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Purification and cloning of glyoxalase II from rat liver

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Abbreviations: CBG, carbobenzoxyglutathione; GLO, glyoxalase; MGO, methylglyoxal; PMSF, phenylmethylsulfonyl fluoride; S-LG, S-lactoylglutathione

Abstract

Glyoxalase (GLO) II, which is a component of GLO system and catalyzes the conversion of S-lactoylglutathione to D-lactate, was purified 1488 fold from rat liver by two steps of Affigel blue and carbobenzoxyglutathione-Sepharose 4B affinity chromatography. The molecular weight of the enzyme was estimated to be 29 kDa which is similar to those from other species. The sequence of N-terminal 9 amino acid residues was determined to be MGIRLLPAT. This was then used to syrnthesize degenerative primers. cDNA clone was isolated by first synthesizing cDNA from RNA and then PCR amplification. The sequence of cDNA clone was determined by serial sequencing analysis.

Keywords: glyoxalase II, liver, purification, sequence

Introduction

The glyoxalase (GLO) system consisting of GLO I (EC 4.4.1.5, lactoylglutathione lyase), GLO II (EC 3.1.2.6, hydroxyacylglutathione hydrolase) and GSH is distributed in all organisms and catalyzes the conversion of methylglyoxal (MGO) to D-lactate through the intermediate S-lactoylglutathione (S-LG) (Racker, 1951; Mannervick, 1980; Thornalley, 1990). The GLO system was known to be involved in detoxification of cytotoxic MGO produced from glycolytic intermediates and threonine and suggested to play a fundamental role in biology (Thornalley, 1990, 1993). However, the exact physiological role of the system is still unknown. Among two enzymic components of the system GLO II has been less studied compared to GLO I that has been extensively studied. In this paper we describe the purification of GLO II from rat liver by two step procedures, determination of N-terminal 9 amino acids sequence of GLO II and cloning of its cDNA.

Materials and Methods

Synthesis of carbobenzoxyglutathione

As an affinity ligand, carbobenzoxyglutathione (CBG), a strong inhibitor of GLO II, was prepared from carbobenzoxy chloride (ICN) and GSH according to the method described by Norton and Hsu (1983) and purified by silica gel TLC (Sigma). The purified CBG was then coupled to activated CH Sepharose 4B (Pharmacia).

Purification of GLO II

Rat (Sprague Dawley) liver (3.7 g) was homogenized in 0.25 M sucrose containing 2 mM 2-mercaptoethanol and the homogenate was subjected on a Affigel blue column (2.5 × 17.5 cm) preequilibriated with 40 mM phosphate buffer, pH 7.2, containing 2 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The column was washed with the buffer to remove the unabsorbed substances and eluted with the buffer containing 0 to 0.5 M KCl gradient. The fraction from the Affigel column was subjected to a CBG-Sepharose column (1.5 × 8 cm) equilibriated with a mixture of the phosphate buffer: glycerol (9 : 1). The protein was eluted using a gradient of 0 to 3 M KCl.

Assay for GLO II activity

The activity of GLO II was analyzed according to the method described by Uolita (1973) or Principato *et al*. (1987). One unit of the activity was defined as the amount of enzyme catalyzing the hydrolysis of 1μmol of S-LG/min or the formation of 1 μmol of 5,5'-dithiobis-(2-nitrobenzoic acid)/min using molar absorption coefficient 3,310 cm^{-1} at 240 nm or 13,600 cm^{-1} at 412 nm.

SDS-PAGE

SDS-PAGE was carried out using 12% gel according to the method of Laemmi (1970). After electrophoresis the gel was stained with Coomassie Brilliant Blue R 250 or subjected to Western blotting with rabbit anti-bovine liver GLO II prepared previousely in this laboratory (Yang *et al*., 1995) as a primary antibody and horseradish peroxidase-conjugated anti-rabbit by immunoglobulin (Amersham) as a secondary antibody. Signal was detected by using ECL reagents (Amersham).

Determination of amino acid sequence

The purified GLO II (0.5 μg) was subjected to SDS-PAGE (12% gel) using Tricine SDS running buffer. Protein on the gel was electrotransfered to PVDF membrane and the protein band corresponding to GLO II after Coomassie Brilliant Blue staining was excised. After removing N-acetyl blocking group of amino terminal with 1 M HCl at 110˚C for 1 h, the protein was subjected to Edman degradation using Beckman LF 3500 gas phase amino acid sequencer. Phenylthiohydantoin derivative after each cycle was analyzed by HPLC.

