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ORIGINAL ARTICLE

Secretory overexpression and isotopic labeling of the chimeric relaxin family peptide R3/I5 in *Pichia pastoris*

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Abstract Relaxin family peptides are a group of peptide hormones with divergent biological functions. Mature relaxin family peptides are typically composed of two polypeptide chains with three disulfide linkages, rendering their preparation a challenging task. In the present study, we established an efficient approach for preparation of the chimeric relaxin family peptide R3/I5 through secretory overexpression in *Pichia pastoris* and in vitro enzymatic maturation. A designed single-chain R3/I5 precursor containing the B-chain of human relaxin-3 and the A-chain of human INSL5 was overexpressed in PichiaPink strain 1 by high-density fermentation in a two-liter fermenter, and approximately 200 mg of purified precursor was obtained from one liter of the fermentation supernatant. We also developed an economical approach for preparation of the uniformly ^{15}N-labeled R3/I5 precursor by culturing in shaking flasks, and approximately 15 mg of purified ^{15}N-labeled precursor was obtained from one liter of the culture supernatant. After purification by cation ion-exchange chromatography and reverse-phase high performance liquid chromatography, the R3/I5 precursor was converted to the mature two-chain form by sequential treatment with endoproteinase Lys-C and carboxypeptidase B. The mature R3/I5 peptide had an α-helix-dominated conformation and retained full receptor-binding and receptor activation activities. Thus, *Pichia* overexpression was an efficient approach for sample preparation and isotopic labeling of the chimeric R3/I5 peptide. This approach could also be extended to the preparation of other relaxin family peptides in future studies.

Keywords Relaxin family peptide · Overexpression · *Pichia pastoris* · Isotopic labeling

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Introduction

Relaxin family peptides are a group of peptide hormones with divergent biological functions (Bathgate et al. 2013; Chan et al. 2011; Ivell et al. 2011). The human genome encodes seven relaxin family peptides, including relaxin-1, relaxin-2, relaxin-3 (also known as INSL7), INSL3, INSL4, INSL5, and INSL6. To date, four G protein-coupled receptors (GPCRs) have been identified as relaxin family peptide receptors, namely RXFP1, RXFP2, RXFP3, and RXFP4 (Kong et al. 2010; van der Westhuizen et al. 2008). Among them, the homologous RXFP1 and RXFP2 are the cognate receptors of relaxin (relaxin-1 and relaxin-2) and INSL3, respectively (Hsu et al. 2002; Kumagai et al. 2002), while the homologous RXFP3 and RXFP4 are the cognate receptors of relaxin-3 and INSL5, respectively (Liu et al. 2003b, 2005a). RXFP1 and RXFP4 can also be activated by relaxin-3 in vitro (Liu et al. 2003a; Sudo et al. 2003). The receptors of INSL4 and INSL6 remain to be identified. In previous work, a chimeric R3/I5 peptide, containing the B-chain of relaxin-3 and the A-chain of INSL5, was found to be a selective agonist of RXFP3 and RXFP4 (Liu et al. 2005b). The europium-labeled R3/I5 peptide was prepared and used in receptor-binding assays due to its low

background binding (Shabanpoor et al. 2011; Zhang et al. 2013).

Most of the relaxin family peptides are typically composed of two polypeptide chains in their mature form. Their mature B-chain and A-chain are linked together by two inter-chain disulfide bonds, and the A-chain also contains an intra-chain disulfide bond. Due to their complex primary structure, preparation of mature relaxin family peptides is a challenging task. Conventionally, chemical peptide synthesis is used to prepare these complex peptides (Shabanpoor et al. 2013a; Wade et al. 2009). Alternatively, we recently established a recombinant expression approach for preparation of the relaxin family peptides through overexpression of designed single-chain precursors in *E. coli* and subsequent in vitro refolding and maturation (Luo et al. 2010a, b; Zhang et al. 2012a, b). In recent years, the *Pichia* expression system has been used for the successful preparation of some disulfide-rich proteins (Cregg et al. 2009; Damasceno et al. 2012; Li et al. 2007; Mattanovich et al. 2012). As the *Pichia* expression system can form correct disulfide linkages in vivo, in the present study, we used it for efficient preparation of the chimeric relaxin family peptide R3/I5 through secretory overexpression of a designed single-chain precursor and subsequent in vitro enzymatic maturation.

Materials and methods

DNA manipulation

The gene encoding the designed single-chain R3/I5 precursor was constructed from four chemically synthesized oligonucleotide primers through annealing and elongation by T4 DNA polymerase. After cleavage by restriction enzyme KpnI, the synthetic R3/I5 gene was ligated into the pPinkα-HC vector (Invitrogen, Carlsbad, CA, USA), which had been pre-cleaved by restriction enzymes StuI (blunt end) and KpnI (sticky end), to generate the pPinkα-HC/R3I5 expression construct. The nucleotide sequence of R3/I5 precursor was confirmed by DNA sequencing.

