Appl Biochem Biotechnol (2010) 162:1547–1557
DOI 10.1007/s12010-010-8936-x

High Enrichment of MMP-9 and Carboxypeptidase A by Tweezing Adsorptive Bubble Separation (TABS)

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Received: 29 October 2009 / Accepted: 17 February 2010 /
Published online: 14 March 2010

Abstract Tweezing adsorptive bubble separation (TABS) was used as a method for the enrichment of matrix metalloproteinases (92-kDa type IV, gelatinase B (MMP-9)) and carboxypeptidase A (CPA) from dilute aqueous solutions. The method is based on the chelation of metalloenzymes applying 2-(carbamoylmethyl-(carboxymethyl)amino)acetic acid (ADA) coupled with an octyl part to form a surface active unit. MMP-9 could be enriched with an enrichment ratio of 12.0 and a recovery of 87.3%, and CPA could be enriched 18.8-fold and with 95.3% recovery. Both enzymes were enriched without significant losses of enzymatic activity. To verify that the enzymes were tweezed by ADA-C8 without abstraction of the zinc ions from the active center, TABS trials were additionally conducted with zinc ions in complex with ADA-C8, which revealed only negligible enrichment ratios of the enzymes (2.2 for MMP-9 and 0.2 for CPA). The results obtained impressively demonstrate that zinc-containing proteases can be enriched selectively and efficiently by TABS.

Keywords Tweezing adsorptive bubble separation · MMP-9 · Carboxypeptidase A · Matrix metalloproteinases · Carboxypeptidases

Introduction

Carboxypeptidases and matrix metalloproteinases (MMPs) belong to the family of medically important metalloproteases with diverse positive and negative functions in the human body [1]. According to classification, the pancreatic carboxypeptidase A (CPA) is

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assigned to the digestive group of enzymes having primarily the function to break down peptides in the gut by exhibiting optimum activity towards aliphatic amino acids containing aromatic hydrocarbon chains [1]. MMPs on the other hand play a central role in the breakdown of extracellular matrix and, therefore, in embryonic development, morphogenesis, reproduction, and tissue resorption as well as remodeling [2–5]. In this respect, the MMP-9 enzyme (92-kDa type IV, gelatinase B) has been intensively studied and found to be negatively involved in a variety of pathological conditions such as cancer, inflammation, infection, brain degeneration, and vascular diseases once its activity is deregulated [6–11]. Recently, blood samples from patients that exhibited acute coronary syndromes have been analyzed for coronary plaques, and MMP-9 was suggested to have the potential as a biomarker [11].

The isolation of these proteases in laboratory applications necessitates their separation from biological materials, for which dialysis and ultrafiltration as common methods are applied, followed by chromatographic steps. The involved cleanup processes, however, are implicated with losses of enzymatic activity [12–17]. To overcome this obstacle, the application of the so-called adsorptive bubble separation (ABS) method as an alternative separation technique can be considered. While the process when normally performed has already been proven as a method of sustainably and efficiently enriching a variety of substances of pharmaceutical and technological interest [18–23], an improvement has been developed and introduced as the "tweezer principle" by Gerken et al. [16], targeting especially useful metalloproteinases (Fig. 1). In general, gas is led into a solution, whereupon a foam column is developed above which is concentrated with surface active substances. The foam then disintegrates back to the so-called foamate which is ready to be of use afterwards. The initial solution contains the active enzyme plus a tweezer that reacts with the enzyme and carries it into the foam phase. The tweezer, by locking on to the enzyme's active center, helps not only to improve the efficiency of the enrichment process but also functions as a foam developer, if the enzyme under investigation does not possess sufficient capacity to form foam. The tweezer therefore consists of a seizing part (carboxyl groups) and an octyl part, which takes over the task of foam development and carriage. For the success of the entire process, it is imperative that the enzyme is available after the enrichment with restored enzymatic activity, which can either be achieved by lowering the pH (precipitation) or simply by filtration.

