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ISOLATION AND PURIFICATION OF RECOMBINANT PUTRESCINE N-METHYLTRANSFERASE

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Putrescine N-metiltransferazele, enzimele-cheie în căile biosintetice conducând de la putrescină până la alkaloizi de natură tropană şi pirolidină, au fost izolate şi purificate din celulele de *E. coli*, transformate cu ajutorul sistemului pQE vector. Preparatele enzimatice native au fost studiate cu ajutorul electroforezei în gel cu gradient de pori. Rezultatele obținute atestă că enzimele recombinante posedă tendința de oligomerizare.

The solanaceous plants belonging to the genera, such as *Atropa, Datura, Nicotiana*, *Solanum, Hyoscyamus, Scopolia, Duboisia* and others, are characterized by the accumulation of the pharmacologically important tropane and pyrrolidine alkaloids, such as anticholinergic alkaloids chyoscyamine and scopolamine. Putrescine N-methyltransferase (PMT, EC 2.1.1.53) catalyses the following initial reaction in the biosynthesis of tropane and pyrrolydine alkaloids from putrescine in roots of plants:

Putrescine + S-Adenosylmethionine (SAM) → N-methylputrescine + S-Adenosylhomocisteine (SAH)

It was of interest to isolate and purify some recombinant PMTs because the purification of PMT from natural sources had turned out to be very difficult [1].

Materials and methods

The chemicals were obtained from commercial sources and were of the highest purity normally available.

The following buffers were used: (A) 50 mM Na-phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM Imidazol; (B) 100 mM HEPES buffer (pH 8.0); (D) 20 mM Na-phosphate buffer (pH 7.8) containing 500 mM NaCl and 20 mM Imidazol; (E) 20 mM Na-phosphate buffer (pH 7.8) containing 500 mM NaCl and 500 mM Imidazol.

Proteins for use as molecular weight standards in native pore gradient gel-electrophoresis [2] from 5% to 25% monomer and 3% cross-linker were supplied by Amersham Biosciences.

For expression PMTs from *Solanum tuberosum* (STPMT), *Datura stramonium* (DSPMT) and *Lycopersicon esculentum* (LEPMT) were cloned into the pQE vector and were transformed into *E. coli*.

Results and discussion

Purification of PMT. All purification procedures were carried out at 4º C. The cells from 250 ml culture medium were precipitated by low speed centrifugation. The pellet was re-suspended in 10 ml buffer A, then 800 µl of buffer A containing 10 mg/ml Lysozym (EC 3.2.1.17) was added and placed on ice for 30 min. After ultrasonication 2x30 s 50 µl of buffer A containing 1 mg/ml DNAse I (EC 3.1.21.1) was added and the suspension was incubated at room temperature for 30 min. The cell debris were removed by centrifugation at 15000 x g for 30 min. After centrifugation the supernatant was applied onto Ni-NTA column that had been prior equilibrated with buffer C. The column was washed with the same buffer until zero protein concentration and the recombinant enzyme was eluted using linear gradient 0 – 100% buffer E in buffer D (Fig.1). Fractions 6-9 were pooled, diluted three-fold with buffer D and used for Ni-NTA re-chromatography as described above.

The Ni-NTA purified enzyme was analyzed by SDS-PAGE (data not shown) where a strong band at 40 kDa indicated that recombinant protein had the same size as other PMTs (for details see ProtParam Q70EW6_DATST, Q2KTH3_CALSE, Q2KTH4_LYCES). In the enzyme preparations traces of other proteins were also found. These may be the result of the co-purification of host proteins, known as chaperonins, which are involved in the correct folding of nascent proteins in *E. coli*. These include, but are not limited to Dna K (70 kDa), Dna J (37 kDa), Grp E (40 kDa), Gro EL (57 kDa), Gro ES (10 kDa). Thus, the additional enzyme purification was necessary.

The Ni-NTA purified enzyme was applied onto a desalting column equilibrated with buffer B. The protein-containing fractions were collected, pooled and applied onto a PBE-94 column, equilibrated with the same buffer. The column was washed with the same buffer and the enzyme was eluted using a linear salt gradient of 0 to 1 M NaCl in the same buffer (Fig.2).

