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Review

Purification methods of mammalian catechol-O-methyltransferases

Carola Tilgmann, Ismo Ulmanen*

Target Protein Laboratory, Orion-Farmos, Orion Research, PL 56, 00014 University of Helsinki, Finland

Abstract

The protein purification strategies used for obtaining homogeneous rat and human soluble catechol-O-methyltransferase (S-COMT) polypeptides are reviewed. Expression and purification of recombinant rat and human S-COMT in *Escherichia coli* and for human S-COMT in baculovirus-infected insect cells made it possible to elucidate the S-COMT polypeptides in more detail. The application of these purification methods has allowed the crystallization of the rat S-COMT protein and the analysis of the kinetic properties of the enzyme in great detail. The availability of the pure S-COMT protein together with the structural data has also greatly enhanced the development of more potent COMT inhibitors.

Keywords: Reviews; Enzymes; Catechol-O-methyltransferase

Contents

1. Introduction

In mammals, catecholamines are metabolized mainly by three enzymes: catechol-O-methyltransferase, (COMT) monoamine oxidase (MAO) and phenolsulphotransferase (PST) [1]. The COMT enzyme and its activity in catalyzing O-methylation of catecholamines were discovered in 1957 by Axelrod

*Corresponding author.

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[2]. The major roles of COMT include its participation in the inactivation of the neurotransmitters in the central nervous system [3–5], and as a barrier enzyme limiting the access of catecholamines to certain biological compartments [6,7]. COMT also has important roles in the metabolism of catecholestrogens and melanin, and in the inactivation of toxic and carcinogenic compounds as well as catechol-containing drugs [8–12].

Since the 1960s the enzyme has been intensively studied, and numerous articles dealing with different aspects of COMT proteins have been published (reviewed in [13–15]). COMT received new attention in the late 1980s when new and more selective inhibitors were discovered. One of the most promising clinical uses of these new inhibitors will probably be in the treatment of Parkinson's disease [15–18].

Developments in molecular biology and gene technology have allowed the production and isolation of large amounts of recombinant proteins for biochemical, pharmacological and structural studies. This has resulted in cloning and characterization of rat and human COMT cDNAs and genes [19–25].

On the basis of the subcellular localization [26,27], cloning and nucleotide sequencing, two major classes of the COMT enzyme, a soluble cytosolic form (S-COMT) and a rough endoplasmic reticulum membrane-bound form (MB-COMT), have been characterized [19–21,27,30,31]. S- and MB-COMT are coded by a single gene using two separate promoters [22,23,28]. The MB-COMT sequence revealed that it contains the S-COMT polypeptide and an amino terminal extension with 17 and 24 amino- acid-long hydrophobic signal-anchor peptides in rat and man, respectively [19–21,29].

Immunocytochemical studies have revealed intensive reactions for COMT in the rat liver and kidney [32], which is in agreement with the high COMT activity in these organs [33]. The localization of COMT in many mammalian organs has been extensively reviewed by Creveling and Hartman [34]. More recent studies of the S-COMT localization, using antibodies against pure recombinant rat S-COMT protein [27], have confirmed and extended the earlier observations [32,35,36]. High levels of both human S- and MB-COMT expressed in *E. coli* [37] and in SF9 insect cells [27] have also allowed more precise and extensive kinetic and structural studies [38,39].

This review will focus on the strategies that have been used to purify rat and human S-COMT polypeptides to elucidate the structure and properties of the COMT enzyme and its gene. The information obtained has also been used to design new and more potent inhibitors for COMT.

2. Measurement of COMT enzyme activity

COMT catalyses the transfer of the methyl group from S-adenosyl-L-methionine (SAM) in the presence of magnesium ions to an acceptor catechol substrate liberating the O-methylated catechol and S-adenosyl-L-homocysteine (SAH) [40]. The methyl group from SAM is transferred predominantly to position 3 of a catechol and a minor proportion of the 4-O-methylated product is formed. COMT activity in biological materials is usually measured in vitro by determination of the amount of the 3- and 4-O-methylated end-products after termination of the enzyme reaction.

Separation of the reaction products by solvent extraction and detection by fluorometry [40] or radiochemical techniques [41–46] have been used. However, the introduction of HPLC techniques coupled with UV detection [47], fluorometric [48,49], radiochemical [50] and electrochemical detection [51,52] have improved the sensitivity and specificity of the analysis. The most recent studies on the purification of S-COMT from different sources for structural studies applied COMT activity assay using reversed-phase (RP)-HPLC. The reaction products were monitored either by UV absorbance at 254 nm or detected electrochemically by redox potential [51,53].

The COMT activity was first detected in rat liver extracts [40]. Earlier studies on the tissue distribution of COMT activity have been extensively reviewed by Guldberg and Marsden [13]. Since then, COMT activity has been identified in various organisms and species: prokaryotes, yeast, plants and different mammals including primates [13,49,54–65]. In mammals, COMT activity has been found in most tissues, the highest levels of the enzyme residing in the liver and kidney. The degree of liver COMT

activity varies among different species being highest in rat and progressively decreasing in cow, pig, mouse, guinea pig, man, cat and rabbit [14].

3. Rat and human native S-COMT

3.1. Purification of S-COMT from rat liver

In spite of the extensive literature on COMT purifications, until 1990 the primary structure of the protein has not been used to establish the homogeneity of the purified COMT. Because most of the COMT activity is generally found in the soluble fraction of the tissue lysates [66], the starting material for COMT purification usually has been the supernatant of the tissue homogenates (cytosolic fraction). Since the highest COMT activity has been measured from rat liver [67], this tissue has also been most frequently used as the source of the material for S-COMT purification [40,67–83]. The COMT enzyme has been enriched by various biochemical separation methods such as differential centrifugation, ammonium sulphate fractionation, adsorption, size exclusion, and anion- or cation-exchange chromatography. The earlier purification data suggested that the lability of the S-COMT protein increases with its specific activity [80,82,84] and therefore more rapid and effective purification methods were needed to recover an active pure protein.

