Combined Modeling and Biophysical Characterisation of CO₂ Interaction with Class II Hydrophobins: New Insight into the Mechanism Underpinning Primary Gushing

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ABSTRACT

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Although there is a common agreement that hydrophobins and CO₂ are responsible for primary gushing of carbonated beverages, the bio-molecular mechanism of this phenomenon is not well understood. Here, hydrophobin HFBII has been produced, extracted, and purified. A gushing test and DLS analysis was performed and allowed the authors to design an MD simulation setup to investigate the interaction of CO₂ molecules with HFBII in time. The results indicate that CO₂ molecules tend to aggregate at the hydrophobic patch of HFBII twice as much as to the rest of the protein. A model is proposed that elucidates the “nanobomb” formation depicting a definite chemical and biophysical description of the primary gushing mechanism.

Keywords: CO₂, Gushing, Hydrophobin, MD simulation, Nanobubble

RESUMEN

Aunque no existe un acuerdo común que hidrofobinas y CO₂ son responsables de chorro primario de las bebidas carbonatadas, el mecanismo de bio-molecular de este fenómeno no se entiende bien. Aquí, hidrofobina

HFBII se fue producido, se extrae y purifica. Una prueba de gushing y el análisis de DLS se realizó y permitió a los autores para diseñar una configuración de simulación MD para investigar la interacción de moléculas de CO₂ con HFBII en el tiempo. Los resultados indican que las moléculas de CO₂ tiende a agregarse en el parche hidrófobo de HFBII dos veces más que para el resto de la proteína. Se propone un modelo que aclara el “nanobomb” formación que representa una descripción definida químicos y físicos del mecanismo de gushing principal.

Palabras claves: CO₂, gushing, hidrofobina, simulación MD, Nanobubble

INTRODUCTION

The scientific description of systematic uncontrolled beer degassing at bottle opening (26), the involvement of organic matter originating from contaminating molds (1), and the hypothesis concerning a solid pellicle around pressurized CO₂ (9,17) as well as the detection of spherical gas structures of about 100 nanometers (the critical diameter of gaseous CO₂ at atmospheric pressure) in contaminated carbonated beverages like beer (13) are the basic statements that report the mechanism of primary gushing of beer.

Indeed, these studies altogether assumed pieces of the puzzle that allowed describing the mechanism of primary gushing, but the biophysical details of the gushing mechanism remain unexplained (53). Previous studies indicated that the hydrophobicity of dissolved gaseous CO₂ was related to the hydrophobic patch of hydrophobins (10,12). This interaction is susceptible to the presence of pure hydrophobic molecules such as unsaturated fatty acids (10) and some plant extracts like hop oils (33,34).

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However, the detailed mechanism of “nanobubble formation” and the formulation of a valid “atomic level hypothesis” on what happens inside a closed contaminated pressurized container remains to be unraveled. These nanostructures were defined as nanobombs as they will explode at the bottle opening (12). Therefore, an in-depth physico-chemical study was conducted to investigate the behavior of hydrophobins in presence of dissolved gaseous CO₂ in an aqueous solution. The specific and different surface properties of Class I hydrophobin SC3 and of Class II sister hydrophobins HFB I and HFB II (the best described hydrophobins) are the basic events to understand the nanoscale modifications that take place during primary gushing. The elasticity of the aggregated nanostructures of hydrophobin is essential to unravel the specific rearrangements at hydrophilic-hydrophobic interfaces (18,19,29,30,50,55). Furthermore, there was an absolute need for understanding the physical laws governing the dissolution of gaseous CO₂ in aqueous solution in presence of potential contaminating agents. Therefore, it is understandable that it has taken about 25 years for scientists to finalize a concrete model that explains step by step formation and disruption of these nano-spherical structures in the liquid.

Hydrophobins are fungal proteins (about 100 amino acids, 7–15 kDa) (36,58) characterized by their ability to form an amphiphilic layer at hydrophobic-hydrophilic interfaces such as the air-water system (19,20). Based on the solubility of the assembled layers, hydrophobins are divided into two classes (19,29,52): Class I hydrophobins assemble into a protein membrane as a rigid film that is difficult to dissolve in water unless by using strong acids (14,54). In contrast, Class II hydrophobins self-assemble into a monolayer as an elastic film which may be dissociated by reagents (8,43,55) or by applying pressure (43). Primary gushing seems to be mostly linked to Class II hydrophobins (60) and a very low amount of them (approximately 3 µg/L) was reported to be sufficient to induce gushing (45).