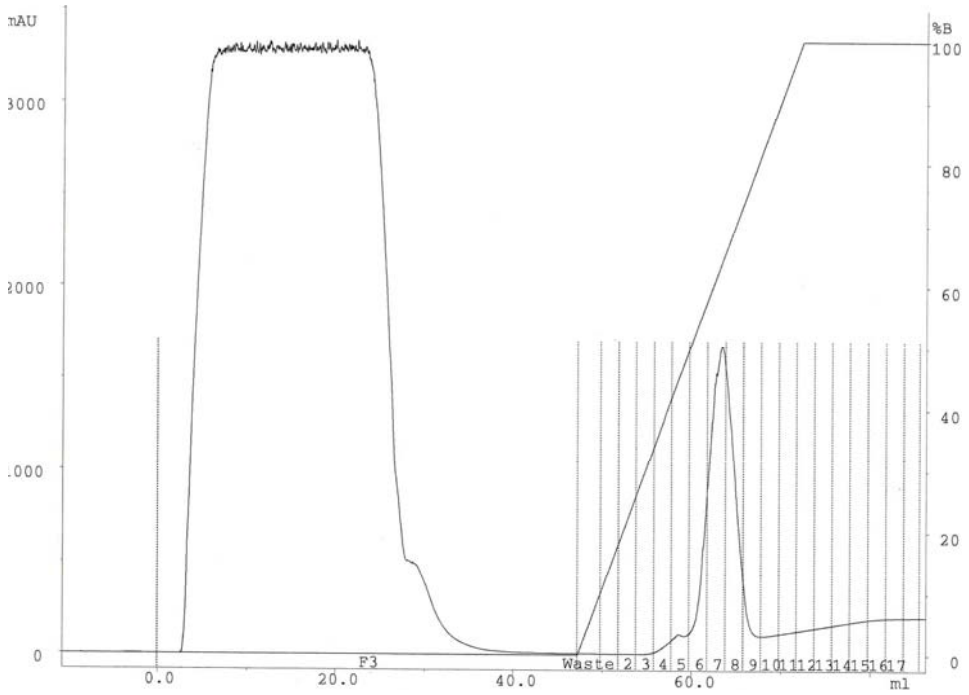

Fig.1. Ni-NTA purification of DSPMT.

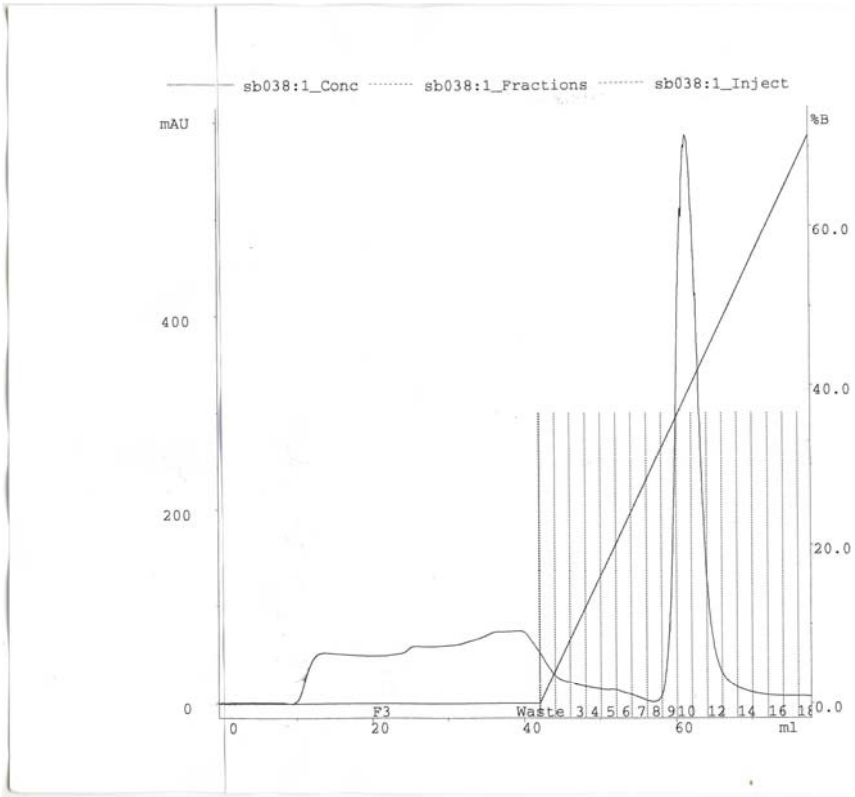

Fig.2. Ion-exchange chromatography of Ni-NTA purified DSPMT.

Native pore gradient gel electrophoresis. The recombinant proteins DSPMT, LEPMT, and STPMT were analyzed by native pore gradient electrophoresis as described by Reva [2] with modifications. The separation gel contained from 5% to 25% monomer and 3% cross-linker. The electrophoresis was carried out in a vertical mini-block.

All of the recombinant proteins studied revealed a single protein band of molecular weight between 67 kDa and 140 kDa (data not shown). For comparison, SDS-PAGE of DSPMT revealed a single band of 40 kDa. The difference in the molecular weight revealed by SDS-PAGE and molecular weight values obtained by native pore gradient gel electrophoresis suggests that the native enzyme is an oligomer.

