens and Zawistowski, 1992). Penicillium roqueforti-imine (PRI) and Penicillium roqueforti-alcohol (PR-OH) were prepared according to the method of Wei *et al.* (1973) and their purity was confirmed by ^{1}H NMR using a NMR spectrometer, Model AM 300 Bruker Spectrospin, Canada, and TLC as described by Siemens (1992).

1.3. Supplies

The 96 well Falcon Microtest III polyvinyl flexible tissue culture plates (ELISA plates) were purchased from Becton Dickinson and Co., Oxnard, CA. The 25 cm^2 polystyrene tissue culture flasks, the 24 and 96 well flat bottom polystyrene tissue culture plates and the 500 ml Erlenmeyer flasks were purchased from Corning Glass Works, Corning, NY. The 1 ml NUNC cryotubes were purchased from A/S NUNC, Kamstrup, Denmark. Normal phase silica gel TLC plates and reversed-phase C_{18} silica TLC plates were purchased from Whatman, USA.

1.4. Instrumentals

The chromatography analyses were carried out on a Waters ALC 204 liquid

chromatography system consisted of a model U6K universal injector, a model 6000A pump, and a model 440 absorbance detector equipped with a 254 nm filter. The column was a pre-packed LiChrosorb reversed-phase C_{18} column (300 mm x 3.9 mm I.D.) of particle size 10 μm from Waters, Division of Millipore, Toronto, Canada. Titertek Multiskan ELISA reader equipped with a 405 nm filter was purchased from Flow Laboratories Inc., McLean, VA. The Mini-Protein II Multi-Casting Chamber and the constant voltage power supplier, Model 1000/500 were obtained from BIO-RAD Laboratories, Richmond, CA.

1.5. Mice

Female BALB/c mice (6-8 weeks old) used for immunization and as feeders, male BALB/c mice (8-12 weeks old) used for ascites production were obtained from the Animal Distribution Centre, University of Manitoba, Winnipeg, MB.

2. Production of PRT

The procedures in this section were performed according to the methods by Wei *et al.* (1973).

2.1. Preparation of stock culture

Penicillium roqueforti was grown on PDA slants for seven days before they were transferred into liquid medium for fermentation. Briefly, 39 g of PDA was dissolved in one litre of distilled water and the mixture was boiled until PDA was dissolved completely. The PDA medium was then pipetted into test-tubes and sterilized at

121°C for 20 min. Slants were made while the medium started to solidify. Spores of *P. roqueforti* ATCC no. 10110 were inoculated onto the slants and incubated at 24°C in darkness for seven days. Next, conidia of *P. roqueforti* were released by flooding the tubes with sterilized 0.05% Tween 80 solution, while shaking the tubes on a vortex mixer. The concentration of the collected conidia was determined using a haemacytometer, using the following formula:

$$\mathbf{N = n \times 10^4 \times DF}$$

Where,
- **N** - the number of spores in 1 ml of solution
- **n** - the number of spores calculated from the square consisted of 25 subsquares on the haemacytometer
- **DF** - the dilution factor

The spore stock solution was diluted to a concentration of 10^6-10^8 spores/ml with sterilized distilled water. The prepared stock culture solution was stored at 4°C until further use.

2.2. Fermentation of *P. roqueforti* culture

The liquid medium used for fermentation consisted of 2% yeast extract and 15% sucrose. Briefly, 20 g of yeast extract and 150 g sucrose were dissolved in one litre of distilled water. The medium was aliquoted in 100 ml fractions into the half-litre Erlenmeyer flasks, and all flasks were stoppered with polyester Dispo plugs. After sterilization (121°C for 20 min), the flasks were placed in an aseptic laminar flow hood, with a positive pressure and UV light on to cool to room temperature. A sterilized 10 ml pipette was used in pipetting 1 ml of stock culture (10^6-10^8 spores) into each flask,

except for the controls. The initial pH of the medium was measured and subsequently, all flasks were incubated in stationary condition at 24°C, in the dark.

3. Isolation and determination of PRT

3.1. Isolation of PRT

Analyses for the presence of PRT in the broths were performed every second day starting from day four. In the sampling process, two flasks were selected randomly for the detection of PRT. Mycelia was recovered by vacuum filtrating using a Whatman No. 3 filter paper. The filtrates were pooled together, and the pH was determined. A 50 ml portion of the media was carefully transferred into a separatory funnel and extracted with two volumes (100 ml) of chloroform. The two chloroform extracts were then evaporated to dryness in a rotary evaporator at 35°C at medium speed. The dried extract was redissolved in 2 ml of chloroform, and transferred into a 5 ml vial. Subsequently, the extract was evaporated to dryness at room temperature under a stream of nitrogen. The dried extract was stored at -25°C until further analysis.

3.2. Determination of PRT using TLC

The presence of PRT in fermenting culture was determined by normal-phase and reversed-phase TLCs. The extracts of cultures from each particular day were dissolved in 1 ml of chloroform, and 10 µl of extract was spotted on the TLC plate. The samples were chromatographed along with the PRT standard. The normal-phase TLC plates were developed in the methanol/chloroform (4:96, v/v) while reversed-

phase TLC plates were developed using methanol/water (70:30, v/v). After drying, the plates were exposed to short-wave UV light for 30 seconds, followed by exposure to long-wave UV light for another 30 seconds or longer. The PRT spots (either bright yellow on normal-phase or dark blue spot on reversed-phase TLC) were located.

In addition, reversed-phase TLC was used as a preparatory step prior to HPLC analyses. The plate was developed as described above and the silica containing the PRT was scraped from the plate, extracted twice with a total of 4 ml methanol. The resulting suspension was centrifuged at 1000 x g, and the supernatant was collected and evaporated to dryness under a stream of nitrogen gas. The dried sample was stored until use.

3.3. Determination of PRT using HPLC

The presence of PRT in fermenting culture was also assessed by a reversed-phase HPLC as described by Siemens and Zawistowski (1992). All dried extracts (see section 3.1) were resuspended with 1 ml of acetonitrile and 20 µl of each samples were injected into the chromatograph. The HPLC analysis was performed using a mixture of acetonitrile and water (65:35, v/v) at flow rate of 1.0 ml/min. Determination of the toxin concentration was based on the peak height measurements, using a standard calibration curve.

4. Production of PRT-MAb

4.1. Preparation of PRT-Protein conjugate

4.1.1. Conjugation of PRT to proteins. Conjugation of PRT to two structurally different protein molecules, bovine serum albumin (BSA) and egg-white ovalbumin (OV) was performed according to the reductive alkylation method of Wei and Chu (1988). Briefly, 25 mg of PRT was dissolved in methanol (5 mg/ml), aliquoted into 1 ml portions and evaporated under a stream of nitrogen. A portion of 5 mg of dried PRT was dissolved in 0.80 ml of methanol-pyridine mixture (1:1, v/v) in a 25 ml round bottom flask. A 50 mg quantity of protein carrier (BSA or OV) was dissolved separately in 5 ml of 0.05 M sodium phosphate buffer, pH 7.2. The protein solution was then added into the flask containing toxin, and the reaction mixture was incubated at 37°C in a water bath for 30 min. A volume of 0.5 mL of 0.026 M sodium borohydride ($NaBH_4$) was added and the mixture was incubated at -5°C for another 30 min. The reaction was terminated by adding 0.5 ml of 0.1 N HCl and the resulting PRT-protein conjugate was dialysed against 4 litres of 0.01 M sodium phosphate buffer containing 0.85% NaCl, pH 7.4, with a total of four changes of PBS over 48 hours at 4°C. The dialysed mixture was aliquoted into 20 µl portions and stored at -85°C, until use.

4.1.2. Determination of protein concentration Protein concentration of both conjugates (PRT-BSA, PRT-OV) was determined using a Bio-Rad protein assay and BSA and OV were used as standards.. The standard curves of both BSA and OV were plotted using known amount of proteins with a range of 5-25 µg/ml.

4.1.3. Confirmational analyses of conjugates

Thin layer chromatography Thin layer chromatography was performed as described in Section 3.2. Approximately 10 µl of each protein standard (BSA and OV), PRT-BSA and PRT-OV conjugates were spotted on the reversed- and normal phase TLC plates. The presence of PRT in conjugates were assessed under long UV light.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 20° C on a Mini-protean II (BIO-RAD) vertical electrophoresis unit using 15 % gel and a discontinuous buffer system (Laemmli, 1970). The stacking gel consisted of 4% acrylamide and 0.1% BIS while the separating gel was prepared with solution of 15% of acrylamide and 0.4% of BIS. Both gels contained 0.1% SDS and were polymerized by the addition of 5 µl TEMED and 50 µl 10% ammonium persulphate (AP) solution (separating gel), or 10 µl TEMED and 50 µl 10% AP (stacking gel) per 10 ml of gel solution. About 3 µg of protein per well (BSA, OV, PRT-BSA, PRT-OV as well as the molecular weight protein standard) was loaded, and the gels were run for 1 hr at a constant voltage of 200 V. The electrode buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS at pH 8.3. All samples were dissolved in 62.5 mM TRIS-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-merceptoethanol and 10% glycerol.

Following electrophoresis, gels were stained for protein by incubation for 30 min in the fixing solution (40% methanol and 10% acetic acid) containing 0.1% Coomassie Brilliant Blue R-250. After fixing, the gel was washed for 2 hr in destaining

solution composed of 40% methanol and 10% acetic acid.

Immunization

Four female BALB/c mice were immunized with 50 μg of PRT-BSA conjugate (on protein weight basis) at approximately three week intervals (Table 2). First, and second injections were performed subcutaneously using the conjugate in 0.15 M phosphate-buffered saline (PBS), pH 7.2, that was emulsified either with Freund's complete or incomplete adjuvant, respectively. The injections were made at two different sites at the back of the mouse neck using no more than 200 μl of solution. The third injection was done interperitoneally using 400 μl of conjugate in PBS without adjuvant. The mice were sacrificed five days after the last injection. Tailbleeding were done before the first immunization to obtain preimmune serum, seven days after each of two subsequent immunizations, and shortly before the mice were killed.

Serially diluted sera were tested for antibodies against PRT by a non-competitive enzyme-linked immunosorbent assay (ELISA) using the PRT-OV conjugate as a coating antigen. The ELISA method is described below. Preimmune serum and standard ovalbumin were used as negative controls. The well containing the highest dilution of mouse antiserum, and giving an absorbance greater than twice the negative control absorbance was taken as the titre of the antiserum.

TABLE 2. A typical immunization schedule for a female BALB/c mouse.

Injections and Bleedings	Day	Injection Volume (μl)	Injection routes
Control (preimmune sera)	0	---	---
50 μg of PRT-BSA in FCA	3	200	subcutaneous
Tailbleeding	10	---	---
50 μg of PRT-BSA in FIA	21	200	subcutaneous
Tailbleeding	28	---	---
50 μg of BSA-PRT in saline	43	400	intraperitoneal
Tailbleeding	50	---	---

FCA Freund's complete adjuvant
FIA Freund's incomplete adjuvant

4.3. Fusion procedure

Fusion was performed as described by Goding (1982). The mouse with the highest titre was killed by a cervical dislocation, and the spleen was removed aseptically. The spleen was suspended in a test-tube containing 10 ml of sterile RPMI-1640. The content of the tube was poured through a tissue dissociator (mesh size 50), and the splenocytes were released into a Petri dish by a gentle pressure asserted on the spleen against the mesh using the glass rod. The spleen tissue remaining on the mesh was washed with 8 ml of fresh RPMI-1640 medium. The splenocytes were transferred from the Petri dish to a test-tube and left for a few minutes to allow large undissociated pieces to settle down. After this time the cell suspension was decanted into a clean test-tube and centrifuged at 300 x g for 10 min at room temperature. The supernatant was discarded and the cells were resuspended in 10 ml of RPMI medium and centrifuged again. The washing procedure was repeated two times.

Murine myeloma cells (P3X63-Ag8.653) which were cultivated in the tissue culture flasks were harvested into three test-tubes and resuspended in 12 ml of RPMI-1640. The washing of cells was performed two times as described above.

