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Inhibition properties of free and conjugated leupeptin analogues

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Running title: Conjugation of leupeptin analogues

Abbreviations: Ahx; 6-aminohexanoic acid, MS:mass spectrometry, SPPS: solid phase peptide synthesis, APTES: aminopropyltriethoxysilane, CDI: carbonyldiimidazole

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Tight binding; Inhibitor design

Enzymes: Trypsin IEC 3.4.21.4, Chymotrypsin IEC 3.4.21.1

Conflict of interest: None

ABSTRACT

Leupeptin is a naturally occurring inhibitor of various proteases, in particular serine proteases. Following its discovery, the inhibitory properties of several other peptidyl argininals have been studied. The specificity of leupeptin is most likely due to the Leu-Leu-Argininal sequence, and its C-terminal aldehyde group has been suggested to enhance the binding efficiency and to be essential for function. The terminal aldehyde group makes the structure less vulnerable to carboxypeptidases. Here, we investigated whether the inhibitory function of leupeptin towards serine proteases is retained after oxidation or reduction of the aldehyde group. The oxidized form, which corresponds to the natural precursor, was shown to be superior to the reduced form in terms of inhibitory properties. However, the original leupeptin possessed enhanced inhibitory properties as compared to the oxidized form.

Based on these results, new synthetic leupeptin analogues, Ahx-Phe-Leu-Arg-COOH and Ahx-Leu-Leu-Arg-COOH, were prepared by solid-phase peptide synthesis using the Fmoc strategy. In these analogues, the N-terminal capping acetyl group was replaced with a 6-aminohexanoyl group to allow conjugation. The structures of the modified leupeptin and the synthetic peptides were confirmed by mass spectrometry (MS).

Determination of the inhibitory properties against trypsin revealed that these further modified tripeptides were tight binding inhibitors to their target enzyme, similar to the natural occurring leupeptin, with K_i values generally in the micromolar range. The Ahx-Phe-Leu-Arg-COOH analogue was selected for conjugation to inorganic oxide nanoparticles and agarose gel beads.

All conjugates exhibited inhibitory activity in the same range as for the free peptides.

INTRODUCTION

Leupeptin (figure 1) is known to be a natural occurring efficient, tight binding inhibitor to various proteases, in particular serine proteases.¹⁻⁸ Leupeptin is produced by various species of actinomycetes and was also confirmed in a number of other families. The production is therefore not species-specific.⁹ Since the discovery of leupeptin, several additional peptidyl argininals have been subject to interest due to the strong and specific inhibition of serine proteases.¹⁰ The specificity of leupeptin is most likely due to the Leu-Leu-Argininal sequence and the C-terminus aldehyde function is suggested to enhance the binding efficiency and to be essential for the function.^{1,11,12} It is also likely that the terminal aldehyde group will make the structure less vulnerable to carboxypeptidases. Aoyagi reported² that the reduction or oxidation of the aldehyde group to alcohol or carboxylate, respectively, strongly impaired the inhibition of plasmin and papain, to the extent that no exact numerical data were given. Our aim here was to investigate whether the inhibitory function towards serine proteases is retained also after oxidation or reduction of the aldehyde group. The biosynthesis of leupeptin does indeed occur via a synthesis of the peptide with a C-terminal carboxyl group, followed by a reduction to aldehyde catalyzed by the enzyme Leupeptin acid reductase with NADH as electron donor and thermodynamically supported by ATP-hydrolysis.¹³ In analogy with that the chemical synthesis of leupeptin is thus more complicated than standard peptide synthesis due to its L-argininal moiety. Many analogues to leupeptin have nevertheless been synthesized giving a broad view of structure-function relations for the inhibition of proteases.^{12,14,15}

Here we are using solid-phase peptide synthesis (SPPS) to synthesize two new leupeptin analogues with an -COOH at the C-terminus instead of an aldehyde, similar to the biosynthetic precursor. Staying at the precursor stage does thus allow a straightforward solid-phase peptide synthesis. One option to synthesize leupeptin to its natural state could actually be based on an enzymatic reduction after the solid-phase peptide synthesis of the oxidized form. As of today, no enzyme that catalyzes the reduction of the C-terminal carboxyl group of the precursor to aldehyde in *Streptomyces* is commercially available.¹³ The N-terminal acetyl group was in these tripeptide analogues of leupeptin replaced with 6-aminohexanoyl group that allows an easy path to conjugation while retaining inhibitory activity. The nucleophilic character of amino groups is frequently used in the conjugation of peptides, amino acids and proteins and a large number of activations of conjugation carriers have been developed.¹⁴⁻¹⁶ The gel support employed here was the bromo activated Workbeads® matrix, where the conjugation results in a secondary amine. For conjugation to inorganic oxide phases, an initial silanisation is often used to introduce suitable functional groups. In this case we have chosen to modify the oxide surfaces with APTES (aminopropyltriethoxysilane), followed by activation of the amino groups by CDI (carbonyldiimidazole). The final result will be a coupling of the peptides by a substituted urea structure. Since the peptides only carry a single primary amino group, the

conjugation is in all cases expected to be uniform and, furthermore, leaving the important arginine accessible.

RESULTS & DISCUSSION

Oxidation and reduction of leupeptin

The molecular weight of oxidized and reduced state of leupeptin was determined by MS analysis and can be seen in figure 2 where the oxidized state shows a peak at 443 m/z and the reduced state shows a peak at 429 m/z with $z=1$ for both peptides. This is in expected accordance with the peak at 427 m/z for natural leupeptin (table 1). Full m/z-spectra can be found in supporting information figure S1a and S1b.