Cloning of GLO II

Primer

Two degenerative primers, RG-N1 and RG-N2 with 24 base size corresponding to N-terminal region, were synthesized and used as primary primer as well as oligo-$p(dT)_{15}$. The secondary primers, RG-N1A and RG-C1A were synthesized from internal sequences after partial sequencing.

Isolation of RNA and synthesis of first strand cDNA

Whole RNA from rat liver (0.5 g) was isolated by routine method. First strand cDNA was synthesized using cDNA synthesis kit (Boehringer Mannheim) in 20 μl reaction mixture containing 5 mM $MgCl_2$, 1 mM deoxynucletide mixture, 1.6 μg oligo-p(dT), gelatin 4 ng, RNase inhibiors 50 units, AMV reverse transcriptase 20 units and 20 μg of the isolated RNA.

PCR

For PCR amplification, 1 μg of first strand cDNA was added in a 20 μl PCR reaction mixture with 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.4 mM of each dNTP, 0.4 μM 5' and 3' primers, and 0.5 units Taq polymerase. Nonspecific PCR amplification was performed 3 cycles at 95˚C for 30 sec for denaturation, at 45˚C for 1 min for annealing, at 72˚C for 1 min for extension and then specific amplification was carried out 40 cycles at 95˚C for 30 sec for denaturation, at 55˚C for 1 min for annealing and at 72˚C for 1 min for extension.

Cloning of PCR product

After purification of DNA from PCR product by 1% LMT agarose gel electrophoresis using QIAEX II kit (Qiagen), the purified DNA was ligated to pT7 blue vector (Novagen). Competent cells were transformed with the ligated vector and incubated in amphicillin-LB-agar plate containing X-Gal and IPTG (Gibco/BRL).

Base sequence analysis

After purification of plasmid from the transformant DNA sequence was analyzed by Sanger's dideoxy method using Delta-Taq cycle sequencing kit (USB) by following the manufacturer's instructions.

In addition to the methods described above GLO II gene cloning using cDNA library of rat liver (Clontech) as starting material was also carried out by library screening according to the method described in Clontech lamda library protocol.

Results and Discussion

Purification of GLO II

After Affigel blue (Figure 1) and CBG affinity (Figure 2) chromatography of rat liver homogenate, GLO II was purified 1488 fold with 21% yield (Table 1), showing that the two steps of affinity chromatography are highly efficient method for GLO II purification. The purified preparation gave a single band at the position corresponding to 29 kDa on SDS-PAGE (Figure 3A) and the same band reacted with rabbit anti-bovine liver GLO II (Figure 3B), suggesting a high homology between rat and bovine GLO II. The presence of a single band of the GLO showing SDS-PAGE suggests that there is no polymorphism in rat liver GLO II.

N-terminal amino acid sequence

The N-terminal amino acid sequence of rat liver GLO II

Table 1. Purification of GLO II from rat liver.Fresh rat liver (3.7 g) was homogenized. Homogenate was chromatographed on Affigel blue column (2.5 × 17.5 cm) and then CBG-Sepharose column (1.5 × 8 cm). The GLO II activity was determined using S-LG as substrate.

Step	Vol. (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold	Yield (%)
Homogenate	16	97.07	287.84	0.34	1.0	100
Affi-gel blue chromatography	102	55.08	47.74	1.154	3.4	57
CBG affinity chromatography	222	0.24	0.04	506	1488	21