The final pellet of both the splenocytes and myeloma cells were resuspended in 5 ml of RPMI-1640 and an aliquot of each cell type was used to count a number of cells with the aid of the Newbauer improved haemacytometer. Briefly, cell suspension (10 µl) was mixed with an equal volume of 0.1% trypan blue solution and the mixture was applied to a haemacytometer. The cells were counted in duplicates under the microscope. The cell concentration was calculated according to the formula mentioned in Section 2.1. Then, the spleen cells (95×10^7) and myeloma cells were mixed in a

ratio of 4:1 in a RPMI-1640 and the mixture was centrifuged at 300 x g for 5 min. The supernatant was removed, and the pellet was gently loosened and placed in the warm water bath. While swirling the tube with the cells in the warm water, a 1 ml of PEG solution was slowly added, dropwise over 1 min.

Solution of PEG was prepared by mixing 2 ml of incomplete medium (serum-free medium) with 2 g of PEG. The PEG solution was autoclaved for 20 min at 121°C, and pH was adjusted to approximate 7.8 by the addition of three drops of 0.1 N NaOH. The PEG solution was kept in the incubator at 37°C until use.

The PEG-cells mixture was diluted with RPMI-1640 medium in two consecutive series. First, 1 ml of warm medium was added over 1 min while the tube was swirled in warm water bath. Then, 8 ml medium was added over a time period of 3 min while swirling the tube. The cells were washed twice as described above. The final pellet was resuspended in a selective medium containing HAT (RPMI-1640 medium with 10% fetal calf serum (FCS) supplemented with 1.0 x 10^{-4} M hypoxanthine, 4.0 x 10^{-7} M aminopterin and 1.6 x 10^{-5} M thymidine) to a concentration of 5 x 10^{5} cells/ml of media. The cell suspension was plated out at 100 µl per well into six 96-well tissue culture plates, containing peritoneal feeder cells suspension at a concentration of 5 x 10^{4} cells/well in complete tissue culture medium (RPMI-1640 and 10% FCS). The plates were incubated at 37°C in a 5% CO_2 environment for approximately two weeks. After two weeks, cells were gradually weaned of aminopterin by replacing HAT medium with HT medium (RPMI-1640 medium with 15% FCS supplemented with 1.0 x 10^{-4} M hypoxanthine and 1.6 x 10^{-5} M thymidine). Cell growth was monitored microscopically and all wells containing developed hybridoma colonies were screened

for the presence of antibodies against PRT using a non-competitive ELISA. The positive hybridomas, which gave an absorbance value greater than 1.5 were further selected for cloning.

4.4. Cloning

Positive hybridomas were cloned by the limiting dilution method as described by Goding (1980). Briefly, cells were counted and diluted with complete medium to 50, 30, and 10 cells/ml. Cell suspension (100 µl) was plated out into the 96-well tissue culture plates containing 100 µl/well of feeder cells. Cells were transferred such that the first two rows of wells contained 5 cells and the remaining 6 rows were equally divided for 3 and 1 cell/well. Cells from each positive well was cloned onto each different plate, and with each plates being pre-incubated with feeder cells at 100 µl/well. The cloning plates were incubated for at least one week and cells were monitored for growth as well as for antibodies reacting with PRT by the non-competitive ELISA. The positive clones that showed an absorbance value greater than 1.0 were selected for the second cloning. Stable clones were propagated either in a 25 cm^2 tissue culture flask or as ascites fluid in male BALB/c mice.

4.5. Production of MAbs *in vitro*

Stable hybridomas were expanded *in vitro* by transferring them to 24-well plates containing 500 µl complete medium per well. Cells were incubated (37° C) until they covered the bottom of the well. During incubation, 500 µl of fresh medium was added into each well to provide nutrients for the growing cells. Then, each clone was

transferred into the 25 cm^2 tissue culture flasks containing 2.5 ml of complete medium and all flasks were incubated for about 5 days. When the growing cells had covered the bottom of the flasks, they were harvested and stored in liquid nitrogen. The spent media (supernatants) were collected, centrifuged at 1500 g for 30 min to remove cellular debris, aliquoted and stored at -20°C for future use. Spent media were used as a source of antibodies.

4.6. Production of MAbs *in vivo*

Stable, positive hybridomas were also expanded *in vivo* as ascites fluid, according to Goding (1980). Briefly, a male mouse BALB/c was primed with 400 µl of 98% pristane (2, 6, 10, 14 - tetramethyl - pentadecane) a week before the injection of hybridoma cells. The cultivated hybridoma cells were counted using the haemacytometer and then, 10^6 cells resuspended in 400 µl of saline were injected peritoneally into the mouse. The built up ascites fluid was noticeable after approximately 10 days, and after that fluid was withdrawn using a 18-gauge needle attached to a 10 ml syringe. The collected fluid was incubated at 37°C for one hour and then at 4°C for overnight, and centrifuged at 3,000 x g for 10 min. Resulting supernatant containing antibodies was aliquoted into 20 µl fractions and stored at - 85°C until use.

5. Screening assays

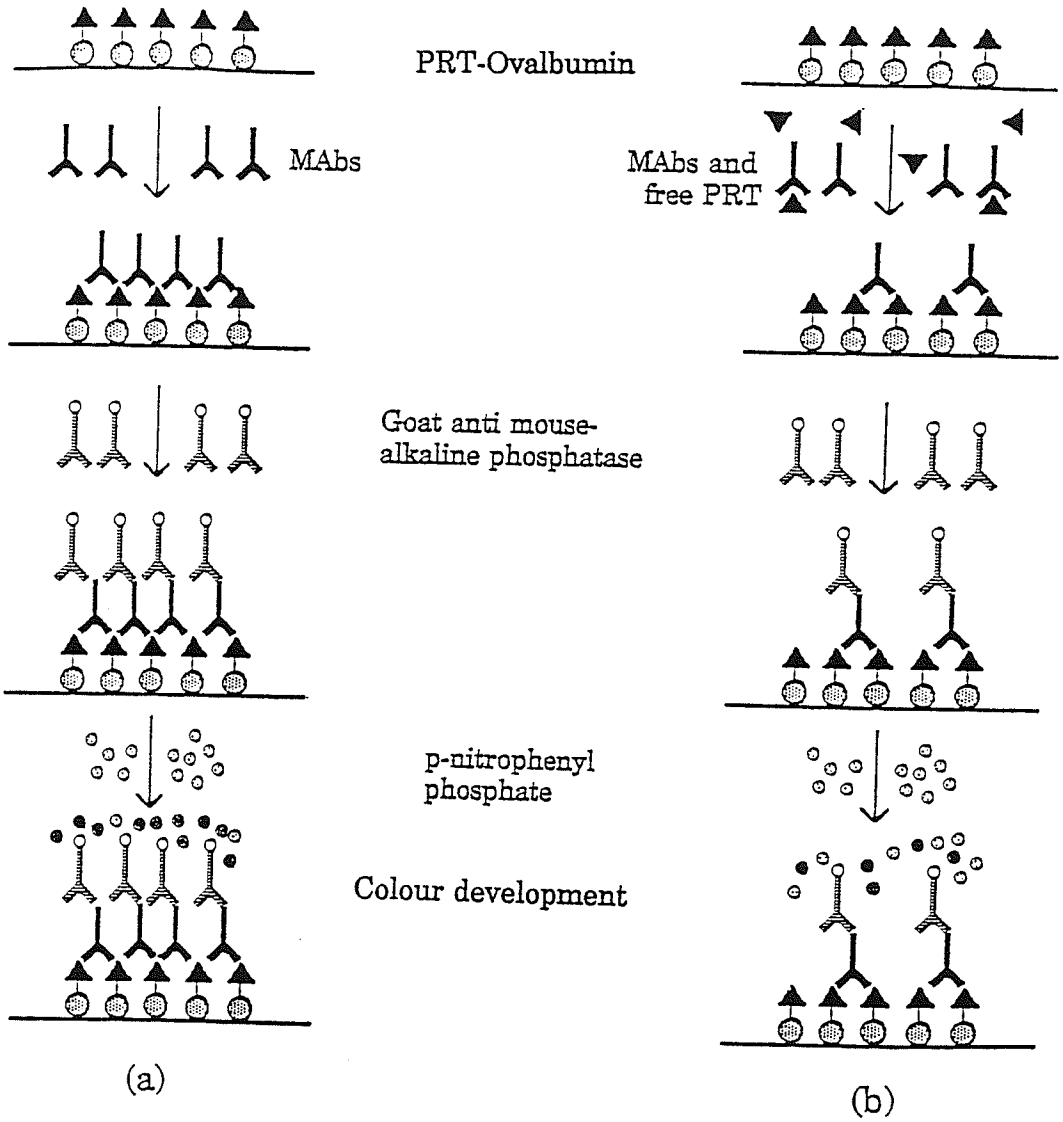
5.1. Indirect non-competitive enzyme-linked immunosorbent assay

An indirect non-competitive ELISA was used to screen antisera, spent media and ascites for the presence of antibodies to PRT. Analyses were performed using samples diluted with PBST (PBS with 0.05% Tween 20), pH 7.2, containing 0.01% skim milk as outlined in Figure 9a. Prior to the screening, the 96-well polyvinyl flexible ELISA plates were coated with 100 µl/well (10 µg/ml) of PRT-OV conjugate in coating buffer (0.1 mM sodium carbonate, pH 9.5) and incubated overnight at 4°C. The plates were washed with excess PBST and incubated with 200 µl/well of a 5% skim milk in PBS for 1 hr at 37°C in order to block sites that are not occupied by conjugates. The blocked plates were washed again and incubated with 100 µl/well of antibody solution (serum, spent medium, or ascites) for 1.5 hr at 37°C. After washing, 100 µl/well of goat anti-mouse IgG alkaline phosphatase conjugate, diluted 1:3000 in PBST containing 0.01% skim milk was added and incubated for one hour at 37°C. After each ELISA step, plates were washed five to six times with PBST to insure total elimination of unbound antibodies. Finally, the plates were incubated at room temperature in darkness with 100 µl/well of enzyme substrate comprised of *p*-nitrophenyl phosphate in 0.1 M diethanolamine containing 1 mM $MgCl_2$. The plates were read at λ_{405nm} using a Titertek Multiskan (Flow Laboratories) ELISA reader.

5.2. Indirect competitive Enzyme-linked Immunosorbent Assay

The protocol for an indirect competitive ELISA (cELISA) was essentially as outlined in Figure 9b. Polyvinyl plates were coated with 100 µl/well of PRT-OV in

Figure 9. The schematic flow diagram of indirect (a) non-competitive, and (b) competitive ELISA.


PRT-Ovalbumin
MAbs
MAbs and
free PRT
Goat anti mouse-
alkaline phosphatase
p-nitrophenyl
phosphate
Colour development
(a)
(b)

coating buffer and blocked as described in section 5.1. The diluted standard toxins (50 µl) at concentration ranging from 0 to 10 µg per well in PBS-methanol or PBS-acetonitrile, together with 50 µl of MAb in PBS (diluted 1:10,000) were added to each well and incubated at 37°C for 1 hr. The addition of second antibodies and substrate were performed as described in Section 5.1.

6. Characterization of PRT-MAb

6.1. Isotyping PRT-MAbs

Both 1H9- and 6F7-MAbs were isotyped using the mouse monoclonal isotyping kit (Serotec Ltd., Oxford, UK) according to the manufacturer recommendations. The Mabs from supernatant (6F7) and ascites (1H9) were diluted 2 and 10 times, respectively, and screened against anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, κ- and λ- light chain antibodies. An agglutination reaction between the tested and anti-mouse antibodies revealed the immunoglobulin class and subclass.

