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Preparation of Disulfide-Bonded Polypeptide Heterodimers by Titration of Thio-Activated Peptides with Thiol-Containing Peptides

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Abstract—Titration of 2-pyridinesulfenyl (SPyr)-protected cysteine-containing peptides with thiol-unprotected peptides has proven to be a superior procedure to selectively generate disulfide-linked peptide heterodimers preventing the formation of homodimers. This procedure was used to synthesize large amounts of highly purified peptide heterodimers consisting of intracellularly active moieties, that were coupled to the third α-helix of the transcription factor Antennapedia, which serves as a 'shuttle tag'. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Receptor-independent uptake of exogenous molecules into living cells provides a powerful tool for studying cellular functions or developing new therapeutic strategies. In a recent preclinical study, the proliferation of cultured leukemic cells from chronic myeloid leukemia (CML) patients was strongly reduced by the application of a heterodimeric membrane-penetrating peptide.[1] A 'shuttle tag' corresponding to the third α-helix of the *Drosophila* transcription factor Antennapedia (**Antp**), the currently best characterized sequence promoting intracellular delivery,[2] was coupled via a disulfide-bond with a biologically active peptide which exhibits binding of high affinity and selectivity to the first SH3 domain [SH3(1)] of the cytoplasmic adapter protein CRKL. By its SH3(1) domain, CRKL can bind to specific sequences of the oncoprotein Bcr-Abl, which is found in over 90% of CML patients. The disruption of Bcr-Abl/CRKL complexes by the application of heterodimeric membrane-penetrating peptides results in a strong reduction of leukemia cell proliferation and thus emphasizes a major role of CRKL in the disease.[3]

To cover the corresponding preclinical assays statistically, the experiments for each patient had to be performed independently several times in a dose-dependent manner. Therefore, heterodimeric peptides—biologically active and inactive (as a control)—were needed in amounts of at least 100 mg. The synthesis of large amounts of disulfide-linked heterodimers usually requires the activation of the thiol function of one of the two peptides followed by the addition of the second peptide with a free thiol function (Scheme 1). The activated compound then undergoes a disulfide exchange reaction to form the peptide heterodimer. Widely used activators are sulfenyl-activating groups, such as 2-nitrophenyl (Nps), 2-pyridinesulfenyl (SPyr) and 3-nitro-2-pyridinesulfenyl (Npys).[4] The main problem during the heterodimer formation is often a considerable generation of homodimers as unwanted by-products.[5] This side reaction appears to be dependent on the sequence of the thiol-containing peptide. For the application in preclinical trials with a high purity of active peptide heterodimers as a prerequisite, the chromatographic purification of a product mixture consisting of homo- and heterodimers can be very difficult or almost impossible when the peptides have similar retention times during RP-HPLC.

In this report, we describe a procedure counteracting the undesired homodimer formation occurring during the disulfide coupling of the **Antp** 'shuttle tag' to the *h*igh *a*ffinity *C*RKL-SH3(1) *b*inding *p*eptide (**HACBP**) and the inactive control peptide (**GGLL**) (Fig. 1).

Results and Discussion

During the disulfide-coupling of the peptides **HACBP** and **GGLL** with the thioactivated **Antp-SPyr**, we observed that under standard conditions (30 min stirring of equimolar amounts in 0.1 M NH_4OAc, pH 6.5) used for the heterodimer formation, homodimers of **HACBP** and **GGLL**

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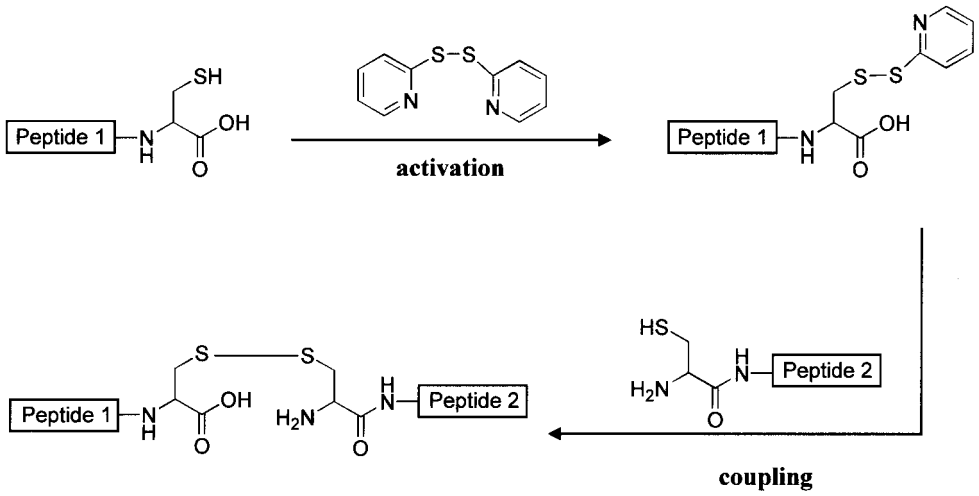

Scheme 1.