A new approach to purify rat liver S-COMT was worked out by Tilgmann and Kalkkinen [85], in order to purify the protein for structural analysis. This purification method is summarized in Fig. 1. Using this scheme about 1330-fold purification of S-COMT with a yield of 11% from rat liver was obtained. SDS-PAGE analysis showed that the final COMT-active fraction of the anion-exchange chromatography Mono-Q (HR5/5, Pharmacia column) still contained several proteins. Further, RP-chromatography (TSK, TMS 250 4×0.46 cm) of the Mono-Q fraction revealed three protein peaks. Highest COMT activity correlated with one of the RP-peaks, which on the basis of one- and two-dimensional SDS-PAGE, contained mainly a $25 \cdot 10^3$ polypeptide (Fig. 2). To indisputably identify the COMT protein, purified by RP-chromatography, N-terminal se-

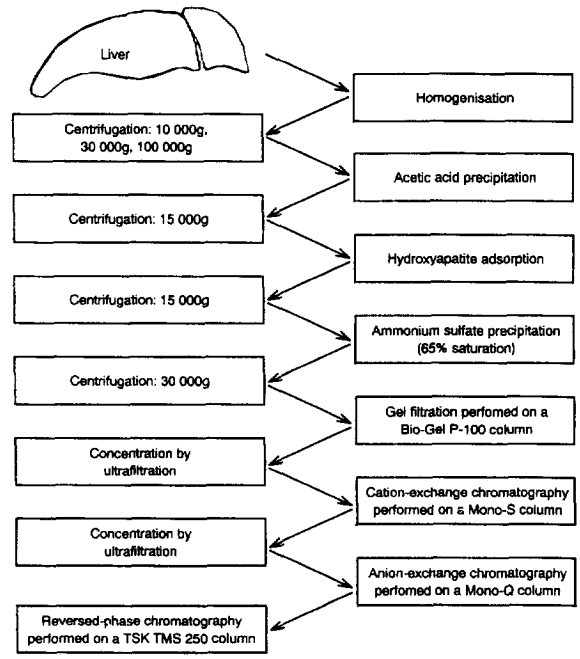

Fig. 1. The outline of the purification steps used for rat liver S-COMT.

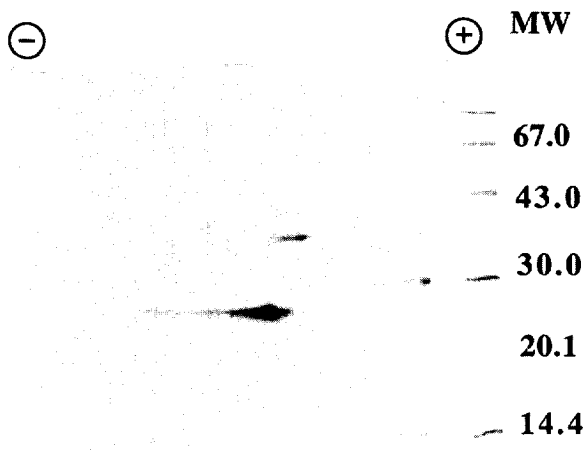

Fig. 2. Two-dimensional gel electrophoresis of enzymatically active rat liver S-COMT fraction eluted from RP-chromatography TSK TMS 250 C1 (3×0.46 cm) column. A 10-μg amount of purified rat S-COMT was separated by isoelectric focusing in capillary rod gels containing pH 4.0–7.5 carrier ampholytes, after which the gels were equilibrated with SDS-PAGE buffer. The second-dimension electrophoresis was performed by a 12.5% SDS-PAGE and the gel was stained by Coomassie Brilliant blue.

quence analysis was carried out. That analysis gave no positive data suggesting that the N-terminus of the protein is blocked. A blocked N-terminus has also been reported for purified S-COMT from pig liver [86].

To determine the primary structure of the COMT enzyme the protein purified by RP-chromatography was alkylated, digested with trypsin and the peptides were separated by RP-chromatography. Ninety-two per cent of the amino acid sequence obtained from the tryptic peptides of the purified rat liver S-COMT corresponded with the nucleotide sequence of putative COMT cDNA clone from rat liver [19,85].

A greatly simplified purification method described by Bertocci et al. [86] employed immunoaffinity chromatography. By these means enough pig liver S-COMT was obtained for amino acid sequence analysis. The drawback of this method was a rather low purification efficiency (154-fold), compared to the 1330-fold purification of rat liver S-COMT obtained with the method described by Tilgmann and Kalkkinen [85]. The purification attempts of native S-COMT from rat and pig liver made it obvious that by this means it was not feasible to obtain enough active protein for structural studies. The S-COMT protein purified by RP-chromatography could be used for amino acid sequence analysis but it was in a denatured state and thus not suitable for kinetic or structural studies [85].

3.2. Purification of human placental S-COMT

Although the main research interest has been directed to rat liver S-COMT, the COMT enzyme has also been partially purified and characterized from human placenta [87,88], liver [89] and brain [90]. According to these studies the human enzyme differs from the rat enzyme in activity, mass, kinetic properties, stability and response to certain enzyme inhibitors [74].

Essentially the same methods which were used for purification of rat liver S-COMT [85] were applied for separation of human placental S-COMT [91]. The minor modifications included omission of the acetate precipitation step and performing chromatographic separations in the presence of 20 m*M* cysteine. After the last non-denaturing chromatographic step (anion-exchange chromatography, Mono-Q column) the human enzyme was 1452-fold purified with a yield of 9%, which was roughly the same as for the rat liver S-COMT. However, the human S-COMT fraction from the Mono-Q column contained several contaminating proteins. Therefore, the COMT enzyme had to be further purified by RP-chromatography. SDS-PAGE analysis revealed that the RP-fraction containing the COMT activity still consisted of two polypeptides of $26 \cdot 10^3$ and $40 \cdot 10^3$. The $26 \cdot 10^3$ protein was identified as COMT based on Western blotting using a specific anti-COMT antiserum [85]. To be able to separate the $26 \cdot 10^3$ COMT protein from the $40 \cdot 10^3$, the RP-fraction containing these two proteins was alkylated before RP-chromatography. Both alkylated and RP-separated 26 and $40 \cdot 10^3$ proteins were cleaved by trypsin, the peptides separated by RP-chromatography and subjected to sequence analysis. The co-eluting $40 \cdot 10^3$ protein was identified as the human sphingolipid activator protein 1 precursor [92].

