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PURIFICATION AND CHARACTERIZATION OF HEPATIC PORCINE GLUCONOLACTONASE

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SUMMARY: A divalent metal dependent gluconolactonase has been isolated from porcine liver and purified to apparent homogeneity. Its molecular weight is estimated at 223,000 and that of the subunits is 37,200 as determined by gel electrophoresis. A K_m value of 6.2 mM was obtained at 27° in 50 mM tris HCl buffer. Gluconolactonase is specific for gluconolactone, and manganese is preferred over magnesium for maximum activity. The hepatic concentration of gluconolactonase is estimated to be 7.2 μmol of enzyme per kg of porcine liver, and a subcellular fractionation study indicates that this enzyme is located primarily within the cytosol.

INTRODUCTION: The enzyme gluconolactonase (E.C.3.1.1.17) catalyzes the hydrolysis of gluconolactone to gluconic acid. The existence of this enzyme in bacteria, yeast and rat liver was established by Brodie and Lipmann(1) who purified the enzyme 36-fold from yeast. Other investigators(2-5) have isolated and partially purified lactonases which utilize 1,5-gluconolactone as a substrate.

In previous studies we have reported the isolation, characterization and kinetic properties of the enzymes glucose dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from hepatic tissue(6-10). Of these three enzymes, only glucose dehydrogenase is localized within the endoplasmic reticulum(8,10). Glucose dehydrogenase converts NAD(P) to NAD(P)H and β-D-glucose to glucono-δ-lactone. It is our purpose to determine the metabolic fate of gluconolactone and to study gluconolactonase from hepatic tissue. In this work we report the isolation, purification, and partial characterization of this enzyme from pig liver.

MATERIALS AND METHODS

Preparation and purification of lactones: D-glucono-1,5-lactone, M. 155°, D-glucurono-1,4-lactone, M. 177-178°, D-galactono-1,4-lactone, M. 135-136°, and

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L-galactono-1,4-lactone, M. 135-136° were recrystallized from ethanol. D-gluconо-1,4-lactone, M. 134-136°, was prepared from D-gluconic acid(11). D-glucoheptono-1,4-lactone, M. 151-152° was used as obtained and D-gulcono-1,4-lactone, M. 186-187°, was recrystallized from methanol. 6-Phosphogluconolactone was prepared by bromine oxidation of glucose-6-phosphate(12). The low pH (4.5) of the resulting solution was raised to pH 7.5 with NaOH immediately before adding the 6-phosphogluconolactone to the enzyme solution.

Lactone assays: The lactone determination was accomplished using either Eisenberg and Field's(13) modification of Hestrin's procedure(14) or a pH method calibrated against the former method(13). The pH method consisted of following the pH change at 27° of a standard reaction mixture using an Orion model 801A digital ionanalyzer equipped with a semi micro pH electrode and an Electro Instruments model 480 recorder. The pH method gave linear plots of ΔpH/min which were calibrated against the spectrophotometric method(13) for each substrate. A typical reaction mixture (3 ml) contained 50 mM pH 7.5 tris HCl buffer, 1.3 mM $MnCl_2$ and 10 units of gluconolactonase. The reaction was initiated by the addition of 8 mg of lactone giving a final lactone conc. of 14.4 mM. Protein concentrations were estimated from measurements of absorbance at 280 and 260 nm according to the method of Warburg and Christian(15) or by the microbiuret method of Goa(16). One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μmole of lactone per min.

Subcellular fractionation: For the determination of the subcellular localization of the enzyme, freshly slaughtered pig liver was homogenized and fractionated according to a modified method of Schneider and Hogeboom(17) as described previously(8). Gluconolactonase, glucose-6-phosphate dehydrogenase(8) and glucose dehydrogenase(8) were assayed in the clear supernatants of the various fractions at 30°.

