African Journal of Biotechnology Vol. 8 (13), pp. 3079-3083, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

High-density fermentation and functional characterization of a recombinant echistatin mutant

Tao Yang[1], Bo Niu[1,2], Niuliang Cheng[1], Jun Xie[1] and Lijun Yang[1]*

[1]Department of Biochemistry and Molecular Biology; Key Laboratory of Cellular Physiology (Shanxi Medical University), Ministry of Education, Taiyuan, Shanxi 030001, China.
[2]Capital Institute of Paediatrics, Beijing 100020, China.

Accepted 12 May, 2009

A disintegrin echistatin mutant, R24K-echistatin, was obtained by overlap extension polymerase chain reaction. It was efficiently expressed as a soluble and fusion protein in *Escherichia coli* at optimized fermentation conditions. The recombinant bacteria had the highest wet weight of 120 g/l in 2×YT medium with IPTG induction. The R24K-echistatin was purified by chitin affinity chromatography and had a yield of 13 mg/l with DTT-cleavage. The purified R24K-echistatin inhibited the aggregation of platelet *in vitro* with IC_{50} of 21 nM which is better efficient than echistatin. This work lays the foundation for further research on specific anti-thrombus disintegrin agents.

Key words: Disintegrin, echistatin, *Escherichia coli*, fermentation, mutant.

INTRODUCTION

Echistatin is a disintegrin isolated from the venom of the viper *Echis carinatus*. It is 49 amino acids long with an RGD motif spanning residues 24 to 26 (Gan et al., 1989). One interesting feature of echistatin is the ability to bind to multiple integrins including alphaIIbbeta3, alphaVbeta-3, and alpha5beta1, which demonstrates the significant role of this disintegrin in cell adhesion, platelet aggregation, cell migration, cell proliferation and differentiation (Wierzbicka-Patynowski et al., 1999; Berman et al., 2003). Based on this feature, echistatin has become a research focus to study and develop disintegrin-derived drugs to block various integrin-mediated physiological and pathological reactions (Belisario et al., 2000; Lu et al., 2003).

However, the RGD motif of echistatin has a pan-inhibitory effect on integrins alphaIIbbeta3, alphaVbeta3, and alpha5beta1 (Foster et al., 1993). The lack of selectivity to a specific hailment greatly limits the application of echistatin as a drug, although theoretically it has anti-thrombosis, anti-tumor metastasis and anti-osteoporosis effects (Meyer et al., 2006).

Recent studies showed that the disintegrins containing the KGD loop showed preferential binding to alphaIIb-beta3 (Hantgan et al., 2006; Minoux et al., 2000). Further study revealed that the mutant KGD disintegrin possesses a much higher anti-platelet aggregative activity than the wild type RGD (Scarborough et al., 1993). Meanwhile, the mutant KGD disintegrin has no effect in restraining new vessel formation (Suehiro et al., 1996). These studies indicate that KGD disintegrins are unique for the development of anti-thrombotic agents.

In a previous study, we performed the optimization of fermentation and purification conditions of echistatin (Yang et al., 2006). In order to improve the specificity of echistatin, we mutated the 24th amino acid Argenine (R) to Lysine (K) (named R24K-echistatin) and compared platelet aggregation inhibition of echistatin and R24K-echistatin. Herein we report the construction of BL21/pTXB1-R24K-echistatin fusion expression system, to achieve high-efficient expression of R24K-echistatin. We subsequently optimized the conditions required for high-density fermentation of the recombinant bacteria in addition to performing purification and biological activity studies to optimize the procedures for large scale production of R24K-echistatin.

MATERIALS AND METHODS

Chemicals and reagents

The expression vector pTXB1, restriction enzymes, and chitin

*Corresponding author. E-mail: biochemyy@126.com. Tel: 86-351-4135452. Fax: 86-351-4135452.

beads for chromatography were purchased from New England Biolabs (Ipswich, MA, USA). The vector pMD-18T, T4 DNA ligase and *Taq* DNA polymerase were obtained from TaKaRa (Otsu, Shiga, Japan). Echistatin was purified in our laboratory as described previously (Yang et al., 2006). The Biostat 5 L fermentor was purchased from B. Braun Biotech International (Melsungen, Germany), and the PACKS-4 platelet aggregometry from Helena (Beaumont, TX, USA).

