JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Structural Characterization of 3-Hydroxypropionaldehyde and Its Derivatives

SABINE VOLLENWEIDER,*,† GUIDO GRASSI,‡ IWO KÖNIG,‡ AND ZDENKO PUHAN†

Laboratory of Food Biotechnology, Institute of Food Science and Nutrition, CH-8092 ETH Zentrum, and Laboratory of Physical Chemistry, CH-8093 ETH Höngg, Zurich, Switzerland

The compound 3-hydroxypropionaldehyde (3-HPA), together with HPA hydrate and HPA dimer, in aqueous solution forms a system with interesting chemical properties. Therefore, 3-HPA has attracted attention by the chemical industry for use as a precursor in the production of plastics, acrylic acid, and 1,3-propanediol and by the food industry, in using 3-HPA-producing *Lactobacillus reuteri* as a probiotic. To produce 3-HPA in high yield from glycerol, *L. reuteri* was used as a biotransformation system. A convenient chromatographic purification method was developed, and purified 3-HPA was analyzed using electrospray ionization mass spectrometry and ^{13}C NMR. Quantitative ^{13}C NMR revealed a concentration-dependent distribution of the three compounds forming the HPA system. At concentrations above 1.4 M, the HPA dimer was predominant. However, at concentrations relevant for biological systems, HPA hydrate was the most abundant, followed by the aldehyde form. Our results indicate that the dimeric form with expected antibiotic properties should not be the active form.

KEYWORDS: **^{13}C NMR; ESI-MS; probiotic; biotransformation; reuterin; 3-hydroxypropionaldehyde**

INTRODUCTION

3-Hydroxypropionaldehyde (3-HPA) was first discovered by Voisenet who observed that it was formed from glycerol during bacterial spoilage of wine by *Bacillus amaracrylus* (*1*). Today several organisms are known to transform glycerol into 3-HPA including bacteria such as *Klebsiella*, *Citrobacter*, and *Lactobacilli* (*2*−*6*). Voisenet postulated in subsequent papers that 3-HPA was produced by dehydration of glycerol, which was confirmed by Abeles et al. (*7*, *8*). Smiley and Sobolov discovered some years later that in living systems a coenzyme B12-dependent glycerol dehydratase converts glycerol into 3-HPA (*9*), which is further reduced to 1,3-propanediol to recover NAD^+ when sufficient glucose is in the medium (*4*, *10*−*15*). In aqueous solution, 3-HPA undergoes a reversible dimerization and hydration (*16*, *17*) (**Figure 1**), thus resulting in an equilibrium of 3-hydroxypropionaldehyde (3-HPA, **1**), 1,1,3-trihydroxypropane (HPA hydrate, **2**), and 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (HPA dimer, **3**), referred to here as HPA or the HPA system. Axelsson et al. found that *Lactobacillus reuteri*, first described by Kandler et al., accumulates an antimicrobial compound in a medium containing glycerol (*18*, *19*). Chemical characterization of the compound led to the HPA system, and on the basis of mass spectrometry data, the antimicrobial compound was identified as the dimeric form of 3-HPA (*20*). The HPA dimer was patented in 1988 under the name reuterin (*21*) and as "antibiotic reuterin" thought to be responsible for the probiotic effects of *L. reuteri.* Probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (*22*). Accordingly, the food industry has started industrial applications with *L. reuteri* to enhance the quality and value of milk products and is interested in using reuterin to better preserve milk, cottage cheese, and meat (*23*−*25*). However, the dynamics of the HPA system have not been studied yet and no data exist that characterize the HPA system under biological relevant conditions. The aim of this study was to clarify the dynamics of the HPA system under biological relevant conditions.

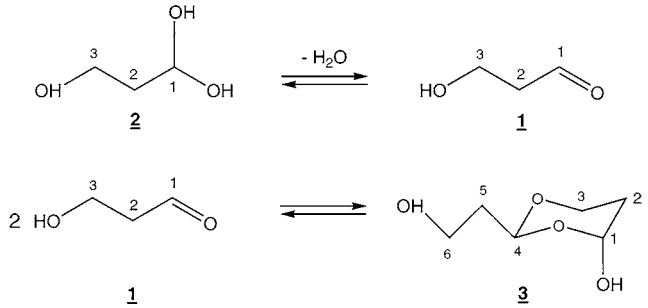

Figure 1. HPA system in aqueous solution determined by ^{13}C NMR (20 °C): 3-HPA (**1**), HPA hydrate (**2**), and HPA dimer (**3**).

MATERIAL AND METHODS

Reagents and Materials. If not otherwise stated, chemicals were purchased from Fluka (Buchs, Switzerland) or Aldrich (Steinheim, Germany). Acrolein was freshly distilled; glycerol, acetone, and ethyl

* To whom correspondence should be addressed. Tel: +41 1 6325369. Fax: +41 1 6321334. E-mail: sabine.vollenweider@ilw.agrl.ethz.ch.
† Laboratory of Food Biotechnology.
‡ Laboratory of Physical Chemistry.

10.1021/jf021086d CCC: $25.00
Published on Web 04/25/2003

acetate were used as received. The D_2O: 99.9% D was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA).

Bacteria Growth Conditions. *L. reuteri* SD 2112 was obtained from BioGaia (Stockholm, Sweden) (*26*). Bacteria were grown in MRS broth (Difco Laboratories, MI) at 37 °C without shaking. Stock cultures were prepared by inoculating skim milk with 6% of the MRS culture and stored at −20 °C. To activate the stock culture, 10 mL of MRS was inoculated with 10% stock culture and grown for 24 h at 37 °C.

