Protein loading into porous CaCO₃ microspheres: adsorption equilibrium and bioactivity retention

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Formulation of proteins into particulate form is a principal strategy to achieve controlled and targeted delivery. It is not only encapsulation under mild conditions but also good control of particle shape and size. For this purpose, the sequential adsorption of polymers, or so-called layer-by-layer (LbL) deposition, can be performed by three different approaches, as reviewed elsewhere: (i) the inclusion of the protein into solid decomposable matrices for further polymer LbL coating, (ii) an approach in which the protein itself is one of the LbL-deposited polymers, and (iii) loading of proteins into preformed polymer particles. One can adjust the size and shape of the protein-containing particles by selection of the size and shape of the decomposable matrices used, or of the polymer particles. For instance, salted-out protein aggregates or insoluble protein–polyanion complexes may be used as matrices for further LbL deposition to form protein-containing particles with rather high polydispersity (particle size in the range 3–10 μm). These particles are intended to be utilized for oral delivery, where particle size distribution is not a critical issue.

Decomposable inorganic and organic matrices such as micro-particles from melamin formaldehyde, polystyrene, PLGA, silica oxide, and carbonates have been used to fabricate protein-containing micro- and nanoparticles with controlled size and shape (spherical). Pulmonary delivery is envisaged in this case. Ten years ago, calcium carbonate vaterite microspheres were developed and have nowadays become one of the most popular decomposable matrices. Together with easy preparation procedure and low costs these microspheres are biocompatible and can be eliminated by mild conditions such as slightly acidic pH.
or EDTA. Another important feature of the microspheres is that they are mesoporous. About half of their internal volume belongs to pores ( pore size in the range 20–60 nm) offering space for encapsulation of macromolecules such as proteins, hormones, enzymes, etc. Protein encapsulation into the pores of the microspheres can be achieved by the following approaches: co-precipitation (entrapment during the microsphere synthesis), infiltration by solvent exchange, and physical sorption.

Despite a progressive increase in the number of scientific reports devoted to protein encapsulation by means of porous CaCO3 microspheres, there is no full understanding of the mechanism of protein binding to the microspheres. Protein activity after microsphere decomposition is also not well studied. The aim of this study is to investigate thermodynamic parameters of protein adsorption on carbonate microspheres in order to understand protein interactions with the microspheres. Model proteins with different molecular masses and charges are employed for this purpose: insulin (Ins), catalase (Cat), aprotinin (Apr), andprotamine (Pro). In addition, biological activities of the adsorbed proteins and those encapsulated after the microsphere dissolution by EDTA are studied.

2. Experimental section

2.1. Materials

CaCl2 – 99%, “Biomedicals” ICN, Inc., USA; Na2CO3 – 99.8%, Pharma, Russia; Pro from salmon and Ct from bovine liver – “Sigma”, Germany; Apr from bovine lung preparation “Ingiprol” (60% active center) Belmedpreparaty, Belarus; trypsin from bovine pancreas (40 U mg⁻¹, 61% active center) – “Fluka”, USA; hydrogen peroxide – “Sigma-Aldrich”, USA; N-benzoyl-l-arginine ethyl ester – “Sigma”, USA; EDTA – “Reakhim”, Russia. Human recombinant Ins zinc salt was kindly provided by the Experimental Biotechnology Plant of Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences. FITC, isomer I – “Sigma”, Germany. All the chemicals were used without further purification. Milli-Q (Millipore, USA) water was used for all experiments.

2.2. Preparation of CaCO3 microspheres

The preparation of CaCO3 microspheres by mixing of CaCl2 and Na2CO3 solutions has been described in detail elsewhere. Briefly, 3 mL of 1 M Na2CO3 solutions were rapidly added to 9 mL of H2O and 3 mL of 1 M CaCl2 and thoroughly agitated on a magnetic stirrer (650 rpm) for 45 s at 20 °C. It is important to properly choose salt concentration as well as stirring time and stirring speed because all these parameters influence the size of the CaCO3 microspheres. After that, the agitation was stopped, and the reaction mixture was left without stirring for 15 min, during which time the formed amorphous primary precipitate of CaCO3 transforms slowly into spherical microspheres. Finally, the particles were separated by centrifugation at 1000 × g, resuspended in 2 mL of H2O and dried at 70 °C for 2 h. Before use, all salt solutions were filtered through a RC 0.20 μm filter (Corning Inc., USA).

