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RESEARCH ARTICLE

Wu Yu, Cui Xiuyun, Wang Jihong, Zhao Peng
Xu Yuefei, Zhao Baochang

Anti-oxidative effect of ribonuclease inhibitor by site-directed mutagenesis and expression in *Pichia pastoris*

Abstract Human placental ribonuclease inhibitor (hRI) is an acidic protein of Mr~50kDa with unusually high contents of leucine and cysteine residues. It is a cytosolic protein that protects cells from the adventitious invasion of pancreatic-type ribonuclease. hRI has 32 cysteine residues, and the oxidative formation of disulfide bonds from those cysteine residues is a rapid cooperative process that inactivates hRI. The most proximal cysteine residues in native hRI are two pairs that are adjacent in sequence. In the present aork, two molecules of alanine substituting for Cys328 and Cys329 were performed by site-directed mutagenesis. The site-mutated RI cDNA was constructed into plasmid pPIC9K and then transformed *Pichia pastoris* GS115 by electroporation. After colony screening, the bacterium was cultured and the product was purified with affinity chromatography. The affinity of the recombinant human RI with double site mutation was examined for RNase A and its anti-oxidative effect. Results indicated that there were not many changes in the affinity for RNase A detected when compared with the wild type of RI. But the capacity of anti-oxidative effect increased by 7~9 times. The enhancement in anti-oxidative effect might be attributed to preventing the formation of disulfide bond between Cys328 and Cys329 and the three dimensional structure of RI was thereby maintained.

Keywords ribonuclease inhibitor, site-directed mutagenesis, cysteine, *Pichia pastoris*, reactive oxygen species

1 Introduction

Human placental ribonuclease Inhibitor (hRI) is a cytosolic acidic glycoprotein with a molecular weight of 50 kDa (Lee et al., 1988). hRI is composed of seven leucine-rich repeats with 57 amino acid residues for each. There is 40% homology among the repeats (Lee et al., 1988; Hofsteenge et al., 1988). RI can bind tightly with bovine pancreatic RNase (RNase A) to form a 1:1 noncovalent complex that competitively inhibits the enzyme activity with a low K_i value (4×10^{-14} mol / L) to control intercellular RNA level (Shapiro and Vallee 1984; Lee et al., 1989). Angiogenin, an activator of angiogenesis, is a protein that has 35 % homology with RNase A (Lee et al., 1989). hRI can combine with angiogenin with a much lower Ki value (7×10^{-16} mol / L). Therefore, hRI has an anti-angiogenesis activity and could be a potential medicine for inhibiting the tumor growth by inhibiting the angiogenesis of tumors (Cui 1991).

hRI contains 32 cysteine residues and at least 30 cysteine residues existing in the reduced states. hRI can be irreversibly inactivated by sulfhydryl reagents. This indicates that the free sulfhydryl groups are essential for its activity (Lee et al., 1988). In 1992, Fominaya studied pig RI and indicated that the oxidation of the 30 cystein residues had a highly cooperative manner. The initial oxidation of cysteine residues could change the conformation of the molecule, thereafter, the other cysteine residues would be oxidized rapidly to form 15 disulfide bonds which inactivated the RI (Forminaya and Hofoteenge 1992). The proximal cysteine residues in native hRI are two pairs that are adjacent in sequence, such as Cys94/Cys95 and Cys328/Cys329. A cystine from such adjacent cysteine residues would likely contain a perturbing *cis* peptide bond within its eight-membered ring that would disrupt the structure of hRI and could facilitate further oxidation (Kim et al., 1999). Our recent study showed that RI also had anti-oxidative function and the free cysteine residues were

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Wu Yu, Wang Jihong
College of Live Sciences, Liaoning Normal University, Dalian 116029, China
Zhao Baochang (✉), Cui Xiuyun, Zhao Peng, Xu Yuefei
Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116027, China
E-mail: zhaobc99@mail.dlmedu.edu.cn

very important for this function (Forminaya and Hofoteenge 1992). In this study, two molecules of alanine were substituted for Cys328 and Cys329, respectively. The cDNA of the double site-directed mutagenesis was constructed into expression vector pPIC9K and expressed in methanol fed *Pichia pastoris* GS115. The effects of the double site-directed mutagenesis of RI cDNA on the affinity of RI for RNase A and on the anti-oxidative function of RI were expected to be revealed.