Production and Purification of 3-HPA. *L. reuteri* cells were produced by inoculating 50 mL of MRS with 1% (v/v) *L. reuteri* stock culture. After 3 h at 37 °C, this culture (50 mL) was added to 1 L of MRS with 20 mM glycerol and incubated overnight to obtain the preculture. The cells were than harvested by centrifugation at 1500*g* for 10 min and washed once with the same volume of potassium phosphate buffer (100 mM, pH 7.0). The cells were resuspended in a total volume of 300 mL of sterile glycerol solution (200 mM), and the biotransformation was allowed to proceed for 2.5 h at 37 °C. The supernatant was recovered after centrifugation at 8000*g* for 10 min and filter-sterilized (FP/30/0.2 CA-S; Schleicher & Schuell GmbH, Einbeck, Germany). A 150 mL amount of the solution was frozen under rotation in a MeOH bath (−50 °C) and lyophilized. The viscous, slightly orange liquid was resuspended with 2 mL of acetone. To eliminate the orange-colored impurities, the sample, containing the HPA, was mixed with 3 g of silica gel 60 and poured in a glass Buchner funnel (2 cm diameter; glass filter no. 3) containing 1 g of silica gel 60. The HPA was eluted with 10 × 5 mL acetone:ethyl acetate (2:1) in a 100 mL pear-shaped glass flask. The elution of the fraction containing the aldehyde (3-HPA) was monitored by adding a droplet of the eluent on a silica gel 60 thin-layer chromtography (TLC) plate (Merck, Darmstadt, Germany), which was immediately dipped into a 2% (w/v) Purpald reagent solution (Aldrich) in 1 M NaOH (*27*). The eluate was concentrated under reduced pressure to ca. 1 mL, loaded on a 47 cm × 2 cm silica gel 60 column, and eluted with a flow rate of 2 mL/min with acetone:ethyl acetate (2:1). Fractions (5 mL) with the highest content of aldehyde indicated by Purpald reagent were combined, and the solvent was evaporated under reduced pressure to yield a colorless, highly viscous HPA solution. Finally, the last traces of acetone and ethyl acetate were removed by a 2-fold evacuation for about 3 min to 10^{-2} mbar under stirring. To obtain a less viscous stock solution, the HPA was diluted with distilled water to about 10 M solution, which was stable for at least 6 months during storage at 4 °C.

Analytical Methods. *Estimation of the Concentration and Purity of 3-HPA.* The concentration of HPA was determined by using a colorimetric method containing tryptophan adapted from Circle et al. (*28*). The standard curves were prepared in 50 mL polypropylene centrifugation tubes. Freshly distilled acrolein (0−6 μmol) was added with a Hamilton syringe to 6 mL of distilled water. Then, 4.5 mL of DL-tryptophan solution (0.01 M solution in 0.05 M HCl; stabilized with a few drops of toluene) and 18 mL of concentrated HCl (37%; 12 N) were added immediately. Samples containing HPA or acrolein were prepared in a total volume of 1 mL with distilled water in round screw cap glass cuvettes. Then, 0.75 mL of DL-tryptophan solution and 3 mL of concentrated HCl were added immediately. Solutions were incubated for 20 min at 37 °C and the absorption was measured at 560 nm. ^{13}C NMR spectra of 1 M solutions containing either acrolein or HPA under test conditions showed the decomposition of the molecules resulting in almost identical spectra (unpublished results). Therefore, this method determined the overall concentration of HPA (**1**−**3**) in aqueous solution. This method (Trp−HCl assay) is specific to 3-HPA and acrolein and shows negligible reaction with metabolites such as glycerol and 1,3-propanediol, as well as with structurally similar molecules such as glyceraldehyde and propionaldehyde (unpublished results).

The purity of the HPA was determined by electrospray ionization mass spectrometry (ESI-MS). The measurement of mass spectra by the electrospray technique using a Finnigan TSQ-7000 mass spectrometer was performed with the stock solution (10 M) diluted to 1 mM in methanol. In the positive ion mode, the signals represent positively charged adducts of the HPA with sodium, potassium, sodium methanolate, and potassium methanolate, respectively, as both sodium and potassium ions are present in the stock solution. Fragmentation in the ESI-MS technique is almost absent.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were recorded on a MSL 200 spectrometer S (Bruker, Manning Park Billerica, MA) at 50 MHz at 20 or 4 °C. Chemical shifts δ (ppm) were expressed in relation to external tetramethylsilane (TMS). Measurements were carried out with the internal ref 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TMSP), whose signal is 2.8 ppm lower than TMS (*29*). Traces of volatile contaminants such as acrolein, acetone, and ethyl acetate were removed from the stock solution under reduced pressure. The 10 M stock solution of HPA was diluted with D_2O. The concentrations of tested solutions were verified by the Trp−HCl assay giving the following values: 4.9, 1.4, 0.15, and 0.03 M, respectively. One milliliter of the solutions was put into 5 mm NMR tubes, sealed, and tested at 20 or 4 °C immediately after mixing. To test the solutions under different conditions, similar aliquots were stored at either 20 or 4 °C. Measurements were performed in the decoupled mode, except for the determination of the multiplicity of the signals. By extending the delay time during the measurements in the decoupled mode, quantitative comparable signal areas of the compounds in a mixture were achieved (*30*). Quantifiable spectra of the HPA system were obtained without adding a relaxation reagent after a delay time of at least 50 s. The delay time for qualitative measurements was 2 s. For the quantitative measurements of 4.9 and 1.4 M or 0.15 M solutions, 4096 or 8192 scans with a delay time of 50 s were recorded, respectively. This gave measuring times from a few hours up to nearly 5 days. For the 0.03 M solution, 65 536 scans and a delay time of 4 s were used to obtain analyzable spectra within 5 days. To determine the time to reach equilibrium, a 4.9 M HPA solution was diluted with D_2O to 1.4 M. Immediately after it was mixed, a spectrum was recorded with a delay time of 2 s and a scan time of 15 min. Measurements were repeated after 1 and 5 h, respectively. The concentrations of these solutions were verified by the Trp−HCl assay after the NMR experiments. To determine the effect of pH and temperature on the HPA system, stock solutions were diluted with D_2O (1.4 M, pH 4.1) or potassium phosphate buffer (0.91 M, pH 4.4, 7.0, 8.9; final potassium phosphate concentration of 120 mM). The final D_2O concentration was at 50%. To obtain a strong acidic solution, HPA was diluted with a deuterium chloride solution (20% DCl) to a HPA concentration of 1 M and a DCl concentration of 12%.

