ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 5, pp. 520-524. © Pleiades Publishing, Inc., 2006.
Published in Russian in Biokhimiya, 2006, Vol. 71, No. 5, pp. 645-650.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-199, February 26, 2006.

Radical Scavenging Activity of Ribonuclease Inhibitor from Cow Placenta

Shuo Wang[1]* and Haiping Li[1,2]

[1]*Tianjin Key Laboratory of Food Nutrition and Safety, Faculty of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300222, P. R. China; fax: (86) 226-060-1456; E-mail: s.wang@tust.edu.cn*
[2]*Faculty of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, P. R. China; fax: (86) 226-060-1456; E-mail: hhppli@163.com*

Received September 11, 2005
Revision received December 2, 2005

Abstract—Cow placenta ribonuclease inhibitor (CPRI) has been purified 5062-fold by affinity chromatography, the product being homogeneous by sodium dodecyl sulfate-gel electrophoresis. The chemiluminescence technique was used to determine the radical scavenging activities of CPRI toward different reactive oxygen species (ROS) including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), lipid-derived radicals ($R^{\cdot}$), and singlet oxygen ($^1O_2$). CPRI could effectively scavenge $O_2^{\cdot-}$, $OH^{\cdot}$, $R^{\cdot}$, and $^1O_2$ at EC_{50} of 0.12, 0.008, 0.009, and 0.006 mg/ml, respectively. In addition, the radical scavenging activities of CPRI were higher than those of tea polyphenols, indicating that CPRI is a powerful antioxidant.

DOI: 10.1134/S0006297906050087

Key words: ribonuclease inhibitor, cow placenta, antioxidant, purification, chemiluminescence

Ribonuclease inhibitor (RI) is a 50-kD scavenger of pancreatic-type ribonucleases (RNases). RI binds pancreatic-type RNases with 1 : 1 stoichiometry and competitively inhibits their ribonucleolytic activity [1-3]. RI is found in the cytosol of mammalian cells and has been purified from many species and tissue types [4, 5]. Its inhibition of ribonucleolytic activity and its cytosolic location have led to the suggestion that RI protects cellular RNA from degradation by invading pancreatic-type RNases [3]. RNases can be cytotoxic by entering the cytosol and degrading cellular RNA. Its ribonucleolytic activity is limited by the presence of excess RI [3, 6]. The noncovalent interactions of the RI–RNase A complex are exceptionally strong ($K_d = 6.7 \cdot 10^{-14}$ M) [7]. RI may have a role in the regulation of RNA turnover in mammalian cells, thus having a potential role in determining levels of gene expression [8].

RI also forms 1 : 1 complexes with angiogenin, a member of the RNase superfamily [4]. Angiogenin is a potent inducer of angiogenesis, a complex process of proliferation and formation of new capillary blood vessels from existing blood vessels, but has only weak ribonucleolytic activities [9]. It has been implicated in wound healing and tumor progression [10]. RI from human placenta can bind with angiogenin with $K_i = 7.1 \cdot 10^{-16}$ M [11] and inhibits its activity, so it may further inhibit the growth of solid tumors [12-14].

An unusual aspect of the RI sequence is the presence of a large number of highly conserved cysteine residues [15]. Each cysteine residue must be reduced for its antiribonucleolytic activity [15, 16]. This feature allows RI to function only in a reducing environment, such as the cytosol. It is well known that reduced glutathione protects organisms from peroxidation by use of its reduced thiol group. Therefore, it is possible that RI has antioxidative activity via its reduced thiol groups. To our knowledge, there has been no report on the antioxidant ability of RI in scavenging reactive oxygen species (ROS) *in vitro*. Here we studied the antioxidant ability of cow placenta ribonuclease inhibitor (CPRI) in scavenging reactive oxygen species using chemiluminescence and compared this with

Abbreviations: CL) chemiluminescence; CPRI) cow placenta ribonuclease inhibitor; DTT) dithiothreitol; EC_{50}) effective concentration for 50% inhibition; $O_2^{\cdot-}$) superoxide anion; $OH^{\cdot}$) hydroxyl radical; $^1O_2$) singlet oxygen; RI) ribonuclease inhibitor; RNase) ribonuclease; ROS) reactive oxygen species; $R^{\cdot}$) lipid-derived radicals.

* To whom correspondence should be addressed.

antioxidant properties of tea polyphenols. This is a basis for the further study of the function of CPRI.

MATERIALS AND METHODS

Purification of CPRI. The rapid procedure described by Blackburn [17] for the purification of RI from human placenta was employed in this study with some modifications.