The hydrophobic patch of the hydrophobin molecule is hypothesized to be responsible for the interaction with gaseous CO₂, resulting in the formation of gaseous CO₂ nanobubbles stabilized by a membrane formed by hydrophobin aggregates with high elasticity (12). To understand such a mechanism requires the use of computational techniques. Molecular dynamic (MD) simulations based on classical Newtonian physics laws were used in this study to investigate the free ligand binding propensity of CO₂ molecules with the hydrophobin HFB II in solvent (water). In aggregate, MD results to support the experimental results and the presented nanobomb hypothesis by characterizing the atomic interaction of the affinity of HFB II with molecular gaseous CO₂.

EXPERIMENTAL

Production of HFB II Hydrophobin

Trichoderma reesei MUCL 44908 was cultivated in a 1 L working volume fermenter (KGW-Isotherm, Schieder GmbH, Germany) equipped with an agitator (RW20 digital, IKA GmbH, Germany) at 25°C for 7 days. The aqueous culture medium consisted of lactose (40.0 g/L), peptone (4.0 g/L), yeast extract (1.0 g/L), KH₂PO₄ (4.0 g/L), (NH₄)₂SO₄ (2.8 g/L), MgSO₄·7H₂O (0.6 g/L), CaCl₂·2H₂O (0.8 g/L) as well as trace solution (2 mL/L) containing FeO₄·7H₂O (0.5 g/L), CoCl₂·6H₂O (3.7 g/L), MnSO₄·H₂O (1.6 g/L) and ZnSO₄·7H₂O (1.4 g/L). Phosphoric acid was used to adjust the pH to 4.5–5 (27). At the end, the mycelium was separated by centrifugation (8,000 × g for 25 min at 6°C, Beckman model J2-21) and the supernatant was stored at 2°C for further analysis.

After the extraction step on the supernatant by foam fractionation, the foamate was filtered on a hydrophilic polyestersulfone filter (PES Millipore; Billerica, MA). Purification was carried out

by liquid chromatography using a RP-LC Source 15RPC column (4.6 × 200 mm; GE Healthcare; Uppsala, Sweden). Elution was done with a linear gradient of acetonitrile (ACN) in MilliQ water containing 0.1% trifluoroacetic acid (TFA) (from 0 to 60% in 55 min) at a flow rate of 1 mL/min and was monitored by UV detection at 214 nm (13).

Identification was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with an Ultraflex II instrument in linear mode and an α-cyano-4-hydroxy cinnamic acid (Brüker Daltonics, GmbH; Bremen, Germany). A microspectrophotometer (NanoDrop ND-1000; Wilmington, DE) (280 nm wavelength, ε = 10) was used for the final quantification of purified hydrophobin HFB II (32).

Gushing Test and DLS Analysis

The gushing test and dynamic light scattering (DLS) analysis were carried out as described previously (13,42). Class II hydrophobin behavior was studied in the presence of gaseous CO₂ in a closed pressurized system under model conditions. For this purpose, a definite amount of pure HFB II was added to sparkling water (7 g/L CO₂) cooled at 2°C to reach the final concentration of HFB II equal to 0, 4.5, 9, and 18 µg/L. The gushing test was then performed and the collected overfoams were further analyzed by DLS.

MD Simulation Parameters

The ultrahigh resolution crystal structure 2B97 (resolution 0.75 Å) was used to simulate the behavior of a Class II hydrophobin as a function of time (19). This crystal structure is a dimeric conformation containing two chains, A and B; only chain A was extracted here. Manganese ions and water molecules were stripped as well. The hydrophobin is a neutral structure. Therefore, no counterions were added to the system. The correct protonation state of the amino acids was verified with the protonate 3D tool in the MOE molecular operating environment software (Chemical Computing Group, Canada). The protein was placed in a dodecahedral box and the distance between the protein and the edge of the box was set to 0.85 nm to ensure that the protein did not interact with its own periodic image during the MD simulation. Considering these settings, the total volume of the box was approximately 90 nm³. Ninety carbon dioxide molecules were randomly added to the box and the remaining parts were filled with 2525 TIP3P water molecules (24). The whole system (protein, water, and CO₂) consisted of approximately 9,000 atoms.

