Ionophoric action of *trans*-isohumulone on *Lactobacillus brevis*

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Compounds (e.g. *trans*-isohumulone) derived from flowers of the hop plant (*Humulus lupulus* L.) protect beer from spoilage. Cells of *Lactobacillus brevis* IFO 3960 did not die when they were exposed to 40 μM *trans*-isohumulone for up to 120 h. At higher concentrations (80, 120 μM) death occurred after a lag period of about 30 h. *trans*-Isohumulone dissipated the transmembrane pH gradient of non-growing cells and reduced their ability to accumulate L-[U- ^{14}C]leucine. The membrane potential was dissipated to a smaller extent. Addition of *trans*-isohumulone to cells that had accumulated L[U- ^{14}C]leucine, under conditions in which no synthesis of protein took place, caused slow leakage of radiolabelled leucine. *trans*-Isohumulone did not inhibit the activity of the proton-translocating membrane ATPase. Potentiometric experiments with resting cell suspensions suggested that *trans*-isohumulone acted as an ionophore of the mobile-carrier type, causing electroneutral exchange of H^+ for divalent cations such as Mn^{2+} . A second monovalent cation (e.g. K^+) was essential for protonophoric activity.

Introduction

Bitter compounds (*cis*- and *trans*-isohumulone and congeners) derived from the hop plant (*Humulus lupulus* L.) have antibacterial properties. They interfere with plasma membrane function in *Bacillus subtilis* (Teuber & Schmalreck, 1973; Schmalreck *et al.*, 1975) but the mechanism by which beer-spoilage bacteria (*Lactobacillus* spp., *Pediococcus* spp.) are inhibited has not been reported. This is relevant since these organisms employ substrate-level phosphorylation (a cytoplasmic process) to generate ATP (Harold, 1986). The undissociated forms of hop compounds and hop-derived compounds exert their antibacterial action on lactic acid bacteria. The ionized forms of such compounds have negligible activity. Divalent cations diminish the activity of *trans*-isohumulone; monovalent cations stimulate it (Simpson & Smith, 1992).

Here, the effects of *trans*-isohumulone (Fig. 1) on cells of *Lactobacillus brevis* IFO 3960 are reported and its ionophoric properties demonstrated. A preliminary report of some of the results has been published previously (Simpson & Hammond, 1991).

Methods

Materials. Chemicals were from Sigma, BDH or Amersham International. *trans*-Isohumulone was prepared and characterized as

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NaDMG, sodium 3,3'-dimethylglutarate.

described previously (Simpson & Smith, 1992). Sodium 3,3'-dimethylglutarate buffer (NaDMG, pH 5.2) was prepared by titrating 0.1 M-NaOH with solid 3,3'-dimethylglutaric acid. The medium of de Man *et al.* (1960) (MRS) was modified by omission of Tween 80 (Simpson & Smith, 1992) and adjusted to pH 5.2 with concentrated HCl. *L. brevis* IFO 3960 from the Institute of Fermentation (Osaka, Japan) was subcultured at three monthly intervals on MRS agar.

Chemical analyses. Concentrations of SO_4^{2-} , PO_4^{3-} , K^+ , Na^+ , NH_4^+ , Mg^{2+} and Ca^{2+} were estimated by ion-exchange chromatography (Simpson, 1991). Cellular ATP was extracted with TCA (Lundin, 1984) or detergents (Simpson & Hammond, 1989) and analysed with the firefly luciferase assay (Simpson & Hammond, 1989).

Effect of *trans*-isohumulone on exponentially growing cells. Stationary-phase organisms (1.5 ml), grown for 3 d at 25 °C in modified MRS, were transferred to fresh medium (150 ml) and incubated at 25 °C with shaking. When the number of organisms reached approximately $4 \times 10^7 \text{ ml}^{-1}$ ($\text{OD}_{560} = 0.5$) *trans*-isohumulone (0–120 μM) was added as a methanolic solution (controls received methanol). Growth was monitored by measurement of OD_{560} , viable cell count (MRS agar) and cellular ATP content. Cell morphology was examined by electron microscopy and light microscopy. Leakage of ATP was assessed by analysis of membrane-filtered growth media.

Fig. 1. Chemical structure of *trans*-isohumulone.

