Experimental procedures:

Cleavage of Asp-Pro bond for the release of peptides attached to a single bead

Two experiments were performed to optimize the recovery of the peptides from a single bead.

(1) Effect of acid concentration and hydrolysis time

In a first experiment, hydrolysis time and formic acid concentration were evaluated. Single beads with similar dimensions carrying the P90 peptide were placed separately in four 500 μL polypropylene Eppendorff tubes. Two of them were hydrolyzed in 10 μL of $H_2{}^{16}O$ containing 0.1% of formic acid for 15 and 25 minutes at 100°C, respectively. The two remaining beads were hydrolyzed in 10 μL of $H_2{}^{18}O$ containing 0.1% of formic acid for 15 and 25 minutes, respectively. To evaluate the effect of hydrolysis time on the relative yield of the released peptide, equal volumes of the hydrolyses solution performed in either normal or ^{18}O-labeled water at different hydrolysis times were mixed and analyzed by ESI-MS. The isotopic ion distribution of the released peptide was analyzed by ISOTOPICA software (4) in order to determine the relative yield of the hydrolysis reactions based on the $^{16}O/^{18}O$ ratio.

To evaluate the effect of acid concentration, a similar experiment was conducted with four additional beads but using higher concentration of formic acid (1%, v/v) in the hydrolysis solution.

(2) Effect of acetonitrile on hydrolysis

The effect of adding acetonitrile to the hydrolysis solution was also evaluated. Single beads of P90 peptide were placed separately in four 500 μL polypropylene Eppendorff tubes and they were hydrolyzed for 25 minutes at 100°C in solutions containing 1% (v/v) of formic acid. However, the composition of the four individual solutions was different. Beads 1 and 2 were hydrolyzed in solutions prepared exclusively with $H_2{}^{18}O$ and $H_2{}^{16}O$, respectively. Beads 3 and 4 were hydrolyzed in solutions prepared with $H_2{}^{18}O$ and $H_2{}^{16}O$, as well, but containing 50% (v/v) of acetonitrile.

Once the hydrolysis concluded, the solutions were cooled to room temperature and centrifuged. Equal volumes of solutions 1 and 4 were mixed and analyzed by ESI-MS, following a similar approach for solutions 2 and 3. The isotopic ion distribution of peptide P90 in both ESI-MS spectra was analyzed with ISOTOPICA (4), using the

determined $^{18}O/^{16}O$ ratio to estimate relative recovery of the peptide for each hydrolysis condition.

Releasing peptides from a single bead

A single bead was placed in a 500 μL polypropylene Eppendorff tube and sequentially washed with 200 μL of water and 200 μL of 50% acetonitrile/water (v/v). Ten microliters of the hydrolysis solution (formic acid 1% (v/v) containing 50% of acetonitrile prepared in a 40/60%, (v/v) mixture of $H_2{}^{18}O/H_2{}^{16}O$) were added and the tube was then sealed and heated for 25 minutes at 100^{o}C. One third of the total volume was used for ESI-MS analysis, separating the remainder for Edman micro-sequencing should a confirmatory experiment become necessary. A blank experiment was also run, to identify undesirable background signals.

Evaluation of hydrolysis conditions

The Asp-Pro linkage is the peptide bond most susceptible to be efficiently cleaved under mild acidic conditions (1-3). Although several experimental conditions have been reported to cleave specifically this peptide bond in proteins, the reported hydrolysis solutions generally contain salts, chaotropic agents, inorganic acids (HCl) (5-6) and generally uses overnight hydrolysis at 100 ^{o}C. These chemical additives are not compatible with ulterior ESI-MS analysis, introducing a desalting step that often leads to undesirable losses. In order to maximize the sensitivity of a valuable experiment that starts from the material attached to a single bead, we focused our attention in the usage of water, formic acid (FA) and acetonitrile (ACN) as the only components of the hydrolysis solution because they are commonly used in the ESI-MS measurements. FA is a rather strong organic acid that might guarantee acidic conditions to cleave efficiently the Asp-Pro bond; it promotes the protonation and aids the ionization of peptides. On the other hand, ACN aids the formation of an efficient spray and improves the solubility of the released peptides, allowing a more efficient recovery. The hydrolysis time was also evaluated and it was shortened as much as possible in order to speed up the analysis and preserve the integrity of the released peptides.

