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ISOLATION OF GLUTATHIONE FROM BOVINE THYMUS AND ITS SIGNIFICANCE TO RESEARCH RELEVANT TO IMMUNE SYSTEMS

by

Karl Folkers, Jan Dahmen, Masahiro Ohta, Henryk Stepien, Johann Leban, Naoki Sakura, Elsa Lundanes and Georg Rampold

Institute for Biomedical Research, The University of Texas at Austin Austin, Texas 78712

and

Yehuda Patt and Ruth Goldman

M.D. Anderson Hospital and Tumor Institute
The University of Texas System Cancer Center
Houston, Texas 77030

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SUMMARY

A peptide was isolated from bovine thymus when a cAMP assay guided fractionation; it was glutathione. Pure glutathione (isolated and purchased) at 1, 10, 20, 40 and 100 μg/ml was active, $P<0.05$-0.01, in the cAMP assay. Glutathione was not active in the mixed lymphocyte culture assay, but was active in assays using T-rosettes. Glutathione may now be separated to avoid its biological interference in assays guiding fractionation to a thymic hormone(s). It is credible that glutathione was in fractions studied biologically and clinically by others. Knowing that glutathione may function in the transport of amino acids across membranes, and that L-alanine is essential for human lymphocytes to respond to mitogenic and allogenic stimulation, it seems possible that glutathione might be functional in the complex immune systems.

INTRODUCTION

Several groups of investigators are currently fractionating extracts from the thymus gland toward isolation of factors or hormones which may have roles in the complex mechanisms of the immune system. Trainin et al., in many publications (exemplified by 1), described a purified fraction designated the thymic humoral factor (THF). A. Goldstein et al. have extensively investigated thymosin Fraction 5 (exemplified by 2), which is a mixture of peptides at an intermediate stage of purification. Low, Goldstein et al. (3) isolated thymosin α_1 from Fraction 5 and established the sequence of its 28 amino acids. Kook et al.(4) described a partial chemical characterization of THF. Kook and Trainin (5) reported on the control exerted by THF on cellular cAMP levels, and considered that THF is a hormone with an action mediated by cAMP. On this basis, the cAMP stimulation could be one biological assay to guide chemical fractionation to a pure thymic hormone.

We have extracted bovine thymus and extensively fractionated the extracts with the initial biological guidance by the cAMP assay. We isolated a peptide

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which unexpectedly was identical with authentic glutathione. Then, we tested pure glutathione (isolated and purchased) in the cAMP assay, the mixed lymphocyte culture assay as well as a T-rosette assay. These are assays which are commonly used in investigations relating the thymus to immune systems. The results are described herein.

MATERIALS AND METHODS

Thymus glands from calves, 4-6 months of age, were immediately frozen and promptly shipped to Austin from the Brown Packing Co. 158 and Greenwood Rd., South Holland, IL, 60473. Precoated plates, Silica Gel 60 (Merck Co. Inc., Rahway, NJ) were used for TLC and precoated cellulose plates (Chromatogram sheet 13255, Eastman Kodak Co., Rochester, NY) were used for TLE. Spots were visualized by spraying with ninhydrin: 0.3 g, n-BuOH:100 ml and HOAc:3 ml)followed by heating. Glutathione, oxidized form (purity grade III) and reduced form (purity grade IV) were purchased from Sigma Chemical Co., St. Louis, MO. Preparative chromatography was performed at 4°C.

cAMP Assay, in vitro

The background of this assay has been described by Gilman (6), Kaneko et al. (7) and Yakir et al. (8). The basis of this assay consists of exposing thymus cells which are suspended in a phosphate buffer-saline solution to the sample for 5 min at 37°C. The cells were then washed with the phosphate buffer-saline solution, and the intracellular content of cAMP was determined.

Cell Preparation. Six-week old C57BL/6 mice, equally of both sexes, were used as donors of thymocytes. Mice were sacrificed by cervical dislocation and their thymus glands were removed. Thymus cells were dispersed in Eagle's minimum essential medium (MEM, Grand Island Biological Co., Grand Island,NY), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C. Enzymatic digestion was terminated by cooling in an ice-water bath. The cells were washed and then resuspended in MEM to a concentration of $3\text{-}4 \times 10^7$ cells/ml.

