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Simple method to assess stability of immobilized peptide ligands against proteases

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Although peptides are used as affinity chromatography ligands, they could be digested by proteases. Usually, peptide stability is evaluated in solution, which differs from the resin-bounded peptide behavior. Furthermore, the study of the degradation products requires purification steps before analysis. Here, we describe an easy method to assess immobilized peptide stability. Sample peptides were synthesized on hydroxymethylbenzamide-ChemMatrix resin. Peptidyl-resin beads were then incubated with solutions containing proteases. Peptides were detached from the solid support with ammonia vapor and analyzed by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry, allowing the detection of the whole peptides as well as their C-terminal degradation products. The method allowed a fast evaluation of peptide ligand stability in solid phase towards proteases that may be present in the crude sample before their use as ligands in affinity chromatography. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: MALDI MS; ESI MS; affinity chromatography; purification

Introduction

Affinity chromatography is the technique of choice to purify macromolecules in low concentration in complex mixtures. Protein affinity ligands such as monoclonal antibodies and protein A are widely used in many protein purification protocols. However, due to their high cost, more economic ligands such as reactive dyes and short peptides have been developed [1]. The construction and screening of combinatorial peptide libraries are widely used to find peptide ligands for any given target [2,3]. Short peptide ligands can be easily produced at high scale by the solid phase peptide synthesis methods introduced by Bruce Merrifield [4]. They are much more physically and chemically stable than antibodies and, if it is necessary to increase their stability, chemical modifications may be easily introduced to them by well-known chemical methods [5]. Additionally, they can be site-directed immobilized in chromatographic supports, and due to their small size, large ligand densities and high adsorption capacities can be achieved [6]. The resultant affinity matrices are much more robust during elution and regeneration as compared to protein-based affinity matrices. Even if any leakage occurs, small peptide molecules can be easily removed from a macromolecular product [7].

When introducing a short peptide as an affinity ligand to purify a target protein, it is very important to evaluate its stability against proteases and peptidases usually present in the crude sample from where the protein is purified. These enzymes may digest

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Abbreviations: *Ac_2O, acetic anhydride; CHCA, α-cyano-4-hydroxycinnamic acid; CHO, Chinese hamster ovary; DIPCDI, 1,3-diisopropylcarbodiimide; DIPEA, N,N-diisopropylethylamine; DMAP, 4-(N,N-dimethylamino)pyridine; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; ESI MS, electrospray ionization mass spectrometry; EPO, erythropoietin; Fmoc, fluorenylmethoxycarbonyl; HMBA, 4-hydroxymethylbenzoic acid; HMBA-CM, Hydroxymethylbenzamide-ChemMatrix; HOBt, 1-hydroxybenzotriazole; Kd, dissociation constant; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NHS, N-hydroxysuccinimide; LP, ; Ac-Phe-His-His-Phe-Ala-His-Ala-Lys; DP, Ac-D-Phe-D-His-D-His-D-Phe-D-Ala-D-His-D-Ala-D-Lys; RP, Ac-D-Ala-D-His-D-Ala-D-Phe-D-His-d-His-D-Phe-D-Lys; rhEPO, recombinant human erythropoietin; SA, spacer arm Ser-Gly-Ala-Ser-Gly; TBTU, 1-[bis(dimethylamino)methylene]-1H–benzotriazolium hexafluorophosphate 3-oxide; tBu, tert-butyl; TFA, trifluoroacetic acid; TIS triisopropylsilane; TOF, time-of-flight;; Z-SA, Z-sinapinic acid*

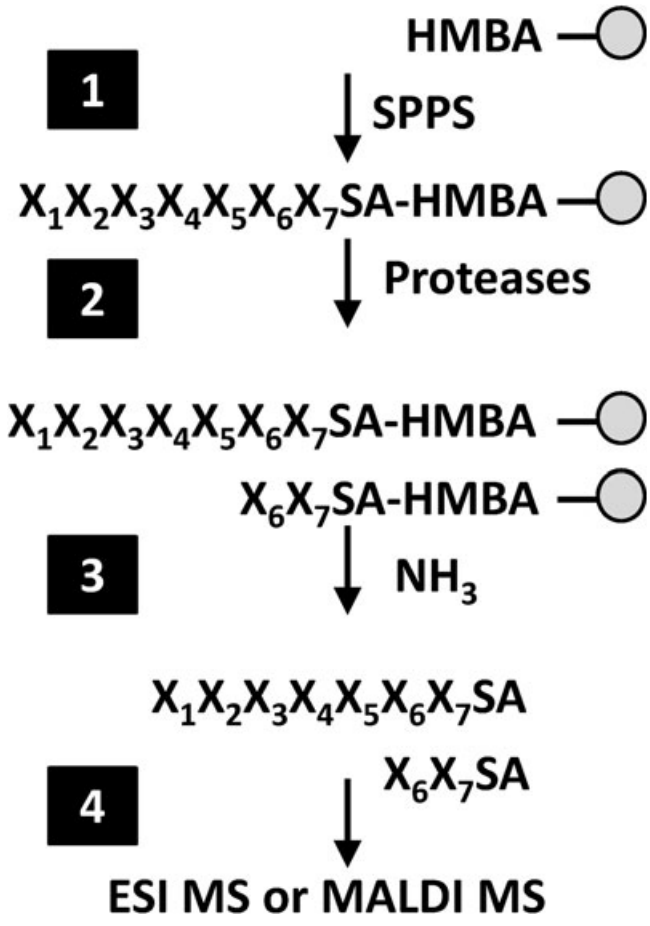
immobilized peptide ligand, thus shortening the useful life of the affinity support. In such cases, slight chemical modifications can be introduced to the peptide ligand to increase its stability [8].