2.3. Protein adsorption

The batch adsorption of model proteins onto mesoporous CaCO3 microspheres (0.005–0.080 mg mL⁻¹) was performed in 0.05 M glycine buffer, pH 8–10, subjected to constant stirring. The proteins used were Pro, Apr, Ins, and Cat. The initial concentration of proteins ranged from 0.1 mg mL⁻¹ to 2 mg mL⁻¹. The suspensions of the microspheres in protein solutions were incubated for 2–60 min under stirring at 23 °C; they were then centrifuged for 5 min at 10000 × g and the supernatant solutions were analyzed. The concentration of Pro was determined using the Lowry method. The concentrations of Apr, Ins, and Cat were measured by UV spectroscopy (Lambda 35, “Perkin-Elmer”) at a wavelength of 280 nm. In the experimental assays, adsorption measurements were conducted at least in triplicate. For protein release experiments, the CaCO3 microspheres with adsorbed protein were washed twice with the same volume of 0.05 glycine buffer in order to remove loosely attached protein molecules.

The amount of adsorbed protein under saturation conditions (equilibrium) was calculated using the following equation:

\[ q_e = \frac{(C_0 - C_e) \times V}{m}, \]

where \( q_e \) is the adsorption capacity (mg g⁻¹), \( C_0 \) and \( C_e \) are the initial and equilibrium protein concentrations (mg mL⁻¹); \( V \) is the volume (mL) of the protein solution; and \( m \) is the mass (mg) of CaCO3.

The following Langmuir isotherm equations were used to fit experimentally obtained adsorption isotherms:

\[ \frac{1}{q_e} = \frac{1}{q_m} + \frac{1}{K_a C_e}, \]

\[ q_e = q_m + K_a C_e, \]

\[ C_e = \frac{1}{K_a q_m} + \frac{C_0}{q_m}, \]

where \( q_m \) is the amount of protein molecules adsorbed to form a monolayer (mg g⁻¹), \( K_a \) is the Langmuir adsorption equilibrium constant (mg g⁻¹), \( q_0 \), and \( K_a \) have been calculated from the plots (i) \( 1/q_e = f(1/C_e) \), (ii) \( q_e = f(q_e/C_0) \), and (iii) \( C_0 q_e = f(C_0) \), which correspond to the equations described above.

Efficiency of protein incorporation was calculated using the following equation:

\[ \eta = \frac{C_0 - C_e}{C_0} \]

2.4. FITC-labeling of proteins

0.1 mg mL⁻¹ FITC solution in 0.5 M carbonate buffer (pH 9.0) was added drop-wise to 2 mg mL⁻¹ protein solution in 0.5 M carbonate buffer under stirring until a FITC:protein molar ratio of 1:5 was achieved. The resulting solution was incubated for 4 h in darkness and dialyzed twice against 50 mM TRIS buffer (dialysis bags with cut-off 8–10 kDa).

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2.5. Optical microscopy
CaCO₃ microspheres were visualized using an optical microscope “Carl Zeiss, JENA” (Germany).

2.6. Confocal laser scanning microscopy (CLSM)
CLSM analysis was done using a Zeiss LSM 510 Meta (Zeiss, Germany) equipped with an oil-immersion objective with 63× magnification and a numerical aperture of 1.4. Standard filter settings for excitation and emission of FITC were used for laser sources, with a wavelength of 488 nm. For studying penetration of proteins into CaCO₃ microspheres, 0.2 mL of 1 mg mL⁻¹ fluorescence-labeled protein solution was added to 0.5 mg of dry CaCO₃ microspheres for imaging after 30 min of incubation.

2.7. Scanning electron microscopy (SEM)
For SEM analysis, the CaCO₃ microsphere suspension in water was placed on a glass slide followed by drying for 1 h at 90 °C. The sample was then sputtered with gold and SEM analysis was conducted using a Gemini Leo 1550VP instrument at an operating voltage of 3 kV.