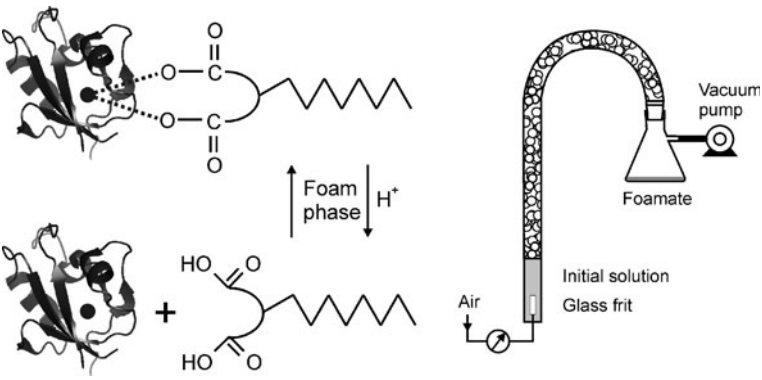

Fig. 1 TABS principle in analogy to Gerken et al. [16]. The tweezer, chelating the metal ion in the metalloenzyme, consists of a seizing part covalently bound to an n-octyl part to enable the carriage into the foam phase. After foam fractionation, the enzyme and the tweezer can be separated from each other by protonation

Under the perspective that both MMP-9 and CPA contain catalytically active metal ions in their centers, it was the purpose of this work to investigate the suitability of TABS to selectively enrich these two medicinally important enzymes without having to accept losses of enzymatic activity. For the chelation and enrichment of the metalloenzymes, *N*-octylcarbamoylmethyliminodiacetic acid (ADA-C8) as the chelator was synthesized. The efficiency values (enrichment ratio and recovery) of both chelation and dechelation were compared to each other and verified by a zinc-containing chelator.

Materials and Methods

All used chemicals were of p.a. quality. MMP-9 (EC 3.4.24.35) was purchased from Roche Diagnostics GmbH (Roche Applied Science, Penzberg Germany) and CPA (EC 3.4.17.1) from Sigma Chemicals, USA. Ellman's reagent was especially prepared and *N*-acetyl-L-phenylalanyl-L-3-thiophenylalanin was synthesized in our laboratory according to the method of Brown et al. [24]. A spectral photometer, type Cecil CE 1021, was used for the UV experiments. ^{1}H- and ^{13}C-NMR spectroscopy (Bruker AC250) was applied for structure elucidation of ADA-C8 and *N*-acetyl-L-phenylalanyl-L-3-thiophenylalanin. ADA-C8 (Fig. 2) was synthesized as described in Gerken et al. [16].

Chelation and Dechelation Experiments

For the chelation of MMP-9 and CPA with *N*-(2-acetamido)iminodiacetic acid (ADA), 1 ml of the chelator was added to the enzyme solutions. The pH value was adjusted between 5 and 7 in order to reach the highest possible stability of the chelate. For the dechelation of the enzyme chelates, the enzymatic solution was mixed with increasing concentrations of zinc(II) sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) until the maximum enzymatic activity was recovered. For the dechelation of the MMP-9 and CPA/ADA-C8 chelates, the pH of the enzymatic solution was lowered to 3.5 by adding HCl. ADA-C8 was eliminated by adding HCl until pH 3 to cause precipitation of the chelator so that it was ready to be eliminated by filtration. For the chelation of ADA-C8 with zinc(II) ions, ADA-C8 was reacted with external zinc(II) ions in equimolar concentrations prior to the addition of the enzymes. For this, 25 mg of $ZnSO_4 \cdot 7H_2O$ and 30 mg of ADA-C8 were dissolved in 10 ml of demineralized water under stirring. Afterwards, the enzymes with different activities were added.

