Purification and characterisation of chicken brain hypoxanthine-guanine phosphoribosyltransferase

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Hypoxanthine-guanine phosphoribosyltransferase enzyme (EC 2.4.2.8) from chicken brain has been purified 10000-fold to homogeneity. The molecular mass of the native enzyme is 85 kDa, with four subunits, each of 26 kDa, and exerts its maximum activity at pH 10.0. The K_m values for hypoxanthine and guanine are 5.2 and 1.8 μM, respectively. The half-life of the enzyme is 30 min at 85°C. Monoclonal antibodies were raised against the native purified enzyme and were used for purification of enzyme to homogeneity. The monoclonal antibody did not bind to the active centre of the enzyme.

Enzymology *HGPRT* *Chicken* *Monoclonal antibody*

1. INTRODUCTION

Hypoxanthine-guanine phosphoribosyltransferase is a salvage enzyme of purine metabolism which catalyses the conversion of hypoxanthine and guanine to IMP and GMP, respectively, by the transfer of phosphoribose from 5-phosphoribosyl 1-pyrophosphate.

The enzyme has particular importance in cell and medical genetics [1]. The enzyme has already been purified and characterized from several species [1–3]. However, little is known about the purified avian enzyme [4–6].

This paper describes the purification, and enzymological and immunological characterization of hypoxanthine-guanine phosphoribosyltransferase from chicken brain.

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Abbreviations: IMP, inosine 5'-monophosphate; GMP, guanine 5'-monophosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HRPO, horseradish peroxidase

2. MATERIALS AND METHODS

2.1. *Enzyme activity assay*

HGPRT activity was measured by the conversion of labelled bases to IMP or GMP according to Beaudet et al. [7]. One unit of enzyme converts 1 μmol hypoxanthine or guanine to IMP or GMP per min at 37°C under standard assay conditions.

2.2. *HGPRT purification and characterization*

All the procedures were carried out at 0–4°C. Fifty chicken brains were homogenized in 3 vols of 50 mM Tris-HCl, pH 7.4; 25 mM KCl; 10 mM $MgCl_2$; 100 mM sucrose; 1 mM DTT and 1 mM PMSF (extraction buffer). The first 3 steps of the purification were carried out according to Hughes et al. [8] except that the heat treatment time was 15 min at 85°C.

After the heat treatment the supernatant was pooled and concentrated by Amicon PM 10 ultrafiltration and dialysed thereafter overnight against 2 × 10 vols of extraction buffer without sucrose.

The dialysed enzyme was loaded (5 ml/h) onto a 10 ml GMP-agarose column, equilibrated and washed with the dialysis buffer, thereafter the pro-

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cedure of Hughes et al. [8] was followed, except that elution was with 1 mM PRPP instead of 5 mM GMP, since unlike GMP it did not have an effect on the measurement of enzyme activity.

Protein was assayed by the methods of Lowry et al. [9] and Bradford [10].

The molecular mass of the native enzyme was determined from its elution volume on a Sephadex G-150 column (2 × 100 cm); 10 μg of the purified chicken HGPRT, together with four molecular mass marker proteins were fractionated. The column was equilibrated with 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM PRPP, and run at a constant flow rate of 5.0 ml/h, 2.5-ml fractions were collected and assayed for HGPRT activity and for absorption at 280 nm.

To determine the molecular mass, and homogeneity of purified enzyme SDS slab gels were run in the discontinuous buffer system of Laemmli [11]. The separation gel was 10 or 12.5% acrylamide while the stacking gel was 5% acrylamide.

Isoelectric focusing was performed in 5% acrylamide gels in the pH range 5–7 as described by O'Farrell [12]. The sample consisting of 10 μg purified chicken HGPRT was lyophilised, dissolved in 30 μl sample buffer and applied to prefocused gels. The second dimension was run on a 12.5% gel, by the method of Laemmli [11]. Non-denaturing isoelectric focusing and developing of the enzyme activity in the gel were done essentially by the method of Chasin and Urlaub [13]. The subsequent Western transfer was according to Towbine et al. [14].

The purified chicken HGPRT enzyme was heat treated at 85°C in the presence of 1 mM PRPP. Samples were taken at different time intervals and the enzyme activity was determined.