Commonly, the verification of stability is evaluated with the peptide in solution, which may differ from the resin-bound peptide behavior [9]. Furthermore, if the peptide to be evaluated is in solution in the reaction mixture, the study of the peptide degradation products will require purification steps before their analysis, making the study much more complicated [10].

In this work, we describe a simple strategy to evaluate the stability of immobilized peptide ligands against proteases using Hydroxymethylbenzamide-ChemMatrix (HMBA-CM) resin and electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS).

Materials

Hydroxymethylbenzamide-ChemMatrix resin was kindly donated by Matrix Innovation Inc. (Montreal, Québec, Canada). Fmoc-amino acids, 1-hydroxybenzotriazole (HOBt) and 1-[bis(dimethylamino) methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (TBTU) were from Peptides International Inc. (Louisville, KY, USA). 1,3-Diisopropylcarbodiimide (DIPCDI), *N,N*-diisopropylethylamine (DIPEA), triisopropylsilane (TIS), piperidine, bovine trypsin, bovine chymotrypsin and α-cyano-4-hydroxycinnamic acid (CHCA) were from Sigma-Aldrich (St. Louis, MO, USA). Chinese hamster ovary (CHO) cell culture supernatant and recombinant human erythropoietin (rhEPO) produced in CHO cells were kindly donated by Zelltek S.A. (Santa Fe, Argentina). *N*-hydroxysuccinimide (NHS) activated Sepharose was from GE Healthcare Life Science (Waukesha, WI, USA). All other reagents were AR grade. *Z*-3,5-Dimethoxy-4-hydroxycinnamic acid (*Z*-sinapinic acid; *Z*-SA) was prepared as described elsewhere [11,12].



Scheme 1. Immobilized peptide stability evaluation. 1) Solid phase peptide synthesis (SPPS) is performed on HMBA-CM resin. A spacer arm (SA) is added to increase the peptide molecular weight; 2) Peptidyl-beads are incubated with proteases; 3) Peptides are separated from the resin with ammonia vapor; 4) Peptides are analyzed by MS.

Methods

Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction.

Fmoc Peptide Synthesis on HMBA-ChemMatrix Resin

The short peptide sequence Ser-Gly-Ala-Ser-Gly (SA) was used as a spacer arm in all the evaluated peptides. This sequence is stable to most proteases described [13]. The spacer arm is necessary to increase the molecular weight of peptides to facilitate their MS analysis and also to facilitate the interaction with both proteases and target proteins.

The SA was synthesized on HMBA-CM resin (35–100 mesh-wet sieved, 100–200 mesh-dry, 0.63 mmol/g). Then, the SA-HMBA-CM resin was divided to synthesize three model peptides (Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His, Ala-Tyr-Val-Lys-His-Arg-Ala-Phe and Val-Arg-His-Tyr-Ser-Phe-Ala-Lys) with known trypsin and chymotrypsin cleavage sites [13] using the Fmoc strategy as previously described [14].

The L-peptide Ac-Phe-His-His-Phe-Ala-His-Ala, with known affinity for rhEPO [15], and two new peptides, one with the same sequence but containing only D amino acids (D analog: Ac-D-Phe-D-His-D-His-D-Phe-D-Ala-D-His-D-Ala) and the other with D amino acids but with inverted sequence (D retroinverse: Ac-D-Ala-D-His-D-Ala-D-Phe-D-His-D-His-D-Phe) were also synthesized by the same method, using the SA-HMBA-CM resin.

Threefold excess of Fmoc-amino acids was used at each coupling step. The C-termini Gly was incorporated with DIPCDI in the presence of 4-(*N,N*-dimethylamino)pyridine (DMAP) in *N,N*-dimethylformamide (DMF) as per Mellor *et al.* [16]. The remaining protected amino acids were incorporated with DIPCDI/HOBt and TBTU/DIPEA. Fmoc removal was performed with piperidine/DMF (1:4). Washings between deprotection, coupling and subsequent deprotection steps were carried out with DMF and dichloromethane (CH_2Cl_2). After peptide elongation, N-terminus acetylation was performed by adding acetic anhydride (Ac_2O) (10 eq) and DIPCDI (10 eq) in CH_2Cl_2. Next, protecting groups were removed with trifluoroacetic acid (TFA)/TIS/H_2O (95:2.5:2.5 v/v/v) during 4 h, leaving the unprotected peptides anchored to the resin.