Adsorptive Bubble Separation Experiments

For running the fractionation trials, the start solutions were mixed with the chelator, or the metal-ADA-C8 chelate, which was used for verification. The amount of 2.75 ml was placed

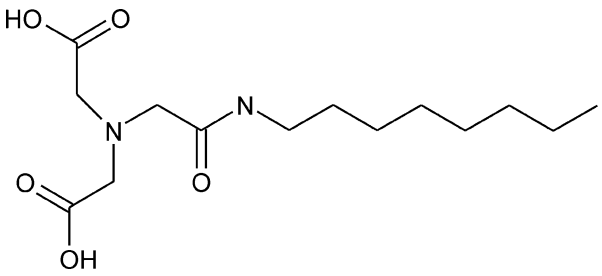

Fig. 2 The chelator *N*-octyl-carbamoylmethyl iminodiacetic acid (ADA-C8); synthesized by *N*-(2-acetamido) iminodiacetic acid (ADA; ID: MHD as to protein data base) bound to an octyl part at the primary nitrogen of the molecule

in a cylindrical glass fractionation column with an ID of 11 mm. The foam developed by introducing compressed air with 1 ml/min flow rate through a glass frit (porosity 3, 16–40 μm pores' size) and afterwards was forced to liquefy by a continuous foam destructor through pressure difference. All trials were performed at room temperature, applying a foaming time of 30 min. The enrichment ratio (ER) and recovery rate (R in percent) as a measure of efficiency were determined under optimized conditions as to gas flow rate, pH of the start solution, initial concentration of the chelator, and foaming time. The values were calculated according to the following equations:

$$\mathrm{ER} = \frac{E_{Af}}{E_{As}} \quad R = \frac{E_{Af} \times V_f}{E_{As} \times V_s} 100\%$$

Where E_{Af} is the enzymatic activity in the foam (U/l), E_{As} is the enzymatic activity in the starting solution (U/l), V_f is the volume of the liquefied foam (l), and V_s is the volume of the starting solution (l).

Enzyme Solutions and Determination of Enzymatic Activity

MMP-9 was obtained as a solution in 20 mM tris and 5 mM $CaCl_2$ at pH 8.0. The specific activity of MMP-9 was ≥200 mU/mg after activation with trypsin. The activity trials were performed according to the manufacturer's instructions (Roche Applied Science Assay Kit, Cat. No. 1 758 900), in analogy to Masui et al. [25]. Bovine pancreatic CPA (C9268-2.5 Ku; 24 mg protein/ml, 71 U/mg) solutions were prepared in LiCl (10%). The protein content was determined at 280 nm absorbency. More dilute solutions were prepared in 1 M Tris–HCl at pH 7.5. When a gradual loss of activity for exceedingly dilute solutions (below 0.1 mg/ml) was observed in the presence of the buffer, bovine serum albumin (BSA) was used for these dilutions at the final concentration of 0.5 mg/ml, which stabilized successfully the enzymatic activity. The hydrolysis at room temperature of *N*-acetyl-phenylalanyl-L-3-thiophenylalanine was monitored at 412 nm using a spectrophotometer. The solution (1 ml) as prepared for the cuvette contained 0.5 M Tris–HCl at pH 7.5, 0.5 mM of Ellman's; and CPA was added to initiate the reaction. The absorbance was read out at 60 s.

When analyzing the foamate, *N*-acetyl-L-phenylalanyl-L-3-thiophenylalanine was used at the final concentration of 0.4 mM and other reactants at the concentrations stated above. The buffer, NaCl, and the foamate (0.1 ml) were mixed and placed in a cuvette. The blank cuvette only contained NaCl and the buffer. Ellman's reagent was added to both cuvettes, and absorbencies were readout at 60-s intervals until no further changes were observed. *N*-acetyl-L-phenylalanyl-L-3-thiophenylalanine was added to both cuvettes, and absorbencies were read out at 5-min intervals. For the ABS experiments, 1.47 ml of the CPA solution (71 U/mg) was diluted with 10% of HCl up to 14.7 ml, and 3 ml of this solution was applied for the foaming experiments.

