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Purification and Kinetic Properties of Human Recombinant Dihydrofolate Reductase Produced in *Bombyx mori* Chrysalides

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Abstract Recent reports describe the inhibition of human dihydrofolate reductase (hDHFR) by natural tea polyphenols. This finding could explain the epidemiologic data on their prophylactic effects for certain forms of cancer, and it raises the possibility that natural and synthetic polyphenols could be used in cancer chemotherapy. In order to obtain larger quantities of hDHFR to support structural studies, we established and validated a baculovirus system for the expression of this protein in *Bombyx mori* chrysalides (pupae of the silkworm enclosed in a cocoon). To isolate the expressed protein, whole infected pupae were homogenized, and the expressed protein was purified by affinity chromatography. Here, we demonstrate the efficient expression of recombinant hDHFR in this model and report that this newly expressed protein has high enzymatic activity and kinetic properties similar to those previously reported for recombinant hDHFR expressed in *Escherichia coli*. The purified protein showed dissociation constants for the binding of natural polyphenols similar to that expressed in *E. coli*, which ensures its usage as a new tool for further structural studies. Although the hDHFR yield per individual was found to be lower in the

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chrysalides than in the larvae of *B. mori*, the former system was optimized as a model for the scaled-up production of recombinant proteins. Expression of proteins in chrysalides (instead of larvae) could offer important advantages from both economic and biosecurity aspects.

Keywords Dihydrofolate reductase · *Bombyx mori* · Chrysalides · Baculovirus · Protein expression

Introduction

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: *nicotinamide adenine dinucleotide phosphate* ($NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) in the presence of the coenzyme NADPH as follows: DHF + NADPH + H^+ → THF + $NADP^+$. This enzyme is necessary for maintaining intracellular pools of THF and its derivatives, which are essential cofactors in one-carbon metabolism. Coupled with thymidylate synthase [1], DHFR is directly involved in thymidylate (dTMP) production through a de novo pathway. DHFR is therefore pivotal in providing purine and pyrimidine precursors for the biosynthesis of DNA, RNA, and amino acids. As a result of its importance in maintaining folate pools in their active reduced state, DHFR has been studied extensively and many compounds have been synthesized and tested as potential drugs that inhibit DHFR function [2–5]. Inhibition of DHFR, resulting in the disruption of DNA biosynthesis, is the basis of the chemotherapeutic action of a range of DHFR inhibitors, generically known as "antifolates." Tumor cells that grow rapidly require a higher concentration of thymidylate than normal cells and therefore are more sensitive to antifolates.

Based on the observation that classical (i.e., methotrexate, or MTX) and nonclassical (i.e., trimethoprim, or TMP) antifolate compounds possess chemical structures similar to that of some tea polyphenols [6], we started to work on the hypothesis that tea catechins could inhibit DHFR activity. Green tea catechins include (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC). EGCG is the most abundant (one 240-mL cup of brewed tea contains up to 200 mg EGCG), and many health benefits, including antioxidant, antibiotic, and antiviral activities, have been attributed to this compound [7, 8]. Suppression of DNA synthesis by tea catechins could explain many of the observed inhibitory effects on cancer of these compounds [9]. Recent studies showed that ester-bonded gallate catechins isolated from green tea (such as EGCG and ECG) are potent inhibitors of DHFR activity in vitro at concentrations found in the serum and tissues of green tea drinkers [6, 10]. EGCG exhibited the kinetic characteristics of a slow-binding inhibitor of DHF reduction with bovine liver DHFR and human DHFR (hDHFR), but it exhibited the kinetic characteristics of a classical, reversible, competitive inhibitor with chicken liver DHFR [6, 10, 11]. Structural modeling showed that EGCG can bind to hDHFR in an orientation similar to that observed for a number of structurally characterized DHFR inhibitor complexes [6]. These results suggest that EGCG could act as an antifolate compound in the same way as MTX and TMP.

Although the expression of hDHFR, a small (~22 kDa) and nonglycosylated protein, does not present significant methodological problems in bacterial systems, we decided to express this enzyme in insect systems for several reasons. First, in order to validate the binding models of novel inhibitors such as the polyphenols from green tea or other natural sources, we needed to produce adequate quantities of hDHFR to support structural studies.

