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Murine Steroid Sulfatase (mSTS): Purification, Characterization and Measurement by ELISA

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The murine steroid sulfatase (mSTS) is a microsomal enzyme, important in steroid metabolism. In the mouse, the gene encoding mSTS is pseudoautosomal and thus escapes X-inactivation. We have purified steroid sulfatase approximately 30-fold from mouse liver microsomes and its properties have been investigated. The major steps in the purification procedure included solubilization with Triton X-100, gel filtration chromatography, DEAE-Sephadex chromatography and HPLC gel filtration chromatography. The purified sulfatase showed a relative molecular weight of 128 kDa on HPLC gel filtration, whereas the enzyme migrated as two bands of 60 and 68 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The isoelectric point of steroid sulfatase was estimated to be 6.2 by column chromatofocusing. Polyclonal antibodies to the purified protein were prepared. An Enzyme Linked Immunosorbent Assay (ELISA) was developed using purified monospecific anti-mSTS antibodies labelled with peroxidase. The standard criteria of precision and reproducibility were satisfied. The assay was applicable to routine determination of mSTS samples in research laboratories. Differences in mSTS liver concentrations were used to identify putative alleles for the mSTS gene (*Sts*). Results in ELISA confirmed the polymorphism previously demonstrated for an enzymatic mSTS activity assay in two inbred mouse strains.

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INTRODUCTION

The microsomal enzyme steroid sulfatase (STS, EC 3.1.6.2) is ubiquitously distributed in mammalian tissues. Moreover, it hydrolyzes several steroid sulfates, especially estrone-sulfate that is a precursor of active estrogens.

In the human species, STS deficiency results in an X-linked ichtyosis syndrome. Human STS has been purified and the gene cloned [1]. It has been mapped to the distal portion of the short arm of the X chromosome that shares no homology with the Y-chromosome. Consequently, there is no functional STS gene on the human Y chromosome [1, 2].

The situation is quite different in mice (Fig. 1). Genetic studies using crosses of a STS-deficient mouse strain with normal animals in various combinations [3] have shown that the STS gene is Y-linked and undergoes obligatory recombination with its X-linked allele [4, 5]. Furthermore, the *Sts* locus escapes X-inactivation [6]. Further support for the pseudoautosomal localization of this gene was given by its linkage to *Crm* [5, 6] that is the most distal marker of the non-pseudoautosomal region of the X-chromosome and to the site of integration of the Moloney virus-15 (*Mov*-15) that is a pseudoautosomal marker [7]. All these studies were performed by estimating the STS enzymatic activity (i.e. the initial velocity of the steroid sulfate hydrolysis) in crude tissue preparation. It has not been confirmed that this activity reflects a function of the gene, since the initial velocity of the substrate hydrolysis depends on several factors: individual tissue preparation, substrate concentration, presence of enzymatic inhibitors and integrity of the membrane structure which regulates the function of the enzyme in its normal state as part of the microsomal membrane [8]. The failure of STS to follow Michaelis–Menten kinetics is probably the reason for the large heterogeneity of the data reported.

The cloned human gene does not cross-hybridize with the non-cloned mouse STS (mSTS) gene [1]. The

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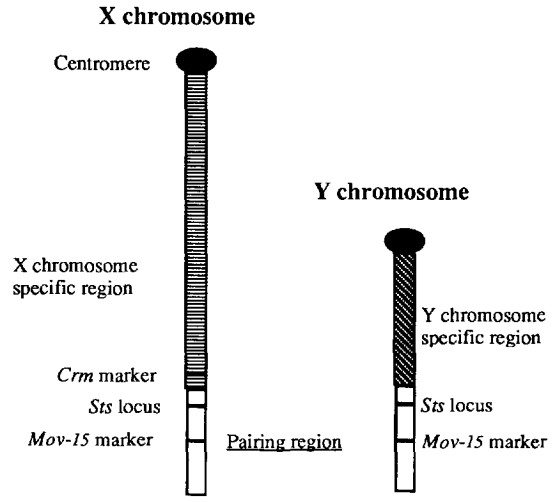

Fig. 1. Localization of the mSTS gene (*Sts*) on the pairing region of X and Y chromosomes and its linkage to the most distal X-chromosome marker *Crm* and to another pseudo-autosomal marker, the *Mov*-15 provirus.

purification of mSTS should thus provide an interesting opportunity into the immunological measurement of the protein itself and pave the way to sequencing the murine gene, that is the only marker of the pseudo-autosomal region in the mouse. The aim of the present study was to purify mSTS from liver microsomes, to produce polyclonal antibodies and to develop an enzyme-linked immunosorbent assay (ELISA) applicable to serial determinations of mSTS concentration in mice tissues. Using this technique, allelic differences for mSTS gene expression in two inbred strains of mice should provide the means to discover functional genes mapping to the pseudoautosomal region of the Y [9].

