PARTIAL PURIFICATION AND DETERMINATION OF MOLECULAR WEIGHTS OF GLYOXALASES BY FILTRATION ON DEXTRAN GEL

Tadeusz JERZYKOWSKI, Wojciech MATUSZEWSKI and Romana WINTER
Department of Biochemistry, Medical Academy, Zabrze, Poland

Received 18 July 1968

1. Introduction

In 1948-1951 it was established that the enzymic conversion of methylglyoxal * into lactic acid takes place in two stages [1-3]. At first the MG reacts with GSH to form lactoyl-GSH, the reaction being catalysed by Gl. I, secondly, lactoyl-GSH is hydrolysed by Gl. II, giving D-lactic acid and regenerating GSH. Since this time many physical and chemical properties of both glyoxalases have been recognized [1-8]. However, to date, to our knowledge, there are no reports in the literature on the molecular weights of glyoxalases. The new methods of molecular weight determination introduced in the last years – gel filtration and sucrose density gradient centrifugation [9-13] – can be applied even to crude preparations of enzyme proteins and consequently to still not highly purified glyoxalases. In the course of our study on this subject it turned out that the molecular filtration on Sephadex permits the separation of both glyoxalases and the partial purification of Gl. II. In this report the results are given of molecular weight determinations of glyoxalases and of chromatography of these two enzymes deriving from various animals and from different tissues.

In the past attention was drawn to the possible role of glyoxalases and MG in the carbohydrate breakdown. However, some new discoveries in metabolism give reason to believe that glyoxalases may play a role in the so-called aminoacetone cycle (see review in ref. [14]). Within the last years renewed interest in the chemistry of MG and glyoxalases has been stimulated also by finding that MG and α-keto-aldehydes are potent inhibitors of protein synthesis and glyoxalase could be involved in the regulation of cell division [15-19].

* Abbrevations used: Gl.I – glyoxalase I (S-lactoyl-glutathione methylglyoxal – lyase, EC 4.4.1.5); Gl.II – glyoxalase II (S-2 hydroxyacyl glutathione hydrolase, EC 3.1.2.6); MG – methylglyoxal; GSH – glutathione; lactoyl-GSH – lactoyl-glutathione.

2. Materials and methods

2.1. *Materials*

MG was prepared from glyceraldehyde [22]. S-lactoyl-GSH was prepared enzymatically [5]. Gl. I for preparation of lactoyl-GSH was obtained from yeast by the method of Racker up to step VIII [13]. GSH was the product of E.Merck-Darmstadt. Sephadex G-75, G-100 and blue dextran were purchased from Pharmacia Fine Chemicals-Uppsala. Protein standards: chymotrypsin (Koch-Light), trypsin (E.Merck), horse heart cytochrome *c* (Fluka), bovine serum albumin (B.D.H.-London), human γ-globulin (Warsaw Serum and Vaccine Plant-Poland). All other reagents were of highest purity commercially available.

2.2. *Methods*

Gl. I activity was assayed by modification of the method of Racker [13]. In this case the cuvet contained (light path 1 cm; final volume 3 ml): phosphate buffer 0.15 mmole – pH 6.8, 0.5 mg GSH, 0.02 – 0.2 ml of enzyme solution, 0.175 mg MG. Reaction was started – after stabilisation of the density – by the addition of MG.

Gl. II assays were carried out by the method of

Racker [13] except that the buffer used was Tris-HCl 0.05 M pH 7.4.

Units of enzyme activity are expressed as μmoles of lactoyl-GSH produced (Gl. I) or decomposed (Gl. II) in one minute (initial velocity) at room temperature under the experimental conditions.

Preparation of homogenates: tissue homogenates (1 : 6 in 0.15 KCl) were prepared by using a Potter-Elvehjem homogenizer and centrifuged with refrigerated centrifuge model MSE Super Speed 25/16 000 rcv. for 30 min. The supernatant from this centrifugation is referred to as 'supernatant'.

