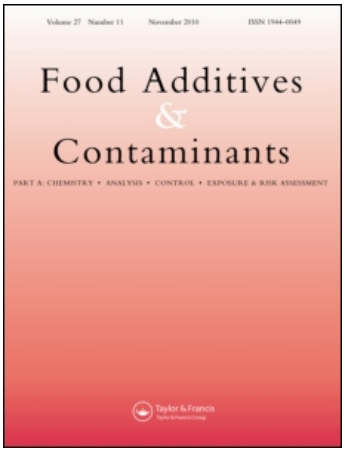

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Effect of novel water-soluble polymeric forms of sorbic acid against *Fusarium oxysporum* f.sp. *radicis-cucumerinum*

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New controlled release water-soluble formulations of sorbic (2,4-hexadienoic) acid were prepared and their inhibitory activity on mycelium growth of Fusarium oxysporum *f.sp.* radicis-cucumerinum *was evaluated. The new products are epoxidized polymers of polyvinylpyrrolidone (PVP) containing covalently bonded sorbic acid (polymeric esters of sorbic acid) and complexes of PVP with hydrogen bonded sorbic acid, characterized by controlled release of sorbic acid. It was shown that the polymeric complexes of sorbic acid with PVP were more effective fungicidal agents than sorbic acid polymeric esters. In all cases the activity of polymeric derivatives (esters and complexes) was increased by lowering the molecular weight of the polymeric carriers. Controlled release formulations of these polymeric derivatives are new promising products due to their low toxicity, wide range of efficient concentrations for application and ability to regulate lyophilicity. Our data contribute to the understanding of the action mechanism of various polymeric sorbic acid formulations and can result in products which are particularly suitable for food and feed protection applications.*

Keywords: sorbic acid, polyvinylpyrrolidone, controlled release formulations, *Fusarium oxysporum*

Introduction

The problem of food contamination by fungi and their toxic metabolites is quite common. Even now, worldwide, 30–40% of food production tests positive for mycotoxins each year. This has a negative effect on the health of the population as well as on the economy of the main animal feed and food producers (Richard *et al.* 1989). A way to deal with this problem is to use organic acids with a small carbon chain, e.g. sorbic acid and propionic acid, as food preservatives. They have been proven to prevent fungal growth and spore germination and to reduce mycotoxin biosynthesis by inhibiting the biological pathways responsible for mycotoxin production and release. They inhibit fungal growth by preventing the permeation of small molecules across membranes as well as by inhibiting some membrane-embedded enzyme systems (Gareis *et al.* 1984, Lennox and Mcelroy 1984).

Sorbic (2,4-hexadienoic) acid is of special interest. It is widely used in different kinds of products (e.g. baked goods, beverages/soft drinks, cheese, fish, fruit juices, fresh fruits and vegetables, tomato juice, margarine, wine, sausage, sea food cocktail, chocolate syrup) for improving taste, for pH balance and as a preservative. Sorbic acid (SA) (E200) and its salts potassium sorbate (E202), sodium sorbate (E201) and calcium sorbate (E203) are also used against bacterial and fungal contamination (Sofos *et al.* 1985, Mahjoub and Bullerman 1986, Skrinjar *et al.* 1995). SA is sparingly soluble in water (0.25 g/100 ml at 30°C), potassium sorbate is freely soluble and sodium sorbate has better solubility than SA but its solid form is unstable and very rapidly undergoes oxidation upon exposure to atmospheric oxygen. The soluble sorbates are preferred when it is desired to use them as preservatives in liquids. SA and sorbates can be directly added into the product. Dusting of food with dry SA is also possible but is less recommended because SA irritates the skin and mucous membranes (Luck 1990). The fungicidal activity of SA is greater

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than that of other monocarboxylic acids (Tzatzarakis *et al.* 2000).

SA possesses low toxicity ($LD_{50} = 7.36$ g/kg after oral administration in rat) and is devoid of carcinogenic activity. It is non-mutagenic and non-clastogenic *in vitro* and *in vivo*. The low toxicity is explained by the fact that SA is metabolized rapidly by similar pathways to other fatty acids. In extreme conditions (high concentrations and temperature) SA may react with nitrite to form mutagenic products (Walker 1990).