Secretory overexpression of the R3/I5 precursor in *Pichia pastoris*

The expression construct pPinkα-HC/R3I5 was linearized by restriction enzyme SpeI and then transformed into the PichiaPink strain 1 using the *Pichia* EasyComp™ Kit (Invitrogen). The transformants on selective PAD plates were then inoculated into 50 ml liquid BMGY medium and induced by methanol for 3 days according to the manual of the PichiaPink™ Expression System (Invitrogen). The secretory expression of the R3/I5 precursor was determined by tricine SDS-PAGE. The colony with the highest expression level was used for fermentation in a two-liter fermenter. The initial fermentation medium contained 10.9 g/l K_2SO_4, 8.9 g/l $MgSO_4$, 0.9 g/l $CaSO_4{\cdot}2H_2O$, 3.0 g/l NaOH, 6 g/l $(NH_4)_2SO_4$, 16 ml/l 85 % H_3PO_4, 20 g/l glycerol, 3 ml/l PTM1 trace elements, and 1.8 ml/l anti-foaming reagent. After autoclave sterilizing and cooling, the pH of the fermentation medium was adjusted to 5.0 by adding appropriate amount of 30 % ammonia solution. Thereafter, 50 ml of the overnight *Pichia* culture broth was seeded into the fermenter and continuously cultured overnight. Next day, 250 ml of 25 % glycerol solution (containing 1 ml of PTM1 trace elements) was added in a fed-batch manner within 8–10 h. Subsequently, approximately 600 ml of methanol (containing 6 ml PTM1 trace elements) was added in a fed-batch manner within 108 h to induce production of the R3/I5 precursor. During fermentation, the temperature was maintained at 30 °C. The pH was maintained at 5.0 before induction and at 3.0 after induction by the addition of 30 % ammonia solution. The dissolved oxygen level was maintained above 10 % by adjusting the stirring speed and aeration rate.

Uniform ^{15}N isotopic labeling of the R3/I5 precursor in *Pichia pastoris*

The *Pichia* colony with the highest expression level was first cultured in 50 ml of liquid YPD medium at 30 °C in a 250-ml flask. The yeast cells were then harvested by centrifugation (5000*g*, 5 min) and washed once with salt medium (4 g/l KH_2PO_4, 4 g/l K_2HPO_4, 0.4 g/l $CaCl_2$, 0.4 g/l NaCl, 2 g/l $MgSO_4$, and 2 ml/l PTM1 trace elements). Subsequently, the yeast cells were resuspended in 250 ml of the salt medium supplemented with 0.5 % $^{15}NH_4Cl$ and 2 % glucose and continuously cultured at 30 °C for 24 h in a two-liter flask. The yeast cells were then harvested by centrifugation (5000*g*, 5 min), resuspended into 250 ml of fresh salt medium supplemented with 0.5 % $^{15}NH_4Cl$, and continuously cultured at 30 °C for 84 h in a two-liter flask with methanol addition according to Fig. 4a. During culture, the salt medium was maintained at pH 3–4 by adding appropriate amount of 1 M KOH solution at 12-h intervals.

Purification of the R3/I5 precursor

The fermentation supernatant was first diluted (1:1) with water, and the pH was adjusted to 2.0 by adding appropriate amount of 1 M HCl solution. Thereafter, the diluted supernatant was loaded onto a cation ion-exchange column (sulfonic ethyl Sephadex) pre-equilibrated with 20 mM glycine buffer (pH 2.0). The supernatant of the ^{15}N-labeling broth was loaded directly onto the ion-exchange column.

After loading, the column was washed with 20 mM glycine buffer (pH 2.0), and the bound R3/I5 precursor was eluted by 20 mM glycine buffer (pH 2.0) containing 1.0 M NaCl. The fraction eluted from the ion-exchange column was then subjected to high performance liquid chromatography (HPLC), and the R3/I5 precursor was eluted from a C18 reverse-phase column by an acetic acetonitrile gradient. The eluted R3/I5 fraction was manually collected and lyophilized.

Enzymatic maturation of the R3/I5 precursor

The purified R3/I5 precursor was dissolved in digestion buffer (50 mM Tris-HCl, 2.0 M urea, pH 8.5) at the final concentration of approximately 5 mg/ml. Endoproteinase Lys-C stock solution (Sigma–Aldrich, St. Louis, MO, USA) was then added (1 U of enzyme to ~20 mg of peptide), and the digestion was carried out at 30 °C overnight. Thereafter, carboxypeptidase B stock solution was added (mass ratio of enzyme to peptide, 1:100), and the digestion was carried out at 30 °C for 2 h. Finally, the reaction mixture was acidified to pH 3.0 by adding appropriate amount of trifluoroacetic acid and subjected to HPLC. The mature R3/I5 peptide was eluted from a C18 reverse-phase column by an acidic acetonitrile gradient, manually collected, and lyophilized.

Circular dichroism spectroscopy

The mature R3/I5 peptides (with or without ^{15}N-labeling) were dissolved in 1.0 mM aqueous HCl solution (pH 3.0) and quantified by ultra-violet absorbance at 280 nm using the extinction coefficient (ε_{280nm}) of 6990 M^{-1} cm^{-1} calculated from the number of tryptophan and tyrosine residues in the peptide. The final peptide concentration was adjusted to 20 μM for circular dichroism measurements performed on a Jasco-715 spectropolarimeter at room temperature. Spectra were scanned from 250 to 190 nm using a quartz cuvette with a 1.0-mm path length. The software J-700 for Windows Secondary Structural Estimation (Version 1.10.00) was used for secondary structure content evaluation from the circular dichroism spectra.