Results and Discussion

Chelation and Dechelation of MMP-9

The enzyme investigated first was MMP-9. Before the foaming trials, it needed to be ensured that the process of chelation and dechelation is working by measurements of the

enzymatic activity. For this, ADA-C8 was added for chelating MMP-9 to cause a decrease of enzymatic activity, which in turn serves as a measure for the extent of chelation. With increasing concentrations of ADA-C8, the initial activity of MMP-9 decreased linearly as can be seen from Fig. 3. The enzyme was inactivated after 50 mg/l of ADA-C8 was added at pH 7.0, which indicates that the enzyme had been completely chelated. At lower pH (pH 5), lesser amounts of the chelator were required for inactivation (only 2 mg/l), which can be ascribed to the increased solubility of the chelate. In order to use the observed chelation characteristic of the enzyme for foam fractionation, it needed to be ensured that the chelation would become reversible so that the enzymatic activity was fully regained. The dechelation of the MMP-9 and ADA-C8 complex was achieved by the addition of zinc(II) ions or by lowering the pH down to 3.5.

Tweezing Adsorptive Bubble Separation of MMP-9

Initial experiments of foam fractionation revealed no enrichment of MMP-9 without a chelator due to the lack of the enzyme to form foam. This normally suggests the application of a surfactant in order to increase the foaming capacity of an enzyme [26]. Previous experience however revealed that, with using ADA alone or synthesized together with an octyl part, the enzyme's foaming capacity is enhanced due to a more unpolar characteristic [16]. Therefore, the ABS experiments were designed so that one process served as a standard for comparison, using a solution containing the enzyme only chelated with ADA (chelate #1), then three solutions using the enzyme in complex with ADA-C8 to observe the tweezing efficiency (chelates #2–#4), and another three solutions using a complex of zinc (II)-ADA-C8 in order to verify that indeed the catalytic center of MMP-9 is involved during the chelation (Fig. 4). The trials were performed at those pH values, where the best chelation properties could be expected. According to the chelation/dechelation experiments, the pH values of 5, 6, and 7 were chosen. Compared to the standard process, applying only ADA as chelator (#1), the enrichment ratio of #4 increased to a maximum of 11.1 with 87.3% recovery at pH 7.

One reason for this significantly higher enrichment can be credited to the increased surface activity of the enzyme ADA-C8 chelate. The octyl part here acts as a surfactant that lowers the polarity of the complex and thus improves the transfer into the foam fraction. Furthermore, it can be assumed that ADA-C8, because of its higher lipophilicity, interacts more efficiently with the enzyme than ADA alone, which is much more polar. For the chelates #2 and #3 at pH 5 and 6, respectively, significant lesser efficiency values were obtained compared to pH 7, which can be ascribed to the lower binding affinity of ADA-C8

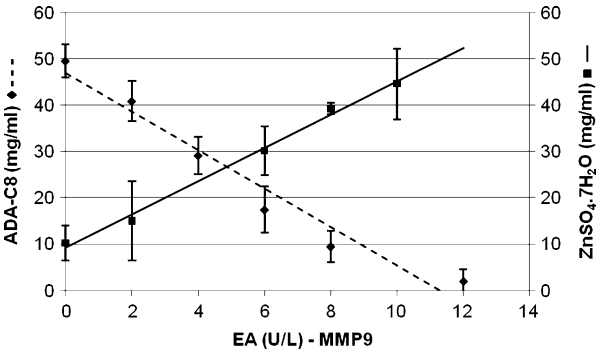

Fig. 3 Enzymatic activity of MMP-9 vs. the concentration of ADA-C8, added for chelation at pH 7 (*diamonds in broken line*), and vs. the concentration of $ZnSO_4.7H_2O$ (*squares in solid line*), added for dechelation at pH 10

