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ORIGINAL PAPER

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Controlled release of water-soluble polymeric complexes of sorbic acid with antifungal activities

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Abstract We synthesized six water-soluble polymeric complexes of sorbic acid with polyvinylpyrrolidone of different molecular weight (mol wt). As shown by infrared absorption spectrum analysis, the complexes were formed by hydrogen bonding. The complexes (SC1, with mol wt=10 kDa, SC2 with mol wt=25 kDa, SC3 with mol wt=30 kDa, SC4 with mol wt=40 kDa, SC5 with mol wt=90 kDa, and SC6 with mol wt=360 kDa) were characterized as low mol wt (SC1, SC2, and SC3) and high mol wt (SC4, SC5, and SC6). The antifungal potencies of the complexes were tested by the macrodilution susceptibility method against environmental and clinically important fungi. Sorbic acid as well as the complexes exhibited minimum inhibitory concentrations (MICs) lower than potassium sorbate against all the strains tested. MICs of SC1, SC2, and SC3 were shown to be 2- to 4-fold lower for yeast and 1.5- to 3-fold lower than those of sorbic acid for moulds, respectively. The MICs of SC4 and SC5 against both of the *Candida* species tested ranged from 500 to 800 µg/ml, whereas for SC6 and sorbic acid they were about 1 mg/ml. The potencies of the high mol wt complexes against moulds were decreased by increasing the mol wt. For both of the moulds tested, the MICs of SC4 were slightly lower than those of sorbate. The MICs of sorbic acid and SC5 were equal to 300 µg/ml and 500 µg/ml respectively for *Aspergillus parasiticus* and for *Penicillum viridicatum*. The susceptibility to SC6 of all of the hyphomycetes tested was higher than that to sorbic acid. The low mol wt complexes and the sorbic acid exhibited minimal fungicidal concentrations (MFCs) 2 and 3 times higher respectively than the MICs. Sorbic acid and SC3 at a concentration of 2.5 mg/ml in an in vitro time kill curve study of *Candida tropicalis* were shown to be fungistatic, whereas SC1 and SC2 were fungicidal at the same concentrations. For *Aspergillus parasiticus* sorbic acid at 2.5 mg/ml was fungistatic for a 24-h period, whereas SC1, SC2, and SC3 were fungicidal.

Keywords Sorbate · Polyvinylpyrrolidone · Antifungal · Controlled release complexes

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Introduction

Weak acid preservatives with a small carboxylic chain, such as sorbic acid (SA) and propionic acid, have been shown to prevent fungal growth and spore germination and to decrease mycotoxin biosynthesis (Beuchat 1981; Lennox and McLeroy 1984; Mahjoub and Bullerman 1986). They prevent growth of fungi by affecting the permeation of membranes to lipophilic acid molecules (Stratford and Rose 1986) and by inhibiting active transport and respiration (Freese et al. 1973).

SA (CH_3-CH=CH-CH=CH-COOH) and its salt potassium sorbate (CH3CH=CH-CH=CH-COOH) are widely used in different kinds of food as preservatives (Chichester and Tanner 1972). Their antibacterial, antifungal, and antimycotoxin activity has been proven in different experimental systems (Aly 1996; Beuchat 1981; Combina et al. 1999; Garza et al. 1993; Mahjoub and Bullerman 1986; Skirdal and Eklund 1993; Sofos and Busta 1981; Sofos and Busta 1983; Stuart et al. 1977). SA is used in poultry feed as a fungal inhibitor, and is beneficial to bird health and growth (Bartov 1985; Sofos et al. 1985). Despite its good antifungal and antimycotoxin activity, SA is slightly soluble in water. For this reason, SA in its crystalline form is used in bakery products (Chichester and Tanner 1972; Sofos and Busta 1983), which leads to problems in homogenization with the substrate. Recently, SA was also used in soft drinks (Chichester and Tanner

1972), but legislation in many parts of the world limits its use.