Inhibitor binding mode

The original natural leupeptin interacts very strongly, but reversibly with trypsin. The backbone of leupeptin forms four hydrogen bonds with trypsin and a fifth hydrogen bond interaction is mediated by a water molecule. The aldehyde carbonyl of leupeptin is shown to form a hemiacetal bond with the side chain oxygen of Ser195 in the active site, in which the hemiacetal oxygen atom is pointing out of the oxyanion hole and forms a hydrogen bond with His57.¹⁷ Radinsky¹⁸ modeled both orientations of the hemiacetal and found that the one where the oxygen atom is facing the oxyanion hole had 15% occupancy whereas the one where oxygen faces the active site His57 had 85% occupancy.

The weaker inhibition by both the oxidized and reduced state of leupeptin can be explained by the loss of the hemiacetal bond possibility in the active site to Ser 195. Tentative binding modes for original and modified leupeptin are shown in figure 3. The stronger inhibition by the oxidized form compared to the reduced form can be explained by the interaction of the negative oxygen in the oxyanion hole, but without the hemiacetal formation. The reduced state of leupeptin does also lack the possibility to form a hemiacetal but can still interact through a hydrogen bond to His57. This could be the reason for the pronounced difference between an apparent K_i of 2.69 μM for the oxidized form and 270 μM for the reduced form (table 1). Based on these data, we decided to synthesize analogues with terminal carboxyl group.

Synthesis of Ahx-Phe-Leu-COOH & Ahx-Leu-Leu-COOH

The synthetic pathway of a serine protease inhibitor of leupeptin type is a challenge due to the argininal residue at the C-terminus of their sequence combined with a low yield as an outcome.¹⁹ The standard arginine peptide, on the contrary, requires only straightforward peptide synthesis and is still a powerful inhibitor. It should also be noted that the biosynthesis proceeds via synthesis of the peptide, followed by a specific enzyme mediated reduction of the terminal carboxylate group to aldehyde.¹³ Conclusively,

chemical synthesis of a precursor peptide combined with a final enzymatic reduction could actually be the preferred strategy for production of leupeptin analogues with a terminal aldehyde group.

Purification and characterization of Ahx-Phe-Leu-Arg-COOH & Ahx-Leu-Leu-Arg-COOH

The two peptides, Ahx-Phe-Leu-Arg-COOH and Ahx-Leu-Leu-Arg-COOH, were purified with preparative HPLC. The yields of the peptides were 36% and 74% respectively, based on the capacity of the resin. The m/z relation were 514 with $z = 1$ for Ahx-Leu-Leu-Arg-COOH and 548.4 with $z = 1$ for Ahx-Phe-Leu-Arg-COOH by LC-MS, which confirmed the molecular masses of the peptides to 513 Da for Ahx-Leu-Leu-Arg-COOH and to 547 Da for Ahx-Phe-Leu-Arg-COOH.

Kinetic measurements of Ahx-Phe-Leu-Arg-COOH & Ahx-Leu-Leu-Arg-COOH

The apparent K_i value of Ahx-Leu-Leu-Arg-COOH (9.48 μM) and Ahx-Phe-Leu-Arg-COOH (3.42 μM) lie in the same micromolar range as oxidized leupeptin (table 1 & 3), confirming that the replacement of the N-terminal capping acetyl group with AHX only has a minor influence. The replacement of one leucine by phenylalanine does, furthermore, lower the K_i value by a factor 3. This is probably due to the fact that phenylalanine is more hydrophobic than leucine and interacts more strongly in the active site of the enzyme. Both the synthesized peptides act as a tight binding inhibitor as can be seen in figure 4. Even though the hemiacetal function is lost by synthesizing them with a carboxylic acid instead of the functional aldehyde, these peptides still show significant inhibition properties.

Conjugated Ahx-Phe-Leu-Arg-COOH

Conjugation of Ahx-Phe-Leu-Arg-COOH

Ahx-Phe-Leu-Arg-COOH was conjugated to three different carriers where the conjugated amount was determined by subtractive absorbance measurements and can be seen in table 2.

Kinetic measurements of conjugated Ahx-Phe-Leu-Arg-COOH

Since the synthetic analogues display the same inhibition mechanisms as the natural leupeptin (tight binding) the apparent K_i values (table 3) were determined by equation 1 and 2. The corresponding graphs can be seen below (figure 5). For details regarding TiO_2 -peptide kinetics, see supporting information figure S2, table S1.

The increase in apparent K_i can be ascribed to sterical inaccessibility due to uneven surface in all three carriers. The peptides can also be conjugated close to each other making the space for several enzyme molecules less accessible. Regarding the gel beads the peptides can be hidden inside the beads which will exclude a number of peptides that are conjugated to those positions and thereby not accessible for the enzymes. The crosslinking that was observed for conjugated protein inhibitors²⁰ is not possible for this peptide since it only has one easily reacting group for the conjugation to take place which, furthermore, is placed opposite to the functional group that is crucial for the inhibition. If the peptide is conjugated in a way that is not sterically inaccessible the inhibition will not be affected by the conjugation. The calculations regarding the efficiency are based on the free Ahx-Phe-Leu-Arg-COOH as a reference, since that is the peptide that is conjugated to all three carriers. The efficiency is easiest regarded as the fraction of inhibitor molecules that are functionally active, rather than a change of intrinsic molecular properties²⁰ and the loss of efficiency can primarily be ascribed sterical inaccessibility. The efficiency is thus calculated from the apparent K_i observed as $\frac{K_{i,free}}{K_{i,app}} = \frac{[I]_{eff}}{[I]_{tot}}$. The conjugation of the peptide is performed not to increase the stability but to merely provide a carrier to the small peptide. This may serve both to remove the inhibitor in a controlled way from a technical sample but also to minimize the risk of the peptide crossing for example the skin barrier if used in protective formulas. As can be seen, in regard to the efficiency, the conjugation of Ahx-Phe-Leu-Arg-COOH to ZnO gives an efficiency of 0.77 which is close to the reference value of 1. The conjugation itself does not affect the peptides inhibition properties. The lower efficiency of the peptide conjugated to agarose gel beads could be explained by the steric inaccessibility for the enzyme, due to the possibility of the peptide hidden inside the gel beads. According to the efficiency about 10% of the conjugated peptides are available for the enzyme.