Antp KKWKMRRNPFWIKIQRC
Antp-SPyr KKWKMRRNPFWIKIQRC(SPyr)
HACBP CGIRVVDNSPPPALPPKRRRSAPSPTRV-$CONH_2$
GGLL CGIRVVDNSPPGALGPLLRRSAPSPTRV-$CONH_2$

Antp-SS-GGLL KKWKMRRNPFWIKIQRC
CGIRVVDNSPPGALGPLLRRSAPSPTRV-$CONH_2$

Antp-SS-HACBP KKWKMRRNPFWIKIQRC
CGIRVVDNSPPPALPPKRRRSAPSPTRV-$CONH_2$

Figure 1. Amino acid sequences of peptides used in this study. Disulfide bonds between the single peptide chains are indicated.

were formed as by-products. Fig. 2A shows that for **Antp-SS-GGLL** about 30% are disulfide-bonded homodimers of the peptide **GGLL**. Peak (1) in Fig. 2A corresponds to the desired heterodimer **Antp-SS-GGLL** with a molecular weight of 5202.3 Da ($mw_{calculated}$=5203.3 Da), Peak (2) corresponds to the unwanted homodimer of peptide GGLL which was identified by the molecular weight of 5768.6 Da ($mw_{calculated}$=5768.8 Da). We therefore optimized the coupling procedure using a titration method where the peptide **GGLL** was dissolved in 0.1% HOAc and added dropwise to a solution of Antp-SPyr over a reduced period of 5 min. The disulfide exchange was then stopped by addition of pure TFA (details see Experimental section). Under these conditions, the reaction resulted almost exclusively in the formation of the desired heterodimer (Fig. 2B). Using the titration procedure, an increased yield of 51% (isolated yield of the coupling reaction) of the heterodimeric product was obtained, while the yield using the conventional method was less than 20%. This synthetic procedure also worked well for the synthesis of biologically active **Antp-SS-HACBP**. Correspondingly, a yield of 72% was obtained (23% by the conventional method). It appears that by using the titration method, the unfavorable homodimer formation by combining equimolar amounts of thio-activated and thiol compound is strongly reduced and results in an at least 2.5-fold increase in yields of purified heterodimers. The titration method was successfully carried out for the generation of further peptide heterodimers, which are currently tested on leukemia cells.

In conclusion, the formation of cysteine-linked heterodimers by titration of thio-activated peptides with thiol peptides provides a simple, rapid and efficient procedure,

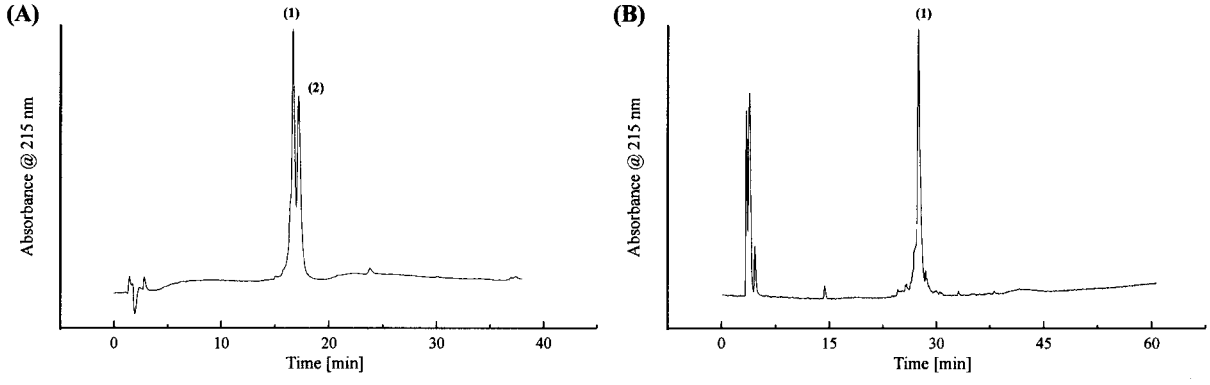

Figure 2. Analytical HPLC profiles of the reaction of **Antp-SPyr** with **GGLL**. (A) after 30 min of stirring equimolar amounts of the peptides in 0.1 M NH_4OAc, pH 6.5. Gradient: 20–70% B in 35 min. (B) After using titration method. Gradient: 10–70% B in 60 min. (1) **Antp-SS-GGLL** (heterodimer desired); (2) homodimer of **GGLL**.

which should be helpful for laboratories where a panel of disulfide-coupled peptides based on a modular system are needed for biological assays or preclinical tests. Moreover, the method should also be generally applicable for the heterodimeric coupling of non-peptide compounds.