Effect of acid concentration and hydrolysis time on the recovery of the P90 peptide

Initially two parameters, the hydrolysis time and FA concentration were studied in order to know their influence on the relative recovery.

Eight beads of the model P90 peptide were independently hydrolyzed in the conditions displayed in the Tables inserted from figures 2A-I to 2A-IV. Four beads (from bead1 to bead4) were hydrolyzed in solutions containing 0.1 % of FA (Figures 2A-I and 2A-II) and the remaining four beads (bead5 to bead8) in 1 % of FA (Figures 2A-III 2A-IV). The hydrolysis solutions were prepared in normal and ^{18}O-labeled water, and mixed conveniently as described in Materials and Methods in order to determine the relative recoveries of P90 based on the $^{16}O/^{18}O$ ratio in the assayed conditions. In each experiment (Figures 2A-I, -II, -III and -IV), one bead was hydrolyzed during 15 minutes and the other during 25 minutes. The hydrolysis temperature in all experiments was 100 ^{o}C as reported in literature (1-3).

Four resultant ESI-MS spectra of this experiment (Figure 2A-I, A-II, A-III and A-IV) and their corresponding expanded regions (Figure 2B-I, B-II, B-III and B-IV) demonstrated that as an average, longer hydrolysis time (25 minutes) yield approximately 1.5 times higher yield of released peptide (59.45 % *vs* 40.55%).

ESI-MS spectra obtained after hydrolysis in a solution containing 1 % of FA (Figure 2A-III and 2A-IV) showed a better S/N ratio compared to those obtained by using 0.1 % of FA (Figure 2A-I and 2A-II), indicating that 1 % of FA improved the yield of the released P90 and perhaps its ionization efficiency as well. Higher concentrations (> 1 %) of FA were not explored in order to preserve the integrity of P90 and also to ensure full compatibility with the ulterior MS analysis.

From these experiments, the best results to release heptapeptides anchored to the Tentagel S NH_2 resin were obtained using 25 minutes of hydrolysis at 100 ^{o}C in an aqueous solution that contains 1 % of FA.

Effect of acetonitrile in the hydrolysis solution for the recovery of P90 peptide

The effect of adding acetonitrile to the hydrolysis solution described in the previous experiment (1 % FA) was also evaluated using additional four beads of P90 (bead9 to bead12, Figure 2A-V and 2A-VI). The hydrolysis were performed using solutions without ACN and containing 50 % of ACN (v/v) prepared with normal and ^{18}O-labeled

water in order to determine the relative recovery of P90 in the two resultant experiments (Figure 2A-V and 2A-VI).

It was evident in both experiments that higher recovery (increased additional by 1.5 folds) was reached in the hydrolysis solutions containing 50 % of ACN (v/v) (Figure 2B-V and 2B-VI). Therefore, the best conditions obtained in these experiments to release and recovered the peptides attached to a single bead were using a hydrolysis solution composed by 1 % FA, 50 % ACN solution prepared with 40 % ^{18}O-labeled water during 25 minutes at 100 ^{o}C.