Effect of trans-isohumulone on non-growing cells. *L. brevis* IFO 3960 was grown in modified MRS (25 °C, 16.5 h with agitation). The exponentially-growing organisms ($OD_{560} = 1.0$) were centrifuged (3000 g, 4 °C; 10 min) and washed twice with NaDMG buffer. They were then resuspended in buffer with glucose (10 g l⁻¹) and *trans*-isohumulone (0–400 µM). The cell suspensions (approximately 5.6×10^7 ml⁻¹) were shaken at 25 °C for 60 min, then analysed for (i) viable cell count on MRS agar, (ii) intracellular ATP content, (iii) cell morphology, and (iv) OD_{560} . Membrane-filtered supernatant fluids were analysed for intracellular materials [ATP, anions, cations, UV (260 nm)-absorbing materials] to check for plasma membrane damage.

Measurement of intracellular pH and membrane potential. Intracellular pH (pH_{int}) and membrane potential ($\Delta\psi$) were estimated from the transmembrane distribution of [¹⁴C]salicylic acid or [³H]tetraphenylphosphonium ions (TPP⁺) respectively (Ten Brink *et al.*, 1985). Cells grown for 3 d in modified MRS were chilled and harvested by centrifugation (3000 g, 10 min, 4 °C). After two washes in cold buffer (NaDMG, containing 10 g glucose l⁻¹) they were resuspended in fresh buffer at 25 °C to a cell density of 8×10^8 ml⁻¹. *trans*-Isohumulone (400 µM) or a combination of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 30 µM) and valinomycin (0.45 µM) were added as methanolic solutions (controls received methanol). The suspensions were then incubated at 25 °C for 60 min before measuring pH_{int} and $\Delta\psi$.

Effect of trans-isohumulone on uptake and efflux of L-[U-¹⁴C]leucine by L. brevis IFO 3960. Cells grown for 3 d at 25 °C in modified MRS were chilled to 4 °C, harvested by centrifugation (3000 g, 4 °C; 10 min) then washed twice with cold buffer (NaDMG containing 10 g glucose l⁻¹). After resuspension in fresh buffer (8×10^8 ml⁻¹) they were incubated for 60 min at 25 °C. The resuspension buffer contained chloramphenicol (50 mg l⁻¹) to prevent incorporation of L-[U-¹⁴C]leucine into protein. L-[U-¹⁴C]leucine (1.85×10^4 Bq, 1.62 µM) was added to portions of the suspension and, at intervals during the incubation (25 °C), the cells were separated from the supernatant by filtration through silicone oil (Ten Brink *et al.*, 1985). The radioactivity associated with each cell pellet was then measured.

The ability of *L. brevis* IFO 3960 to retain pre-loaded L-[U-¹⁴C]leucine in the presence of CCCP, valinomycin and *trans*-isohumulone was studied. Cells were loaded with L-[U-¹⁴C]leucine for 20 min. Immediately before the end of the 20 min incubation, the suspension was divided into portions (1.2 ml each). The following additions (6 µl each) were made to each tube: (i) methanol, (ii) *trans*-isohumulone (80 mM), (iii) CCCP (60 mM), and (iv) valinomycin (90 µM) and CCCP (6 mM). Samples (200 µl) were removed from each tube at 5, 10, 20 and 30 min, and the radioactivity in the cell pellet was measured.

Effect of trans-isohumulone on activity of the membrane-bound ATPase. The proton-translocating ATPase of *L. brevis* IFO 3960 was isolated and its activity assessed (Bender *et al.*, 1986). The reaction mixture contained NaDMG buffer (16 ml, 50 mM, pH 4.5), MgSO₄ (10 mM), glycerol (10 %, v/v) and 54 mg membrane protein l⁻¹. It was warmed to 30 °C and divided into two portions. Methanol (40 µl) was added to one portion and *trans*-isohumulone (60 µM) to the other. The reaction was initiated 10 min later by addition of ATP (6 mM). Samples (1 ml) were withdrawn from the mixture immediately and at 3 min intervals up to 30 min. The phosphate content of each sample was then determined (Serrano, 1978). Membrane protein content was measured as described by Spector (1978) using bovine-γ-globulin as standard.