The sequences obtained from the $26 \cdot 10^3$ protein were 82% identical with the sequences of rat and pig liver S-COMT, confirming that the purified protein was human S-COMT. N-terminal sequence analysis of the purified human placental S-COMT showed no positive data, suggesting that its N-terminus is blocked, like that of rat and pig liver S-COMT [85,86].

3.3. Properties of the native rat and human S-COMT proteins

According to several reports the purified COMT enzyme is unstable and loses 50–70% of its activity at 4°C within 24 h [68,72,93]. However, Tilgmann and Kalkkinen [85] reported that the semi-purified rat liver S-COMT protein (material in the second-last purification step) without addition of reductants was stable for about 96 h at room temperature. A half-life of 5.5 days at 4°C for the highly purified rat liver S-COMT recorded by Korkolainen and Nissinen [83] is in accordance with the observed high stability of the enzyme.

The purification of human placental S-COMT showed that it is distinctly less stable than the rat liver enzyme. In practice it turned out that human S-COMT activity was completely lost at the very

Table 1
Comparison of the amino acid sequences of the human (H), rat (R) and porcine (P) MB- and S-COMT amino acid sequences derived from the cloned cDNAs

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      MB-COMT →
R  -  MP------LAAVSLGLLLLA-LLLLLRHLGWGLVTIFWFEYVLQPVHNLI  -43
      ::      :::: :::.::  :::::::: ::::  : : :..:::.:::.
H  -  MPEAPPLLLAAVLLGLVLLVVLLLLLRHWGWGLCLIGWNEFILQPIHNLL  -50

      S-COMT →
R  -  MGDTKEQRILRYVQQNAKPGDPQSVLEAIDTYCTQKEWAMNVGDAKGQIM  -93
      ::::::::::  : : : ::  :::::::::::: :::::::::: :: :.
H  -  MGDTKEQRILNHVLQHAEPGNAQSVLEAIDTYCEQKEWAMNVGDKKGKIV  -100
                                          :: :: :: ::: ::
P  -                                      KERAMHVGRKKCQIV  -15

R  -  DAVIREYSPSLVLELGAYCGYSAVRMARLLQPGARLLTMEMNPDYAAITQ  -143
      :::: :  ::..:::::::::::::::::::: ::::::.:.:.::: :::::
H  -  DAVIQEHQPSVLLELGAYCGYSAVRMARLLSPGARLITIEINPDCAAITQ  -150
      :.:.::  :::::::::::::::::::::: : :::.:::.::: :::.:
P  -  DTVVQEQRPSVLLELGAYCGYSAVRMARLLLPSARLLTIELNPDNAAIAQ  -65

R  -  QMLNFAGLQDKVTILNGASQDLIPQLKKKYDVDTLDMVFLDHWKDRYLPD  -193
       :. :::. :::::.. ::::::::::::::::::::::::::::::::::
H  -  RMVDFAGVKDKVTLVVGASQDIIPQLKKKYDVDTLDMVFLDHWKDRYLPD  -200
       .::::::. :.::.::::::::::::::::::::::::::::::::::::
P  -  QVVDFAGLQDRVTVVVGASQDIIPQLKKKYDVDTLDMVFLDHWKDRYLPD  -115

R  -  TLLLEKCGLLRKGTVLLADNVIVPGTPDFLAYVRGSSSFECTHYSSYLEY  -243
      ::::: ::::::::::::::::: ::.::::: ::::: :::::: :.:::
H  -  TLLLEECGLLRKGTVLLADNVICPGAPDFLAHVRGSSCFECTHYQSFLEY  -250
      :::::::::::::::::::::::::::::::::    :::::. :.:::
P  -  TLLLEECGLLRKGTVLLADNVICPGAPDFLAHVRGCGRFECTHFSSYLEY  -186

R  -  MKVVDGLEKAIYQGPSSPDKS  -264
        :::::::::: :: :
H  -  REVVDGLEKAIYKGPGSEAGP  -271
        .::::::::.::::::: : :
P  -  SQMVDGLEKAVYKGPGSPAQP  -186
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The putative signal peptides of the MB-COMT are underlined. The translation-initiation methionines for the MB- and S-COMT are boxed. Identical amino acids are shown by two dots and similar amino acids by one dot.

beginning of the purification if cysteine (20 m*M*) was omitted from the buffers [91]. Similarly the use of reducing agents like mercaptoethanol, dithiothreitol or cysteine by other investigators has stabilized the enzyme and allowed the purification and partial characterization of human COMT [68,82,87,93–95]. It can be concluded that since reducing agents can restore COMT activity, the observed rapid inactivation of purified S-COMT is most likely a result of the oxidation of sulphydryl group(s) of the protein molecule [68].

The protein purification has revealed some heterogeneity in COMT activity. Multiple COMT forms have also been demonstrated for a variety of tissues and animal species, suggesting the presence of isozymes [96,97]. In most cases the degree of homogeneity of the COMT enzyme has been demonstrated by SDS-PAGE or by sedimentation analysis in the ultracentrifuge. On the basis of analysis by SDS-PAGE, size exclusion chromatography or sucrose gradient centrifugation, the S-COMT from the rat liver, pig liver and pig brain has a molecular mass of about $24 \cdot 10^3$ [68,77,98]. Molecular masses of $52 \cdot 10^3$ [87] and $49 \cdot 10^3$ [88] for human placental S-COMT enzyme have also been reported. Both human placental and rat liver S-COMT-activity eluted on size exclusion chromatography as a single symmetrical peak of $25 \cdot 10^3$. Analysis with native PAGE showed a molecular mass of $25 \cdot 10^3$ for purified rat liver S-COMT and $26 \cdot 10^3$ for the human placental S-COMT. These techniques, and more recently, chemical cross-linking, indicated that S-COMT is a monomeric enzyme [85,91,99].

