The specific plasminogen activator from *Trimeresurus stejnegeri* venom (TSV-PA) is a serine proteinase presenting 23% sequence identity with the proteinase domain of tissue type plasminogen activator, and 63% with batroxobin, a fibrinogen clotting enzyme from *Bothrops atrox* venom that does not activate plasminogen. TSV-PA contains six disulfide bonds and has been successfully overexpressed in *Escherichia coli* (Zhang, Y., Wisner, A., Xiong, Y. L., and Bon, C. (1995) *J. Biol. Chem.* 270, 10246–10255).

To identify the functional domains of TSV-PA, we focused on three short peptide fragments of TSV-PA showing important sequence differences with batroxobin and other venom serine proteinases. Molecular modeling shows that these sequences are located in surface loop regions, one of which is next to the catalytic site. When these sequences were replaced in TSV-PA by the equivalent batroxobin residues none generated either fibrinogen-clotting or direct fibrinogenolytic activity. Two of the replacements had little effect in general and are not critical to the specificity of TSV-PA for plasminogen. Nevertheless, the third replacement, produced by the conversion of the sequence DDE 96a-98 to NVI, significantly increased the $K_m$ for some tripeptide chromogenic substrates and resulted in undetectable plasminogen activation, indicating the key role that the sequence plays in substrate recognition by the enzyme.

The plasminogen activator from *Trimeresurus stejnegeri* venom (TSV-PA) is a 234-residue single chain glycoprotein with an apparent molecular weight of 33 kDa (1). Like physiological tissue type plasminogen activator (t-PA), TSV-PA specifically cleaves the Arg$^{561}$-Val$^{562}$ plasminogen bond to generate two-chain plasmin, a key enzyme in fibrinolysis (2, 3). Sequence homology with trypsin and other venom serine proteinases (4) indicates that TSV-PA belongs to the family of serine proteinases (5). Trypsin-like serine proteinases, which cleave the peptide bond following arginine or lysine, display very different substrate and inhibitor specificities. They were among the first enzymes to be studied extensively (6). In particular, the role of the “specificity pocket” in determining the “primary”, or $P_1$, specificity of the enzyme has long been recognized (7). The existence and critical importance of other additional structural determinants of proteinase specificity have also been established (8), but both their location and their role remain unknown.

The sequence of TSV-PA exhibits a high degree of identity (60–66%) with other snake venom proteinases, such as *Vipera russelli* venom factor V activator (9), batroxobin from *Bothrops atrox* venom (10), and *Agkistrodon contortrix* venom protein C activator (11), which present considerable differences in their substrate specificities. For example, TSV-PA, which efficiently activates plasminogen, does not clot or degrade fibrinogen and does not activate or degrade factor X, prothrombin, and protein C (1). On the other hand, batroxobin, which shows a thrombin-like activity (12), does not act on plasminogen. TSV-PA shares a weak sequence identity with the serine proteinase domain of human t-PA (23%) and urokinase type plasminogen activator (u-PA) (21%) yet is functionally analogous to them. Moreover, in common with the highly nonspecific trypsin, TSV-PA is a one-chain enzyme, and it possesses six disulfide bonds, of which five are topologically equivalent; the sequence identity with trypsin is 40%.

For the expression and refolding of recombinant TSV-PA (rTSV-PA) in bacteria we used a strategy similar to the one reported by Maeda *et al.* (13) for batroxobin. This led us to undertake structure-function studies on the substrate specificity of TSV-PA by site-directed mutagenesis to examine the role of particular residue(s) or region(s). We thus looked at three peptide fragments (residues SNNFQ 60–64, DDE 96a-98, and SWRQV 173–176) that show quite different amino acid sequences between TSV-PA and batroxobin. These fragments are located in solvent-exposed loops in a sequence homology trypsin-based three-dimensional model of TSV-PA. Consequently, we exchanged the TSV-PA sequence by the equivalent batroxobin residues by site-directed mutagenesis. A TSV-PA variant whose modifications are located close to the catalytic site lost its plasminogen activation properties.

**EXPERIMENTAL PROCEDURES**

*Materials—* Human thrombin, human Lys-plasminogen, and urokinase (two-chain form, $M$, 33,000) were obtained from Sigma. Bovine factor Xa was from Pierce. Human fibrinogen (grade L) from Kabi Vitrum was pretreated with diisopropyl fluorophosphate according to the instructions of the manufacturer. Natural TSV-PA was purified from *T. stejnegeri* venom as described before (1). The concentration of...
plasminogen was determined by measuring the absorption at 280 nm, using an absorption coefficient ε280 of 16.8 (14).