Polyvinylpyrrolidone (PVP) is a polar, amphoteric soluble polymer. It is used in medical and pharmaceutical fields as an excipient and plasma expander (Seale *et al.* 1992). More specifically, it was found that PVP may be used to improve the dissolution rate of a poorly water-soluble drug, the bioavailability of products (Sugano and Shinogi 1999, Tantishaiyakul *et al.* 1999), the physicochemical stability of new vesicle systems (Amemiya *et al.* 1999) and as a carrier molecule for drug delivery systems (Chary *et al.* 1999, Kamada *et al.* 1999).

The last few decades have witnessed concerted efforts to enhance the effectiveness of drugs used in therapeutic and preventive veterinary medicine by improving their selectivity and solubility and lowering their toxicity and side biodegradation. The use of bioactive substances in controlled release forms eliminates these disadvantages. Diffusion systems, liposomes, systems with hydrolysable bonds between a bioactive ligand and a polymer carrier etc. are such formulations. The aim of the present work was to investigate the activity of two types of polymeric derivatives of SA with controlled release action—polyvinylpyrrolidone (PVP) esters of this acid and its PVP complexes—on mycelial growth of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. *Fusarium oxysporum* is a common mould that contaminates tomato and potato plants, wheat and corn (Chu *et al.* 1995, Seo *et al.* 1996).

Materials and methods

Reagents and chemicals

The solvents used (acetone, dimethylformamide (DMF), dimethylsulphoxide (DMSO) and ethanol) were trade products of analytical grade. Dextrose and agar were provided by Merck. Chloroacetamide, SA and polyvinylpyrrolidone (PVP) with molecular weights (MW) of 10 kDa, 40 kDa and 360 kDa respectively were provided by Sigma.

Water-soluble sustained release formulations of SA (type A and type B)

Formulation of type A: SA covalent bonded to PVP. Polymeric carriers for the covalent immobilization of SA (PVP containing epoxy groups) were prepared by the reaction of PVP with chloroacetamide in the presence of Na ethylate in ethanol (Devarajan *et al.* 1992) (figure 1). Twenty-five g (0.225 base moles) of PVP with Mw of 10 kDa, 40 kDa and 360 kDa and 7.0 g (0.076 moles) of chloroacetamide were introduced in 200 ml of dry ethanol and then 5.0 g (0.217 moles) of sodium were introduced and the reaction continued at 10–12°C for 4 h. The resulting precipitate was filtered, washed by ethanol, reprecipitated from dry DMSO in dry acetone and was

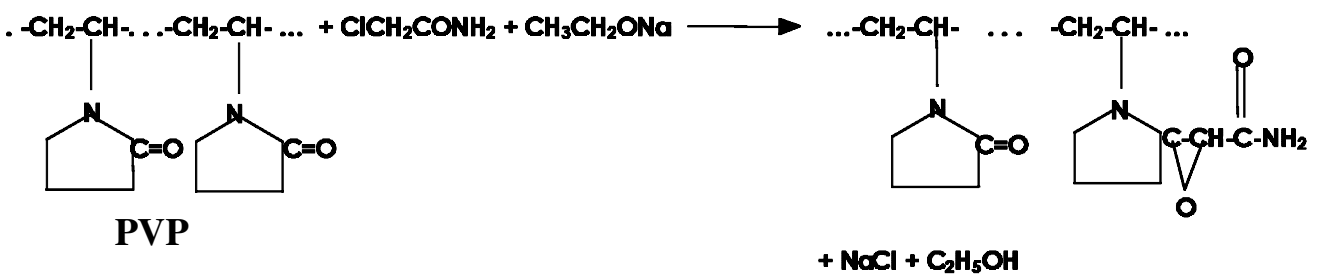


Figure 1. The synthesis of epoxidized polyvinylpyrrolidone (PVP) after the reaction with chloroacetamide in the presence of Na ethylate in ethanol.

Table 1. Molecular weight, content of epoxide groups in epoxidized polyvinylpyrrolidone (PVP) and content of sorbic acid (SA) in polymeric esters.

Polymeric esters	Epoxidized PVP			
	Molecular weight (kDa)	Content of epoxide group (mole %)	Content of SA	
			(mass %)	(mole %)
Polymeric ester 1	10	11.2	4.6	5.1
Polymeric ester 2	40	10.1	4.5	4.9
Polymeric ester 3	360	7.2	3.2	3.4

finally dried in vacuum. Epoxide content was determined by functional analysis (table 1).

Polymeric esters of SA were prepared by the reaction of acid (5.0 g, 0.045 moles) with PVP (5.0 g, 0.045 base moles) containing epoxy groups in dry DMF (50 ml) at 90°C for 4 h. Polymeric esters were precipitated in ethanol, washed by ethanol and dried in vacuum. The content of SA was determined spectrophotometrically at 254 nm (figure 2).