Activity assays of the mature R3/I5 peptide

The receptor-binding assay was carried out according to a previously described procedure using DTPA/Eu^{3+}-labeled R3/I5 as a tracer (Zhang et al. 2013). The receptor activation assay was carried out as previously described using a cAMP response element-controlled nanoluciferase as a reporter (Wang et al. 2014; Zhang et al. 2014).

Results and discussion

Design of an R3/I5 precursor for secretory overexpression in *Pichia pastoris*

For secretory overexpression of R3/I5 in *Pichia pastoris*, we designed a single-chain R3/I5 precursor in which the B-chain of human relaxin-3 and the A-chain of human INSL5 were joined by a short peptide linker (Fig. 1a). A negatively charged tag, which improves the expression of single-chain insulin in yeast (Kjeldsen et al. 1999), was fused to the B-chain N-terminus of the R3/I5 precursor (Fig. 1b). The gene encoding the R3/I5 precursor was chemically synthesized using *Pichia*-optimized codons to improve the expression level. This synthetic gene was ligated into the pPinkα-HC expression vector, which encodes the leading sequence of the α-mating factor for secretory expression (Fig. 1c). This leading sequence would be removed in vivo by enzymatic cleavage after the dibasic site to allow secretion of the intact R3/I5 precursor into the medium. The R3/I5 precursor can be converted to the two-chain mature form in vitro by sequential endoproteinase Lys-C digestion and carboxypeptidase B treatment after purification from the fermentation supernatant (Fig. 1b).

Secretory overexpression, purification, and maturation of the R3/I5 precursor

After transformation of the pPinkα-HC/R3I5 construct into the PichiaPink strain 1, several transformants were used to test the expression level. After methanol induction, secretory expression of the R3/I5 precursor was confirmed by SDS-PAGE analysis, which also revealed variation in the expression levels of the transformants (data not shown). To achieve high-level expression, the *Pichia* transformant exhibiting the highest expression level in the small-scale test was cultured in a two-liter fermenter for 6 days (Fig. 2a). In the fermenter, the *Pichia* cell density (OD_{600nm}) reached approximately 450 at the final stage of fermentation (Fig. 2b). After harvesting by centrifugation, approximately 670 ml of supernatant and approximately 400*g* of wet cell pellet were obtained from one liter of the fermentation broth. Tricine SDS-PAGE (Fig. 2b, inner panel) analysis showed that the expression level of the R3/I5 precursor in the supernatant increased gradually after methanol induction, with a final yield of approximately 300 mg/l estimated from the band density compared with the purified R3/I5 precursor standard. Thus, the *Pichia* expression system was efficient for secretory overexpression of the designed single-chain R3/I5 precursor.

The R3/I5 precursor in the fermentation supernatant was purified according to a two-step procedure (Fig. 2a). First,