Inhibition activity analysis of free and conjugate peptide in gelatin layer

The gelatin erosion method that simulates a surface with a protective cover layer is here used to evaluate the function of the peptide in its different conjugation states where the relative area increase rate is a plotted

against the increasing inhibitor concentration in figure 6. The curves obtained as a function of concentration for the free peptide and the TiO₂-conjugated peptide, respectively, are virtually identical. The data obtained for the peptide-ZnO conjugate, on the other hand, result in a notably steeper slope at lower inhibitor concentration, suggesting a stronger binding. This may tentatively be explained by a model where the enzyme molecules, once “captured” by the immobilized peptides, also interact nonspecifically with the ZnO-surface, resulting in addition of binding energies and thus also cooperative binding. As has been noted earlier by us²¹ the well area converges to an asymptotic nonzero value “plateau”, at high inhibitor concentration that is virtually identical for the different cases. The limit may actually be set by the size of the droplet applied and initial enzyme diffusion. However, the similar limiting values further confirm that the inhibition modes are identical for free and conjugated inhibitor. The control experiments carried out with non-conjugated oxide particles resulted in plateau values close to unity, compared to the value of 0.42-0.50 for the inhibitor formulations (table 4). The plateau value of ZnO-particles decreases, however, to 0.86 suggesting a certain inhibitory effect by the oxide itself. This is actually in accordance with the pattern observed for the ZnO conjugate. Taken together, the patterns, for both slope and plateau, confirm that the conjugation does not influence the peptide function. For details regarding the gelatin erosion method, see supporting information figure S3, table S2.

CONCLUSIONS

After background experiments where the aldehyde group of leupeptin was converted to carboxylate or alcohol group, respectively, we could design two new leupeptin analogue protease inhibitors by the use of solid-phase peptide synthesis. This design of peptides, using SPPS, is useful in order to create smaller peptides with inhibitory effect in a straightforward and easy synthesis. The two new peptides had a new functional group, -COOH, instead of the original aldehyde group of the natural state leupeptin. The N-terminal acetyl group was also replaced by the commonly used conjugation spacer Ahx as an extra handle in the other end of the peptide for an easier conjugation path. With these modifications the peptides gave an apparent K_i value in the micromolar range. The conjugation of Ahx-Phe-Leu-Arg-COOH to inorganic particles and agarose gel beads gave a retained apparent K_i value in the micromolar range for all conjugates. The conclusions from these data are, first, that the leupeptin analogs still are functional inhibitors, with a more retained function for the oxidized form. Furthermore, the replacement of the original N-terminal acetyl group with Ahx did not have a large impact on the inhibitory properties but allowed well defined and sterically favorable conjugation of the peptide. The data further show that the properties are retained when the peptide is immobilized to a soluble or particulate carrier. It is possible that the peptide efficiency may be further improved by application of the natural enzyme aided maturation reaction of natural leupeptin to generate the aldehyde function. As a consequence, and in a more general context,, peptide inhibitors that are designed for conjugation may facilitate the use of their inhibitory

properties both in medical formulations and as enzyme scavengers in biotechnical applications. In the latter case it allows easy removal of conjugated inhibitors from the sample by filtering or centrifugation. The highly developed technology for peptide synthesis does also allow both rational and virtually unlimited combinatorial approach far beyond canonical amino acids for the design of inhibitors.

MATERIALS & METHODS

Materials

Leupeptin (L2884), AgNO₃, sodium borohydride, N α -Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPA), trypsin from porcine pancreas type IX-S, titanium(IV) oxide, zinc oxide, 1,1'-carbonyldiimidazole (CDI), triethylamine, (3-aminopropyl)triethoxysilane (APTES), dimethylsulfoxide (DMSO), Acetonitrile (ACN), formic acid (FA), acetic acid, O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HCTU), N,N-diisopropylethylamine (DIPEA), dichloromethane (DCM), piperidine, acetic anhydride, trifluoroacetic acid (TFA), triisopropylsilane (TIS), Fmoc-Leu-OH, Fmoc-Phe-OH and Boc-Ahx-OH were all purchased from Sigma-Aldrich. WorkBeads™ 40/1000 ACT was a kind gift from Bio-Works Sweden AB, Uppsala.

Methods

Oxidation of leupeptin using Tollen's test and confirmation of reaction with mass spectrometry (MS)

0.25 gram of AgNO₃ was dissolved in 15 mL milliQ H₂O. 0.5 mL 1M NaOH was added creating a brown solution, which precipitated. The addition of 0.5 mL of 25% NH₃ (\approx 14M) resulted in a clear solution containing Ag(NH₃)₂⁺. The function of the solution was confirmed by reaction with benzaldehyde. 100 μ L of 5.3 mM leupeptin was mixed with 0.5 mL Ag(NH₃)₂⁺ incubated in water bath (70°) for 5 min. The oxidized inhibitor (figure 7) was desalted by chromatography on ISOLUTE® SPE C18. Peptides (625 nM) were dissolved in 50% ACN 0.1% FA and directly infused by a syringe pump (Harvard apparatus, Holliston, MA, USA) at a flow rate of 4 μ L/min. Infused peptides were ionized by electrospray using an Ion Max Source (ThermoFisher Scientific, Bremen, Germany). Peptide mass spectra were recorded over a period of 5 min, resulting in >570 spectra per peptide with a LTQ Orbitrap Velos Pro (ThermoFisher Scientific) in the m/z 150-2000 range using the Orbitrap (Fourier transform) analyzer set to 30 000 resolution. The ionization potential was +4.2 kV, no sheath gas flow was used and the inlet capillary temperature was set to 300° C. Instrument calibration was carried out according to standard operating

procedures using Pierce™ LTQ Velos ESI Positive Ion Calibration Solution (Thermo Fisher Scientific) to assure high mass accuracy.