Chromatography – unless otherwise stated – was performed according to the method described by Andrews [1] and density gradient centrifugation according to the method of Martin and Ames [12]. Some details of these methods in our experiments have been given in the text.

3. Results and discussion

3.1. *Separation of glyoxalases*

Racker [3] and Wieland et al. [4] have elaborated the methods of preparation of glyoxalases. The preparation of Gl. I from yeast gives very good results. But some difficulty consists in the purification of Gl. II. The preparations of this enzyme are of low specific activity and they often lose activity very rapidly, even when kept frozen. In this paper we present a very simple procedure of partial purification of Gl. II by the use of filtration on dextran gel. As is shown in fig. 1 the fractions with maximal activities of Gl. II

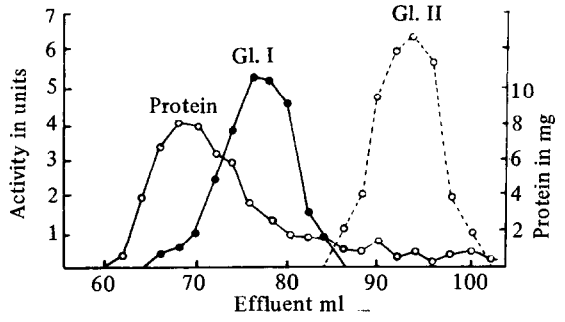

Fig. 1. Sephadex chromatography of 2 ml of supernatant from homogenate of frozen beef liver. Column (1.6 X 50 cm) equilibrated and eluted with 0.067 M Na_2HPO_4 in 2 ml fractions.

are separated from a great deal of proteins and from Gl. I. In the case of preparation of Gl. II from beef liver the highest purification coefficient (in a single run) was about 1 : 30 against approximately 1 : 15 after the fifth step of purification with the classical method. The chromatography of supernatants of chicken kidney gives better results in specific activity (table 1).

Table 1

Results of two typical Sephadex G-75 (virus) of supernatants from chicken kidney and beef liver. Details are given in the text and in the legend of fig. 1. Activity is given in units.

	Chicken kidney		Beef liver	
	Gl. I	Gl.II	Gl. I	Gl. II
Whole activity (in extracts)	10.80	37.80	22.20	30.06
Recovery (sum of activity in all fractions)	about 100% in all samples			
Specific activity (in samples)	0.28	0.97	0.42	0.56
Specific activity (in fractions with highest activity)	1.20	20.40	1.49	17.07

It must be emphasized that after purification on Sephadex only very small losses of enzymatic activity were observed during the chromatography procedures.

To check whether the preparations of Gl. II and also of Gl. I from columns were satisfactory, the course of the reactions catalysed by the glyoxalase system was

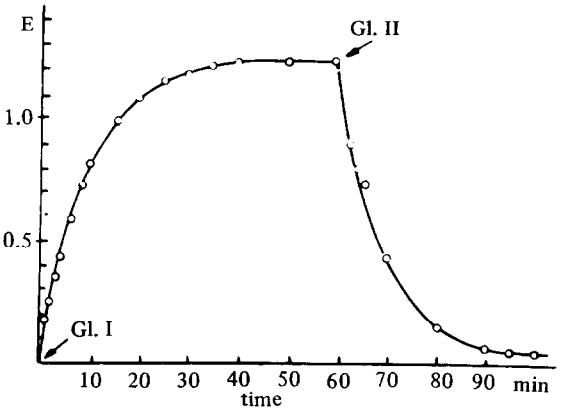

Fig. 2. Formation and breakdown of lactoyl-GSH catalysed by the glyoxalase system. 0.05 ml of fraction with max. activity of Gl.I (Sephadex G-75 chromatography) added to 0.5 mg of GSH and 0.175 mg of MG in 3 ml phosphate buffer 0.05 M pH 6.8. After stabilisation of the density 0.05 ml of fraction with max. activity of Gl. II was added (marked with arrow).

followed from the light absorption in the region of 240 mμ according to Racker [3]. Fig. 2 shows that the glyoxalase system functioned in accordance with theory: the two enzymes enabled the lactoyl-GSH to be formed and decomposed.