In addition to the difference in molecular mass between the rat liver and human placental S-COMT, chromatofocusing revealed that the two proteins have slight difference in isoelectric points (5.1 and 5.3; [85,91]). Earlier other investigators have reported isoelectric points from 4.6 to 5.3 for rat liver S-COMT [74,79,97,100,101] 5.5 for human liver S-COMT [74] and 5.0 for human brain S-COMT [90]. The differences in size and isoelectric point between human and rat liver S-COMT proteins obviously reflect the differences in the primary structures of the polypeptides — direct sequence analysis of the tryptic peptides from both enzymes indicated 80% homology (Table 1).

Quantitative amino acid analysis has suggested that the pig liver COMT contains five cysteines [98] and the rat liver COMT contains one [7]. However, the protein sequence analysis has established the presence of four conserved cysteine residues in the rat and human S-COMT proteins [85,91]. In addition to this the human S-COMT has three more cysteines. The 165 amino acids predicted from the porcine incomplete cDNA clones [86] show 82% identity with the corresponding human sequence. Three of the cysteine residues are also found in the porcine sequence (Table 1). Comparison of the cDNA sequence of rat liver S-COMT [19] with the human placental S-COMT cDNA sequence [20] showed that in these polypeptides altogether 44 amino acids are different. Computer based sequence comparisons of methyltransferases have revealed two consensus sequences (LDv/IGXGXG and LRPGGXL) in mammalian methyltransferases, corresponding to the amino acids LELGAYCG and LRKGTVL in rat and human S-COMT [102,103].

COMT has been proposed to be associated with a carbohydrate moiety [104], which was later reported to be a short polysaccharide [7]. However, SDS-PAGE analysis before and after endoglycosidase-H treatment suggested that no N-linked glycans exist in rat or human COMT enzyme (Tilgmann and Kal-

Table 2
Plasmids and bacterial strains used for the expression of rat and human recombinant S-COMT

Construct	Bacterial strain	Induction	Product	Ref.
pRCX2	BL-21(DE3)	IPTG	Rat-S-COMT	[31]
pHCX12			Human S-COMT (authentic)	
pOGL495	DH-5	IPTG	Human S-COMT (fusion)	Lundström et al., unpublished data
pDS56/RBSII	SG13009	IPTG	Human S-COMT[a]	[105]

[a] Contains 24 amino acids from the MB-COMT sequence in the amino terminus.

kkinen, unpublished results). This is in agreement with the observed inability of the endoglycosidase treatment to alter the mobility of porcine liver S-COMT in SDS-PAGE [86]. The deduced sequences of the S-COMT clones reveal no N-glycosylation sites, confirming that the protein is non-glycosylated.

4. Expression of recombinant S-COMT proteins

4.1. Expression of recombinant S-COMT proteins in E. coli

E. coli is a promising host for expression of the recombinant S-COMT protein since it completely lacks endogenous COMT enzyme. The human and rat S-COMT expression plasmids have been constructed containing the S-COMT-coding sequence from rat liver cDNA [19] and human placental cDNA clones [20,21] as summarized in Table 2.

The bacteria carrying the rat COMT expression construct revealed a $25 \cdot 10^3$ intensively stained polypeptide (representing about 10% of the stained proteins) which reacted with the anti-COMT antiserum in Western blotting. Similarly bacteria containing the human COMT vectors produced a CBB-stained and immunoreactive $25–26 \cdot 10^3$ polypeptide [37,105]. All the recombinant S-COMT enzymes produced in bacteria showed COMT enzyme activity.

4.2. Expression of recombinant human S-COMT fusion protein in E. coli

To enhance the purification of human S-COMT, we have expressed recombinant human S-COMT in *E. coli* using the GST-fusion protein system (Tilgmann, Lundström and Ulmanen, unpublished results). The coding sequence of the human placental S-COMT [20] was inserted into the pGEX-2T vector (Pharmacia, Biotech). SDS-PAGE analysis of the IPTG-induced bacterial lysates revealed that the GST-COMT fusion protein was expressed at high levels (Fig. 3). Densitometric scanning of the stained gels indicated that about 10–15% of the bacterial protein consisted of the recombinant polypeptide. The fusion polypeptide was fully soluble and enzymatically active.

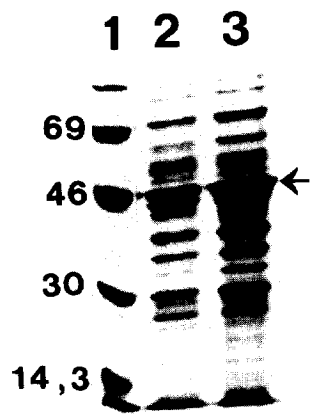

Fig. 3. SDS-PAGE of the human S-COMT-GST fusion protein. Lane (1): molecular mass standard; lane (2): uninduced bacteria; lane (3): bacteria induced with 0.5 m*M* IPTG for 3 h. An aliquot of the bacterial lysate was analysed in a 10% SDS-PAGE and the gel was stained with Coomassie Brilliant blue.

4.3. Expression of recombinant human S-COMT in insect cells

The human S-COMT sequence [20] was expressed in insect cells using the baculovirus expression system [27,106,107]. The infected cells were grown as described [27]. The baculovirus expression system produced enzymatically active recombinant S-COMT and the protein also reacted with COMT antiserum in Western blotting [27].