Reduction of leupeptin and confirmation of reaction with MS

A 100-fold molar excess of NaBH₄ was added to a solution of 0.53 μM leupeptin dissolved in milliQ-H₂O. The reduction (figure 8) took place over a time period of 2 hours, followed by desalting with SPE C-18 and was confirmed with MS-detection using the same protocol as in “Oxidation of leupeptin”.

Synthesis of Ahx-Phe-Leu-Arg-COOH & Ahx-Leu-Leu-Arg-COOH

Solid-phase peptide synthesis & purification

The peptides were synthesized using Fmoc chemistry on a 50 μmol scale, starting from Fmoc-Arg(Pbf) Wang resin (70 mg, 0.7 mmol/g). Deprotection of the Fmoc groups was carried out using 20% piperidine in dimethyl formamide (DMF). Amino acid couplings were performed in DMF using amino acid:HCTU:DIPEA (5:4:10), with coupling times of 30 min to 1h. Peptides were quantified using Kaisers test.²² Total deprotection and simultaneous cleavage from the solid support was achieved with a mix of TFA/TIS/H₂O (95/2.5/2.5%, v/v, 5 mL) for 1.5 h with agitation. After filtration and evaporation of TFA by N₂(g) bubbling, the peptides were precipitated and washed once with cold diethyl ether (Et₂O), followed by lyophilization. For details see supporting information.

The crude peptide was purified with reversed phase HPLC (Varian 940-LC) using a semi preparative Grace Vydac C8-column (22 mm x 150 mm, 10 μm, 300 Å) with a gradient of ACN (acetonitrile):H₂O, containing 0.1% TFA, from 5% (2 min) to 40% ACN in 20 minutes at a flow rate of 15 mL/min. UV detection was done at 220 nm. The peptide identities were confirmed by LC-MS using an instrument constellation consisting of a Waters 2700 sample manager, an Agilent 1100 series HPLC/UV-VIS diode-array detector, and Waters Micromass ZQ mass detector in negative ion mode. The measured m/z values (546.3 and 512.3, respectively) were in accordance with the calculated masses (figure 4). Fractions containing the desired peptides (figure 9) were pooled, evaporated, freeze-dried and stored at -20 °C until further use.

Conjugation of Ahx-Phe-Leu-COOH

Inorganic particle carriers

All derivatization and immobilization steps were performed at room temperature and in plastic Falcon tubes or 2 mL plastic Eppendorf tubes. 0.5 grams of the inorganic particulate carriers (TiO₂ & ZnO) was first silanized using 100 mM APTES in 10 mL ACN while stirring for 24 hours. The particles were then centrifuged followed by removal of ACN, washed 3 times with EtOH, one final time with acetone and then dried at 65 °C for 5 hours. Following that, the particles were activated using 120 mg CDI and 0.72 mmol triethylamine in 5 mL ACN while stirring for 2 hours. The particles were then centrifuged followed by removal of ACN, washed 3 times with EtOH, one final time with acetone and then dried at 65 °C o/n. Conjugation of Ahx-Phe-Leu-Arg-COOH was carried out by stirring a controlled amount of peptide overnight with 50 mg activated TiO₂ or ZnO in 0.1M NH₄HCO₃ buffer pH 7.52. The particles were then centrifuged, and the absorbance of the supernatants was measured at 259 nm, allowing a subtractive quantification of conjugated peptide using the extinction coefficient of 195 M⁻¹cm⁻¹ for Phe residues²³. The conjugation was terminated by washing with 0.1M NH₄HCO₃ pH 7.52 and the conjugated particles (figure 10) were stored at 4 °C until further use.

Agarose gel beads

WorkBeads™ 40/1000 ACT from Bio-Works with an average particle size of 45 µm and reactive group content of 200 µmol/mL were used when coupling Ahx-Phe-Leu-Arg-COOH to agarose gel beads (resin). 1 mL resin was washed with deionized water on a glass filter and dried using suction. 0.5 mL 4.38 mM of peptide 1 in 0.1M NH₄HCO₃ pH 7.52 was then added to 0.5 mL dried resin. The slurry mixture was incubated at RT overnight while stirring. Following that, the slurry mixture was centrifuged, and absorbance was measured on the supernatant. The amount conjugated was determined as mentioned above in 'Inorganic particle carriers'. The immobilization was terminated by first washing the resin with 0.1M NH₄HCO₃ pH 7.52 and drying by suction followed by incubation of the resin in 1M ethanolamine-HCl pH 9.5 at RT overnight while stirring to block the remaining reactive groups. The blocking agent was finally removed by washing the gel beads as described above. The suspension of the conjugate (figure 11) was stored at 4°C in 0.1M NH₄HCO₃ pH 7.52 until further use.

