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Sequence-specific Ni(II)-dependent peptide bond hydrolysis for protein engineering: Active sequence optimization

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ABSTRACT

In previous studies we showed that Ni(II) ions can hydrolytically cleave a peptide bond preceding Ser/Thr in peptides of a general sequence R_N–(Ser/Thr)–Xaa–His–Zaa–R_C, where R_N and R_C are any peptide sequences. A peptide library screening, assisted by accurate measurements of reaction kinetics for selected peptides, demonstrated the preference for bulky and aromatic residues at variable positions Xaa and Zaa [A. Krężel, E. Kopera, A.M. Protas, A. Wysłouch-Cieszyńska, J. Poznański, W. Bal, J. Am. Chem. Soc., 132 (2010) 3355–3366]. In this work we used a similar strategy to find out whether the next residue downstream to Zaa may influence the reaction rate. Using an Ac–Gly–Ala–Ser–Arg–His–Zaa–Baa–Arg–Leu–NH_2 library, with Zaa and Baa positions containing all common amino acids except of Cys, we found a very strong preference for aromatic residues in both variable positions. This finding significantly limits the range of useful Xaa, Zaa and Baa substitutions, thus facilitating the search for optimal sequences for protein engineering applications [E. Kopera, A. Belczyk-Ciesielska, W. Bal, PLoS One 7 (2012) e36350].

1. Introduction

The sequence specific cleavage of the peptide bond has been an issue of interest in protein biotechnology. We thoroughly studied the hydrolysis assisted by Ni(II) ions in R_N–(Ser/Thr)–Xaa–His–Zaa–R_C peptide sequences. First, we found that hydrolysis occurred when Xaa was Ala or His, Zaa was Lys, and R_N and R_C were nonspecific N-terminal and C-terminal peptide sequences. The R_N–(Ser/Thr) peptide bond was uniquely hydrolyzed in these peptides in the presence of Ni(II) ions above pH 7 [1–4]. Aiming to find a general set of rules relating the substitutions at Xaa and Zaa and the rate of hydrolysis, we prepared a combinatorial library of R_1–(Ser/Thr)–Xaa–His–Zaa–R_2 peptides. In this library Xaa residues included 17 common R-amino acids (except Asp, Glu, and Cys), and Zaa residues included 19 common R-amino acids, except Cys. The leaving group R_1 was Ac–Gly–Ala, and the invariable C-terminal tail R_2 was Lys–Phe–Leu–NH_2. Matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry was used for screening the hydrolysis of peptides. The statistical analysis of screening results indicated that bulky and hydrophobic amino acids were preferred in Xaa and Zaa positions, and Ser was preferred over Thr in the alternative position preceding Xaa [5]. These results were confirmed by accurate studies of reaction kinetics for selected peptides. In the follow-up study, we deciphered the multi-step mechanism of this reaction. The crucial element of this mechanism is the formation of the intermediate ester, resulting from the acyl shift from the Ser/Thr amide side chain hydroxyl. This ester hydrolyzes spontaneously in water, yielding two peptide products R_1 and (Ser/Thr)–Xaa–His–Zaa–R_2, with the Ni(II) ion bound to the latter peptide. The role of the Ni(II) ion in the reaction is structural, rather than catalytic [6].

According to those results the optimal condition of Ni(II)-dependent peptide bond hydrolysis is pH of 8.2 or above and temperature of above 40 °C. We demonstrated the usefulness of this reaction in protein purification, as a tool for the specific removal of affinity tag from a thermostable fusion protein [7]. However, to enable broader biotechnological use of this method further sequence optimization is required, so that milder reaction conditions could be applied. We therefore decided to systematically review the effects of substitutions in positions Zaa and Baa on the rate of hydrolysis of peptide bond preceding Ser in R_N–Ser–Arg–His–Zaa–Baa-sequences (Baa was invariably Lys in R_1–(Ser/Thr)–Xaa–His–Zaa–R_2 peptides). For this purpose, we synthesized a combinatorial library of R_1–Ser–Arg–His–Zaa–Baa–R_3 peptides, where R_1 is Ac–Gly–Ala, and R_3 is Arg–Leu–NH_2, studied their Ni(II)-related hydrolysis using MALDI-TOF mass spectrometry, and performed a thorough statistical analysis of relations between the reaction rate and the peptide sequence. The reason for changing the C-terminal dipeptide from the previous Phe–Leu to Arg–Leu was the necessity to assure the presence of the positive charge in all peptides to enable time-of-flight detection in the MALDI experiment. In the previous library it was accomplished by the Lys residue, which became the variable Baa

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residue in the current study. We verified the library screening results for selected R_1–Ser–Arg–His–Zaa–Baa–R_3 peptides using HPLC. In order to provide correspondence with the previous library study [5] a second set of peptides was studied, differing by the C-terminal substitution. These peptides contained the last two amino acids of the previously used R_2 sequence, R_4: Phe–Leu–NH_2. Eventually, we modeled the lowest energetic structures of the peptide characterized by the highest Ni(II) dependent hydrolysis rate.

2. Experimental

2.1. Materials

N-α-9-fluorenylmethyloxycarbonyl amino acids (F-moc amino acids) were purchased from Sigma-Aldrich and Fluka Co. Trifluoroacetic acid (TFA), piperidine, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), triisopropylsilane (TIS), N,N-diisopropylethylamine (DIEA) and Ni(II) chloride hexahydrate were obtained from Sigma-Aldrich. TentaGel® S RAM resin was obtained from Rapp Polymer Inc. Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. Pure sodium hydroxide was obtained from Chempur. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (≥99.5%) was purchased from Carl Roth GmbH.