Peptidyl-Resin Incubation with Trypsin, Chymotrypsin and CHO Extract

Peptide beads were washed successively with CH_2Cl_2, DMF, H_2O: DMF (3:7; 5:5; 7:3 v/v) and H_2O. Then, 100 mg of each peptidyl-resin (approximately 10 000 peptidyl-beads) was incubated with solution A (40-μM trypsin in 40 mM Tris–HCl, pH 8.1, 10 mM $CaCl_2$ buffer), B (40-μM chymotrypsin in 40 mM Tris–HCl, pH 8.1, 10 mM $CaCl_2$ buffer) or C (CHO cell culture supernatant) overnight at 37 °C. Bead

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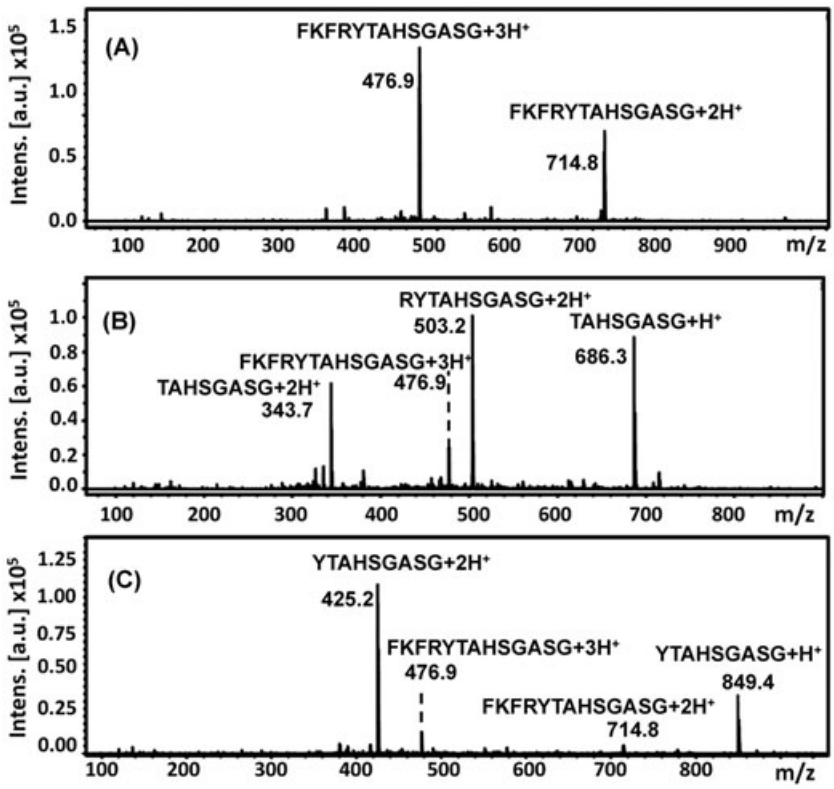

Figure 1. ESI mass spectra of model peptide H-Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2 (FKFRYTAHSGASG) before (A) and after treatment with chymotrypsin (B) and trypsin (C). The signals corresponding to the whole peptide at m/z 476.9 (FKFRYTAHSGASG + $3H^+$) and 714.8 (FKFRYTAHSGASG + $2H^+$), and the signals corresponding to degradation products at m/z 343.7 (TAHSGASG + 2H), 686.3 (TAHSGASG + H^+), 503.2 (RYTAHSGASG + $2H^+$), 425.2 (YTAHSGASG + $2H^+$) and 849.4 (YTAHSGASG + H^+) are observed.

samples were taken after 1, 4, 6 and 24 h of incubation and then thoroughly washed with H_2O, H_2O:DMF (7:3; 5:5; 3:7 v/v), DMF and CH_2Cl_2. Finally, they were air dried.

Peptide Cleavage and Elution from the Beads

Peptides were detached from the resin with ammonia vapor as has been previously described [17]. Samples of 0.2 mg of each peptidyl-resin were placed into separate micro tubes, which were placed in a drying chamber together with a flask containing 30% NH_4OH. The drying chamber was clamped shut and left to stand overnight at room temperature. Released peptides were eluted by adding 1 ml of acetic acid:acetonitrile (CH_3CN):H_2O (3:4:3 v/v/v).

Mass Spectrometry Analysis of the Eluted Peptides

Eluted peptides were analyzed by ESI MS and MALDI MS.

Electrospray ionization mass spectrometry was recorded in a Bruker microTOF-Q II (Bruker Daltonics GmbH, Leipzig, Germany). A 5-μl aliquot of eluted peptide was injected and analyzed in positive mode. The tip voltage was 3.5 KV, and the flow rate was 180 μl/h.

Matrix-assisted laser desorption/ionization mass spectrometry were recorded in an Ultraflex II TOF/TOF (Bruker Daltonics GmbH, Leipzig, Germany). A 1-μl aliquot of eluted peptide was loaded onto the sample plate, air-dried at room temperature, and then 1 μl of MALDI matrix solution was added on the sample dried layer (successive-dry-layers deposit method). Two matrix solutions were assayed: (i) α-cyano-4-hydroxycinnamic acid (CHCA) 5 mg/ml in CH_3CN/H_2O (1:1, v/v) with 0.1% TFA and (ii) *Z*-sinapinic acid (*Z*-SA) [11,12] 5 mg/ml in methanol (CH_3OH)/H_2O (1:1, v/v). Mass spectra were acquired in the MS reflector positive ion mode.