Kinetics

Real time measurements

Kinetic parameters were obtained by measuring initial velocities in the presence of selected concentrations of natural leupeptin (0.14-1.62 µM), oxidized leupeptin (8-127 µM), reduced leupeptin (10-413 µM) and peptide 1 & 2 (8-254 µM). All reactions were performed in 0.1 M NH₄HCO₃ pH 7.52 and 2% (v/v) DMSO

using concentrations of BAPA and trypsin fixed at 1 mM and 0.25 μM , respectively. The reaction was monitored at 410 nm in a Shimadzu UV-1601 UV-vis spectrophotometer.

End point measurements

Kinetic parameters were obtained by mixing substrate, enzyme and conjugate in a plastic Eppendorf tube with varying concentrations of TiO_2 -peptide 1 0-70 μM , ZnO -peptide 1 0-60 μM and gel beads conjugated to peptide 1 0-60 μM and incubating with end-over-end rotation. 150 μL aliquots were withdrawn at 5 time points for each reaction and the reaction was stopped in 100 mM acetic acid pH 3. Absorbance was measured at 410 nm in a UV-1601 UV-vis spectrophotometer (Shimadzu).

For real time and end point measurements the K_i value was estimated by the graphical method of Dixon²⁴ and Morrison²⁵ (equation 1). K_i was extracted after fitting the data to equation 1 and equation 2 using GraphPad Prism 8. A K_M of 0.82 mM was used for the calculations.

$$\frac{v_i}{v_o} = 1 - \frac{([E] + [I] + K_i^{app}) - \sqrt{([E] + [I] + K_i^{app})^2 - 4[E][I]}}{2[E]} \quad (\text{Eq. 1})$$

The form of K_i^{app} varies with the type of inhibitor. For competitive inhibitors, as in the case of leupeptin, the K_i value can be calculated from equation number 2:

$$K_i^{app} = K_i \left(1 + \frac{[S]}{K_M}\right) \quad (\text{Eq. 2})$$

Inhibition activity analysis of free and conjugate peptide in gelatin layer

The experiments were performed as described earlier.²¹ The concentration of the free inhibitor Ahx-Phe-Leu-Arg-COOH was ranging from 5 to 50 μM , for ZnO -conjugate 1-15 μM and for TiO_2 -conjugate 1-25 μM .

AUTHOR CONTRIBUTION

GJ designed the project, served as senior and corresponding author, academic supervisor for EB.

EB shared the design planning, wrote the first draft of the manuscript, performed or participated in most experiment and participated in the final writing steps.

JV provided expertise in designing and leading the peptide synthesis work and participated in the writing of the manuscript.

SL planned and performed MS experiments and participated in the writing of the manuscript.

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Figure Legends

Figure 1. Leupeptin in its natural state (Ac-Leu-Leu-Arg-CHO)

Figure 2. (a) MS spectra of oxidized leupeptin with a peak at 443 m/z corresponding to the molecular ion [M+H], (b) MS spectra of reduced leupeptin with a peak at 429 m/z corresponding to the molecular ion [M+H] confirming the identity of both products.

Figure 3. Tentative comparison of active site binding mode for original leupeptin, reduced and oxidized leupeptin, respectively.

Figure 4. The ratio v_i/v_0 is plotted as a function of the inhibitor peptide Ahx-Phe-Leu-Arg-COOH to the left and Ahx-Leu-Leu-Arg-COOH to the right. The conditions for the experiments and calculations were $[Trypsin] = 0.25 \mu M$, $[BAPA] = 1 \text{ mM}$, $K_m = 0.82 \text{ mM}$. Error bars represent SD

Figure 5. v_i/v_0 is plotted as a function of the conjugated TiO_2 -Ahx-Phe-Leu-Arg-COOH (a), ZnO-Ahx-Phe-Leu-Arg-COOH (b) and WorkBeads™-Ahx-Phe-Leu-Arg-COOH (c). The conditions for the experiments and calculations were $[Trypsin] = 0.25 \mu M$, $[BAPA] = 1 \text{ mM}$, $K_m = 0.82 \text{ mM}$. Error bars represent SD

Figure 6. Relative area increase rate for the free peptide 1, conjugated peptide 1 and free particles.

Figure 7. The oxidation of leupeptin using Tollen's Test.

Figure 8. The reduction of leupeptin using sodium borohydride.

Figure 9. Structure of the two tripeptides.

Figure 10. Conjugate of Ahx-Phe-Leu-Arg-COOH to TiO_2 particles, where $R = (CH_2)_3NHC(=O)$ -Ahx-Phe-Leu-Arg-COOH.

Figure 11. Conjugate of agarose gel bead and peptide 1. The gel bead in comparison with the peptide is about 40 000 times larger and the gel bead can conjugate a number of peptides. This figure only demonstrates the conjugation between the bead and the peptide itself and the components are not in correct scale.

Supporting information

Supplement titles

Figure S1a. MS spectra of oxidized leupeptin.

Figure S1b. MS spectra of reduced leupeptin.

Figure S2. Kinetic measurements of TiO_2 -peptide conjugate.

Figure S3. Erosion well formation rate for the free Ahx-Phe-Leu-Arg-COOH and in its conjugated state.

Table S1. Extracted values from figure S2.

Table S2. The rates for the gel experiment for free Ahx-Phe-Leu-Arg-COOH and its conjugated state to TiO₂ and ZnO.

Data accessibility

For information about raw data, i.e. direct instrumental output, contact gunnar.johansson@kemi.uu.se

Table 1. Summary of the representative results from the reduction and oxidation of leupeptin, including the obtained kinetic parameters for the different state of the inhibitors.

	State	Functional group	K_i	m/z observed	z	M_w (Da)
Leupeptin	Natural	-CHO	88 ± 8 nM	427.3	+1	426.3
	Oxidized	-COOH	2.7 ± 0.1 μ M	443.3	+1	442.3
	Reduced	-CH ₂ OH	$270 \pm$ 100 μ M	429.3	+1	428.3

Table 2. Summarizes the amount of Ahx-Phe-Leu-Arg-COOH immobilized onto the chosen carriers.