**Methods**

Construction of a TSV-PA Expression Plasmid—The pET expression vector (Novagen) was used for expression of TSV-PA in *Escherichia coli*. To subclone the TSV-PA open reading frame from the cDNA clone D16 into the pET17b vector, a polymerase chain reaction was conducted with a forward primer, En, and a reverse primer, Ecr, in the presence of an adapter primer, E. Primer E contains the following elements: 1) a BamHI site to facilitate subcloning (italicized); 2) the coding sequence for the C-terminal recognition site derived from factor Xa (boldface); and 3) the coding sequence for the 5 C-terminal residues of TSV-PA (underlined). The product, amplified with primers En and Ecr, was first subcloned into a pGEM vector (Promega). After digestion with BamHI and EcoRI, the recovered TSV-PA open reading frame was ligated into the pET17b vector at BamHI and EcoRI sites, to generate the expression plasmid, designated pET(tsvpa). The construct was sequenced on both strands to ensure that the coding sequence of TSV-PA was correct.

**Site-specific Mutagenesis**—The construction of expression plasmids for TSV-PA variants was followed by overlap expansion using polymerase chain reaction techniques, mainly as described by Ho et al. (15). Using pET(ts vacpa)/15 as the template, one fragment was amplified between primer Vn, in the sense direction, containing specific alterations in the nucleotide sequence and an antisense Tn, terminal primer (GCTAGTTATTGCTCAGCGG), located downstream of the TSV-PA coding sequence. Another fragment was amplified between the antisense primer Vnr (reverse complementary to Vn) and a sense primer located in the Tn, promoter sequence (TAATACGACTCACTATAGGG). The resulting fragments were purified by gel electrophoresis on polyacrylamide gels and combined in a subsequent “fusion” reaction in which the overlapping ends annealed. Polymerase chain reaction amplification was then carried out between the Tn, promoter and terminal primers. The resulting 1-kilobase pair fragment was also purified by gel electrophoresis, digested by BamHI and EcoRI, and ligated into the pET17b vector. The entire sequence encoding mutated TSV-PA was then verified by sequencing both DNA strands. The synthetic oligonucleotide used to generate the TSV-PA variants are listed in Table I.

**Expression and Renaturation of Wild Type and TSV-PA Variants**—The *E. coli* strain BL21(DE3) was transformed with plasmid of wild type, pET(ts vacpa), or of TSV-PA variants. Cells were grown at 37 °C up to an optical density of about 0.6 at 600 nm and then induced with 0.4 mM isopropyl-1-thio-β-D-galactoside and cultivated for an additional period of 3 h at 37 °C. After cell lysing, inclusion bodies containing TSV-PA or mutated TSV-PA were recovered by centrifugation, washed three times with 50 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, and then dissolved in 8 M urea in 50 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, and incubated in the buffer for 20 min at room temperature. Refolding was started by diluting the sample 50 times in 50 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, and incubated in the buffer for 20 min at room temperature. Refolding was allowed to proceed at room temperature and was monitored by assaying the amidoactivity of TSV-PA on chromogenic substrate S-2238. When the enzyme activity reached a plateau, after about 30–48 h, the refolding mixture was stored at 4 °C for subsequent purification. N-terminal sequence determinations of the recombinant protein were performed by Edman degradation with a 470A gas phase sequencer.

**Purification of Recombinant Wild Type and Mutated TSV-PA**—The purification of refolded wild type and mutated proteins was performed according to the purification steps used for natural TSV-PA; And 2) the purified mutant TSV-PA was subjected to site titration with 4-methylumbelliferyl p-guanidinobenzoate (16), using a Kontron spectrofluorimeter. Titrations were performed in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl.

**SDS-Polyacrylamide Gel Electrophoresis—** SDS-PAGE was performed on a Phastsystem apparatus (Pharmacia) following the manufacturer's directions.

**Immunochromatographic Analysis of rTSV-PA**—Purified rTSV-PA was tested by enzyme-linked immunosorbent assay using rabbit polyclonal antibodies raised against natural TSV-PA. Competition experiments were performed as described by Choumet et al. (17). Briefly, various concentrations of natural or rTSV-PA were incubated for 12 h at 4 °C with a fixed dilution of anti-TSV-PA antibodies as deduced from preliminary tests. Subsequent steps were performed as described above. The concentration of natural or rTSV-PA that gave half of the maximal response (IC50) was determined.