Synthesis of the Peptide Affinity Chromatographic Matrix

Peptide ligand with affinity for rhEPO Ac-Phe-His-His-Phe-Ala-His-Ala-Lys (LP) [15] and its corresponding D analog and D retroinverse: Ac-D-Phe-D-His-D-His-D-Phe-D-Ala-D-His-D-Ala-Lys (DP) and Ac-D-Ala-D-His-D-Ala-D-Phe-D-His-D-His-D-Phe-D-Ala-Lys (RP), were synthesized by the Fmoc chemistry on Rink-amide resin, as described by Chan and White [14]. The spacer arm was not incorporated in the peptide because the NHS-Sepharose provides a spacer arm [18]. Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Peptides were synthesized using threefold excess of each Fmoc-amino acid at each coupling step incorporated with DIPEA/TBTU. Fmoc removal was performed with piperidine/DMF (1:4). Washings between deprotection, coupling and subsequent deprotection steps were carried out with DMF and CH_2Cl_2. After completion of elongation, acetylation of the N-terminus was performed by adding acetic anhydride (Ac_2O) (10 eq) and DIPCDI (10 eq) in CH_2Cl_2. A Lys was incorporated at the C-terminus to allow the peptide to be coupled through its side-chain amine group, thus assuring the same peptide orientation in the Sepharose support as that in the HMBA-CM resin. The peptide amide was synthesized to prevent peptide polymerization during coupling. Peptides were separated from the resin, and their side-chain protecting groups were removed by treatment with a mixture of TFA/TIS/H_2O (95:2.5:2.5) for 2 h.

Peptides were isolated by precipitation with cold diethyl ether and then dissolved in CH_3CN/H_2O (1:1) and lyophilized. These peptides were then immobilized on NHS activated Sepharose, as described by Hermanson *et al.* [6]. A threefold excess of each ligand peptide LP, DP and RP dissolved in 1-ml dimethyl sulfoxide (DMSO) was added to 1-ml NHS-Sepharose provided with a spacer arm with a ligand density of 16–23 μmol NHS/ml drained medium. Anhydrous triethylamine was added to a level equimolar to the amount of peptide charge. The gel/peptide slurry was stirred for 4 h at room temperature, and afterwards the LP-Sepharose, DP-Sepharose and RP-Sepharose matrices were washed with DMSO. Any remaining reactive group was blocked by addition of a slight excess of ethanolamine at the end of the reaction.

rhEPO Adsorption Isotherm Determination

Chromatographic matrix aliquots, 30 μl, were put into tubes containing 1-ml pure rhEPO solution at different concentrations in 20 mM sodium phosphate buffer, pH 4. The suspension was gently shaken overnight at 24 °C to enable the system to reach its equilibrium. Protein concentration was determined with Bradford reagent. The equilibrium concentration of rhEPO bound to the matrix was calculated as the total amount of rhEPO present at the beginning of the experiment less the amount still in the soluble phase at equilibrium. Dissociation constant (Kd) for each matrix was calculated using a one-to-one Langmuir binding model as described by Chase [19], using the SigmaPlot 2001 regression program (2001 SPSS Inc).

Results and Discussion

In this work, a strategy to assess immobilized peptide ligand stability against proteases using HMBA-CM resin and ESI MS or MALDI MS was developed. Scheme 1 resumes the method.

ChemMatrix resin was used as the solid support. Its amphiphilicity allowed elongation of the peptide sequence via Fmoc-synthesis in organic solvents, and, afterwards, stability assays performed by incubation of the peptidyl-resin in aqueous buffer with proteases [20].

While the peptidyl-resin was incubated with aqueous solutions of proteases, samples of beads were taken subsequently. Proteases, N-terminal degradation products and buffer were removed easily by washing and filtration processes. Because the peptide and their C-terminal degradation products were covalently attached to the beads, there was no need of arduous purification steps.

The next step consisted in the cleavage of the peptides with ammonia vapor for their MS analysis. For that purpose, the linker 4-hydroxymethylbenzoic acid (HMBA) was used to introduce a cleavage site between the peptide and the resin [21,22]. As has been previously reported [23], the ammonia vapor was easily removed by evaporation to avoid its interference in the MS peptide analysis. The chemical structure of CM, composed entirely of polyethyleneglycol (PEG) monomers that contain exclusively primary ether bonds, made it chemically stable to ammonia thus avoiding sample contamination that would affect MS analysis.

Figure 1 shows an example of the ESI MS mass spectra obtained before and after subjecting the peptide-beads to chymotrypsin or trypsin digestion when model peptides were used. Electrospray ionization mass spectrometry allowed the detection of the whole peptides (Figure 1(A)) as well as their C-terminal enzymatic degradation products (Figure 1(B) and (C)) obtained after incubation with trypsin or chymotrypsin (other mass spectra are shown in the Supporting Information).

Although both, ESI MS and MALDI MS are useful for peptide analysis, less time is necessary for MALDI MS than for ESI MS. Furthermore, unlike ESI MS, MALDI MS is an off-line technique in which samples are spotted to a target plate prior to ionization and therefore they can be reanalyzed.