Over the last few decades polymeric molecules have been used to enhance the effectiveness of drugs used in therapeutic, preventive, and veterinary medicine by improving their selectivity and solubility and lowering their toxicity and biodegradation.

Recent advances in polymer science have resulted in interesting findings concerning the properties and/or applications of 1-vinyl-2-pyrrolidone (vinylpyrrolidone) polymeric molecules. Polyvinylpyrrolidone (PVP) is a polar, amphoteric water-soluble polymer, which forms water-soluble complexes with a wide variety of substances. It is used in medical and pharmaceutical fields as an excipient. PVP has been used to improve the bioavailability of products (Fang et al. 1999; Hammouda et al. 1999; Sugano and Shinogi 1999; Tantishaiyakul et al. 1999). It was also designed as a carrier molecule for various drug delivery systems in humans (Cable and Lloyd 1999; Chary et al. 1999; Kamada et al. 1999).

PVP is currently used as a food additive (Golubkina et al. 1990; Gomez-Plaza et al. 2000; Kawasaki et al. 1995). Typically, as reported in a final report on the safety assessment, PVP K-30 [average molecular weight (mol wt) 40,000] is used in cosmetic formulations, food additives, and pharmaceuticals (Anonymous 1998).

The aim of the present study was to investigate the in vitro antifungal activity of new PVP derivatives of SA synthesized in our laboratory.

Materials and methods

Drugs, reagents, media, and stock solutions

SA was from Sigma (St Louis, Mo., USA). Peptone yeast extract with 0.2% glucose (PYG) pH 6.4 was a gift from Bioprepare (Papanicolaou, N. Pchyhiko, Athens, Greece).

SA solution at 10 mg/ml was prepared in 0.250 M phosphate buffer pH 6.4. Potassium sorbate solution was prepared by neutralization of 0.746 g of SA with 1 M potassium hydroxide solution and dissolving in 50 ml of distilled water at a final concentration of 20 mg/ml. Solutions were stored at room temperature.

General procedure for synthesis of SA-PVP complexes

Complexes were prepared as follows. SA (1 g) and 40 g of PVP (mol wt=10 kDa or mol wt=25 kDa or 30 kDa or 40 kDa) were suspended in 50 ml and 150 ml of sterile, distilled water, respectively. For the 90-kDa or 360-kDa complexes, 0.5 g SA and 9 g PVP of 90 kDa or 360 kDa mol wt were suspended respectively in 80 ml and in 150 ml of water. The solutions were heated at 95°C until complete dissolution. To form each complex, SA solution and the respective PVP solution were immediately mixed and the temperature was adjusted to 100°C. SA and PVP are not highly soluble in water unless heated. Heating enhances the solubility of the compounds and enables them to react. The reaction leads to a clear solution after the drop in temperature, which is an indication that all the SA is attached on the polymer. The final pH was adjusted to 6.4 with 0.5 M phosphate buffer. The newly synthesized sorbate-PVP complexes were stored in water at room temperature at a final concentration of 10 mg/ml. The complexes were compared with PVP and SA by infrared (IR) absorption spectrum analysis.

Fungal strains and growth conditions

The fungi tested included two environmental strains of hyphomycetes (moulds) and two clinical strains of *Candida* as follows. The strain *Aspergillus parasiticus* 1130 was kindly provided by Professor S.W. Peterson (National Centre for Agricultural Utilization Research, Ill., USA); the strains *Penicillium viridicatum* CA 100, *Candida albicans* TEI 101, and *Candida tropicalis* TEI 184 were from our collection (The Technological Institution of Athens Collection). Both of the hyphomycetes were phenotypically identified. *A. parasiticus* 1130 and *P. viridicatum* CA 100 strain were grown at 25°C and at 30°C, respectively. The *Candida* strains were identified by the API system. *C. albicans* TEI-101 and *C. tropicalis* TEI-184 were isolated from cervical and blood cultures, respectively, and were grown at 35°C. All strains were grown on Sabouraud dextrose agar unless otherwise specified.