Second, we needed an easily expressed protein to validate our expression system in chrysalides (pupae of the silkworm enclosed in a cocoon) of *Bombyx mori*. The *B. mori* nucleopolyhedrosis virus (BmNPV) expression system can take advantage of the inexpensive, convenient, and high-level production of heterologous proteins in silkworm larvae. This expression system has been successfully applied to produce a large amount of foreign proteins such as human vascular endothelial growth factor [12] and different interferon isoforms from bovine or canine species [13–15]. Therefore, the baculovirus expression system might be considered one of the most effective expression tools in silkworm larvae [15, 16]; however, its use to infect chrysalides of this insect is less known. Although baculovirus-mediated expression in *B. mori* pupae has been described before [17–20], the efficiency of this system remains unknown. Thus, in this study, the baculovirus-mediated recombinant protein expression in *B. mori* larvae and pupae is directly and quantitatively compared. Expression of recombinant proteins in the chrysalides of *B. mori* would present biosecurity and economic advantages during the scaling up of the production of these proteins. Here, a detailed study of the production of hDHFR in chrysalides of *B. mori* (hDHFR-cbm) is presented, which confirms the integrity and functionality of this enzyme by comparing its kinetic properties with those obtained from hDHFR expressed in *Escherichia coli* (hDHFR-ec).

Materials and Methods

Materials

The human DHFR gene containing the complete open reading frame was purchased from Invitrogen (Barcelona, Spain). hDHFR-ec was a gift from Berry Birdsall at the National Institute for Medical Research (London, UK). A hybrid silkworm strain (commercial name *Baiyu* × *Qiufeng*) was used in this research, and the larvae were reared with mulberry leaves at 25 to 27 °C. Highly purified tea polyphenols EGCG (>95%), ECG (>98%), EGC (>98%), and EC (>98%) were purchased from Sigma Chemical Co. (Madrid, Spain). Quercetin-3-β-D-glucoside (Qglc), quercetin-3-D-xyloside (Qxyl), quercetin-3-rhamnoside (Qrha), and quercetin-3-D-galactoside (Qgal) were form Fluka (Madrid, Spain).

Cloning of the hDHFR Gene and Construction of Recombinant Baculovirus

The human DHFR gene was cloned by polymerase chain reaction (PCR) using pOTB7-hDHFR as the template. The primers were designed as follows: the sense primer was 5′-GCT GAT ATC ATG GTT GGT TCG C-3′ (*Eco*RV), and the antisense primer was 5′-ACC AAG CTT TTA ATC ATT CTT CTC-3′ (*Hin*dIII). The PCR product was analyzed by electrophoresis on a 1% agarose gel and subsequently subcloned into the TA cloning vector pGEM-T (Promega Corporation, Madison, WI). The sequence was confirmed by the dideoxy-chain termination method with a nucleotide sequencer. For the construction of recombinant baculovirus, the BmNPV/Bac-to-Bac expression system developed recently by our group was used [21]. This system can generate recombinant baculovirus rapidly in bacteria through gene transposition by the transposon Tn7. The hDHFR gene was cut from the above vector by digestion with *Eco*RV and *Hin*dIII and subsequently cloned into the *Ehe*I/*Hin*dIII sites of the donor vector pFastBacHTshort, which contains an expression cassette within the left and right arms of the Tn7 transposon. The recombinant pFastBacHTshort-hDHFR was subsequently transformed into DH10BmBacmid containing

a reconstructed BmNPV genome. About 1 ng of the recombinant donor plasmid was transformed into 100 μL of the competent DH10BmBacmid cells. The mixture was incubated at 37 °C for 4 h to achieve transposition. Then the cells were diluted serially using SOC medium [Super Optimal Broth (SOB) with added glucose] and spread evenly on the plates. The transformants were screened by growth on LB agar plates containing kanamycin (50 μg/mL), tetracycline (7 μg/mL), X-gal (100 μg/mL), and isopropyl-β-D-thiogalactoside (40 μg/mL). After 48 h of incubation at 37 °C, the white colonies were selected and cultured overnight in medium containing kanamycin. The recombinant Bacmid DNA was subsequently extracted and transfected into BmN cells to generate the recombinant baculovirus. Five days later, the medium supernatant was collected as stock virus and used to infect silkworm larvae or chrysalides. Baculovirus titer was calculated using a standard viral plaque assay [22].

Insect Growth Conditions and Inoculation

Newly molted fifth instar silkworm larvae were used as hosts for virus infection and expression. In order to reduce their movement, they were placed on a bed of crushed ice for 10 min before inoculation. About 10^6 plaque forming units of the recombinant virus (20 μL) were injected subcutaneously into the larvae with a syringe (100 heads/batch). Half an hour after the injection, the larvae were fed with mulberry leaves and then reared at 25 to 27 °C. During the first 3 days, no obvious symptoms were observed. By the fourth day, the larvae began to lose appetite and displayed infection symptoms. Ninety-six hours after infection, no more feed was given to the larvae to clean their guts, and subsequently, the larvae were collected. Alternatively, silkworm pupae were also inoculated with the vector in order to assess whether the expression level of the recombinant protein is similarly efficient in the two platforms. For that, pupae were extracted from the cocoon 1 week after spinning was completed. Pupae were injected in the dorsal side, in the thoracic-abdominal junction with the same inoculums used for larvae. After injection, they were kept undisturbed in an incubator at 25 °C for 5 days, and after that, they were frozen and kept at −80 °C until processing.

