

The Profibrinolytic Enzyme Subtilisin NAT Purified from *Bacillus subtilis* Cleaves and Inactivates Plasminogen Activator Inhibitor Type 1*

Received for publication, February 26, 2001, and in revised form, April 26, 2001
Published, JBC Papers in Press, April 26, 2001, DOI 10.1074/jbc.M101751200

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In this report, we demonstrate an interaction between subtilisin NAT (formerly designated BSP, or nattokinase), a profibrinolytic serine proteinase from *Bacillus subtilis*, and plasminogen activator inhibitor 1 (PAI-1). Subtilisin NAT was purified to homogeneity (molecular mass, 27.7 kDa) from a saline extract of *B. subtilis* (*natto*). Subtilisin NAT appeared to cleave active recombinant prokaryotic PAI-1 (rpPAI-1) into low molecular weight fragments. Matrix-assisted laser desorption/ionization in combination with time-of-flight mass spectroscopy and peptide sequence analysis revealed that rpPAI-1 was cleaved at its reactive site (P1-P1': Arg³⁴⁶-Met³⁴⁷). rpPAI-1 lost its specific activity after subtilisin NAT treatment in a dose-dependent manner (0.02–1.0 nM; half-maximal effect at ~0.1 nM). Subtilisin NAT dose dependently (0.06–1 nM) enhanced tissue-type plasminogen activator-induced fibrin clot lysis both in the absence of rpPAI-1 (48 ± 1.4% at 1 nM) and especially in the presence of rpPAI-1 (78 ± 2.0% at 1 nM). The enhancement observed in the absence of PAI-1 seems to be induced through direct fibrin dissolution by subtilisin NAT. The stronger enhancement by subtilisin NAT of rpPAI-1-enriched fibrin clot lysis seems to involve the cleavage and inactivation of active rpPAI-1. This mechanism is suggested to be important for subtilisin NAT to potentiate fibrinolysis.

Subtilisin NAT (1) (formerly designated BSP, or nattokinase), a serine proteinase from *Bacillus subtilis*, has been reported to have potent fibrinolytic activity (1, 2). The enzyme is composed of 275 amino acids with a molecular mass of 27.7 kDa in its mature form (1). DNA sequence analysis showed that subtilisin NAT was 99.5 and 99.3% homologous to subtilisins E and Amylosacchariticus, respectively (3). It is also homologous to other members of the subtilisin family (BPN' 86% and Carlsberg 72%), and sequences are conserved especially around the three amino acids (serine 221, histidine 64, and aspartic acid 32) essential for the catalytic center of serine proteinases.

The mechanism for this enzyme to potentiate fibrinolysis is

* This work was supported in part by Grant-in-aid for Scientific Research 10670040 from the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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not fully understood. Subtilisin NAT is reported not to possess plasminogen activator activity but appears to directly digest fibrin by limited proteolysis (4). However, this direct cleavage of fibrin does not seem to account for all of the enhancement of the fibrinolytic activity that has been observed without affecting the fibrinolytic cascade. To explore other possible mechanisms, we have looked for interactions between subtilisin NAT and the physiological inhibitors of fibrinolysis, plasminogen activator inhibitor type 1 (PAI-1)¹ (5) and α_2 -antiplasmin (α_2 -AP) (6). These inhibitors are both members of the serine protease inhibitor superfamily (SERPINs). The SERPINs are proteolytically cleaved and inactivated by a variety of proteases including members of the subtilisin family (7).

PAI-1 is the primary inhibitor of tissue-type plasminogen activator (tPA) and regulates fibrinolytic activity in the vasculature at the initial step of the fibrinolytic cascade (5). Evidence for the significance of PAI-1 in the regulation of fibrinolysis has been documented by both epidemiologic studies and experimental animal models including transgenic (8) and gene knockout animals (9). High PAI-1 activity is directly related to impaired fibrinolysis (10), and low activity is associated with bleeding disorders (11, 12). The fibrinolytic activity determined by the balance between tPA and PAI-1 can be altered by changing the gene expression of either molecule under a variety of physiological or pathological conditions (13). Their balance could also be altered as a consequence of the interaction of PAI-1 with serine proteases other than plasminogen activators in plasma (14). Like other SERPINs, PAI-1 inhibits plasminogen activator activity by forming a stoichiometric complex through its reactive site in the C-terminal region, the so-called strained loop (15, 16). The strained loop of SERPINs is exposed to the outside of the molecule and is very susceptible to proteolytic digestion as shown for both PAI-1 (17) and α_1 -antitrypsin (18) using neutrophil elastase and for other members using snake venom and bacterial metalloproteases (19).

In the present study we examine the possible interaction between subtilisin NAT and PAI-1 or α_2 -AP to clarify the mechanism for subtilisin NAT to enhance fibrinolytic activity. We found that subtilisin NAT cleaved PAI-1 at the P1-P1' peptide bond by limited proteolysis, resulting in the effective enhancement of tPA-induced clot lysis of PAI-1-enriched fibrin.

¹ The abbreviations used are: PAI-1, plasminogen activator inhibitor 1; α_2 -AP, α_2 -antiplasmin; rpPAI-1, recombinant prokaryotic PAI-1; SERPIN, serine protease inhibitor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; PAGE, polyacrylamide gel electrophoresis; MS, mass spectroscopy; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight.