2.2. Peptide library synthesis

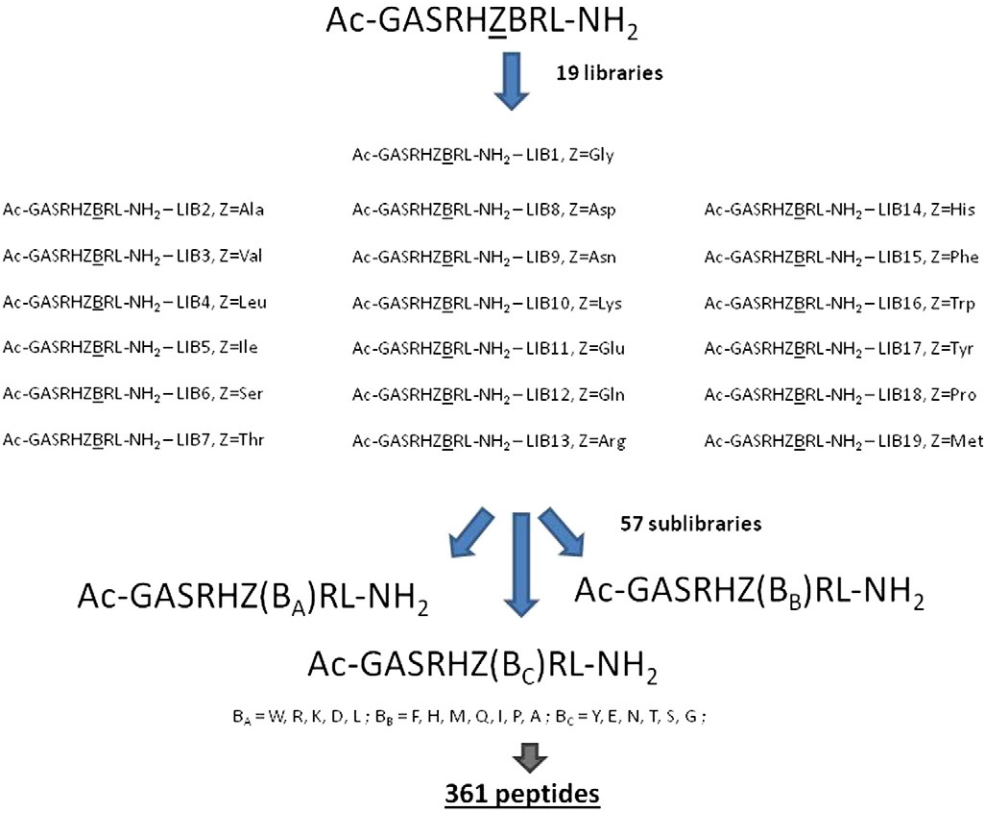
Solid-phase peptide synthesis of the library was performed automatically (Prelude Peptide Synthesizer, Protein Technologies) by the Fmoc/t-Bu strategy [8]. The general formula of the library, illustrated in Scheme 1, was Ac-GASRHZBRL-NH_2 with variable positions Z and B (Z corresponds to the three-letter label Zaa, and B corresponds to Baa). The peptides were synthesized with Z known and B randomized in three batches containing equal amounts of respective amino acids. Batch A = (W, R, K, D, L, V), Batch B = (F, H, M, Q, I, P, A), and Batch C = (Y, E, N, T, S, G) were chosen to provide the minimal possible overlap of peptide signals in the MALDI-TOF spectrum. This approach generated 57 sublibraries, which were desalted and purified by HPLC (Breeze, Waters) using an analytical C_{18} column (ACE, 250 × 4.6 mm) monitored at 220 and 280 nm. The eluting solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in water, and solvent B was 0.1% (v/v) TFA in 90% (v/v) acetonitrile. The sequences were confirmed using a Q-Tof1 ESI-MS spectrometer (Waters).

2.3. Peptide synthesis

Individual peptides were synthesized in the solid phase according to the F-moc protocol [8]. All syntheses were accomplished on TentaGel S RAM resin using HBTU as the coupling reagent, and Fmoc-AA, both in the presence of DIEA (1:1:2 in DMF). In all cases the cleavage was done manually by a reagent composed of 95% TFA, 2.5% TIS and 2.5% water. The acetylation of the N-terminus was carried out in 20% of acetic anhydride in dichloromethane (DCM). The peptides were purified by HPLC (Breeze, Waters) using an analytical C_{18} column (ACE, 250 × 4.6 mm) monitored at 220 and 280 nm. The eluting solvent A was 0.1% (v/v) TFA in water, and solvent B was 0.1% (v/v) TFA in 90% (v/v) acetonitrile. The sequences were confirmed using a Q-Tof1 ESI-MS spectrometer (Waters). Sequences of these individually synthesized peptides are shown in Table 1.

2.4. MALDI-TOF screening of hydrolysis progress in sublibraries

The hydrolysis tests were performed in 20 mM HEPES at pH 8.2 and temperature of 37 °C in the thermoblock (J.W. Electronics), controlled within ±0.2 °C. The samples contained 1 mM peptide sublibrary, 0.17 mM Internal Standard Peptide (ISP) (FTPPVQAAYQK) and



Scheme 1. The strategy of library synthesis. There are 361 Ac–Gly–Ala–Ser–Arg–His–Zaa–Baa–Arg–Leu–NH_2 peptides in 57 sublibraries, generated by three batches of Baa residues for each of 19 Zaa residues.