Effect of trans-isohumulone and ionophores on the passive proton permeability (Harold & Baarda, 1968). Cells of *L. brevis* IFO 3960 were grown in modified MRS for 16.5 h at 25 °C with shaking, harvested by centrifugation (3000 g, 4 °C; 30 min), washed twice with cold deionized water and stored at 4 °C. They were resuspended to a concentration of about 1×10^9 organisms ml⁻¹ in an unbuffered medium containing KCl

(150 mM) and MgCl₂ (2 mM) at a range of temperatures (0–24 °C). Cells were transferred to a vessel (25 ml capacity) and sparged with O₂-free N₂ gas. The suspension pH was monitored with a pH meter and adjusted to 6.5 with NaOH. A pH gradient was imposed by addition of HCl (100 µM). Antibacterial agents (CCCP, 15 µM; valinomycin, 0.45 µM or *trans*-isohumulone, 100 µM) or methanol alone were then added. Further additions were made as appropriate. The methanol concentration did not exceed 0.2 % in any experiment. Occasionally deionized water was used as the suspending medium and pH was not adjusted. In some experiments KCl, NaCl, CaCl₂, MgCl₂ or MnCl₂ were added to the suspensions. In others the medium was analysed for Na⁺, K⁺, NH₄⁺, Ca²⁺ and Mg²⁺, before and after treatment with *trans*-isohumulone (100 µM).

Interaction between trans-isohumulone and Mn²⁺. The interaction between *trans*-isohumulone and Mn²⁺ in methanol was studied by UV spectroscopy (Pfeiffer *et al.*, 1974).

Results and Discussion

Effect of trans-isohumulone on growing organisms

There was a concentration-dependent reduction in growth rate in the presence of *trans*-isohumulone. The growth of cells exposed to 40 µM-*trans*-isohumulone was arrested immediately after it was added to the cell suspension. Growth resumed after a lag period of 7 h if the organisms were resuspended in fresh growth medium. The lag phase of untreated organisms was 1 h. No death occurred when the cells were exposed to 40 µM-*trans*-isohumulone for up to 120 h. At higher concentrations (80, 120 µM) death occurred after a lag of about 30 h. The rates of death induced by 80 and 120 µM-*trans*-isohumulone were similar. Some cell lysis occurred after about 60 h contact with 80 and 120 µM-*trans*-isohumulone at 25 °C, as shown by a reduction in OD_{560} , ATP leakage and microscopy. In addition, after 40 h exposure to 80 µM-*trans*-isohumulone, the length of treated cells was 25.5 ± 6.8 µm (mean $\pm$ SD, $n = 100$), whereas that of the untreated organisms was 40.3 ± 9.4 µm ($n = 100$) as estimated by electron microscopy. The organisms did not aggregate in the presence of *trans*-isohumulone.

In the early stages of contact with 40 µM-*trans*-isohumulone there was a reduction, followed by a three-fold stimulation, in ATP content (Fig. 2). Higher concentrations of *trans*-isohumulone (80, 120 µM) reduced the ATP content of cells. No ATP leaked from treated organisms when *trans*-isohumulone was added, indicating that the integrity of the cell membrane was maintained. At concentrations of *trans*-isohumulone that caused a reduction in viable count, the reduction in ATP content was small.

Effect of trans-isohumulone on non-growing organisms

The viability of *L. brevis* IFO 3960 was not affected by 60 min exposure to 400 µM-*trans*-isohumulone, equivalent to 10 × the MIC under these conditions. However,

Fig. 2. Effect of *trans*-isohumulone on cellular ATP content of growing organisms. Immediately before addition (arrow) of 40 (■), 80 (▲) or 120 (●) μM -*trans*-isohumulone (final concentration), the exponentially growing culture contained approximately 3×10^7 organisms ml^{-1} , each of which contained 2×10^{-17} mol ATP. The data shown represent the mean of duplicate determinations. The error did not exceed 10% of the mean in any case.