2 Materials and methods

2.1 Materials

2.1.1 Bacterium strains and plasmids

Escherichia coli strain DH5α, pET23b-ri, and pPIC9K were available in the laboratory. Yeast *Pichia pastoris* strain GS115 was from TakaRa Biotechnology (Dalian) Co., Ltd.

2.1.2 Reagents

Restriction endonucleases, *Taq* polymerase, T4 DNA ligase, Fragment Recovery Kit, Agarose Gel DNA Purification Kit, and MiniBEST Plasmid Purification Kit were purchased from TakaRa Biotechnology (Dalian) Co., Ltd. MOPS, RNase A, peptone Y, yeast nitrogen base, and CNBr activated sepharose 4B were purchased from Gibco and Invitrogen, respectively. Goat anti-rabbit IgG-HRP was from Shanghai Huamei Biotechnology Co. Ltd. hRI antibody readily available in the lab.

2.1.3 Equipment

PCR thermal cycler (Perkin Elmer Cetus) was purchased from Thermo Life Sciences (HK) Ltd. Electroporator was from Eppendorf AG22331 Hamburg, Germany. Spectrophotometer 754 was from Shanghai Third Analyzer Factory.

2.1.4 Primers

Primers were synthesized by TakaRa Biotechnology (Dalian) Co.,Ltd. Primer 1: 5'-CGGAATTCAGCCTGGACATCCAG-3' (*Eco*R I restriction site underlined); Primer 2: 5'-AGCCGCGGCGGCTGTGAAGCTGCA-3' (*Sac* II restriction site underlined); Primer 3: 5'-ACCCGCGGCCT-CCCACTTCAGCTCA-3' (*Sac* II restriction site underlined); Primer 4: 5'-TTCGGCCGTCAGTGGTGGT-GGTGGTGCGCAAGCTT3' (*Eco*52 I restriction site underlined).

2.2 Methods

2.2.1 Double site-specific mutagenesis of hRI

Fragments of hRI gene containing the desired double site–specific mutations (Ala328 and Ala329 substituted for Cys328 and Cys329, respectively) were generated by PCR using pET-23b-ri as a template. Primers 1 and 2 were used in PCR to amplify the 1 121 bp long fragment, whereas primers 3 and 4 were used in PCR to amplify the 383 bp long fragment. The two fragments were digested with *Eco* R I/*Sac* II and *Sac* II/*Eco*52 I, respectively. After 1% agarose gel electrophoresis, the two bands were collected and ligated with *Eco*R I/*Eco*52 I digested plasmid pPIC9K under the catalysis of T4 DNA ligase. The recombinant constructed pPIC9K-mri was transformed into *E. coli* DH5α. The positive colonies were identified by *Eco*R I/*Eco*52 I double restriction hydrolysis and PCR.

2.2.2 Transformation to Pichia pastoris

The recombinant yeast expression vector pPIC9K containing mutant hRI cDNA was linearized by *Bgl* II and transformed into yeast *Pichia pastoris* GS115 by electroporation (~7500 V/cm for 10 min). The bacterium was pretreated with 1mol/L sorbitol. After electroporation, 200-600 µL of the bacterium was spread onto a plate with MD medium and incubated at 30 ℃ for 72 hrs.

2.2.3 Screening the phenotypes

The positive transformant is transferred to MM and MD plates, respectively, and incubated at 30℃. The bacterium growing at the same speed on both MM plate and MD plate had Mut$^+$ phenotype. If the growing speed of the bacterium on the MD plate is higher than that on the MM plate, the transformant would belong to Mut^s phenotype.

2.2.4 Identification of the positive colony

The screening of the strains was done with His$^+$ Mut^s phenotype and the genomic DNA was isolated and identified by PCR.