EXPERIMENTAL

Materials

Reagents for buffers, horseradish peroxidase, *O*-phenylenediamine (OPD), phenylmethanesulphonyl fluoride (PMSF), dithiotreitol and anti-rabbit IgG peroxidase conjugate were obtained from Sigma Chemical Company (St Louis, MO, U.S.A). Glutaraldehyde, glycerol, sodium dodecyl sulfate, hydrogen peroxide solution, Tween 20, isopentane and gelatine were obtained from Merck (Darmstadt 1, Germany). Bovine serum albumin (BSA) was from Pierce (Rochford, II, U.S.A.). Triton X-100 was from Amresco (Solon, OH, U.S.A). Sephacryl S-300, Sephadex S-200, DEAE Sephadex and Polybuffer exchanger columns (PBE 94) were purchased from Pharmacia Ltd (Uppsala, Sweden). The tritiated steroid [6,7-^{3}H]estrone sulfate (sp. act. 47.70 Ci/mmol) was from DuPont NEN products (Boston, MA, U.S.A.) and purified by partition chromatography. Equipment for electro-elution (Biotrap BT 1000) was obtained from Schleicher and Schuell (Dassel, Germany). Equipment for concentration was from Millipore Products Division (Bedford, MA, U.S.A.). All other chemicals used in the experiments were reagent grade and were obtained from commercial suppliers.

Preparation of liver extracts

Adult male mice of the NZB/BINJ/GNC and CBA/H/GNC inbred strains (abbreviated N and H hereafter) were obtained from identified breeders after 127 and 114 brother x sister generations of mating, respectively, and maintained in our laboratory using the same mating system for 8 further generations [10].

Tissue preparation for enzymatic activity: adult male animals were killed and the liver was removed immediately, washed in cold saline, frozen in liquid nitrogen, and stored at −78°C until assayed. For the assay, a liver was diced and homogenized in cold Tris–HCl buffer (5 vol., 50 nmol/l, pH 7.6). The cold homogenate was sonicated (3 × 20 s) and centrifuged (8000 *g*, 15 min) at +4°C to obtain the supernatant containing the crude preparation of mSTS.

Tissue preparation for purification (microsomal fraction): livers were sliced, and homogenized in 1 vol. ice-cold 0.25 M sucrose. After centrifugation at 12,500 *g* for 20 min, the supernatant containing the bulk of the mSTS activity was collected. After centrifugation at 105,000 *g* for 1 h, the pellets containing the microsomes were washed three times in 0.05 M Tris–HCl (pH 7.6), and resuspended in 0.05 M Tris-HCl (pH 7.6) containing 1% Triton X-100. This suspension was sonicated 10 times with a 15 s burst of sonic wave produced by a sonifier cell disruptor Model 1850 (Heal-System-Ultrasonics, Inc.). Insoluble material was removed by centrifugation at 105,000 *g* for 90 min.

Tissue preparation for ELISA: livers were homogenized with 5 vol. ice-cold PBS containing Triton X-100, 0.5% (v/v) and an antiprotease (PMSF, 50 μl/10 ml) in a Potter–Elvehjem homogenizer with 10 strokes. The homogenate was sonicated 10 times in an ice bath with a 30 s burst of sonic wave produced by the same sonifier cell disruptor and then centrifuged at 12,500 *g* for 30 min. The samples used for the calibration curve were prepared from liver extracts from the N strain frozen (−50°C) in isopentane and stored for several months at the same temperature. Comparisons of fresh and stored tissue showed that no alteration occurred.