Formulation of type B: SA complexes with PVP. The SA complexes prepared contained SA attached to the polymer via a hydrogen bond (figure 3).

Complexes were prepared in aqueous solution. One g of SA in 50 ml of water and 40 g of PVP (MW = 10 or 40 kDa) in 100 ml of water or 18 g of PVP (Mw = 360 kDa) in 100 ml of water respectively were prepared separately. Both solutions were heated until completely dissolved. The above solutions were mixed and the temperature was adjusted to 100°C. The reaction led to a clear solution after lowering the temperature, which is an indication that all SA was attached to the polymer.

In vitro *experiment*

Fungus. The isolate AFu-68 of *Fusarium oxysporum* f.sp. *radicis-cucumerinum,* used in this study as a model biological material, was obtained from a cucumber plant with 'root and stem rot' symptoms (Vakalounakis 1996).

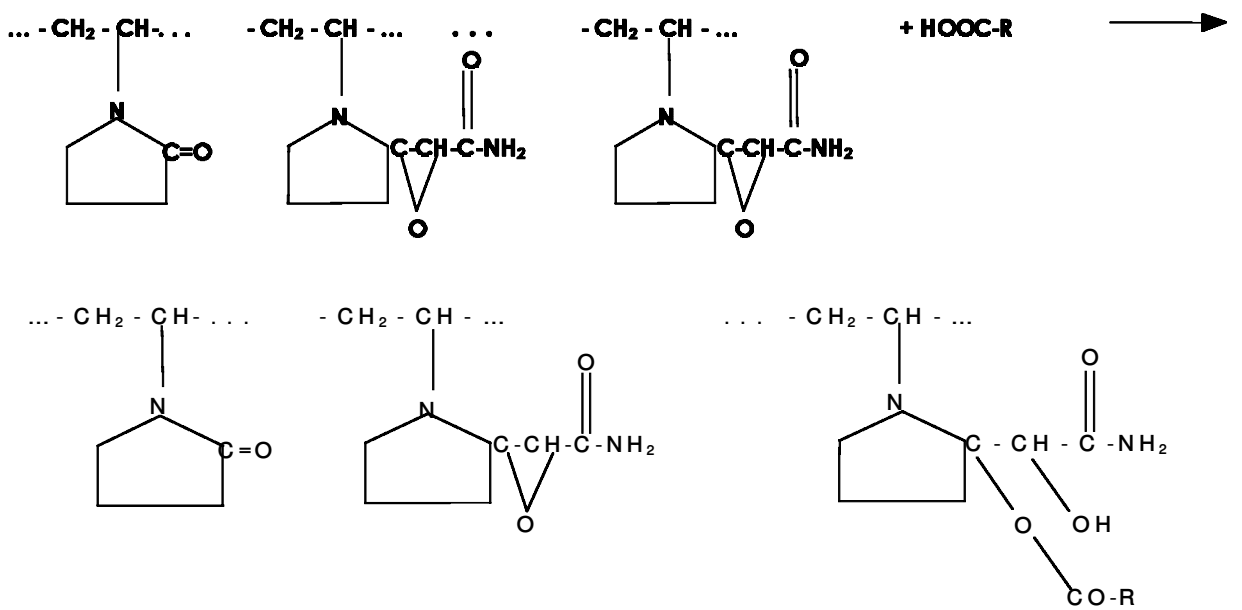
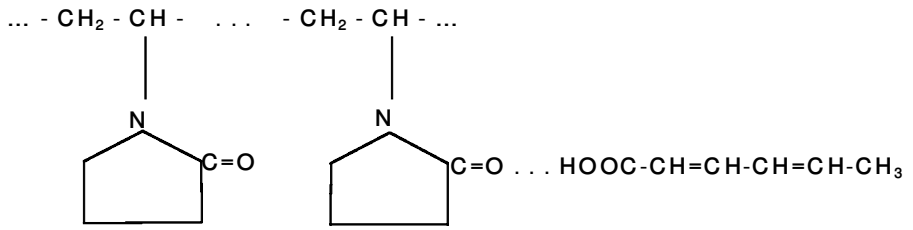


Figure 2. The proposed structure of polymeric ester of sorbic acid (SA) (SA covalent bonding to polyvinylpyrrolidone (PVP)).