6.2. Specificity of monoclonal antibodies

The specificity of MAbs was tested using an indirect competitive ELISA (cELISA) as outlined in Figure 9b. The polyvinyl plates were coated with PRT-OV and blocked with PBST-milk as discussed earlier (Section 5.1). The addition of monoclonal antibodies, however, was accompanied with the addition of the competitive mycotoxins. The standard ELISA curves for the monoclonal antibody against PRT were made by using the competitive PRT amount ranges from 1 ng to 10 µg dissolved in either methanol (20%, 40%, and 50%) or acetonitrile (10% and 20%). In cross-reactivity

studies of monoclonal antibodies, the competitive mycotoxins at different amount (1 to 10^6 ng/assay) such as, PRT, penicillic acids, patulin, ochratoxin A, aflatoxins B_1, B_2, G_1, G_2, PR-Imine, and PR-OH were added onto the plates by dissolving them either in 40% methanol or 10% acetonitrile. The amount of PRT, which caused 50% inhibition in absorbance served as a reference to the amount of other toxins required to yield 50% inhibition of MAbs. Detection of antibodies in cELISA was performed as mentioned in Section 5.1.

7. Development of ELISA for the detection of PRT

7.1. The effect of solvents on cELISA

Methanol and acetonitrile were used as solvents for free PRT in the cELISA. Effects of solvent concentration on the performance of the ELISA system was investigated in three experiments. In experiment 1, 20% and 40% methanol were used; in experiment 2, the same concentration (20%) of methanol and acetonitrile used while in experiment 4, 20% of acetonitrile was compared with 10%. Both solvents were prepared in aqueous solutions.

7.2. The effect of incubation time on cELISA

The effect of incubation time on the performance of cELISA was investigated in experiment 3. Standard PRT dissolved in 20% of acetonitrile was mixed with 50 µl of MAb in PBS (1:1). The mixture (100 µl/well) was applied into ELISA plates (Section 5.2) and incubated either for 45 or 90 min at 37°C. The plates were developed as

described in Section 5.1.

7.3. Determination of detection limit and accuracy for ELISA

A standard curve of PRT was generated by plotting absorbance values obtained from cELISA tests vs. log of PRT concentration. The detection limit of cELISA was determined directly from the standard curve. The calibration curve was constructed using acetonitrile/PBS (10:90, v/v) as solvent, and an incubation time of 90 min. The known amount of PRT ranging from 5 to 1,000 ng was used. The accuracy of the cELISA was assessed using a reverse-phase HPLC system, where acetonitrile/water (65:35, v/v) were used as solvent system at a flow rate of 1.0 ml/min.

IV. RESULTS AND DISCUSSIONS

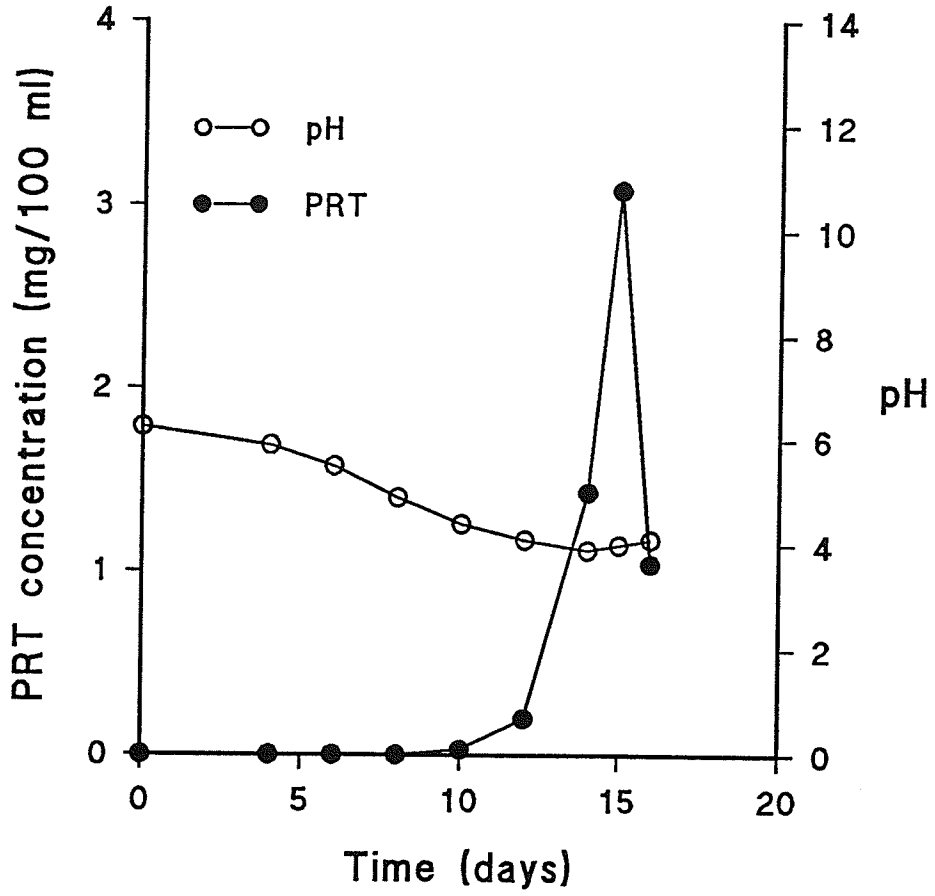
1. Production of PRT

The YES medium used as a substrate is easy to handle and contributes little extraneous material to chloroform extracts of culture filtrates (Still, 1973). Figure 10 shows a typical profile of PRT production by *P. roqueforti*.

The presence of PRT appeared on day 8 and increased up to day 15, when maximum production occurred (31 mg/L), and the amount of PRT decreased thereafter. The maximum amount of PRT produced varied from one batch to the other, although all growth conditions (temperature, pH) were kept constant. The maximum level of PRT produced in various batches was observed on day 15 ranging from 0.2 to 31 mg/L. Since the maximum level of PRT dropped dramatically after the 15th day, all processes were stopped on day 18. Among the total of six fermentation batches, the highest level (31 mg/L) of PRT was obtained in Batch A (Figure 10). Nevertheless, this amount was still about 10 times smaller than the amount of this toxin reported by Wei and Liu (1978) for the same strain of *P. roqueforti*.

The decrease in PRT production after 15 days was also encountered by Chang *et al.* (1991). Chang *et al.* (1991) proposed that prolonged storage of *P. roqueforti* spores in a freeze-dried state and the numerous transfer of culture on synthetic media might account for poor growth conditions of the culture and eventually could lead to the decrease in PRT production. Although *P. roqueforti* ATCC no. 10110 has not been

Figure 10. Time course production of PRT in YES medium (2% yeast extract and 15% sucrose) at 37°C.

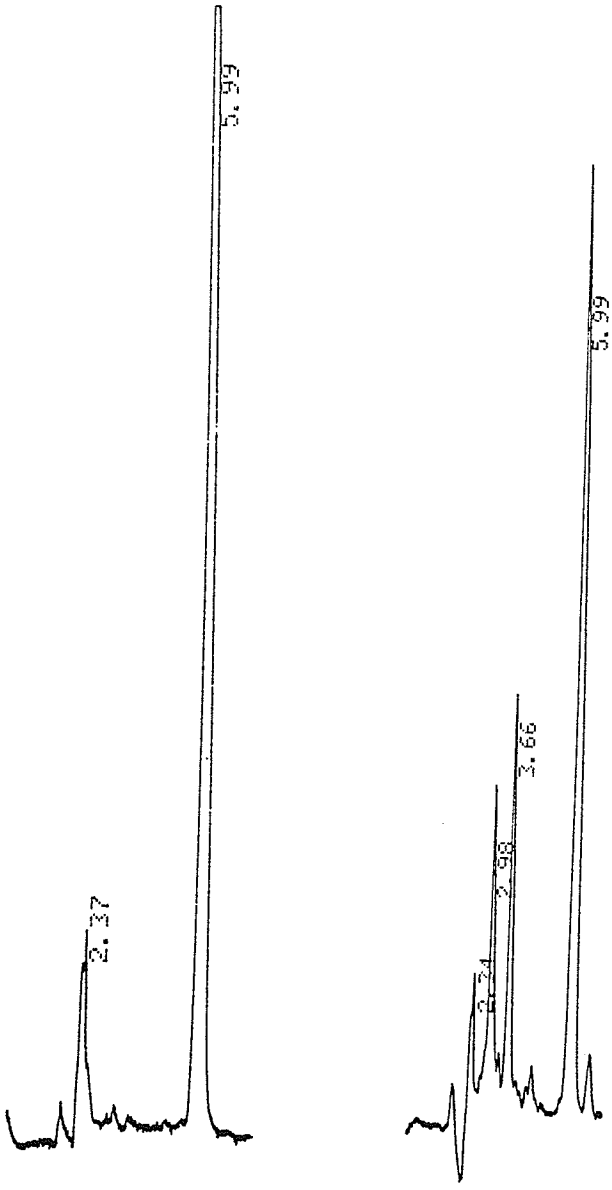

PRT concentration (mg/100 ml)
pH
PRT
Time (days)
0
1
2
3
4
0
5
10
15
20
0
2
4
6
8
10
12
14
pH

discussed by Chang *et al.* (1991), the outlined reasons might account for the similar decrease in PRT production, since the fungal spores used in this experiment had been stored in a freeze-dried form for more than ten years. More tests, however, are required to further confirm this hypothesis.

The pH of the medium is a detrimental factor on the production of PRT. The optimal production of this toxin was in the range of acidic pH, with the optimal pH value of 5.5 (Wei and Liu, 1978). The pH value for PRT production in all batches indicated that the original pH of 6.25 dropped steadily to 4.0 within 15 days. The maximum PRT production occurred on day 15 showed to have pH value of 4.0, and the pH remain at the same level up to 18 day.

The presence of PRT in fermenting broth was confirmed by TLC analysis, while the amount of toxin produced was quantified by the RP-HPLC. A typical PRT chromatogram is shown in Figure 11. The PRT peak was eluted at retention time of 5.99 min. Concentration of PRT in samples was determined using the peak height measurements. The calibration curve for PRT was linear over the tested concentration range (0.125-5 ng/μl) with correlation coefficient of 0.9993 (Appendix I). Since the PRT peak could be identified clearly from the chromatogram, purification of the toxin was not necessary for the purposes of identification. However, a purified form of PRT was needed for conjugation with a protein carrier. Scott *et al.* (1977) employed a total of two purification steps in order to obtain a pure form of PRT. In an attempt to purify the toxin using a normal-phase silica gel mini column, the recovery rate after each purification was 80%. However, since the attempt of producing PRT resulted in very low amount even before purification, the PRT from commercial source was used

Figure 11. The HPLC analysis of (a) PRT standard and (b) PRT produced in YES medium, on the 15th day of fermentation using a solvent system of acetonitrile:water (65:35, v/v) at flow rate of 1.0 ml/min.



(a) (b)

in the further experiments.

2. Conjugation of PRT to proteins

PRT was conjugated to proteins (BSA, OV) according to the reductive-alkylation method proposed by Wei and Chu (1988) as outlined in Figure 4. An aldehyde group at position 12 was utilized as a PRT site of conjugation to a protein carrier. Although PRT in addition to this group contains other functional groups such as an acetoxy group, an α-, β-unsaturated ketone, methyl and epoxide groups, the aldehyde group is a convenient site for conjugation since it can be utilized without prior chemical modification. This group forms a Schiff-base with the ε-NH_2 group of lysine and, the free sulfhydryl group of cysteine residues present in protein molecules (Shaw *et al.*, 1984). The formed adduct was further stabilized by reduction with sodium borohydride ($NaBH_4$). The reduction of the conjugate is essential, since it enhances a molar ratio of toxin to protein carrier (Shaw *et al.*, 1984) and produces an excellent immunogen (Wei and Chu, 1988).