MD Simulations

All simulations were performed using the GROMACS package (version 4.5.3) (22) in conjunction with the OPLS-AA force field (25). Partial charges of the CO₂ molecules were taken from a previous study (23). The system was relaxed by executing 2,000 steps of steepest decent and subsequently 2,000 conjugated gradient energy minimization. Two rounds of equilibration were performed, first 100 ps at constant volume (NVT ensemble) and the next 100 ps at constant pressure (NPT ensemble). All heavy protein atoms were kept fixed with position restraints during the equilibration steps. Next, position restraints were removed and the system was simulated for 100 ns in NPT ensemble. A snapshot of the system was saved every 4 ps. The simulation temperature was kept constant by coupling protein and solvent separately to an external bath temperature at 298 K and by using a coupling constant of 0.1 ps with the V-rescale thermostat (7). The simulation pressure was kept constant in the NPT ensemble by coupling protein and solvent separately to an external bath pressure at 4 atm by using a coupling constant of 1 ps with the Parrinello-Rahman barostat (38). The particle-mesh Ewald method was used for calculation of the long-range electrostatics (15). A cutoff of 1.4 nm

Fig. 1. Reverse phase liquid chromatography chromatogram of foamate containing hydrophobin HFBII.

was used for the van der Waals interaction. The LINCS algorithm was used to ensure a 2 fs integration step by constraining all bonds in the system (22).

Measurements

We calculated the amount of CO₂ molecules per Å² accessible surface area (ASA) within a sphere of 10 Å near the hydrophobic patch compared to the rest of the protein surface. The following 18 residues were defined as the hydrophobic patch: Gly6, Leu7, Leu12, Val18 until Val24, Val54 until Ala58, and Ala61 until Cys64.

RESULTS AND DISCUSSION

Characterization of HFBII

An example of RP-LC chromatograms generated during purification is shown in Figure 1. After analysis of several collected fractions using MALDI-TOF, a molecular weight equal to 7.042 kDa was obtained, which corresponds to complete HFBII molecule minus the last amino acid (phenylalanine) (37). This protein is collected in 40–50% ACN fractions. The removal of the last amino acid could be explained by biodegradation as this effect has been observed for mycotoxins as well (2).

The gushing test and the DLS analysis (Fig. 2) were positive for all bottles containing HFBII hydrophobin. As described by Deckers et al (13), DLS analysis is positive when particles with a diameter of at least 100 nm (i.e., critical diameter of gaseous CO₂ at atmospheric pressure) are detected. This diameter allowed the determination of the number of CO₂ molecules used in the MD simulations (for details, see also supplementary data, calculation S1).

Molecular Dynamics Simulations

Gushing is most relevant to beer, although gushing has also been described in different carbonated beverages (39). Hydrophobins and CO₂ are the two main factors responsible for primary gushing. When HFBII is added to sparkling water, gushing is observed just like in beer, underlining the role of both hydrophobins and CO₂ in the gushing mechanism (13). Although it is now widely regarded that hydrophobin is the main factor responsible for gushing, we suggest here that CO₂ plays the major role. It was observed that gushing completely disappears with time if carbonated beverages are bottled in PET (polyethylene terephthalate) bottles (data not shown). This effect can be explained by the permeability of PET for CO₂, which results in decreasing and finally stopping the gushing potential.

Fig. 2. Particle size distributions measured in sparkling water containing 0, 4.5, 9, and 18 µg of HFBII/L. For clarity, only the interesting part of the curve around 100 nm diameter (critical diameter of CO₂ at atmospheric pressure) is shown.

When CO₂ dissolves in water, the following equilibria take place:

Where k_H is the Henry's constant, k_S is the dissolution constant, and k_{a1} and k_{a2} represent the two dissociation constants of carbonic acid.