Purification of hDHFR by MTX Affinity Chromatography

To increase the recovery of recombinant protein, the whole bodies of infected larvae or pupae were homogenized with ice-cold extraction buffer (50 mM Tris-HCl, pH 7.9, 1 M NaCl, 1 mM DL-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/mL DNase and proteases inhibitors cocktail), and then the mixture was centrifuged at 10,000×g for 30 min at 4 °C to remove large debris and lipids. The supernatant was filtered to remove the remaining lipids, and the filtrate was centrifuged again at 14,000×g for 30 min at 4 °C. The supernatant was collected as the crude extract of recombinant hDHFR. To remove other insect proteins, the crude extracts were incubated with 14% (vol/wt) polyethylene glycol (PEG) 6000, stirred for 1 h at 4 °C and centrifuged at 12,000×*g* for 20 min. The supernatants were applied to an MTX-agarose (Sigma) column equilibrated with 50 mM potassium phosphate buffer, pH 6.5, containing 100 mM KCl. The column was then washed with 200 mL 50 mM potassium phosphate buffer, pH 6.5, containing 2 M KCl. The enzyme was eluted using 10 mL 50 mM Tris-HCl, pH 8.6, containing 1 M KCl and 2 mM TMP. To remove TMP, the samples were loaded on a Sephadex G-25 chromatographic column. Fractions containing DHFR activity were combined, dialyzed overnight against 3×2-L buffer B, concentrated in an Amicon concentrator (YM-10 membrane) and stored at –80 °C. The hDHFR concentration was determined using the Bio-Rad protein assay

procedure with bovine serum albumin (BSA) as the standard. The homogeneity of the enzyme samples was confirmed by the presence of a simple band in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels with silver staining, and the identity of the protein was confirmed by trypsin digestion and high-performance liquid chromatography (HPLC)/mass spectroscopy (MS) analysis.

Electrophoresis and Western Blot Assays

Proteins were separated by SDS-polyacrylamide gels (12.5%), transferred to nitrocellulose membranes and analyzed by immunoblotting (ECL Plus; GE Healthcare, Barcelona, Spain). A specific antibody against hDHFR (D0942) was purchased from Sigma.

hDHFR Trypsin Digestion and HPLC/MS Analysis

Crude protein chrysalide extracts (25 μg per lane) were resolved in 12.5% SDS-polyacrylamide gels, which were then stained with ProteoSilver Plus (Sigma). Selected bands were excised and destained using the same kit, and the samples were digested with trypsin using standard procedures [23]. Separation and analysis of the tryptic digests were performed with an HPLC/MS system consisting of an Agilent 1100 Series HPLC connected to an Agilent Ion Trap XCT Plus MS using an electrospray interface. Dry samples from in-gel digestion were resuspended in 10 μL water/acetonitrile/formic acid (94.9:5:0.1) and injected onto a thermostatted Zorbax SB-C18 HPLC-column (5 μm, 150×0.5 mm) at a flow rate of 10 μL/min at 40 °C. The digested peptides were eluted using a linear 0 to 80% gradient of water/acetonitrile/formic acid (10:89.9:0.1) for 120 min. The MS was operated in the positive mode with a capillary spray voltage of 3,500 V and a scan speed of 8,100 (*m*/*z*)/s from 300 to 2,200 *m*/*z*. The nebulizer gas pressure was set to 15 psi, and the drying gas flow was set to 5 L/min at 350 °C. MS/MS data were collected in an automated data-dependent mode. The most intense ions were sequentially fragmented using helium collision-induced dissociation with an isolation width of 2 and relative collision energy of 35%. Data processing was performed with the DataAnalysis program for LC/MSD Trap v.3.2 (Bruker, Madrid, Spain) and Spectrum Mill MS Proteomics Workbench (Agilent Tech., Santa Clara, CA).