**Chromogenic Assays and Kinetic Analyses**—The amidolytic activity of the enzymes was measured as described before (1). The kinetic parameters Kcat and kcat were determined by analysis of double-reciprocal plots of the initial velocity as a function of substrate concentration. The concentrations of the chromogenic substrates S-2238 and S-2251 varied from 0.3 mM to 0.01 mM in assays of all enzymes. The enzyme concentrations used were between 8 and 17 nM, except in the case of the TSV-PA variant D96A/N97V/E98I, which was 55 nM. S-2302 and S-2444 substrate concentrations varied from 0.03 mM to 0.4 mM, and the final enzyme concentrations varied from 26 to 55 nM.

**Plasminogen Activation Assays and Kinetic Analysis**—The initial rate of plasminogen activation was measured with Lys-plasminogen as described previously (1). For kinetic analyses, Lys-plasminogen concentrations varied from 10 to 100 nM in the case of natural and recombinant wild type TSV-PA, the final concentration of TSV-PA being 10 nM. In the case of TSV-PA variants, Lys-plasminogen concentrations varied from 50 to 1000 nM, and the final concentration of the enzyme varied from 10 to 20 nM.

**Topological Alignment and Modeling of TSV-PA**—Modeling of the three-dimensional structure of TSV-PA (computations, visualization and analysis) was performed using the BIOPOLYMER and HOMOLGY modules of the BIOSYM Technologies Inc. (San Diego, CA) software package.

The set of structurally conserved regions (SCRs) thus determined was based on the crystal structure of trypsin, using the Bio-Rad protein assay. To determine the molar concentration of the active site enzyme, wild type and mutated rTSV-PA were subjected to site titration with 4-methylumbelliferyl p-guanidinobenzoate (16), using a Kontron spectrofluorimeter. Titrations were performed in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl.

**RESULTS**

**Expression of TSV-PA in E. coli**—After overexpression of TSV-PA in *E. coli*, a major protein of 30 kDa could be detected in whole cell extracts by SDS-PAGE. After denaturation, the...
under reducing conditions (5% b-mercaptoethanol); in lane 4, it was performed under nonreducing conditions. Proteins were stained with Coomassie Brilliant Blue.

renaturation process yielded 20% of active enzyme. Active rTSV-PA was purified from misfolded protein and from contaminant bacterial proteins by chromatography in a Mono-Q anion exchange column (Fig. 1). The protein corresponding to peak 2 was able to hydrolyze chromogenic substrates and possessed plasminogen activation activity. This fraction corresponded indeed to rTSV-PA. The inset in Fig. 1 shows an SDS-PAGE analysis of the different fractions obtained by expression of TSV-PA in E. coli. Purified rTSV-PA reacted with polyclonal antibodies prepared against the natural enzyme. The I50 determined through competition experiments was similar for recombinant and natural TSV-PA (5-10^-7 M; data not shown).

Activity Assays and Kinetic Analyses of rTSV-PA—Titration of the active site in each molecule of natural and rTSV-PA gives a ratio of active site concentration to protein concentration of 0.97 ± 0.02 for both, indicating that rTSV-PA was fully active.

The activity of rTSV-PA on chromogenic substrates was characterized by the K_m and k_cat parameters obtained from Lineweaver-Burk plots for the synthetic substrates S-2238, S-2231, S-2302, and S-2444. Table II shows that the kinetic parameters of rTSV-PA with these substrates, when determined under the same conditions in parallel experiments, were not significantly different from those of natural TSV-PA. We also analyzed the kinetics of plasminogen activation by TSV-PA. TSV-PA activates Lys-plasminogen with a second order rate of 3.8 μM^-1 min^-1 and with small Michaelis and catalytic constants (K_m = 53 μM, k_cat = 0.2 min^-1). We obtained the same result for rTSV-PA (Table II). K_m of TSV-PA on Lys-plasminogen activation is 35 times smaller than that of u-PA (K_m = 1.9 μM; Ref. 23). On the other hand, TSV-PA cleaves plasminogen with a k_cat 100 times and 50 times lower than that of u-PA and t-PA, respectively (24, 25).