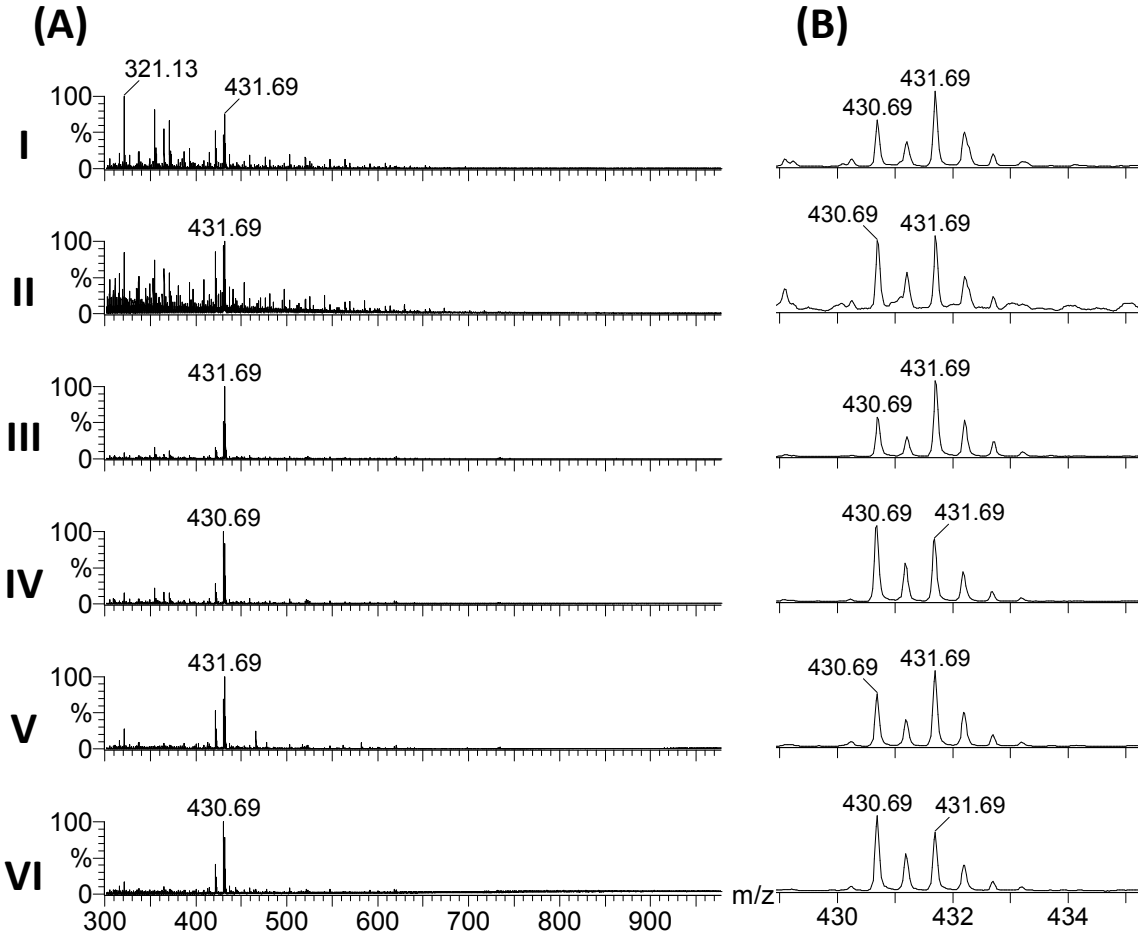

Figure 2. *Effects of formic acid and acetonitrile concentrations and hydrolysis time on the relative recovery of P90 peptide. Each ESI-MS spectra shown in the (A) column from I to VI were obtained after mixing equal volumes of two separated hydrolysis of P90 beads in the conditions described in the corresponding inserted tables (from A-I to A-VI). The expanded regions shown in (B) column from I to VI correspond to P90 peptide detected from A-I to A-VI experiments. The tables shown in (B) column display the*

relative recovery of P90 peptide obtained from two different assayed hydrolysis conditions. The relative recovery expressed as %, was determined by using ISOTOPICA (4) after analyzing the $^{16}O/^{18}O$ ratio of the resultant P90. All hydrolyses were carried out at 100^{o}C.

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