RNase-A-Sepharose was prepared by coupling 10 mg RNase A (Roche Co., Switzerland) with 3.5 ml CNBr-activated Sepharose 6B (Sigma, USA). RNase A solution was prepared by dissolving 8 mg RNase A in 4 ml 0.1 M $NaHCO_3$, pH 9.5, and precooled to 3°C.

An isoelectric precipitation step was added after ammonium sulfate saturation to remove many non-inhibitor substances. The ammonium sulfate saturation precipitate was redissolved in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 5 mM dithiothreitol (DTT), and 10% glycerol (v/v). The solution was adjusted to pH 4.5-4.7 by slowly adding 0.01 M glacial acetic acid. Then it was centrifuged at 16,000*g* for 25 min to obtain the isoelectric precipitate. The isoelectric precipitate was immediately resuspended to a minimum volume of 45 mM KH_2PO_4/45 mM K_2HPO_4, pH 6.4, and dialyzed in the same buffer twice for 8 h. The dialyzed solution was centrifuged at 48,000*g* for 1 h before being applied to the affinity column.

During the purification procedure, protein was determined according to Lowry et al. [18] with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis of purified CPRI was performed following a standard procedure.

Evaluation of the radical scavenging activity of CPRI by chemiluminescence analysis. Radical scavenging activities of CPRI were assessed using a bioluminescence meter LB 9507 (Berthold, Germany). This meter is composed of three parts: an automatically rotating sample support in which two sample cells (glass tube, diameter 10 mm, height 80 mm) can be placed, a chemiluminescence monitor, and a data processor. Each reaction solution can be mixed in turn and each sample cell can rotate and cross the monitor at a set time interval according to a program of our design. When testing, the chemiluminescence (CL) intensity of a reaction system can be recorded in the data processor at a set time interval. The radical scavenging activities of tea polyphenols were assessed under the same condition as a control.

CPRI sample was diluted into four different concentrations of solutions (0.1, 0.2, 0.01, and 0.02 mg/ml) prepared in 20 mM acetate (pH 5.0), containing 3.0 M NaCl and 15% (v/v) glycerol for the following test. Tea polyphenols were prepared in 0.1 and 0.01 mg/ml solution with distilled water.

$O_2^{\cdot-}$ was generated [19] by autooxidation of pyrogallol. The reaction mixture contained 50 μl pyrogallol (10^{-3} M), 700 μl Tris-HCl (20 mM, pH 8.5), and 20 μl luminol (10^{-3} M; Fluka, USA). A sample cell was first placed in the bioluminescence meter with 100 μl sample solution. When the cell crossed the monitor, other reaction solutions loaded in the two bottles in the bioluminescence meter were injected into the cell *in situ*. The CL was simultaneously recorded in the processor and then was recorded once every 5 sec (Tris-HCl instead of the specimen was present in the control).

$OH^{\cdot}$ was assigned [19] after generation by a Fenton-type reaction. The reaction mixture included 20 μl $FeCl_2$ (10^{-3} M), 30 μl 1,10-phenanthroline (10^{-3} M; Sigma), 800 μl Tris-HCl (20 mM, pH 7.38), and 50 μl H_2O_2 (0.6%).

$R^{\cdot}$ was assigned after lipid peroxidation generated by the oxidation of primrose oil (unsaturated fatty acid content > 80%), induced by H_2O_2. The reaction mixture contained 100 μl evening primrose oil, 800 μl Tris-HCl (20 mM, pH 7.38), and 100 μl H_2O_2 (0.6%) in sample cells.

$^1O_2$ was assigned [19] after singlet oxygen was chemically generated by the reaction between NaClO and H_2O_2 in a solution of pH 8.0 at 37°C. The reaction mixture included 200 μl of 1.4 mM NaClO, 800 μl Tris-HCl (20 mM, pH 8.0), and 50 μl H_2O_2 (0.6%). The testing procedure for $OH^{\cdot}$, $R^{\cdot}$, and $^1O_2$ was similar to that for the $O_2^{\cdot-}$ assay.

Chemiluminescence data reported as mean values ± S.D. were processed using SPSS software. Every experiment was repeated three times.

RESULTS AND DISCUSSION

Purification of CPRI. The purification procedure is summarized in Table 1. The preparation of the inhibitor thus obtained yielded a single band after electrophoresis in the presence of sodium dodecyl sulfate (SDS) on a 10% polyacrylamide gel. The molecular weight estimated by SDS-gel electrophoresis was 50 kD.