2.3. *Immunological methods*

A × DBA_2/F_1 mice were immunized on the 1st, 3rd, 7th and 30th day with 10 μg purified chicken HGPRT mixed with complete Freund's adjuvant.

Spleen cells from hyperimmunized A × DBA_2/F_1 mice were fused with Sp-2/O-Ag myeloma cells [15] 4 days after the last booster to obtain monoclonal antibodies. The antibody production of the hybrids was evaluated by enzyme-linked-immunosorbent assay (ELISA) [17] and by double immunoprecipitation [16]. To obtain monoclonal antibodies in large amounts, 10^6 hybrid cells were injected intraperitoneally into A × DBA_2/F_1 mouse. Ascites fluid was collected 4–6 weeks later.

The IgG subclass of the monoclonal antibody was determined by ELISA, using HRPO-conjugated specific rabbit antibodies against mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM (Miles) as a second antibody.

Immunoaffinity column for the immunopurification was prepared from IgG against chicken HGPRT purified from ascites fluid and precipitated by ammonium sulphate (33%) as described [17]. A crude enzyme supernatant was prepared as previously, except that the acid precipitation step was omitted, and the concentrated supernatant was dialysed against PBS. The enzyme was loaded onto a 5 ml immunoaffinity column, washed with PBS and PBS + 0.5% NP10; finally the bound enzyme was eluated with 3 M NH_4SCN, and quickly dialysed against 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, 1 mM DTT.

3. RESULTS AND DISCUSSION

3.1. *Purification and enzymological characterization of the enzyme*

The purification of HGPRT from chicken brain is summarized in table 1. The enzyme was purified

Table 1

Purification of HGPRT from chicken brain

Procedure	Volume (ml)	Protein (mg)	Total activity (μM/min)	Spec.act. (μM/min per mg protein)	Yield (%)	Purification
S-100 supernatant	450.0	3375.2	228.5	0.0677	100	–
pH 5.0 supernatant	450.0	2475.8	217.3	0.0878	95.1	1.3
Heat denaturation (85°C)	420.0	562.8	196.9	0.349	86.1	5.2
Affinity chromatography	5.75	0.148	103.0	698.1	45.7	10300.0

more than 10000-fold applying 4 successive steps. The overall recovery of activity was 45%.

Molecular mass determinations were made both by SDS-polyacrylamide gel electrophoresis and molecular sieve chromatography. As shown in fig.1 on the SDS gel a single protein band of 26 kDa can be seen. The molecular mass of native HGPRT was estimated to be 85 kDa on a Sephadex G-150 column (fig.2). These results are in good agreement with those obtained in the case of mammalian HGPRT [1] where a tetrameric structure was proposed [18]. Isoelectric focusing of

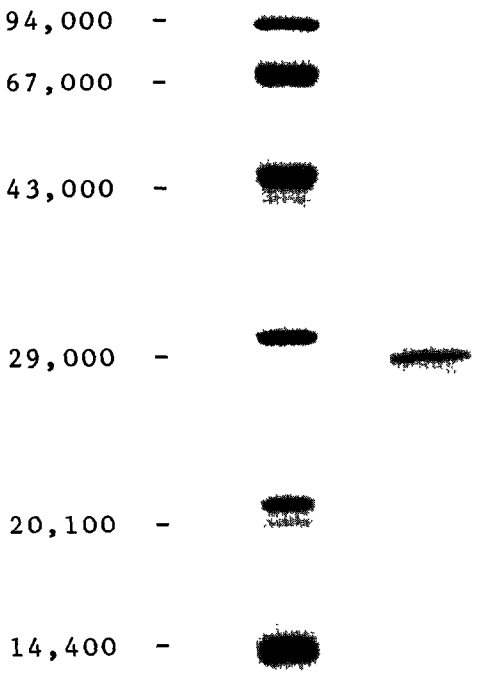

Fig.1. SDS-polyacrylamide gel electrophoresis of chicken brain HGPRT. Standards and their molecular masses were as follows: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). The gel was stained by the silver staining procedure [23].