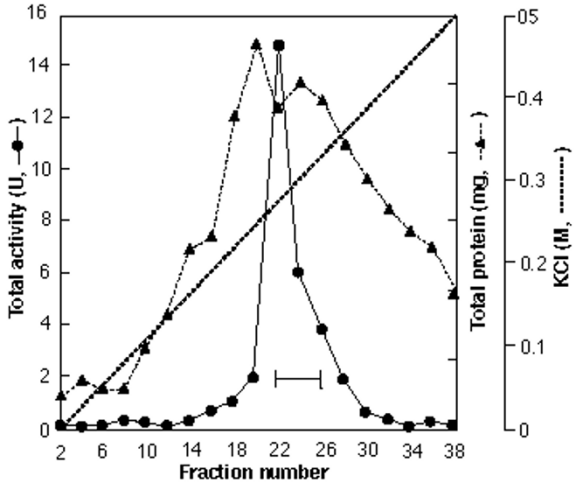

Figure 1. The profile of Affigel blue chromatography. Rat liver homogenate was applied to Affigel column (2.5 × 17.5 cm) equilibriated with 40 mM potassium phosphate, pH 7.2 containing 2 mM 2-mercaptoethanol and 0.1 mM PMSF. The column was washed with 270 ml of the buffer. The column was then eluted with a gradient of 0 - 0.5 M KCl in 400 ml of the same buffer and fractions indicated with bar were pooled.

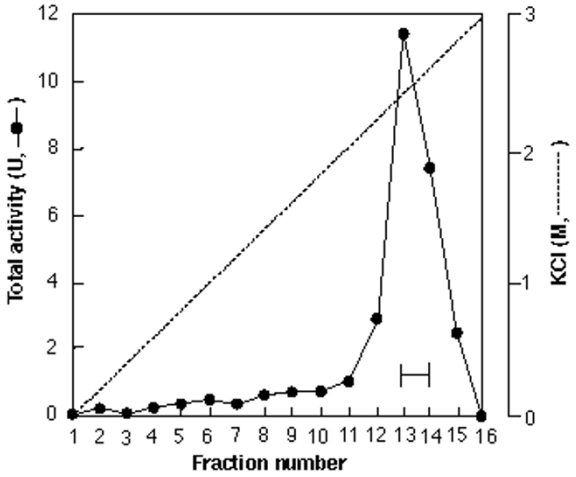

Figure 2. The profile of CBG affinity chromatography. Partially purified GLO II was loaded onto CBG-Sepharose 4B column (1.5 × 8 cm) equilibriated with buffer (9 parts of 40 mM potassium phosphate, pH 7.2, containing 2 mM 2-mercaptoethanol and 0.1 mM PMSF : 1 part of glycerol). The column was washed with the buffer and then eluted with a gradient of 0 - 3 M KCl in 80 ml of the same buffer and fractions indicated with bar were pooled.

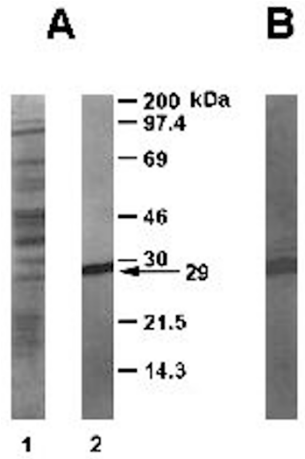

Figure 3. SDS-PAGE of purified GLO II. A. Coomassie Brilliant Blue R 250 staining: lane 1, rat liver whole homogenate; lane 2, purified GLO II. B. Western blot analysis of purified GLO II. Anti-GLO II antibody and peroxidase-conjugated anti-rabbit IgG were used as primary and secondary antibody, respectively. Signal was shown by using ECL reagents.

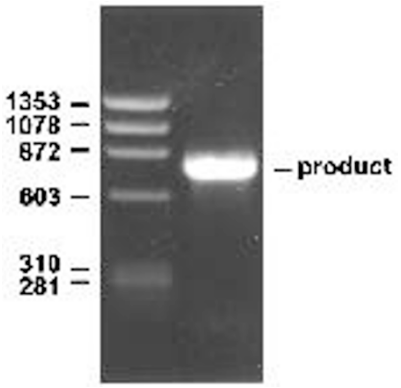

Figure 4. Agarose gel electrophoresis of PCR products with primers RG-N1, RG-N2 and oligo-p(dT)$_{15}$.

was determined by 10 cycles of Edman degradation and HPLC. The sequence of N-terminal 9 amino acid residues was Met-Glu-Ile-Arg-Leu-Leu-Pro-Ala-Thr. Recently, Ridderström e*t al.* (1996) has reported the whole amino acid sequence of human liver GLO II. When the N-terminal 9 amino acid sequence of rat liver GLO II was compared with that of human liver GLO II, it showed deletion of one amino acid residue at position 9 and 50% homology i.e. substitutions are seen at residues 2, 3, 4 and 5 (Table 2).