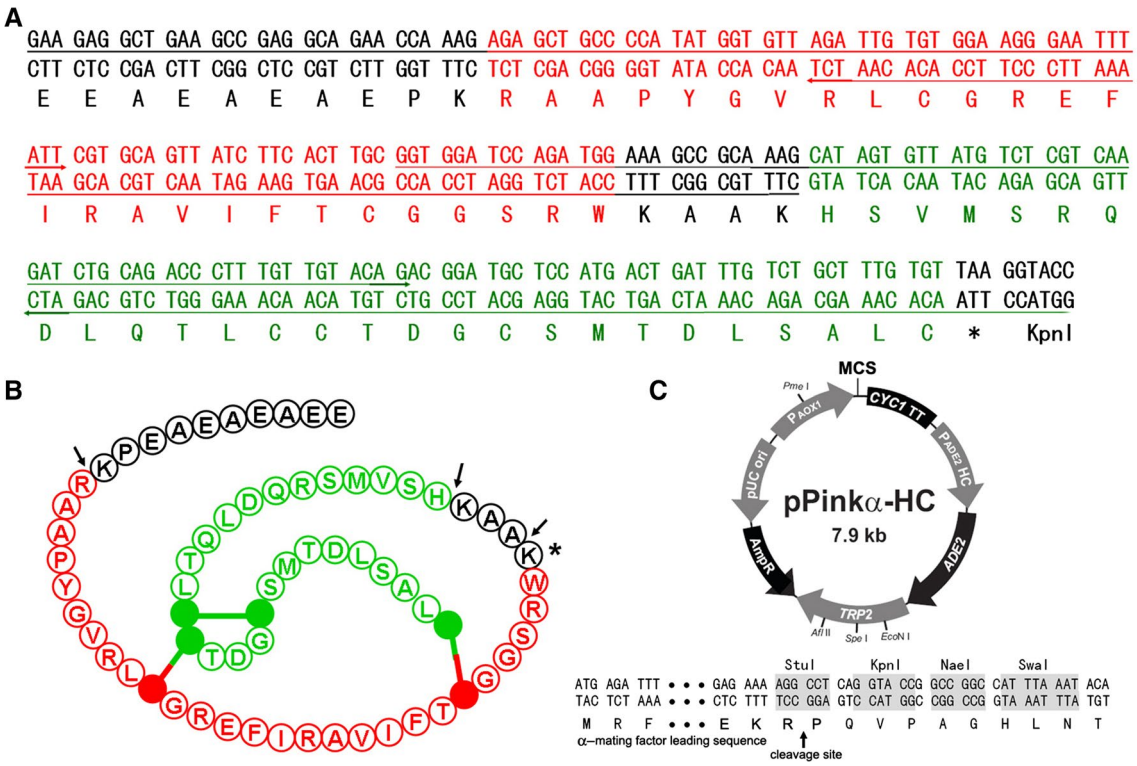

Fig. 1 **a** The nucleotide and amino acid sequences of the designed single-chain R3/I5 precursor for secretory overexpression in *Pichia pastoris*. The four nucleotide primers used for construction of the synthetic R3/I5 gene are *underlined*. The B-chain of human relaxin-3 is shown in red and the A-chain of human INSL5 in green. **b** A schematic presentation of the designed single-chain R3/I5 precursor. Cysteine residues are shown as *filled circles*, and disulfide bonds are shown as *sticks*. The endoproteinase Lys-C cleavage sites are indicated by *arrows*. The additional B-chain C-terminal Lys residue removed by carboxypeptidase B after Lys-C cleavage is indicated by an *asterisk*. **c** Vector map and multiple cloning site of pPinkα-HC vector for cloning of the R3/I5 precursor. The dibasic cleavage site of the α-mating factor leading sequence is indicated by an *arrow*

the fermentation supernatant was diluted (1:1) with water to decrease the ionic strength and then subjected to cation ion-exchange chromatography because the R3/I5 precursor was positively charged under a low pH condition. As analyzed by tricine SDS-PAGE (Fig. 3a, inner panel), the R3/I5 precursor (indicated by an asterisk) was present in the eluted fraction, while most of other proteins flowed through the ion-exchange column. Thereafter, the R3/I5 precursor eluted from the ion-exchange column was subjected to HPLC (Fig. 3a): a major peak (indicated by an asterisk) was eluted from a C18 reverse-phase column by an acidic acetonitrile gradient. After the major peak fraction (assuming the R3/I5 precursor) was lyophilized, approximately 200 mg of white powder was typically obtained from one liter of the fermentation supernatant.

The above lyophilized powder was then treated sequentially by endoproteinase Lys-C and carboxypeptidase B and then subjected to HPLC (Fig. 3b): a major peak (indicated by an asterisk) was eluted from a C18 reverse-phase column after enzymatic treatment. Mass spectrometry analysis (Fig. 3b, inner panel) confirmed that the major peak was the mature R3/I5 fraction (measured value 5956.0, theoretical value 5955.9). Approximately, 100 mg of mature R3/I5 peptide was typically obtained from one liter of the fermentation supernatant. Thus, the present *Pichia* overexpression and in vitro enzymatic maturation approach were efficient for preparation of the mature R3/I5 peptide.

Preparation of uniformly ^{15}N-labeled R3/I5 using the *Pichia* expression system

Nuclear magnetic resonance (NMR) technology has been widely used for solving protein structures. Incorporation of ^{15}N and/or ^{13}C isotopes can facilitate the protein structure solving. In a long-term goal, we hope to solve the structure of the receptor-bound R3/I5 peptide in order to identify its key residues interacted with the receptor, thus we need an efficient approach for preparation of the stable isotope-labeled R3/I5 peptide. We had tried the *E. coli* expression system, but the expression level of the R3/I5 precursor was very low in the labeling medium. In this study, we have shown that the R3/I5 precursor can be efficiently overexpressed in *Pichia pastoris*, which grows well in salt medium suitable for stable isotope incorporation. Thus, we