5. Recombinant S-COMT proteins

5.1. Purification of recombinant S-COMT protein from E. coli for structural and functional studies

Cultivation of 8 l of *E. coli* containing the pRCX2 (rat) and pHCX12 (human) expression constructs yielded about 16 g wet weight of bacterial cells, which was the starting material for the recombinant protein purification (the purification scheme used for recombinant S-COMT protein purification is outlined in Fig. 4). Briefly, the bacterial cells were disrupted by sonication and the resulting lysates were clarified by ammonium sulphate precipitation or ultrafiltration. The concentrated samples were fractionated by size exclusion chromatography, from which the COMT activity of rat and human recombinant pro-

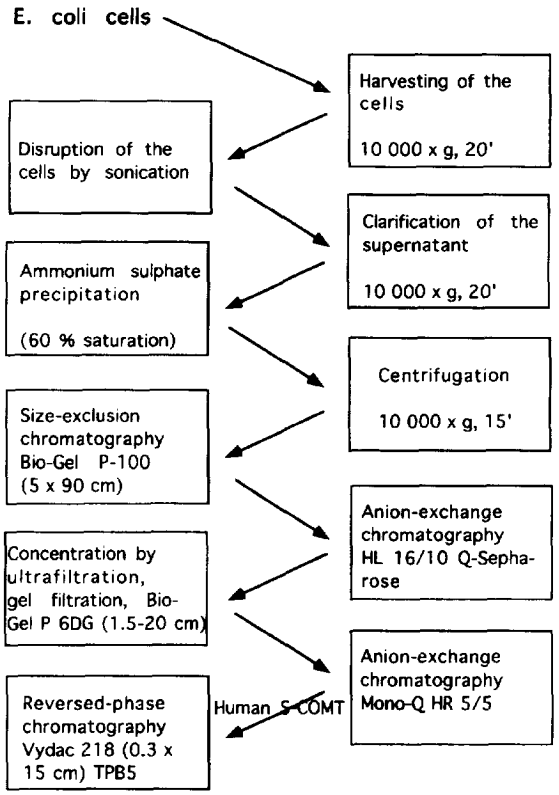

Fig. 4. Purification scheme of recombinant rat S-COMT protein produced in *E. coli*.

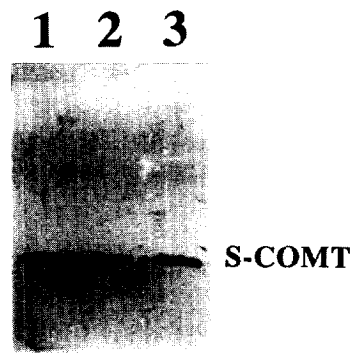

Fig. 5. Western blot analysis of three different fractions of recombinant rat S-COMT eluted from an anion-exchange chromatography column (Mono-Q HR 5/5, Pharmacia). SDS–PAGE was run on a 12.5% gel and the protein detected by anti-COMT antiserum.

teins eluted as single symmetrical peaks corresponding to a molecular mass of about $25 \cdot 10^3$. Due to large quantities of contaminating protein remaining after the size exclusion chromatography, a high capacity anion-exchange chromatography (HL 16/10 column, Pharmacia) was carried out before the final purification step on a high resolution anion-exchange chromatography (Mono-Q HR 5/5, Pharmacia) [37]. The recombinant rat S-COMT eluted as three separate peaks from the anion-exchange Mono-Q column having slightly different specific activities. In SDS-PAGE, all three peaks gave a single band with a mobility of $25 \cdot 10^3$. The $25 \cdot 10^3$ protein from all three peaks also reacted with anti-COMT antiserum on Western blots (Fig. 5). In RP-chromatography, the three enzymatically active fractions from the Mono-Q column gave single peaks with identical retention times (Fig. 6).

To determine the atomic structure of the S-COMT, a highly pure, homogeneous and enzymatically active protein was required. Thus, for crystallization of rat recombinant S-COMT, the anion-exchange chromatography fraction from Mono-Q-column containing the highest specific COMT activity was used. Although all protein purification parameters applied indicated that recombinant S-COMT protein was in a homogenous form, the isoelectric focusing gel analysis of the pure S-COMT revealed multiple protein bands. The different, possibly conformational, forms were not analysed further, but the material was used for crystallization. To increase the structural homogeneity of the S-COMT protein and to enhance the formation of suitable crystals, the crystallization was performed in the presence of a COMT inhibitor 1,5-dinitrocatechol, magnesium and the co-substrate S-adenosyl-L-methionine. These procedures led to the successful crystallization and subsequent solving of the atomic structure of recombinant rat S-COMT at 2.0 Å resolution [38,108].

Purification of *E. coli*-produced human recombinant S-COMT revealed that in contrast to the corresponding rat protein, the activity of the human S-COMT eluted as a single peak from the anion-exchange chromatography Mono-Q column [37]. However, the human S-COMT protein was still contaminated with bacterial proteins and was purified further by RP-chromatography. In RP-chromatography, three dominant peaks were eluted and the