RESULTS

Production and Purification of 3-HPA. 3-HPA was produced by a method adapted from Talarico and Dobrogosz (*20*). The *L. reuteri* strain SD 2112 (*31*), used by the food industry, was used for the biotransformation of glycerol. The highest amount of 3-HPA was formed when *L. reuteri* was incubated in the growth medium (MRS) with glycerol, but the sample contained undesirable byproducts after purification. Therefore, biotransformation was conducted in distilled water containing 200 mM glycerol. Glycerol (20 mM) in the preculture increased the production of 3-HPA (*32*). Within 2.5 h of incubation, 170 mM 3-HPA could be produced, representing a transformation of 85%. After elimination of the cells by centrifugation, the filter-sterilized and lyophylized supernatant could be used directly for the purification step. A method for preparative column purification with acetone/ethyl acetate on a silica 60 gel support was developed. About 1 g of pure HPA could be recovered from the column, which corresponds to a yield of 60% for the purification. The overall yield was 45% pure HPA from the initial glycerol used. To determine the HPA concentration, a method (Trp−HCl assay) for acrolein determination developed by Circle et al. (*28*) was adapted and allowed the accurate molar quantitation of HPA using acrolein as standard (unpublished results). The Trp−HCl assay allowed the determination of overall molar concentration of the HPA system, including **1**−**3**. The purified HPA can be stored for at least 6 months at 4 °C without any loss or transformation (results not shown). Purified HPA was free of contaminants, especially glycerol and 1,3-propanediol, as verified with ESI-MS (with a detection limit of 10^{-6} M for a 10^{-3} M solution, **Figure 2**) and

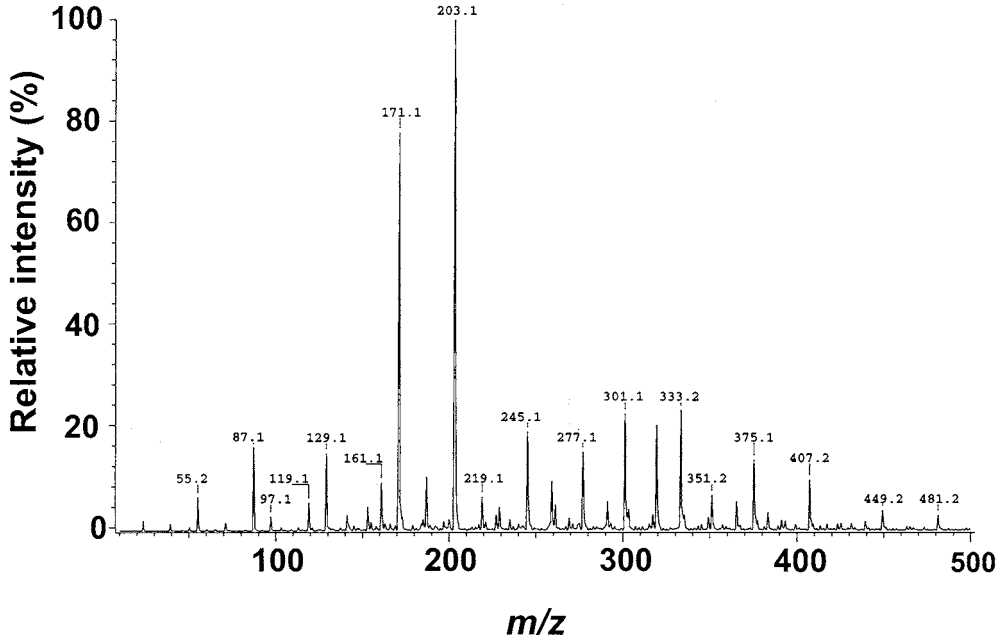

Figure 2. ESI-MS spectra of purified HPA (1 mM) in methanol. Values of *m/z* 23, 39, 55, 71, 87, 119, and 141 correspond to Na^+, K^+, and their CH_3OH adducts, respectively. A detailed description of the *m/z* values is given in **Table 1**.

Table 1. *m/z* Values of Purified HPA (1 mM) in Methanol Measured by ESI-MS

1	**3**	HPA trimer (**6**)	HPA tetramer (**7**)
74 $[C_3H_6O_2]$	**148** $[C_6H_{12}O_4]$	**222** $[C_9H_{18}O_6]$	**296** $[C_{12}H_{24}O_8]$
97 $[+ Na]^+$	**171** $[+ Na]^+$	**245** $[+ Na]^+$	**319** $[+ Na]^+$
129 $[+ CH_3OH + Na]^+$	**187** $[+ K]^+$	**261** $[+ K]^+$	
	203 $[+ CH_3OH + Na]^+$	**277** $[+ CH_3OH + Na]^+$	**351** $[+ CH_3OH + Na]^+$
	219 $[+ CH_3OH + K]^+$	**293** $[+ CH_3OH + K]^+$	**367** $[+ CH_3OH + K]^+$

^{13}C NMR (with a detection limit of 3 mM in a solution of 4.9 M HPA, **Figure 3**).