When analyzing the peptides and their degradation products with MALDI MS, α-cyano-4-hydroxycinnamic acid matrix clusters interfered in MALDI MS analysis of low molecular weight products. On the other hand, a minor number of cluster signals were observed when using *Z*-sinapinic (*Z*-SA) acid matrix previously described [12] as the matrix of choice for short peptide analysis (Figure 2).

In a previous work, the synthesis and screening of combinatorial peptide libraries to find peptides with affinity for rhEPO had been described. In that work, the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Lys (LP) proved to be a suitable affinity ligand for rhEPO purification by affinity chromatography from crude CHO cell cultures [15]. Therefore, as a proof of concept, the method herein described was applied to assess the stability of the peptide LP. In addition, due to the stereochemical selectivity of proteases, its corresponding D analog (DP) and D retroinverse (RP) were also evaluated [24]. Figure 3 shows the MALDI mass spectra of the L peptide after incubation with chymotrypsin. The signals corresponding to the entire L-peptide and the C-terminal

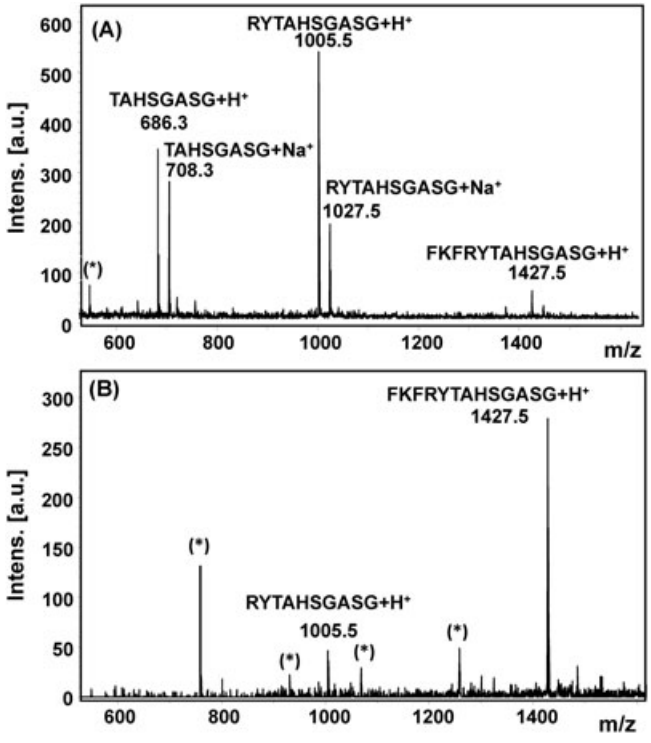

Figure 2. MALDI mass spectra of model peptide H-Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2 (FKFRYTAHSGASG) after treatment with chymotrypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signal corresponding to the whole peptide at m/z 1427.5 (FKFRYTAHSGASG + H^+) and the signals corresponding to degradation products at m/z 1005.5 (RYTAHSGASG + H^+), 1027.5 (RYTAHSGASG + Na^+), 686.3 (TAHSGASG + H^+) and 708.3 (TAHSGASG + Na^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

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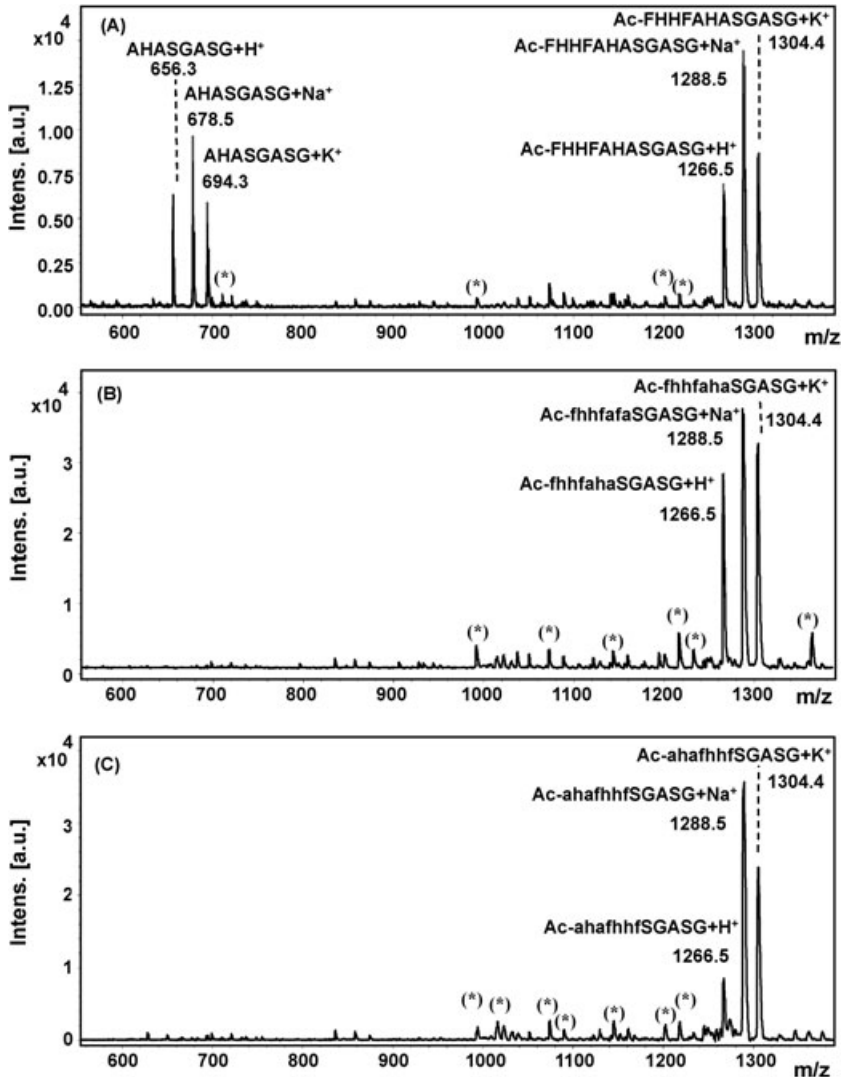

