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Use of liquid chromatography–mass spectrometry coupling for monitoring the serralysin-catalyzed hydrolysis of a peptide library

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Abstract

The use of a peptide library of limited size, is considered to be more appropriate for studying a protease with a complex specificity, but very sensitive and efficient analytical techniques must be used. We have designed and synthesized a 49-peptide library of the type *Z*-AlaXXAla(amide) (X=Ala, Leu, Val, Phe, Ser, Arg, Glu) for studying the *Pseudomonas aeruginosa* serralysin specificity. All compounds of the peptide library could be identified by a LC–MS procedure. After hydrolysis of the library by pseudomonal serralysin, the LC–MS procedure also allowed the identification of the hydrolysis products and the different cleavage sites of the substrates.

Keywords: Peptide library; Serralysin

1. Introduction

The alkaline protease of *Pseudomonas aeruginosa*, also named serralysin, due to its similarity to the enzyme from *Serratia marcescens*, has a quite broad specificity [1–4]. Numerous studies with various synthetic substrates could not precisely define the amino acids which are specific to the P_1 or P_1' position (according to the nomenclature of Schechter and Berger [5]). This representation assumes that the scissible bond of a peptide is located between the subsites P_1 and P_1'. The amino acids preceding this bond are named P_2, P_3, etc...(N-terminal side) and the ones following the bond are named P_2', P_3', etc...(C-terminal side). Furthermore, it has also been demonstrated that the enzyme presents a substrate specificity similar to that of plasmin, which is a serine protease. Indeed, it may hydrolyze peptidyl-paranitroanilides, in which the S_1 site is preferentially occupied by an arginine residue [6,7]. Considering this quite surprising result and in order to precise the mode of action of the enzyme, we have recently studied, qualitatively and quantitatively, the hydrolysis of several biologically important peptides [4]. From our results, it seems unlikely that *Pseudomonas* serralysin may only be considered as a protease specific to arginine in the P_1 position. Then, it appeared relevant to compare simultaneously the hydrolysis of different similar peptides, by use of a peptide library, as previously described for other enzymes [8,9].

Nevertheless, due to the broad specificity of

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Pseudomonas serralysin, it is necessary to choose very sensitive methods for both separation and identification of hydrolysis products. Electrospray-mass spectrometry (ES-MS) was recently demonstrated to be an efficient method for analysis of peptide libraries [10,11]. We have then developed a liquid chromatography (LC)–MS procedure in order to separate the hydrolyzed peptides and to identify the sites of cleavage. Furthermore, as a large library including all possible amino acids would lead to ambiguous results, we have decided to limit it's size. We have then synthesized a library corresponding to the general formula: *Z*-AlaXXAla(amide), in which X may be any of the following amino acids: Ala, Leu, Val, Phe, Ser, Arg and Glu, representative of the different classes of amino acids and allowing possible separation on the basis of hydrophobicity. The two mixture positions result in a total of 49 individual peptides.

2. Experimental

2.1. Reagents

2.1.1. Reagents and solvents for peptide synthesis

*N*α-9-Fluorenylmethoxycarbonyl-*O-tert.*-butyl-L-serine-α-pentafluorophenyl ester [Fmoc-Ser(tBu)-OPfp], Fmoc-Ala-OPfp, Fmoc-Val-OPfp, Fmoc-*N*-2,2,5,7,8-pentamethylchroman-6-sulfonyl-L-arginine [Fmoc-Arg(Pmc)-OH], diisopropylcarbodiimide (DIPCDI), dimethylaminopyridine (DMAP), dimethylformamide (DMF)–20% piperidine were obtained from PerSeptive Biosystem (France). Fmoc-Glu(γ-*tert.*-butyl ester)-OPfp [Fmoc-Glu(Ot-Bu)OPfp], Fmoc-Phe-OPfp, Fmoc-Leu-OPfp, *N*α-benzyloxycarbonyl-alanine (*Z*-Ala-OH) and resin for peptide amides were from Bachem (Voisins-le-Bretonneux, France). Hydroxybenzotriazole (HOBt) was from Sigma (L'Isle d'Abeau, France). Trichloroacetic acid and solvents used for peptide synthesis (DMF, dichloromethane and diethyl ether) were from SDS (Peypin, France).

2.1.2. Reagents for enzymatic activity

Methanol was purchased from Fluka (L'Isle d'Abeau, France), Tris from Boehringer Mannheim (Meylan, France). Crystallized *P. aeruginosa* alkaline protease (AP) (M_r=49 500; 5.01 mPU/mg protein) was obtained from Nagase (Osaka, Japan). Its purity was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Stock solution was prepared in 0.1 *M* NaCl to a concentration of about 50 μ*M*. The final concentration was spectrophotometrically determined at 280 nm ($E^{1\%}_{280\ nm}$= 16.0) and the solution was stored at −20°C.