Cloning of GLO II

The sequences of two degenerative primers, RG-N1 and RG-N2, synthesized from the amino acid sequence of N-terminal region and two internal primers of RG-N1A and -C1A obtained from the partial sequence are shown in Table 3.

Electrophoretic pattern of the isolated RNA from rat liver showed clearly two ribosomal 28S and 18S RNA bands, suggesting that RNA extraction was achieved without degradation (data not shown). First strand cDNA was obtained from whole RNA by AMV reverse transcriptase and was amplified by PCR using RG-N1, -N2

Figure 5. Nucleotide sequence of cDNA and the deduced amino acid sequence of GLO II from rat liver. Amino acid sequence of N-terminal region was directly determined from purified GLO II. Base sequences indicated with underlined Italic were used as secondary primers. The sequence of C-terminal region was failed to be determined in this study.

Table 2. N-terminal amino acid sequence of rat liver GLO II

Rat	M	G	I	R	L	L	P	A	-	T
Human[1]	M	K	V	E	V	L	P	A	L	T

[1] Cited from Ridderström e*t al.* (1996).

Table 3. Primers used for PCR.

RG-N1[1]	ATGGGTATCGAGCTGCTGCCGGCG
RG-N2[1]	ATGGGCATTGAACTCCTCCCAGCT
RG-N1A[2]	TCACACACCTTTCCACAC
RG-C1A[2]	GTGTATTCATGGCCACAG

[1] Primary degenerative primers synthesized from the N-terminal amino acid sequence.

[2] Secondary primers synthesized after partial base sequencing.

and oligo-p$(dT)_{15}$ primers. The resulting PCR product showed a band of GLO II cDNA with 800-900 base pairs (Figure 4). After the ligation of the obtained GLO II cDNA with pT7 blue vector and transformation of competent cells with the ligated product, the cloned GLO II cDNA was obtained and subjected to the second PCR. From sequence analysis of the first PCR product, the sequences of about 300 base pair length of 5' and 3' regions were determined. As shown in Figure 5, 735 base pair sequence out of entire GLO II cDNA was determined using internal primers, RG-N1A and RG-C1A, obtained from partial sequence. Considering the N-terminal 9 amino acid sequences (shown in Table 2) and full base sequence of human GLO II (783 base pair), the sequence of 5'- end with 24 bases corresponding the N-terminal region and 3'-end with about 24 bases were failed to be determined directly in this study. Cloning by library screening using

cDNA library gene showed the same result.

Entire amino acid sequence of rat liver GLO II deduced from the base sequence including the N-terminal region of nine amino acids which were determined by Edman degradation (see Table 2) is presented in Figure 5.

GLO II cDNAs from human and *Arabidopsis thaliana* have recently been cloned and sequenced (Ridderström *et al*., 1996; Ridderström and Mannervik, 1997). Comparison of the two cDNA nucleotide and amino acid sequences deduced from the cDNA sequences shows 60% and 54% identity, respectively and certain limited regions shares 100% identity, indicating that they are derived from the same ancester. However, their entire sequences are quite divergent, reflecting their evolutional distance.

Comparison of rat GLO II cDNA and amino acid sequence obtained in this study with those from human demonstrated 87% and 90% identity, respectively. Particularly, two segments 45-70 and 130-152 of rat GLO II were 100% identical with corresponding sequences in the human enzyme. The higher homology in nucleotide and amino acid sequence of GLO II between human and rat than between human and plant is well in accord with their evolutional closeness.

The first identical region 45-70 contains four histidine residues. On considering that histidine residue is involved in the catalytic acivity of the enzyme (Ball and Vander Jagt, 1981), the region seems to be important in the activity. Analysis of the three-dimensional structure from the deduced amino acid sequence using protein analysis program of Macvector showed that the region 45-70 is buried in the protein to form the active site pocket. The domain also had partial flexibility, which may have the advantage for being used as the active site. These analyses support the assumption that the region is participated in the enzyme activity (data not shown).

Although this study provides a little understanding on the primary structure and active site of GLO II, further molecular biological studies using the cloned GLO II cDNA is remained to be done to elucidate the biological role and catalytic mechanism of the enzyme.

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