Antifungal susceptibility studies

Susceptibility testing was performed by the broth macrodilution method as described previously following the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 1997) but with some modifications. For hyphomycetes, the minimum inhibitory concentrations (MICs) were measured according to NCCLS recommendations (NCCLS, 1998), but with one modification. PYG (potato yeast extract glycose) broth was used, instead of RPMI 1640. The MIC procedure was as follows. Briefly, 7-day *A. parasiticus* 1130 or 10-day *P. viridicatum* CA 100 conidia were collected in sterile saline. The spores were then washed in the same medium, were counted using a hemocytometer, and were adjusted to a density of 6×10^3 colony-forming units (cfu)/ml. The required concentrations of the drugs were prepared in PYG medium (0.5 ml) by serial dilution in sterile 6-ml polystyrene tubes and inoculated with an equal volume (0.5 ml) of the conidial suspension. The serial dilutions of drug and *A. parasiticus* or *P. viridicatum* were incubated for 24 h at 37°C and at 30°C, respectively.

To determine the exact MICs, intermediate dilutions of sorbate and its PVP complexes were prepared by adding the appropriate amount of compound required in a final PYG volume of 0.5 ml. Then 0.5 ml of the conidial suspension was added to give a final conidia concentration of 3×10^3/ml. Growth was recorded after gentle vortexing of the dilution tubes. The MIC was defined as the lowest concentration of the drug that inhibited visible growth by 80% (compared with the drug-free control) after 24 h of incubation at 37°C. The minimal fungicidal concentration (MFC) was determined as follows; 100 µl from each tube, in which no visible growth was scored, was washed twice in order to remove the antifungal agent, plated on Sabouraud agar, and incubated at 37°C for 24 h. The lowest drug concentration at which six or fewer colonies grew was recorded as the MFC.

For determinations of *C. albicans* and *C. tropicalis* MICs, 24-h colonies were suspended in PYG broth at pH 6.4 at a final inoculum of 4×10^3 cfu/ml. After the addition of an appropriate amount of antifungal agent, the MICs were determined in a final volume of 1 ml of PYG broth. The MICs were defined as the lowest concentration of the drug that inhibited growth by 90% compared with the drug-free control after 24 h of incubation at 37°C. The MFCs were determined by the same procedure as used for hyphomycetes.

Time-kill procedures

C. tropicalis

Cultures of *C. tropicalis* (24-h) were diluted to obtain an approximate final concentration of 4×10^3 cfu/ml in PYG after the addition of the drug. Aliquots (4 ml) were incubated with 2.5 mg/ml of sorbate, or SC1, or SC2, or SC3, or with 20% PVP for 24 h at 37°C.

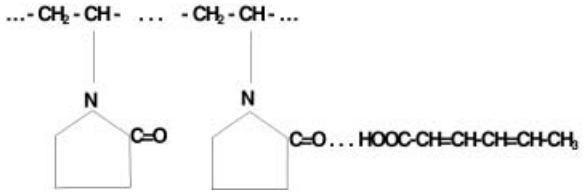

Fig. 1 The suggested structure of hydrogen bonding complex of sorbic acid (*SA*) with polyvinylpyrrolidone (*PVP*)

At different time intervals, 100 µl of the cultures was removed and, after serial dilution, 0.1 ml was spread on Sabouraud agar plates in duplicate. The plates were incubated at 35°C for 24 h and the numbers of cfu were determined.

A. parasiticus

Conidial suspension in PYG broth (4 ml), about 4.5×10^4 conidia/ml was incubated at 37°C in the presence of 2.5 mg/ml final concentration of SA or SC1, or SC2, or SC3. Control tubes contained about 4.5×10^4 conidia/ml. The effect of the compounds was monitored over a 24-h period as described for *C. tropicalis*.