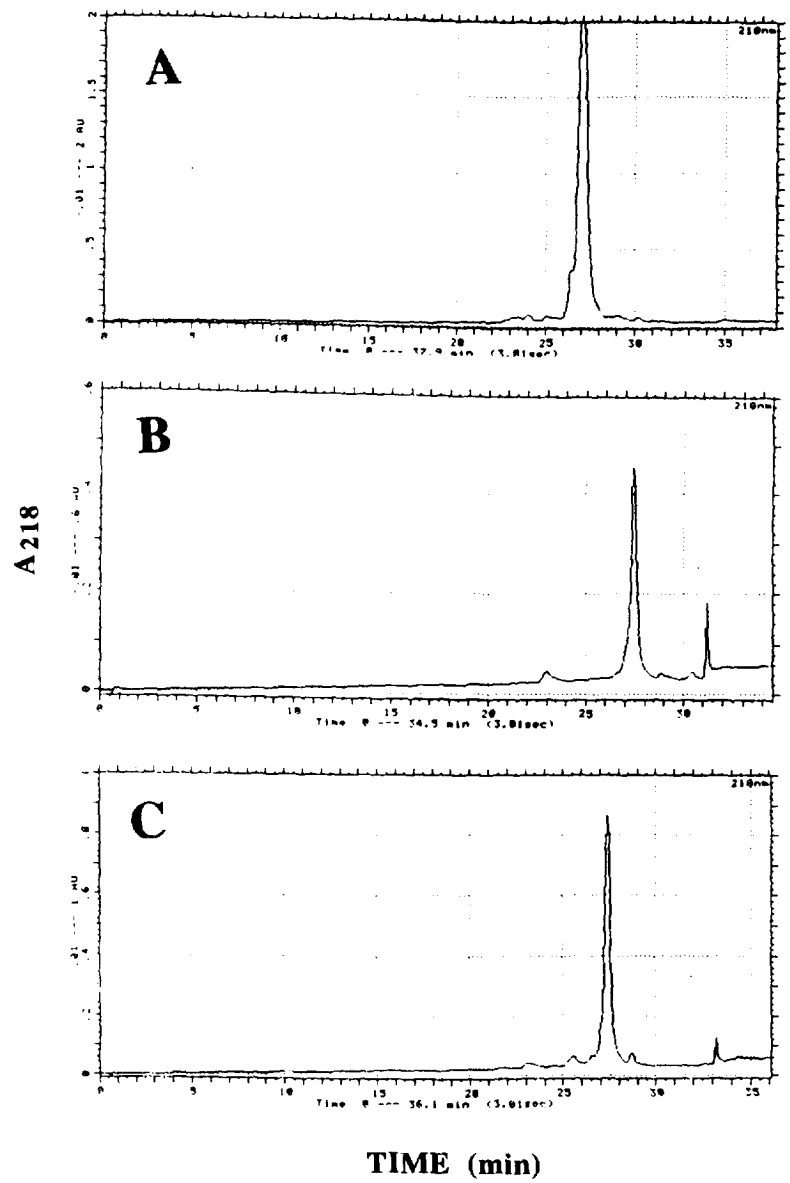

Fig. 6. RP-chromatography of three different fractions of purified rat recombinant S-COMT protein eluted from anion-exchange chromatography (Mono-Q HR 5/5, Pharmacia) column. RP-chromatography was performed on a TSK TMS 250 C1 (0.46×3 cm) column. A linear gradient of acetonitrile (20–60% in 40 min) in 0.1% trifluoroacetic acid was used for elution with a flow of 1.0 ml/min. Chromatography was monitored at the sensitivity of −0.01–1.0 AUFS.

presence of S-COMT protein was identified by Western blotting. The immunologically active RP-peaks gave a single band of 26 kDa in Coomassie Brilliant blue stained SDS-PAGE. Internal amino acid sequence analysis from the tryptic peptides also confirmed the identity of the recombinant S-COMT proteins. Unfortunately the recombinant human S-COMT could not be purified by these means to homogeneity in active form, and thus did not allow crystallization attempts.

5.2. Purification of recombinant human S-COMT from GST-fusion protein

To obtain sufficient amounts of recombinant human S-COMT for structural studies, human S-COMT was expressed as a glutathione S-transferase (GST) fusion protein in *E. coli*. After the induction with IPTG, the bacteria from a 5-l flask culture medium were harvested by centrifugation. The cells were disrupted in MTPBS buffer (phosphate buffered saline with 1% Triton X-100, 0.2 m*M* phenylmethylsulphonyl fluoride, 0.1% β-mercaptoethanol) by sonication at 100 watts (at 4°C) and the lysate was clarified by centrifugation (10 000 *g*, 20 min at 4°C). The lysate was applied on a glutathione–Sepharose 4B-affinity-column (20 ml, Pharmacia) in MTPBS buffer and the column was washed with 10 column volumes of the same buffer. Recombinant S-COMT was then cleaved from the fusion protein in the column with 40 U thrombin for 1 h and with additional 20 U thrombin for 1 h. Fusion protein was eluted from the column with the cleavage buffer containing 50 m*M* Tris–HCl, (pH 8.0), 150 m*M* NaCl, 20 m*M* L-cysteine and 0.1% β-mercaptoethanol. Aliquots from the eluted fraction were analysed by SDS-PAGE and stained with Coomassie Brilliant blue (Fig. 7). The fractions containing the fusion protein were pooled, concentrated, and the buffer changed by ultrafiltration (Omega Filter NMWL, Filtron).

Further purification was performed on an anion-exchange Mono-Q chromatography column (HR5/5, Pharmacia). The column was equilibrated with 20 m*M* Tris-HCl, (pH 6.05), containing 0.1% β-mercaptoethanol, and the protein eluted with a linear gradient of NaCl (0–0.7 *M*) in 40 min (Fig. 8). The Mono-Q fractions were analysed by 12.5% SDS-PAGE and the peak containing COMT-activity and a single $26 \cdot 10^3$ band (Fig. 9, lane 2) was analysed further by RP-chromatography. From the RP-column, the human recombinant S-COMT eluted as a single peak (Fig. 10), which by analysis in SDS-PAGE showed only the $26 \cdot 10^3$ S-COMT protein (Fig. 10, insert). The active homogeneous recombinant human S-COMT peak from the Mono-Q column is used for ongoing crystallization studies.

The other fractions eluted from the Mono-Q column contained two species of polypeptides with

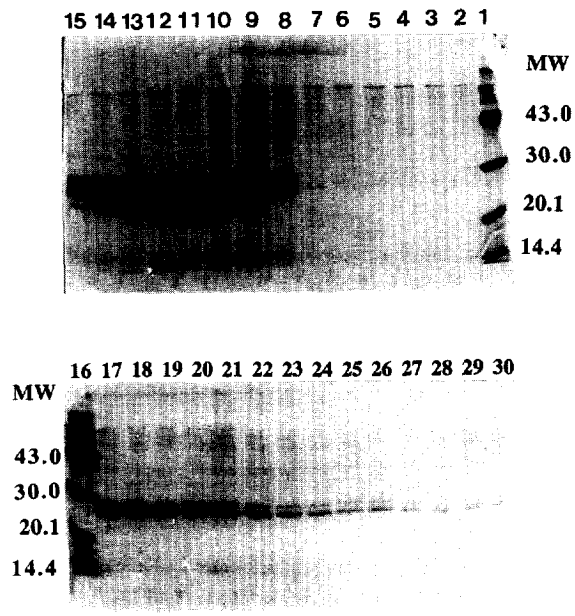

Fig. 7. SDS-PAGE analysis of recombinant human S-COMT protein eluted from glutathione-Sepharose affinity chromatography column (Pharmacia). The bound GST-COMT fusion protein was eluted from the column by the cleavage of the fusion protein with thrombin and every third fraction eluted was analysed by a 12.5% SDS-PAGE. Lanes 1 and 16: low molecular mass standard (Pharmacia). Lanes 2–7: fractions collected during the washing of the column prior to the enzyme cleavage. Lanes from 8 on: fractions eluted after the enzymatic cleavage with thrombin. The gel was stained by Coomassie Brilliant blue.