3.2. *Molecular weight determinations*

The isolated fractions with highest activities after chromatography of glyoxalases were used to determine molecular weights on Sephadex G-75 and G-100. Each enzyme was chromatographed together with cytochrome *c* or γ-globulin (Sephadex G-100 column). Moreover on the same columns there were chromatographed individually trypsin, chymotrypsin and bovine serum albumin. Molecular weights of standard proteins were taken as indicated in parenthesis: cytochrome *c* (12 400), chymotrypsin (25 000), trypsin (24 000), bovine serum albumin (67 000), human globulin (150 000). The values calculated for the two enzymes studied on Sephadex G-75 column were 45 800 and 23 000 for Gl. I and Gl. II respectively. Similar results were obtained after chromatography on Sephadex G-100 column: 51 300 and 22 400 for Gl. I and Gl. II respectively. These results represent the means of several determinations *.

All preparations from several sources that we have examined gave similar elution patterns. Within the experimental limits of the technique no differences could be detected among the enzymes from the following animals and tissues: beef (liver, kidney, brain, pancreas), pig (liver, kidney), guinea pig (liver, kidney, pancreas, brain), mouse (liver, brain), rat (liver, brain, pancreas, muscle – only Gl. I), chicken (liver, kidney). The elution volume of each enzyme was checked several times with calibrated Sephadex G-75 columns and did not change in phosphate buffer pH 7.4 with different ionic strength: 0.002; 0.02; 0.2; 1.2 and 2.2. We have found no evidence for the existence of forms with multiple molecular weights.

In order to confirm the molecular weights of glyoxalases, we determined these values by the method of Martin and Ames [12]. Sedimentation determinations in sucrose gradients were performed on using M.Christ (Osterode, W. Germany) ultracentrifuge Omega with swinging bucket rotor No. 0.001. 4.5 ml linear gradients of sucrose were prepared from 5.0 and

* The exact experimental data are taken from a dissertation submitted by W.Matuszewski to the Medical School in partial fulfilment of the requirements for the Doctor's degree.

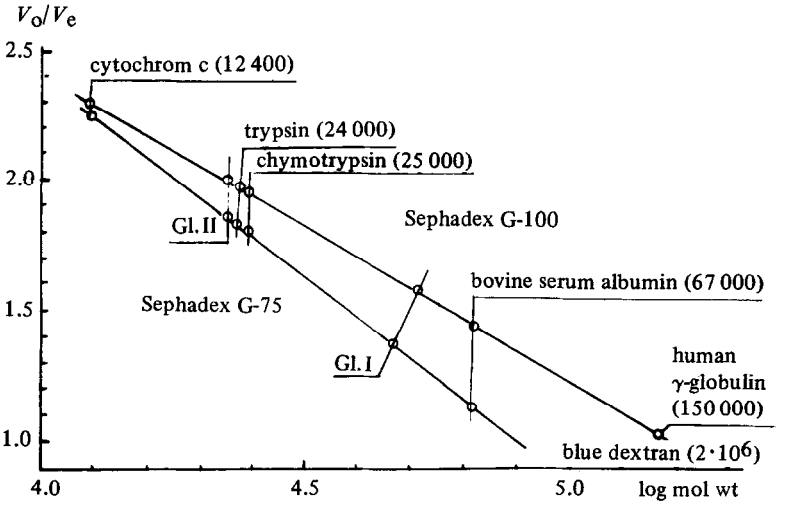

Fig. 3. Molecular weights of glyoxalases from beef liver as determined by gel filtration on Sephadex G-75 and G-100. Plot of the ratio V_o/V_e versus log of molecular weights. Columns (1.6 × 50 cm) equilibrated and eluted with 0.05 M Tris-HCl buffer containing 0.1 M KCl. Both glyoxalases were from chromatographic fractions with the highest concentrations. Molecular weights of standard proteins indicated in parenthesis.