In preparing the affinity column, RNase A was coupled with CNBr-activated Sepharose 6B (RNase A/Sepharose 6B, 10 mg/3.5 ml) with coupling over 85%. Compared with the method described by Blackburn (RNase A/Sepharose 4B, 50 mg/40 ml) [17], the affinity capacity of the column is higher.

After isoelectric precipitation, the applied sample volume *prior to* the affinity column decreased and CPRI activity increased. Thus the affinity chromatograph was easy, time-saving, and expensive-reagent-saving. The decrease in the non-inhibitor substances in the applied sample prolonged the use of the affinity column because many non-inhibitor substances were hard to elute and harmful to the column.

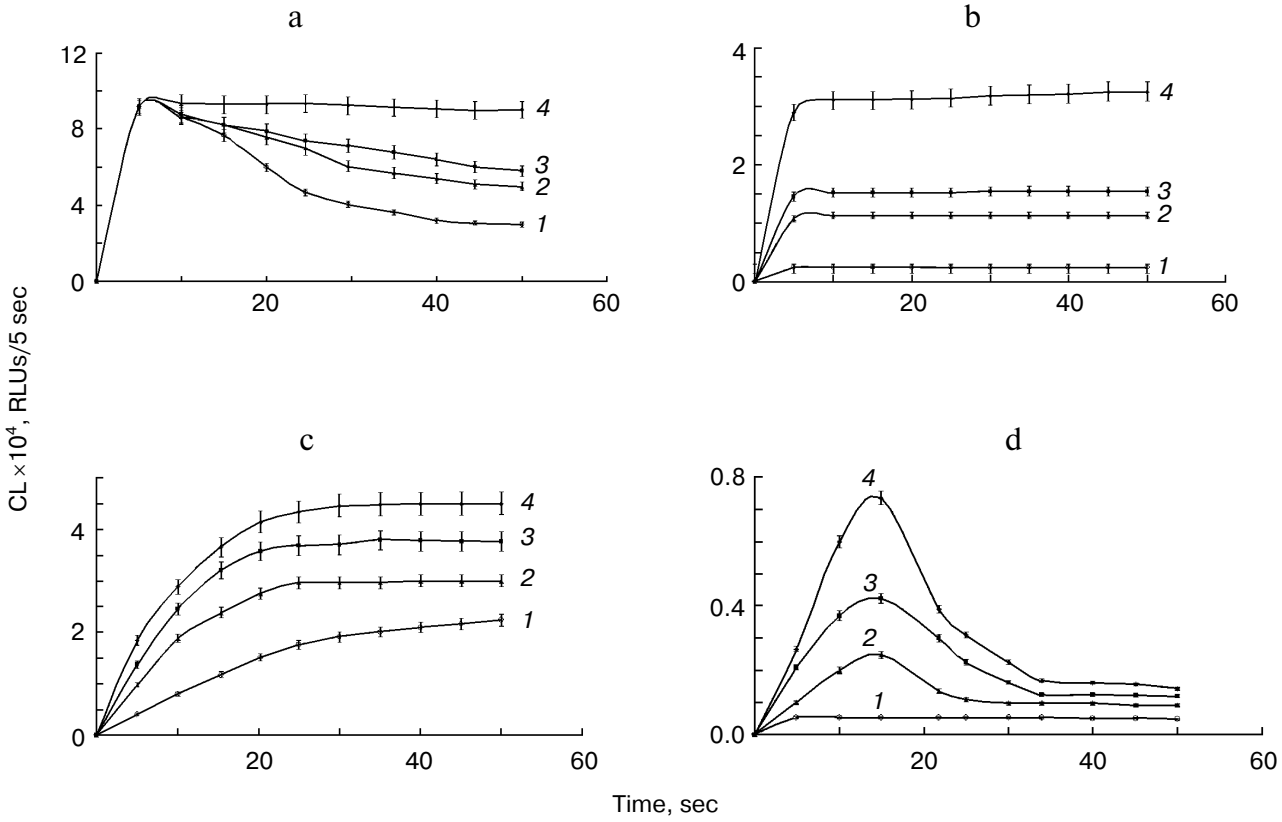
Radical scavenging activities of CPRI. Panel (a) of the figure shows the time-dependent scavenging effect of