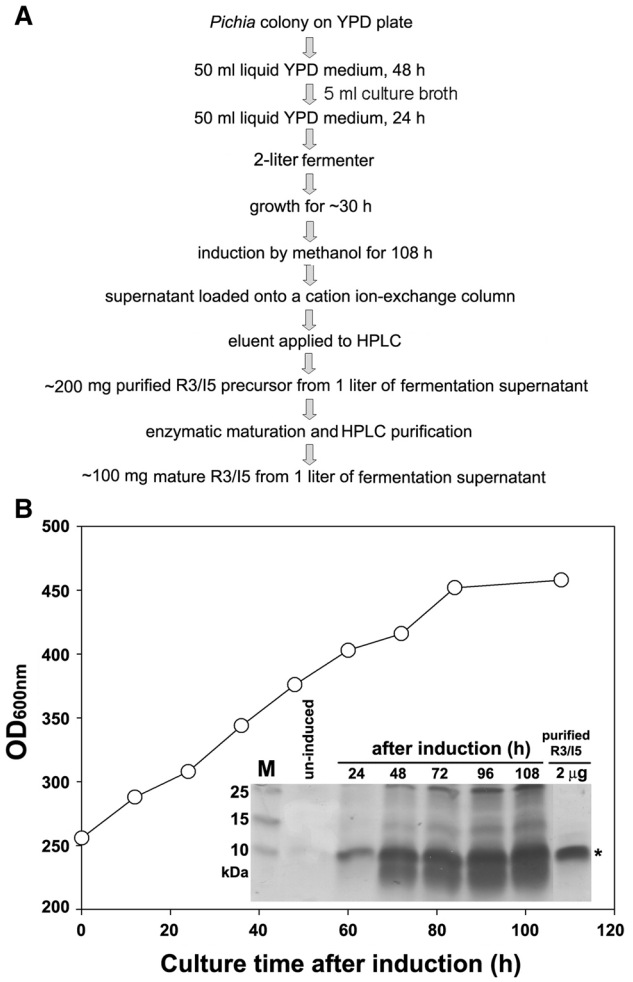

Fig. 2 **a** The procedure for preparation of the mature R3/I5 peptide through overexpression of a single-chain precursor in *Pichia pastoris* and in vitro enzymatic maturation. **b** The cell density curve during the methanol induction stage of fermentation. *Inner panel* tricine SDS-PAGE analysis of the R3/I5 precursor expression during fermentation. An aliquot (10 µl) of the fermentation supernatant was loaded onto a 16.5 % tricine SDS-polyacrylamide gel, which was stained with Coomassie brilliant blue R250 after electrophoresis

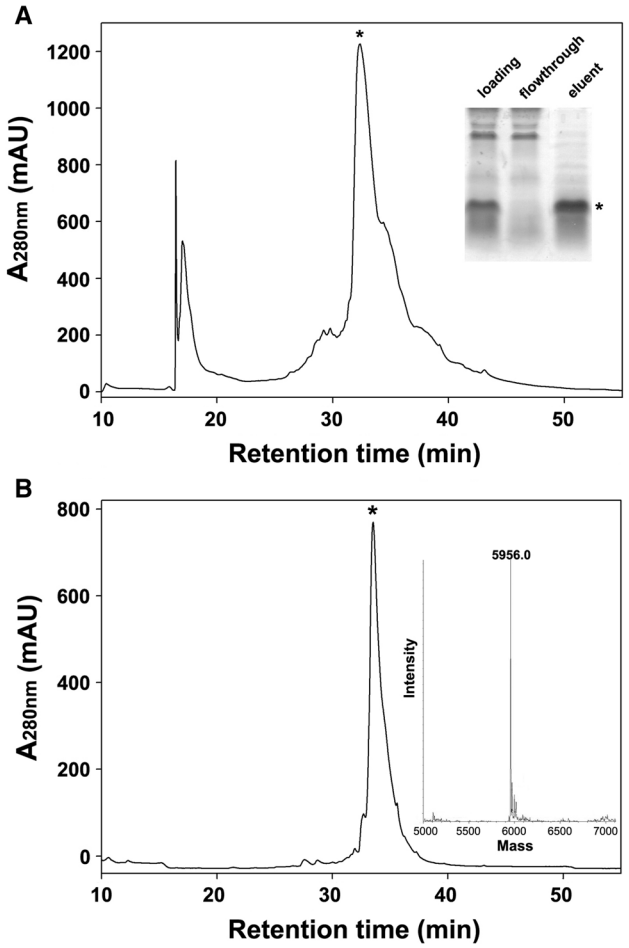

Fig. 3 **a** HPLC purification of the R3/I5 precursor. The R3/I5 precursor fraction eluted from the ion-exchange chromatography was loaded onto a C18 reverse-phase column and eluted by an acidic acetonitrile gradient. The R3/I5 precursor fraction is indicated by an *asterisk*. *Inner panel* tricine SDS-PAGE analysis of the flow through and eluent fractions of the ion-exchange chromatography. The band of the R3/I5 precursor is indicated by an *asterisk*. **b** HPLC purification of the mature R3/I5 peptide after endoproteinase Lys-C and carboxypeptidase B treatment. The digestion mixture was loaded onto a C18 reverse-phase column and eluted by an acidic acetonitrile gradient. The mature R3/I5 peptide fraction is indicated by an *asterisk*. *Inner panel* mass spectrometry analysis of the mature R3/I5 peptide

attempted to prepare the ^{15}N-labeled R3/I5 peptide using the *Pichia* expression system.

High-density fermentation can improve the protein expression level, but this approach has high consumption of nitrogen (such as NH_4Cl) and carbon (CH_3OH) sources, which are very expensive in the ^{15}N and ^{13}C forms. Thus, we developed an economical approach for ^{15}N isotopic labeling of the R3/I5 precursor by culturing the yeast cells in shaking flasks (Fig. 4a). The yeast cells grew well in the labeling medium, although the cell density was not very high (Fig. 4b). SDS-PAGE analysis showed that the R3/I5 precursor was expressed after methanol induction (Fig. 4b, inner panel). The ^{15}N-labeled R3/I5 precursor was also purified using the two-step procedure (ion-exchange chromatography and HPLC). The eluted fraction from the ion-exchange column was then subjected to HPLC (Fig. 5a): a major peak (indicated by an asterisk) was eluted from a C18 reverse-phase column. This fraction was lyophilized and then sequentially treated with endoproteinase Lys-C and carboxypeptidase B. After enzymatic treatment, a major peak (indicated by an asterisk) appeared on HPLC (Fig. 5b). The measured molecular mass of the major peak was 6033.0 (Fig. 5b, inner panel), which had a molecular mass increase of 77.0 compared with the measured molecular mass (5956.0) of the unlabeled mature R3/I5. Thus, all nitrogen atoms (77 in total) in the labeled R3/I5 were