The carbon dioxide reacts chemically with water molecules to form carbonic acid, an unstable and weak diprotic acid, but the reactivity is clearly in favor of dissolved CO₂ as the dissolution constant k_S equals 1.7×10^{-3} at 25°C. However, H₂CO₃ concentration is dependent upon pH. At pH 7.0, about 80% of carbonic acid is mono-dissociated in HCO₃⁻ as $pK_{a1} = 6.4$ at 25°C (39). In order to obtain a CO₂ concentration of approximately 7 g/L, a pressure is applied to increase the CO₂ solubility as stated by Henry's Law (28,46). The more the CO₂ is dissolved in water, the more the H₂CO₃ is formed and ionized in HCO₃⁻. Moreover, increasing the concentration of H⁺ results in pH decrease. When the pH is lower than the pK_{a1} value, the equilibria are displaced toward the left side and H₂CO₃ is finally dissolved in CO₂ (5,44). Sparkling water has a final pH value of 4.0 so that the main species is dissolved CO₂. The beer case is not so much different. Beer can be considered as a phosphate buffer and has a pH value of 4.00–4.30. The equilibria are also directed toward the dissolved CO₂.

Previously, multiple MD simulations were performed to study the hydrophobin behavior such as folding (59) and the interaction with both hydrophobic and hydrophilic interfaces (16,35). Here, we conducted MD simulations to study CO₂ clustering at the hydrophobic patch of a class II hydrophobin in solvent. This patch contains exclusively hydrophobic aliphatic side chains and is formed by two loop regions in the central β-barrel structure. As an initial test to verify the CO₂ parameters, 170 carbon dioxide molecules and 2,641 water molecules were added randomly to a box of

Fig. 3. Example of CO₂ aggregation in bubble-like structures. CO₂ molecules are shown as spheres. Water molecules are depicted in stick representation.

Fig. 4. Normalized CO₂ molecules near the hydrophobic patch. Number of CO₂ molecules per square Å in a sphere of 10 Å near the hydrophobic patch as a function of the MD trajectory. ASA: Accessible surface area.

Fig. 5. **A**, The structure of HFBII alone. **B**, Example of CO₂ clustering near the hydrophobic patch. **C**, Snapshots at 1, 50, and 100 ns of simulation time. CO₂ molecules are shown is sphere representation. Solvent molecules are omitted for clarity and the hydrophobic patch is shown in darker color.

Fig. 6. Schematic description of the formation and the stabilization of CO₂ nanobubble by elasticity of the Class II hydrophobin film in successive phases. **A**, Hydrophobins are present in the liquid. **B**, Hydrophobins migrate to the interface where they will become all contaminated. **C**, The concentration at the interface results in the formation of the crystalline layer. **D**, Disturbance of the Henry's equilibrium by energy incorporation. **E**, Return to equilibrium by nanobubble formation. **F**, Closing of the nanobubble. The values of tensioactivity were reported by Bamforth (4).

approximately 90 nm³ and this system was simulated for 100 ns. As expected, the hydrophobic CO₂ molecules quickly diffuse to each other and form both local and global aggregates in the system. An example of this aggregation at 10 ns is shown in Figure 3 (A movie is available on the following website: <http://www.chem.kuleuven.be/research/bio/bmm/index.html>).

Next, a system with HFBII hydrophobin, CO₂, and water molecules was simulated for 100 ns. This simulation was conducted with water molecules without any other components in order to work with the simplest model occurring in nature. Figure 4 graphically displays the amount of CO₂ molecules within the vicinity (10 Å) of the hydrophobic patch. The CO₂ per Å² ASA amount was followed during the whole trajectory and compared with the rest of the protein. It can be seen that in the vicinity of the hydrophobic patch, the amount of CO₂ molecules per Å² ASA is more than twice as large as in the vicinity of the rest of the protein surface. This effect is the strongest after 20 ns simulation time,

which can be considered as an equilibration time necessary to cluster the CO₂ molecules on the protein surface.