Since PRT has the ability to fluoresce under UV light, fluorescence property of this compound was followed in the conjugates. Two protein molecules (BSA, OV) and their conjugates (PRT-BSA and PRT-OV) were spotted onto TLC plates. The results indicated that only the conjugates, which contain PRT molecules, yielded fluorescence. Although the detection of fluorescence in the conjugates showed that PRT molecules were bound to proteins, the type of binding involved could not be confirmed. Successful conjugation should involve covalent binding, however, the possibility of hydrophobic interaction or other non-covalent bindings cannot be

eliminated when evidence was based solely on the above obtained results. Therefore, both conjugates were further analyzed by SDS-PAGE in order to find whether they have higher molecular weight than their respective protein carriers, which could provide indication of successful conjugation. Samples of PRT-BSA, and PRT-OV and their corresponding protein standards, BSA and OV, were separated by SDS-PAGE using 15% gel system and the resulted relative mobilities (R_f) values were determined for each compound after staining the gel with CBB. The electrophoretic bands of both conjugates exhibited lower R_f values than their corresponding protein standards. This indicated that molecular weights of conjugates were higher than their protein carriers as a result of conjugations with PRT molecules. Wei and Chu (1988) have reported that conjugation of PRT to BSA *via* the reductive-alkylation procedure may result in binding up to 26 PRT molecules to one molecule of protein. Subsequently, this may yield in a molecular weight increase of about 8,000.

The PRT-BSA conjugate which yielded a soluble antigen was used in the immunization process while PRT-OV was used as a coating antigen for characterization of antibodies produced in later stages. The PRT-BSA conjugate was used as an immunogen but not PRT-OV since the former one is more soluble. In other words, PRT-BSA has the ability to retain its native form, which enables the antigenic determinant of PRT to be prominently exposed (Erlanger, 1980). The subsequent immunogenic response is the production of antibodies specific to PRT. The resulted antibodies could then be easily characterized in later stage by ELISA, using another conjugate (PRT-OV) as a coating antigen.

3. Immunization

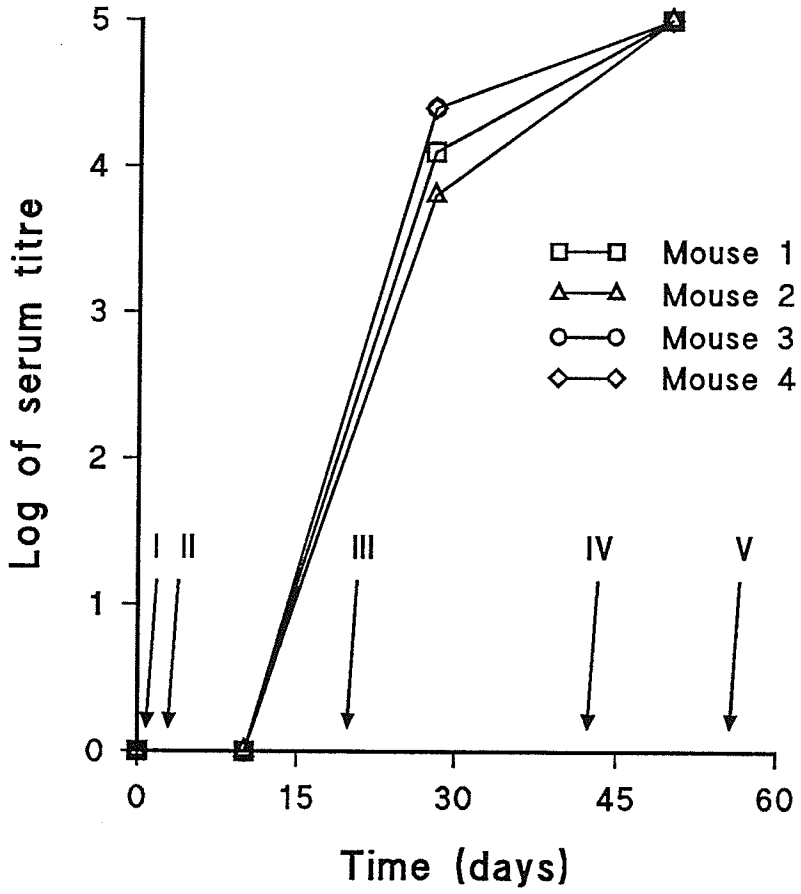
Four BALB/c female mice were immunized with 50 μg of PRT-BSA conjugate (Material and Methods). The protein concentration in conjugates was assessed by the BIO-RAD protein assay kit (see standard curve in Appendix II).

The typical immunization schedule for one mouse is shown in Table 2, where immunizations were performed at 3 week intervals. The first and second injections were performed using Freund's complete and incomplete adjuvants, respectively, and the third injection was performed without adjuvant. Usually, antigen is administered subcutaneously in Freund's complete adjuvant containing attenuated *Mycobateria* and frequent booster injections are given in Freund's incomplete adjuvant. This regime produces polyclonal hypergammaglobulinemia accompanied by high level of specific antibody against the administered antigen. In addition, the mycobacterial component used in the primary immunization induces predominantly antibodies of the IgG_1 isotype (Hadjipetrou-Kouronakis and Moller, 1984).

Both first and second injections were performed subcutaneously, while the third and the last injections were performed intraperitoneally. According to Stahli *et al.* (1980), the final boost is most effective when given intravenously. However, it is considered safer and as nearly effective if the final boost is given intraperitoneally (Goding, 1983). Therefore, the final injection was performed intraperitoneally without the adjuvant.

The sera titres of four mice were monitored using the non-competitive ELISA with PRT-OV as a coating antigen, and the results are presented in Figure 12. The PRT-OV conjugate was used as the coating antigen for two reasons. Firstly, PRT alone

Figure 12. Antibody titre of mice sera raised against PRT over a period of time. I. Pre-bleeding of mice; II. subcutaneous injection of PRT-BSA with FCA; III. subcutaneous injection of PRT-BSA with FIA; IV. intraperitoneal injection; V. Fusion. About 100 μl of blood was collected from the lateral tail vein.


Log of serum titre
5
4
3
2
1
0
I
II
III
IV
V
Mouse 1
Mouse 2
Mouse 3
Mouse 4
0
15
30
45
60
Time (days)

cannot be used for coating onto ELISA plates since it is poorly absorbed to polyvinyl chloride. Thus attachment of toxin to the plate was accomplished through a protein carrier *via* hydrophobic interactions. Secondly, the conjugate must contain protein carrier (eg. OV) other than the one that is present in the conjugate used for mice immunization (BSA), in order to eliminate antibodies produced against BSA.

Moreover, during screening for antibodies against PRT, negative controls of preimmune sera and OV standard were also included in each ELISA test to eliminate non-specific antibodies such as those that were produced against BSA, which may cross-react with OV due to structural similarities of both proteins.

After the first two injections, all mice showed a weak antibody responses with titres ranging from 6,400-25,000. However, after the third injection, all four mice exhibited a dramatic increase in titre that exceeded 100,000. Since mouse no. 3 consistantly exhibited the highest titre throughout the whole immunization process, it was chosen for fusion.

4. Hybridoma Production

Spleen cells from BALB/c mouse immunized with PRT-BSA were fused with P3X63-Ag.8.653 myeloma cells. The resulting hybridomas were cultured in 96-well microculture plates. Growing hybridomas were observed in 75% of the wells. A fusion frequency of 1.2×10^{-5} was calculated on the basis of all splenocytes (5×10^{7}) being used in the fusion. The fusion resulted in 20 cell clones secreting antibodies that exhibited high reactivity with PRT giving absorbance values over 1.0 as assessed by

non-competitive ELISA (Table 3).

The 20 positive hybridomas were selected and propagated into 24-well microculture plates prior to further cloning. Among these, 6 clones were chosen for the first cloning, since they exhibited good growth and high reactivity with PRT. These cell lines were designated as 6E7, 6F7, 1G10, 1H9, 6C3, and 5C7 (Table 3). The quantity of positive hybridomas in the first screening, was considerably reduced during the course of subcloning because of insufficient growth or lost ability to produce antibodies. A rigorous selection during the early screening phase is important to eliminate unsatisfactory clones. Selected clones should exhibit high reactivity with the antigen of interest as well as good stability. In this study, the stability of cells was defined by its ability to yield 100% cell growth recovery. Moreover, they shall be able to secrete antibodies that give high absorbance value (> 1.00) upon reacting with PRT when assessed by the ELISA test. These stability characteristics are crucial since these cell clones may become the potential stock cell culture for *in vitro* and/or *in vivo* production of MAbs. After the first cloning, only two hybridomas, the 1H9 and 6F7 showed relatively stable characteristics. Line 1H9 resulted in 12 positive clones while 6F7 yielded only one clone. Among the above positive cell lines, the 1H9-H5 and 6F7-B6 were further cloned.

The resulting positive clone from 6F7-B6 was designated as E8 and the cells were further propagated in the tissue culture flask, with a total volume of 10 ml/flask, in order to produce MAb. The resulting subclones from line 1H9-H5 were as many as 5, and among them, G7 showed the greatest stability. Moreover, only G7 appeared as a single colony within the well, indicating that the cells could be derived from a single

TABLE 3. Hybridoma production and the selection of subclones

Hybridoma lines (after fusion)	Absorbance (λ_{405nm})	Hybridoma lines (first cloning)	Hybridoma lines (second cloning)
1C6	1.186		
1E6	1.375		
1G10	2.188		
1H3	1.376		
1H9	1.949 (12 positives)	**H5** (8 positive)	**G7**
1H11	1.071		
2F8	2.158		
3C6	2.096		
4F8	2.202		
5C7	2.120		
6A3	2.140		
6C3	1.590		
6C4	1.144		
6D3	1.282		
6D7	1.811		
6E3	1.385		
6E4	1.081		
6E7	2.216		
6F7	2.152 (1 positive)	**B6** (1 positive)	**E8**
6H3	1.012		

cell which are able to produce a single type of MAb. Therefore, 1H9-H5-G7 (1H9) had been chosen as the cell line that provide that PRT-MAb for future experiment.

5. Assessment of MAb-PRT

5.1. Analysis of MAb titre The hybridoma clone 1H9 manifested high stability, and consequently used for the production of MAb. The hybridomas were cultivated *in vitro* as spent medium as well as they were used for the production of MAb *in vivo* as ascites fluid. The titre of spent media and ascites were compared, using non-competitive ELISA (Fig. 13). The titre of ascites (1:102,400) produced using 1H9 cells was about sixty-four fold higher than the titre of spent media (1:1,600). Higher titre of ascites was predictable since antibodies concentration in ascites is usually higher than those in the spent media. Kohler (1980) has also reported that the titre of ascites is usually 10^2-10^3 fold higher than spent media. Ascites antibodies exhibited a high absorbance value (over 1.0) when diluted up to 1:3,200, while further dilution caused a dramatic drop in the absorbance value (Fig. 13).

5.2. Isotyping immunoglobulin subclasses Both 1H9 and 6F7 were isotyped using a BIO-RAD mouse isotyping kit (Material and Method). The results of the isotyping tests indicated that the antibodies of both cell lines belong to the IgM immunoglobulin subclasses and both contain the κ light chains (Table 4). The

Figure 13. The absorbance value of MAbs (1H9) from ascites and spent medium over a series of dilution.

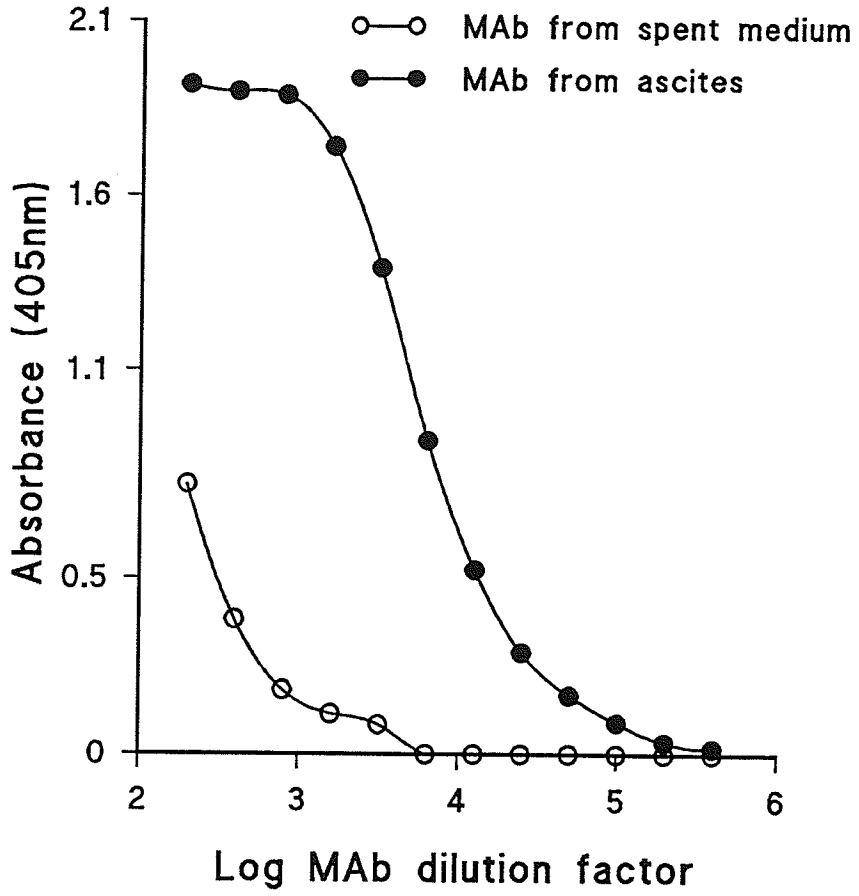

MAb from spent medium
MAb from ascites
Absorbance (405nm)
2.1
1.6
1.1
0.5
0
2
3
4
5
6
Log MAb dilution factor

TABLE 4. Reactivity of developed MAbs with antibodies to different subclasses of mouse immunoglobulins as assessed by a BIO-RAD isotyping assay.