2.1.3. Reagent for LC–MS analysis

Acetonitrile (LiChrosolv) was supplied by Merck (Nogent-sur-Marne, France) and ammonium acetate from Sigma. Water was prepared by filtering demineralized water through a Milli-Q filtration system (Millipore, Saint-Quentin, France).

The dipeptide amides AlaAla, AlaLeu, AlaPhe, AlaArg and the *Z*-protected *Z*-AlaAla, *Z*-AlaPhe, *Z*-AlaArg, *Z*-AlaSer were obtained from Bachem. *Z*-AlaVal, *Z*-AlaLeu and *Z*-AlaGlu were obtained from Sigma. The dipeptide amides AlaGlu, AlaSer and AlaVal were synthesized in our laboratory by the solid-phase method, using Fmoc strategy.

2.2. Synthesis of the 49-peptide library

The 49-peptide library *Z*-AlaXXAla(amide) (X= Ala, Leu, Val, Phe, Ser, Arg, Glu) was synthesized by the solid-phase method using Fmoc strategy. Fmoc-Ala-resin amide was prepared by coupling a resin amide (0.5 mequiv./g) and Fmoc-Ala-OPfp (excess five-fold) in the presence of DMAP (0.3 *M*) and HOBt (0.3 *M*) in DMF. The Fmoc-Ala-resin amide was split into seven packs for the coupling of each Fmoc-X-OPfp amino acid (excess five-fold) in the presence of HOBt (0.3 *M*) or Fmoc-Arg-OH amino acid (excess five-fold) in presence of HOBt (0.3 *M*) and DIPCDI (excess five-fold). The seven packs were combined, mixed and split again before coupling of the second X amino acid. The *Z*-AlaXXAla-resin amide was prepared from Fmoc-XXAla-resin amide and *Z*-Ala-OH (excess five-fold) in presence of HOBt (0.3 *M*) and DIPCDI (excess five-fold). Final cleavage of the 49-peptide library was done with 95% TFA and the product precipitated with diethyl ether. Between each step, the resin was washed with DMF and the Fmoc deprotection was realized with DMF–20% piperidine.

2.3. Hydrolysis of the 49-peptide library

The *Z*-AlaXXAla(amide) solutions were prepared in methanol with a concentration of about 5.6 g/l. The enzymatic hydrolysis of the library was carried out in 5 m*M* Tris–HCl buffer, pH 8.6 at 30°C with a substrate concentration of about 0.56 g/l (≈10 m*M*) (methanol 10%) and with an enzyme concentration about 0.3 μ*M*. After 2 h, the enzymatic reaction was blocked by thermal denaturation of the enzyme (10 min at 65°C) and stored at −20°C.

2.4. HPLC–MS method

The system consists of an HP 1100 series liquid chromatograph–mass spectrometer (Hewlett-Packard, France), with binary pump, vacuum degasser, autosampler, thermostated column compartment, diode-array detector, LC–MS interface and mass spectrometer. LC–MS was used with electrospray ionization (ESI). Complete system control and data analysis were done on a HP ChemStation for LC–MS.

The column was an Inertsil ODS 3 (150×3 mm, particle size 5 μm) thermostated at 40°C, with a pre-column (10×3 mm) with the same phase (Chrompack France, Les Ulis, France). Gradient elution was performed with (A) 10 m*M* ammonium acetate buffer, pH 6.6 and (B) acetonitrile as mobile phase. After 10 min of isocratic condition in A, the gradient was run from A to 30% B in 30 min and then in 5 min to 100% B. The flow-rate was 0.4 ml/min and the injection volume was 5–20 μl. The MS conditions are described in Table 1.

Table 1
MS conditions

Source	ESI
Ion mode	Positive
Capillary voltage	1800 V
Nebulizer pressure	40 p.s.i. (1 p.s.i. = 6894.76 Pa)
Drying gas flow (N_2)	11 l/min
Drying gas temperature	350°C
Scan	[50/600] u
Gain	5
Collision induced dissociation	35 V

3. Results and discussion

3.1. Design and synthesis of the peptide library

Concerning our previous results on serralysin specificity [4], we have decided to synthesize a medium-size library in which two alanines are specifically defined in the supposed P_2' and P_2 positions and randomized amino acids in P_1' and P_1 positions. The chosen library can be represented by the formula *Z*-AlaXXAla(amide). It was designed from our previous results concerning the synthesis and the evaluation of efficient substrates of thermolysin and an other metalloprotease from *Pseudomonas aeruginosa*, pseudolysin [12]. Such a library should then be suitable for further comparison of the activities of different bacterial proteases of these families. The choice of amino acids was made on the basis of molecular masses and hydrophobicity in order to be representative of all different classes of amino acids and having different molecular masses in order to avoid ambiguities in data analysis. We have then chosen Ala, Leu, Val, Phe, Ser, Arg and Glu, leading to a 49-mer library corresponding to 28 different molecular masses. The synthesis was performed as described in Experimental, using a solid-phase procedure according to Fmoc strategy.