Steroid sulfatase activity

Sulfatase activity was measured by desulfation of [6,7-^{3}H]estrone-sulfate. Enzymatic activities of liver extracts were assayed according to Prost *et al.* [11, 12] with minor modifications. The substrate used for the assay, tritiated estrone-sulfate (E_1-S) ammonium salt, was purified by partition chromatography (celite, isooctane-ethylacetate gradient) to remove the labelled free estrone. The specific activity of the labelled steroid was adjusted with unlabelled estrone-sulfate to 20 mCi/mmol. Since an excess of substrate inhibits

sulfatase activity [9], each crude preparation of mSTS was assayed in dulpicate at three substrate concentrations (0.5, 2.5, and 5.0 μmol/l). Before each assay, the substrate was preincubated for 30 min at 37°C. The reaction was then begun by the addition of the crude preparation of mSTS (10 μl, 0.2 mg protein) to the preincubated substrate. The enzymatic reaction was performed at 37°C for 20 min in Tris–HCl buffer, pH 7.6, 50 mmol/l (500 μl). The reaction was stopped by addition of Na_2CO_3 (1 ml, 0.1 mol/l). The free labelled estrone was extracted in 4 ml petroleum benzine. Radioactivity, measured by scintillation counting, was converted to free estrone concentration. K_M and V_{max} were calculated from Lineweaver–Burk reciprocal plots. V_{max}, a measure of enzymatic activity, is reported as pmol min^{-1} mg $prot^{-1}$. Sample assays with K_M below 10 μmol/l were excluded from the data analysis.

A simplified assay of enzyme activity was used for further purification. The enzyme suspension (50 μl) was mixed with 2 μmol/l E_1-S (containing approx. 3×10^4 dpm of tritiated E_1-S), dissolved in a 50 mM Tris–HCl buffer (pH 7.6), and incubated at 37°C for 30 min. The reaction was terminated by adding 1 ml 0.1 M Na_2CO_3. The free estrone produced by the reaction was extracted using 4 ml petroleum benzine. The petroleum benzine extract was transferred to a scintillation counting vial, mixed with 10 ml Pico-fluor 30 scintillation solution, and the radioactivity was measured by a Searle analytic scintillation counter. Enzyme activity was reported in CPM.

mSTS purification

The microsomal fraction was fractionated at 4°C by gel filtration on a Sephacryl S-300 column (3.5 × 78 cm) at a flow rate of 17 ml/h with: 50 mM Tris–HCl, pH 7.6; 0.15 M, NaCl; 0.5% Triton X-100 as elution buffer (buffer A). Fractions (3.5 ml) were collected and stored at 4°C prior to enzymatic assay. This step was followed by ion-exchange chromatography. A DEAE-Sephadex column (2.4 × 14 cm) was washed with 50 vol. Buffer A. The active fractions were pooled after gel filtration, loaded onto the column and washed with buffer A. The mSTS was not retained. A new DEAE-Sephadex column (0.5 × 12 cm) (DEAE II) was equilibrated with buffer B (Tris–HCl, 50 mM, pH 7.6; 0.10 M, NaCl; 0.25% Triton X-100). The eluent of the first DEAE was concentrated with an Amicon membrane (Diaflo type, Amicon), dialyzed with buffer B, and loaded onto the column. After washing with buffer B, the biological material was eluted from the column with 0.15 M NaCl in buffer B at 8 ml/h. The eluent was collected in 2 ml fractions. This step was followed by HPLC chromatography. A TSK G3000SWXL column (0.8 × 30 cm) was equilibrated with buffer C (Tris–HCl 50 mM, pH 7.6; 0.15 M, NaCl; 0.12% Triton X-100). The active fractions after DEAE II were pooled, dialyzed with buffer C, and loaded onto the column. The biological material was eluted from the column with buffer C at 0.5 ml/min. Enzyme activity was measured for each fraction (0.25 ml/tube).

Sodium dodecyl sulfate(SDS)–polyacrylamide slab gel electrophoresis was performed on a 10% polyacrylamide gel by the Laemmli method [13]. Nondenaturing electrophoresis was carried out on a 7.5% cylindrical polyacrylamide gel (5 mm i.d.) containing 2% Triton X-100 according to Davis [14]. Protein bands were visualized by staining with the silver stain technique reported by Dubray and Bezard [15].

The relative molecular weight of mSTS was estimated by gel filtration chromatography. The purified mSTS preparation was loaded onto a column of G-200 sephadex (1.4 × 85 cm) together with a small amount of standard proteins and eluted with the same buffer. Enzymatic activity was measured in each fraction (0.3 ml/tube) and molecular weight was calculated with cytochrome C, egg albumin, transferrin, and β-galactosidase as calibration standard proteins.

The isoelectric point of mSTS was estimated by chromatofocusing through a polybuffer exchange column (PBE 94, 1 × 21 cm) equilibrated with buffer I (Imidazole–HCl, 25 mM; 1% Triton X-100, pH 7.6). The mSTS sample was eluted at 20 ml/h with 71 ml buffer II (12.5% polybuffer 74; 1% Triton, pH 4) and enzyme containing fractions were collected after thorough washing with 2 bed vol. of the same buffer. An aliquot of each fraction was drawn to determine mSTS activity.