2.8. Protein biological activity
The specific activity of Cat was measured by monitoring the rate of hydrogen peroxide decomposition.²⁷ The specific activity of Apr was measured by inhibition of trypsin using N-benzoyl-L-arginine ethyl ester as a substrate.²⁸ To analyze the activity of Apr and Cat, CaCO₃ microspheres were washed twice in 0.05 M glycine buffer (pH 9.0). The absorbance was monitored at 280 nm.

2.9. Measurement of the hydrodynamic radius of proteins
Sizes of proteins were determined by photon correlation spectroscopy (DLS) using a zeta-sizer (Nano ZS, Malvern, UK). 1 mL of filtered 1 mg mL⁻¹ protein solution in 0.05 M glycine buffer (pH 9.0) was injected into the cell and measured for a period of 9 min.

2.10. Measurement of zeta-potential
The zeta-potential of proteins and of CaCO₃ microspheres was measured by a dynamic laser light scattering method on a zeta-sizer (Nano ZS, Malvern, UK). 1 mg mL⁻¹ of protein or 0.5 mg mL⁻¹ of CaCO₃ suspension in 0.0125 M glycine buffer (pH 9.0) was injected into a measuring cell and data were collected for 30 s.

2.11. Liquid penetration chromatography
Gel filtration of proteins was carried out using the Smartline system (“Knauer”, Germany), using a high-pressure column packed with Biofox 17 SEC 9 (8 × 30 mm), previously calibrated with standard proteins: cytochrome c (Mₗ 12.4 kDa), carbonic anhydrase (Mₗ 29 kDa), bovine serum albumin (Mₗ 66 kDa) and amylase (200 kDa). The column was loaded with 0.5 mL of 0.5 mg mL⁻¹ protein in 0.05 M glycine buffer (pH 9.0); the process was conducted at a flow rate of 0.5 mL min⁻¹ in 0.05 M glycine buffer (pH 9.0). The absorbance was monitored at 280 nm.

3. Results and discussion
CaCO₃ microspheres with an average diameter of 4.8 µm and a low degree of aggregation were obtained by crystallization from supersaturated solution according to previous reports.¹⁵,¹⁶ As can be seen from Fig. 1, the microspheres show a highly developed porous internal structure. The microspheres are composed from channel-like spherulitic nanocrystals of a size of tens of nanometers. As reported in a previous study,¹⁴ the average specific surface area of the microspheres is 8.8 m² g⁻¹, their density is 1.6 g cm⁻³, and the average pore size is in the range 20–60 nm. The particles used in this study were prepared using the same protocol and possessed the same properties. Subsequently, these parameters allow us to estimate the area occupied by adsorbed proteins. The pI of CaCO₃ microspheres is in the range 8–9.¹⁵ The zeta-potential of CaCO₃ microspheres in 0.05 M glycine buffer at pH 9.0 was found to be slightly positive (5.0 ± 0.3 mV), which might be due to the closeness to the pI.

We focused further on protein interaction with the porous carbonate surfaces so as to understand the mechanism of protein adsorption to the particles, because both protein affinity to the particle surface and steric limitations on diffusion through the pores may play significant roles in determining protein loading into the particles. Table 1 shows the physical–chemical properties of the proteins used in this study. Molecular weights, diameters known from the literature and measured in glycine buffer, as well as isoelectric points (pI), are included in the table. Glycine buffer was incubated with the CaCO₃ microspheres (40 mg mL⁻¹), and the microspheres were then separated by centrifugation. This ensured that the conditions used for measurement of the protein solutions were equivalent to the conditions used for protein adsorption to the microspheres. As found by DLS measurements, Pro, Apr, and Cat were present in solution as monomers and Ins...
Table 1  Physical–chemical properties of model proteins used in this study. Measurements of zeta-potential and hydrodynamic diameter were carried out in 0.05 M glycine buffer (pH 9.0) and in 0.05 M glycine buffer (pH 9.0) incubated with CaCO₃ microspheres (40 mg mL⁻¹), respectively