Purification: Pig liver fresh from the slaughterhouse was cut into 2-cm cubes. 50 g amounts were added to 150 ml of distilled water, and homogenized in a Waring Blender for 60 s. The homogenate was filtered through cheesecloth and then centrifuged at 600xg for 1 h at 4°. The supernatant was heated to 55° for 10 min, cooled to 4°, and stored overnight. The pH was lowered to 5.8 with 5% acetic acid, and centrifuged for 1 h at 6,000xg at 4°. The resulting supernatant was made 60% saturated in ammonium sulfate, centrifuged for 30 min at 10,000xg, and the resulting precipitate discarded. The solution was then made 80% saturated in ammonium sulfate and kept at 4° for 30 min. This solution was then centrifuged at 10,000xg at 4° for 30 min and the precipitate dissolved in 50 mM pH 7.5 tris HCl buffer with a final protein conc. of at least 35 mg/ml. This extract was made 2 x 10^{-5} M in p-toluene sulfonyl chloride and stored at 4°.

Column separations: All column chromatography was done at room temperature as separations at 4° gave repeatedly poor results. 5 ml of the extract was loaded on either a Sephadex G-150 or Sephacryl S-200 column (2.6 x 38 cm) and eluted with 5 mM pH 7 phosphate buffer containing 0.002% sodium azide. The most active fractions from two such column elutions were pooled and concentrated two-fold with Lyphogel. The resulting condensed fraction (10 ml) was loaded onto a Cellex-P column (1.3 x 2.8 cm) and negatively chromatographed with 1 mM pH 6.0 potassium phosphate buffer while contaminant protein remained on the column. The most active fractions were again pooled and condensed 2-fold with Lyphogel. The resulting solution (7 ml) was loaded onto a spheroidal hydroxylapatite column (0.9 x 8 cm) and negatively chromatographed with 1 mM pH 6.5 phosphate buffer. The maximum specific activity fractions were combined (6 ml) and chromatographed by absorption on a second Cellex-P column (0.8 x 8 cm) previously equilibrated with 1 mM pH 6.0 potassium phosphate buffer. The column was then washed with an additional 100 ml of 1 mM pH 6.0 potassium phosphate buffer resulting in the elution of contaminant protein. Finally, the addi-

TABLE I: Purification Procedure and Results

Step	Activity units/ml	Protein mg/ml	Volume ml	Specific Activity units/mg	Overall Purification fold
1. Homogenization	450	98.5	3400	4.2	----
2. Heat and Acid treatment	522	18.2	1350	28.7	6.8
3. Ammonium Sulfate fractionation	938	35.3	60	26.6	6.3
4. Sephacryl 200 or Sephadex G-150	353	2.05	108	172.3	41.0
5. Cellex-P	446	1.00	76	446	106.2
6. Spheroidal hydroxylapatite	356	0.75	65	475	113.1
7. Cellex-P	114	0.21	87	1040	247.6

tion of 100 ml of the same buffer containing 20 mM KCl resulted in the elution of a single protein peak which contained virtually all of the lactonase activity.

RESULTS

I. Purification procedure: A typical set of purification results is given in Table I. The final specific activity was greater than 1000 units per mg protein and the enzyme is apparently homogeneous as indicated by the results of 4% acrylamide gel and SDS gel electrophoresis. A single band with a molecular weight of 223,000 (±5,000) was obtained on 4% gels using the method of Ornstein(18) and Davis(19). Standards used included catalase (240,000), pyruvate kinase (237,000), glucose oxidase (148,000), and bovine serum albumin (67,000). Sodium dodecyl sulfate electrophoresis according to the method of Weber and Osborn(20) gave a single subunit band of 37,200 (±500). Standards used included bovine serum albumin (67,000) and molecular weight markers (14,300, 28,600, 42,900, 57,200, 71,500 and 85,800) furnished by Gallard-Schlesinger Chemical Corp. These results are consistent with gluconolactonase being a hexamer whose subunits are virtually identical in size.