Type B

Figure 3. The proposed structure of hydrogen bonding complex of sorbic acid (SA) with polyvinylpyrrolidone (PVP).

Stock solutions. Stock solutions of the PVP–SA complexes and polymeric esters of SA were prepared with distilled water to a final concentration 10 mg/ml. One g of SA was suspended in 10 ml of water. All solutions were kept at 4°C.

Culture conditions. *F. oxysporum* f.sp. *radicis-cucumerinum* was grown on potato dextrose agar (PDA) in 9 cm petri dishes containing 20 ml of nutrient medium adjusted to pH 6.4 with phosphate buffer. The medium was sterilized by autoclaving at 121°C for 20 min. When PDA had been cooled down to 50°C the growth inhibitors were added to give the required concentrations. PDA without growth inhibitors as well as PVP without SA served as controls. The dishes were inoculated centrally with 5 mm diameter agar plugs taken from the periphery of young, 5–6-days-old cultures of the fungus grown at 25°C in the dark. The cultures were incubated at 27°C for 6 days in the dark. Colony diameters were measured daily in two directions. The experiment was performed three times with four replicate dishes per treatment.

Results and discussion

Characterization of SA-controlled release formulations

Formulation of type A. The content of epoxide groups in all the polymers prepared by the reaction of PVP with chloroacetamide in the presence of Na ethylate was 7.2–11.2 mole%, while the content of SA attached to the epoxidized polymers ranged between 3.4 and 5.1 mole% (table 1). All these polymers were soluble in water. The epoxide group, which did not participate in the reaction with SA, was hydrolysed once the polymer had been dissolved in water. Polymeric esters with greater amounts of bonded SA can lose solubility as a result of cross-linking of polymer via polymerization of bonds of acid residues.

Formulation of type B. The content of SA in the complexes of molecular weight 10 kDa and 40 kDa was 2.5 mass% while the content of the complex of molecular weight 360 kDa was 5.5 mass%.

In vitro *activity of SA and of SA sustained release formulation*

All complexes as well as the polymer ester with MW = 10 kDa showed a higher inhibitory effect than SA in water on linear growth of *F. oxysporum* f.sp. *radicis-cucumerinum* at 800 ppm (figure 4). However, all complexes were more effective than the polymeric esters. In particular, the inhibition of mycelial growth of SA at 800 ppm was 11.6%, while the inhibition of polymeric ester 1 was 14.0%, of polymeric ester 2 1.7% and of polymeric ester 3 0.5%. In contrast, the inhibition of complex 1 at 800 ppm was 39.4%, of complex 2 33.0% and of complex 3 22.4%.

The dosage–mycelial growth inhibition curves of SA and its complexes were linear. The ED_{50} (dosage for 50% inhibition of mycelial growth) value of complex 1 was 1089 ppm, of complex 2 1478 ppm and of complex 3 2169 ppm, while the ED_{50} value of SA was 5592 ppm (table 2, figure 5). PVP without SA had no effect on mycelial growth of *F. oxysporum* f.sp.

radicis-cucumerinum. The slope of the curves decreased with increasing molecular weight of the complex, while the smallest value was observed with SA (table 2). In table 2 the ED_{50} values obtained from three different experiments are illustrated. The % values of the coefficient of variation (CV%) are small (4.58%–19.93%), confirming the good reproducibility of the experiments. The ED_{50} values obtained by all