<table>
<thead>
<tr>
<th>Protein</th>
<th>( M_w ) (kDa)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Experimental data</th>
<th>pI</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>5.0</td>
<td>3.0(^{29})</td>
<td>4 ± 1</td>
<td>11.0</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Apr</td>
<td>6.5</td>
<td>2.9(^{30})</td>
<td>3 ± 1</td>
<td>10.5</td>
<td>−4.2 ± 0.3</td>
</tr>
<tr>
<td>Ins</td>
<td>5.8</td>
<td>2.7 (monomer), 5.1 (hexamer)(^{31})</td>
<td>4 ± 1</td>
<td>5.3</td>
<td>−14.1 ± 0.3</td>
</tr>
<tr>
<td>Cat</td>
<td>250</td>
<td>10.5(^{32})</td>
<td>15 ± 1</td>
<td>5.4</td>
<td>−9.9 ± 0.3</td>
</tr>
</tbody>
</table>

was present as a hexamer. Gel-filtration chromatography confirmed that Ins was mostly present as a hexamer (Fig. S1, ESI†). It is noteworthy that the hydrodynamic diameters of three proteins (Pro, Apr, and Ins) were similar to each other (3–4 nm) whereas that of Cat was much larger (15 nm), as shown in Table 1. The zeta-potentials of the proteins used were measured by DLS and the results are presented in Table 1. Pro was positively charged and Apr possessed a rather low negative charge. In contrast, the zeta-potentials of Ins and Cat were much higher, with a negative charge.

Firstly, adsorption isotherms were studied at room temperature. For this, a rather high concentration of CaCO₃ microspheres (40 mg mL⁻¹) and a long incubation time (30 min) were chosen because under these conditions sufficient protein molecules may be loaded so as to saturate the microspheres (Fig. S2 and S3, ESI†). The Langmuir isotherm is usually used to describe adsorption of a solute from a liquid solution as follows:\(^{33}\)

\[
q_e = \frac{q_m K_a C_e}{1 + K_a C_e},
\]

where \( q_e \) is the equilibrium adsorption capacity (mg g\(^{-1}\)), \( C_e \) is equilibrium protein concentration (mg mL\(^{-1}\)), \( q_m \) is maximum adsorption capacity or the amount of molecules adsorbed to form a monolayer (mg g\(^{-1}\)), and \( K_a \) is the Langmuir adsorption equilibrium constant (mL mg\(^{-1}\)).

Fig. 2 shows adsorption isotherms of the proteins used in this study. To obtain \( q_m \) and \( K_a \), the experimentally determined adsorption isotherms have been presented in three different ways. They correspond to the following functions: \( 1/q_e = f(1/C_e) \), \( q_e = f(q_m/C_e) \), and \( C_e/q_e = f(C_e) \). The resulting graphs for the example of Cat fitted to linear curves are shown in Fig. 3a–c, respectively.

The coefficient of determination was calculated for the three plots for all model proteins used in this study in order to find the best correlation of experimental data:

\[
r^2 = \frac{\sum (q_m - \overline{q})^2 - \sum (q_m - \overline{q_e})^2}{\sum (q_m - \overline{q})^2},
\]

where \( q_m \) is the equilibrium capacity obtained from the isotherm model, \( \overline{q_e} \) is the equilibrium capacity obtained by experiment, and \( \overline{q_e} \) is the average of \( q_e \).

Table S1 (ESI†) contains the calculated \( q_m \) and \( r^2 \) values for the proteins used. To compare adsorption parameters for four model proteins, the best fit (highest \( r^2 \)) has been chosen for each and every protein. Table 2 shows \( q_m \), \( K_a \), and \( 1/K_a \) for the proteins.

Maximum adsorption capacity (\( q_m \)) in terms of protein mass is much higher (more than an order of magnitude) for Cat and Ins, as compared to Pro and Apr (Table 2). However, if considering this value as the number of protein molecules adsorbed, there is no significant difference between these four proteins (Table 2). This means that the number of adsorption centers for all the model proteins studied is similar. Larger proteins such as Cat and Ins (hexamer) show a higher adsorbed amount in terms of mass.