Fluorescent Labeling of hDHFR in *B. mori* Chrysalides

Chrysalides were sliced into two pieces and fixed with cold acetone for 10 min (−20 °C). The slices were rinsed three times with ice cold phosphate-buffered saline (PBS) and incubated in 3% BSA in PBS for 30 min to block nonspecific binding of the antibodies. Then the samples were washed three times with PBS. To reveal differences in hDHFR content, the samples were incubated overnight at 4 °C with anti-hDHFR antibody (Sigma D0942) diluted 1:500 in 1% BSA–PBS. The next day, the chrysalides were rinsed in PBS and incubated for 1 h at room temperature with secondary antibody goat antirabbit IgG Alexa fluor 633 (Molecular Probes, Invitrogen) diluted 1:1,000 in 1% BSA–PBS. Finally, the samples were rinsed in PBS and examined with a Typhoon 9410 laser scanner imager (GE Healthcare). Control experiments were performed in which the first or second antibodies were omitted.

DHFR Assays

DHF was obtained from Aldrich Chemie GmbH (Madrid, Spain) and NADPH from Sigma. DHFR activity was determined by following the decline of NADPH and DHF by

absorbance measurements at 340 nm (ε = 11,800 M^{-1} cm^{-1}) using a Perkin-Elmer Lambda-2 spectrophotometer with 1.0-cm light-path cuvettes. The temperature was maintained at 25 °C using a Haake D1G circulating bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of ±0.1 °C. One unit (U) corresponds to the amount of enzyme that converts 1 μmol of DHF and NADPH to THF and NADP per minute at pH 7.5 and 25 °C. Experiments were performed in a buffer (pH 7.5) containing 2-(*N*-morpholino)ethanesulfonic acid (Mes, 0.025 M), sodium acetate (0.025 M), *tris* (hydroxymethyl)aminomethane (Tris 0.05 M), and NaCl (0.1 M). The pH of the reaction was measured before and after the experiment. The assays were started by adding the enzyme. In the absence of the enzyme, the rate of change of absorbance was negligible. The values of the maximum steady-state rate (V_{max}) and the Michaelis constant of DHFR for DHF $\left(K_m^{DHF}\right)$ and NADPH $\left(K_m^{NADPH}\right)$ were determined from the curvature evident in the plots of NADPH and DHF disappearance versus time. For K_m^{DHF} and K_m^{NADPH} determinations, the initial concentration of saturating NADPH (100 μM) or DHF (200 μM) was considered constant during the overall consumption of 10 μM DHF or 20 μM NADPH by the enzyme, respectively. Data were fitted by nonlinear regression to the integrated form of the Michaelis equation [24] using Marquart's algorithm [25] implemented in Sigma Plot 2.01 for Windows (Sigma Plot SPSS Inc. Chicago, IL).

Polyphenol Binding Assays

The fluorescence of DHFR is reduced upon binding of substrates and inhibitors, and this property may be used as a convenient method for determining both the enzyme concentration and the dissociation constants of enzyme–ligand complexes. Dissociation constants for the binding of polyphenols to free hDHFR were determined by fluorescence titration in an automatic-scanning Perkin-Elmer LS50B spectrofluorimeter with 1.0-cm light path cells and equipped with a 150-W xenon (XBO) light source. The formation of the binary complex between the enzyme and the ligand was followed by measuring the quenching of tryptophan fluorescence of the enzyme upon addition of microliter volumes of a concentrated stock solution of ligand. Fluorescence emission spectra were recorded when hDHFR fluorescence was excited at 290 nm. All measurements were corrected for dilution, and the data from the titration curves were fitted as described previously [26, 27]. Titrations were performed at pH 7.4 in the same buffer used for DHFR assays.

Statistical Analysis

Kinetic constants of hDHFR on their substrates were calculated in 10 determinations (n= 10) from plots of NADPH and DHF disappearance versus time. Dissociation constants for the binding of polyphenols to hDHFR were calculated from five independent fluorescent titration experiments (n=5). In all cases, the mean ± standard deviation (SD) is presented.

Results and Discussion

Efficient Expression of hDHFR in *B. mori* Chrysalides

A recombinant baculovirus containing the hDHFR gene was generated by using the BmNPV/Bac-to-Bac expression system [15], and the insertion of this gene into the BmBacmid was confirmed by PCR analysis. This recombinant virus was used to infect

silkworm chrysalides. Fluorescence labeling of hDHFR in the chrysalides of *B. mori* was carried out by immunofluorescence using appropriate primary and secondary antibodies (Fig. 1a). The efficient expression of this protein was also confirmed by SDS-polyacrylamide gels followed by Western blotting as well as by trypsin digestion of crude extract proteins coupled to HPLC/MS analysis. Crude extract of chrysalides infected with the recombinant virus showed an intense band after Western blotting with hDHFR-specific antibodies that was absent in crude extracts of noninfected chrysalides (Fig. 1b). Other SDS-polyacrylamide gels were silver-stained and several protein bands in the range of 20 to 30 kDa were extracted from the gels and analyzed by MS of their tryptic digest products (Fig. 2). Crude extract of infected chrysalides presented an approximately 23.0-kDa protein tryptic digestion of which yielded nine peptides corresponding to hDHFR (NCBInr code AAH70280), and among them the *N*-[(-)MVGSLNCIVAVSQ-NMGIGK(N)] and C-terminal [(K)YKFEVYEKND(-)] fragments were identified. This protein was absent in noninfected chrysalides (Fig. 2).