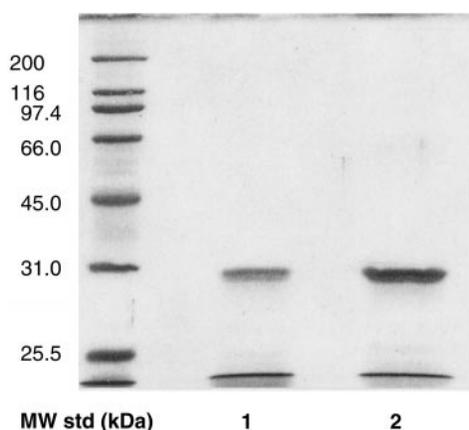


FIG. 1. Polyacrylamide gel electrophoresis of purified subtilisin NAT. Purified subtilisin NAT was subjected to 10% SDS-PAGE under either nonreduced (lane 1) or reduced (lane 2) conditions.

EXPERIMENTAL PROCEDURES

Purification of Subtilisin NAT—Subtilisin NAT was purified from a saline extract of *B. subtilis* (*natto*) as previously reported (1) by techniques of ammonium precipitation and column chromatography using butyl-Toyopearl (Tosoh Co., Tokyo, Japan), CM-Toyopearl (Tosoh Co.), and Sephadex G-50 (Amersham Pharmacia Biotech). After the purification to homogeneity, the protein concentration was determined by the BCA protein assay reagent kit from Pierce.

Purification of Active Recombinant Prokaryotic PAI-1 (rpPAI-1)—The cultivation of bacteria expressing PAI-1 and the purification of active non-glycosylated rpPAI-1 have been previously described (20, 21). Purified active rpPAI-1 was stored at -80°C before use. The concentration of active rpPAI-1 was determined by titration with two-chain uPA (20) of which the active site concentration was previously determined by titration with 4-methyl-umbelliferyl-*p*-guanidinobenzoate (22).

Other Proteins and Materials—Glu-plasminogen was prepared from freshly frozen plasma by lysine-Sepharose affinity chromatography (23). Human fibrinogen was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN), and trace amounts of contaminated plasminogen and plasmin were removed by passage through lysine-Sepharose. After treatment with 1 mM (final concentration) phenylmethylsulfonyl fluoride (purchased from Sigma), the material was dialyzed exhaustively against 50 mM Hepes (analytical grade and purchased from Sigma) buffer containing 100 mM NaCl. α_2 -AP was purchased from Biopool (Umeå, Sweden). Human thrombin was purchased from Welfide Corp. (Osaka, Japan). Single-chain tPA was kindly provided by Dai-ichi Pharmaceutical Co. (Tokyo, Japan). The chromogenic substrate S-2444 (L-pyrroglutamyl-glycyl-L-arginine-*p*-nitroanilide) and human plasmin were purchased from Chromogenix AB (Mölnådal, Sweden).

Analysis of the Proteolytic Action of Subtilisin NAT on Active and Latent rpPAI-1—rpPAI-1 (1.7 μM) was incubated with subtilisin NAT (0.5 nM) for various intervals (0, 10, 30, and 60 min) at 37°C , and the reaction was stopped by the addition of the sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE). The samples were then subjected to 10% SDS-PAGE. The latent form of rpPAI-1 was obtained by the incubation of active rpPAI-1 at 37°C for 48 h (24), and its specific activity was confirmed to be undetectable after the treatment. The latent form of rpPAI-1 was also treated by subtilisin NAT as mentioned above, and the proteolytic action of subtilisin NAT was analyzed by SDS-PAGE.

Measurement of PAI-1 Activity after Treatment with Subtilisin NAT—The specific activity of rpPAI-1 toward plasminogen activators after interaction with subtilisin NAT was estimated by a two-step assay (25). rpPAI-1 (10 nM) was incubated with increasing concentrations of subtilisin NAT for 1 h at 37°C in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 0.1% bovine serum albumin. After the addition of two-chain urokinase (final concentration, 100 IU/ml, ~ 10 nM), the mixture was incubated for another 30 min at 37°C . S-2444 (final concentration, 1.0 mM) was then added, and its hydrolysis was continuously monitored by absorbance at 405 nm. The PAI-1 activity was inversely proportional to the amount of uPA preserved in the incubation mixture. The amount of S-2444 hydrolyzed by subtilisin NAT was estimated using the above assay conducted in the absence of uPA, and this value was subtracted although it was almost negligible.

