CHINESE JOURNAL OF CATALYSIS
Volume 31, Issue 12, 2010
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Catal, 2010, 31: 1445–1451.

RESEARCH PAPER

Possible Role of Carboxyl and Imidazole Groups in the Catalysis of Pummelo Limonoid Glucosyltransferase

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Abstract: Limonoid bitterness is a serious problem in the citrus industry worldwide. Limonoid glucosyltransferase is an enzyme that catalyzes the conversion of bitter limonoid into non-bitter limonoid glucoside while retaining the health benefit of limonoids in the juice. The immobilization of this enzyme in a column can solve the juice bitterness problem. More information about the catalytic residues of the enzyme is needed in this immobilization process. Glutamate/aspartate, histidine, lysine, tryptophan, serine, and cysteine residues were chemically modified to investigate their roles in the catalytic function of limonoid glucosyltransferase. Inactivation of the enzyme following modification of carboxyl and imidazole moieties was a consequence of a loss in substrate binding and catalysis in the glucosyltransfer reaction. The modification of a single histidine residue completely destroyed the ability of limonoid glucosyltransferase to transfer the D-glucopyranosyl unit. Tryptophan seemed to have some role in maintaining the active conformation of the catalytic site. Lysine also seemed to have some direct or indirect role in this catalysis but the modification of serine and cysteine did not have any effect on catalysis. Therefore, we conclude that the carboxyl and imidazole groups containing amino acids are responsible for the catalytic action of the enzyme.

Key words: chemical modification; catalytic residue; carboxyl group; imidazole group; uridine diphosphate glucosyltransferase; pummelo albedo

Chemical modification has assumed a prominent role among the many special techniques that have been developed recently to assist in determining the structure-function relationships of proteins. Sophisticated techniques such as X-ray crystallography and site directed mutagenesis have reduced the importance of chemical modification techniques. However, they are still used in fundamental studies of structure-function relationships of enzymes and other proteins as well as in studies related to the industrial requirements of foods, nutrition, and pharmacology [1–5]. In the absence of crystallographic data, the chemical modification of amino acid side chains provide information about the groups involved in the catalytic functioning of enzymes.

In this study, chemical modification was used to identify and quantify catalytically essential amino acid residues of limonoid glucosyltransferase (LGTase). This enzyme catalyzes the transfer of a glucose unit from uridine diphospho glucose (UDPG) to limonoids to yield nonbitter glucosides, which reduces limonoid bitterness in citrus. Identification of the essential residues is necessary for the future application of this enzyme in debittering citrus juice or in the industrial production of limonoid glucoside (LG) through immobilization. The purification and characterization of this enzyme from pummelo albedo and its immobilization have been reported [6,7]. No report exists wherein the active site of this enzyme was studied. Considering the medicinal value of LG, a chemical modification study of LGTase was carried out with special emphasis on the structure-function relationship. This paper describes the identification of some amino acid residues that appear to play a direct or indirect role in the catalysis of the glucosyltransfer reaction.

1 Experimental

Diethylpyrocarbonate (DEPC), *p*-chloromercuribenzoate (PCMB), 3-nitro-L-tyrosine ethylester (NTEE), and 1-ethyl-3-

Received date: 19 May 2010.

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Foundation item: Supported by: Ministry of Education, Culture, Sports, Science and Technology of Japan.

DOI: 10.1016/S1872-2067(10)60130-1

(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Wako Pure Chemical Co. Ltd. Diisopropyl fluorophosphates (DFP), iodoacetamide, N-bromosuccinimide (NBS), and citraconic anhydride (CA) were purchased from Sigma Chemical Co. Ltd. Limonoid glucosyltransferase was purified from pummelo albedo tissue [6].

1.1 Chemical modification of the amino acid residues

1.1.1 Histidine modification

LGTase (20 μg, 4.32 pkat) was incubated with 2 mmol/L DEPC in 20 mmol/L sodium phosphate buffer, pH 7.0 at 25 °C for 20 min to a final volume of 40 μl. Modification was measured by monitoring the absorbance at 240 and 280 nm to discriminate between the modification of histidine and tyrosine residues, respectively [8–10]. The modified enzyme was then incubated with the substrate under standard conditions using a 100 μl reaction volume and the residual activity was assayed. For an analysis of the time course of modification the enzyme was incubated with various concentrations of DEPC from 0.25 to 2.0 mmol/L at different time intervals. Subsequently, aliquots from each reaction were added to the substrate solution at 2 min intervals for catalysis.