PCR amplification of R24K-echistatin gene

Six gene fragments were designed according to the amino-acid sequence of echistatin in the GenBank (AAB33186.1). Their sequences were as follows: F1: atg gaa tgc gaa tcc ggt ccg tgc tgc cgt *aac tgc aaa ttc ctg aaa gaa*; F2: *ttg acg ttt aag gac ttt ctt* cca tga tag acg TTT GCA CGA TTC CCA CTG CTG; F3: AAA CGT GCT AAG GGT GAC GAC atg gac gac tac *tgc aac ggt aaa act tgc gac*; F4: *acg ttg cca ttt tga acg ctg* acg ggc gca ttg ggc gtg ttt cca ggc cga tga; F5: ag *cat atg* gaa tgc gaa tcc ggt ccg tgc; F6 : ta *gaa gag c* tta agt agc cgg acc ttt gtg cgg. Fragments F1, F2, F3, and F4 consisted of the full-length se-quence of mutant echistatin. F1 and F2 contained a complementary sequence of 21 bp in size (shown in italic) and so did F2 and F3 (shown in capital). The 15th codon in F2 was mutated from gca to ttc, which encodes lysine (K). The complementary sequence bet-ween F3 and F4 was also 21 bp in size (shown as italic). Fragment 5 was a 5-terminal specific primer, including a *Nde* I restriction site and an initiation codon ATG; fragment 6 was a 3-terminal specific primer, including a *Sap* I restriction site and a termination codon TAA. The R24K-echistatin gene was amplified by overlap extension PCR (94°C for 3 min; 94°C for 30 s, 55°C for 30 s, and 72°C for 15 s, 35 cycles; 72°C for 25min).

Construction of R24K-echistatin-expressing recombinant bacteria

The amplified DNA was cloned into the plasmid pMD-18T and used to transform *Escherichia coli* JM109. The transformants were selected by blue/white screening and cultured in the presence of 100 μg ampicillin/ml. The plasmid containing the R24K-echistatin was recovered and digested with *Nde* I and *Sap* I. The DNA fragment containing the R24K-echistatin was purified by gel electrophoresis and inserted into the plasmid pTXB1. The resultant plasmid was transformed into *E. coli* BL 21(DE3). The transformants were selected using medium containing 100 μg ampicillin/ml. Positive clones were identified by PCR analysis and DNA sequencing. *E. coli* cells harboring expression vector pTXB1-R24K-echistatin were grown in LB medium at 37°C, 250 rpm overnight. The resultant preculture (200 ml) was inoculated into 4 L of 2×YT fermentation medium.

High-density fermentation and expression of the R24K-echistatin fusion Protein

E. coli cells were grown in 2×YT medium at 37°C, 400 rpm and fed-batch fermentation was carried out in a 5 L jar fermentor. The expression of R24K-echistatin fusion protein gene was induced by adding 0.08 mM IPTG. The optimized culture time and induction time were determined according to expression level of the target protein.

Purification of the R24K-echistatin

Cells were collected by centrifugation, re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and disrupted by sonication. Lysate was centrifugated at 18,000 *g*, for 25 min and the supernatant was filtered through a 0.45 μm membrane. The filtrate was 10-fold diluted with balancing buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH 8.0) and applied to a chitin bead column. The flow-through fractions were collected and analyzed by 18% SDS-PAGE. The column was washed with 3 ml of balancing buffer containing 1 M NaCl and also with 3 ml of 3 column volumes of balancing buffer containing 50 mM of DTT for a minute each. The column was capped and placed at 4°C for 20-40 h. After washing with balancing buffer on the following day, fractions were collected and analyzed with 18% SDS-PAGE. The chitin column was eluted and regenerated with 3 ml of 3 column volumes of 0.3 M NaOH for a minute.