The adsorption equilibrium constant \( K_a \) was also much higher for Cat and Ins compared to the other proteins. As a result, the Gibbs free energy \( \Delta G \) calculated by the equation below was lower for Cat and Ins, indicating a higher affinity of these proteins for the microsphere surface compared to Apr and Pro:

\[
\Delta G = -RT \ln K_a.
\]
centers are occupied. These values are much lower for Cat and Ins compared to Apr and Pro. Thus, affinities of Cat and Ins for carbonate microspheres are higher than those of Apr and Pro. We believe that the higher affinity of Cat and Ins than Pro and Apr can be explained by the impact of electrostatic forces involved in the adsorption process. Cat and Ins are negatively charged, which may drive their interaction with positively charged CaCO₃ microspheres. In contrast, Apr has a very low but negative zeta-potential and Pro has a positive zeta-potential that may reduce the interaction of these proteins with the microspheres by electrostatic binding.

The porous structure of the CaCO₃ microspheres allows one to load a large amount of adsorbing molecules, due to the high surface area available in the mesoporous structure. We have evaluated the input of the porous structure to the amount of adsorbed proteins. Fig. 4 presents theoretical values for maximum protein adsorption, assuming monolayer formation for non-porous (gray) microspheres and porous (light gray) ones, as used in this study (diameter of 4.8 μm and surface area of 8.8 m² g⁻¹). The surface area occupied by a protein molecule has been chosen to be a circle with a diameter equal to the hydrodynamic diameter determined in this study by DLS (Table 1). The capacity for maximal theoretical protein adsorption to porous microspheres is much higher (about an order of magnitude) than to non-porous microspheres, due to the large surface area. Experimentally observed amounts of proteins adsorbed in porous microspheres (Fig. 4, black columns) were always below the theoretically calculated maximum values. However, the experimentally found values differ from one protein to another. Ins and Cat occupied 94% and 82% of the theoretical maximum values, and Apr and Pro just 8% and 33%, respectively. To understand why Apr and Pro have much lower affinity for the carbonate microspheres, we studied in more detail protein penetration into the microspheres and protein distribution inside them.

To follow protein diffusion into the microspheres and protein distribution inside the microspheres, we have used CLSM. For this analysis, all four proteins were labeled by FITC by the procedure described elsewhere. However, Apr-FITC and Pro-FITC aggregated in the presence of supernatant of CaCO₃, probably because of low colloidal stability of the proteins in the pH range close to their pI (pH 9, glycine buffer). Another explanation could be that the aggregation was caused by significant changes of the hydrophilic–hydrophobic balance of these rather small proteins, even when one protein molecule binds one molecule of FITC during the labeling procedure. Cat and Ins (hexamer) are much larger and one FITC molecule does

\[ R = \frac{8.314 \text{ J mol}^{-1} \text{ K}^{-1}}{\text{mol L}^{-1}} \]

where \( R \) is the universal gas constant (8.314 J mol⁻¹ K⁻¹) and \( T \) is the absolute temperature in K.

Negative values of \( \Delta G \) mean that for all four model proteins, the equilibrium in protein adsorption is shifted towards adsorbed protein molecules. \( 1/K_a \) represents an equilibrium concentration of protein molecules when half of the adsorption

\[ q_m = f(1/C_e) \]

\[ q_a = f(q_e/C_e) \]

\[ C_e = f(q_a) \]