Confirmation of the HPA Structure and Purity by ESI-MS. Analysis of 1 mM HPA methanolic solution with ESI-MS (**Figure 2**) showed only signals of compounds with a *m/z* 74 such as 3-HPA monomers, dimers, trimers, tetramers, and different Na^+ and K^+ adducts (**Figure 2**). HPA hydrate could not be detected with this method. **Table 1** gives the mass to charge (*m/z*) ratio of purified HPA. Under these conditions, the HPA dimer form was the most abundant HPA as shown by peaks with the highest intensity with *m/z* of 171 corresponding to the Na adduct of $C_6H_{12}O_4$ (*m/z* 148 + Na) and *m/z* of 203 corresponding to the CH_3OH-Na adduct of $C_6H_{12}O_4$ (*m/z* 148 + CH_3OH + Na). A smaller amount of 3-HPA was found (*m/z* 97 + Na and *m/z* 129 + CH_3OH + Na). No impurities such as glycerol (*m/z* 92) and 1,3-propanediol (*m/z* 76) or their sodium and sodium methanolate adducts (*m/z* 115 and 147; 99 and 131, respectively) were observed in the spectrum.

^{13}C NMR of Aqueous HPA Solutions. The ^{13}C NMR of HPA solution (1.4 M) in D_2O showed that 3-HPA and 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (HPA dimer) were the main components. The 1,1,3-trihydroxypropane (HPA hydrate) was also detected. The chemical shifts and the coupling constants of the HPA system (4.9 M) are given in **Table 2**. The values for 3-HPA and HPA dimer are in agreement with the values cited in the literature (*17*, *33*). To find the best conditions for studying the HPA system by ^{13}C NMR, experiments were performed to determine the time required for the HPA system to reach its equilibrium. The diluted HPA solution (1.4 M, pH 4.1) was analyzed by ^{13}C NMR immediately after dilution of the 4.9 M solution, after 1 and 5 h, as well as after 5 days. All solutions gave exactly the same spectrum (**Figure 3A**). This means that the equilibrium state had been reached within about 15 min (time of scanning after the mixture) after dilution. To determine the composition of the HPA system under different conditions, spectra of HPA solutions were recorded in D_2O (pH 4.1), in 12% DCl, and at three different pH values (pH 4.4, 7.0, 8.9). The ^{13}C NMR spectra of HPA solutions (0.91 M) in a pH range from 4.4 to 8.9 recorded immediately after preparation showed no influence of pH on concentration of the three main compounds of the HPA system (data not shown). In contrast, pH had a significant effect on the stability of these compounds for longer incubation periods (*34*). Spectra of HPA solution at pH 4.1 (1.4 M) and 4.4 (0.91 M) showed no change for up to 7 days storage at 20 °C (measurement at 20 °C). Afterward, signals for acrolein were detected in samples. However, when HPA solutions were stored and measured at 4 °C, no acrolein was observed, even after 150 days of storage. Under strong acid conditions (12% DCl), the HPA system broke down into other substances, within a few hours, which could not be identified, as shown in **Figure 3B**. In the buffered HPA solution at pH 8.9 stored at 20 °C, signals for acrolein and new signals were already detected after 2 days and there was a change in the ratio of the main components of the HPA system (**Figure 3C**). The signals of acrolein (199, 141.7, and 137.4 ppm, respectively, not shown in these figures) and HPA dimer disappeared after 7 and 14 days (results not shown), respectively. The spectra in **Figure 3A**,**C** showed a splitting of signals in the range of 30−40 ppm into 3−4 new substances, which indicates an exchange of H/D in the HPA compounds.

Composition of Aqueous HPA Solutions by Quantitative ^{13}C NMR. Figure 4 shows the ^{13}C NMR spectra of purified

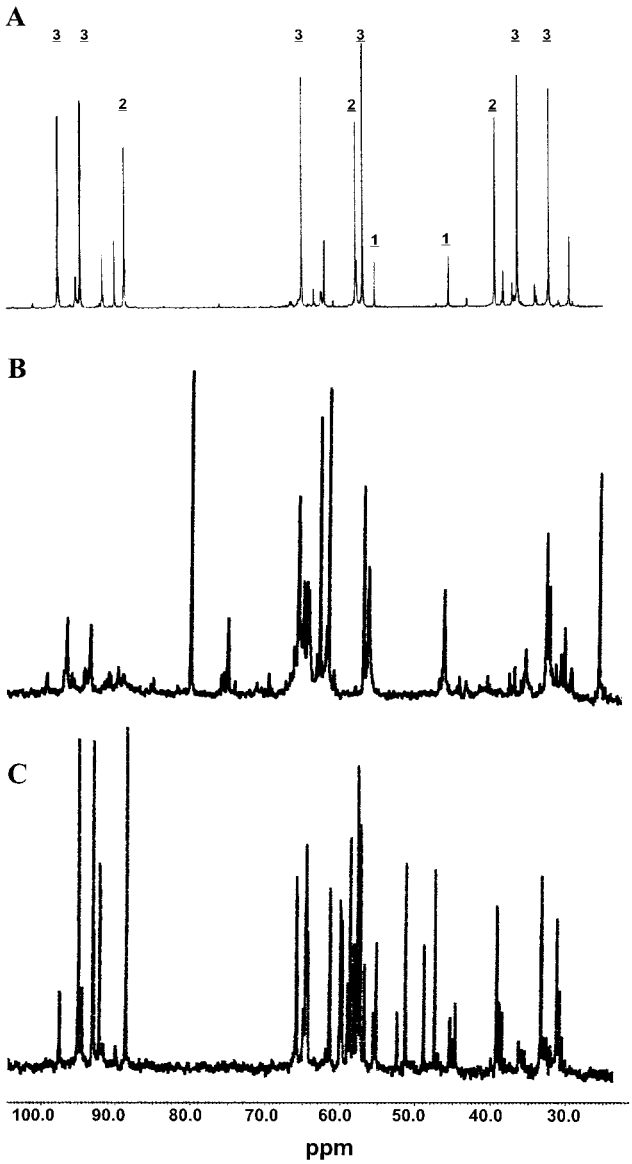

Figure 3. Detail of the ^{13}C NMR spectra of HPA solutions at different pH values (20 °C): (**A**) HPA (1.4 M, pH 4.1) in D_2O after 2 days, (**B**) HPA (1 M, 12% DCl) after 1 day, (**C**) HPA (0.91 M, pH 8.9) in buffer with D_2O after 7 days.