The glycosylation rate of mSTS was studied by enzymatic digestion by a *N*-glycosidase F (PNGase). The mSTS bands revealed by SDS–polyacrylamide slab gel electrophoresis, were cut and electro-eluted with a Biotrap BT 1,000. The eluent volume (400 μl) was concentrated 4-fold with Ultrafree MC Millipore. Eluent was incubated 24 h at 37°C in Tris–glycine buffer with 1 μl mercaptoethanol and 0.1 unit of PNGase, and loaded on a SDS polyacrylamide slab gel.

Protein content was estimated according to the procedure of Shaffner [16], using bovine serum albumin as standard protein.

Preparation of mSTS polyclonal antibodies

Three-month-old female NZW rabbits were used. Two rabbits were immunized once i.d. with 50 μg pure mSTS emulsified in Freund complete adjuvant followed by immunizations with 25 μg mSTS emulsified in Freund incomplete adjuvant every 10 days. Specific antibodies were detected by dot–blot assay: a sample containing mSTS was blotted onto immobilization membrane for protein (ProBlott, Applied Biosystem). The membrane was soaked in 5% (w/v) of non-fat dry milk in TN buffer (25 mM, Tris–HCl; 0.5 M, NaCl, pH 7.5) for 45 min at room temperature to prevent non-specific binding. The ProBlott was incubated with rabbit antiserum in TN buffer for 1 h at room temperature. The ProBlott was washed extensively in

Table 1. Summary of purification of the microsomal mouse steroid sulfatase

	Total protein (mg)	Total activity (CPM × 10^{-3})	Specific activity (CPM × 10^{-2}/mg prot.)	Purification ratio
Solubilized microsomes	48.95	3441	151.6	1.0
Gel filtration pool	14.80	1073	725.0	4.8
DEAE I	7.20	697	968.7	6.4
DEAE II	1.05	390	3696.7	24.0
Gel filtration HPLC	0.24	109	4558.8	30.0

TNT buffer (0.1% Triton X-100 in TN buffer) for 3 × 10 min and incubated with a secondary antibody (Horseradish peroxidase-conjugated goat anti-rabbit IgG) in TN buffer for 45 min at room temperature. The membrane was washed extensively in TNT buffer and soaked in diaminobenzidine-H_2O_2 solution at room temperature for colour development. The reaction was stopped by washing in distilled water. Immunoglobulins were purified from antisera by affinity chromatography through protein-A sepharose (Pharmacia, Uppsala, Sweden).

ELISA

The enzyme-antibody conjugate was prepared by coupling horseradish peroxidase to purified anti-mSTS immunoglobulins with glutaraldehyde, as described previously [17].

An ELISA sandwich technique was performed using 96-well microtiter plates (Immunoplate Maxisorp, Nunc). The phosphate-buffered saline (PBS) used contained 10 mM sodium phosphate, pH 7.2 and 150 mM NaCl. The microtiter plates were coated with 100 μl anti-mSTS (10 mg/ml) in PBS and incubated 2 h at 37°C. The plates were then blocked by incubation for 30 min at 37°C with 200 μl blocking buffer (PBS containing 1% bovine serum albumin) per well and the plates were washed with washing solution (PBS containing Tween 20, 0.5 ml/l). In the first step, 100 μl/well of liver sample in duplicate, diluted in PBS containing 0.5% gelatin (w/v) and 0.5% Tween 20, were added and incubated for 2 h at room temperature. After washing 5 times with 0.01 M PBS, pH 7.4 containing 0.05% Tween-20 (v/v), 100 μl of peroxidase-labelled anti-mSTS labelled with peroxidase were added and incubated for 2 h at room temperature with continuous shaking. Incubation was stopped by emptying and washing wells with distilled water. The peroxidase enzyme reaction was started by addition of 100 μl substrate solution containing 1,2-phenylenediamine 0.4 g/l in 0.1 M sodium citrate buffer, pH 5.2, at 0.1% (v/v) H_2O_2 30%. The enzymatic reaction was stopped by addition of HCl 3 N (50 μl/well). Absorbance was measured at 490 nm in an automatic dual wavelenth plate reader (Dynatech MR 7,000). A set of blanks ('no antigen', 'no antibody' and 'no antibody or conjugate') were run for each ELISA and the highest blank absorbances subtracted from the absorbances obtained with the liver extracts.