2.2.5 Induced of expression and identification of the recombinant protein

The PCR-identified positive recombinant was transferred into a 100 mL BMGY medium and incubated at 30℃, till the OD_{600} = 2~6. Thereafter, it was centrifuged at 1500×g for 5 min. The cells were collected and suspended into BMMY medium. Methanol was added in 24 hrs intervals and the expression was induced by methanol in a 0.5%

medium. After inducing for 144 hrs, the supernatant was assayed for the expressed mutant protein by SDS-PAGE and Western blot.

2.2.6 Purification of mutant protein

The mutant protein was subjected to an affinity chromatographic column packed with CNBr activated Sepharose 4B with RNase A acting as the ligand of the chromatography. Non-specific starting buffer (0.1 mmol / L Tris-HCl buffer, pH 8.0, 0.05 mmol / L EDTA, 15% glycerol, and 100 mmol / L β-mercaptoethanol) was used to wash the column for removing non-specifically bound protein, till A260 reached to 0. The specific bound protein hRI was eluted with the elution buffer (20 mmol / L acetate, pH 5.0, 3 mol / L NaCl, 1 mmol / L EDTA, and 15 % glycerol) and the protein was purified at 4 ℃ (Cui et al., 2003). After being concentrated by dialysis, the sample was subjected to SDS-PAGE electrophoresis.

2.2.7 Determination of the inhibitory effect of mutant hRI on RNase activities

The enzymatic activity of RNase A was measured spectrophotometrically with cyclic 2’, 3’-CMP as substrate. 0.99 mL reaction system included 0.2 mL of 0.5 mol / L Tris-acetate buffer, pH 6.5, 5 mmol / L EDTA, 0.1 mL of 10 mmol / L cyclic 2’, 3’-CMP, and 1~40 μL purified hRI and the reaction proceeded at 25℃ for 3min. After that, 10 μL (1 μg) RNase A was added to the reaction system. The inhibitory ability of hRI to the hydrolytic effect of 1 μg RNase A on cyclic 2’, 3’-CMP was assayed at 286 nm (Peter 1979). One unit of the hRI was defined as the amount of hRI required for inhibiting by 50% of the activity of 5 ng RNase A.

2.2.8 Determination of anti-oxidative capability of hRI

The enzymatic activity of RNase A was measured spectrophotometrically with yeast RNA as the substrate. Various concentrations (0.0005%—2%) of H_2O_2 were added to 5 ng RNase A in 0.5 mL buffer containing 50 mmol / L MOPS, pH 7.5, 5 mmol / L $MgCL_2$,50 μL 2% yeast RNA, and 25 μL purified hRI. It was then incubated at 37℃ for 15 min. The reaction was terminated by adding 0.5 mL of 10% TCA. The assay proceeded at A_{280} (Peter 1979)

3 Results

3.1 Construction of yeast expression vector pPIC9K-mhRI

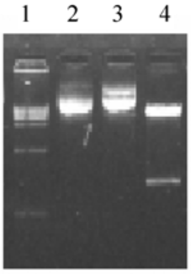
Fragments of hRI gene containing the desired double site-directed mutations (Ala328 and Ala329 substituted for Cys328 and Cys329, respectively) were generated by PCR (Fig. 1) and these products were sequenced accordingly. The construction of yeast expression vector pPIC9K carrying mutant hRI (pPIC9K-mhRI) was showed by double restriction cutting (Fig. 2). Results indicated that the double site-directed mutagenesis of hRI was done successfully.

3.2 The induced expression of mutated hRI in *Pichia pastoris* GS115 and its purification

The recombinant yeast expression vector pPIC9K containing the mutant hRI was linearized by restriction endonuclease *Bgl* II and transformed into yeast *Pichia pastoris* GS115 cells by electroporation. The positive colonies with phenotype His^+ Mut^s were then screened. The induced expression was carried out for 144 hrs to reach the maximum expression (Fig.3). The result of the Western blot is shown in Fig. 4. In the recombinant expression plasmid pPIC9K linearized by *Bgl* II the AOX1 gene was replaced by expression cassette. This disruption of the AOX1 gene forced this bacterium to rely on the transcriptionally weaker AOX2 gene for growth on methanol. As a result, the strain had a Mut^s phenotype with reduced ability to grow on methanol. The mutated hRI was purified by affinity chromatography packed with CNBr activated Sephrose-4B-RNase A and a single band on SDS-PAGE was identified (Fig.5). The activity was measured to be 200 U / mL.