3.2. LC–MS analysis of the library before hydrolysis

The library was first analyzed before hydrolysis, according to the methodology described in Experimental. The obtained chromatogram is shown in Fig. 1. The experimental elution conditions had been determined after different preliminary experiments, in order to allow the highest level of resolution. Identification of the peaks was made on the basis of ESI-MS data. Nevertheless, no obvious correlation could be observed between total ionic current (TIC) and UV data, mainly due to the fact that chromatographic peaks may contain several different peptides. In order to solve some possible ambiguities, we have compared the experimental elution times with values predicted by a commercial software (Peptide Companion, Ver. 1.24 CoshiSoft/PeptiSearch, Tucson, AZ, USA) using the methods developed by Guo et al. [13]. Fig. 2 demonstrates that there is a good

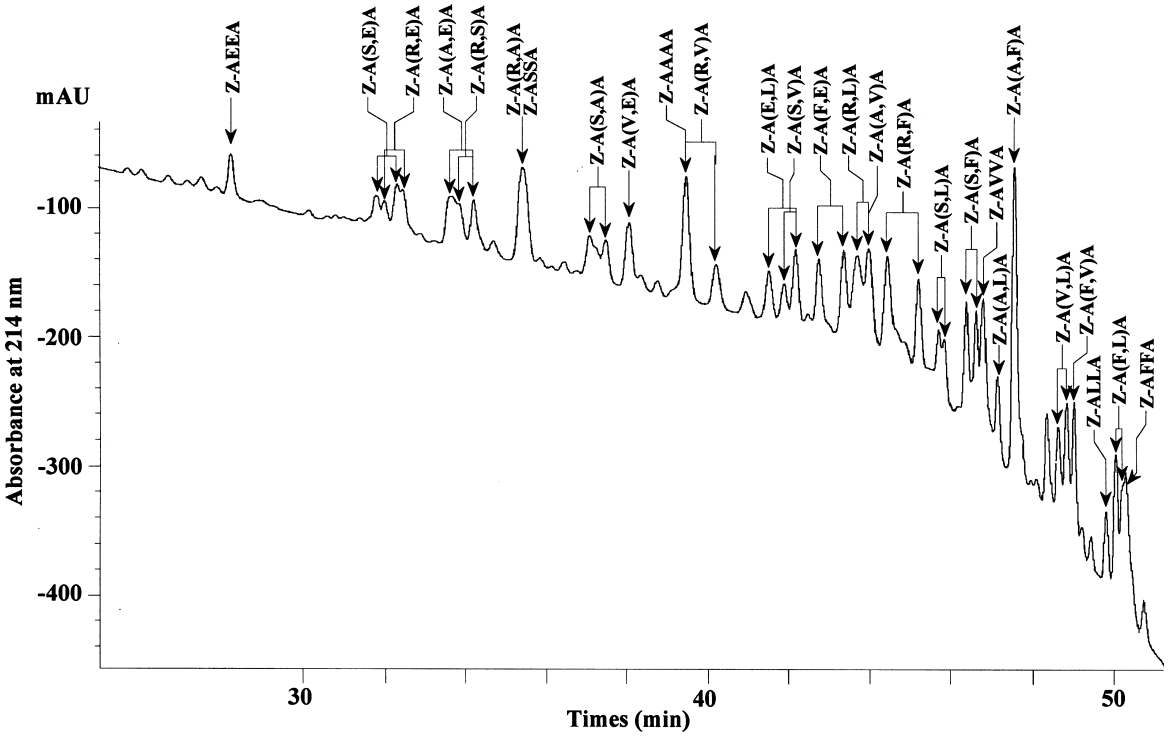


Fig. 1. HPLC of LC–MS analysis of 49-peptide library (0.56 g/l) in 5 m*M* Tris–HCl buffer, pH 8.6, 10% methanol. HPLC conditions: 0% B/A $\overset{10'}{\rightarrow}$0% B/A $\overset{30'}{\rightarrow}$30% B/A $\overset{5'}{\rightarrow}$100% B/A (A: 10 m*M* ammonium acetate buffer, pH 6.6; B: acetonitrile).

relationship between the experimental and predicted orders of elution. The only exception occurs for serine containing peptides which elute at later retention times than those predicted, but in this series, the order of elution is in good agreement with the hydrophobicity of the second varying amino acid. Assignment of the peptides of the library are indicated in Fig. 1.