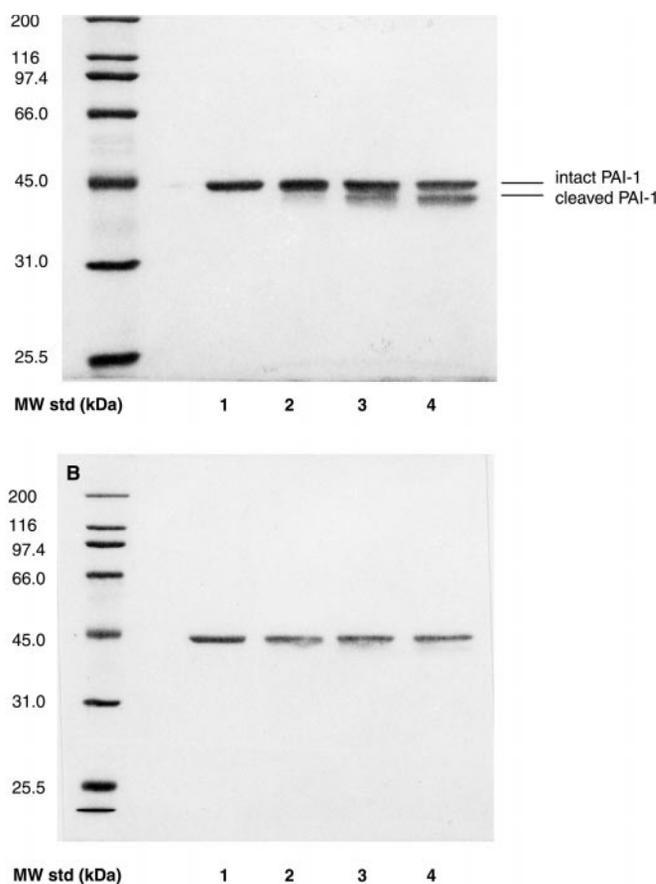


FIG. 2. Cleavage of rpPAI-1 by subtilisin NAT. rpPAI-1 either in the active form (A) or in the latent form (B) was incubated with catalytic amounts of subtilisin NAT for different time periods (lanes 1–4 indicate 0, 10, 30, and 60 min, respectively) and was subjected to 10% SDS-PAGE without reduction.

The rate of the hydrolysis of S-2444 in the presence of rpPAI-1 and absence of subtilisin NAT was considered to represent the rate at full activity of rpPAI-1 (0% inhibition). The rate of the hydrolysis in the absence of rpPAI-1 was considered to represent fully inactivated rpPAI-1 (100% inhibition).

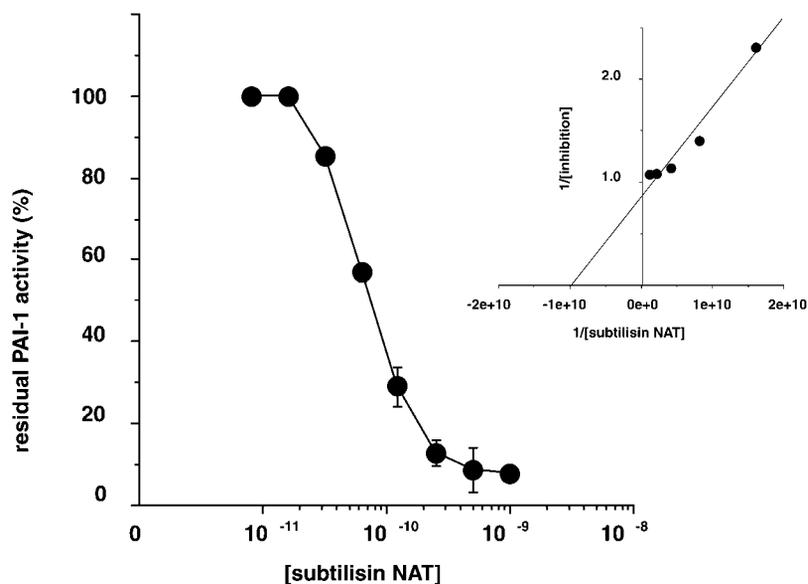
SDS-PAGE—SDS-PAGE was performed according to Laemmli (26), and protein bands were stained by Coomassie Brilliant Blue. A broad range molecular weight standard was purchased from Bio-Rad.

Measurement of Clot Lysis—96-well microtiter plates were used for the clot lysis assay. 2 μM fibrinogen, 1 μM Glu-plasminogen, 0.2 nM tPA, 4 nM rpPAI-1, and various concentrations of subtilisin NAT (0, 0.016, 0.032, 0.063, 0.13, 0.25, 0.5, and 1 nM) were added to individual wells, and the clot formation was initiated by the addition of 0.2 unit/ml human thrombin. Absorbance at 405 nm in each well was measured every 15 min until the time of complete clot lysis employing an automatic microtiter plate reader (Plate Analyzer ETY-300, TOYO, Tokyo, Japan). To analyze the possible effect of subtilisin NAT on direct fibrin dissolution, the clot lysis time was determined as mentioned above in the absence of tPA. Another set of similar experiments without rpPAI-1 was also conducted employing a much lower ($1/100$) concentration of tPA (2 μM) to analyze the effect of subtilisin NAT on fibrin clot lysis in the absence of PAI-1. All of the assays were conducted at 37°C .

Analysis of the Proteolytic Action of Subtilisin NAT on Other Proteins Involved in Fibrinolysis— α_2 -AP (2.0 μM) was incubated with subtilisin NAT (0.5 nM) for various intervals (0, 10, 30, and 60 min) at 37°C , and the reaction was stopped by the addition of the sample buffer for SDS-PAGE. The samples were then subjected to 10% SDS-PAGE. Fibrinogen (2.0 μM) was also treated with subtilisin NAT (0.5 nM) in a similar way, and its cleavage was analyzed by 10% SDS-PAGE after reduction.