Figure 3. MALDI mass spectra of peptides (A) Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG), (B) Ac-D-Phe-D-His-D-His-D-Phe-D-Ala-D-His-D-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-fhhfahaSGASG) and (C) Ac-D-Ala-D-His-D-Ala-D-Phe-D-His-D-His-D-Phe-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-ahafhhfSGASG) after treatment with chymotrypsin using *Z*-sinapinic acid matrix. The signals corresponding to the whole peptides at m/z 1266.5 (Ac-FHHFAHASGASG + H^+, Ac-fhhfahaSGASG + H^+ and Ac-ahafhhfSGASG + H^+), 1288.5 (Ac-FHHFAHASGASG + Na^+, Ac-fhhfahaSGASG + Na^+ and Ac-ahafhhfSGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+, Ac-fhhfahaSGASG + K^+ and Ac-ahafhhfSGASG + K^+) and the signal corresponding to the degradation product at m/z 656.3 (AHASGASG + H^+), 678,5 (AHASGASG + Na^+) and 694.3 (AHASGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

degradation product AHASGASG were clearly detected. On the other hand, only the signals corresponding to the entire peptide were observed when analyzing DP and RP even after 24 h of incubation due to their higher stability towards proteases.

The three peptide ligands were immobilized on Sepharose and their affinity towards rhEPO were evaluated by adsorption isotherm experiments (Figure 4). The isotherms showed a good fit of experimental data to a Langmuir-type isotherm. The

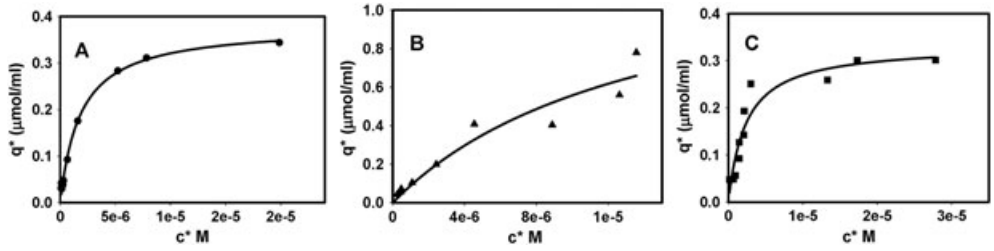

Figure 4. Equilibrium adsorption isotherm for the binding of rhEPO to (A) LP-Sepharose, (B) DP-Sepharose and (C) RP-Sepharose. Chromatographic matrix aliquots, 30 µl, were put into tubes containing 1-ml pure rhEPO solution at different concentrations. The suspension was gently agitated overnight at 24 °C. Protein concentration was determined with Bradford reagent. Kd values of 1.8 µM, 12 µM and 2.4 µM were obtained for LP-Sepharose, DP-Sepharose and RP-Sepharose respectively.