Experimental

General

Fmoc-amino acids (L-configuration) used in this study were obtained from Orpegen (Heidelberg, Germany) and PerSeptive Biosystems (Wiesbaden, Germany). Protective groups were: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Gln(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc). HBTU was obtained from Perkin–Elmer/ABI (Weiterstadt, Germany), NMP was purchased from Merck (Darmstadt, Germany). Other reagents and solvents were of analytical or higher grade and obtained from Merck or Fluka (Neu-Ulm, Germany). Analytical HPLC was performed on a Vydac C18 column (The Separations Group, Hesperia, CA, 5 μm, 300 Å, 4.6×250 mm), solvent A: 0.07% TFA; solvent B: 0.05% TFA in 80% acetonitrile; flow rate 0.8 ml/min; UV-detection at 215 and 230 nm. Acetonitrile and TFA were purchased from Merck (Darmstadt, Germany) and were of gradient grade.

Peptide synthesis

Synthesis were carried out on an ABI433A (Perkin Elmer, Weiterstadt, Germany) synthesizer using standard Fmoc(9-fluorenylmethoxycarbonyl)/*tert*-butyl-based solid-phase peptide chemistry. Acylations were carried out using HBTU/HOBt activation in NMP at a scale of 0.1 mmol using a preloaded TentaGel-S-Trt-Cys(Trt)-Fmoc resin (Rapp Polymere, Tübingen) for **Antp**, and a Rink amide MBHA-resin (Novabiochem, Bad Soden) for **HACBP** and **GGLL**. After the chain assembly, the peptides were cleaved from the resin and deprotected using a cleavage cocktail consisting of 94% TFA, 3% ethandithiol and 3% H_2O (vol) for 120 min. The crude peptides were precipitated in ice-cold *tert*-butylmethylether (MTBE), washed twice with MTBE, and were vacuum-dried. After analyzing by reversed-phase HPLC on a Vydac C18 column (250×4.6 mm) and electrospray mass spectrometry (ES/MS) (Sciex API III, Perkin–Elmer) the crude peptides were used directly for the activation and coupling procedure. **Antp**: $mw_{calculated}$=2317.9 Da, mw_{exp}=2318.6 Da; **HACBP**: $mw_{calculated}$=3023.5 Da, mw_{exp}=3024.0 Da; **GGLL**: $mw_{calculated}$=2885.4 Da, mw_{exp}=2885.2 Da.

Antp-SPyr. 20 mg (92 μmol) 2,2′-dithiodipyridine (Aldrithiol™) were dissolved in 50 ml isopropanol (solution A) and 214 mg (92 μmol) **Antp** were dissolved in 400 ml 2 M HOAc (solution B). The solutions A and B were mixed and after 30 min stirring, the mixture was analyzed by HPLC and ES/MS, diluted with H_2O (1:2), and the activated product **Antp-SPyr** was purified by HPLC (0–80% solvent B in 80 min) on a preparative Vydac C18 column (47×250 mm, 300 Å, 15–20 μm, flow rate 40 ml/min, UV-detection at 215 nm). Fractions containing the pure product were pooled and lyophilized. **Antp-SPyr**: $mw_{calculated}$=2426.9 Da, mw_{exp}=2427.5 Da.

Antp-SS-GGLL and Antp-SS-HACBP. For the heterodimer formation under standard conditions, equimolar amounts (25 μmol) of **Antp-SPyr** and the peptide **GGLL** or **HACBP** were dissolved in 50 ml of 0.1 M NH_4OAc (pH 6.5) and stirred for 30 min.

For the heterodimer formation by titration, 25 μmol of the peptide **GGLL** or **HACBP** were dissolved in 10 ml 0.1% HOAc and added dropwise to a solution of **Antp-SPyr** (25 μmol dissolved in 40 ml of 1 M NH_4OAc, pH 6.0 over a reduced period of 5 min. 4 ml of pure TFA were then added to stop the disulfide exchange reaction. Thereafter, the mixture was analyzed by HPLC, the peaks which appeared were collected and their molecular weight was determined by ES/MS. The desired heterodimer was subsequently purified using preparative HPLC on a Vydac C18 column (47×250 mm, 300 Å, 15–20 μm, flow rate 40 ml/min, UV-detection at 215 nm). Fractions containing the pure products were pooled and lyophilized. **Antp-SS-GGLL**: $mw_{calculated}$=5203.2 Da, mw_{exp}=5202.3 Da; **Antp-SS-HACBP**: $mw_{calculated}$=5341.4 Da, mw_{exp}=5340.9 Da.

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