20.0 % (w/v) sucrose solutions in buffer; 0.05 M Tris-HCl pH 7.0 *. A solution of 0.1 ml of glyoxalase (from tube with highest activity after chromatography) and about 1 mg of human hemoglobin in 0.05 M Tris-HCl buffer was layered on the gradient. The tubes were centrifuged for 14.5 hr at 38 000 rev/min at 3°C. 52 fractions were collected and all fractions were tested for Gl. I or Gl. II and the presence of standard protein (at 407 mμ). By comparison of the sedimentation rate the molecular weights of glyoxalases were calculated. By this method Gl. I and Gl. II appeared to have 46 200 mol wt ($S^{o}_{20,w} = 3.476$) and 21 420 ($S^{o}_{20,w} = 2.332$) respectively. These values based on two runs, are in good agreement with values obtained with the gel filtration method. It should be pointed out that the combination of these two methods allowed a much more reliable estimation of molecular weights of glyoxalases [13].

4. Summary

Glyoxalase I and glyoxalase II (EC. 4.4.1.5 and EC 3.1.2.6) were separated by gel filtration on Sephadex G-75 and G-100. This simple procedure permitted also the partial purification of glyoxalase II. The purification coefficient in a single run from supernatant from beef liver was about 1 : 30 compared with 1 : 15 after the fifth step of purification with classical methods.

* The devices employed for preparation of the gradients and collections of fractions were kindly provided by Prof. Dr. Ostrowski from the Medical Academy in Cracow.

The molecular weights of glyoxalase I and glyoxalase II were estimated by gel filtration and found to be in the range of 45 800-51 300 and 22 400-23 000, respectively. These values were confirmed by the sucrose density gradient centrifugation (mol weights 46 200 and 21 420, respectively). The elution volumes of glyoxalases from all animal sources examined were equal, as were those of the enzymes chromatographed in phosphate buffer of different ionic strength.

References

[1] F.G.Hopkins and E.J.Morgan, Biochem. J. 42 (1948) 23.
[2] E.M.Crook and K.Law, Biochem.J. 46 (1950) 37.
[3] E.J.Racker, J.Biol.Chem. 190 (1951) 685.
[4] T.Wieland, G.Pfleiderer and H.Lau, Biochem.Z.327 (1956) 393.
[5] T.Wieland, B.Sandmann and G.Pfleiderer, Biochem. Z. 328 (1956) 239.
[6] W.O.Kermack and E.Mathesson, Biochem.J.65 (1957) 48.
[7] I.A.Rose, Biochem.Biophys. Acta 25 (1957) 214.
[8] E.E.Cliffe and S.G.Waley, Biochem.J.73 (1959) 25 P.
[9] J.R.Whiteaker, Anal. Chem. 35 (1963) 1950.
[10] T.Wieland, P.Deusberg and H.Determann, Biochem. Z. 337 (1963) 303.
[11] P.Andrews, Biochem. J. 91 (1964) 222.
[12] R. Martin and B.Ames, J. Biol. Chem. 236 (1961) 1372.
[13] L.M.Siegel and K.J.Monthy, Biochem. Biophys. Acta 112 (1966) 346.
[14] M.L.Green and W.H.Elliott, Biochem. J. 92 (1964) 537.
[15] A.Szent-Györgyi, L.G.Együd and J.A.McLaughlin, Science 155 (1967) 539.
[16] L.G.Együd and A.Szent-Györgyi, Proc. Natl. Acad. Sci. U.S. 55 (1966) 388.
[17] H.Otsuka and L.G.Együd, Cancer Res. 27 (1967) 1498, Part 1.
[18] A.Szent-Györgyi, Proc.Natl.Acad.Sci.U.S.57 (1967) 1642.
[19] L.G.Együd, J.McLaughlin and A.Szent-Györgyi, Proc. Natl. Acad. Sci. U.S. 57 (1967) 1422.
[20] I.W.Patterson and A.Lazarow, in: Methods of Biochemical Analysis, vol.2, ed.D.Glick (Interscience, New York, 1955) p. 270.