approximately 1000 Da difference in the apparent molecular mass (Fig. 9, lane 3). We assume that the polypeptide with the higher molecular mass has an extended C-terminus of ca. 7 amino acids. This heterogeneity is probably due to the suppression of the S-COMT translation stop codon, which on the basis of DNA-sequence, could lead to the extension of 7 amino acids and the termination of translation in the stop-codon of the pGEX-2T vector sequence. Similar heterogeneity for this reason has been reported in the case of the human recombinant Troponin C protein produced by the same GST-fusion-system [109]. Another possibility is the extension of the C-terminus of the bacteria-produced S-COMT by ribosomal frame-shifting as observed by Vilbois et al. [110].

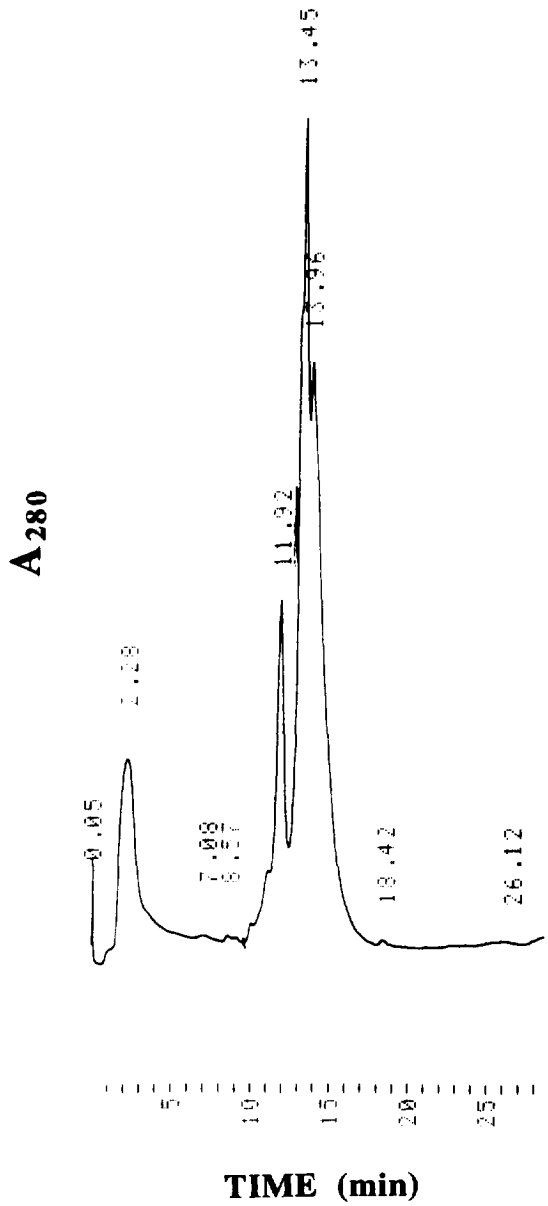

Fig. 8. Anion-exchange chromatography of the recombinant human S-COMT eluted from the glutathione–Sepharose affinity chromatography column. The Mono-Q, HR 5/5 column (Pharmacia) was eluted with a linear gradient of NaCl (0–0.7 *M* in 40 min) in 20 m*M* Tris-HCl, (pH 6.05), containing 0.1% β-mercaptoethanol with a flow of 1.0 ml/min. Chromatography was monitored at the sensitivity of 0.25 AUFS.

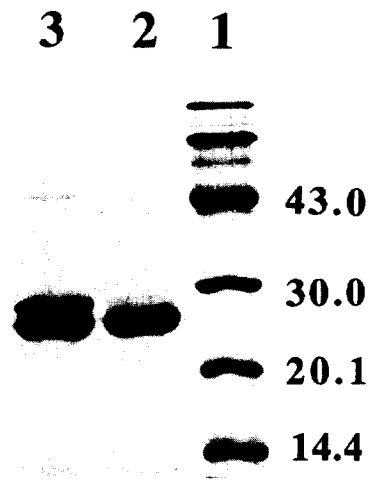

Fig. 9. SDS-PAGE analysis of recombinant human S-COMT enzyme eluted from the anion-exchange chromatography shown in Fig. 8. The fractions were analysed on a 12.5% SDS-PAGE followed by Coomassie Brilliant blue staining. Lane (1): low molecular mass standard (Pharmacia); lane (2): the fraction from the peak 1; lane (3): the fraction from the peaks 2 and 3.

5.3. Purification of recombinant human S-COMT from insect cells

In order to obtain sufficient amounts of eukaryotic recombinant human S-COMT protein for biochemical analysis and enzyme studies, recombinant baculoviruses were used to infect SF9 cells. SDS-PAGE analysis of the proteins from infected cells, followed by staining with Coomassie Brilliant blue, indicated that the cells infected with the S-COMT vector produced high amounts of $25 \cdot 10^3$ protein [27]. The purification was started by harvesting the cells from 1 l of culture medium (5×10^6 cells/ml) by centrifugation. The cells were disrupted in 50 m*M* sodium phosphate buffer containing 150 m*M* NaCl and 0.1% Triton X-100. The homogenate was centrifuged and purified by size exclusion chromatography on a Bio-Gel P-100 (2.5 cm×90 cm) in 20 m*M* bis-Tris-HCl (pH 5.8) [85]. The eluted fractions were pooled and purified as described previously for the *E. coli*-produced human S-COMT [37]. In the last purification step by RP-chromatography a single band of about $26 \cdot 10^3$ was seen by SDS-PAGE. This $26 \cdot 10^3$ polypeptide reacted with the anti-COMT serum in Western blots, and the 100 000 *g* superna-