Antibodies to mouse Immunoglobulin subclasses	Abs producing cell lines	
	1H9-H5-G7	6F7-B6-E8
IgG_1	-	-
IgG_{2a}	-	-
IgG_{2b}	-	-
IgG_3	-	-
IgM	+	+
λ	-	-
κ	+	+

\- Absorbance values were lower than 0.200.

\+ Absorbance values were higher than 1.500.

antibodies did not cross-react with other subclasses which may indicate monoclonal characteristics of antibodies.

Immunoglobulin of IgM class is a pentameric structure that have the molecular weight of 900,000. The pentameric molecule consists of five, four-chained structures that are held together by disulfide bonds between their F_c portions and a polypeptide J-chain. Although each pentameric IgM has 10 antigen receptors, it does not usually have a functional valency of 10 due to the planar configuration of the structure. When one F_{ab} is bound to antigen, the other neighbouring site is blocked, thus making the molecule appear pentavalent, or less (Benjamini and Leskowitz, 1988). Even then, it is important to have appropriate dilution of MAb in the competitive ELISA since free toxin is used to compete with the pentameric structure of MAb for the bound antigen. When the concentration of MAb is too high, the pentameric IgM may interlock with each other, thus making the antigenic receptors unavailable to antigens. However, it is also important that a high enough concentration of MAb is available to bind with free antigen, making a substantial difference in absorbance value for the antigen detection. Therefore, a working range of 1:10,000 dilution of ascites was chosen since for this dilution correlation between titre and absorbance is linear (Fig. 13).

5.3. Cross-reactivity studies of PRT-MAb Cross-reactivity of PRT-MAb was studied using a competitive ELISA (cELISA) with PRT-OV (1 µl/well) as a coating antigen. The protein concentration of PRT-OV was assessed by BIO-RAD protein assay standard curve (see Appendix III). Variety of mycotoxins and other compounds that are structurally similar to PRT were used as competitive antigens. In this study, cross-

reactivity of MAb to other compounds was calculated as compared to PRT according to the formula:

$$CR = \frac{\text{PRT concentration needed for 50\% inhibition of absorbance}}{\text{concentration of other compounds needed for 50\% inhibition}} \times 100\%$$

The inhibitory effect of competing free PRT and PRI with coated PRT-OV for binding to MAbs is shown in Figure 14. These curves were plotted using PRT, and PRI in the concentration range from 5 to 10,000 ng/assay, and 5 to 100,000 ng/assay, respectively. Regression analysis of the linear portion of these curves revealed a good correlation of 0.9914 and 0.9998 for PRT and PRI, respectively (Appendix IV).

The amount of toxin yielding 50% inhibition was calculated for PRT to be 2.2 µg. This cross reactivity study showed that PRT-MAb only cross react with PR-imine (PRI). The amount of PRI required to cause 50% inhibition was 3.2 µg, indicating a 70% cross-reactivity.

Other toxins such as PR-alcohol (PR-OH), ochratoxin A, penicillic acid, patulin, aflatoxin B_1, B_2, G_1, G_2 (in concentration range of 5-100,000 ng/assay) did not show cross reactivity with PRT-MAb. Among the two derivatives of PRT tested, only PRI but not PR-OH cross-reacted with PRT-MAb. This indicates that the epitope recognized by the developed antibodies is located on the left side of the toxin molecule and most probably is composed of the acetoxy group at position 3 (Fig. 15). This is supported by the similarities and differences between the chemical structures of all three toxin molecules. The left side of PRT and PRI molecules are identical, while the structure of PR-OH is varied in that the acetoxy group is replaced by the hydroxyl

Figure 14. The inhibitory effects of free PRT and PRI on the binding of PRT-MAb in the cELISA. Both PRT and PRI were dissolved in 10% acetonitrile and incubated for 90 min at 37°C.

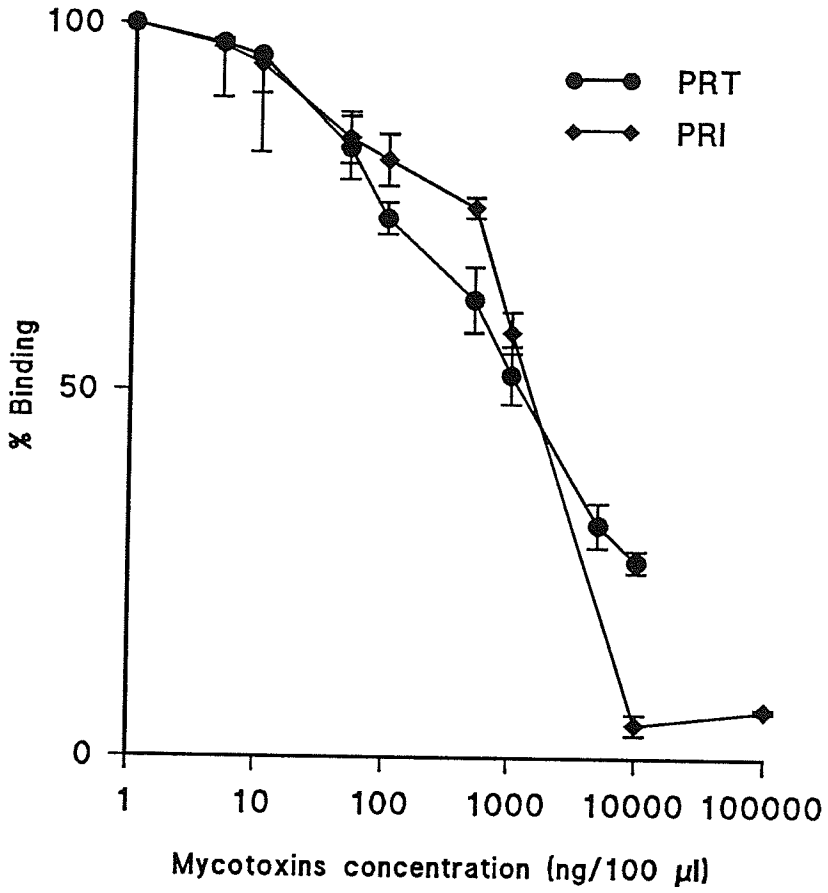
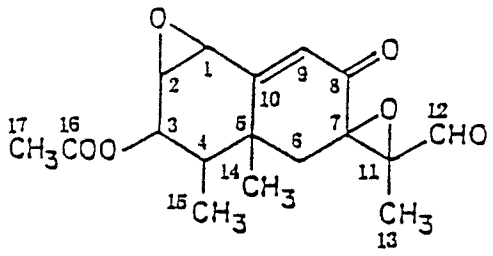
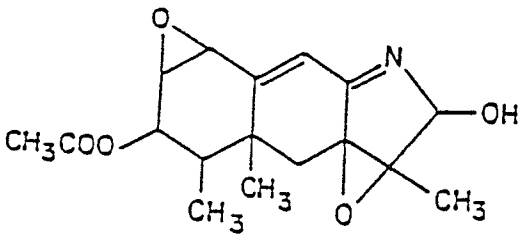

100
50
0
% Binding
PRT
PRI
1
10
100
1000
10000
100000
Mycotoxins concentration (ng/100 µl)

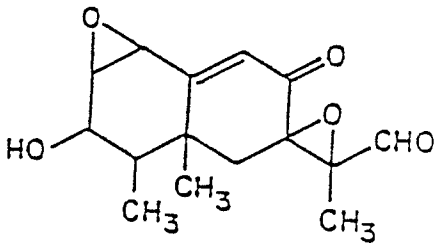
Figure 15. The chemical structure of *Penicillium roqueforti* mycotoxins.



PR toxin



PR-imine



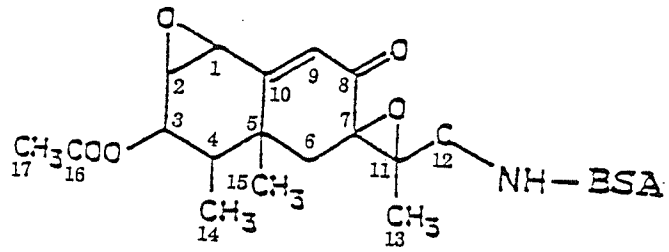
PR-OH

moiety. Our results are also in agreement with the hypothesis given by Erlanger (1980), in that, specificity of anti-hapten antibodies is directed primarily at the side of the molecule that is furthest removed from the point of conjugation to the protein carrier. Moreover, conjugation of PRT to BSA resulted in the production of two conjugate forms, one being compound A, where the aldehyde group at position 12 of PRT formed a bridge with amino group of BSA; and the other being compound B where the hydroxyl group at position 12 formed an additional five carbon member ring through rearrangement with the α, β-unsaturated ketone at position 8 (Fig. 16). Since the results indicated that the MAb has highest specificity towards PRT, the synthesized immunogen would most likely have the structure of conjugate A.

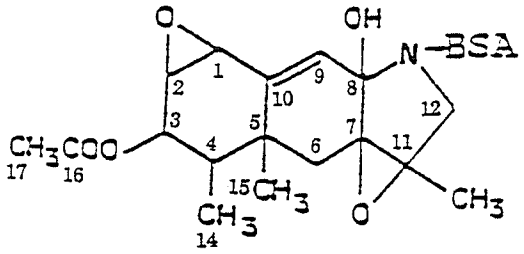
In contrast, Wei and Chu (1988) have suggested that the conjugate B (Fig. 16) was the predominant form used for the production of rabbit polyclonal antibodies against PRT, since their antibodies were most specific to PRI (100%), which structure resembles conjugate B. Antibodies produced by these researchers exhibited only 70% of cross-reactivity with PRT, which resembles conjugate A, and showed insignificant cross-reactivity with PR-OH.

The structures of other toxins, such as ochratoxin A, penicillic acid, patulin, aflatoxin B_1, B_2, G_1, G_2, do not resemble to that of PRT, and would have expected to have insignificant cross-reactivity with PRT-MAb. The highest amount of toxins used in these cross-reactivity studies was 100,000 ng/assay since the amount of each toxin available for testing was limited. Therefore, the resulting insignificant cross-reactivities could only be reported as less than 2.2% (Table 5).

Figure 16. Two forms of PRT-BSA conjugates produced *via* the reductive alkylation process.



Conjugate A



Conjugate B

TABLE 5. Cross-reactivity of MAb 1H9 with PRT and other mycotoxins

Toxins	*Amount of toxins yielding 50% inhibition (μg)	% Cross-reactivity
PR Toxin	2.2	100.0
PR-imine	3.2	70.0
PR-OH	>100.0	<2.2
Ochratoxin A	>100.0	<2.2
Penicillic acid	>100.0	<2.2
Patulin	>100.0	<2.2
Aflatoxin B_1	>100.0	<2.2
Aflatoxin B_2	>100.0	<2.2
Aflatoxin G_1	>100.0	<2.2
Aflatoxin G_2	>100.0	<2.2

* The maximum amount of toxins used in this cross-reactivity study was 100 μg/well. Therefore, any toxin that showed no inhibition at 100 μg was reported as less than 2.2% cross-reactivity with PRT-MAb (1H9).