RESULTS

mSTS purification

Solubilization by Triton X-100 and sonication allowed mSTS purification and characterization from mouse liver microsomes. This pretreatment was necessary since mSTS is tightly bound to the membranes, and gave a yield of 80–90% of the enzymatic activity in a soluble form. The present success in solubilizing mSTS from microsomes is the consequence of the four-step purification method described in Table 1.

mSTS must be maintained in detergent buffers at all stages of purification to avoid aggregation and surface absorption. Sonication is essential to make the protein non-sedimental in chromatographic procedures as well as in analytical electrophoresis.

The relative molecular weight of the intact enzyme has been estimated by gel filtration to be 128,000 Da. In denaturing conditions (SDS–PAGE), the pure mSTS resolved into two subunits exhibiting M_r of respectively 60,000 and 68,000 Da (Fig. 2). Because mSTS binds to concanavalin A sepharose and can be eluted by α-methyl glucoside, we concluded that it was a glycoprotein, and we studied the deglycosylation of the two subunits separately as follows: the two bands revealed by SDS–PAGE were cut, electro-eluted and digested by *N*-glycosidase F (PNGase). The product was loaded on a SDS–polyacrylamide slab gel. A single band, corresponding to a M_r of 60 kDa, was observed after migration therefore revealing that mSTS is a homodimer with differential glycosylation.

The isoelectric point of mSTS has been estimated to be slightly acid (I = 6.2) by chromatofocusing, probably because the covalently bound carbohydrate side chains contain few sialic acid residues.

ELISA

The amount of enzyme-conjugate was directly proportional to the amount of mSTS present in each well. Our data indicate that in the case of mSTS the procedure used will yield linear standard curves from which unknown concentrations of mSTS can be

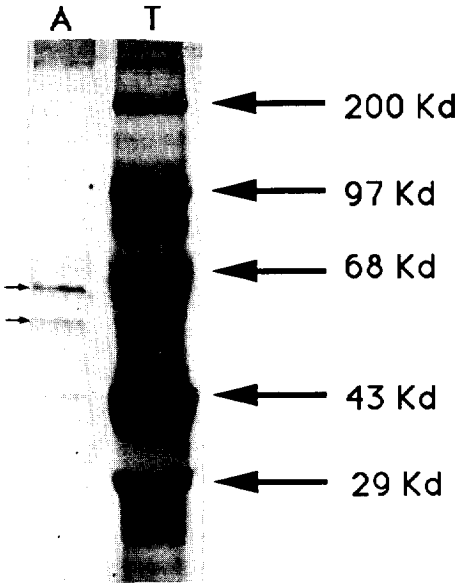

Fig. 2. SDS–PAGE of purified steroid sulfatase (mSTS). Lane T, Marker proteins; lane A, protein pattern of the mouse steroid sulfatase after the purification steps.

determined. Each plate was standardized by running a standard curve in parallel with the samples.

The pure antigen, mSTS, was not required to construct the standard curve as is the case for radioimmunoassay. A stock preparation of liver extracts, specially preserved by freezing in isopentane (see Experimental) and standardized in ELISA against pure mSTS, was used for this purpose in routine determinations. Figure 3 shows a typical calibration curve using diluted samples of this standard. The limit of detection was shown to be 9 pmol of mSTS: this sensitivity is within the range of the enzymatic activity assay [9]. A blank of only 0.1 absorbance units at 490 nm was obtained when samples are replaced by buffer with all the other components remaining the same. This blank value was automatically substracted since a double-beam spectrophotometer was used for the data shown in Fig. 3.

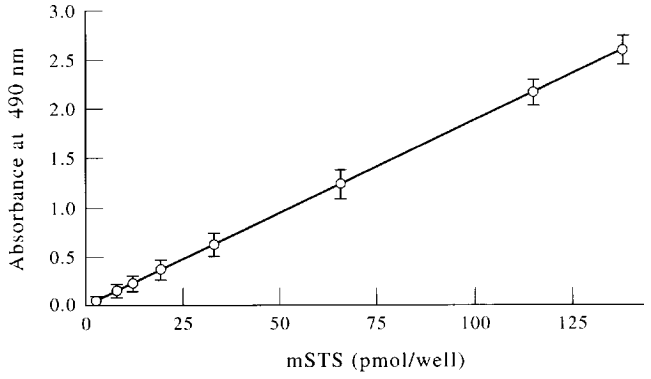

Fig. 3. Calibration curve for the enzyme immunoassay of murine STS. Each point shows mean ± SEM for 10 runs done in duplicate.