Molecular Modeling—The topology-based sequence alignment between TSV-PA and trypsin is shown in Fig. 2. Fig. 3 shows a MOLSCRIPT representation (26) of the trypsin-like three-dimensional model of TSV-PA. As in all members of the family of serine proteinases (21), the topology of TSV-PA consists of two subdomains of antiparallel β-barrel structure (three strands in each subdomain) and a C-terminal helical segment. The spatial organization of the catalytic triad residues His^57, Asp^102, and Ser^195 (7) at the active site cleft between the two subdomains is preserved in TSV-PA. The overall dimensions of TSV-PA are slightly larger than those of trypsin but smaller than those of u-PA. His^217α appears to reduce solvent accessibility to the gorge of the S1 specificity pocket. In the S2 pocket, a valine replaces the imidazole ring of His^99 in u-PA. As a consequence, small hydrophobic residues usually found at the P2 position of known TSV-PA substrates can be accommodated. The C-terminal peptide of TSV-PA, contiguous to the large 234–243 helix, comprises a β-turn for residues 243–245a with Gly^244 in the i + 1 position and an extended conformation for the Asp-Pro-Pro segment, which lies approximately perpendicular to the axis of the helix. This spatial arrangement will ensure the formation of the Cys^91/Cys^245 disulfide bridge characteristic of TSV-PA. All amide bonds are in trans.

Sequence comparison between TSV-PA and batroxobin identifies several regions where the two enzymes present a significant difference in amino acid sequence. Among those, the three segments, A, B, C (Table I), are exposed to the solvent in our three-dimensional model (Fig. 3). The loop ending at Val^99 is smaller than that of u-PA and corresponds to region B of TSV-PA, made up of the charged peptide KKDDE 95–98, while the more hydrophobic SADTLA 95–98 segment lines the S2 pocket of u-PA. Region B is in close spatial proximity to the catalytic triad of the molecule, and the active site is thus slightly more accessible in TSV-PA than in u-PA, but less so than in trypsin. The S3 pocket is essentially composed of Gly^216, and the 95–99 loop does not extend to it as in u-PA. Upon imposing the canonical conformation of positions P5 to P1 of the inhibitors complexed to trypsin, human α-thrombin, and u-PA (Protein Data Bank codes 1MCT, 1AJB, and 1LMW, respectively) to the corresponding sequence of plasminogen, two features become apparent. First, the cyclic side chain of position P3 (Pro^559) points away from region B, and only the main chain-main chain hydrogen bond with Gly^216 remains. Second, positions P6 (Lys^556) and P5 (Lys^557) of plasminogen, located next to the small disulfide loop containing the Arg^561-Val^562 scissile bond (27) lie proximal to Asp^97 of TSV-PA. Finally, the Cys^128–Cys^232 disulfide bond in trypsin is replaced by a stacking interaction between Pro^128 and Phe^232 in TSV-PA, thus helping stabilize the long Pro^128–Val^135 unstructured segment.

Other features of the three-dimensional model of TSV-PA common to the family of serine proteinases include the glycine at position 69, the characteristic CGG 42–44 sequence, and an internal salt bridge between the amino terminus and the side chain of the catalytic site residue Asp^194. Interestingly enough, in contrast to all trypsin-like serine proteinases that possess a glycine 193 at the S1 subsite, TSV-PA has a phenylalanine.

Mutagenesis of TSV-PA—To examine whether the three regions mentioned above might be implicated in the differential substrate specificities of TSV-PA, the variants constructed by replacing the corresponding amino acid residues by those of batroxobin (Table I), were expressed and purified as for the wild type enzyme. Under the same conditions, the renaturation yield of the variants varied from 7 to 20% and their elution from the Mono-Q column was slightly shifted according to their electric charge (results not shown).

For each TSV-PA variant the active site to protein concentration ratio per molecule, as determined by the Bio-Rad protein assay, was close to 1, as was the case for wild type rTSV-PA. Immunochemical analysis of TSV-PA variants by enzyme-
linked immunosorbent assay showed that the IC_{50} values measured with each variant were similar to that of natural and rTSV-PA, suggesting that the mutations performed did not affect the epitope recognized by anti-TSV-PA antibodies and that the structural changes are due only to segment replacement and not to folding differences.

**Activity of TSV-PA Variants on Chromogenic Substrates—**All TSV-PA variants hydrolyzed S-2238, S-2444, and S-2251 but with significant quantitative differences (Table III). Detailed kinetic analyses revealed that replacement of residues 60–64, SNNFQ by RRFMR (mutant A), did not significantly modify the \( K_m \) and \( k_{cat} \) values of TSV-PA for these three substrates.