The hDHFR yield after infection of silkworm chrysalides or larvae with the recombinant baculovirus is summarized in Table 1. The yield of hDHFR in larvae was very high, with an average of 582 μg per larva, which is comparable with the yield of other recombinant proteins using this baculovirus system [15]. Although the yield of hDHFR in chrysalides was approximately three times lower (190 μg per chrysalide) than in the larvae, the results indicated that this decrease was not a consequence of a decrease in the infection efficiency of the virus in this expression system. As can be observed in Table 1, the lower hDHFR yield in infected chrysalides was directly related to their lower body weight. Thus, the same yield of this recombinant protein was observed when chrysalides and larvae were compared with respect to their body weight.

Purification of hDHFR From *B. mori* Chrysalides

Recombinant proteins from silkworms are often difficult to purify because of the many types of storage proteins derived from their hemolymph that bind nonspecifically to purification columns. Similar purification problems were observed when chrysalides were used as a source of recombinant protein. To solve these problems, PEG 6000 protein precipitation was used [28]. In order to optimize the concentration of PEG 6000 different concentrations of this chemical was employed to precipitate proteins in the crude extract of silkworm chrysalides infected with the recombinant virus. As observed in Fig. 3a, PEG 6000 at 14% precipitated most of the extract proteins but not hDHFR, which remained in

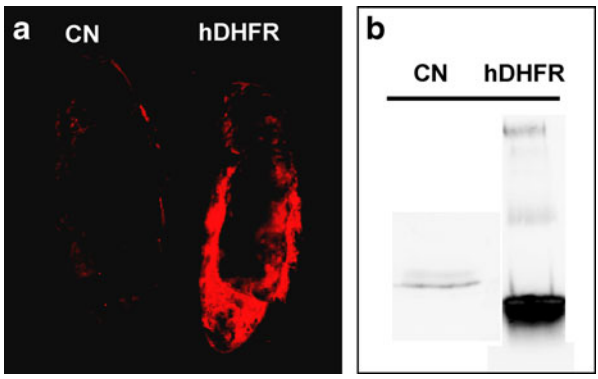


Fig. 1 Expression of hDHFR in *B. mori* chrysalides. **a** Immunofluorescence of hDHFR in noninfected chrysalides (CN) and those inoculated with the hDHFR recombinant virus (hDHFR). The image is representative of five experiments with similar results. **b** Western blot analysis of chrysalide crude protein extracts (CN, not infected with the recombinant virus, and hDHFR, infected with the recombinant virus)

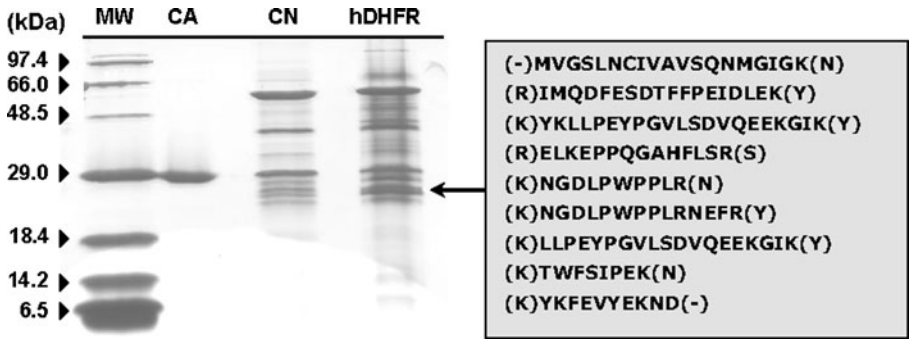


Fig. 2 Silver-stained SDS-polyacrylamide gel containing crude protein extracts of control chrysalides (CN) and chrysalides infected with the hDHFR recombinant virus (hDHFR). The *lane CA* contained pure carbonic anhydrase with a molecular weight of 29 kDa. The *inset panel* shows the fragments detected by MS after tryptic digestion of the 23.0-kDa protein in the hDHFR lane. All fragments have a sequence corresponding to hDHFR