It was of interest to study the conditions of oligomer dissociation. Proteins can sometimes be dissociated from oligomers using common agents as Triton X-100, Nonidet P-40, guanidine hydrochloride or others. The enzyme preparations obtained in this study were treated with different agents at various concentrations and then subjected to native pore gradient gel electrophoresis. For example, Fig.3 shows electrophoretical pattern of DSPMT treated with Triton X-100 (in the presence of 2-mercaptoethanol) at various concentrations of the detergent. As can be seen, electrophoresis revealed - at various concentrations of the detergent - only a single band of molecular weight of approximately 80 kDa.

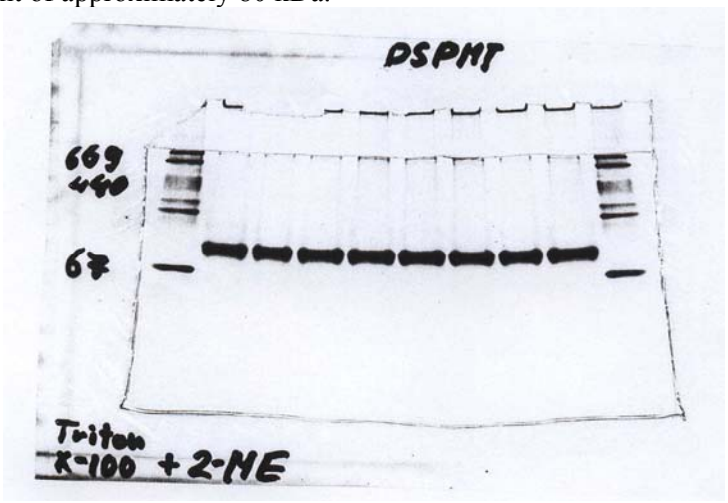

Fig.3. Native pore gradient gel electrophoresis of DSPMT treated with Triton X-100 at various final concentrations. M – molecular weight markers (from 67 kDa to 669 kDa), 1-8 – DSPMT treated with Triton X-100 at final concentrations from 0.1% to 0.8%, respectively.

Similar data were obtained by electrophoresis of DSPMT preparations treated with Nonidet P-40 (the final concentration up to 0.8%), Betaine (the final concentration up to 1%).

In some instances, dissociation of DSPMT was achieved in the presence of 2 M Guanidine hydrochloride (Fig.4). At this concentration (and higher) of Guanidine hydrochloride the quaternary and tertiary structures of proteins are complete destroyed and refolding products migrate in the electric field very slowly, due to frictional restrictions.

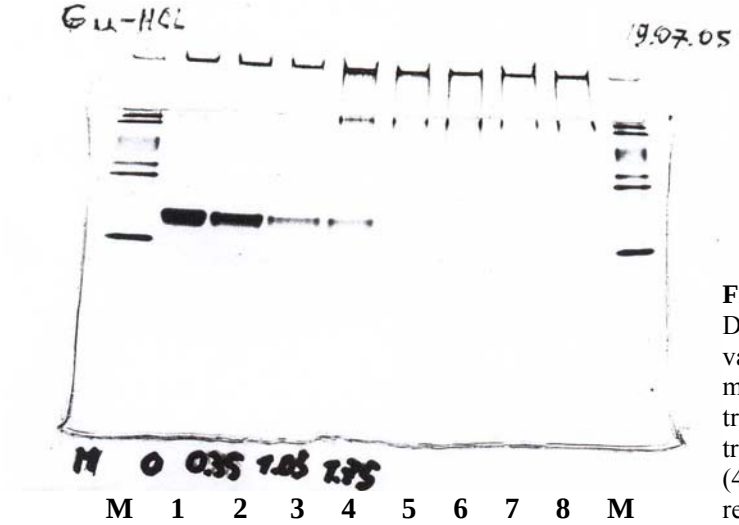

Fig.4. Native pore gradient gel electrophoresis of DSPMT treated with Guanidine hydrochloride at various final concentrations. M – molecular weight marker (from 67 kDa to 669 kDa), 1-8 – DSPMT treated Guanidine hydrochloride at final concentrations from 0 (1), 0.35 M (2), 1.05 M (3), 1.75 M (4), 2.45 M (5), 3.15 M (6), 3.9 M (7), 4.6 M (8), respectively.

According to the model proposed [3], PMT active site is constituted by three sub-sites: A, B, C. In this model, putrescine is proposed to bind with one amino group at substrate-binding sub-site A and the other amino group at the catalytic sub-site C where N-methylation takes place. Sub-site B binds the hydrocarbon unit in putrescine. Sub-site C has a positive charge, provided by the enzyme's amino acid residue(s) or bound SAM, which would dispel an incoming substrate amino group. The relatively high pH optimum for PMT may help to reduce this positive charge and also to convert the amino group of putrescine to a non-protonated form. Thus, according to this model, ionic interactions play an essential role in enzyme function. They also may contribute to the process of protein oligomerisation *in vitro*.

References:

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