Replacement of the distinctive electrically charged TSV-PA DDE 96a-98 region by the neutral sequence of batroxobin (NVI), mutant B, significantly increased the \( K_m \) value for S-2238 and S-2251 and abolished the activity on S-2444. We thus prepared the single point mutants B1 (D96aN), B2 (D97V), and B3 (E98I). As shown in Table III, the \( K_m \) values increased in some cases and decreased in others. The changes in \( k_{cat} \) displayed the same behavior too. For example, the \( k_{cat} \) value of D97V for S-2238 was about 8-fold lower than that of the wild type enzyme, whereas it was more than 3-fold higher for S-2238.

In mutant C, replacement of the 173–176 peptide (SWRQV to NGLPA) modified slightly the \( k_{cat} \) value of the enzyme for these substrates, increasing it for S-2238 and S-2251 and decreasing it for S-2444 by about a factor of 2. The double mutant displayed the combined effects of individual replacements for S-2238, with slight increases in the \( K_m \) values of all three substrates, and 2–3-fold increases in the \( k_{cat} \) values of S-2238 and S-2251.

**Activity of TSV-PA Variants on Plasminogen—**Replacement of the 60–64 and 173–176 peptide (mutants A and C, and double mutant AC) did not abolish plasminogen activation by TSV-PA (Table III). However, the \( K_m \) for plasminogen was increased 4-fold over that of the wild type TSV-PA for mutant C and 22-fold for mutant AC, resulting in a significant change of the enzymatic activity of plasminogen. Interestingly enough, mutant B showed no detectable plasminogen activation activity.
ity, even when the sensitivity of the assay was increased 1000 times. Mutants B1, B2, and B3 retained some plasminogen activation activity but with significantly decreased affinity and rate constants. The most important effect was observed for B2, for which $K_m$ increased by a factor of 25. For mutants B1, B2, and B3, the observed plasminogen activation changes widely (from 10- to 60-fold) with respect to the tripeptides (Table III).

Like TSV-PA, the variants did not show any direct fibrinolytic activity and did not clot a solution of purified fibrinogen, indicating the absence of thrombin-like activity (data not shown).

**DISCUSSION**

Studies of structure-function relationships and of potential thrombolytic activities *in vivo* require in general large amounts of protein difficult to obtain by purifying TSV-PA from its natural source; *T. stejnegeri* snakes are rather rare, and their venom is quite expensive. To overcome this obstacle, we prepared rTSV-PA by production in the efficient *E. coli* expression system. With 12 cysteines and six disulfide bridges, the successful expression of rTSV-PA relied on the isolation of the protein from inclusion bodies, followed by a denaturation-renaturation process in appropriate redox conditions to allow for the correct formation of disulfide bridges. The kinetic parameters of rTSV-PA with synthetic substrates and plasminogen were like those of natural TSV-PA and were not influenced by the absence of glycosylation. Anti-TSV-PA antibodies recognized the natural and the recombinant TSV-PA with comparable sensitivity.

Trypsin and the serine proteinases of snake venoms share a common structure and are believed to have evolved from a common ancestor (5). While the central region surrounding the catalytic triad is highly conserved structurally (28), the surfaces of snake venom serine proteinases may show considerable shape differences given the wide variations in loop sequence and length. The arrangement of disulfide bonds is identical to that of trypsin, except that the Cys128–Cys232 bond of trypsin is replaced by one involving the C-terminal cysteine (4), i.e. the Cys91–Cys245e bond in TSV-PA. The functional significance of this modification, as well as that of the 6–7-residue insertion at the C terminus, is unknown.

As opposed to trypsin, which displays a broad substrate specificity, venom serine proteinases, whose primary structure is highly conserved (60–75% identity among members of the

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**TABLE I**

Structure-function analysis of TSV-PA: site-directed mutagenesis

The TSV-PA variants were constructed by polymerase chain reaction techniques as described under “Experimental Procedures.” Only the sense primers are listed here; the VnR primers were exactly reverse complementary.