the supernatant fraction. For further purification, affinity chromatography on MTX-agarose was necessary to obtain homogeneous preparations of hDHFR. Gel filtration on Sephadex G-25 was employed to remove TMP, a DHFR inhibitor used for elution of the protein during the affinity step. As a result, up to 217-fold purification of the enzyme was achieved (Table 2). Preparations were judged to be homogeneous when a single band was observed on a silver-stained reducing SDS-PAGE gel (Fig. 3b). The native Mr of the enzyme estimated by the gel filtration method (M r=22,500±1,500, mean ± SD, n=3) was consistent with the SDS-PAGE results and similar to that for hDHFR expressed in other expression systems [29].

Kinetic Properties of hDHFR Expressed in *B. mori* Chrysalides

As one of the objectives of this research was to obtain sufficient amounts of recombinant hDHFR for further structure/function studies, hDHFR-cbm was kinetically characterized, and the results were compared with those obtained with hDHFR expressed in *E. coli*. The Michaelis constants (K_m) values for both substrates, NADPH and DHF, were determined using the purified enzymes (Table 3). Due to the very low K_m values, the integrated Michaelis equation was used for their calculation (see "Materials and Methods"). The K_m of hDHFR-cbm for its substrate was essentially the same as that calculated using hDHFR-ec (Table 3) and was similar to the K_m values of hDHFRs obtained from other studies [30].

Table 1 hDHFR yield after infection of larvae and chrysalides of *B. mori* with the recombinant virus.

	Number of individuals inoculated[a]	Average group body weight (g)	Total hDHFR yield (mg)[b]	Average hDHFR yield per individuals (μg/per individual)	Average hDHFR yield per body weight (μg/g body weight)
Larva	50	216.4	29.1	582	134.6
Chysalide	50	74.4	9.5	190	127.8

[a] For this experiment, larvae or chrysalides were collected together

[b] The yield of hDHFR (mg) in crude extracts was calculated based on DHFR activity (U) measurements. Calculated U in crude extracts was extrapolated to standard curves of U vs. mg of hDHFR obtained with a homogenized purified samples.