**Table 2**Parameters of protein adsorption (\( q_m, K_a, 1/K_a \)) found from best fits to the Langmuir equation (Table S1, ESI), as well as Gibbs free energy (\( \Delta G \)), for the model proteins used in this study.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( q_m ) (mg g⁻¹)</th>
<th>( q_a ) (mol g⁻¹)</th>
<th>( K_a ) (L mol⁻¹)</th>
<th>( 1/K_a ) (mol L⁻¹)</th>
<th>( \Delta G ) (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>3.4 ± 0.5</td>
<td>(6.9 ± 0.5) × 10⁻⁷</td>
<td>(5 ± 1) × 10⁻⁴</td>
<td>(1.9 ± 0.2) × 10⁻⁴</td>
<td>−21 ± 1</td>
</tr>
<tr>
<td>Apr</td>
<td>1.1 ± 0.3</td>
<td>(1.7 ± 0.3) × 10⁻⁷</td>
<td>(11 ± 2) × 10⁻³</td>
<td>(0.9 ± 0.1) × 10⁻⁴</td>
<td>−23 ± 1</td>
</tr>
<tr>
<td>Ins</td>
<td>23.0 ± 2.0</td>
<td>(6.5 ± 0.5) × 10⁻⁷</td>
<td>(60 ± 5) × 10⁻¹</td>
<td>(1.7 ± 0.2) × 10⁻⁵</td>
<td>−27 ± 1</td>
</tr>
<tr>
<td>Cat</td>
<td>34.0 ± 2.0</td>
<td>(1.5 ± 0.2) × 10⁻⁷</td>
<td>(2380 ± 150) × 10⁻⁵</td>
<td>(4.2 ± 0.5) × 10⁻⁷</td>
<td>−36 ± 2</td>
</tr>
</tbody>
</table>
not change the balance significantly. Ins and Cat were stable and their interaction with CaCO₃ microspheres was studied. Both proteins were found to be distributed within the whole volume of the CaCO₃ microspheres, indicating no limitations on diffusion through the microsphere pores (Fig. 5). However, Ins was distributed more homogeneously inside the microspheres compared to Cat. This may be explained by the larger size of Cat molecules (hydrodynamic diameter of 15 nm against 4 nm of Ins). Thus, although some pores were not available for Cat molecules (pores in the range 20–60 nm), larger pores did not present any diffusion barrier and Cat molecules could penetrate through the whole volume of the microspheres. We believe that there would be no diffusion limitation for unlabeled Apr and Pro, because their size is similar to the size of Ins molecules. Very low occupation of microsphere molecules by Apr and Pro compared to Ins and Cat may be explained by low affinity of Apr and Pro for the carbonate surface, but not by diffusion limitations on penetration through the microsphere pores. This fact is supported by lower $K_a$ and higher Gibbs free energy for Apr and Pro compared to Ins and Cat.

Binding of the adsorbed proteins could be studied by determining the amount of adsorbed protein molecules washed out after rinsing with buffer solution. Table 3 shows the amount of protein included in the microspheres after adsorption and the amount after two washing steps in buffer solution. Protein retention was calculated from these values. Cat demonstrated very strong binding to the microsphere surface and very little was released after washing. Other proteins were released more significantly, probably because of lower (weaker) binding compared to Cat. Pro and Ins were also retained to a large

![Fig. 4](image-url) Maximum protein adsorption calculated for porous (light gray) microspheres used in this study and non-porous (gray) microspheres with the same diameter (4.8 μm). The measured amount of proteins adsorbed ($q_m$) on porous microspheres is presented as black columns.

![Fig. 5](image-url) LSM images of CaCO₃ microspheres after incubation with Ins-FITC (a–c) and Cat-FITC (d–f). (a, d) fluorescent images, (b, e) transmission images, (c) and (f) fluorescence profiles for the images (a) and (d), respectively.

**Table 3** Characteristics of protein adsorption in CaCO₃ microspheres and activity retention after microsphere dissolution

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein content (mg g⁻¹)</th>
<th>Protein retention after two washing steps, % from adsorbed protein</th>
<th>Retention of specific activity after dissolution of CaCO₃ in EDTA, % of initial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>1.5 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>74</td>
</tr>
<tr>
<td>Apr</td>
<td>0.6 ± 0.1</td>
<td>0.10 ± 0.02</td>
<td>16</td>
</tr>
<tr>
<td>Ins</td>
<td>11.0 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>64</td>
</tr>
<tr>
<td>Cat</td>
<td>20.3 ± 1.0</td>
<td>20.0 ± 1.2</td>
<td>99</td>
</tr>
</tbody>
</table>

* Adsorption conditions: CaCO₃ and protein concentration are 40 and 1 mg mL⁻¹, respectively, incubation time 30 min, glycine buffer pH 9.0.
extent in the microspheres after washing, which may be caused by the large size of the Ins hexamer and the rod-like structure of peptide Pro. The Gibbs free energy values for Apr, Ins, and Pro are about two times higher than that of Cat; this may explain the much stronger binding of Cat.