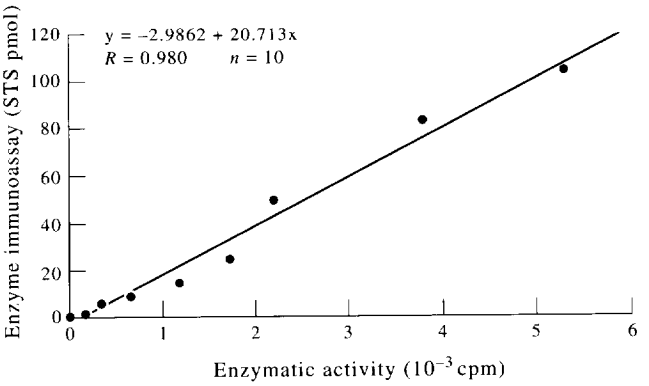

Fig. 4. Comparison of the enzyme immunoassay with the enzymatic activity assay. Each point represents the mean of duplicates.

The data (Fig. 4) indicate complete correlation between the enzymatic activity assay, performed in duplicate at three substrate concentrations, and the immunoassay, indicating that the two assays are interchangeable. Table 2 shows the reproducibility of the assay when performed on the same or different days using different ranges of mSTS concentration. The precision was adequate in the case of intra-assay conditions; however, the inter-assay data indicate that the assay cannot be compared from day to day using the same standard curve. This variation is due to the different adsorbent capacities of the microtiter plastic plates. It is, therefore, necessary to standardize each plate with a standard curve.

This immunoassay measuring the mSTS liver concentrations was subsequently used to compare the two inbred mice strains, N and H, which are known to exhibit polymorphisms for the *Sts* gene [9]. The results were in agreement with the enzymatic assay as shown in Table 3.

DISCUSSION

Previous attempts to purify mSTS have been hampered by the difficulty to solubilize the enzyme in an aqueous system, without aggregation of the protein. Complete purification was achieved with conventional techniques and produced monospecific antibodies by our solubilization protocol.

*Table 2. Coefficient of variation (C.V.) of the enzyme immunoassay**

STS (pmol)	C.V.† "within-run" (%)	C.V.‡ "between-run" (%)
95	5.5	6.5
65	6.6	11
25	8.9	23

*The standard curve of Fig. 3 was used to attain STS values.

†Eleven replicates on the same day.

‡Ten replicates on each of 6 different days.

Table 3. mSTS activity (pmol/min/mg protein) and enzyme immunoassay (pmol/mg protein) in two inbred strains of mice. The difference between N and H is significant: $F_{(2,26)} = 23.48$ *(*$P < 0.001$*)*

Group	Activity ± SEM	ELISA ± SEM
NZB	167.2 ± 29.1	688 ± 11.9
CBA/H	77.8 ± 16.7	115 ± 8.2

mSTS is an homodimer that exhibits a relative molecular weight of 128 kDa. The micro-heterogeneity of the two subunits, visualized by the SDS–gel electrophoretic pattern, may be attributed to differences in carbohydrate content since the two bands are resolved in a unique band when the sugar side chains are removed. The molecular organization of mSTS shows very close similarity to human STS in spite of the absence of immunological cross-reactivity of the two proteins.

The genetic structure of the pseudoautosomal region of the Y chromosome has been extensively described in the human species [18] whereas this region remains unknown in the mouse genome where the *Sts* gene is the only available marker. Previously published papers [19, 20] focused on the activity of the STS enzyme and not on the STS protein itself. We have presented here the first technique to measure this protein. Several papers have reported strain differences for the enzymatic activity. In addition, the comparison of differences for the mSTS protein itself between strains of mice may shed light on the evolution of the pseudoautosomal region. The measurement of the protein itself should help to overcome the difficulties of analyzing the data obtained from crosses between strains selected for low vs high activity of the mSTS enzyme and to understand the behavior of the *Sts* alleles at the male meiosis. The relative simple method developed here to measure the enzyme as a function of the protein concentration can be used to test a large number of individuals resulting from informative meioses that is a prerequisite for linkage detection of candidate genes on the pseudoautosomal region using the *Sts* gene as a marker. Moreover, the purification of the protein should provide the means to sequence the gene and thus to enter the pseudoautosomal region of the mouse.

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