Determination of Molecular Mass—The molecular mass of the intact and the cleaved forms of rpPAI-1 was determined using matrix-assisted laser desorption/ionization (MALDI) in combination with a time-of-flight (TOF) mass analyzer (AXIMA-CFR, Shimadzu Solutions for Science/Kratos Analytical, Kyoto, Japan) in the Life Science Department,

FIG. 3. **Dose-dependent inactivation of rpPAI-1 by subtilisin NAT.** Active rpPAI-1 (10 nM) was incubated for 1 h with increasing concentrations of subtilisin NAT (*abscissa*), and its residual activity toward uPA (*ordinate*) was determined. *Inset*, a double-reciprocal plot of subtilisin NAT concentration and its inhibitory effect.



Kratos PC Axima CFR V2.0.0: Mode linear-new, Power: 54, P.Ext. @ 40000 (bin 234)
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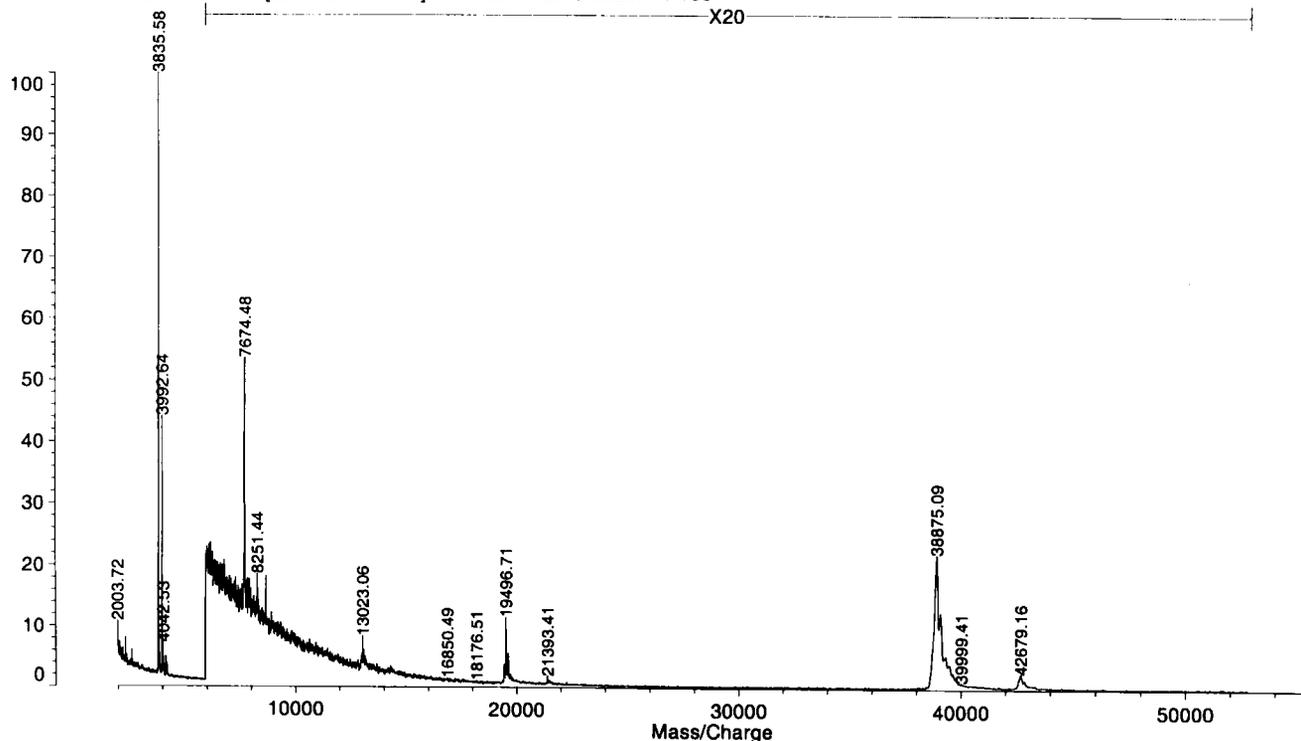


FIG. 4. **MALDI-TOF/MS spectra of subtilisin NAT digest of active rpPAI-1.** Active rpPAI-1 was incubated with catalytic amounts of subtilisin NAT for 24 h at 37 °C (see Fig. 5) and was subjected to MALDI-TOF/MS spectrum analysis.

Analytical Instruments Division, of Shimadzu Co. The instrument was externally calibrated using apomyoglobin (horse) and its dimer form. Sinapinic acid in 0.1% trifluoroacetic acid and acetonitrile (70:30) was used as matrix. A 0.7- μ l protein sample (\sim 1.2 pmol of rpPAI-1) and 0.7 μ l of matrix solution were mixed on the sample plate and were subjected to analysis.

N-terminal Sequence Analysis—Automatic amino acid sequence analyses were performed by using an Applied Biosystems protein sequencer (model 476A, Applied Biosystems, Foster City, CA) according to the established principles. For N-terminal sequence analysis, rpPAI-1 (1.8 μ M) was incubated with 0.5 nM subtilisin NAT in a final volume of 100 μ l for 24 h at 37 °C. The reaction was stopped, and the sample (\sim 180 pmol of rpPAI-1) was lyophilized and loaded onto a polyvinylidene difluoride membrane (ProSpin, Applied Biosystems) after reconstitution and desalting. Data were analyzed by the Applied Biosystems model 610A data analysis program.