Alternative	Carriers	Conjugated amount of Ahx-Phe-Leu-Arg-COOH
Inorganic particles	TiO ₂	6.1 nmol/mg particle
	ZnO	4.4 nmol/mg particle
Gel beads	WorkBeads™	150 nmol/ml beads

Table 3. Summary of the apparent K_i values from the different states of inhibitors and the efficiency where free Ahx-Phe-Leu-Arg-COOH is used as the reference. *reference value

State	Carrier	Peptide	K_i	Efficiency	K_i stored
Free	-	Leupeptin	88 ± 8 nM	-	
		Leupeptin-COOH	2.7 ± 0.1 μ M	-	
		Ahx-Leu-Leu-Arg-COOH	9.5 ± 1.0 μ M	-	
		Ahx-Phe-Leu- Arg-COOH	3.4 ± 0.2 μ M	1.00*	
Conjugated	TiO ₂		11 ± 2 μ M	0.32	7.1 ± 1.1 μ M
	ZnO	Ahx-Phe-Leu- Arg-COOH	4.4 ± 1.3 μ M	0.77	7.6 ± 1.5 μ M
	WorkBeads™		29 ± 4 μ M	0.12	53 ± 9 μ M

Table 4. The relative asymptotic plateau values extracted from GraphPad Prism 8, where the resulting value shows the asymptotic area ratio obtained at virtually maximal inhibition.

State of peptide 1	Plateau
Free Ahx-Phe-Leu-Arg-COOH	0.50 ± 0.04
Only ZnO-particles	0.86 ± 0.07
ZnO-Ahx-Phe-Leu-Arg-COOH	0.46 ± 0.02
Only TiO ₂ -particles	1.24 ± 0.04
TiO ₂ -Ahx-Phe-Leu-Arg-COOH	0.42 ± 0.21