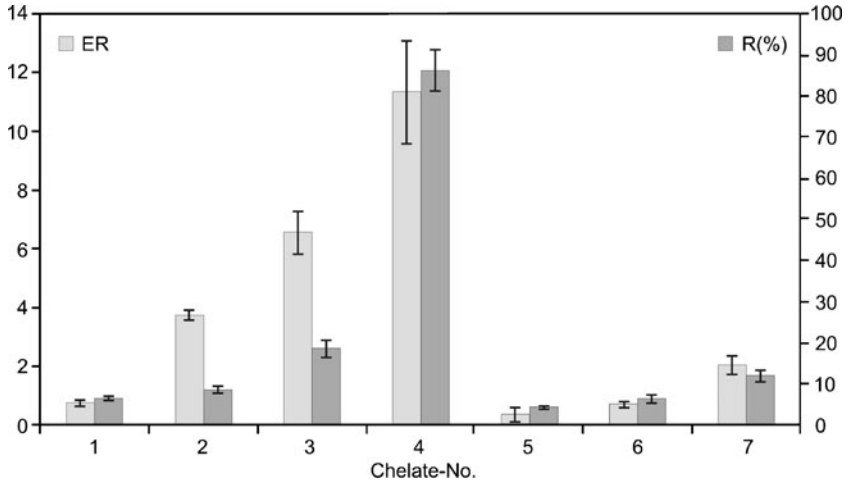

Fig. 4 Enrichment ratios (*ER*) and recovery rates (*R*) of MMP-9 in complex with ADA (#1 at pH 7), ADA-C8 (#2 to #4; at pH 5, 6, and 7, respectively), and zinc(II)-ADA-C8 complexes (#5 to #7; at pH 5, 6, and 7, respectively)

towards the enzyme at these two lower pH values. Gerken et al. [16] made the same observation when applying TABS for the enrichment of laccase C and horseradish peroxidase. Both enzymes were enriched with highest efficiency values at the pH where complete inactivation was achieved.

To confirm that indeed the active zinc(II) ion in the center of MMP-9 is responsible for the chelation and thus enrichment via TABS, a bound complex of zinc(II) and ADA-C8 was used for foam fractionation at different pH values. Here, only an enrichment factor of 2.2 and a recovery of 12.2% were obtained at pH 7 (Fig. 4, #7), which points out that ADA-C8 couples entirely with MMP-9 to form an easily foamable complex. As, potentially, also the calcium ions in MMP-9 can become involved in the chelation with ADA-C8, calcium ions were added to the foam fractions after the experiments, and the enzymatic activity was measured. As only negligible activity values were determined, it can be assumed that the addition of zinc(II) completely induced the dechelation process.

Chelation and Dechelation of Carboxypeptidase A

To substantiate the findings made with MMP-9, carboxypeptidase A, an enzyme showing also an active zinc(II) in the center, was applied next for TABS. The enzyme was likewise inactivated by the addition of ADA-C8 (Fig. 5). With increased concentrations, the initial activity of the enzyme decreased linearly and reached complete inactivation at 101 mg/l (also at pH 7). Analogous to MMP-9, it can be assumed that carboxypeptidase A was entirely chelated by ADA-C8. At lower pH values, down to 6.0 or 5.0, the amount of ADA-C8 required for inactivation remained the same. Dechelation in turn was again accomplished by the addition of zinc(II) or by lowering the pH down to 3.5.

Tweezing Adsorptive Bubble Separation of Carboxypeptidase A

Likewise to MMP-9, an enrichment of CPA without a chelator could not be achieved. In analogy to the previous experiments, the ABS trials were performed at pH 5, 6, and 7. In

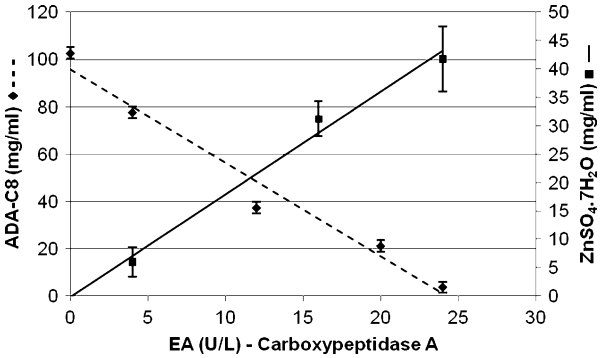

Fig. 5 Enzymatic activity of carboxypeptidase A vs. concentration of ADA-C8, added for chelation at pH 7 (*diamonds in broken line*), and vs. the concentration of $ZnSO_4 \cdot 7H_2O$, added for dechelation at pH 10 (*squares in solid line*)