Table 2. ^{13}C NMR Chemical Shifts[a] and Coupling Constants[b] of the HPA System

	compd no.				
carbon	**1**	**1**[c,1]	**2**	**3**	**3**[c,2]
atom	ppm (Hz)	ppm	ppm (Hz)	ppm (Hz)	ppm
C-1	206.3 (d, 87.6)	203.3 (d)	88.6 (d, 162.1)	97.5 (d, 159.0)	100.4
C-2	45.4 (d, 22.2; t, 126.0)	43.1 (d)	39.3 (t, 125.5)	32.1 (t, 129.8)	30.1
C-3	55.2 (t, 143.2)	60.4 (t)	57.7 (t, 142.4)	65.0 (t, 147.2)	69.1
C-4				94.5 (d, 159.6)	97.3
C-5				36.4 (t, 126.7)	34.9
C-6				56.8 (t, 143.0)	59.7

[a] Chemical shift δ in ppm related to TMS. [b] Coupling constants J in hertz (Hz). [c] Values cited in **1** and **2** in relation to TMSP: 1, ref *17*; 2, ref *29*. For comparisons with the given values (in relation to TMS), 2.8 ppm should be added (*33*).

HPA in D_2O. According to the intensity of the signals at a concentration of 4.9 M (**Figure 4A**), the HPA dimer (**3**) was predominant as compared with the HPA hydrate (**2**) and 3-HPA (**1**). Beside these main compounds, other unidentified signals

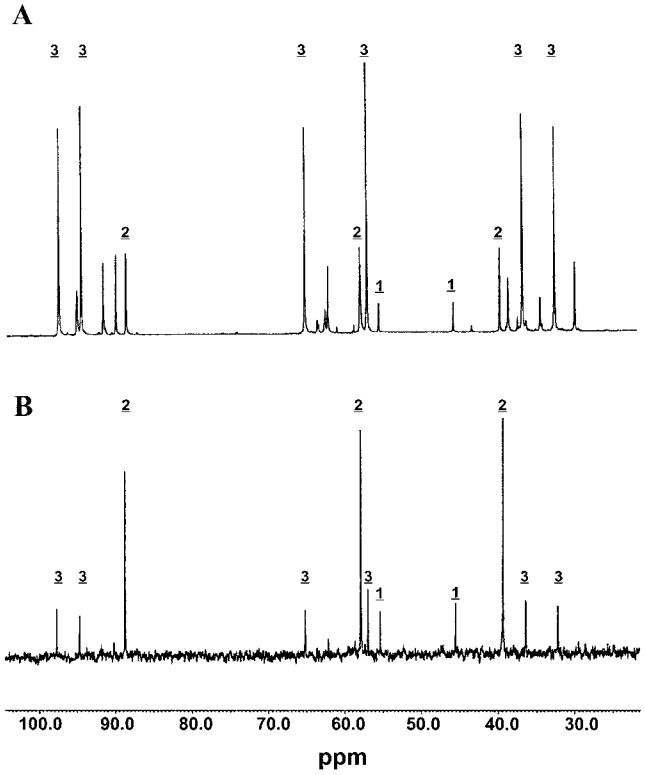

Figure 4. ^{13}C NMR spectra of HPA solutions in D_2O at different concentrations (20 °C): (**A**) 4.9 M and (**B**) 0.15 M.

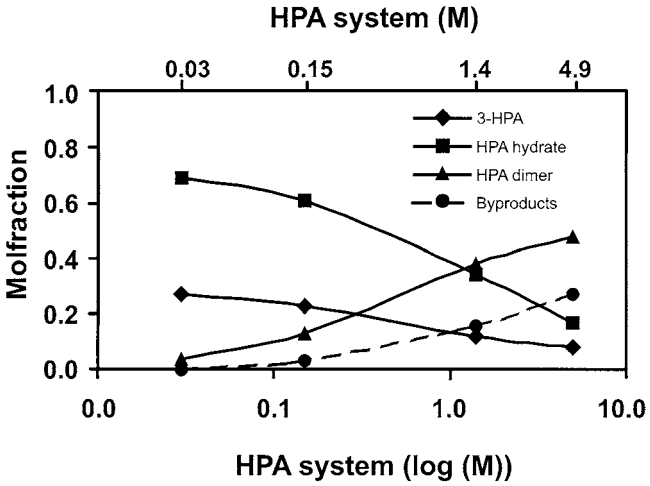

Figure 5. Concentration-dependent distribution of the HPA system compounds in D_2O at 20 °C (logarithmic scale).

were also recorded. However, at a lower concentration of 0.15 M, HPA hydrate was predominant. Signals for HPA dimer and 3-HPA were very much decreased, and those of other compounds nearly disappeared (**Figure 4B**).