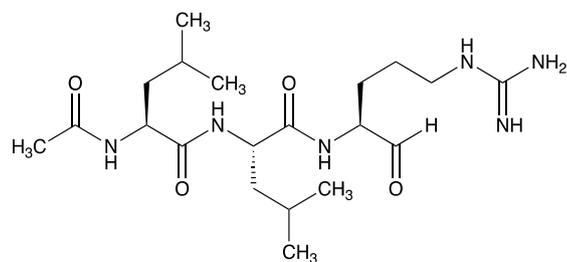
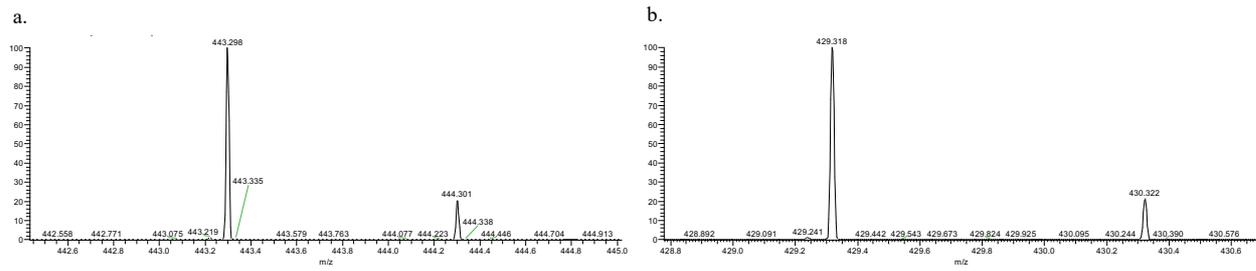
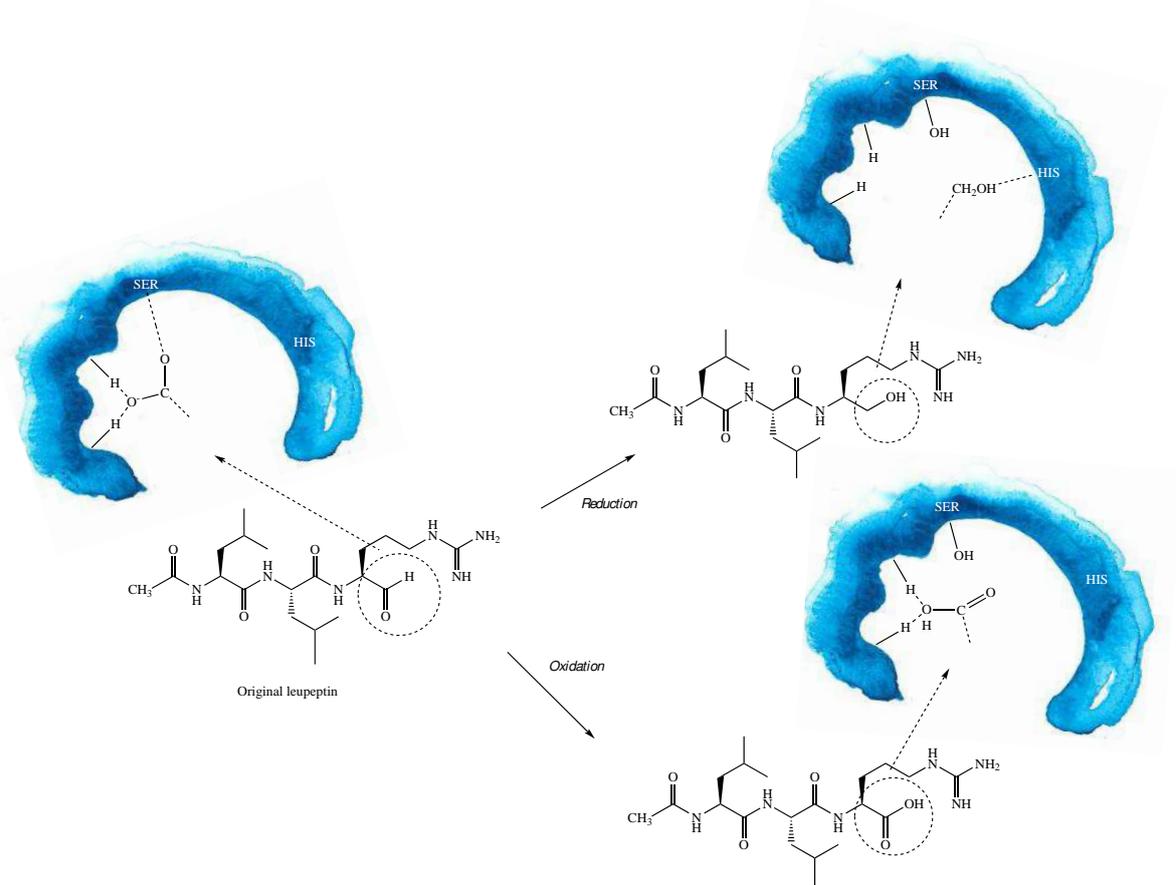
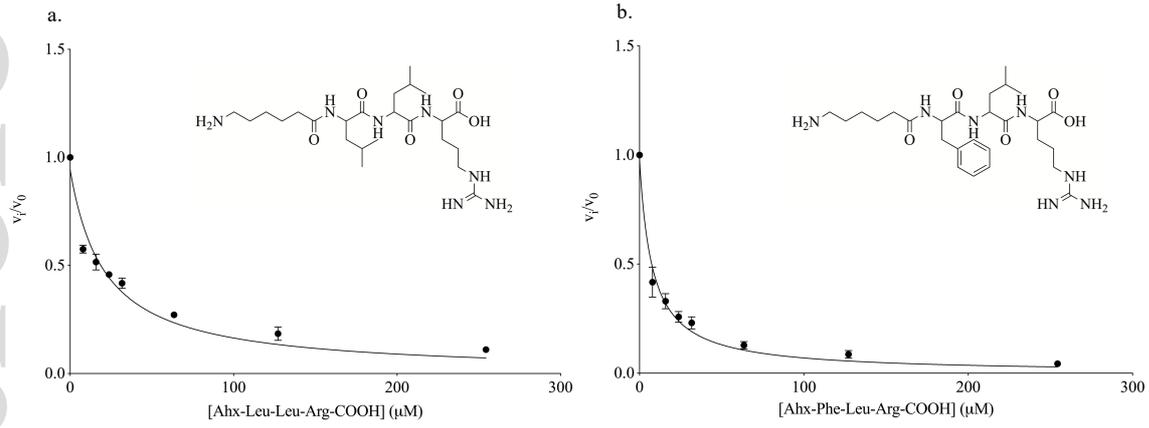
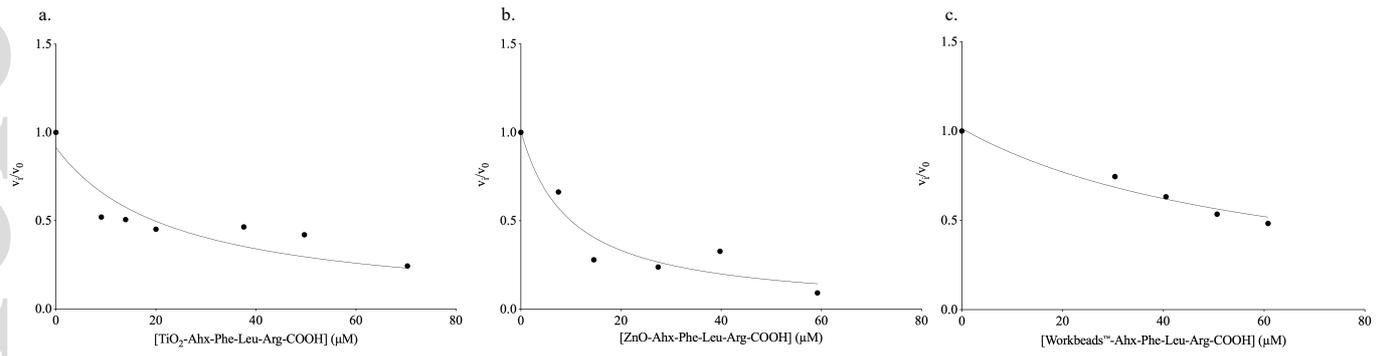


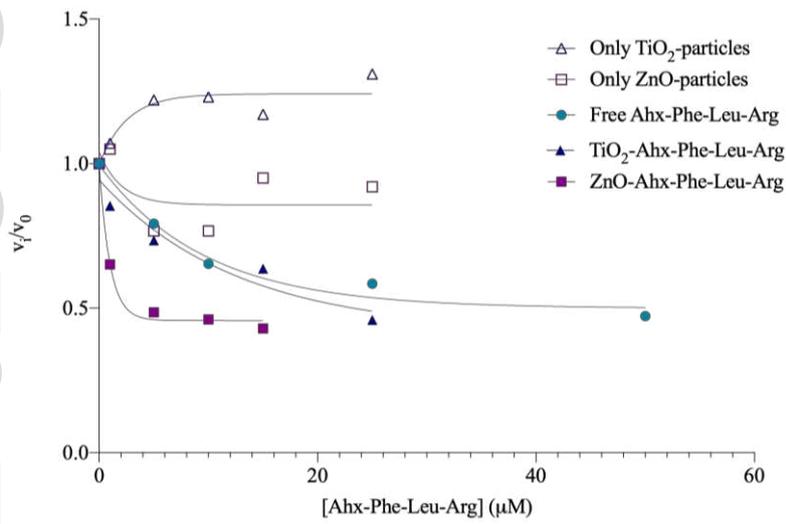
Figure 1. Leupeptin in its natural state (Ac-Leu-Leu-Arg-CHO)