Results

Characterization of sorbate-PVP complexes

Figure 1 shows the expected molecular structure of a basic unit of sorbate-PVP molecule. A PVP is a polymeric molecule from one repeated unit; in the newly synthesized sorbate-PVP complexes, SA is attached by hydrogen bonds at a specific position of the repeated polymeric unit. The mol wts of SA polymeric forms ranged from 10 kDa to 360 kDa. The SA content of the complexes is 2.5% w/w for the first four complexes, namely SC1 (10 kDa), SC2 (25 kDa), SC3 (30 kDa), and SC4 (45 kDa), and 5.5% w/w of SA in the SC5 (90 kDa) and SC6 (360 kDa).

Figure 2 shows IR spectra of SA (panel a), PVP (10 kDa) and SC1 (panel b). The peak area at 2,500–2,800 cm^{-1} in SC1 corresponds to the formation of hydrogen bonds (Pecsok et al. 1980). Similar spectra were detected for SC2–SC6 compounds. Free SA was detected at peak area of 2,200 cm^{-1} (Pecsok et al. 1980).

Antifungal activities of SA and SA-PVP complexes

The range of antifungal activity of sorbate-PVP complexes was first estimated by the double dilution method. Table 1 shows the MICs of the different fungi tested. In all the cases SA was shown to be more active than potassium sorbate.

For *A. parasiticus* 1130 and *P. viridicatum* CA 100, the MICs of the complexes SC1–SC5 ranged from 3 times lower up to the same level of sensitivity as that of SA. The susceptibilities to SC6 for both the moulds tested were about 1.5 times higher than those of sorbate. In all cases the MICs were inoculum dependent. By using twice as much inoculum the MICs of sorbate and of the complexes were equally increased by 2 times (data not shown).

The MFCs of SC1, SC2, and SC3, for both of the moulds tested, were 2 times higher than the MICs, whereas the MFCs of SA were 3 times higher than the MICs. The MFCs for the high mol wt compounds were 3 times higher than the MICs. By doubling the inoculum size, the MFCs of SA and of the low mol wt complexes increased 4 and 2 times respectively compared with the MICs (data not shown).

C. albicans TEI 101 and *C. tropicalis* TEI 184 showed higher MICs to SA than the hyphomycetes by the macrodilution method. However, susceptibilities of *C. tropicalis* TEI 184 to SC1, SC2, and SC3 were higher than those of *C. albicans* TEI 101. The MFCs for *C. albicans* and *C. tropicalis* to the low mol wt compounds were 2 times and the MFCs to the high mol wt compounds 4 times higher respectively than the MICs. Potassium sorbate exhibited the lowest antifungal activity against all of the strains tested. The stability of the complexes was checked over an 8-month period, by performing the susceptibility testing at 2-month intervals; during this period the biological activity was unchanged.

Time-killing curves

Figure 3 shows the effect of exposure of *C. tropicalis* TEI 184 to SA and its derivatives SC1, SC2, and SC3 over a 24-h period. The effect of SA and SC3 is fungi-

Table 1 Antifungal activities of sorbic acid (*SA*), potassium sorbate (*PS*), and of six sorbate-polyvinylpyrrolidone complexes (*MIC* minimum inhibitory concentration)[c]

Microorganism	MIC (µg/ml)							
	SA[a]	PS	SC1	SC2	SC3	SC4	SC5[b]	SC6[b]
Candida albicans TEI 101	1,000	>1,000	250	300	400	500	700	1,000
Candida tropicalis TEI 184	1,000	>1,000	300	400	500	600	800	>1,200
Aspergillus parasiticus 1130	300	500	100	150	200	250	300	500
Penicillum viridicatum CA 100	500	750	200	250	300	350	500	750

[a] SA in 0.250 M phosphate buffer

[b] There is some uncertainty about the MICs of these compounds, because of difficulty in handling (high viscosity) them

[c] *Results shown are from a typical experiment. MIC determinations were repeated three times with similar results, for the same inoculum concentration*