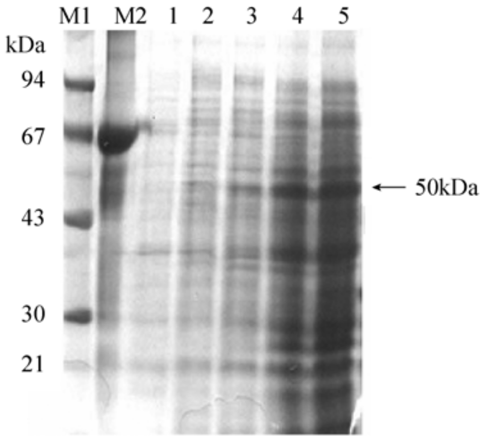
1: DNA marker; 2: PCR product

Fig.1 1% agarose gel analysis of PCR product from recombinant plasmid in *E. coli* DH5α



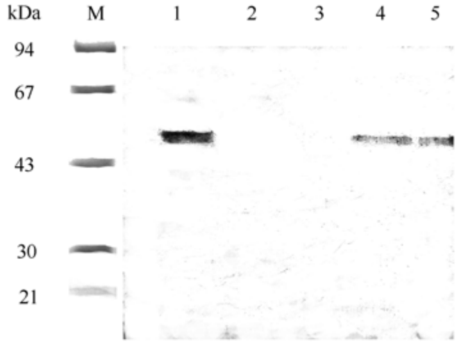
1: DNA marker; 2,3: recombinant plasmid;
4: fragment of recombinant plasmid (EcoR I / Eco52 I)

Fig.2 1% agarose gel analysis of restriction enzyme digested recombinant plasmid in *E. coli* DH5α



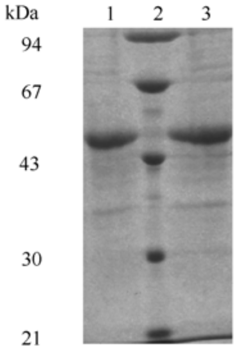
M1: molecular marker; M2: BSA marker (6.9kDa);
1-5: GS115 with pPIC9K-mutant hRI induced for 48~144 hours

Fig.3 SDS-PAGE analysis of the total proteins prepared from GS115 harboring plasmid pPIC9K-mutant hRI



M: Molecular marker; 1: Positive control; 2: Negative control;
3: Un-induced GS115 with pPIC9K-mutant hRI; 4: Induced GS115 with pPIC9K-mutant hRI for 144hours; 5: Induced GS115 with pPIC9K-hRI for 144hours

Fig.4 Western blot analysis of induced GS115 with pPIC9K-mutant hRI



1: Wild type hRI; 2: Low molecular protein marker;
3: Mutant type hRI

Fig.5 SDS-PAGE analysis of purified hRI protein

3.3 Dixon plot for the determination of K_i

Dixon plot showed that the K_i value of mutant hRI for RNase A was 3.24×10^{-14} mol / L; K_i value of native hRI for RNase A was 2.88×10^{-14} mol / L (Fig. 6). Results indicated that there was no significant difference between the K_i values of mutant hRI and native hRI. The mutation of hRI did not change the affinity for RNase A.

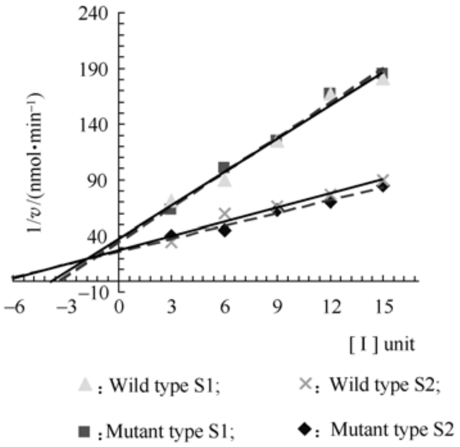


Fig.6 Dixon plot of inhibitory kinetics of the wild and the mutant hRI

3.4 Anti-oxidative effects of mutant hRI

The anti-oxidative properties of mutant and wild hRI were examined. 0.006% (v/v) H_2O_2 could reduce the activity of wild hRI by 50% but if the concentration of H_2O_2 was increased to 0.05%, the activity of mutant hRI was reduced by50%. Results showed that the ability of anti-oxidation against H_2O_2 of mutant hRI was increased by 7~9 times if compared with wild hRI (Fig. 7).