contrast to the enrichment of #1, applying the standard process with only ADA as chelator, the highest enrichment factor of 18.8 was achieved for chelate #4 at pH 7, along with a nearly quantitative recovery of 95.3% (Fig. 6) and, furthermore, without a significant loss of enzymatic activity. Analogous to MMP-9, a strong interaction between ADA-C8 and CPA by its active zinc(II) in the center can be assumed. The experiments with zinc(II)-ADA-C8 chelates confirmed that it was not possible to enrich CPA under these conditions. Only an enrichment factor of approximately 0.2 along with 5.5% recovery was determined for chelate #7, pointing out that CPA forms indeed a complex with ADA-C8, which very efficiently can be transferred into the foam phase.

Postulated Structure of the Enzyme Chelates

Matrix metalloproteinases are multidimensional proteins possessing a rather complex structure. The MMP-9 as a gelatinase comprises the propeptide N-terminal domain with its cysteine-switch motif and the hemopexin C-terminal domain which bridges towards the catalytic domain. Another characteristic of the gelatinase is that the catalytic center contains the fibronectin domain [27, 28]. The active zinc(II) atom is coordinated by a catalytic water molecule (activated by Glu402) and by the histidine side chains His405 and His401 that

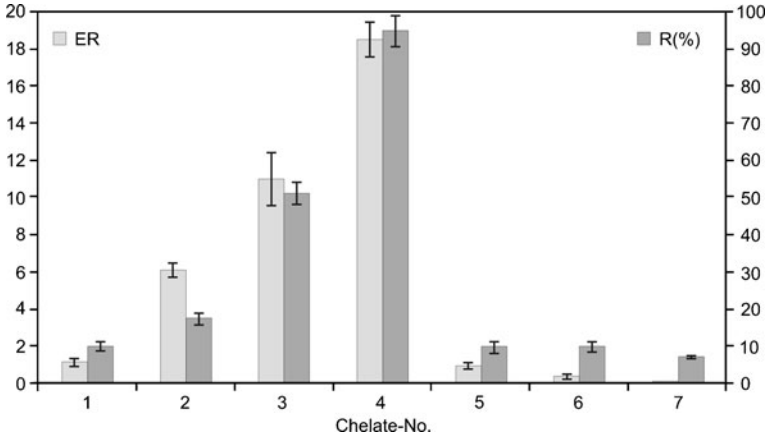

Fig. 6 Enrichment ratios (*ER*) and recovery rates (*R*) of carboxypeptidase A in complex with ADA (#1 at pH 7), ADA-C8 (#2 to #4; at pH 5, 6, and 7, respectively), and zinc(II)-ADA-C8 (#5 to #7; at pH 5, 6, and 7, respectively)

reach from the αB-helix into the center. Another coordinator is the residue His411, which turns from the sequence Gly408 to Ser412 into the active site. Besides catalytic ion, a structural zinc ion is also contained in the active center, as well as in total five calcium ions, which however have not been reported to play a role in biologically relevant processes [28]. Another important characteristic of MMPs, and thus also of MMP-9, is the hydrophobic S1′-pocket which binds P1′-substrates and is partially composed as a wall by the segments Pro421-Tyr423.

There have been many studies so far about the structure of MMPs and their inhibitors, mainly for the purpose of targeting diseases by specifically designed inhibitors. Rowsell et al. [29] studied the crystal structure of pharmaceutically useful reverse hydroxamate inhibitors in complex with MMP-9. The authors found that the coordination of the catalytic zinc by the histidine side chains was completed by the oxygen atoms of the inhibitor. Tochowicz et al. [30] followed the same prospects and X-ray studied the complexation of five novel inhibitors with a truncated MMP-9 active site that lacked the fibronectin and hemopexin domains. For all compounds, the active site of MMP-9 was involved by a compromise between active zinc coordination, favorable hydrogen bonding, or a flexible accommodation of hydrophobic P1′ groups in the flexible S1′ cavity of the enzyme.