In vitro platelet aggregation analysis of R24K-echistatin

Aspirin-free blood was collected from healthy donors in 3.8% (w/v) sodium citrate (1:9 ratio) and used to obtain platelet-rich plasma (Foster et al., 1993). A 0.9% NaCl solution containing 400 μl of platelet-rich plasma and 50 μl of purified R24K-echistatin or echistatin was incubated at 37°C for 2 min while stirring. Then, 50 μl of 20 μM adenosine diphosphate (ADP) was added to each reaction, platelet aggregation was monitored for 5 min, and aggregation curves were recorded with 0.9% NaCl as negative control.

RESULTS AND DISCUSSION

Construction of R24K-echistatin expression plasmid

R24K-echistatin showed a band of ~ 170 bp on DNA agarose gel upon PCR amplification and digestion by *Nde* I/*Sap* I (data not shown). These observations indicated that the R24K-echistatin was inserted into pTXB1 vector. PCR studies and DNA sequencing confirmed that pTXB1-R24K-echistatin contained the identical sequences as reported by Gan (1989) with point mutation at the 24th amino acid (R→K).

Optimization of the fermentation conditions and expression of the R24K-echistatin fusion protein

Induction of fusion protein was initiated at 1 h interval of cultivation time and the best results with (OD_{600} = 15 ± 2) were obtained when expression was induced after 7 h (Figure 1). In Figure 2, expression of R24K-echistatin fusion protein stabilized after 4 h of IPTG induction. Previous study has demonstrated that in addition to proper culture medium, optimized culture conditions, and induction time changes in the parameters in fermentation process also should be carefully monitored (de Maré et al., 2005). According to our results, one drop of 10% ammonia water increases the pH by 0.01. Under optimal conditions, a cell density of about 120 g wet cell weight/l was obtained. The expected target protein bands with molecular weights of 32 kDa appeared clearly in lanes, as indicated by the arrows (Figure 3).

DTT-mediated cleavage and purification of R24K-echistatin

R24K-echistatin protein was cleaved from the fusion pro-

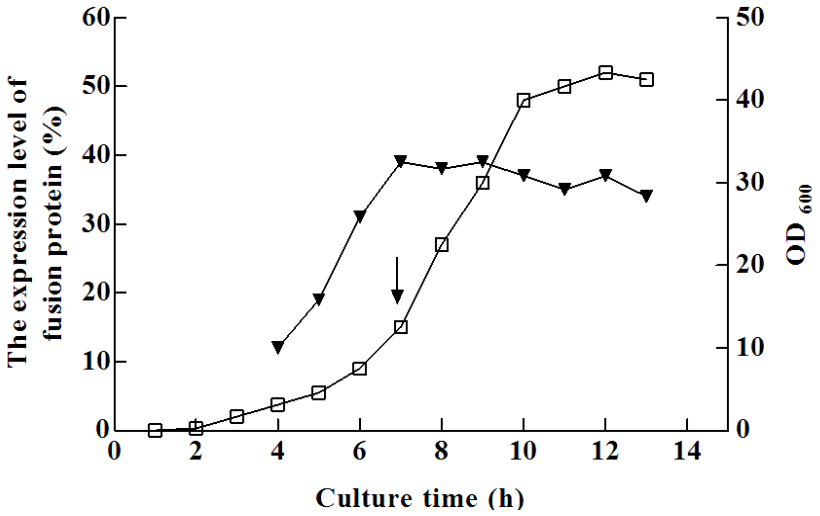

Figure 1. Profile of expression level of R24K-echistatin fusion protein by a fed-batch fermentation of *Escherichia coli* BL21(DE3). The composition of the initial fermentation 2×YT medium was: 16 g Tryptone/l, 10 g yeast extract/l, 5 g NaCl/l; and that of the feeding solution was: 200 g yeast extract/l, 10 g $MgSO_4 \cdot 7H_2O$/l, 20 g NaCl/l, 50% glycerol (v/v), 10% ammonia water (v/v), antifoam 289. The time courses of biomass OD_{600} is shown by square line graph (□), expression level of fusion protein are shown by delta (▼). Aeration and pH were controlled at 60-80% and 7.2-7.4, respectively. The time of IPTG addition is indicated by the arrow.