Fig. 3. Effect of *trans*-isohumulone on the intracellular ATP content of non-growing cells of *L. brevis* IFO 3960. A standardized suspension of organisms (approximately $5.6 \times 10^7 \text{ ml}^{-1}$) was prepared in NaDMG buffer containing glucose (10 g l^{-1}), exposed to different concentrations of *trans*-isohumulone for 60 min and their ATP content then assayed.

the ATP content of the cells was reduced (Fig. 3). Microscopy, OD_{560} and measurement of their ability to retain a pool of ATP and UV absorbing material indicated that the structural integrity of the plasma membranes was not impaired.

Uptake of L-[U- ^{14}C]leucine by *L. brevis* IFO 3960 was inhibited by the combined action of CCCP and valinomycin and, to a lesser extent, by CCCP alone.

Leucine uptake in this organism was probably mediated by a proton motive force (Δp)-dependent carrier that was energized by both ΔpH and $\Delta\psi$ components. *trans*-Isohumulone also inhibited uptake of L-[U- ^{14}C]leucine (Fig. 4a). Furthermore, application of *trans*-isohumulone (or CCCP and valinomycin) to cells that were pre-loaded with L-[U- ^{14}C]leucine caused them to slowly lose the label (Fig. 4b). A concentration of *trans*-isohumulone of $400 \mu\text{M}$ was equivalent approximately to the MIC for growth at this cell concentration (as estimated from the effect of *trans*-isohumulone on ATP content).

trans-Isohumulone at $400 \mu\text{M}$ dissipated the transmembrane pH gradient (ΔpH) of *L. brevis* IFO 3960. Control cells incubated for 60 min in NaDMG containing glucose had a ΔpH of 0.52 ± 0.002 (mean $\pm$ SEM; $n = 3$). Those treated with $400 \mu\text{M}$ *trans*-isohumulone had a ΔpH of 0.09 ± 0.09 ($n = 3$). The effects on $\Delta\psi$ were less pronounced: the values of $\Delta\psi$ were $-71.5 \pm 3.1 \text{ mV}$ and $-42.7 \pm 9.9 \text{ mV}$ ($n = 3$), obtained for control organisms and those treated with *trans*-isohumulone, respectively.

Ionophoric activity of trans-isohumulone.

In lactic acid bacteria, ΔpH is an important component of Δp , providing a mechanism by which generation of energy (ATP) and its utilization for nutrient transport can be coupled (Kashket, 1987). In addition, the intracellular pH influences nutrient transport and metabolic processes indirectly (Poolman *et al.*, 1987). The ability of an antibacterial agent to dissipate ΔpH can result from any of several mechanisms: (i) it may inhibit the activity of the proton-translocating ATPase, (ii) it may inhibit energy generation causing a reduction in ATPase activity due to a lack of ATP, or (iii) it may decrease the natural impermeability of the cell membrane to H^+ or other cations. This may result from significant physical disruption of the membrane or from effects on its permeability to specific ions. The activity of the membrane-bound ATPase of *L. brevis* IFO 3960 was not affected by $60 \mu\text{M}$ -*trans*-isohumulone at pH 4.5. An ATPase activity of $266 \text{ nmol phosphate (mg protein)}^{-1} \text{ min}^{-1}$ was obtained in the presence and absence of *trans*-isohumulone. Also, ATP limitation was not responsible for growth inhibition since the cellular concentration of ATP increased when growth was inhibited.

Ionophores transport ions across membranes (Harold, 1986). True ionophores move freely through membranes, whereas quasi-ionophores form a stationary pore. The activity of true ionophores is temperature-dependent, unlike that of quasi-ionophores. The permeability of resting cell suspensions of *L. brevis* IFO 3960 to protons can be expressed in terms of their equilibration times

Fig. 4. Effect of *trans*-isohumulone on uptake and efflux of L-[U-¹⁴C]leucine by *L. brevis* IFO 3960. (a) *trans*-Isohumulone [0 (■), 40 (▲), 80 (●), 200 (□) and 400 (△) µM] was added to organisms (approx. 8×10^8 ml⁻¹) suspended in NaDMG buffer (containing glucose). After 5 min, L-[U-¹⁴C]leucine was added and its uptake by the cells monitored. Chloramphenicol was present to prevent incorporation of L-[U-¹⁴C]leucine into protein. (b) Organisms which had been pre-loaded with L-[U-¹⁴C]leucine for 20 min were exposed to 400 µM-*trans*-isohumulone (●), 300 µM-CCCP (▲) or 30 µM-CCCP and 0.45 µM-valinomycin (■). Control organisms (□) received solvent only. The efflux of radiolabel from the cells was then monitored. In both experiments each point represents the mean of four determinations. The error in each case was no greater than 5% of the mean.