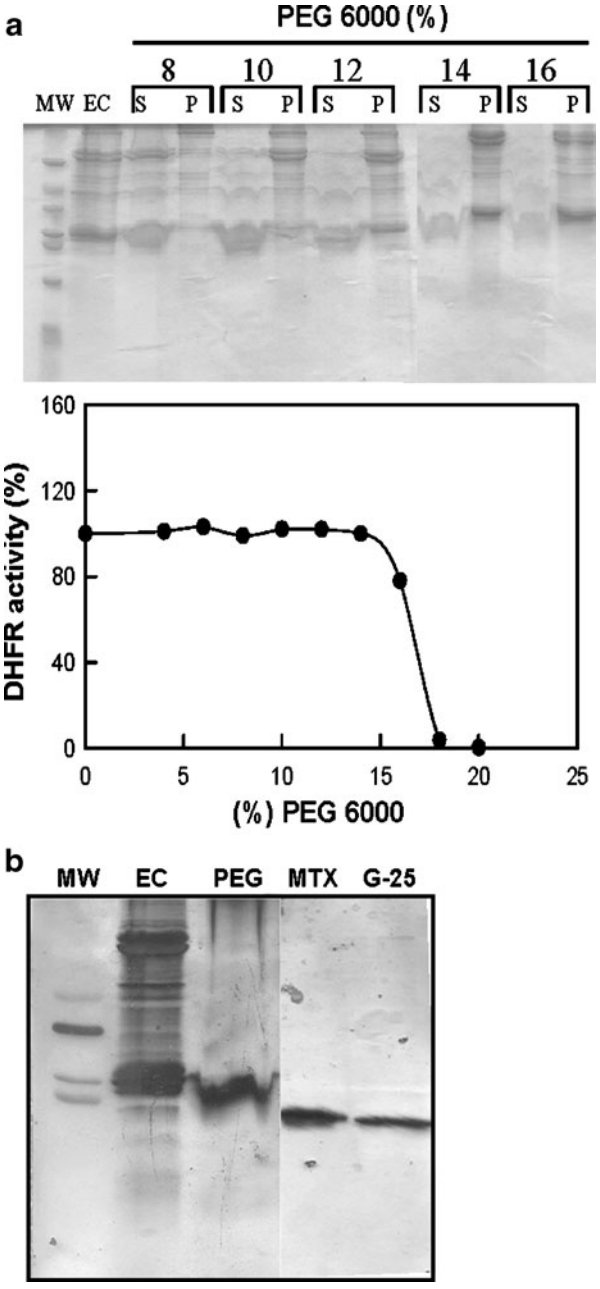


Fig. 3 Purification of hDHFR from *B. mori* chrysalides. **a** PEG 6000 fractionation of hDHFR and chrysalide crude protein extracts. The *upper panel* presents a Coomassie blue-stained gel containing supernatant (S) and pellet (P) fractions obtained after precipitation with different concentrations of PEG 6000. The *lower panel* shows the DHFR activity in the supernatants of chrysalide crude extracts precipitated with different concentrations of PEG 6000. *MW* molecular weight markers; *EC* crude extract. **b** SDS- polyacrylamide gel analysis of the fractions obtained during hDHFR purification. PEG, after PEG 6000 precipitation; MTX, after MTX affinity chromatography; G-25, after Sephadex G-25 gel filtration

From the calculated V_{max} value, a catalytic constant of 51±4 s^{-1} was determined for hDHFR-cbm, and this was similar to the catalytic constant for hDHFR-ec (Table 3). Altogether, the results indicate that the BmNPV/Bac-to-Bac expression system using *B. mori* chrysalides as a host is an adequate system for the effective production of high amounts of hDHFR for kinetic and structural studies.

Table 2 Summary of the purification procedure of hDHFR from 50 chrysalides of B. mori.

	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	2,025	18.3	9×10^{-3}	100	1
14% PEG 6000	501	15.9	0.032	86.9	3.56
MTX Agarose[a]	1.88				
Sephadex G-25	1.49	2.9	1.95	15.8	217

[a] The activity in this fraction could not be determined due to the presence of TMP.

Probing hDHFR-cbm for Polyphenol Binding Studies

Finally, this newly expressed hDHFR-cbm was used for binding studies with tea catechins and flavonoids. Green tea extracts containing significant amounts of tea catechins strongly inhibited the activity of bovine liver DHFR [11]. In order to detect which components of these extracts were responsible for this inhibition, hDHFR-cbm activity was assayed in the presence of EC, EGC, ECG, and EGCG. The results showed that both ECG and EGCG were potent inhibitors of this human enzyme, while polyphenols lacking the ester-bound gallate moiety (e.g., EGC and EC) did not inhibit it. The effective binding of ECG to free hDHFR-cbm was determined by following the decrease in enzyme fluorescence that occurs after formation of the enzyme–inhibitor complex (Fig. 4). When DHFR fluorescence is excited at 290 nm, its emission spectrum shows a maximum at 340 to 350 nm. The binding of ECG quenched this fluorescence, and the data showed that the dissociation constant of this enzyme–inhibitor complex is 2.01±0.1 μM (Fig. 5). Similar results were obtained for EGCG (Fig. 5); however, EC did not modify the emission spectrum of hDHFR-cbm (Fig. 4). These results indicate that the ester-bound gallate moiety is essential for the inhibition of this enzyme, as has been determined in the case of the bovine enzyme [11]. The high structural similarity between quercetin glucuronides and ester-bound gallate tea catechins (Fig. 5) prompted us to study the binding of these compounds to hDHFR-cbm. The four quercetin glucuronides used in this study have the same core structure (quercetin) but differ in the sugar moiety and in the number and spatial position of the OH groups linked to the sugar ring. The affinity of hDHFR for these compounds will help us to identify the optimal conformation for the interaction and inhibition of hDHFR by these quercetin glucuronides. Figure 5 presents the calculated dissociation constants for hDHFR-cbm, which were essentially the same as those obtained using hDHFR-ec. Qxyl bound hDHFR much stronger than the other three quercetin glucuronides, with a dissociation constant of 0.51±0.08 μM. Qxyl and ECG have similar chemical structures (Fig. 5) and similar dissociation constants for their binding to hDHFR (Fig. 5); however, the presence of

Table 3 Kinetic characterization of hDHFR.

Enzyme	K_m^{DHF} (μM)	K_m^{NADPH} (μM)	k_{cat} (s^{-1})	Catalytic efficiency[a] (s^{-1} μM^{-1})
hDHFR-cbm	1.9±0.6	7.4±1.2	51±4	28
hDHFR-ec[b]	1.6±0.5	7.2±1.0	49±4	31
hDHFR-ec[c]	2.7±0.5	5.7±0.9	40±2	15

[a] Catalytic efficiency with respect to DHF

[b] Calculated in this study

[c] Calculated by Cody et al. [30].