TABLE II: Subcellular Localization of Hepatic Porcine Gluconolactonase

Fraction	Glucose-6-phosphate Dehydrogenase Activity Units	%	Glucose Dehydrogenase Activity Units	%	Gluconolactonase Activity Units	%
Mitochondrial	0	0	203	14.4	17.0	0.4
Microsomal	0	0	287	20.4	36.1	0.9
Soluble supernatant ppt.	2990	100	920	65.2	3853	98.6

II. Cellular concentration: Hepatic tissue concentrations may be calculated (21-23) from the following:

$$\frac{\text{Units/kg tissue}}{\text{Units/mg pure enzyme}} \times \frac{1000}{\text{M.Wt.}} = \mu\text{mol/kg tissue}$$

Using activity measurements from crude extracts along with the specific activity and molecular weight of porcine gluconolactonase, a value of 7.2 μmol of enzyme per kg of tissue is obtained.

III. Subcellular fractionation: The results of this experiment are given in Table II. It is apparent that the majority of gluconolactonase is localized in the soluble portion of the cytoplasm. There is a small amount of activity in the microsomal fraction, which suggested a repeat of the fractionation experiment in the presence of Triton X-100. A similar experiment was carried out by Cutroneo, et al.(24) to determine the location of prolyl hydroxylase. It was concluded that an increase in microsomal activity was consistent with a model in which prolyl hydroxylase is located in vesicles on the surface of the endoplasmic reticulum. We repeated the fractionation process, incubating the microsomes with 0.1% Triton X-100, and observed virtually no activity for gluconolactonase.

IV. Kinetic studies: The pH of maximum activity was determined to be 7.5 in 50 mM tris HCl buffer at 27°. A number of lactones (see Materials and Methods) were tried with little success. Of all the 1,4-lactones tried only glucono-

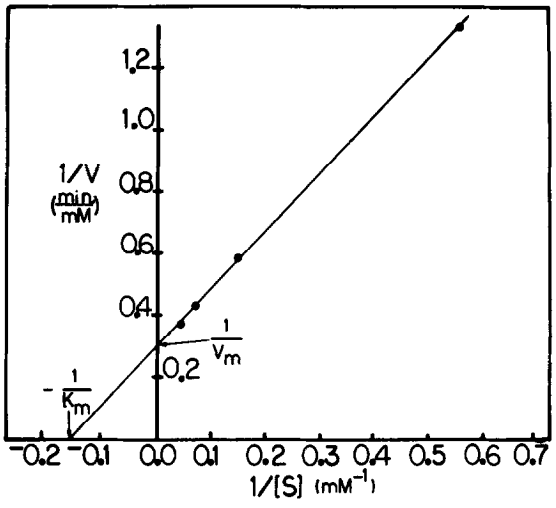

Figure 1

Lineweaver-Burk plot of gluconolactonase reaction at pH 7.5 with D-glucono-1,5-lactone at 27°.

lactone served as a substrate, and its activity was only twenty-five percent that of the glucono-1,5-lactone. 6-Phosphogluconolactone was seen to hydrolyze with a half life of approximate 90 s in 50 mM pH 7.5 tris HCl buffer, a result similar to that obtained previously(12) in 70 mM pH 7.4 glycylglycine buffer. Gluconolactonase did catalyze the conversion of the 6-phosphogluconolactone to 6-phosphogluconate, although it was impossible to determine a K_m value with any degree of accuracy.

Fig. 1 is a Lineweaver-Burk plot of gluconolactonase activity with glucono-1,5-lactone as the substrate in pH 7.5 50 mM tris HCl buffer at 27°. K_m and V_m values of 6.2 mM and 10 μmoles/min were obtained from this data.

V. Effect of metals: As was the case with other lactonases(1-3), gluconolactonase loses activity during the purification procedure and this activity is readily restored by adding Mn^{2+}. Activity can also be restored by adding Mg^{2+}, however only approximately one-fourth of the original activity is restored after the enzyme is dialyzed against 0.1 mM EDTA and then excess Mg^{2+} added. In a similar experiment with Mn^{2+}, it was possible to restore all of the initial enzymatic activity.

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