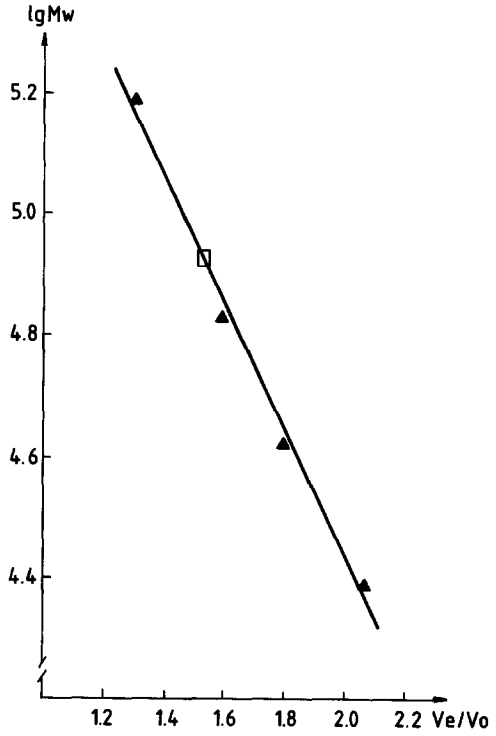

Fig.2. Gel filtration of chicken HGPRT on a Sephadex G-150 column. The ratio of the elution volume (V_e) to the void volume (V_o) was determined with blue dextran. Protein standards (▲) in order of decreasing molecular mass were: aldolase 158 kDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa and chymotrypsinogen A 25 kDa. Protein standards were located by the absorbance at 280 nm, and HGPRT (□) was located by the assay of enzyme activity.

purified HGPRT in the pH range 5–7 resulted in 3 major bands corresponding to isoelectric points of 6.2, 5.9 and 5.7 (fig.3). Similarly, 3 main subunit types were also found in the case of human enzyme [19] with slightly different p*I* values. The enzyme was active over a broad pH range, with optimum activity at pH 10.

Lineweaver-Burk plots for hypoxanthine and guanine were used to determine the K_m values for guanine and hypoxanthine as 1.8 and 5.2 μM, respectively. These are in the same range as the K_m values of the Chinese hamster enzyme [20]. The K_m value of 1.8 μM for hypoxanthine was in good agreement with that previously reported from a partially purified chicken liver extract [2]. The K_m values for PRPP in the presence of hypoxanthine

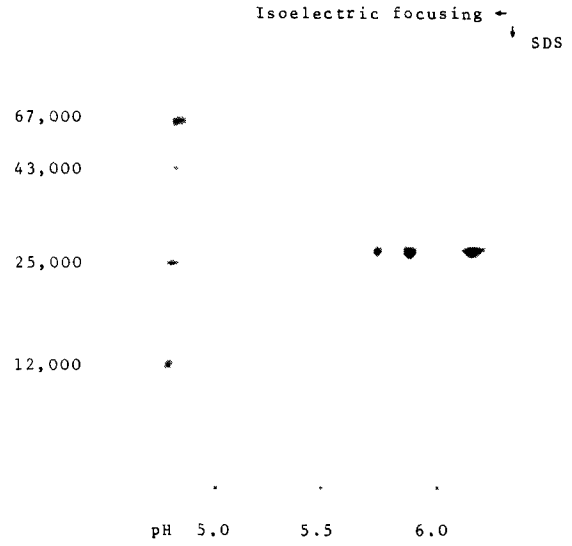

Fig.3. Two-dimensional gel electrophoresis of purified chicken HGPRT. Gels were focused in the pH range 5–7 as described. Second dimension was done by the method of Laemmli. Molecular mass markers: bovine serum albumin 67 kDa, ovalbumin 43 kDa, chymotrypsinogen A 25 kDa and ribonuclease 12 kDa.

and guanine were estimated to be 20–50 μM. The K_m for PRPP varied widely depending on the concentration of $MgCl_2$ (not shown).

There is an extreme heat stability of the enzyme; in the presence of PRPP the half-life is 30 min at 85°C. Heat inactivation values obtained from crude extracts of mouse brain and L cells were of the same order of magnitude [21]. On the other hand, partially purified enzyme from Chinese hamster ovary cells retained 50% of the original activity after 7.5 min at 80°C [22].