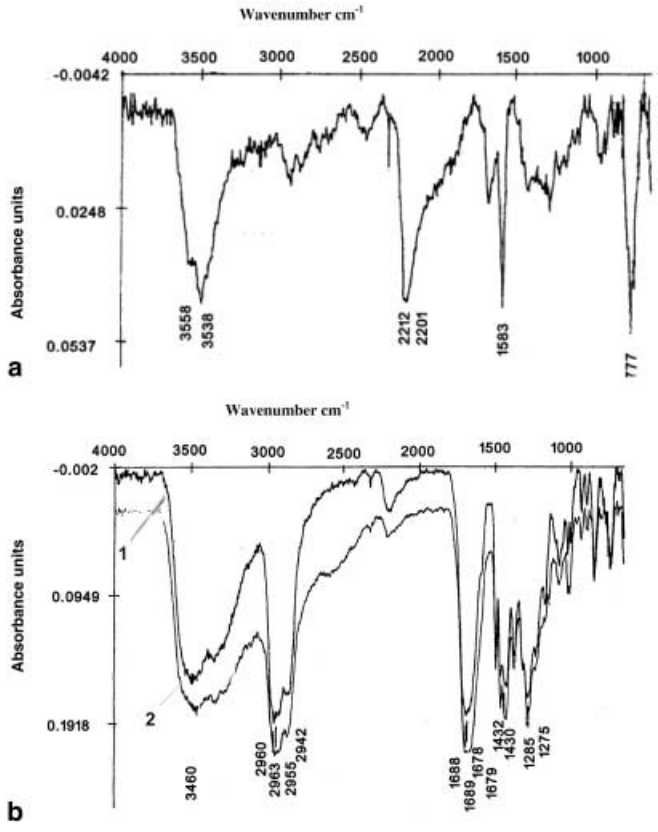

Fig. 2 The infrared absorption spectra of SA **(a)**, PVP (mol wt=10,000), line (1) and SC1, line (2) **(b)**

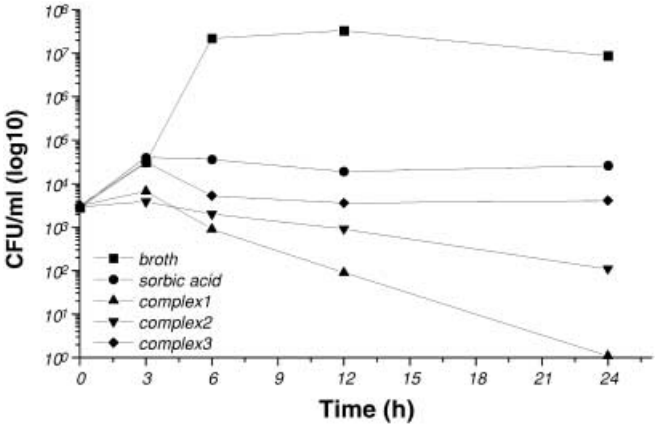

Fig. 3 Comparison of the fungicidal or fungistatic activities of SA complex 1 (SC1), complex 2 (SC2), and complex 3 (SC3) at 2.5 mg/ml each, against *Candida tropicalis* TEI 184. Each point represents the mean of two independent determinations. Experiments were repeated twice with similar results

static. Moreover, the fungistatic activity of SC3 is slightly higher than that of SA. In the presence of SA and SC3 the decrease in cell yield did not exceed one log over a period of 24 h. SC1 and SC2 are fungicidal, but SC1 provided ≥99.9% killing within 24 h. Detection level was 1 colony/ml of broth culture plated.