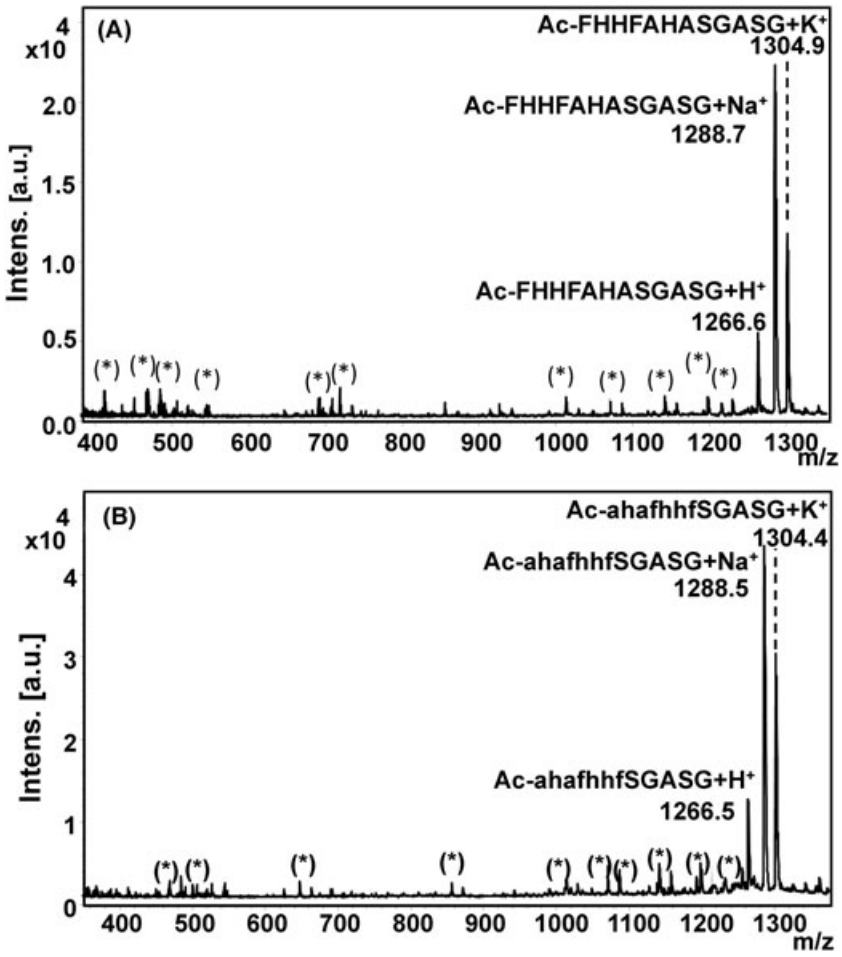

Figure 5. MALDI mass spectra of peptides (A) Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) and (B) Ac-D-Ala-D-His-D-Ala-D-Phe-D-His-D-His-D-Phe-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-ahafhhfSGASG) after treatment with CHO cells extract during 24 h at 24 °C. The matrix used was *Z*-sinapinic acid in both cases. The signals at m/z 1266.5 (Ac-FHHFAHASGASG + H^+ and Ac-ahafhhfSGASG + H^+), 1288.5 (Ac-FHHFAHASGASG + Na^+ and Ac-ahafhhfSGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+ and Ac-ahafhhfSGASG + K^+) correspond to the whole peptide. No degradation products are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

dissociation constant (Kd) of the LP-Sepharose (Kd = 1.8 ± 0.1 μM) was similar to RP-Sepharose (Kd = 2.4 ± 0.6 μM), while DP-Sepharose showed a higher Kd (12 ± 7.0 μM). The apparent binding constant (1/Kd) of the immobilized peptides were 5.2×10^5 l/mol and 4.2×10^5 l/mol for LP-Sepharose and RP-Sepharose, respectively, and 8.3 10^4 l/mol for DP-Sepharose. As has been previously described [25–27], one drawback of using a D-analogue is the affinity loss towards the target. On the other hand, the retroinverse peptide, due to its similar side chain topology to the parent peptide, exhibited similar affinity with an enhanced resistance to proteolysis, and therefore increased stability.

Although both LP and RP are suitable ligands for rhEPO purification, RP is much more expensive due to the use of D-amino acids in its synthesis. When working on an industrial process, such as the production of rhEPO for therapeutic use, expressed in recombinant CHO cells, the cost must be minimized. The stability of both peptides was also evaluated with CHO cell extracts. The MS spectrum of LP (Figure 5) as well as of RP did not show C-terminal degradation products after 24 h of incubation with the extract thus proving that the cheaper LP-Sepharose previously described [15] can be used to purify rhEPO from recombinant CHO cell culture.

Conclusion

The method here developed allowed a fast assessment of the peptide ligands stability in solid phase towards proteases that may be present in the crude sample, before their use in affinity chromatography. Due to the high sensitivity of MS, only a small sample of peptidyl-resin is required to perform the evaluation. As the enzymatic digest is performed in solid-phase, no hard purification protocols are needed before the analysis because the degradation products are still attached to the resin. Furthermore, this method uses techniques and reagents frequently used in all peptide laboratories, and therefore it is friendlier than other current strategies. In conclusion, we have optimized the peptide-stability analysis after protease interaction using a new method that can be applied in a wide range of laboratories.

Acknowledgements

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. ESI mass spectra of peptide H-Ala-Tyr-Val-Lys-His-Arg-Ala-Phe-Ser-Gly-Ala-Ser-Gly-NH_2 (AYVKHRAFSGASG) after treatment with chymotrypsin (A) and trypsin (B). The signals corresponding to the whole peptide at m/z 449.9 (AYVKHRAFSGASG + $3H^+$) and 674.8 (AYVKHRAFSGASG + $2H^+$) and the signals corresponding to the degradation products 377.2 (SGASG + H^+), 595.3 (AFSGASG + H^+) and 888.4 (HRAFSGASG + H^+) are observed.