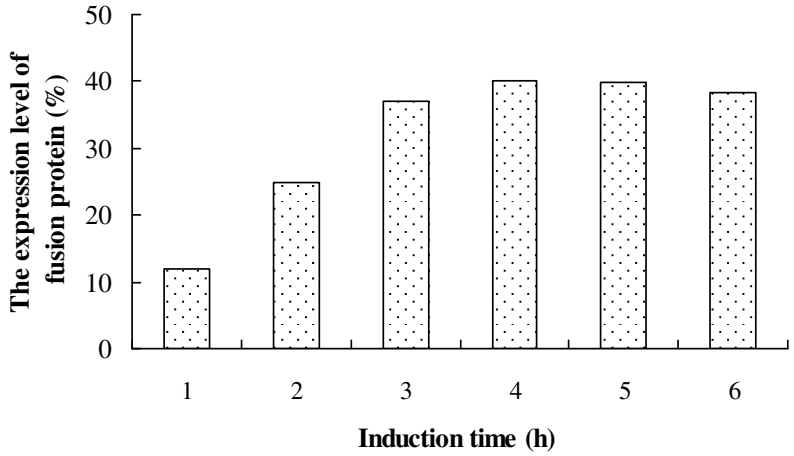

Figure 2. Effects of different durations of induction time on R24K-echistatin fusion protein expression. Following 7 h cultivation (OD_{600}=15 ± 2), 0.08 mM IPTG was added and cultured for various periods of time. The fusion protein expression stabilized after 4 h of induction.

tein by DTT treatment on the chitin column to give a molecular weight of 5.4 kDa, as shown in Figure 4. The yield of R24K-echistatin was 13 mg/l. Here we used the IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system, which incorporates an intein element and a chitin binding domain (CBD) for affinity purification (Szweda et al., 2001). The method of intein fusion and chitin affinity purification facilitates a high level of expression as well as one-step purification of R24K-echistatin.

Anti-aggregation effect of R24K-echistatin

As shown in Figure 5, both R24K-echistatin and echistatin inhibited the platelet aggression in a dose-dependent manner. The IC_{50} of R24K-echistatin was 21 nM, which was much lower than that of echistatin 320 nM.

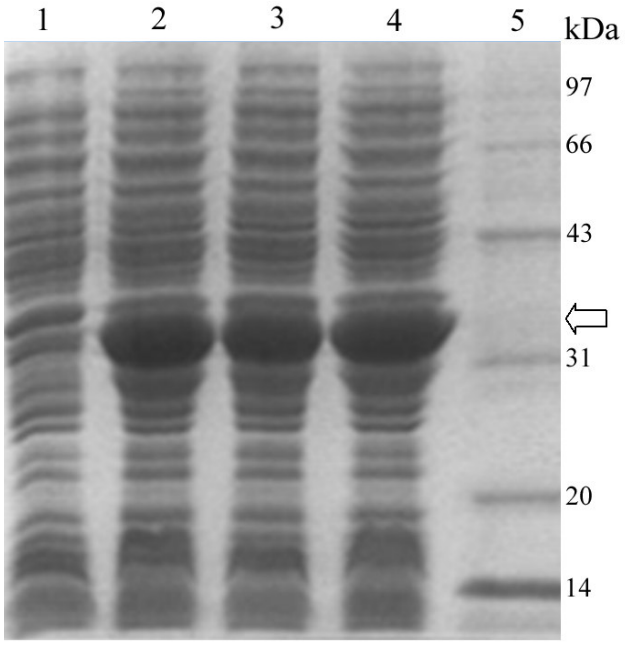

Figure 3. SDS-PAGE analysis of the fermentation lysates of the R24K-echistatin bacterial strain. Lane 1, the lysates of BL21/pTXB1-R24K-echistatin before induction; lanes 2-4, the lysates of BL21/pTXB1-R24K-echistatin after induction; lane 5, protein marker. The arrowhead indicates the band with a molecular weight of 32 kDa. The production accounted for 40% of the total cell protein as determined by an electrophoresis imaging system.