RESULTS

Cleavage of rpPAI-1 by Subtilisin NAT—Subtilisin NAT was purified to homogeneity with a molecular mass of \sim 28 kDa (Fig. 1). Peptide sequence analysis after trypsin digestion revealed that the protein was subtilisin NAT. The specific activity for hydrolysis of S2251 (0.5 mM) was 20.7 mmol/min/mg. In a fibrin plate assay, which was calibrated using human plasmin, the specific activity was 55.8 casein units/mg.

After incubation with a catalytic amount of subtilisin NAT (\sim 0.5 nM), active rpPAI-1 (1.7 μ M) was cleaved into smaller fragments in a time-dependent manner (Fig. 2A). The molecular mass of the main fragment was \sim 39 kDa, which is similar to the size of the cleaved form of PAI-1 generated by plasmin-

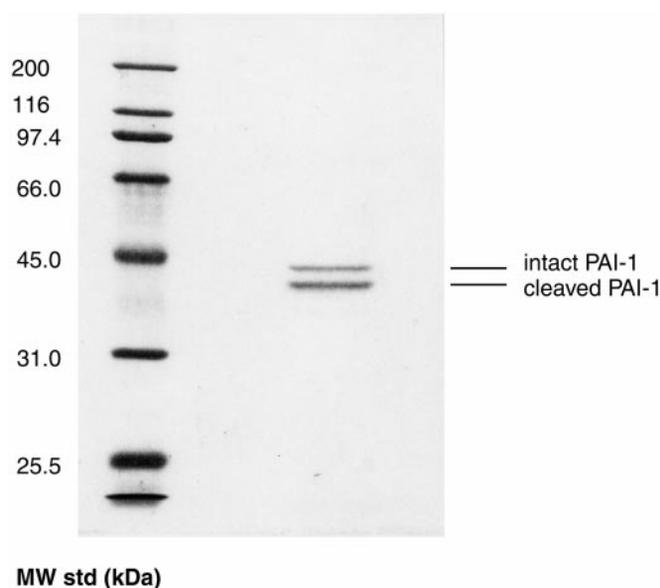


FIG. 5. SDS-PAGE of subtilisin NAT digest of active rpPAI-1 used for peptide sequence analysis and MALDI-TOF/MS spectrum analysis.

ogen activators through cleavage of its reactive site (21, 27). Similar cleavage was observed even in the presence of vitronectin, a protein cofactor known to maintain PAI-1 activity (data not shown).

We also analyzed the interaction between the latent form of rpPAI-1 and subtilisin NAT to see a possible difference in its susceptibility to subtilisin NAT because the latent form of PAI-1 adopts a unique conformation in which its reactive site is buried inside the molecule (28). The latent form of rpPAI-1 appeared to be more resistant to subtilisin NAT digestion with only a small fraction being cleaved into the expected fragments (Fig. 2B). Thus, complete insertion of the reactive site loop into the molecule seems to make it resistant to cleavage by subtilisin NAT.

Inactivation of rpPAI-1 Activity by Subtilisin NAT—Active rpPAI-1 was incubated with different concentrations (0.02–1.0 nM) of subtilisin NAT for 1 h, and the residual PAI-1 activity was estimated by its specific activity toward uPA. rpPAI-1 lost its specific activity after incubation with subtilisin NAT in a dose-dependent manner (Fig. 3). Half-maximal inhibition was obtained at ~0.1 nM subtilisin NAT by a double-reciprocal plot of subtilisin NAT concentration and its inhibitory effect. This inactivation of PAI-1 activity by subtilisin NAT suggests that rpPAI-1 was proteolytically cleaved at a site close to its reactive center.

Analysis of the Subtilisin NAT Cleavage Site in rpPAI-1—The subtilisin NAT cleavage site in rpPAI-1 was analyzed by automated Edman degradation and MALDI-TOF/MS (Fig. 4) using an unfractionated subtilisin NAT digest of rpPAI-1 (Fig. 5). Automated sequence analysis of the subtilisin NAT digest of rpPAI-1 gave two residues of an approximately 1:0.5 ratio for the first seven steps. One sequence was consistent with the N-terminal sequence of PAI-1 (N-Val-His-His-Pro-Pro-Ser-Tyr; average, 58.8 pmol), and the other was a new sequence consisting of N-Met-Ala-Pro-Glu-Glu-Ile-Ile (average, 26.8 pmol). The latter sequence originates from the C-terminal portion of the intact PAI-1 molecule and corresponds to amino acid residues 347–353. The subtilisin NAT cleavage site in PAI-1 appears to be between the residues Arg³⁴⁶-Met³⁴⁷, which forms its reactive site (P1-P1').