Incubation with Test Samples. Cell suspensions were divided into culture tubes, and the incubations were performed in triplicate. Pre-incubation was for 30 min in MEM, containing theophyllin (10^{-2} M/ml, Sigma Chemical Co.). Test samples, dissolved in 10 µl MEM, were added, and then there was an incubation at 37°C for 30 min. The incubations were terminated by washing with large volumes of phosphate buffered saline, and the cells were frozen using dry ice.

Intracellular cAMP Determination. To the cells, was added 0.1 ml of 1 M HCl, followed by heating in a boiling water bath for 3 min. The mixture was then immediately cooled in ice-water bath. After adjusting the pH to 7.4 by adding 0.25 M Tris/20 mM EDTA buffer, pH 1233, supernatants were subjected to cAMP assay using Amersham's cAMP assay kit (Amersham Corp., IL).

Isolation of Glutathione

Since glutathione is a known substance, and has been isolated many times from tissues, it seems unnecessary to describe its isolation from thymus in complete detail. However, the general nature of the isolation may be of interest to the widespread investigations on the chemical fractionation of thymus extracts and the diverse biological assays which are being conducted.

The isolation of glutathione resulted from the following sequence of steps: (a) Frozen thymus glands, received soon after slaughter, were cut into pieces and lyophilized; (b) The lyophilized tissue was defatted with methylene chloride; (c) The residue was extracted with methanol, and the methanol was removed in vacuo; (d) Water was added to the methanol extractives, and the mixture was centrifuged; (e) The supernatant was lyophilized to yield a residue;

TABLE 1. cAMP Assay

Sample	Level	cAMP, pM/10^7 cells mean value ±SD	P
Control		2.57 ± 0.09	
GSH[1]	100 μg/ml	3.97 ± 0.28	<0.01
Control		4.01 ± 0.35	
GSH	10 μg/ml	5.61 ± 0.61	<0.02
GSSG[2]	10 μg/ml	5.75 ± 1.02	<0.05
Control		3.55 ± 0.58	
GSH	10 μg/ml	4.91 ± 0.55	<0.05
	1 μg/ml	5.19 ± 1.41	<0.05
Control		3.84 ± 0.68	
GSSG	40 μg/ml	8.41 ± 2.26	<0.05
	20 μg/ml	6.48 ± 1.35	<0.05
Control		6.55 ± 1.03	
GSSG	10 μg/ml	9.32 ± 0.65	<0.02
	1 μg/ml	9.25 ± 1.03	<0.05
Control		4.01 ± 0.35	
GSSG	1 μg/ml	5.00 ± 0.31	<0.05

(1) GSH: Glutathione, reduced form
(2) GSSG: Glutathione, oxidized form

(f) The residue was subjected to gel filtration on Sephadex G-15; (g) Selected fractions were lyophilized to yield a residue which significantly increased the cellular levels of cAMP in mouse thymocytes at levels of 100 μg/ml; (h) Purification on Sephadex LH-25 yielded fractions which increased the intracellular level of cAMP in mouse thymocytes at levels of 20 μg/ml; (i) The activity was consistently found in certain fractions which were pooled and subjected to ion exchange chromatography on DEAE-Sephadex A-25; (j) Active fractions were collected, lyophilized and desalted on Sephadex G-10; (k) Chromatography was repeated on DEAE-Sephadex A-25; (l) lyophilization of active fractions and desalting yielded glutathione in the oxidized form.

Amino acid analysis after hydrolysis by constant boiling HCl, 24 hrs, 110°C: Glu, 2.15; 1/2 Cys, 1.77; Gly, 2.09. $[\alpha]_D^{21}$ -99.5°(c 1.2, H_2O). Hunter and Eagles (9). $[\alpha]_D^{27}$ -93.9°(c 1, H_2O).

The isolated compound was indistinguishable from authentic glutathione by TLE on cellulose at pH 6.5 and 3.5 (400 V/cm, 30 min); TLC on silica gel(EtOAc-HOAc-H_2O-n-BuOH, 1:1:1:1, Rf 0.16) and by HPLC μ-Bondapak, 3.9x300 ml, 0.1 M KH_2PO_4, pH 3.0, V_R 6.5 ml.