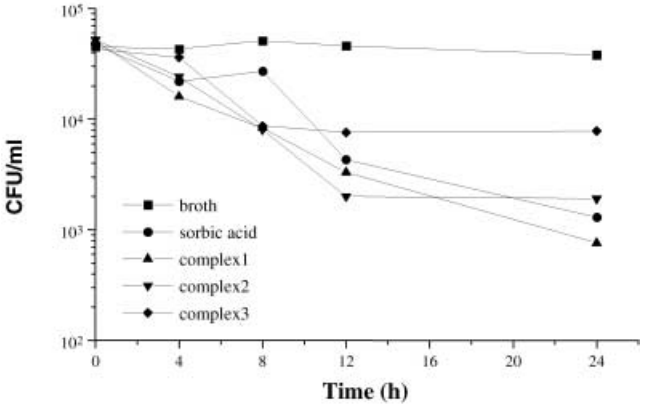

Fig. 4 Comparison of the fungicidal or fungistatic activities of SA complex 1 (SC1), complex 2 (SC2), and complex 3 (SC3) at 2.5 mg/ml against *Aspergillus parasiticus* 1130. Each point represents the mean of two independent determinations. Experiments were repeated twice with similar results

The three complexes SC4, SC5, and SC6 at 2.5 mg/ml showed a reduction in cfu/ml of *C. tropicalis* TEI 184 with time. However, with all of the three high mol wt compounds the growth of the organism after 24 h was inhibited only about 60% compared with the drug-free control (data not shown).

The effects of SA, SC1, SC2, and SC3 at 2.5 mg/ml extracellular concentration over a 24-h period on the ability of *A. parasiticus* conidia to produce colonies are shown in Fig. 4. In the presence of SA the decrease of cell yield in culture did not exceed one log, which is correlated with a fungistatic activity, whereas the activities of SC1, SC2, and SC3 in the same in vitro model were shown to be fungicidal (Fig. 4). The concentration of the drugs used for the kill curve studies was two- to fivefold higher than the MICs of the drugs for efficient killing. The addition of 20% PVP to the growth medium promoted the growth of both *A. parasiticus* and *C. tropicalis* by about 8%.

Discussion

SA is a weak acid preservative with antifungal activity, but its low solubility in water limits its use. In the present study, we constructed new polymeric complexes of SA with antifungal activities and compared the in vitro potencies against different fungal species.

The most-pronounced activity of SA was seen against *A. parasiticus* and *P. viridicatum*; lesser activity was observed against *Candida* species. Similar results concerning the spectrum of activities of SA and potassium sorbate for hyphomycetes have been reported (Vinas et al. 1990; Wallhauser and Luck 1978), but because of the different experimental conditions and different fungal strains tested, comparisons are difficult.

Some interesting findings concerning the biological activity of SA were recently reported. Lambert and Stratford (1999) demonstrated by using a thermodynamic-

kinetic approach that inhibition depends more on the degree to which individual preservatives are concentrated within yeast cells, rather than on the undissociated acid concentration per se. Secondary toxic actions for SA have been suggested, such as inhibition of glycolysis (Azukas et al. 1961) or changing of the permeability of the plasma membrane (Strattford and Anslow 1996; Strattford and Anslow 1998). Thus, the exact mode of action of SA has not been fully elucidated.

PVP by itself promotes fungal growth (our data), but when the SA molecule is attached by hydrogen bond, a polymeric structure is formed with controlled-release antifungal properties. In the newly synthesized SA polymeric formulations the antifungal ligand is attached by hydrogen bond to the polymer in a controlled-release form. As shown by the MICs determinations and killing curves, the activities of the complexes are decreased by increasing the mol wt. Low mol wt complexes have shown inhibition of *A. parasiticus* conidia germination as well as killing activities against *C. tropicalis*. Although the modes of action of these polymers are not fully understood, it is believed that the conformation of the complexes and their controlled release play a major role in their function. In all of the complexes some free SA was detected by IR spectroscopy. However, the contribution of the free SA in the complexes to their antifungal activity is unclear.

Probably, facilitated release and other, as yet unknown, biological properties of the SA-PVP complexes diminish cell yield in cultures and decrease the MIC of SA-PVP compared with sorbate. However, it is necessary to study further the physicochemical properties of the complexes to confirm the mode of action and resistance of SA-PVP complexes (Tsatsakis et al. 2000).

In conclusion, the results obtained in the present study suggest that SA-PVP complexes may serve as an effective novel SA delivery system with potential antifungal activities.

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