Figure 5 demonstrates the presence of a CO₂ aggregate bound to the hydrophobic patch of the protein during the MD trajectory (A movie is available on the following website: <http://www.chem.kuleuven.be/research/bio/bmm/index.html>). As such, the hydrophobic patch has a tendency to cluster the hydrophobic CO₂ molecules close to the protein surface. It can be hypothesized that this effect could be enhanced when multiple hydrophobins are present and polymerize with each other, thereby increasing the hydrophobic patch area and reducing contact with other parts of the protein. It is shown from the simulation as well that CO₂ binding does not happen exclusively at the hydrophobic patch but also on other hydrophobic areas of the protein, as is visible in Figure 5. Possibly, these areas could be buried upon oligomerization of multiple hydrophobins, thereby reducing binding of CO₂ at these sites. Of note, during the simulation certain CO₂ molecules are

occasionally captured in internal cavities of the hydrophobin and stay encapsulated within the protein for multiple nanoseconds.

Nanobubble Formation and Gushing Mechanism

Strong interaction between CO₂ and hydrophobin resulting in formation of stabilized nanobubbles is required for the gushing phenomenon. The formation of these stabilized nanobubbles in a closed container can be described in six successive phases (Fig. 6). In the first phase, hydrophobins which are contaminated by CO₂ (i.e., extracted during gaseous CO₂ injection) and non-contaminated hydrophobins are present in the liquid. Secondly, because hydrophobins are amphiphilic, they migrate to the hydrophilic-hydrophobic liquid-gaseous interface (47,48) where they all become contaminated because of the contact with a pure CO₂ atmosphere. Thirdly hydrophobins self-assemble at this interface, forming a crystalline monolayer (11) when the critical concentration is reached (crystallization) (50). Applying energy (e.g., shaking) to this closed system will disturb the equilibrium established by Henry's Law and the hydrophobin film enters the liquid. This will result in the attraction between the hydrophilic glass wall of the bottle and the "hydrophilic part" of the hydrophobin layer (41) and the hydrophobic part will act as an anchorage place or a pit for the nanobubble formation (39) by accumulation of gaseous CO₂ molecules. In fact, to return to the equilibrium, exchange of CO₂ molecules between the gaseous and the liquid phase occurs. Because of their ability to reduce surface tension from 72 to 30 mJ/m² (11), but also because of the elasticity of their structure (49), class II hydrophobins are able to accumulate gaseous CO₂ molecules and finally form a stabilized nanobubble closed by the lateral force resulting from the Young-Laplace's Law.

After the nanobubble formation, the gushing phenomenon can be considered in five stages: in a closed bottle, disruption and explosion, nucleation, foaming, and degassing. In closed bottle, gaseous CO₂ nanobubbles stabilized by the crystalline layer of hydrophobins are insulated from the beer. The internal pressure of the stabilized nanobubbles is equal to the internal pressure of the bottle (around 4 bars), and these pressurized nanobubbles will act as nanobombs when opening the bottle. When the bottle is opened, the pressure drop provokes the disruption of the elastic hydrophobin structure and the nanobubbles explode (28,57). This explosion provides the required energy for nucleation and releasing of dissolved CO₂ as it can be compared to the shock given to an opened beer bottle (31). The bubbles resulting from the explosion represent a local high concentration of gaseous CO₂. The bubbles will grow by diffusion of dissolved CO₂ because the beer is oversaturated according to the fact that solubility of gas in liquid is directly proportional to the partial pressure of that gas above the liquid (Henry's Law) (6,21,27,46,56). As they ascend to the surface, the growing bubbles attract proteins and create overfoaming (40). If the overfoaming and the remaining beverage is let to be degassed at atmospheric pressure, nanobubbles stabilized by hydrophobin film will be reformed with a diameter equal to a critical diameter at atmospheric pressure which are detectable by DLS (equals 100 nm for CO₂ as reported by Deckers et al (13)).

CONCLUSION

For the first time, MD simulations were carried out in order to reveal the biophysical details of the primary gushing mechanism. By this technique we demonstrated that the hydrophobic interaction between CO₂ and hydrophobic patch of Class II hydrophobins causes the formation of CO₂ nanobombs with the ability to result in vigorous overfoams when the pressure is released. This discovery allowed us to consider the formation of these nanobombs in several steps: first, contamination of hydrophobins by CO₂; secondly, migration of hydrophobin to the interface;

then, crystallization when reaching the critical concentration, followed by the exchange of CO₂ molecules due to the disturbance of equilibrium by applying energy to the system; and finally, by the nanobubble formation.