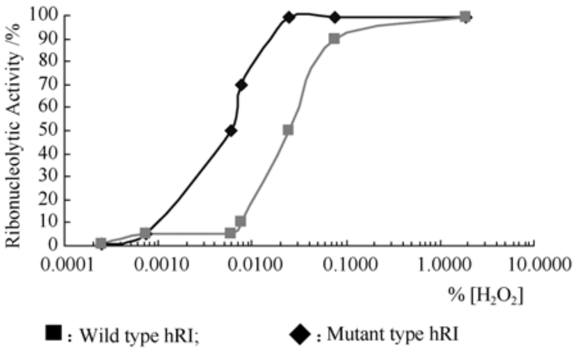


Fig.7 The resistant effect of hRI on hydrogen peroxide injury

4 Discussion

Using genetic engineering, the double site-directed mutagenic hRI cDNA produced Cys328Ala and Cys329Ala. The fragment was constructed into expression vector pPIC9K and integrated into the genome of *Pichia pastoris*. Because of the homology of linearized pPIC9K-mhRI hydrolyzed by *Bgl* II with *AOX*1, genomic *AOX*1 of the transformer was replaced by this fragment producing a Mut^s phenotype. The disruption of genomic *AOX*1 forced the bacterium to rely on the transcriptionally weaker *AOX*2 gene for growth on methanol (Cregg et al., 1989); hence, this phenotype reduced the ability to grow on methanol. This gene replacement strain utilized less methanol and sometimes expressed higher levels of foreign protein than wild-type (Mut^+) strains (Cregg and Madden 1987).

In 1993, Kobe and Deisenhofer discovered the horseshoe structure of pig RI (PRI) by X ray diffraction (Kobe and Deisenhofer 1993). This structure was also demonstrated in PRI-angiogenin complex (Papageorgiou 1997) and it was constructed by the arrangement and folds of 16 homologous Leucine–rich repeats (LRR). Each repeat contained a short β chain which arranged on the inner surface of the horseshoe to form a parallel β-pleated sheet and a long α-helix which decorated the outer surface.

The sequence of the human ribonuclease inhibitor (hRI) contained 32 cysteine residues present in the reduced form. The peptide bonds in proteins were *trans* configuration rather than *cis* configuration. The deviation between the two configurations in free energy was 3 kcal /mol (Scherer et al., 1998). A cystine formed from such adjacent cysteine residues would likely contain a perturbing *cis* peptide bond within its eight–membered ring. *Cis* peptide bond in the eight-membered ring was more stable and had lower free energy than that of *trans* peptide bond by 9.2 kcal / mol (Turner and Meador 1957). In the eight-membered ring formed by the formation of cystine Cys-Cys, the atom C and atom N in the peptide bond were linked as sp^2 hybrid bond. Moreover, the configurational energetics suggested that a peptide bond within a cystine formed from adjacent cysteine residues would be convenient in the *cis* configuration.

The *cis* peptide bond in the eight-membered ring could distort and fold the conformational structure of the protein and promote the oxidation of other cysteine residues to inactivate the protein (Kin et al., 1999). In the present paper, alanine is substituted for Cys328 and Cys329 and the anti-oxidation of the mutant RI to H_2O_2 is promoted for 7-9 times. The affinity for RNase A did not affect the reaction. The enhancement of anti-oxidative ability of the mrRI can accord with the substitution of Ala for Cys328-Cys329 which avoided the formation of disulfhydryl bond between the two positions and at the same time increased the stability of the molecule (Shapiro and Vallee 1984; Polakwski et al., 1993). The anti- angiogenic ability can be inactivated in aerobic conditions. Mutated hRI can be expected to efficiently inhibit angiogenesis produced by angiogenin if compared with wild hRI. This strategy of replacing adjacent Cysteine may be utilized in other proteins to enhance their stability.

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