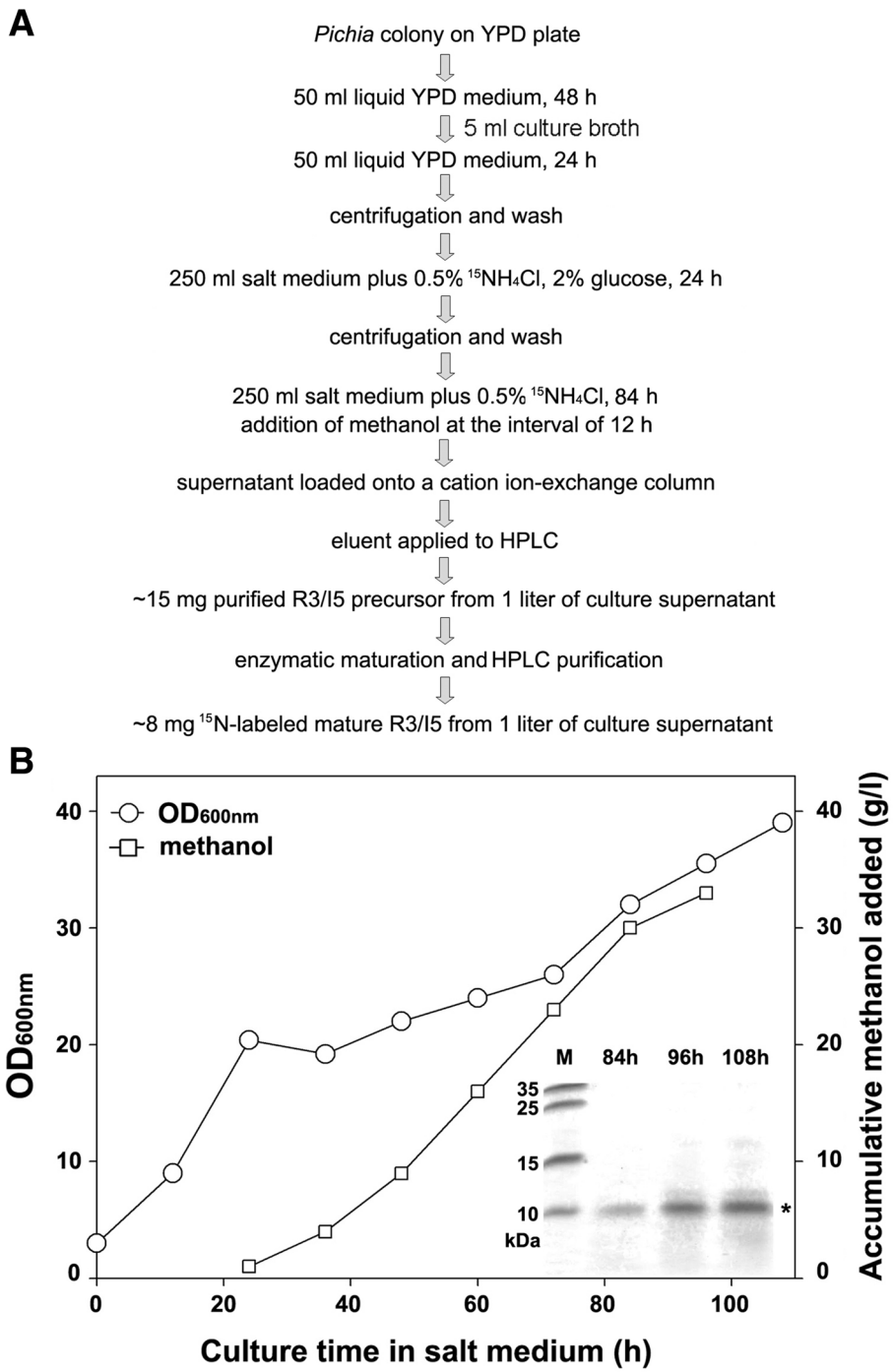

Fig. 4 **a** The procedure for preparation of the uniformly ^{15}N-labeled R3/I5 peptide. **b** Cell density curve and methanol addition curve. *Inner panel* tricine SDS-PAGE analysis of the ^{15}N-labeled R3/I5 precursor expression. An aliquot (20 µl) of the culture supernatant was loaded onto a 16.5 % tricine SDS-polyacrylamide gel, which was stained with Coomassie brilliant blue R250 after electrophoresis

occupied by heavy ^{15}N atoms, confirming the high efficiency of ^{15}N incorporation using the described economical approach. After purification and maturation, approximately 8 mg of mature ^{15}N-labeled R3/I5 peptide was typically obtained from one liter of the labeling broth. This procedure is compatible with uniform isotopic labeling by ^{13}C or by both ^{15}N and ^{13}C. Thus, our present work provided an efficient approach for preparation of the stable isotope-labeled R3/I5 peptide at a low cost.