Regarding the structure of MMP-9 and ADA-C8, due to the high reactivity of ADA-C8 towards zinc(II) and because of the activity reduction trials that demonstrated the blockage of zinc(II) by ADA-C8, we postulate that indeed the catalytic zinc ion is the most suitable link (Fig. 7), as the structural zinc ion, with its three histidine side chains (His175, His190, and His203) at the β-strand in the neighborhood and Asp177 as the fourth ligand, is well protected against any additional ligands such as ADA-C8. As a possible binding mode, one carboxylate of the chelator connects to the active zinc ion by hydrogen bonds while the oxyl and primary N-group at the backbone side may find coordinations to, e.g., Leu188 and Pro421, respectively, in the S1′ pocket of the enzyme. A likewise inhibition mode was observed from Rowsell et al. [29] when studying the structure of MMP-9 and reverse hydroxamate as inhibitor. The NO_2 moiety bonded with zinc and the backbone nitrogen and oxygen atoms structured with these amino acids located in the S1′ cavity of MMP-9.

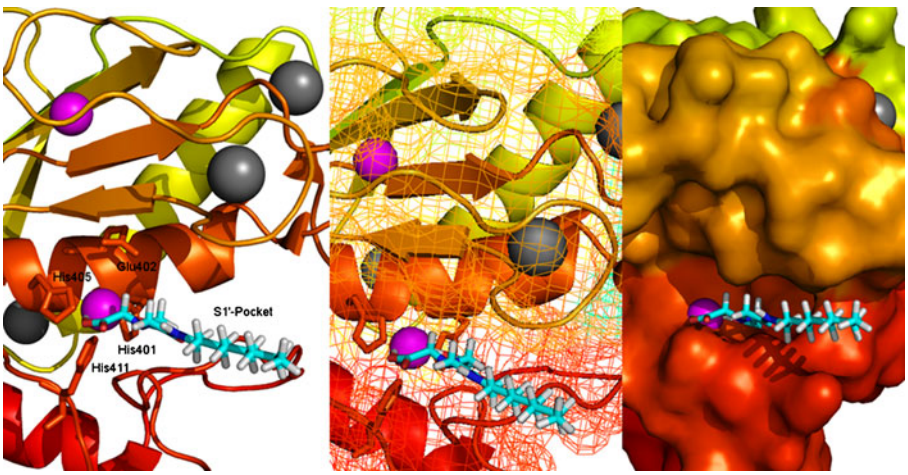

Fig. 7 Postulated structure of the active site of MMP-9 and ADA-C8 as inhibitor (*stick representation*). Active and structural zinc ions are indicated in *magenta* and calcium ions in *gray*. Created with PyMOL software, DeLano Scientific LLC., based on the pdb-file 1GKC

The catalytic center of carboxypeptidase A is composed of the active zinc ion which is tightly coordinated by three amino acid side chains (His196, His69, and Glu72) and a water molecule. Other residues that may play an important role in the catalytic mechanism are Arg127, Arg145, Tyr248, and Glu270 [31]. In particular, the guanidinium moiety of Arg145 and the carboxylate of Glu270, with their location near the surface of the enzyme, are noteworthy as taking part in inhibitor actions. In the core of the enzyme, there is also the round-shaped primary recognition pocket located, which mainly accommodates aromatic moieties such as phenyl rings. It is clear that CPA hydrolyses amino acids from polypeptide substrates at their C-terminal [1, 32]. The hydrolysis pathway as debated may either follow an attack of the carboxylate of Glu270 or an attack of the water molecule provided by the zinc ion in collaboration with the Glu270 carboxyl group [31, 32]. Besides generating a hydroxyl group that attacks the carbonyl carbon of the peptide bond, the zinc ion functions as a stabilizer of the transition state that is caused thereby. Other possible binding sites can be the Arg145 moiety by forming hydrogen bonds with the terminal carboxylate of the substrate or the primary substrate recognition pocket (S1′ pocket) [33].