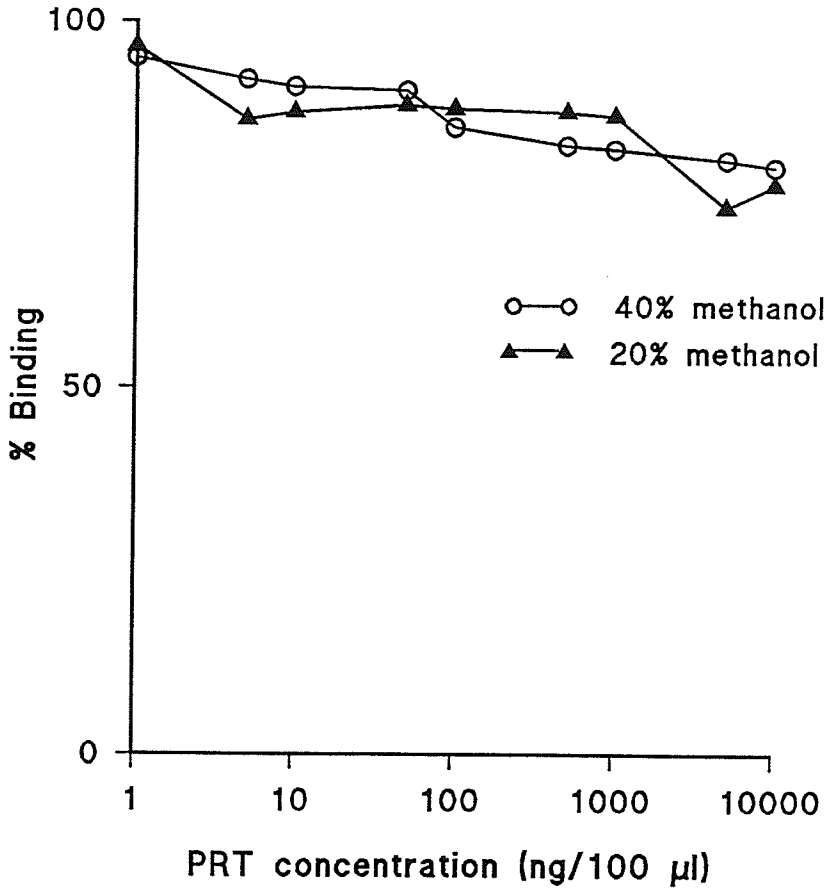
6. Development of MAb-based ELISA for the detection of PRT

The PRT-MAb (1H9) was utilized to develop a competitive ELISA (cELISA) for the detection of PRT. The PRT-OV conjugate was used as a coating antigen, while a free standard PRT was used as a competitive antigen (Materials and Methods). Since PRT is not soluble in aqueous buffer, organic solvents such as methanol, chloroform, acetone and acetonitrile are usually employed to dissolve this toxin. Most organic solvents at high concentration, however, are not compatible with antibodies. They may cause significant interference in ELISA due to denaturation of antibodies.

In order to establish proper conditions for the ELISA test, several experiments were conducted to investigate the effects of two solvents : methanol, and acetonitrile on monoclonal antibodies. The effect of solvent concentration was investigated using 20 and 40% methanol (Experiment 1), 20% methanol and 20% acetonitrile (Experiment 2), and 10 and 20% acetonitrile (Experiment 4). In addition, the effect of incubation time of free PRT (competing antigen) dissolved in 20% acetonitrile, on the ELISA test was also conducted (Experiment 3).

6.1 Experiment 1 Figure 17 shows the effect of methanol on the inhibitory behaviour of PRT in the competitive ELISA. For this test PRT was dissolved either in 20 or 40% methanol, added into ELISA plates previously coated with the PRT-OV conjugate and incubated for 45 min at 37°C. An almost lack of response to the PRT was observed for the tested toxin in the range of 1 to 10,000 ng/assay for both methanol concentrations. The highest concentration of PRT resulted only in 20% inhibition. It is possible that instability of PRT in aqueous

Figure 17. The effect of methanol concentration on the inhibitory behaviour of PRT in the cELISA. Toxin was dissolved in 20 and 40% methanol and incubated for 45 min at 37°C.


100
50
0
% Binding
40% methanol
20% methanol
1
10
100
1000
10000
PRT concentration (ng/100 µl)

solution of methanol is accounted for these negative results. Siemens and Zawistowski (1992) have shown that storage of PRT in methanol/water (70:30) even at lower temperature (5°C) than that used in the ELISA test, resulted in a significant deterioration of this toxin.

6.2 Experiment 2 In contrast to the above results, Danieli *et al.* (1980) has reported that PRT is relatively stable in aqueous solution of acetonitrile. In order to compare the effects of both solvents on the inhibitory behaviour of PRT, methanol and acetonitrile at the same concentration (20%) were used to dissolve PRT for ELISA. Different concentration of PRT in both solvents were incubated for 45 min at 37°C. The effect of both solvents is shown in Figure 18. Acetonitrile appeared to be a better solvent for PRT than methanol, since overall competing effect of PRT for antibodies was improved. At highest concentration, PRT dissolved in 20% acetonitrile yielded about 40% inhibition, which was two fold higher than the inhibition obtained when PRT was dissolved in 20% methanol. Although acetonitrile appeared to be a better solvent for PRT, the short incubation time could be accounted for the low inhibitory effect of PRT. Subsequently, the time of incubation was investigated using PRT dissolved in 20% acetonitrile.

6.3 Experiment 3 Figure 19 shows the effect of incubation time (45 and 90 min) on the inhibitory behaviour of PRT in the competitive ELISA. Longer incubation time of PRT with antibodies yielded significant improvement of the ELISA performance resulting in 50% inhibition at the highest PRT concentration (10,000 ng).

Figure 18. The effect of methanol and acetonitrile on the inhibitory behaviour of PRT in the cELISA. Toxin was dissolved in 20% of methanol or acetonitrile and incubated for 45 min at 37°C.

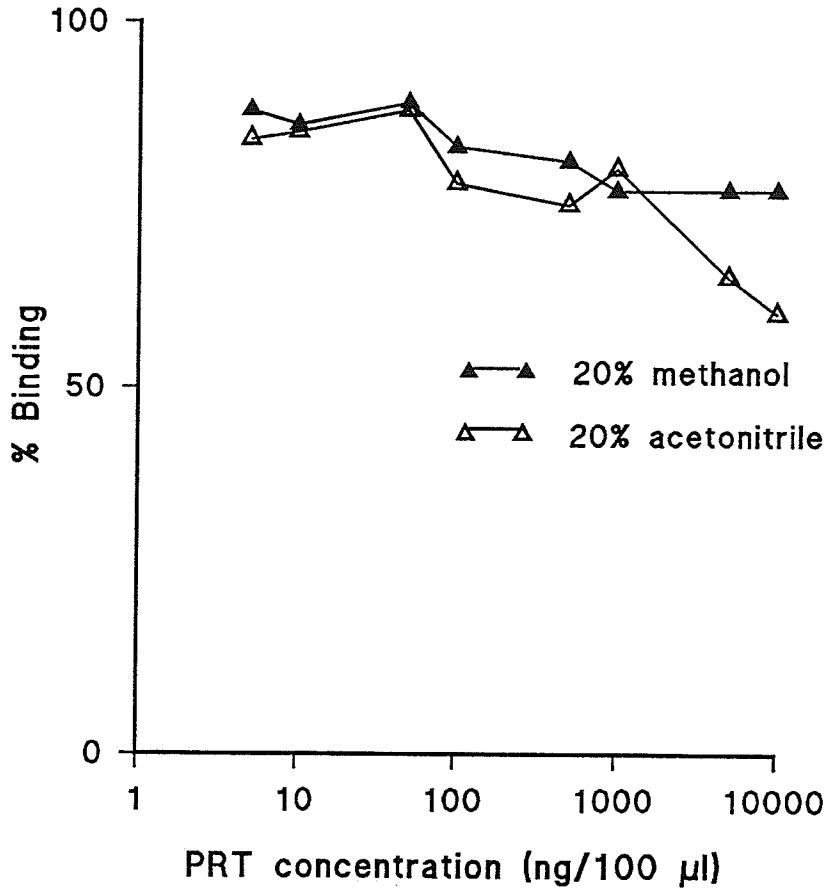
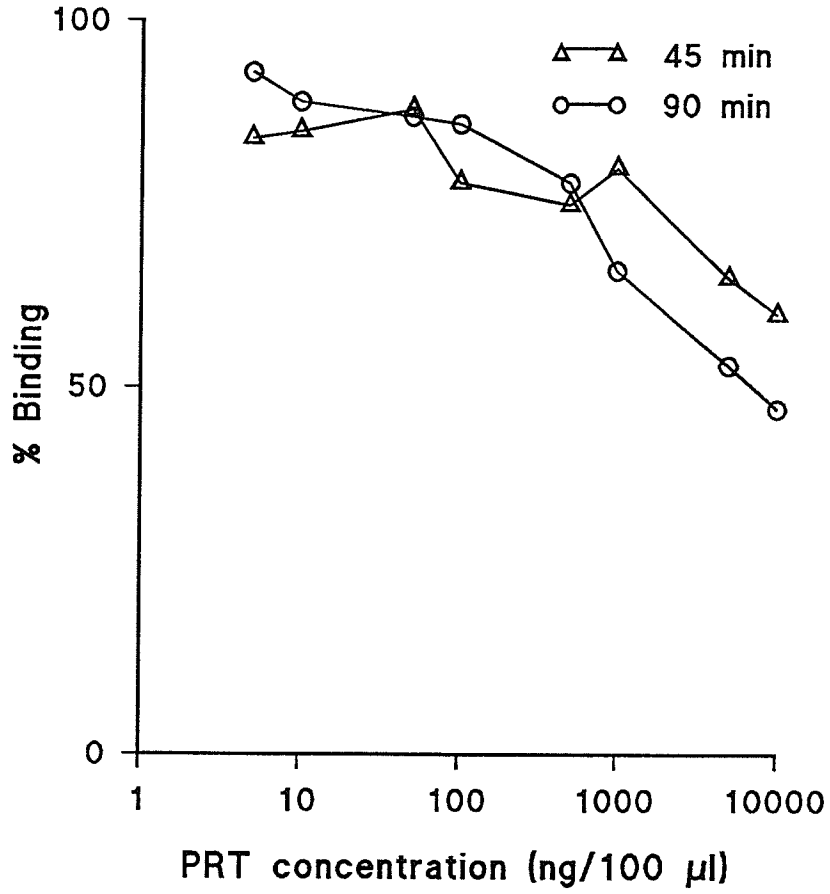

100
50
0
% Binding
1
10
100
1000
10000
PRT concentration (ng/100 µl)
20% methanol
20% acetonitrile

Figure 19. The effect of incubation time on the inhibitory behaviour of PRT in the cELISA. Toxin was dissolved in 20% acetonitrile and incubated for 45 or 90 min at 37°C.