Composition of the HPA System. According to the spectra shown **Figure 4A**,**B**, important differences in composition of the HPA system as a function of concentration could be observed. The distribution of the compounds forming the HPA system in aqueous solution was largely dependent on the concentration of HPA. Quantitative ^{13}C NMR of the concentration-dependent distribution of HPA system compounds was carried out with HPA solutions of 4.9, 1.4, 0.15, and 0.03 M in D_2O. The concentration of each individual compound in the HPA system (**1**−**3**) and byproducts was determined (**Figure 5**). At a concentration of 4.9 M, the HPA system consisted mainly of the HPA dimer. Signals of byproducts were detected at this

concentration, but their structure was not determined. These products decreased continuously with a progressive dilution of the HPA solution and were undetectable at a concentration of 0.03 M HPA. At concentrations lower than 1.4 M, a rapid decrease of dimer concentration was observed, which was mainly transformed to HPA hydrate. The HPA hydrate concentration increased steadily until the lowest concentration (0.03 M) of HPA solution was tested in these experiments.

DISCUSSION

Production and Purification of HPA. The most frequent method for producing 3-HPA is still the catalytic hydration of acrolein in strong acid conditions (*16*). However, this method results in modest yields with a lot of contaminants. The biotransformation of glycerol into 3-HPA using *L. reuteri* (SD 2112) enabled us to increase the yield and the purity of 3-HPA. The addition of glycerol (20 mM) in the preculture increased the production of 3-HPA (*32*); however, more sophisticated experiments are required to clarify the influence of glycerol on *L. reuteri* productivity and to know whether the glycerol conversion pathway is induced in the lactobacilli and how (*2*). The production of 3-HPA was carried out without glucose to avoid further reduction of 3-HPA to 1,3 propanediol (*14*) and to favor the accumulation of 3-HPA (*4*, *14*, *15*). Our method allowed a rapid production of 3-HPA in sufficient quantities for characterization, without the need for extraction prior to purification. This is another important advantage of this method as compared with the catalytic hydration method. Current methods for purification of HPA use, for example, buffers containing a coupling reagent to trap 3-HPA, which is difficult to recover, or HPLC, which is a more analytical method (*3*, *34*, *35*). Therefore, a new preparative purification column with acetone:ethyl acetate on a silica 60 gel support was developed. This quick and simple purification method allowed HPA to be obtained in high yield (45% pure HPA from the initial glycerol added) without any impurities, as shown by ESI-MS (**Figure 2**). This method can be easily scaled-up if larger amounts of HPA are needed. The limiting factor would be the column size for purification. In the present experiments, ca. 2 g of sample was used to charge a glass column with 65 g of silica gel.

Chemical Characterization. The HPA system is complex, and its chemical characterization is difficult. Most analytical results have been obtained in experimental conditions that were irrelevant to biological systems. Nielsen et al. (*17*) recorded ^{13}C NMR spectra after heating a sample containing ca. 2 M acrolein in 0.5 N H_2SO_4 to 50 °C for 4 h. Talarico and Dobrogosz (*20*) analyzed HPA by ^{13}C NMR in D_2O and in deuterated methanol but without specifying the concentrations. Barbirato et al. (*3*) analyzed HPA by ^{1}H NMR in D_2O (10%) also without indicating the concentrations. The spectra from the two latter studies were quite similar. Talarico and Dobrogoz (*20*) discovered HPA hydrate as a third form, in addition to the already identified 3-HPA and HPA dimer (*16*), but each form was detected by a different method. To analyze the HPA system, we combined the following methods: a highly sensitive ESI-MS method to verify the purity of HPA preparation; a specific Trp−HCl assay, which successfully determined the molar concentrations of the HPA system (**1**−**3** together); and a qualitative and quantitative ^{13}C NMR. NMR is the method of choice for in depth structural and chemical characterization. Despite its relatively low sensitivity, which depends additionally on the scanning time, this method has some main advantages. It does not interfere with the sample; samples can be measured in a desired medium, and different compounds can be observed simultaneously in one sample, which makes it the ideal method for determining the dynamics of the HPA system in aqueous solution under biologically relevant conditions. Quantitative ^{13}C NMR studies led to the first quantitative data on the composition of the compounds forming the HPA system. Chemical characterization of the HPA system was performed by ^{13}C NMR in aqueous solution in relation to concentration, pH, and temperature with HPA concentrations that were close to the concentrations used in bioassays. ^{13}C NMR studies confirmed the presence of the three HPA forms and revealed little influence of moderate changes in pH (4.1−7.0) on the distribution of the different HPA compounds. However, under strong acidic conditions (12% DCl) or at pH 8.9, the HPA system was destroyed after 1 day, respectively, 7 days. A low temperature of 4 °C had no influence on the composition (150 days measured), whereas long incubation at 20 °C favored the production of acrolein, which is also observed during alcoholic fermentation, containing glycerol as a byproduct, or during the storage and maturation of alcoholic products (*1*, *4*, *14*, *36*). This result indicates the importance of temperature controlling during the production and storage of alcoholic beverages, but further work is needed to fully elucidate this problem.