The modified LGTase was treated with hydroxylamine hydrochloride at a concentration of 250 mmol/L for 16 h at pH 7.0 and at 4 °C for a reversal of the modification [9–11]. The activity of the untreated and the hydroxylamine treated enzyme was then determined for further analysis.

1.1.2 Tryptophan modification

The tryptophan residue was modified with NBS. The modification was carried out by titrating 1 ml of LGTase (0.5 mg) in 0.1 mol/L sodium phosphate buffer, pH 7.0 and at 30 °C with freshly prepared 20 to 50 mmol/L NBS. The modification was followed by monitoring the changes in absorbance at 280 nm. The reagent was added in six installments of 10 μl each. After each addition, an aliquot (10 μl) was removed and quenched by the addition of 20 μl of 25 mmol/L L-tryptophan and checked for residual enzyme activity under standard assay conditions. The K_m and k_{cat} values were also determined for the partially modified enzyme. The number of tryptophan residues oxidized per molecule of LGTase was calculated from the decrease in absorbance at 280 nm by assuming a molar absorption coefficient of 5 500 $(mol/L)^{-1}\cdot cm^{-1}$ [12]. The enzyme incubated in the absence of NBS was used as a control. The loss of LGTase activity by NBS treatment was partially protected by pre-incubating the enzyme with excess limonin.

1.1.3 Carboxyl-group modification

The carboxyl group was modified with EDC and NTEE. The enzyme (0.5 mg) was incubated with 35 mmol/L EDC and 21 mmol/L NTEE in 1 ml of 50 mmol/L Mes/Heps buffer (75/25, v/v), pH 6.0 and at 25 °C for 30 min. Subsequently, the reaction was terminated by the addition of 10% (w/v) TCA and the precipitated protein was collected by centrifugation, washed extensively with chilled acetone, air dried, and dissolved in 0.5 ml of 100 mmol/L NaOH. An aliquot of the reaction mixture was withdrawn before the addition of TCA and assayed for residual enzyme activity. The number of incorporated nitrosyl groups was determined spectrophotometrically at 430 nm using a molar absorption coefficient of 4 600 $(mol/L)^{-1}\cdot cm^{-1}$ [13]. An enzyme sample incubated in the absence of EDC/NTEE served as a control. K_m and k_{cat} values were also determined for the partially modified enzyme.

1.1.4 Lysine modification

The lysine residue was modified with CA according to the method of Dixon and Perham [14]. The enzyme (100 μg) was treated with 100 μl of 10 mmol/L CA in 1 ml of 100 mmol/L sodium bicarbonate buffer, pH 8.4 and at 25 °C. The reagent was added in 5 installments while maintaining a pH of 8.4. An aliquot of the reaction mixture was withdrawn after each addition of CA and the residual activity was determined under standard assay conditions. An enzyme sample incubated in the absence of CA served as a control. De-citraconylation was achieved by incubating the modified enzyme samples at pH 4.0 and at 4 °C for 15 h followed by an assay of the residual enzyme activity.

1.1.5 Cysteine modification

PCMB and iodoacetamide were used separately to modify the cysteine residue. Modification of 50 μg of the enzyme (10.8 pkat) was carried out with 300 μmol/L of PCMB in 100 μl of 50 mmol/L phosphate buffer, pH 7.2 and at 25 °C for 30 s. The treated sample was dialyzed against the same buffer at 4 °C for 4 h to remove the reagent before determining the residual LGTase activity.

The modification of LGTase (50 μg, 10.8 pkat) with 2 mmol/L iodoacetamide was performed in 100 μl of 20 mmol/L phosphate buffer, pH 7.7 and at 25 °C for 1 min. The treated sample was dialyzed against the same buffer at 4 °C for 4 h to remove the reagent and the residual enzyme activity was determined.

1.1.6 Serine modification

DFP was used to modify the serine hydroxyl group. The enzyme solution (0.5 mg in 100 μl of 200 mmol/L phosphate buffer, pH 7.7) was incubated with sufficient DFP to give a concentration of 0.2 to 1 mmol/L at 25 °C for 30 min. The residual enzyme activity was determined under standard assay