Characterization of the mature R3/I5 peptide

The secondary structure of the recombinant R3/I5 peptide was assessed by circular dichroism (Fig. 6a). Both the unlabeled and the ^{15}N-labeled R3/I5 peptides showed an α-helix-dominated conformation (the estimated α-helix content for both peptides was approximately 45 %), suggesting that the recombinant R3/I5 peptides had native conformation with correct disulfide linkages. In

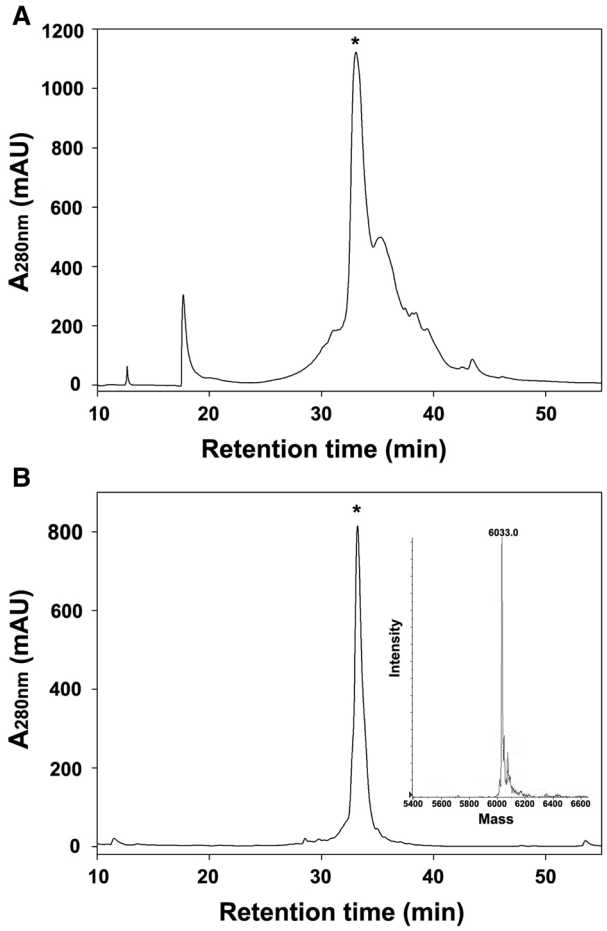

Fig. 5 **a** HPLC purification of the uniformly ^{15}N-labeled R3/I5 precursor. The R3/I5 precursor fraction eluted from the ion-exchange chromatography was loaded onto a C18 reverse-phase column and eluted by an acidic acetonitrile gradient. The ^{15}N-labeled R3/I5 precursor fraction is indicated by an *asterisk*. **b** HPLC purification of the uniformly ^{15}N-labeled mature R3/I5 peptide after endoproteinase Lys-C and carboxypeptidase B treatment. The digestion mixture was loaded onto a C18 reverse-phase column and eluted by an acidic acetonitrile gradient. The mature ^{15}N-labeled R3/I5 peptide fraction is indicated by an *asterisk*. *Inner panel* mass spectrometry analysis of the uniformly ^{15}N-labeled mature R3/I5 peptide

receptor-binding assays (Fig. 6b) and receptor activation assays (Fig. 6c), the measured pIC_{50} and pEC_{50} values of the unlabeled and the ^{15}N-labeled mature R3/I5 peptides were similar to those of the *E. coli* overexpressed relaxin-3, suggesting that both the unlabeled and the ^{15}N-labeled mature R3/I5 peptides retained full activities toward receptor RXFP3. Therefore, the *Pichia* overexpression system provides an efficient approach for preparation of the fully active mature R3/I5 peptide at low cost.

In the competition receptor-binding assays (Fig. 6b), IC_{50} values are conventionally used to measure the relative receptor-binding potencies of different ligands. IC_{50} is the ligand concentration that can inhibit 50 % binding of the tracer with the receptor. Thus, the measured IC_{50} is related to the tracer concentration used in the assays, and it does not mean the receptor-binding affinity that is conventionally measured by the dissociation constant (K_d). The K_d of relaxin-3 with receptor RXFP3 is approximately 300 pM measured using the ^{125}I-labeled relaxin-3 (Liu et al. 2003b). In our present assay, a non-radioactive europium-labeled R3/I5 was used as a tracer. When nanomolar range of the europium-labeled R3/I5 was used in the competition assays, the measured IC_{50} values of relaxin-3 and R3/I5 with receptor RXFP3 were typically 10-30 nM, assayed either in our laboratory or in other laboratories (Shabanpoor et al. 2012, 2013b; Zhang et al. 2012b, 2013, 2014). In the receptor activation assays (Fig. 6c), EC_{50} values are conventionally used to measure the relative receptor activation potencies of different ligands. EC_{50} is the ligand concentration that can cause 50 % of the maximal response. In our present work, HEK293T cells transiently transfected with receptor RXFP3 and a cAMP response element-controlled NanoLuc reporter were used for the receptor activation assays (Zhang et al. 2014). Due to high sensitivity of the NanoLuc reporter, the measured EC_{50} values of relaxin-3 to receptor RXFP3 were typically ~30 pM, suggesting that activation of a small percent of the overexpressed RXFP3s could reach half of the maximal effect. Thus, the measured EC_{50} of relaxin-3 to RXFP3 was ~10 % of the K_d value of relaxin-3 with receptor RXFP3.