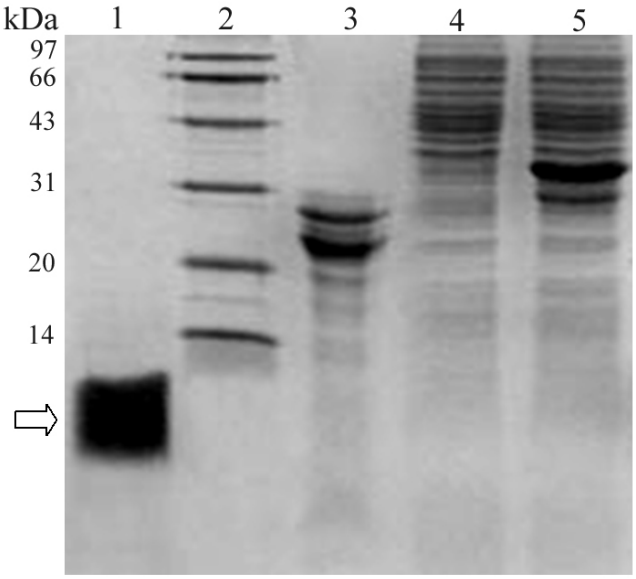

Figure 4. SDS-PAGE and coomassie blue staining of fractions following purification of the R24K-echistatin fusion protein. Lane 1, elution fraction with DTT-cleavage; lane 2, protein marker; lane 3, NaOH elution fraction; lane 4, flow-through fraction; lane 5, input lysate for chitin affinity purification. The R24K-Echistatin protein was cleaved from fusion protein by DTT on chitin column, to give a molecular weight of 5.4 kD, as indicated with an arrow.

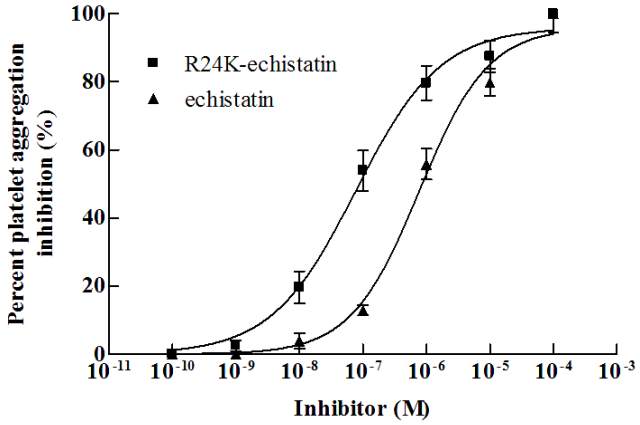

Figure 5. Platelet aggregation analysis for treatments with R24K-echistatinand wild-type echistatin. The inhibition rate is $[(A_0–A)/A_0] \times 100\%$, in which A_0 is the maximum aggregation of negative control and A is the maximum aggregation of the experiment groups treated with R24K-echistatin and wild-type echistatin (Osende et al. 2001).

This indicates that the inhibitory effect of R24K-echistatin was more efficient than that of echistatin. These results suggest that the KGD motif in R24K-echistatin may be more effective in binding to $\alpha_{IIb}\beta_3$ integrin and have implications in medicinal research as it may be used to efficiently inhibit thrombosis of multiple pathological origins.

Conclusion

A more efficient *E. coli* expression system of echistatin mutant (R24K-echistatin) was established and optimized fermentation conditions for the high wet weight recombinant bacteria (120 g/l) were established. The echistatin mutant was affinity purified with chitin column and DTT-cleavage to yield 13 mg/l, which shows higher activity of inhibition of platelet aggregation compared to echistatin. This study provides a foundation for further biological and structural studies of R24K-echistatin.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (30600226) and the Patent Spreading Grant of Shanxi Province (091005). We thank Lecturer Longguang Niu at Department of Pharmacology, Shanxi Medical University, China, for statistical analysis.