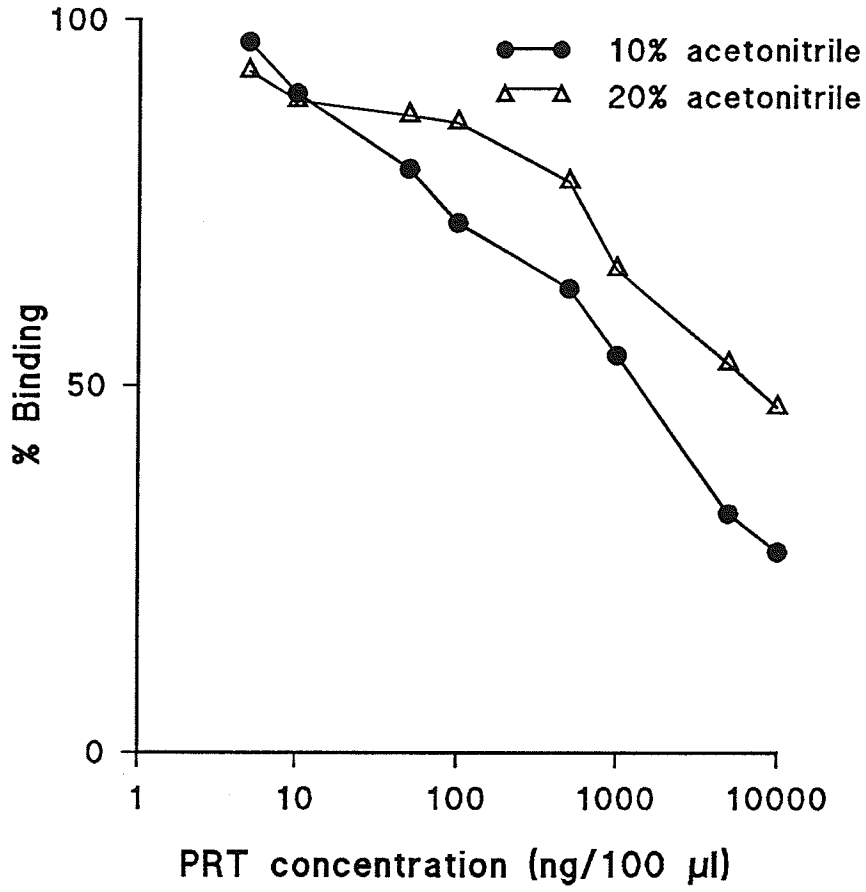

100
50
0
% Binding
45 min
90 min
1
10
100
1000
10000
PRT concentration (ng/100 µl)

The inhibition was improved by 10% over inhibition obtained for a 45 min incubation.

It seems possible, that the prolonged time of incubation of PRT with antibodies provided a better exposure of paratopes of the pentameric MAb to toxin molecules thus yielding multivalent binding between both compounds and consequently resulted in a higher avidity of a formed complex. Consequently, the last experiment was devised to optimize concentration of acetonitrile for the ELISA system.

6.4 Experiment 4 Figure 20 shows the effect of acetonitrile on the inhibitory behaviour of PRT in the cELISA. Toxin was dissolved either in 20% or 10% acetonitrile and incubated for 90 min. The highest inhibition (approximately 75%) was exhibited by PRT dissolved in 10% acetonitrile, which was higher than that obtained in the previous experiments. Using 10% acetonitrile solution to dissolve PRT and increasing time of incubation of this toxin with MAbs yielded a significant improvement of the ELISA performance. Although acetonitrile is a good solvent for PRT, at a higher concentration it could denature the protein carrier (OV) of the coating conjugate (PRT-OV) causing unfolding of protein molecules and consequently resulting in a better presentation of conjugated PRT molecules for the MAb binding (Scopes, 1982). This may result in the decrease of the inhibitory behaviour of free PRT.

Figure 20. The effect of acetonitrile concentration on the inhibitory behaviour of PRT in the cELISA. Toxin was dissolved in 20% or 10% acetonitrile and incubated for 90 min at 37°C.


100
50
0
% Binding
10% acetonitrile
20% acetonitrile
1
10
100
1000
10000
PRT concentration (ng/100 µl)

7. Standard ELISA curve for PRT

The calibration curve of the inhibitory effect of PRT on PRT-MAb was plotted using the conditions set up in experiment 4. Toxin was dissolved in 10% acetonitrile and incubated with PRT-MAbs for 90 min at 37°C. The resultant curve is shown in Figure 21. The highest inhibition of 75% was achieved by using 10,000 ng/assay of PRT with a detection limit (10% inhibition) of 10 ng/assay of PRT. The developed ELISA system is shown to be not as sensitive as the polyclonal antibody-based RIA reported by Wei and Chu (1988). However, it provides greater specificity towards PRT than the former assay system.

8. Accuracy of ELISA system

The accuracy of the developed ELISA system was compared with a reversed-phase HPLC that employed acetonitrile/water (65:35, v/v) at a flow rate of 1.0 ml/min. The amount of PRT used ranged from 5 ng to 1,000 ng. Good reproducibility was obtained for ELISA results when analyzed on HPLC. When both set of results where compared, a good correlation between ELISA system and HPLC was found with correlation coefficient of 0.9840 (Fig. 22).

Figure 21. The standard ELISA curve for PRT. Toxin was dissolved in 10% acetonitrile and incubated for 90 min at 37°C.

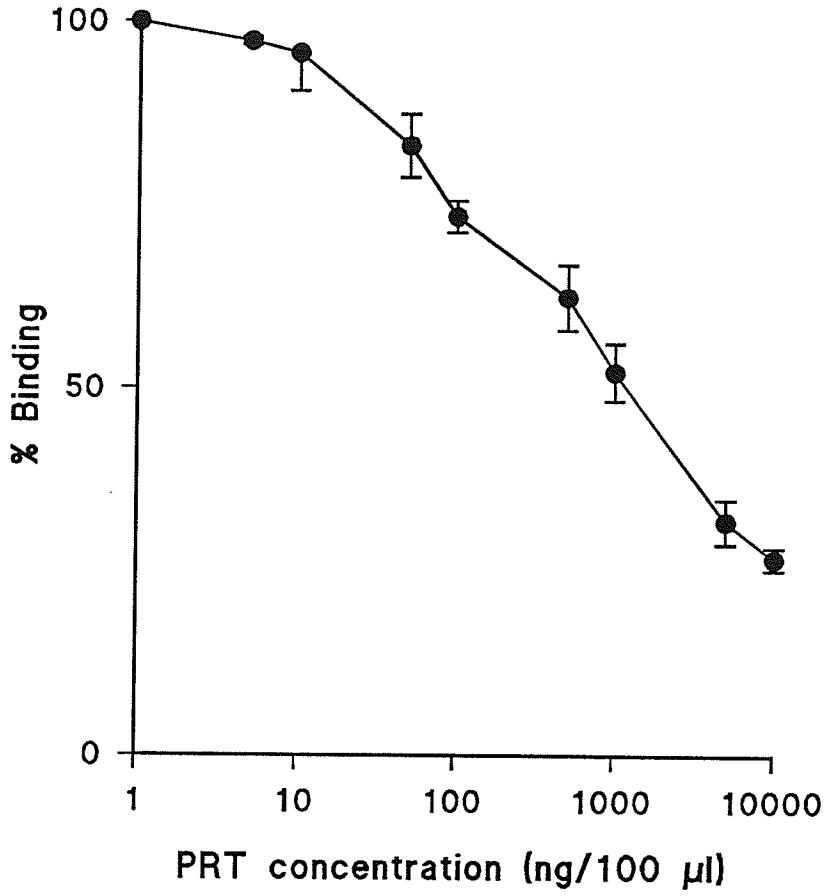
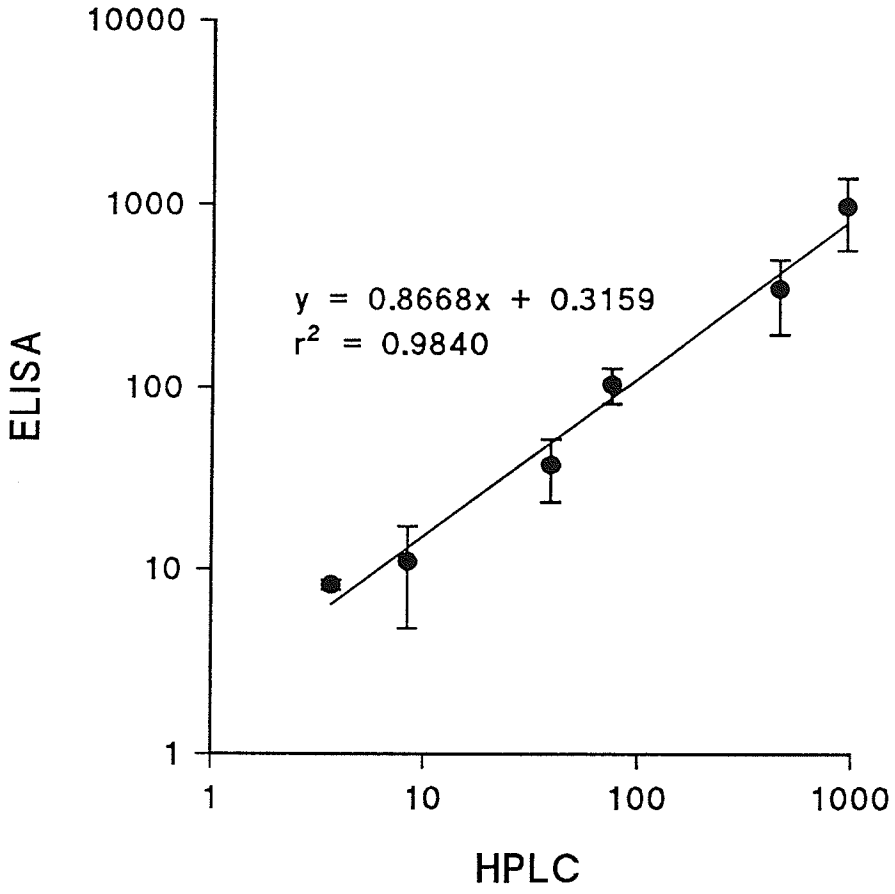

100
50
0
% Binding
1
10
100
1000
10000
PRT concentration (ng/100 µl)

Figure 22. Correlation between cELISA and reversed-phase HPLC of standard PRT. ELISA was performed using PRT dissolved in 10% acetonitrile and incubated for 90 min at 37°C. A solvent system of acetonitrile:water (65:35, v/v) at a flow rate of 1.0 ml/min was used for HPLC analysis.


10000
1000
100
10
1
ELISA
y = 0.8668x + 0.3159
r² = 0.9840
1
10
100
1000
HPLC

V. CONCLUSIONS AND RECOMMENDATIONS

The purpose of this research was to develop a monoclonal antibody specific to PRT and its use in an enzyme-linked immunosorbent assay (ELISA) for the detection of this toxin.

A PRT-producing strain of *Penicillium roqueforti* (ATCC no. 10110) was cultivated in a synthetic medium containing 2% yeast extract and 15% sucrose. Although PRT was produced, the levels were relatively low ranging from 0.20 to 31 mg/L in six different batches. Consequently, a commercial PRT, was used for further research. The prolonged storage of *P. roqueforti* spores (10 years) and the high frequency of mould transfer to maintain cultures, could account for the relatively low production of toxin. Therefore, for the future production of PRT, it is recommended that fresh isolates of different *P.roqueforti* strains are used in order to select the highest PRT-producing strain. Moreover, corn extract can be used in conjunction with YES medium in order to increase toxin production level as reported by Chang *et al.* (1991).

Monoclonal antibodies were prepared by immunization of mice with a PRT-BSA conjugate and subsequent fusion of mouse spleen cells with a nonsecretor myeloma cell line.

The 1H9 MAb developed during the course of this research was highly reactive with PRT (100%), cross-reactive with PRI (70%) and exhibited no reactivity with

PR-OH. Although the 1H9 MAb can be utilized of the detection of PRT and PRI, discrimination between both toxic forms would not be possible due to the MAb broad specificity. In order to improve MAb specificity to PRT, it is necessary to prepare different immunogen that one used in the above studies. Toxin should be conjugated to protein carrier *via* acetoxy group at position 3. This group is on site that is farthest removed from the toxic site of the toxin molecule, and is structurally different in both toxins: PRT and PRI. Since this part of the molecule would be primarily exposed to trigger an immune response, monoclonal antibodies that are synthesized against this part of hapten, should have a high specificity to PRT.

The developed PRT-MAb was shown to be of IgM class. This antibody class is relatively unstable and therefore could account for the low sensitivity of the assay. Thus, a future production of monoclonal antibody is recommended to be of IgG class, since it is a more stable type of immunoglobulin. In such studies, a screening assay that employs protein A could aid in identifying IgG during the course of MAb production.