MALDI-TOF/MS analysis gave two high molecular mass peaks of 42679.2 and 38875.1 Da and a low molecular mass

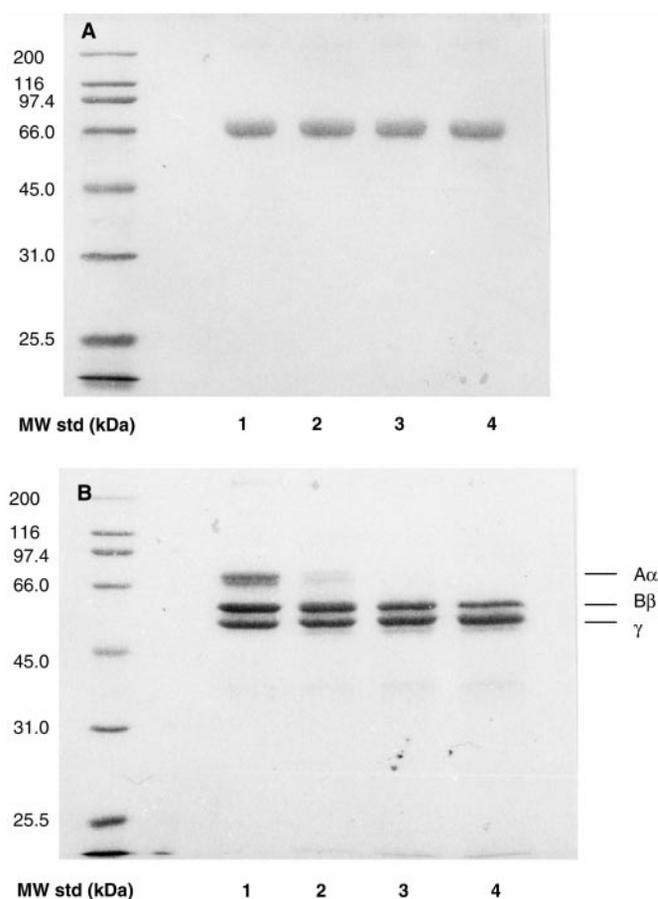


FIG. 6. Proteolytic action of subtilisin NAT on α_2 -AP and fibrinogen. α_2 -AP and fibrinogen were treated with catalytic amounts of subtilisin NAT for different time periods (lanes 1–4 indicate 0, 10, 30, and 60 min, respectively), and the reaction mixtures were then subjected to 10% SDS-PAGE either without reduction (A, α_2 -AP) or with reduction (B, fibrinogen).

peak of 3835.6 Da (Fig. 4). These peaks correspond to intact rpPAI-1 (calculated mass, 42769.3 Da, –90.1 Da), the N-terminal fragment of cleaved rpPAI-1 (1–346) (calculated mass, 38983.8 Da, –108.7 Da), and the C-terminal fragment of cleaved rpPAI-1 (347–379) (calculated mass, 3803.6 Da, +32.0 Da), respectively. This was consistent with the results obtained by peptide sequence analysis of the rpPAI-1 digest and confirmed that the cleavage site was the P1-P1' site. The difference between the measured molecular mass and the theoretical mass (10 to ~100 Da) was within the accuracy range for this size of protein (>30,000 Da), although a mass accuracy of $\pm 0.05\%$ is normally obtained for peptides with this instrument. Minor peaks of 21393.41 and 19496.71 Da correspond to doubly charged species of 42679.2 and 38875.1 Da, respectively. Another minor peak of 7674.48 Da corresponds to a dimer of the 3835.6-Da peptide and is probably formed as an artifact of the MS analysis.

Proteolytic Action of Subtilisin NAT on Other Proteins Involved in Fibrinolysis— α_2 -AP, another important inhibitor of fibrinolysis and member of the SERPIN family, did not appear to interact with subtilisin NAT (no complex formation or cleavage products) (Fig. 6A). The A α chain of fibrinogen was cleaved, although the B β and γ chains were resistant to subtilisin NAT under the conditions employed (Fig. 6B).

Effect of Subtilisin NAT on tPA-induced Fibrinolysis—The fibrin clot lysis time induced by 0.2 nM tPA was 0.40 ± 0.02 h and was prolonged by the addition of 4 nM active rpPAI-1 to 29.1 ± 2.4 h. Subtilisin NAT dose dependently shortened clot lysis time, reaching 7.6 ± 0.6 h at 1 nM subtilisin NAT (Fig. 7A).