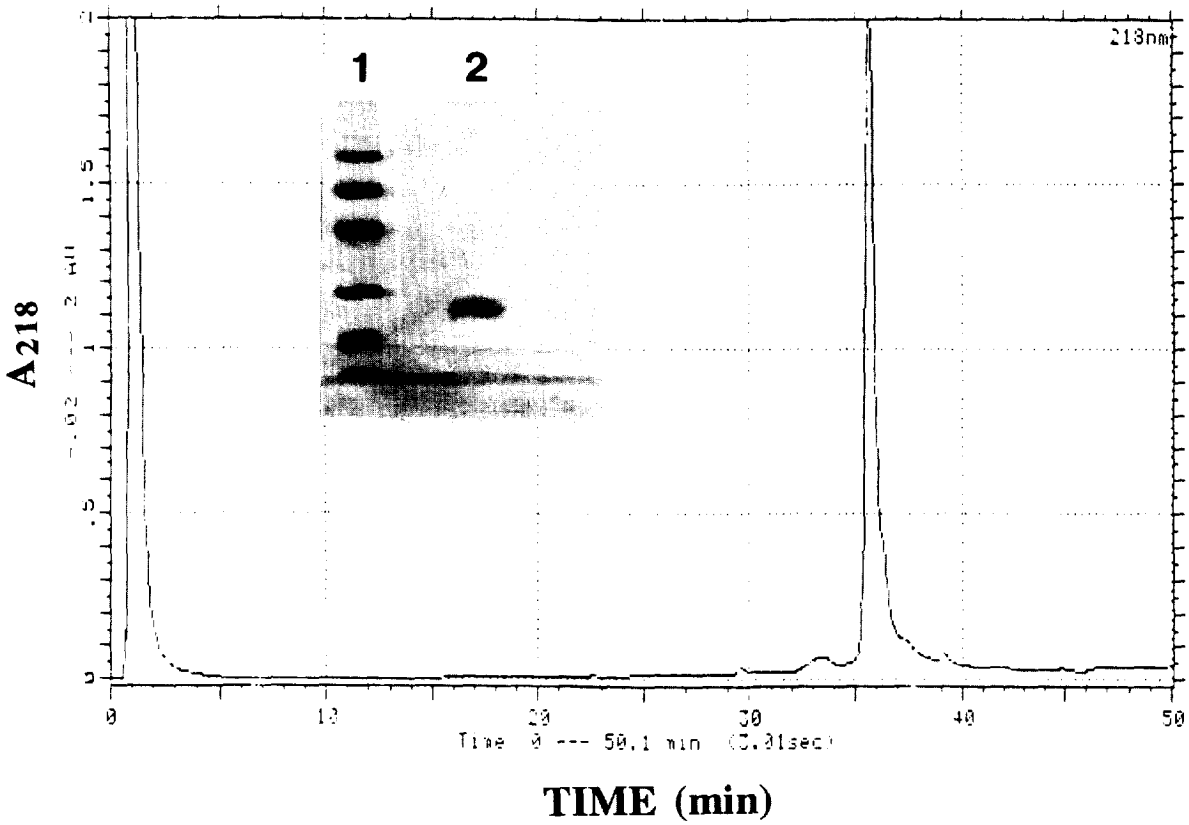

Fig. 10. Characterization of the purified recombinant human S-COMT protein by reversed-phase chromatography and SDS-PAGE. The peak 1 from the anion-exchange chromatography shown in Fig. 8 was analysed on a TSK TMS 250 C1 (0.21×3 cm) column. The protein was eluted with a linear gradient of 3–100% of acetonitrile in 60 min, with a flow of 0.5 ml/min. Chromatography was monitored at the sensitivity of −0.02–2.0 AUFS. The collected peak from RP-chromatography was further analysed by a 12.5% precasted Phast SDS-PAGE followed by Coomassie Brilliant blue staining (insert). Lane (1): low molecular mass standard (Pharmacia); lane (2): the peak collected from RP-chromatography.

tant fraction of the lysates from infected cells showed COMT enzyme activity confirming that the recombinant product was human S-COMT [27].

6. Conclusions

The purification methods described here have allowed more detailed characterization and provided the tools for molecular cloning of the COMT cDNAs and genes. Analysis of the gene structure and expression have revealed how the synthesis of COMT polypeptides is regulated. Highly purified COMT proteins have been used to raise specific antisera, which in immunohistochemical studies have given new information on the localization of COMT polypeptides in tissues and cells (reviewed in [31]).

Both human and rat recombinant COMT proteins expressed in *E. coli* were enzymatically active, soluble and the specific activities of the proteins were similar to that of the authentic enzymes [37]. Purification of the recombinant rat S-COMT allowed recently its crystallization together with the 1,5-dinitrocatechol inhibitor. The three-dimensional structure could be determined at 2.0 Å resolution [38,108]. The observed kinetic behaviour of the enzyme with different substrates could be explained

on the basis of the atomic structure of recombinant rat S-COMT [39]. The atomic structure and the sequence comparison reveal that all residues that are important for the binding of the substrates and for the catalytic reaction are conserved in human and rat COMT proteins [38,108].

Until recently, the kinetic constants of the COMT enzyme have not been established. The use of tight-binding inhibitors and recombinant S-COMT protein, has allowed the determination of the actual enzyme concentrations and the accurate measurement of the kinetic parameters and the substrate selectivity [39]. This study proposed a new kinetic reaction mechanism for the COMT enzyme. Understanding of the structure and the catalytic mechanism of COMT enzyme will help the development of more potent and clinically interesting COMT-inhibitors.

7. List of abbreviations

COMT	Catechol-O-methyltransferase
S-COMT	Soluble COMT
MB-COMT	Membrane-bound COMT
cDNA	Complementary DNA
RP	reversed-phase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
GST	Glutathione S-transferase
IPTG	Isopropyl-β-D-galactopyranoside
CBB	Coomassie Brilliant blue

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