| TSV-PA variants | Oligonucleotides |
|-----------------|-----------------|
| A S60R/N61R/N62F/F63M/Q64R | CACTGCGACAGGAGATTTATGCGGTTGCTGTTT |
| C S173N/W174G/R174aL/Q175P/V176A | AACAGCTTATAATGGGCTGCCGGCGGCAAACACA |
| AC S60R/N61R/N62F/F63M/Q64R, S173N/W174G/R174aL/Q175P/V176A | CACTGCGACAGGAGATTTATGCGGTTGCTGTTT |
| B D96aN/097V/E98I | AACACCTTTAATGGGCTGCCGGCGGCAAACACA |
| B1 D96aN | CACTGCGACAGGAGATTTATGCGGTTGCTGTTT |
| B2 D97V | AACACCTTTAATGGGCTGCCGGCGGCAAACACA |
| B3 E98I | AACACCTTTAATGGGCTGCCGGCGGCAAACACA |

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**TABLE II**

Enzymatic activity: comparison of natural TSV-PA and recombinant TSV-PA

The methods used for kinetic analyses are described under “Experimental Procedures.” The data represent the mean of three determinations, which differed by 5–15%.

| Substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------|-------|----------|---------------|-------|----------|---------------|
| H-o-Phe-Pip-Arg-p-NA* (S-2238) | 16 | 746 | 46.6 | 18 | 833 | 46.3 |
| H-o-Val-Leu-Lys-p-NA (S-2251) | 370 | 339 | 0.92 | 405 | 340 | 0.85 |
| H-o-Pro-Phe-Arg-p-NA (S-2302) | 170 | 103 | 0.61 | 330 | 100 | 0.30 |
| H-o-Glu-Gly-Arg-p-NA (S-2444) | 2500 | 568 | 0.23 | 2000 | 500 | 0.25 |
| Lys-plasminogen | 0.053 | 0.2 | 3.8 | 0.050 | 0.2 | 4.0 |

* p-NA, para-nitroaniline.
Snake Venom Plasminogen Activator

Kinetic analysis of TSV-PA variants on chromogenic substrates and plasminogen activators

| TSV-PA variant | S-2238 (H-D-Phe-Pip-Arg-p-NA) | S-2251 (H-D-Val-Leu-Lys-p-NA) | S-2444 (H-D-Glu-Gly-Arg-p-NA) | Lys-plasminogen |
|---------------|-------------------|-------------------|-------------------|----------------|
|               | $K_a$  | $k_{cat}$ | $k_{cat}/K_a$ | $K_a$  | $k_{cat}$ | $k_{cat}/K_a$ | $K_a$  | $k_{cat}$ | $k_{cat}/K_a$ | $K_a$  | $k_{cat}$ | $k_{cat}/K_a$ |
| Recombinant   | 18     | 833     | 46           | 405   | 340     | 0.85         | 2000  | 500      | 0.25         | 0.050 | 0.2      | 4.0          |
| A             | 20     | 735     | 37           | 560   | 440     | 0.79         | 1900  | 500      | 0.24         | 0.065 | 0.09     | 1.4          |
| C             | 91     | 1520    | 17           | 300   | 625     | 1.7          | 2500  | 195      | 0.078        | 0.20  | 0.18      | 0.9          |
| AC            | 63     | 1294    | 21           | 1800  | 992     | 1.8          | 2900  | 157      | 0.12         | 1.11  | 0.13      | 0.12         |
| B             | 105    | 744     | 37           | 1900  | 357     | 0.070        | ND    | ND       | ND           | ND    | ND        | ND           |
| B1            | 67     | 1492    | 22           | 2000  | 750     | 0.75         | 690   | 253      | 0.37         | 0.62  | 0.08      | 0.13         |
| B2            | 667    | 2850    | 4.3          | 3000  | 720     | 0.58         | 1400  | 65       | 0.046        | 1.25  | 0.04      | 0.032        |
| B3            | 17     | 868     | 51           | 900   | 290     | 0.37         | 1000  | 123      | 0.12         | 0.77  | 0.06      | 0.078        |

$^a$ p-NA, para-nitroaniline.

$^b$ ND, no detectable enzymatic activity.

TSV-PA shows a high specificity for plasminogen and promises to be an interesting biochemical and pharmacological tool for investigating fibrinolysis in vitro and in vivo. Altogether, our results suggest that, in analogy to t-PA (8), the interaction of TSV-PA with macromolecular substrates such as plasminogen is of a complex nature and depends on secondary site binding. Finally, plasminogen activators seem to show no apparent correlation between their activity toward small substrates on one hand and plasminogen on the other. Comparative structural studies of t-PA, u-PA, and TSV-PA may help to further understand the nature of this discrimination for plasminogen.

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