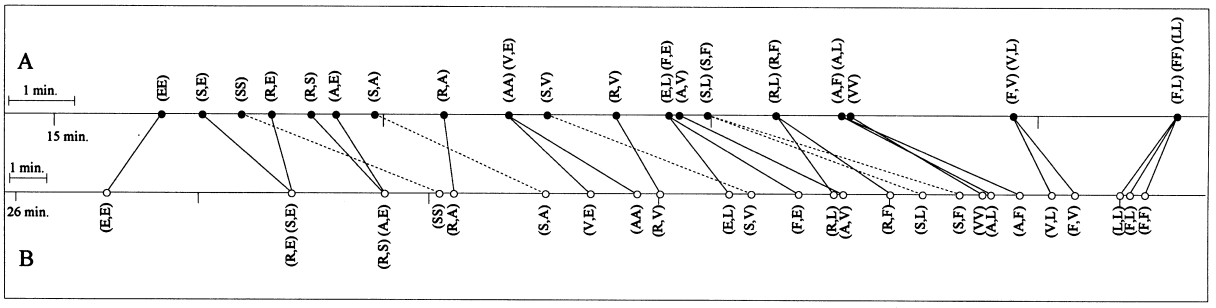


Fig. 2. Comparison of the retention times of *Z*-AlaXXAla(amide) either predicted using the methods of Guo et al. (A) measured in this study (B). The *Z*-AlaXXAla(amide) peptides are indicated only by the (X,X) amino acid residues.

3.3. LC–MS analysis of the library after hydrolysis

The hydrolysis pattern, after a 2-h hydrolysis is quite complex (Fig. 3), confirming the broad specificity of the enzyme. Nevertheless, LC–MS appears very useful for the identification of cleavage sites. Arrows on the figure indicate the identified products of hydrolysis. On the N-terminal side of the peptides of the library, *Z*-AlaSer, *Z*-AlaArg, *Z*-AlaAla, *Z*-AlaLeu and *Z*-AlaPhe appear to be quite easily split, whereas *Z*-AlaVal and *Z*-AlaGlu could not be evidenced. This result is confirmed by the fact that, for an elution time of 35.4 min, the peak of low intensity corresponds to *Z*-AlaSerSerAla(amide) alone, whereas in the initial library, *Z*-Ala(Arg,