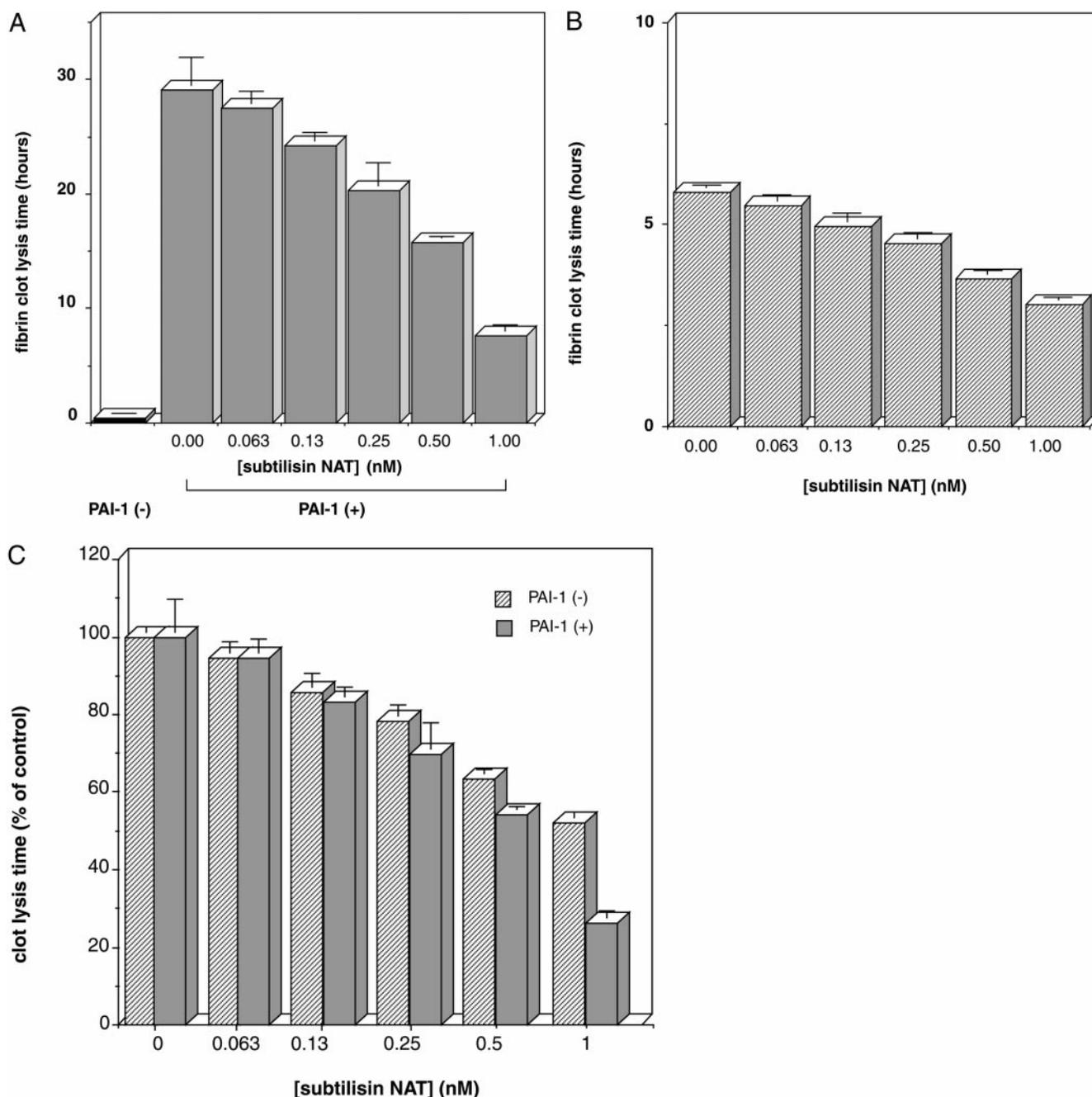


FIG. 7. Potentiation of tPA-induced fibrin clot lysis time by subtilisin NAT. The profibrinolytic activity of subtilisin NAT was evaluated either in rpPAI-1-enriched fibrin clots (A) or in fibrin clots without rpPAI-1 (B). The concentrations of tPA employed were 0.2 nM in rpPAI-1-enriched fibrin clot lysis and 2 μ M in rpPAI-1-depleted fibrin clot lysis. The clot lysis time obtained at each subtilisin NAT concentration, shown in A and B, was expressed as a percentage over the corresponding lysis time in the absence of subtilisin NAT (C).

To evaluate the direct influence of subtilisin NAT on fibrin dissolution, similar clot lysis assay was conducted in the complete absence of tPA. The dissolution of the fibrin clot, however, was not observed over a period of 40 h even in the presence of the highest concentration of subtilisin NAT (1 nM) (data not shown). We then analyzed the effect of subtilisin NAT on rpPAI-1-depleted fibrin clots, employing a lower concentration of tPA ($\frac{1}{100}$, 2 μ M) to make the clot lysis time long enough (5.8 ± 0.1 h). The clot lysis was also shortened by subtilisin NAT in a dose-dependent manner (3.0 ± 0.1 h at 1 nM subtilisin NAT), albeit to a lesser extent (Fig. 7B). This enhancement of clot lysis time in the absence of rpPAI-1 is most likely caused by direct digestion of fibrin as previously reported (1, 29). The clot lysis times both in the presence and absence of rpPAI-1 were expressed as a percentage of the time obtained without subtili-

sin NAT in each assay and were compared (Fig. 7C). The extent of the enhancement was more dramatic in the presence of rpPAI-1, suggesting that the inactivation of PAI-1 by subtilisin NAT plays a crucial role in the effective lysis of PAI-1-enriched fibrin clots.

DISCUSSION

The subtilisin-like serine protease (subtilase) superfamily is one of two large serine protease superfamilies (the other being the (chymo)trypsin-like serine protease family) (30). This subtilase superfamily contains large numbers of proteases of extremely widespread occurrence in plants, bacteria, yeast, and eukaryotes. It contains a large group of proprotein convertases, which have attracted a great deal of attention recently (31–33).

Both chymotrypsin and subtilisin have well conserved ar-

rangements of catalytic His, Asp, and Ser residues, but they are located in radically different protein scaffolds, β/β for chymotrypsin and α/β for subtilisin (30). Subtilisin NAT is a member of the subtilisin family, whose members have rather broad substrate specificity due to larger and more hydrophobic S1 and S4 binding sites (34). Its potential to enhance fibrinolytic activity was first reported by Sumi *et al.* (2). The analyses of its nucleotide sequence (3) and amino acid sequence (1) confirmed that this enzyme belonged to the subtilisin family, showing strong homology with both subtilisin E (from *B. subtilis*) (35) and subtilisin J (from *Bacillus stearothermophilus*) (36).