3.2. *Immunological characterization of chicken HGPRT enzyme*

The immunologic characteristics of chicken HGPRT have been examined and compared to those of hamster. As shown in fig.4a the conventional mouse antiserum raised against chicken HGPRT cross-reacts with the hamster enzyme, indicating that there is a homology between the antigen determinants of the two enzymes. Our data are in contradiction with a previous publication where the antibody against chick liver HGPRT did not cross-react either with human, Chinese hamster or mouse HGPRT enzyme [6].

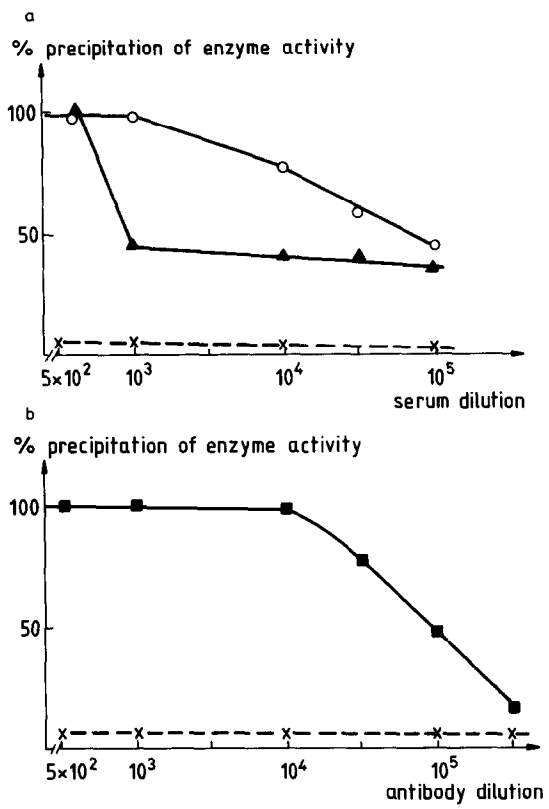

Fig.4. (a) Specificity of anti-chicken HGPRT serum. 0.1 unit of HGPRT from chicken (○—○) and Chinese hamster (▲—▲) was reacted with decreasing concentration of antiserum produced against chicken HGPRT. Immunoprecipitation and enzyme assay were done as described in section 2. (×—×) Control serum. (b) Immunoprecipitation of chicken HGPRT (■—■) with monoclonal antibody. The reaction conditions are the same as above. (×—×) Control supernatant.

There is a great difference, however, between the chicken and hamster enzymes regarding the specificity to the antiserum. The immune serum caused complete precipitation of chicken HGPRT at 1000-fold dilution, while in the case of hamster enzyme the precipitation was only 40%, at the same antibody dilution.

One of the 200 growing hybridomas produced HGPRT-specific monoclonal antibodies. This monoclonal antibody reacted only with the chicken enzyme, and did not give any cross-reaction with purified Chinese hamster HGPRT. As shown in fig.4b the monoclonal antibody has a high specifi-

Table 2

Immunopurification of chicken HGPRT

Procedure	Volume (ml)	Protein (mg)	Total activity (M/min)	Spec.act. (M/min per mg protein)	Yield (%)	Purification
S-100 supernatant	280.0	2187.8	146.19	0.0649	100	–
Heat denaturation (85°C)	215.0	420.5	131.4	0.312	89.8	4.8
Affinity chromatography (immune affinity column)	3.5	0.140	98.7	705.0	67.5	10862.0

city; it precipitates completely the enzyme at 10000-fold dilution. This antibody recognizes the native form of the enzyme since in the case of Western blotting of the enzyme from native and SDS-polyacrylamide gel, the antibody reacted only with the native enzyme (not shown). The monoclonal antibody belongs to IgG subclass 1, and can be bound to protein A.

The monoclonal antibody was used to prepare an immunoaffinity column and for subsequent immunopurification of the enzyme. This method allowed us to purify the chicken enzyme to homogeneity, and with the same specific activity as with the GMP-agarose affinity column (table 2). The immunoaffinity column serves as a new and easier approach to purify the enzyme. Our data further support the previous suggestions that there is a great similarity in the characteristics of the HGPRT enzymes, regardless of the species from which they are purified.

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