DISCUSSION

Our isolation of glutathione from bovine thymus was independent of information described by Rotter and Trainin (10) that they had observed glutathione--"which appeared frequently in the vicinity of the elution pattern of G-10-IV". Therefore, they tested glutathione as a control under conditions where G-10-IV elicited responses to both PHA and ConA. Glutathione was inactive at those levels which were tested in their assays.

Both reduced and oxidized forms of glutathione were tested at levels of 1, 10, 20, 40 and 100 μg/ml in our cAMP assay (Table 1). In all six assays, glutathione was active at all five concentrations, P<0.05-0.01.

In the mixed lymphocyte culture assay, glutathione was inactive. The control value for comparison with glutathione at a level of 100 μg/ml was 8560±994.

When glutathione (by purchase) was assayed using T-rosettes, at concentrations of 10, 100 and 200 μg, the assay values were 30.7±1.7% and 48.2±1.9%, and 44.2±12.7%, respectively. The increases in T-rosettes at levels of 100 and 200 μg of glutathione in comparison with 27.2±4.5%, are significant, $P<0.005$.

An important aspect of the presence of glutathione in the fractionation of thymus extracts towards a pure hormone(s) is knowing that glutathione can be responsible for activity in certain assays. Steps can be taken to remove glutathione to avoid its interference in bioassays.

It is credible that glutathione may be present in thymosin Fraction 5, described by Goldstein et al. (2), and in the fraction which Kook, Yakir and Trainin (4) obtained after the step of dialysis and which was designated as "CTO" by Rotter and Trainin (10). Fraction 5 is still under extensive biological and clinical investigation. "CTO" has been studied clinically by Varsano et al. (11) and by Zaizov et al. (12).

Glutathione is known to be a coenzyme for certain enzymes including glyoxalase and maleyacetoacetate isomerase. Glutathione is also a coenzyme for the oxidation of formaldehyde to formate. It has been proposed that glutathione may also function in a transport of amino acids across membranes (13). This latter role is provocative in contemplating a function for glutathione in the thymus. Concerning amino acids, Rotter, Yakir and Trainin (14) found L-alanine is essential for human lymphocytes to respond to mitogenic and allogeneic stimulation.

Glutathione may merit new biological studies for a possible role in the complex mechanisms of the immune systems.

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REFERENCES

1. Trainin, N., Small, M., Zipori, D., Umiel, T., Kook, A.I. and Rotter, V. (1975) The Biological Activity of Thymic Hormones, pp. 117-144, Kooyker Scientific Publications, Rotterdam (D.W. van Bekkum, ed.).
2. Goldstein, A.L., Slater, F.D., White, A. (1966) Proc. Nat'l. Acad. Sci. USA 56, 1010-1017.
3. Low, T.L.K., Thurman, G.B., McAdoo, M., McClure, J., Rossio, J.L., Naylor, P.H., Goldstein, A.L. (1979) J. Biol. Chem. 254, 981.
4. Kook, A.I., Yakir, Y. Trainin, H. (1976) Adv. Exp. Med. Biol. 66, 215-220.

5. Kook, A.I., Trainin, N. (1974) J. Exp. Med. 139, 193-207.

6. Gilman, A.G. (1970) Proc. Nat'l Acad. Sci. USA 74, 725.

7. Kaneko, T., Saito, S., Oka, H., Oda, T., Yanaihara, N. (1973) Metabolism 22, 77.

8. Yakir, Y., Kook, A.I., Trainin, N. (1978) J. Exp. Med. 148, 71.

9. Hunter, G. and Eagles, B.A. (1927) J. Biol. Chem. 72, 147-166.

10. Rotter, V. and Trainin, N. (1979) J. Immunol. 122, 414.

11. Varsano, I., Schonfield, T.M., Matoth, Y., Shohat, B., Englander, T., Rotter, V. and Trainin, N. (1977) Acta Paediatr. Scand. 66, 329.

12. Zaizov, R., Vogel, R., Cohen, I., Varsano, I., Shohat, B., Rotter, V. and Trainin, N. (1977) Biomedicine 27, 105.

13. Miura, R. and Metzler, D.E. (1976) Biochemistry 15, 283-290.

14. Rotter, V., Yakir, Y., Trainin, N. (1979) J. Immunol. 123, 1726.