It is assumed, due to the complete inactivation of the enzyme as determined before, that indeed one carboxy group of ADA-C8 bridges to the active zinc ion by oxygen binding and that the other C-terminal coordinates to either one of the histidine side chains Arg127, Arg 71, and Glu270 (Fig. 8). These ligands are closely located to the active zinc ion near the surface and thus are as easily accessible. A similar binding mode was reported by Cho et al. [34] who investigated the inhibition of CPA by an enantiomeric pair of N-(hydroxyaminocarbonyl)phenylalanine. The authors found that the L-enantiomer was bound to the zinc atom by coordination with its carbonyl oxygen and additionally formed a hydrogen bond with the carboxylate of Glu270. Both enantiomers were bound to CPA with their phenyl rings at the substrate recognition pocket and showed hydrogen bonds of the carboxylate towards the Arg147 and Arg127 moieties.

It can further be assumed as a possible binding mode that the octyl part of ADA-C8 connects to the primary recognition pocket of carboxypeptidase A and/or that the NH-fragment of ADA-C8 is coordinated by the Tyr248 moiety. These interactions would contribute to the stability of the complex, which has been demonstrated also by Kim and Libscomb [35] who studied a more complex but stable phosphonate inhibitor, consisting of

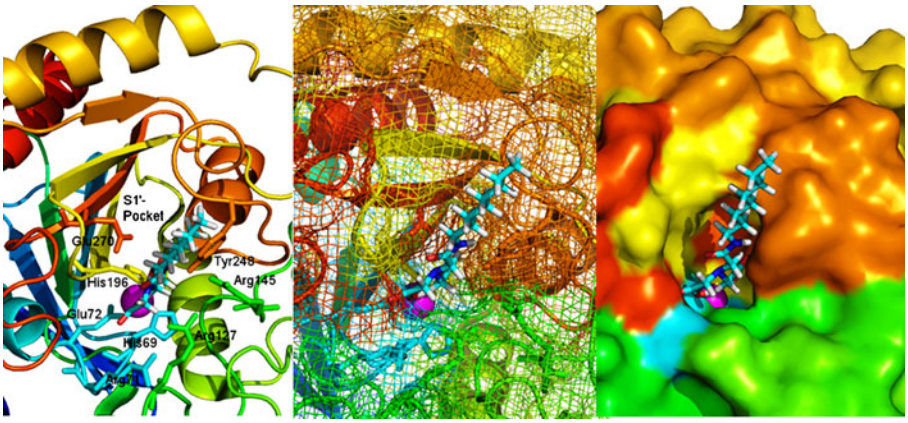

Fig. 8 Postulated structure of carboxypeptidase A with its catalytic zinc ion (*magenta*) and ADA-C8 as inhibitor (*stick representation*). Created with PyMOL software, DeLano Scientific LLC., based on the pdb-file 6CPA

benzyloxycarbonyl, alanine, alanine P, and (0)phenylalanine (OPL). While the phenyl ring of the OPL part occupied the hydrophobic pocket, the C-terminal formed hydrogen bonds to the moieties Asn144, Arg145, Tyr248, and Arg127. The phosphinyl group coordinated the zinc atom by oxygen bonding. From the amino to the carboxyl end (electrophilic side of the zinc sphere), one phosphinyl oxygen bound the Arg127 moiety and another one the Glu270 moiety.

Conclusion

TABS, which is based on the selective chelation of metalloenzymes in order to achieve higher efficiency values of the process, increased significantly the enrichment of the investigated MMP-9 and carboxypeptidase A metalloproteinases. Future investigation on TABS with respect to MMP-9 or CPA enrichment should be performed in regards to the possibility of introducing TABS in method designs that are currently applied for purifying these kinds of metalloenzymes.

Acknowledgement Financial support by the German Federation of Industrial Research Associations "Otto von Guericke" (AiF) through the Research Association of the German Food Industry (FEI) is gratefully acknowledged (project 121 ZN).

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