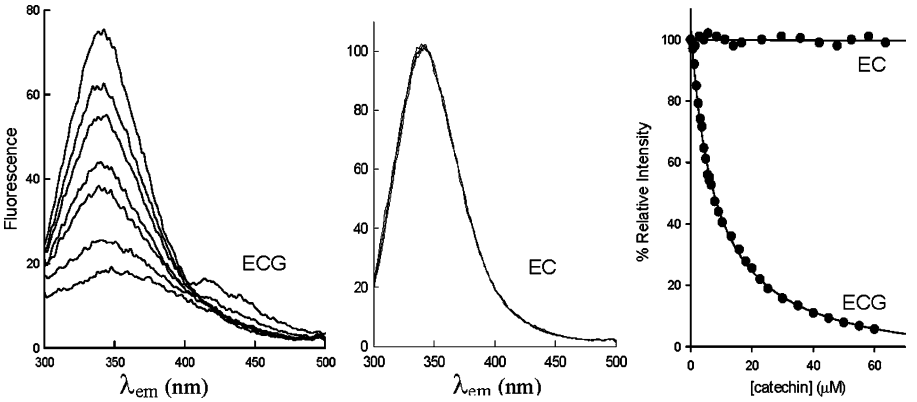
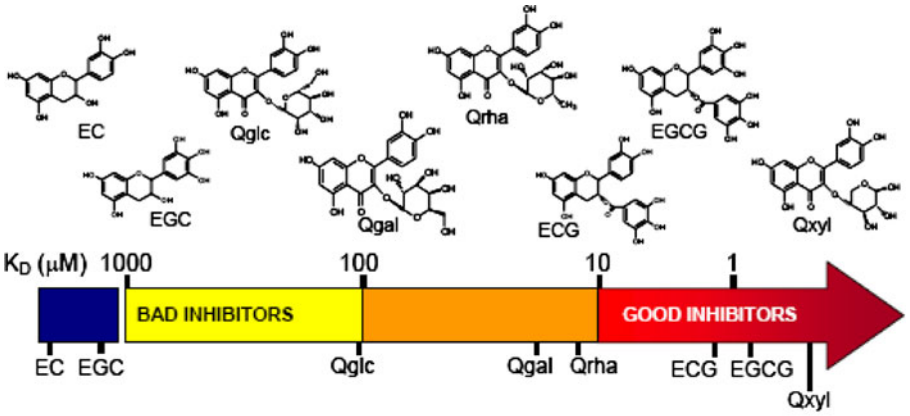


Fig. 4 Titration fluorescence experiments for the binding of ECG and EC to hDHFR-cbm. In the *left panel*, the points are experimental (after correction for enzyme dilution), and the *lines* are best-fit theoretical curves. The enzyme concentration was 0.1 μM

a sugar ring in Qxyl instead of the highly reactive phenolic gallate moiety in ECG could represent an advantage with respect to its stability and bioavailability, which could improve the effectiveness of Qxyl as an antifolate agent.

Conclusions

In this paper, we describe the use of a BmNPV/Bac-to-Bac expression system to generate recombinant hDHFR in *B. mori* chrysalides and the procedures for the efficient purification



Dissociation constants for the binding of polyphenols to hDHFR (μM)								
Enzyme	EC	EGC	Qglc	Qgal	Qrha	ECG	EGCG	Qxyl
hDHFR-cbm	nd	nd	106	21.5	20.3	2.01	0.87	0.51
h-DHFR-ec	nd	nd	102	22.8	17.2	1.78	0.92	0.59

Fig. 5 Dissociation constants (K_D) for the binding of tea catechins and quercetin-related flavonoids to hDHFR expressed in different systems. EC and EGC did not modify the fluorescent spectrum of the enzyme, and therefore, the dissociation constants for these compounds could not be determined (nd)

of this protein. The purified protein showed dissociation constants for the binding of natural polyphenols similar to that expressed in *E. coli*, which ensures its usage as a new tool for further structural studies. Although the expression of hDHFR in silkworm larvae was found to be three times more efficient than in pupae (considering the yield of hDHFR per individual), this latter system offers biosecurity and economic advantages. Companies dedicated to large-scale production of proteins in this insect may need to express a wide variety of protein products. Mobile silkworm larvae should be continuously fed several days after virus inoculation, and therefore, care should be exercised during these operations in order to avoid virus dissemination and possible cross contamination to other insect batches infected with viruses containing other desired proteins. In contrast, the immobility of the pupae and the absence of feeding guarantee a safer process in which infected individuals can be safely stored without further manipulations. Furthermore, expressing protein in chrysalides may also have important economic advantages. For example, since chrysalides are infected after formation of their silk cocoons, the cocoons could be used to produce silk-related products.

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