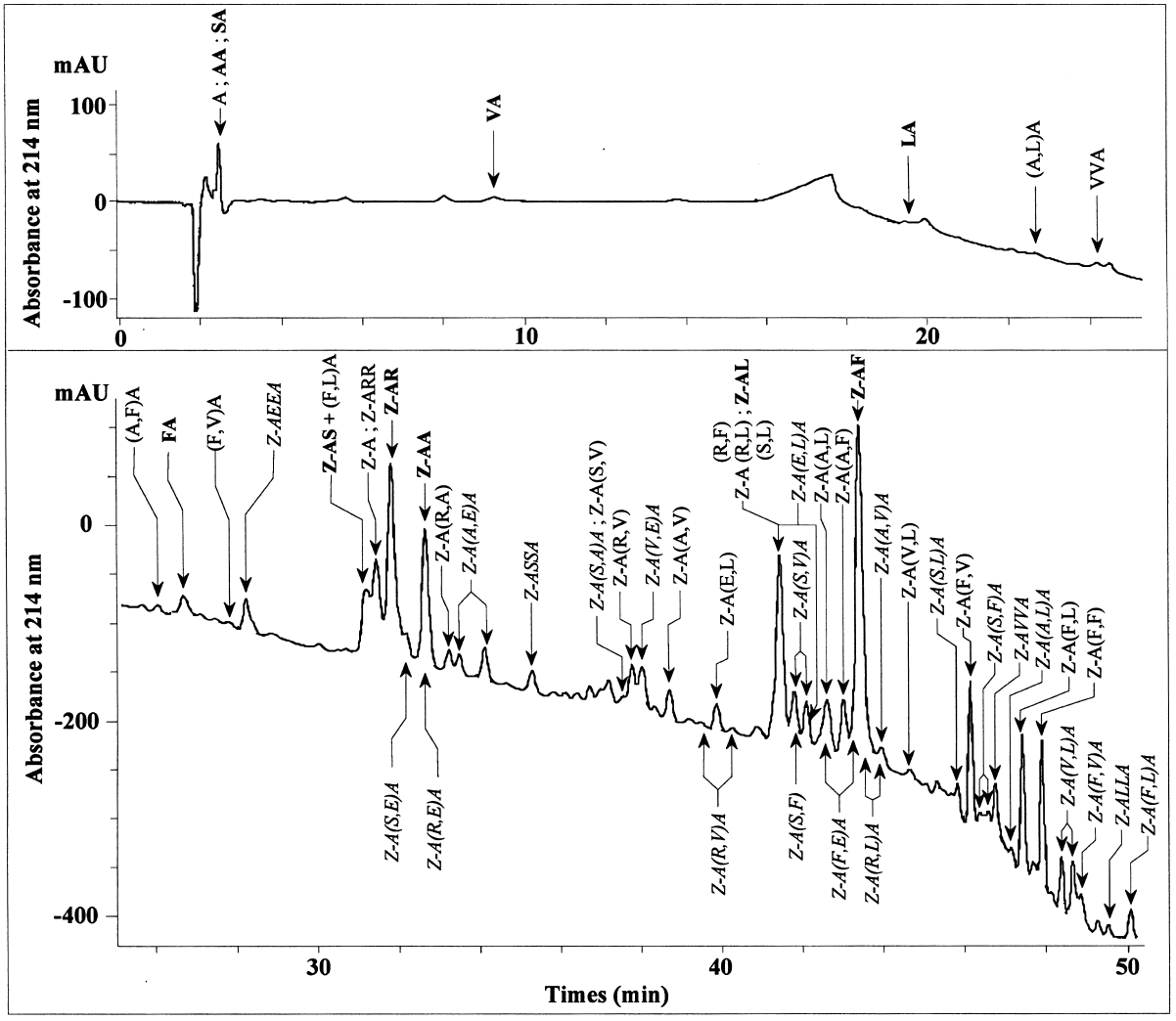
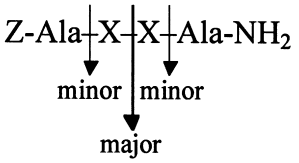


Fig. 3. HPLC of LC–MS analysis of 49-peptide library (0.56 g/l) hydrolysis by alkaline protease (0.3 μM) in 5 mM Tris–HCl buffer, pH 8.6, 10% methanol, after 2 h of enzymatic reaction. HPLC conditions: 0% B/A $\underset{10'}{\rightarrow}$ 0% B/A $\underset{30'}{\rightarrow}$ 30% B/A $\underset{5'}{\rightarrow}$ 100% B/A (A: 10 mM ammonium acetate buffer, pH 6.6; B: acetonitrile).

Ala)Ala(amide) are also present. The absence of this substrate in the hydrolysate, together with the identification of the presence of *Z*-AlaArg, *Z*-Ala(Arg, Ala), AlaAla(amide) and Ala(amide) confirm the cleavage of Arg–Ala and Ala–Ala bonds in the library. We may propose that *Z*-AlaArg-AlaAla(amide) is mainly hydrolyzed in *Z*-AlaArg and AlaAla(amide) and may be at a less extent, in *Z*-AlaArgAla and Ala(amide). Considering *Z*-AlaAlaArgAla(amide), cleavage in *Z*-AlaAlaArg and Ala(amide) were evidenced, but no data support a cleavage into *Z*-AlaAla and ArgAla(amide). Another important information derived from the LC–MS analysis is that minor cleavage sites may be evidenced after the first or before the last alanine, leading to the following hydrolysis pattern:



This result clearly indicates that two subsites allow a possible hydrolysis and that in this case the P_2 or P_2' position may be occupied either by the Z group or by the amide group, respectively. This information is supported by the identification of *Z*-Ala and of Ala(amide) even if their amounts are quite low.

In conclusion, considering the broad specificity of serralysin and the difficulties to study simultaneously hydrolysis of several substrates, due to the high number of cleavage sites, the use of a limited-size library which includes all classes of amino acids is of a great interest. Analysis of hydrolysis by LC–MS unambiguously identify the different cleavage sites and may allow a comparison of the specificity of two closely related proteases by discriminating some minor differences in their specificity. Furthermore, the analysis of the evolution of the chromatogram with time may be quantitatively used for measurement of hydrolysis rates at given cleavage sites. Such an analysis is currently in progress in the case of serralysin, in order to clarify the specificity and to compare the order of range of major and minor cleavage hydrolysis sites.

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