Concentration-Dependent Distribution of the HPA System Compounds. Despite the different degrees of hydration and the ability of 3-HPA to dimerize, calculations of concentrations in the HPA system, determined by the Trp−HCl assay, which determines the concentration of the three HPA forms together, were based on the molecular mass of the aldehyde form (3-HPA; MR, 74.08) (*3*), as is commonly done for other aldehydes. There is a definite advantage for using solutions with defined molar HPA concentrations as compared to determinations of HPA concentration by minimum inhibitory concentration or activity units, which give only relative concentrations (*25*, *31*). The use of defined molar HPA solutions will also allow the direct comparison of the antimicrobial activity with other substances such as hydroxyurea on a molar basis (*25*, *37*). In this study, the comparison of aqueous HPA solutions with defined molar concentrations allowed the study of the dynamics of the HPA system. Saturated, especially short chain aldehydes, tend to be hydrated in aqueous solutions depending on their chemical structure. Acrolein (C_3H_4O) shows a 2.0% degree of hydration (the amount of hydrate), and propanal (C_3H_6O) shows a 47.1% (*38*) degree of hydration, as cited by Schauenstein et al. (*39*). At concentrations up to approximately 1 M (pH 5.0), formaldehyde is present in aqueous solutions primarily or exclusively in the hydrated form, methylene glycol (*39*). As other aldehydes such as malonaldehyde (*40*), HPA exists in various forms. Although the formation of the different malonaldehyde forms is dependent on pH of the solution, the composition of the HPA system was found to be only dependent on concentration and not on the pH, as shown by quantitative ^{13}C NMR. At a high concentration of 4.9 M, the HPA dimer was predominant. At concentrations lower than 1.4 M, a rapid decrease in dimer concentration was observed, which was mainly replaced by HPA hydrate when HPA concentration decreased from 1.4 to 0.03 M, the lowest tested concentration. Therefore, hydration of HPA in aqueous solution increased with dilution. At a HPA concentration of 4.9 M, the amount of HPA hydrate was 17% and as high as 70% at 0.03 M. **Figure 5** shows (by extrapolating the curve) that concentrations lower than 0.03 M are likely to have similar amounts of HPA hydrate. In some studies, HPA concentrations in the micromolar range have been calculated using the molecular mass of the dimer form (*37*, *41*), whereas our results suggest that HPA exists mainly as a

monomer at this concentration. This implies that the concentrations used by these authors were twice as high as those reported. Our results indicate that almost no HPA dimer exists in the HPA system at concentrations used for bioassays demonstrating inhibitory effects on the growth of other organisms (0.03 M (*3*)). Consequently, our data indicate that the HPA dimer could not be the active compound responsible for the antimicrobial function (*21*). In this paper, authors stated that the dimeric form of the HPA may specifically inhibit the action of the ribonucleotide reductase, the key enzyme in DNA synthesis. They also found an inhibition of thioredoxin. Both of these enzymes have sulfhydryl groups, and the reaction of HPA may be directed toward these chemically unstable groups (*20*). HPA is an aldehyde, which is known to react with biological molecules. HPA was shown to be an effective agent in the sterilization and fixation of biological tissues (*42*). HPA is also known to be transformed into acrolein, a very toxic aldehyde (*4*, *5*, *9*, *14*). In these works, acrolein was found in alcoholic beverages; in the present work, preliminary results showed that the formation of acrolein may be influenced by temperature. Further work is needed to address the question of how HPA interacts with biological molecules and how it can inhibit the growth of microorganisms.

LITERATURE CITED

(1) Voisenet, M. E. Formation d'acroléine dans la maladie de l'amertume des vins. *C. R. Acad. Sci.* **1910**, *t. 150*, 1614−1616.

(2) Slininger, P. J.; Bothast, R. J. Optimizing aerobic conversion of glycerol to 3-hydroxypropionaldehyde. *Appl. Environ. Microbiol.* **1985**, *50*, 1444−1450.

(3) Barbirato, F.; Grivet, J. P.; Soucaille, P.; Bories, A. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. *Appl. Environ. Microbiol.* **1996**, *62*, 1448−1451.

(4) Mills, E.; Baugh, W. D.; Conner, H. A. Studies on the formation of acrolein in distillery mashes. *Appl. Microbiol.* **1954**, *2*, 9−13.

(5) Serjak, W. C.; Day, W. H.; Van Lanen, J. M.; Boruff, C. S. Acrolein production by bacteria found in distillery grain mashes. *Appl. Microbiol.* **1954**, *2*, 14−20.

(6) http://brenda.bc.uni-koeln.de/php/result_flat.php3?ecno=4.2.1.30, 2002.

(7) Voisenet, M. E. Sur un ferment, contenu dans les eaux, agent de déshydratation de la glycérine. *C. R. Acad. Sci.* **1914**, *158*, 195−197.

(8) Abeles, R. H.; Brownstein, A. M.; Randles, C. H. β-Hydroxypropionaldehyde, an intermediate in the formation of 1,3-propanediol by *Aerobacter aerogenes*. *Biochim. Biophys. Acta* **1960**, *41*, 530−531.

(9) Smiley, K. L.; Sobolov, M. A cobamide-requiring glycerol dehydrase from an acrolein-forming *Lactobacillus*. *Arch. Biochem. Biophys.* **1962**, *97*, 538−543.

(10) Johnson, E. A.; Lin, E. C. *Klebsiella pneumoniae* 1,3-propanediol: NAD^+ oxidoreductase. *J. Bacteriol.* **1987**, *169*, 2050−2054.

(11) Schütz, H.; Radler, F. Anaerobic reduction of glycerol to propanediol-1.3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. *Syst. Appl. Microbiol.* **1984**, *5*, 169−178.

(12) Abbad-Andaloussi, S.; Amine, J.; Gerard, P.; Petitdemange, H. Effect of glucose on glycerol metabolism by *Clostridium butyricum* DSM 5431. *J. Appl. Microbiol.* **1998**, *84*, 515−522.

(13) Sobolov, M.; Smiley, K. L. Metabolism of glycerol by an acrolein-forming *Lactobacillus*. *J. Bacteriol.* **1960**, *79*, 261−266.

(14) Sauvageot, N.; Gouffi, K.; Laplace, J. M.; Auffray, Y. Glycerol metabolism in *Lactobacillus collinoides*: production of 3-hydroxypropionaldehyde, a precursor of acrolein. *Int. J. Food Microbiol.* **2000**, *55*, 167−170.

(15) El-Ziney, M. G.; Arneborg, N.; Uyttendaele, M.; Debevere, J.; Jakobsen, M. Characterization of growth and metabolite production of *Lactobacillus reuteri* during glucose/glycerol cofermentation in batch and continuous cultures. *Biotechnol. Lett.* **1998**, *20*, 913−916.