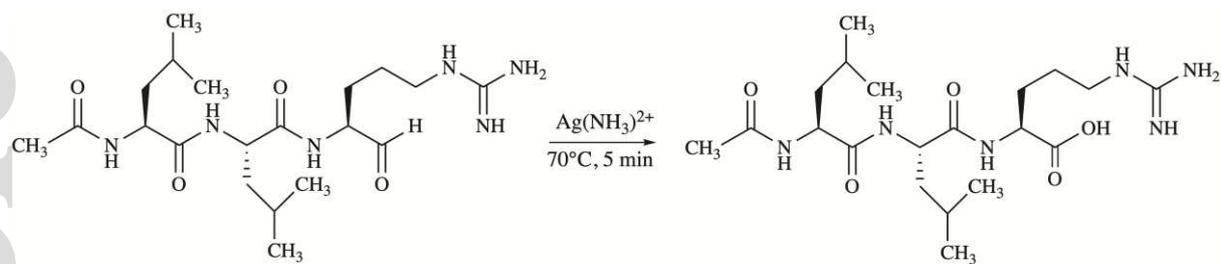


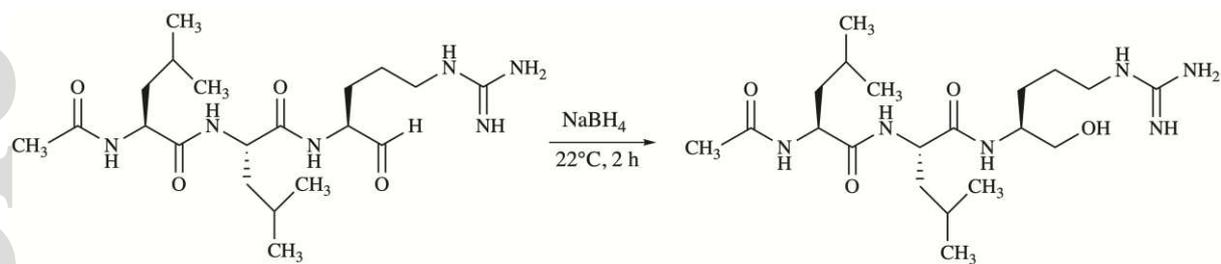


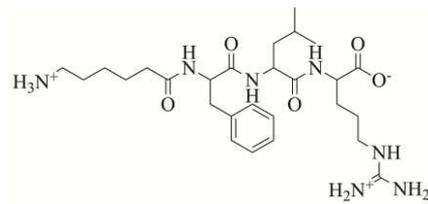






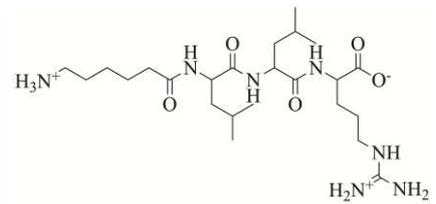






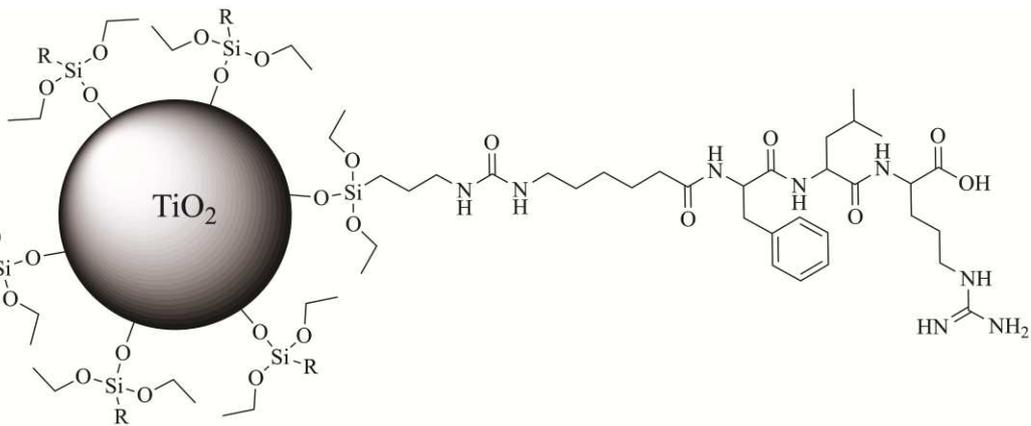
Ahx-Phe-Leu-Arg-COOH

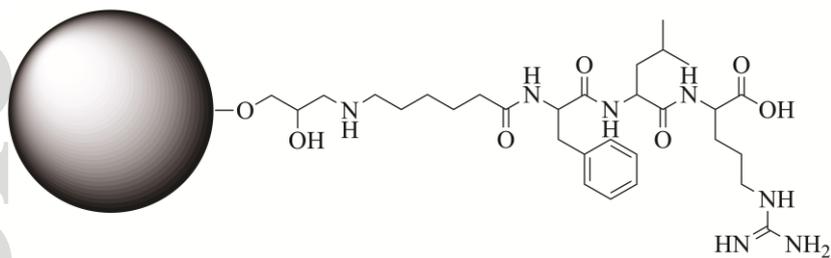
547.7 Da



Ahx-Leu-Leu-Arg-COOH

513.7 Da





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