PERSPECTIVES

Despite the Class II, Class I hydrophobin was not reported as gushing inducer up to now. However the mechanism described here seems to be the same as described by Wang et al (51) where the rigid film formed by Class I SC3 hydrophobin is able to stabilize oil droplets. But in the case of gushing, the hydrophobic component is gaseous CO₂ and is unable to extract rodlet structure as oil can do in order to form stabilized nanobubbles. However, this statement needs further practical and computational experiments because several parameters can influence the surface properties. The demonstrated interaction also supports the new approach of preventing gushing by employing hydrophobic molecules to extract hydrophobins from wort of beer.

SUPPLEMENTARY DATA

In order to simulate precisely the interaction between the hydrophobin HFBII and CO₂ molecules, the amount of CO₂ molecules placed in the box for the MD simulations has to be calculated. These calculations are based on the experiments carried out with DLS and the observation of CO₂ nanobubble particle size at atmospheric pressure. Detailed calculations are given.

Calculation S1: Detailed Calculation for the Number of CO₂ Molecules Placed in the Box for the MD Simulation

As detected by DLS, the critical diameter of CO₂ at 1 atm (i.e., opened bottle) is 100 nm (i.e., radius (r) = 50 nm). The volume of one bubble (V) equals:

$$V_{1\text{atm}} = \frac{4}{3} \times \pi r^3 = 5.24 \times 10^5 \text{ nm}^3$$

By application of the general law of gas, the volume of one bubble and the critical diameter of CO₂ at 4 atm (i.e., closed bottle) can be determined as follows:

$$\frac{P_{1\text{atm}} \times V_{1\text{atm}}}{n_{1\text{atm}} \times T_{1\text{atm}}} = \text{constant} = \frac{P_{4\text{atm}} \times V_{4\text{atm}}}{n_{4\text{atm}} \times T_{4\text{atm}}} \Leftrightarrow V_{4\text{atm}} = \frac{P_{1\text{atm}} \times V_{1\text{atm}}}{P_{4\text{atm}}} = 1.31 \times 10^5 \text{ nm}^3$$

$$r_{4\text{atm}} = \sqrt[3]{\frac{3V_{4\text{atm}}}{4\pi}} = 31.5 \text{ nm} \Rightarrow d_{4\text{atm}} = 63 \text{ nm}$$

The volume of one CO₂ molecule (V_{CO₂}) was estimated to be 1.35 nm³ using the solvent accessible area. Combining the volume of the bubble (V_{4atm}) and of the CO₂ molecules, the number of CO₂ molecules in one bubble was estimated as follows:

Number of CO₂ molecules in one bubble =

$$\frac{V_{4\text{atm}}}{V_{\text{CO}_2}} = 97,000 \text{ molecules}$$

This amount has to be converted to the box size used in the simulations. Knowing the surface of the bubble (S_{4atm}), knowing that the solvent accessible area of the hydrophobic patch (S_{patch}) of HFBII is 7.4 nm² (19), and supposing that only 2/3 of the surface of the bubble is covered, the amount of hydrophobin monomers covering the surface of the bubble is determined as follows:

$$S_{4\text{atm}} = 4\pi \times r_{4\text{atm}}^2 = 1.2 \times 10^4 \text{ nm}^2$$

Amount of monomer of HFBII covering one bubble =

$$\frac{2}{3} \times \frac{S_{4\text{atm}}}{S_{\text{patch}}} = 1,081 \text{ monomers of HFBII}$$

The proportion between CO₂ and HFBII at 4 atm then equals:

$$\text{Proportion} = \frac{\text{Number of CO}_2 \text{ molecules in one bubble}}{\text{Amount of monomer of HFBII covering one bubble}} = 90$$

It means that there are 90 CO₂ molecules per monomer of HFBII, i.e., 90 molecules of CO₂ in the simulation box at 4 atm.

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