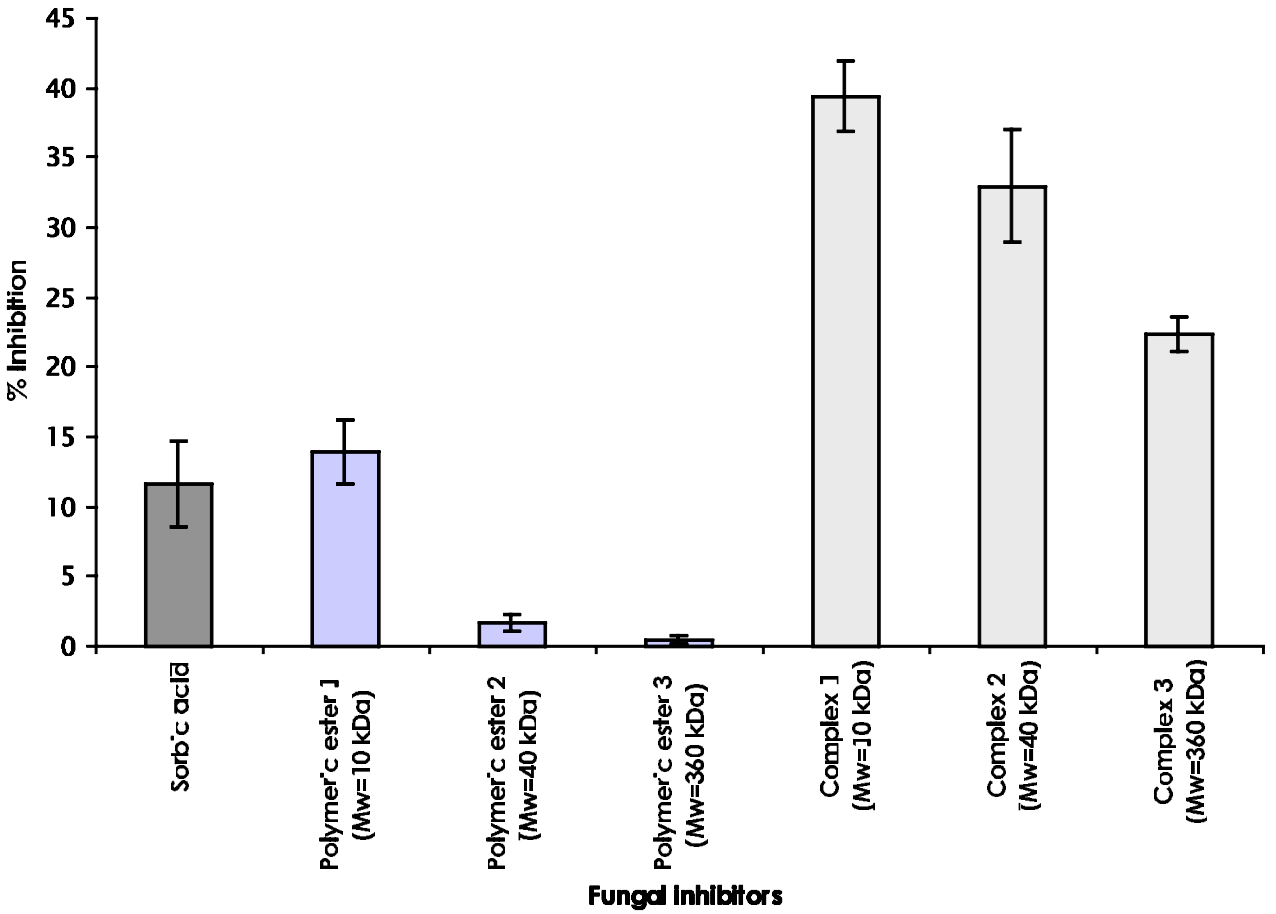


Figure 4. Inhibitory effect of sorbic acid (SA) and the polymeric derivatives. Vertical bars indicate standard errors of the mean on mycelium growth of Fusarium oxysporum *f.sp.* radicis-cucumerinum *at 27°C for 6 days, pH = 6.4 at 800 ppm.*

Table 2. ED_{50}[a] values (in ppm) of the complexes of sorbic acid (SA) on linear growth of Fusarium oxysporum *f.sp.* radicis-cucumerinum.[b]

Bioactive	Exp. 1	Exp. 2	Exp. 3	Mean	SD[c]	CV(%)[d]	Slope	r^2	p
Complex 1 (MW = 10 kDa)	1147	1059	1063	1090	± 49.9	4.58	0.0397	0.9702	< 0.001
Complex 2 (MW = 40 kDa)	1585	1701	1148	1478	± 291.7	19.73	0.0310	0.9719	
Complex 3 (MW = 360 kDa)	2690	2188	1629	2169	± 530.4	24.45	0.0204	0.9625	
Sorbic acid	4515	5520	6742	5592	± 1114.8	19.93	0.0090	0.9802	

[a] ED_{50} = dosages (ppm) for 50% inhibition of mycelial growth.
[b] The content of SA in the first two complexes is 2.5 mass %, while in the third complex is 5.5 mass %.
[c] Standard deviation.
[d] Coefficient of variation.