Application of the *Pichia* expression system to other relaxin family peptides

In our present study, the chimeric relaxin family peptide R3/I5 was efficiently overexpressed in *Pichia pasteris*, with the highest expression level reported so far. After purification using a simple two-step procedure, the single-chain precursor was enzymatically converted to the fully active mature R3/I5 peptide with a final yield of approximately 100 mg per liter of the fermentation supernatant. After further improvement of the fermentation conditions, the yield might be further increased. The *Pichia* expression system is suitable for large-scale fermentation because the high yield transformant is stable due to integration of the foreign gene into the host genome. Furthermore, the *Pichia* cells grow rapidly in cheap salt medium and can reach high cell density. Thus, the method established in the present work is suitable for large-scale preparation of R3/I5 peptide at low cost. Additionally, we also provided an economical procedure for preparation of the uniformly stable isotope-labeled R3/I5 peptide using the *Pichia* overexpression system. In future work, other relaxin family peptides could also be overexpressed and isotopically labeled using the *Pichia* expression system for ligand-receptor interaction studies.

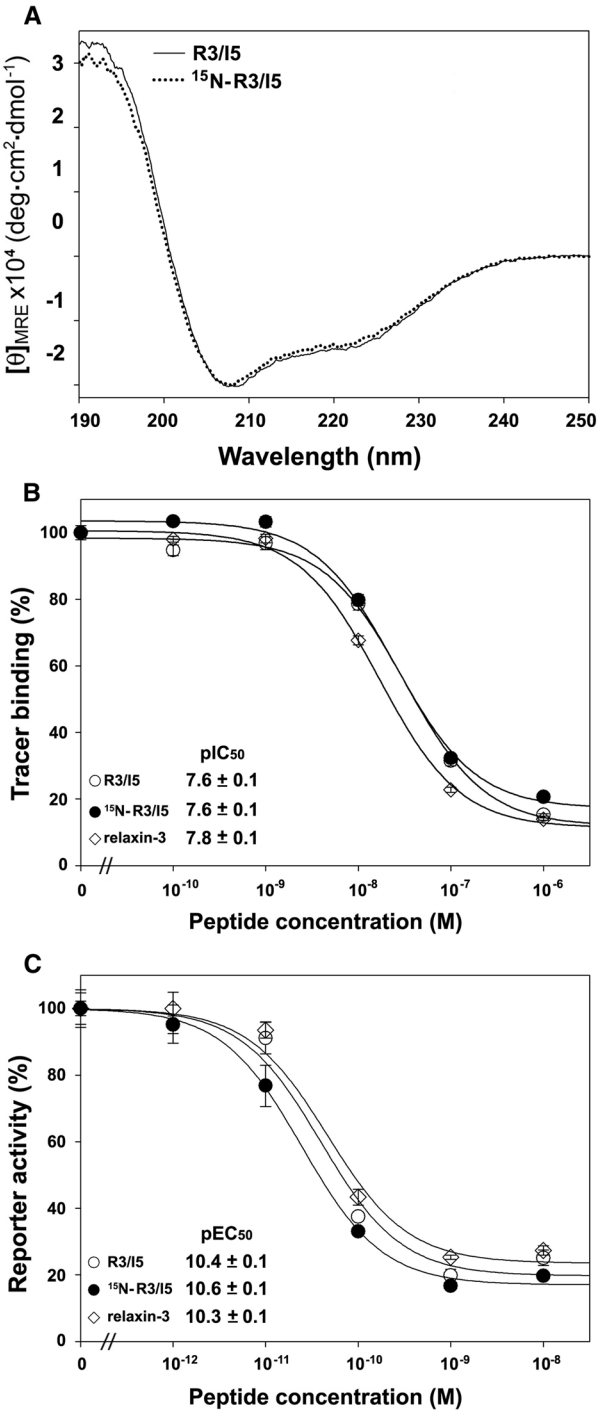

Fig. 6 Characterization of the unlabeled and the uniformly ^{15}N-labeled mature R3/I5 peptides. **a** Circular dichroism spectroscopy. **b** RXFP3-binding assays. The DTPA/Eu^{3+}-labeled R3/I5 was used as a tracer, and the Chinese hamster ovary (CHO) cells stably expressing human RXFP3 were used as a receptor source. The measured data were expressed as mean ± SE ($n = 3$) and fitted to sigmoidal curves using the software SigmaPlot 10.0. **c** RXFP3-activation assays. The human embryonic kidney (HEK) 293T cells transiently transfected by human RXFP3 and a cAMP response element-controlled nanoluciferase were used for the activation assay. The measured data were expressed as mean ± SE ($n = 3$) and fitted to sigmoidal curves using the software SigmaPlot 10.0

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Conflict of interest The authors declare that they have no conflict of interest.

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