REFERENCES

Belisario MA, Tafuri S, Di Domenico C, Della Morte R, Squillacioti C, Lucisano A, Staiano N (2000). Immobilised echistatin promotes platelet adhesion and protein tyrosine phosphorylation. Biochim Biophys Acta. 2: 227-236.

Berman AE, Kozlova NI, Morozevich GE (2003). Integrins: structure and

signaling. Biochem. (Mosc). 12: 1284-1299
de Maré L, Velut S, Ledung E, Cimander C, Norrman B, Karlsson EN, Holst O, Hagander P (2005). A cultivation technique for E. coli fed-batch cultivations operating close to the maximum oxygen transfer capacity of the reactor. Biotechnol. Lett. 14: 983-990.
Foster MR, Hornby EJ, Brown S, Kitchin J, Hann M, Ward P (1993). Improved potency and specificity of Arg-Gly-Asp (RGD) containing peptides as fibrinogen receptor blocking drugs. Thromb Res. 3: 231-245.
Gan ZR, Condra JH, Gould RJ, Zivin RA, Bennett CD, Jacobs JW, Friedman PA, Polokoff MA (1989). High-level expression in Escherichia coli of a chemically synthesized gene for [Leu-28] echistatin. Gene. 1: 159-166.
Hantgan RR, Stahle MC, Connor JH, Horita DA, Rocco M, McLane MA, Yakovlev S, Medved L (2006). Integrin alphaIIbbeta3: ligand interactions are linked to binding-site remodeling. Protein Sci., 8: 1893-1906.
Lu X, Lu D, Scully MF, Kakkar VV (2003). Modulation of integrin-binding selectivity by mutation within the RGD-loop of snake venom proteins: a novel drug development approach. Curr. Med. Chem. Cardiovasc Hematol Agents, 2: 189-196.
Meyer A, Auernheimer J, Modlinger A, Kessler H (2006). Targeting RGD recognizing integrins: drug. development, biomaterial research, tumor imaging and targeting. Curr. Pharm. Des. 22: 2723-2747.
Minoux H, Chipot C, Brown D, Maigret B (2000). Structural analysis of the KGD sequence loop of barbourin, an alphaIIbbeta3-specific disintegrin. J. Comput. Aided Mol. Des., 14: 317-327.
Osende JI, Fuster V, Lev EI, Shimbo D, Rauch U, Marmur JD, Richard M, Varon D, Badimon JJ (2001). Testing platelet activation with a shear-dependent platelet function test versus aggregation-based tests: relevance for monitoring long-term glycoprotein IIb/IIIa inhibition. Circulation, 11: 1488-1491.
Scarborough RM, Naughton MA, Teng W, Rose JW, Phillips DR, Nannizzi L, Arfsten A, Campbell AM, Charo IF (1993). Design of potent and specific integrin antagonists. Peptide antagonists with high specificity for glycoprotein IIb-IIIa. J. Biol. Chem. 2: 1066-1073.
Suehiro K, Smith JW, Plow EF (1996). The ligand recognition specificity of beta3 integrins. J. Biol. Chem. 17: 10365-10371.
Szweda P, Pladzyk R, Kotlowski R, Kur J (2001). Cloning, expression and purification of the Staphylococcus simulans lysostaphin using the intein-chitin-binding domain (CBD) system. Protein Exp. Purif., 3: 467-471.
Wierzbicka-Patynowski I, Niewiarowski S, Marcinkiewicz C, Calvete JJ, Marcinkiewicz MM, McLane MA (1999). Structural requirements of echistatin for the recognition of alpha(v)beta(3) and alpha(5)beta(1) integrins. J. Biol. Chem. 53: 37809-37814.
Yang LJ, Yang T, Cheng NL, Xie J, Niu B (2006). Fermentation and purification of echistatin fusion protein expressed in Escherichia coli. Wei Sheng Wu Xue Bao. 1: 95-98