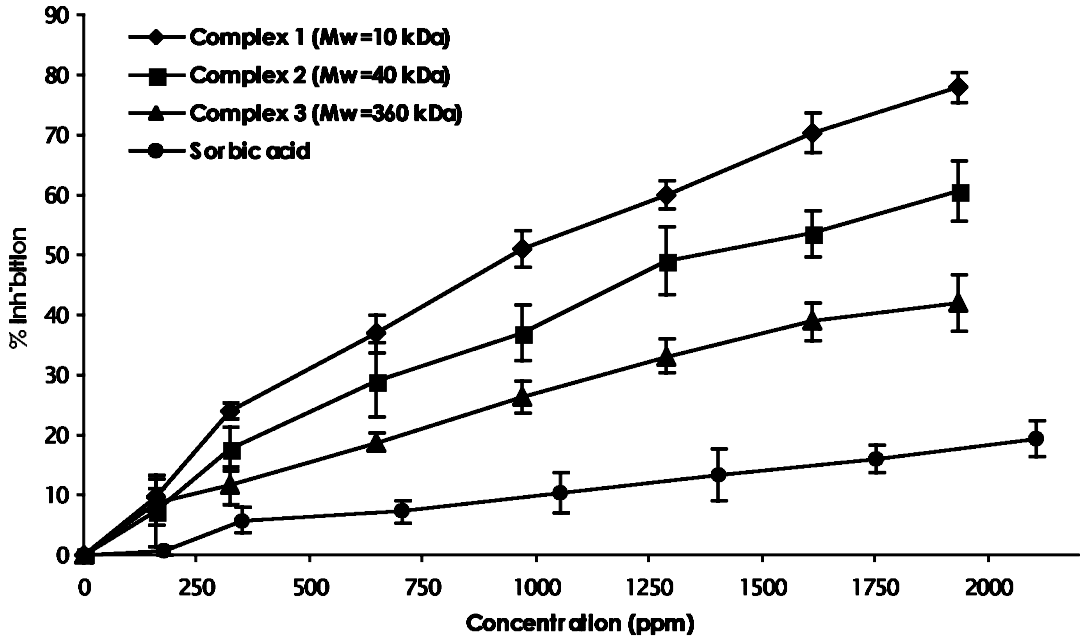


Figure 5. Inhibition of water solutions of sorbic acid (SA) and its polyvinylpyrrolidone—SA complexes on mycelium growth of Fusarium oxysporum *f.sp.* radicis-cucumerinum *at 27°C, pH = 6.4.*

the mycostatic agents were significantly different (by one-way ANOVA, $p < 0.001$, $F_{obs} = 31.461 > F_{crit} = 4.066$).

The inhibitory effect of polymeric esters and complexes of SA on mycelial growth of F. *oxysporum* f.sp. *radicis-cucumerinum* was increased in a manner dependent on the molecular weight of PVP. The lower the molecular weight the higher the inhibition. This is probably due to the easier release of SA from PVP with a lower molecular weight, which consequently acts upon the fungus in a higher concentration. It has been shown that in a polymeric system the bioactivity is attributed to the release of the bioactive substance (e.g. SA) from the polymer. The hydrolysis rate of the bioactive ligand from the system is associated with the entire expression of bioactivity. A moderate hydrolysis rate indicates optimum bioactivity results (Shtilman *et al.* 1998).

The higher inhibitory effect of SA complexes than that of polymeric esters on mycelial growth of *F. oxysporum* f.sp. *radicis-cucumerinum* may be attributed to the structure of the hydrogen bonding in the SA complex with PVP, which is weaker than the SA–PVP covalent bond of the polymeric esters.

PVP is an inert molecule, but SA is an effective fungistatic agent. Gareis (1984) suggested that aflatoxin and T-2 toxin production may be stimulated by an amount of SA near to the minimal inhibitory concentration, but may be reduced at higher concentrations of SA. It is suggested that a specific concentration of SA is able to decrease the activity of the tricarboxylic acid cycle, leading to an increase of the intracellular acetyl coenzyme A level, which is further associated with fungal inhibition (Shantha and Murthy 1981). The formulations of PVP–SA prepared not only possess low toxicity to mammalian cells, but increase the fungistatic activity of SA.

Conclusions

Polymeric formulations of SA, in which the bioactive ligand is attached to the polymer carrier by different hydrolysable bonds, exerted an inhibitory effect on the mycelial growth of *Fusarium oxysporum* f.sp. *cucumerinum*. It was shown that fungicidal activity of polymeric complexes of SA with PVP, in which the SA was connected with the polymeric carrier with

hydrogen bond, was greater than that of polymeric esters of SA prepared from PVP containing epoxide groups. The bioactivity was higher for polymers with lower molecular weight, probably because of the higher hydrolysis rate of SA from the polymeric systems.

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