Table 1. Purification of ribonuclease inhibitor from cow placenta

Step	Protein, mg	Activity, units	Volume, ml (g)	Specific activity, units/mg	Purification, fold	Recovery, %
Placenta homogenate	$8.1 \cdot 10^4$	$2.58 \cdot 10^6$	1500 ml	32	1	100
Ammonium sulfate precipitate	$1.5 \cdot 10^4$	$2.12 \cdot 10^6$	30 g	142	4.4	82
Isoelectric precipitation	$1.1 \cdot 10^4$	$1.86 \cdot 10^6$	18 g	164	5.1	72
48,000*g*, 1 h	$9.1 \cdot 10^3$	$1.79 \cdot 10^6$	100 ml	199	6.2	69
Affinity chromatography	8.3	$1.34 \cdot 10^6$	40 ml	$1.62 \cdot 10^5$	5062	52

CPRI on $O_2^{\cdot-}$. In the present system, $O_2^{\cdot-}$ generated from pyrogallol autooxidation could oxidize luminol to produce a strong chemiluminescence signal that was proportional to the concentration of $O_2^{\cdot-}$. Therefore, the scavenging effect of CPRI on $O_2^{\cdot-}$ could be obtained by observing its inhibition of the CL. The CL of the control increased quickly and then remained relative stable. After adding CPRI, the CL decreased slowly and dose-dependently, and tended to be flat after 50 sec. The effective concentration (EC_{50}), which was defined as the concentration to inhibit by 50% the chemiluminescence intensity, was usually used to express the radical scavenging activities.

Panel (b) of the figure shows the time-dependent scavenging effect of CPRI on $OH^{\cdot}$. $OH^{\cdot}$ generated in a Fenton-type reaction could oxidize luminol to produce a



Radical scavenging effect of CPRI. a) Time-dependent scavenging effect of CPRI on $O_2^{\cdot-}$ with 0.2 mg/ml (*1*) and 0.1 mg/ml CPRI (*2*). Tea polyphenol (0.1 mg/ml) was taken as a positive control (*3*). b-d) Time-dependent scavenging effect of CPRI on $OH^{\cdot}$ (b), $R^{\cdot}$ (c), and $^1O_2$ (d) with 0.02 mg/ml (*1*) and 0.01 mg/ml CPRI (*2*). Tea polyphenol (0.01 mg/ml) was taken as a positive control (*3*). a-d) Tris-HCl instead of specimen was present in blank control (*4*)

strong chemiluminescence signal proportional to the concentration of $OH^{\bullet}$ [20]. The CL of the control quickly increased with time and reached a plateau after 7 sec. After addition of CPRI, the CL intensity was decreased in a dose-dependent fashion.

Panel (c) of the figure shows the time-dependent scavenging effect of CPRI on $R^{\bullet}$. $R^{\bullet}$ generated in an H_2O_2/evening primrose oil system could produce relatively strong CL not depending on luminol. The CL of the control increased with time and reached a plateau after 40 sec. The CL intensity dose-dependently decreased after the addition of CPRI.

Panel (d) of the figure shows the time-dependent scavenging effect of CPRI on $^1O_2$. $^1O_2$ was generated in the reaction between NaClO and H_2O_2 in a solution of pH 8.5 and detected by the CL technique. The CL of the control produced a peak at 17 sec and then decayed quickly with time. The CL dose-dependently decreased with the addition of CPRI.

The higher the EC_{50} value, the lower were the radical scavenging activities. Referring to EC_{50} (Table 2), it could be observed that the radical scavenging activities of CPRI towards ROS decreased in the sequence $^1O_2 > OH^{\bullet} > R^{\bullet} > O_2^{\bullet-}$ which is consistent with the decreasing sequence of ROS activities. Table 2 also compares the EC_{50} of CPRI and tea polyphenols and indicates that the radical scavenging activities of CPRI were higher than those of tea polyphenols, which are well-known antioxidants, showing that CPRI is a powerful ROS scavenger.

ROS in the form of $O_2^{\bullet-}$, $OH^{\bullet}$, $^1O_2$, and $R^{\bullet}$ are highly reactive and potentially damaging transient chemical species formed in all cells as unwanted metabolic byproducts of normal aerobic metabolism. Cells are protected from ROS induced damage by a variety of endogenous ROS scavenging enzymes and chemical compounds [21]. ROS attack biological molecules leading to cell or tissue injury. The role of ROS in tissue damage in various human diseases, cancer, and aging is becoming increasingly recognized [22].

Moenner et al. showed that significant amounts of RI are present in human and rat erythrocytes that are essentially devoid of ribonuclease or functional RNA. They suggested a role for RI in the metabolism and aging process of erythrocytes [5]. Cui's investigation showed that activities of RNases increased with aging, while the activities of RI decreased with aging [23]. Cui's study showed that the exogenous placental RI could inhibit the growth of mouse tumors, such as sarcoma S-180. The inhibitory effects of RI on the tumor could be associated to its antioxidative effects as well as its anti-angiogenic role [24]. Cui's experiment also suggests that RI has a protective role against mouse hepatic damage induced by CCl_4 [25]. Our results, showing that CPRI can scavenge all the ROS studied powerfully, has further proved that the mechanism for the function of RI in cancer and aging is closely related with its antioxidative activity.