Although subtilisin NAT was shown to enhance fibrinolysis, its precise mechanism is not known. It does not activate plasminogen but is reported to directly digest fibrin especially in its cross-linked form (29). In the present study we found a second mechanism by which subtilisin NAT enhances fibrinolysis through cleavage and inactivation of PAI-1. Because PAI-1 is the primary inhibitor of fibrinolysis and regulates total fibrinolytic activity by its relative ratio with tPA, its inactivation is directly related to the enhancement of fibrinolysis. Cleavage of PAI-1 at its reactive center loop has been demonstrated for activated coagulation factors (37) and neutrophil elastase (17), both members of the (chymo)trypsin serine protease family. The interaction of SERPINs with other members of the subtilisin family has also been reported. Subtilisins BPN' (38) and Carlsberg (7), which are both homologous to subtilisin NAT, cleave and inactivate α_1 -antitrypsin. The latter was shown to cleave α_1 -antitrypsin at two distinct sites within the reactive site loop including its P2 site (7). Although it does not possess inhibitory activity, ovalbumin, another SERPIN, was also shown to be cleaved by subtilisin Carlsberg (39). This cleavage was not observed when conformationally modified ovalbumin (S-ovalbumin), in which the reactive center loop is fully inserted into β -sheet A, was treated with subtilisin Carlsberg. This is consistent with data in our present study that showed active rpPAI-1 was more susceptible to subtilisin NAT digestion than the latent form whose conformation is known to be similar to that of S-ovalbumin (28).

The reactive center loop of active SERPINs is exposed above the plane of the molecule and is essential for forming a stoichiometric complex with their target serine proteases, providing a "bait" residue (P1 residue) in the strained loop that acts as a pseudo-substrate for the target protease (16). An interesting feature of PAI-1 is that the active molecule spontaneously converts into a latent molecule (40), in which the strained loop is fully inserted into the molecule as a central strand (28) generating an apparently smaller Stokes' radius (40). The decreased susceptibility of latent rpPAI-1 to subtilisin NAT digestion, therefore, appears to be due to its compact conformation with the reactive site loop inserted into the molecule.

In addition to the plasminogen activators, there are a number of serine proteases that can reduce PAI-1 activity through complex formation or proteolytic cleavage. As a result of these interactions the fibrinolytic balance is shifted toward increased tPA activity (14, 17, 37, 41). This is based on the fact that tPA is a unique enzyme possessing intrinsic specific activity in its single-chain form (42) unlike other members of the (chymo)trypsin serine protease family. The amount of active enzyme activity in this case is simply determined by its relative concentration over its specific inhibitor. Several activated coagulation factors, including factor XIa (37), calcium-bound factor Xa and thrombin (41), and neutrophil elastase (17), have the ability to interact with PAI-1. The inactivation of PAI-1 by these activated coagulation factors is considered to be one of the mechanisms responsible for the well known phenomenon of coagulation-associated enhancement of fibrinolysis (14). In the

present study subtilisin NAT was also shown to be able to enhance tPA-induced clot lysis of rpPAI-1-enriched fibrin by inactivating PAI-1.

Subtilisin NAT also shortened tPA-induced fibrin clot lysis time in the absence of rpPAI-1, although to a lesser extent. The effect was likely caused by direct digestion of fibrin by subtilisin NAT as previously demonstrated both *in vitro* (29) and *in vivo* (4). This is consistent with the fact that subtilisin NAT shows similar activity both in plasminogen-rich and plasminogen-poor fibrin plates. We also demonstrated subtilisin NAT-induced partial digestion of the A α chain of the fibrinogen molecule. Although we did not analyze the cleavage site in fibrinogen, lysine is a candidate for the P1 residue of the cleavage site, since P1-lysine is a characteristic of preferable substrate for subtilisin NAT (1, 2). Because similar partial digestion of fibrin by plasmin is known to enhance fibrinolysis by providing a C-terminal lysine in the A α chain that binds to both plasminogen and tPA (43), this may be another mechanism for subtilisin NAT to enhance fibrinolysis.

The fact that subtilisin NAT, like plasmin, is able to directly digest fibrin and the finding that subtilisin NAT preferably hydrolyzes a synthetic substrate for plasmin (H-D-Val-Leu-Lys-p-nitroanilide) (1) prompted us to analyze a possible interaction between subtilisin NAT and α_2 -AP. Subtilisin NAT, however, neither formed a high molecular weight complex with α_2 -AP nor cleaved it. Therefore, inactivation of α_2 -AP does not seem to be involved in the enhancement of fibrinolysis by subtilisin NAT. This, in turn, suggests that the cleavage of PAI-1 by subtilisin NAT is a rather specific phenomenon.

In the present study we have shown that subtilisin NAT inactivates PAI-1 by limited proteolysis of its reactive site. This mechanism seems to allow this profibrinolytic enzyme to initiate effective lysis of PAI-1-enriched fibrin clots.

Acknowledgments—We thank Michio Fujie and Masako Suzuki for invaluable technical assistance.

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The Profibrinolytic Enzyme Subtilisin NAT Purified from *Bacillus subtilis* Cleaves and Inactivates Plasminogen Activator Inhibitor Type 1

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J. Biol. Chem. 2001, 276:24690-24696.

doi: 10.1074/jbc.M101751200 originally published online April 26, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M101751200](https://doi.org/10.1074/jbc.M101751200)

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