Prepared monoclonal antibodies were applied to develop a competitive ELISA for the detection of PRT. Two major factors that effect ELISA such as, solvent system and incubation time, were investigated. Since PRT is not soluble in aqueous solution, organic solvents (methanol and acetonitrile) were used to dissolve PRT for the assay. Although PRT dissolves equally well in both methanol and acetonitrile, the results indicated that PRT is relatively unstable in methanol. In contrast, PRT showed a good stability in acetonitrile. Among several sets of conditions tested for compatibility with MAbs, solvent containing 10% acetonitrile in PBS buffer and incubation time of 90 min

was chosen since this set has resulted in the best standard curve for the PRT determination. The proposed ELISA had a detection limit (10% inhibition) of 10 ng PRT per assay. Comparison of the ELISA with reversed-phase HPLC method for the quantification of PRT in the range of 5 to 10^3 ng gave a good correlation with correlation coefficient of 0.9840.

The established MAb-based ELISA has been tested only in a model system containing PBS buffer. Therefore, it is neccessary to evaluate the performance of this assay by testing different samples such as, blue-cheeses, salad dressing containing blue-cheese and fermenting broths, for the presence of PRT. Testing should include validation of method with emphasis on accuracy and reproducibility.

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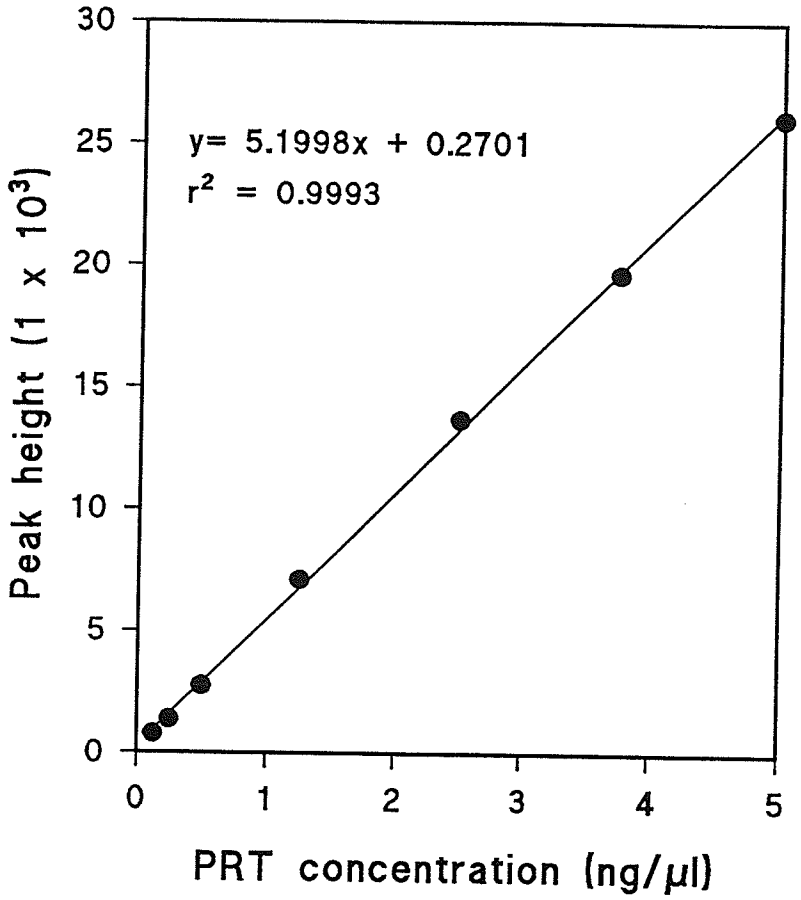
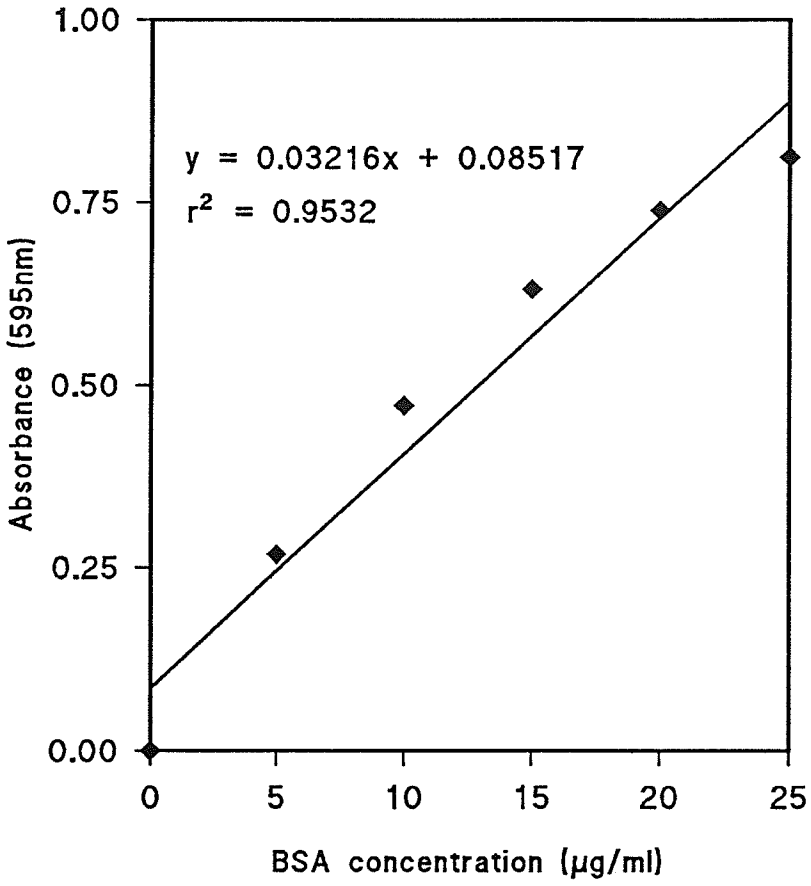
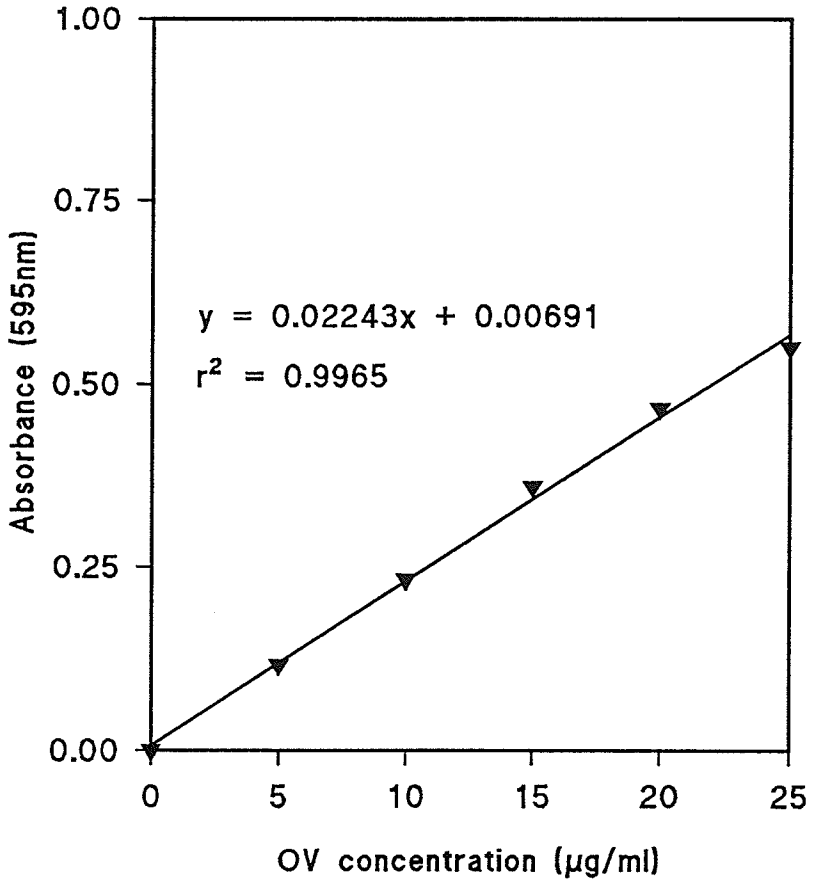
APPENDIX I - Correlation of PRT concentration and HPLC peak height. PRT standard was dissolved in acetonitrile and the analysis was carried out using acetonitrile:water (65:35, v/v) at a flow rate of 1.0 ml/min.


y= 5.1998x + 0.2701
r² = 0.9993
Peak height (1 x 10³)
PRT concentration (ng/µl)
0
5
10
15
20
25
30
0
1
2
3
4
5

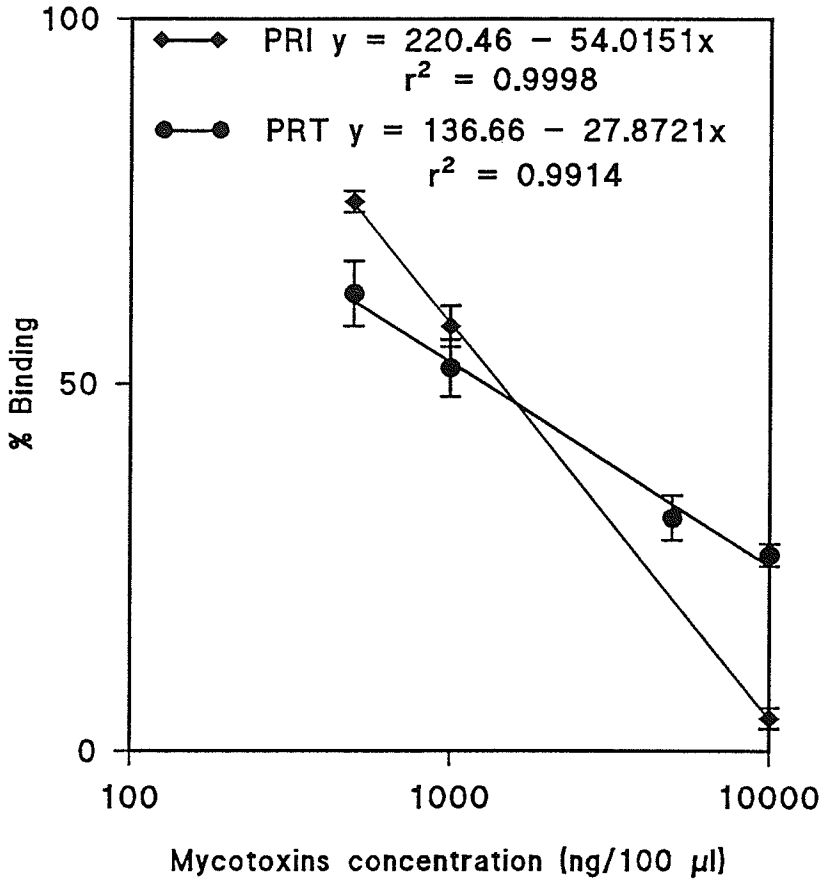
APPENDIX II - Standard curve of correlation between bovine serum albumin (BSA) concentration and absorbance values for protein analysis by BIO-RAD assay.


1.00
0.75
0.50
0.25
0.00
Absorbance (595nm)
y = 0.03216x + 0.08517
r² = 0.9532
0
5
10
15
20
25
BSA concentration (µg/ml)

APPENDIX III - Standard curve of correlation between egg white albumin (OV) concentration and absorbance values for protein analysis by BIO-RAD assay.


1.00
0.75
0.50
0.25
0.00
Absorbance (595nm)
y = 0.02243x + 0.00691
r² = 0.9965
0
5
10
15
20
25
OV concentration (µg/ml)

APPENDIX IV - Standard ELISA curves for PRT and PRI. Toxins were dissolved in 10% acetonitrile and incubated for 90 min at 37°C.


100
50
0
% Binding
PRI y = 220.46 − 54.0151x
r² = 0.9998
PRT y = 136.66 − 27.8721x
r² = 0.9914
100
1000
10000
Mycotoxins concentration (ng/100 µl)