Figure S2. MALDI mass spectra of peptide H-Ala-Tyr-Val-Lys-His-Arg-Ala-Phe-Ser-Gly-Ala-Ser-Gly-NH_2 (AYVKHRAFSGASG) after treatment with trypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the degradation products at m/z 888.4 (HRAFSGASG + H^+) and 911.4 (HRAFSGASG + Na^+), 595.3 (AFSGASG + H^+), 618.3 (AFSGASG + Na^+) and 634.3 (AFSGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S3. MALDI mass spectra of peptide H-Ala-Tyr-Val-Lys-His-Arg-Ala-Phe-Ser-Gly-Ala-Ser-Gly-NH_2 (AYVKHRAFSGASG) after treatment with chymotrypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the degradation products at m/z 1115.6 (VKHRAFSGASG + H^+), 1138.6 (VKHRAFSGASG + Na^+) and 1154.6 (VKHRAFSGASG + K^+), 377.2 (SGASG + H^+), 400.2 (SGASG + Na^+) and 416.2 (SGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S4. ESI mass spectrum of peptide H-Val-Arg-His-Tyr-Ser-Phe-Ala-Lys-Ser-Gly-Ala-Ser-Gly-NH_2 (VRHYSFAKSGASG) after treatment with chymotrypsin. The signal at m/z 682.85 corresponds to the whole peptide VRHYSFAKSGASG + $2H^+$. The signals at m/z 576.3 and 810.4 correspond to the degradation products SFAKSGASG + H^+ and KSGASG + H^+ respectively.

Figure S5. MALDI mass spectra of peptide H-Val-Arg-His-Tyr-Ser-Phe-Ala-Lys-Ser-Gly-Ala-Ser-Gly-NH_2 (VRHYSFAKSGASG) after treatment with trypsin using as matrix (A) Z-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the degradation product at m/z 377.2 (SGASG + H^+), 400.2 (SGASG + Na^+) and 416.2 (SGASG + K^+) are observed. The signals

marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S6. MALDI mass spectra of peptide H-Val-Arg-His-Tyr-Ser-Phe-Ala-Lys-Ser-Gly-Ala-Ser-Gly-NH_2 (VRHYSFAKSGASG) after treatment with chymotrypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the degradation product at m/z 810.4 (SFAKSGASG + H^+), 833.4 (SFAKSGASG + Na^+) and 849.4 (SFAKSGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S7. MALDI mass spectra of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) before treatment with chymotrypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the whole peptide at m/z 1266.5 (Ac-FHHFAHASGASG + H^+) 1288.5 (Ac-FHHFAHASGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S8. MALDI mass spectra of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 1-h treatment with chymotrypsin using as matrix (A) Z-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the whole peptide at m/z 1266.5 (Ac-FHHFAHASGASG + H^+) 1288.5 (Ac-FHHFAHASGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+) and the signal corresponding to the degradation product at m/z 656.3 (AHASGASG + H^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S9. MALDI mass spectra of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 4-h treatment with chymotrypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the whole peptide at m/z 1266.5 (Ac-FHHFAHASGASG + H^+) 1288.5 (Ac-FHHFAHASGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+) and the signals corresponding to the degradation product at 656.3 (AHASGASG + H^+), 678.5 (AHASGASG + Na^+) and 694.3 (AHASGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S10. MALDI mass spectra of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 6-h treatment with chymotrypsin using as matrix (A) *Z*-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals corresponding to the whole peptide at m/z 1266.5 (Ac-FHHFAHASGASG + H^+) 1288.5 (Ac-FHHFAHASGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+) and the signals corresponding to the degradation product at m/z 656.3 (AHASGASG + H^+), 678.5 (AHASGASG + Na^+) and 694.3 (AHASGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S11. MALDI mass spectrum of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 24-h treatment with chymotrypsin using α-cyano-4-hydroxycinnamic acid as matrix. The signals corresponding to the whole peptide at m/z 1266.5 (Ac-FHHFAHASGASG + H^+) 1288.5 (Ac-FHHFAHASGASG + Na^+) and 1304.4 (Ac-FHHFAHASGASG + K^+) and the signals corresponding to the degradation product at m/z 656.3 (AHASGASG + H^+), 678.5 (AHASGASG + Na^+) and 694.3 (AHASGASG + K^+) are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S12. MALDI mass spectra of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 1-h treatment with CHO cell extract using as matrix (A) Z-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals at m/z 1266.5, 1288.5 and 1304.4 correspond to the whole peptide as Ac-FHHFAHASGASG + H^+, Ac-FHHFAHASGASG + Na^+ and Ac-FHHFAHASGASG + K^+ respectively. No degradation products are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S13. MALDI mass spectra of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 6-h treatment with CHO cell extract using as matrix (A) Z-sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid. The signals at m/z 1266.5, 1288.5 and 1304.4 correspond to the whole peptide as Ac-FHHFAHASGASG + H^+, Ac-FHHFAHASGASG + Na^+ and Ac-FHHFAHASGASG + K^+, respectively. No degradation products are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.

Figure S14. MALDI mass spectrum of the peptide Ac-Phe-His-His-Phe-Ala-His-Ala-Ser-Gly-Ala-Ser-Gly-NH_2 (Ac-FHHFAHASGASG) after 24 h treatment with CHO cell extract using α-cyano-4-hydroxycinnamic acid as matrix. The signals at m/z 1266.5, 1288.5 and 1304.4 correspond to the whole peptide as Ac-FHHFAHASGASG + H^+, Ac-FHHFAHASGASG + Na^+ and Ac-FHHFAHASGASG + K^+, respectively. No degradation products are observed. The signals marked with an asterisk (*) correspond to clusters of the MALDI matrix used.