(16) Hall, R. H.; Stern, E. S. Acid-catalysed hydration of acrylaldehyde: Kinetics of the reaction and isolation of β-hydroxypropionaldehyde. *J. Chem. Soc.* **1950**, 490−498.

(17) Nielsen, A. T.; Moore, D. W.; Schuetze, A., Jr. ^{13}C and ^{1}H NMR study of formaldehyde reactions with acetaldehyde and acrolein. Synthesis of 2-(hydroxymethyl)-1,3-propanediol. *Pol. J. Chem.* **1981**, *55*, 1393−1403.

(18) Axelsson, L.; Chung, T. C.; Dobrogosz, W. J.; Lindgren, S. E. Production of a broad-spectrum antimicrobial substance produced by *Lactobacillus reuteri*. *Microb. Ecol. Health Dis.* **1988**, *2*, 131−136.

(19) Kandler, O.; Stetter, K.-O.; Lagnado, J. R. *Lactobacillus reuteri* sp. nov., a new species of heterofermentative Lactobacilli. *Zbl. Bakt. Hyg., I. Abt. Orig. C* **1980**, *1*, 264−269.

(20) Talarico, T. L.; Dobrogosz, W. J. Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. *Antimicrob. Agents Chemother.* **1989**, *33*, 674−679.

(21) Dobrogosz, W. J.; Lindgren, S. E. Antibiotic reuterin. PCT/US88/01423, 1988.

(22) Tannock G. W. *Probiotics: A Critical Review*; Horizon Scientific Press: Wymondham, Norfolk, U.K., 1999.

(23) Daeschel, M. A. Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol.* **1989**, *43*, 164−167.

(24) El-Ziney, M. G.; Debevere, J. The effect of reuterin on *Listeria monocytogenes* and *Escherichia coli* O157:H7 in milk and Cottage cheese. *J. Food Prot.* **1998**, *61*, 1275−1280.

(25) El-Ziney, M. G.; van-den-Tempel, T.; Debevere, J.; Jakobsen, M. Application of reuterin produced by *Lactobacillus reuteri* 12002 for meat decontamination and preservation. *J. Food Prot.* **1999**, *62*, 257−261.

(26) Kandler, O. Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List No. 8. *Int. J. Syst. Bacteriol.* **1982**, *32*, 266−268.

(27) Rahn, C. H.; Schlenk, H. Detection of aldehydes with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol as spray reagent. *Lipids* **1973**, *8*, 612−616.

(28) Circle, S. J.; Stone, L.; Boruff, C. S. Acrolein determination by means of tryptophane. *Ind. Eng. Chem.* **1945**, *17*, 259−262.

(29) Hoffman, R. E.; Davis, D. B. Temperature dependence of NMR secondary references for D_2O and $(CD_3)_2SO$ solutions. *Magn. Reson. Chem.* **1988**, *26*, 523−525.

(30) Braun, S.; Kalinowski, H.-O.; Berger, S. *100 and More Basic NMR Experiments*; Verlag Chemie: Weinheim, 1996.

(31) Chung, T. C.; Axelsson, L.; Lindgren, S. E.; Dobrogosz, W. J. *In vitro* studies on reuterin synthesis by *Lactobacillus reuteri*. *Microb. Ecol. Health Dis.* **1989**, *2*, 137−144.

(32) Luthi-Peng, Q.; Scharer, S.; Puhan, Z. Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 73−80.

(33) Nakano, T.; Takanobu, T.; Yasutaka, I.; Masaya, O. Chemoselective oxidation of the primary alcohol function of diols catalyzed by zirconocene complexes. *Synthesis* **1986**, *9*, 774−776.

(34) Talarico, T. L.; Casas, I. A.; Chung, T. C.; Dobrogosz, W. J. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. *Antimicrob. Agents Chemother.* **1988**, *32*, 1854−1858.

(35) Vancauwenberge, J. E.; Slininger, P. J.; Bothast, R. J. Bacterial conversion of glycerol to β-hydroxypropionaldehyde. *Appl. Environ. Microbiol.* **1990**, *56*, 329−332.

(36) Claisse, O.; Lonvaud-Funel, A. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. *J. Food Prot.* **2001**, *64*, 833−837.

(37) Yunmbam, M. K.; Roberts, J. F. The *in vitro* efficacy of reuterin on the culture and bloodstream forms of *Trypanosoma brucei brucei*. *Comp. Biochem. Physiol., C*: *Pharmacol., Toxicol. Endocrinol.* **1992**, *101*, 235−238.
(38) Le Hénaff, P. Sur les équilibres et les vitesse d'hydratation et d'hemiacétalisation des aldéhydes aliphatiques. *C. R. Acad. Sci.* **1967**, *t. 265*, 175−178.
(39) Schauenstein, E.; Esterbauer, H.; Zollner, H. Saturated aldehydes. *Aldehydes in Biological Systems: Their Natural Occurrence and Biological Activities*; Pion: London, 1977.
(40) Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **1991**, *11*, 81−128.
(41) Yunmbam, M. K.; Roberts, J. F. *In vivo* evaluation of reuterin and its combinations with suramin, melarsoprol, DL-alpha-difluoromethylornithine and bleomycin in mice infected with *Trypanosoma brucei* brucei. *Comp. Biochem. Physiol., C: Pharmacol., Toxicol. Endocrinol.* **1993**, *105*, 521−524.
(42) Chen, C. N.; Sung, H. W.; Liang, H. F.; Chang, W. H. Feasibility study using a natural compound (reuterin) produced by *Lactobacillus reuteri* in sterilizing and cross-linking biological tissues. *J. Biomed. Mater. Res.* **2002**, *61*, 360−369.

Received for review October 30, 2002. Revised manuscript received February 25, 2003. Accepted February 27, 2003.

JF021086D