Retention of protein biological activity after elimination of decomposable CaCO₃ microspheres is crucial for protein encapsulation using the microspheres. Cat retained about 80% of its initial activity after adsorption into the microspheres followed by microsphere dissolution in 0.2 M EDTA (Table 3). Taking into account that Cat does not change its activity in solution in the presence of 0.2 M EDTA (data not shown), the reduction of Cat activity by 20% might be related to partial protein denaturation, either due to adsorption onto the carbonate surface or the rather high pH of the adsorption conditions (pH 9.0). We have tested the effect of pH on Cat activity in solution and found the activity of Cat to be pH-dependent (Fig. 6). After 1 h incubation, Cat activity was reduced by more than half at pH 9.0. This means that a change of Cat secondary structure is not significant after adsorption onto CaCO₃ microspheres and further microsphere dissolution; the protein loses activity due to the rather high pH values during the adsorption experiment. Apr fully retained its biological inhibition activity after adsorption followed by microsphere dissolution in 0.2 M EDTA. Apr activity does not depend on pH (Fig. 6). This again proves that adsorption of these proteins (Cat and Apr) followed by their release (microsphere decomposition) does not significantly affect the protein secondary structure. This is very important and shows that decomposition by EDTA might be used to eliminate carbonate microspheres aiming at protein encapsulation by CaCO₃ microsphere templating.

pH has a strong effect not only on protein activity but also on protein adsorption. Furthermore, we have studied how incubation pH affects protein binding and subsequent protein release during a washing step. This is important for the encapsulation procedure, because washing-out of poorly bound protein molecules or layer-by-layer polymer coating of protein-containing CaCO₃ microspheres includes multiple washing steps. For higher pH values (ranging from 8 to 10), the amount of protein adsorbed decreased for all proteins except Cat (Fig. 7a). It is probable that the very strong binding of Cat compared to other proteins (indicated by much lower Gibbs free energy, Table 2) makes Cat adsorption pH-independent. The amount of protein remaining after adsorption followed by washing with buffer is presented in Fig. 7b. For most of the proteins, the amount of protein released after washing increased for higher pH values. This means that there is a direct correlation between protein binding and release as a function of pH. The stronger the protein binding, the fewer protein molecules will be released after a washing step. If a protein is bound very strongly, such as Cat in this study, its binding and release might be pH-independent. In general, one can consider the CaCO₃ particles as matrices to separate proteins by employing the different affinities of the adsorbing proteins to the particles at various pH values (in the range above neutral pH, so as to keep the particles stable). This may give an option for reuse of the particles and make use of their expanded surface area for the adsorption of large amounts of proteins.

4. Conclusions

Protein adsorption on porous vaterite CaCO₃ microspheres has been investigated for the following proteins having different molecular weights and isoelectric points: Pro, Apr, Ins, and Cat. Adsorption isotherms have been constructed and parameters of adsorption equilibria such as $q_{m}$, $K_a$, $\Delta G$ have been calculated. The adsorption isotherms fit well to the model of monomolecular Langmuir adsorption. Values of Gibbs free energy were found to be in the range from $-40$ to $-20$ kJ mol⁻¹. This shows that the adsorption equilibrium for all the studied proteins is shifted towards the adsorbed protein. Cat and Ins have lower values of $\Delta G$, which may explain why these two proteins have stronger interaction with the microspheres. Ins and Cat occupied
94% and 82% of the theoretically calculated monolayer and Apr and Pro just 8% and 33%, respectively. This means that protein adsorption is not limited by steric hindrance of protein diffusion through the mesoporous structure of the microparticles but depends on protein affinity to the carbonate surface. No diffusion limitations may occur because the dimensions of pores of the microparticles (20–60 nm) are larger than the hydrodynamic diameters of the proteins (15 nm for Cat and about 4 nm for other proteins studied). Cat has the highest affinity for the microparticles (lowest $\Delta G$ of $-36$ kJ mol$^{-1}$) and was almost not released after adsorption followed by multiple washing steps; other proteins show release of 30–70% of the initially adsorbed amount. Moreover, the stronger the protein binding, the less the protein molecules will be released after washing steps, as found in the pH range 7–10. Proteins (Cat and Apr) keep their biological activity after adsorption followed by microsphere dissolution in EDTA. This demonstrates high promise for CaCO$_3$ microparticles as templates for protein encapsulation.

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References