Table 2. EC_{50} comparison between CPRI and tea polyphenols (mg/ml)

ROS	CPRI	Tea polyphenols
$O_2^{\bullet-}$	0.12	0.16
$OH^{\bullet}$	0.008	0.01
$R^{\bullet}$	0.009	0.020
$^1O_2$	0.006	0.009

The fact that aging and cancer have relevance to peroxidative lesions has been well proved. This suggests that the radical-scavenging effects of CPRI may contribute to its function in the cell protection from peroxidative injuries unrelated to inhibition of RNase A. The antioxidative mechanisms of RI may be associated with the multiple thiol groups in its molecule, which can play a role just like reduced GSH. Our results seem to suggest further studies are needed on the biological function and development of RI-based medicine.

REFERENCES

1. Blackburn, P., Wilson, G., and Moore, S. (1977) *J. Biol. Chem.*, **252**, 5904-5910.
2. Lee, F. S., Shapiro, R., and Vallee, B. L. (1989) *Biochemistry*, **28**, 225-230.
3. Hofsteenge, J. (1997) in *Ribonucleases: Structures and Functions* (D'Alessio, G., and Riordan, J. F., eds.) Academic Press, New York, pp. 621-658.
4. Lee, F. S., and Vallee, B. L. (1993) *Progr. Nucleic Acid Res. Mol. Biol.*, **44**, 1-30.
5. Moenner, M., Vosoghi, M., Ryazantsev, S., and Glitz, D. G. (1998) *Blood Cells Mol. Dis.*, **24**, 149-164.
6. Blackburn, P., and Moore, S. (1982) in *Pancreatic Ribonuclease* (Boyer, P. D., ed.) *The Enzymes*, Vol. XV, Pt. B, Academic Press, New York, pp. 317-433.
7. Vicentini, A. M., Kieffer, B., Matthies, R., Meyhack, B., Hemmings, B. A., Stone, S. R., and Hofsteenge, J. (1990) *Biochemistry*, **29**, 8827-8834.
8. Kim, J. S., Soucek, J., Matousek, J., and Raines, R. T. (1995) *J. Biol. Chem.*, **270**, 31097-31102.
9. Shapiro, R., and Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2238-2241.
10. Holloway, D., Shapiro, R., Hares, M. C., Leonidas, D. D., and Acharya, K. R. (2002) *Biochemistry*, **41**, 10482-10489.
11. Rajashekhar, G., Loganath, A., Roy, A. C., and Wong, Y. C. (2002) *Mol. Reprod. Dev.*, **62**, 159-166.
12. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. (1999) *N. Engl. J. Med.*, **324**, 1-8.
13. Shapiro, R. (2001) *Meth. Enzymol.*, **341**, 611-628.

14. Gho, Y. S., and Chae, C. B. (1997) *J. Biol. Chem.*, **272**, 24294-24299.
15. Blazquez, M., Fominaya, J. M., and Hofsteenge, J. (1996) *J. Biol. Chem.*, **271**, 18638-18642.
16. Kim, B. M., Schultz, L. W., and Raines, R. T. (1999) *Protein Sci.*, **8**, 430-434.
17. Blackburn, P. (1979) *J. Biol. Chem.*, **254**, 12484-12487.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
19. Yu, W., Zhao, Y., Xue, Z., Jin, H., and Wang, D. (2001) *J. Am. Oil Chem. Soc.*, **78**, 697-701.
20. Yildiz, G., and Demiryurek, A. T. (1998) *J. Pharmacol. Toxicol. Meth.*, **39**, 179-184.
21. Halliwell, B., and Gutteridge, J. M. C. (1990) *Meth. Enzymol.*, **186**, 1-85.
22. Halliwell, B., Gutteridge, J. M. C., and Cross, C. E. (1992) *J. Lab. Clin. Med.*, **119**, 598-620.
23. Cui, X. Y. (1994) *Tumor (in Chinese)*, **14**, 52-154.
24. Cui, X. P., and Cui, X. Y. (1999) *Pract. Oncol. (in Chinese)*, **14**, 23-25.
25. Cui, X. Y., Fu, P. F., Pan, D. N., Zhao, Y. T., Zhao, J., and Zhao, B. C. (2003) *Free Rad. Res.*, **37**, 1079-1085.