Fig. 5. *trans*-Isohumulone causes an influx of protons into *L. brevis* IFO 3960. Organisms (approximately 1×10^9 ml⁻¹) were suspended in KCl (150 mM), and MgCl₂ (2 mM). Net proton movements were monitored by changes in extracellular pH. HCl (100 µM) and *trans*-isohumulone (100 µM) were added at the stages indicated.

($T_{1/2}$, the time required to dissipate one-half of the difference between the starting and equilibrium pH values). Control suspensions had a $T_{1/2}$ of 2.8 min. CCCP or valinomycin only reduced $T_{1/2}$ slightly (2.5 and 2.3 min respectively). This was because the ability of CCCP to catalyse transmembrane proton movements was soon limited by the development of a membrane potential. When both CCCP and valinomycin were present, CCCP-induced proton movements were balanced by

valinomycin-induced K⁺ movements and the resulting H⁺ ingress was rapid ($T_{1/2} = 0.3$ min). *trans*-Isohumulone caused an increase in proton permeability ($T_{1/2} = 0.3$ min; Fig. 5), suggesting that it acts as an ionophore. The process was apparently electroneutral since the proton movements were not limited by development of a membrane potential. Also, relief of electrical constraints by valinomycin did not affect the rate of proton movements.

The direction of proton ingress could be reversed by addition of 0.8 mM-Mn²⁺ to the *trans*-isohumulone-treated cell suspension. This effect was not apparent when monovalent cations (Na⁺, K⁺) or other divalent cations (Ca²⁺, Mg²⁺) were added instead of Mn²⁺. Analysis of extracellular fluids by ion-exchange chromatography showed that leakage of Na⁺, NH₄⁺, Ca²⁺ and Mg²⁺ did not occur (leakage of K⁺ could not be tested for since it was present in the suspending medium). The high concentration of manganese in lactic acid bacteria such as *L. brevis* (typically 30 mM; Archibald & Fridovich, 1981) may favour its participation in the ionophoric action of *trans*-isohumulone.

At 25 °C, *trans*-isohumulone only displayed protonophoric activity when a second monovalent cation (K⁺ or Na⁺) was present in the suspending medium. It was inactive when the cells were suspended in water ($T_{1/2} = 2.8$ min). The ability of *trans*-isohumulone to interact with the plasma membrane may be enhanced by monovalent cations. It may be relevant that, in aqueous

solution, *trans*-isohumulone is unable to chelate K⁺ unless divalent or trivalent cations are present (W. J. Simpson & P. S. Hughes, unpublished data).

The effects of *trans*-isohumulone were dependent on temperature. With 100 µM-*trans*-isohumulone, T_{1/2} values of 30, 5.6, 2.0 and 0.3 min were obtained at 0, 5, 10 and 20 °C, respectively. Similar results were obtained regardless of whether *trans*-isohumulone was added before or after the temperature shift.

The ionophoric properties of *trans*-isohumulone clearly depend on its ability to complex metal ions. This property has been known for some time (Hudson & Rudin, 1959) but the interaction of *trans*-isohumulone with Mn²⁺ had not been demonstrated. Spectroscopic experiments showed that, in methanol, *trans*-isohumulone binds to Mn²⁺ ($K_d < 10^5$). Other experiments (P. S. Hughes & W. J. Simpson, unpublished data) have shown that its affinity for other physiologically significant divalent cations (e.g. Mg²⁺, Ca²⁺) is less than that for Mn²⁺.

These results imply that *trans*-isohumulone acts as a mobile-carrier of ions. Thus, at temperatures above the 'melting point' of the membrane lipids, it traps protons at one membrane surface and exchanges them for Mn²⁺ (or other divalent cations) at the other. The stoichiometry of the process is unknown. At lower temperatures, the mobility of *trans*-isohumulone in the cell membrane is reduced, thus restricting ionophoric activity. Experiments using liposomes, or other model membranes, will be necessary to confirm the ionophoric properties of *trans*-isohumulone.

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