J Gastroenterol 2002; 37:928–934

Journal of Gastroenterology

Functional analysis of recombinant pancreatic secretory trypsin inhibitor protein with amino-acid substitution

Kinuko Kuwata[1], Masahiko Hirota[1], Hiroyuki Shimizu[2], Masanori Nakae[3], Shoji Nishihara[3], Akio Takimoto[3], Kenji Mitsushima[3], Norihisa Kikuchi[3], Kazuaki Endo[4], Masayasu Inoue[5], and Michio Ogawa[1]

[1] Department of Surgery II, Kumamoto University Medical School, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[2] Diagnostics Department, Shionogi, Osaka, Japan
[3] Discovery Research Laboratory, Shionogi, Hyogo, Japan
[4] Clinical Research Depertment, Shionogi, Osaka, Japan
[5] Department of Biochemistry I, Osaka City University Medical School, Osaka, Japan

Background. We hypothesized that mutation of the pancreatic secretory trypsin inhibitor (*PSTI*) gene may promote a predisposition to pancreatitis, possibly by reducing the inhibition of trypsin activity. Based on this hypothesis, we performed a biochemical analysis of recombinant PSTI protein. ***Methods.*** The trypsin inhibitory activity of the recombinant protein was analyzed. The activity of PSTI protein with a point mutation of the most common type: ^{34}Asn (AAT)-to-Ser (AGT)(101A>G N34S: N34S) in exon 3, was compared with that of the wild type. ***Results.*** The function of N34S PSTI remained unchanged under both the usual alkaline and acidic conditions compared with the wild-type PSTI. The calcium concentration did not affect the activity of recombinant PSTI. The trypsin susceptibility of the N34S protein was not increased either. ***Conclusions.*** Mechanisms other than the conformational change of PSTI associated with amino-acid substitution, such as abnormal splicing, may underlie the predisposition to pancretitis in patients with the N34S mutation.

Key words: PSTI, recombinant protein, trypsin, familial pancreatitis, chronic pancreatitis

Introduction

Pancreatic acinar cells synthesize various types of proteolytic enzymes, including trypsin and chymotrypsin, which are stored as inert proenzymes, such as trypsinogen and chymotrypsinogen. Trypsin, activated from trypsinogen by enterokinase in the intestine, or partially by trypsin itself in the pancreas, acts as the triggering enzyme, which leads to the activation of many of the pancreatic digestive proenzymes.

Pancreatic secretory trypsin inhibitor (PSTI) is synthesized in the acinar cells of the pancreas,[1] and acts as a potent protease inhibitor that inhibits the intrapancreatic activation of trypsin to prevent the occurrence of pancreatitis.[2] Human PSTI is a single-chain polypeptide consisting of 79 amino acids, including 23 amino acids of signal peptide. The human *PSTI* gene is approximately 7.3 kb long and involves four exons.[3]

Recent studies in this and other laboratories reported that some patients with chronic pancreatitis had a point mutation in the *PSTI* gene: ^{34}Asn(AAT)-to-Ser(AGT) (101A>G N34S: N34S) in exon 3.[3–7] Chen et al.[8] reconfirmed the association of the N34S mutation with pancreatitis, though they presented a rather negative view of the causative relation between the N34S mutation and pancretitis in a previous report.[5] Witt et al.[4] and Pfützer et al.[6] also found a significant association of the N34S mutation with the pathogenesis of chronic pancreatitis. We also found the N34S mutation in two Japanese chronic pancreatitis patients in the same family.[3] Genetic mutations in the *PSTI* gene seems to promote a predisposition to pancreatitis by lowering the threshold for pancreatitis caused by other factors. Based on this hypothesis, we analyzed the recombinant N34S protein biochemically.

Materials and methods

Chemicals

Human and bovine trypsin were purchased from Sigma Chemical (St Louis, MO, USA). *N*-Benzoyl-L-arginine *p*-nitroanilide (L-BAPA) was purchased from the Protein Research Foundation (Osaka, Japan). DNA-modifying enzymes and pUC118 were obtained from

Received: January 10, 2002 / Accepted: May 17, 2002
Reprint requests to: M. Ogawa

Takarashuzo (Kyoto, Japan). All other reagents used were of analytic grade.

Construction of the expression plasmid for human PSTI and site-directed mutagenesis

Various human mutant *PSTI* genes, including the R67S mutant, have been constructed and expressed in an *Escherichia coli* host-vector system.[9] To achieve overexpression of the genes, we further developed an expression system with *Saccharomyces cerevisiae* and constructed an expression plasmid pRS5402 for the R67S mutant PSTI. The plasmid pRS5402 contains the mature region of the R67S mutant gene fused to the secretion signal sequence of mating factor alpha-1 from *S. cerevisiae* under the control of the galactose-inducible *GAL1* promoter in yeast. A 0.5-kb portion of *Bam*HI-*Sal*I fragment containing the R67S human *PSTI* mutant gene was isolated and subcloned to the *Bam*HI-*Sal*I site of pUC118 containing the *lac* promoter and ampicillin-resistance gene. Site-directed mutagenesis was performed by the method of Taylor et al.,[10,11] using the reagents and protocol of the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The oligonucleotides used for mutagenesis were: 5′-GAAAATCGGAAACGCCAGACTTCT ATCC-3′ (Ser-67→Arg: wild type) and 5′-GGCCAAATGTTACAGTGAACTTAATGG-3′ (Asn-34→Ser), in which the nucleotide change for the mutation is underlined. For the construction of the N34S mutant, the *Bam*HI-*Bsm*I fragment of N34S plus the R67S double mutant, and the *Bsm*I-*Sal*I fragment of wild-type *PSTI* were fused (Fig. 1). The mutated DNA sequences were confirmed by the dideoxy method. Each of the resultant *Bam*HI-*Sal*I fragments was replaced to the corresponding sites of pRS5402, and the expression plasmids obtained were designated pST5401 for the wild type and pST5401NS for the N34S mutant.

Gene expression and purification of mutant PSTIs

To express the wild-type and N34S mutant *PSTI* gene, the host strain *S. cerevisiae* BJ1991 was transformed with pST5401 or pST5401NS by the method of Ito et al.[12] The recombinant cells were grown in 1 liter of YP-Gal medium (2% yeast extract, 4% Bacto-peptone, 4% galactose, pH 7.2) at 30°C for 72 h by flask culture. Because the PSTI produced in the cells was secreted into the culture medium as a mature form, the culture supernatant was prepared from the whole culture broth by centrifuging. After filtration with a 0.22-μm membrane, the supernatant, adjusted to pH 3.0 with HCl, was loaded onto a 50-ml SP-Sepharose Fast Flow column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM acetic acid (pH 3.0) and the column was washed with 50 mM sodium acetate buffer, pH 4.0. The PSTI was eluted with a linear gradient of 0−0.5 M NaCl and precipitated by 75% saturated ammonium sulfate from active fractions. The precipitate was dissolved in a small volume of 50 mM sodium acetate buffer, pH 4.0, and subjected to reverse-phase HPLC, using an ODS-A column (YMC, Kyoto, Japan) with a constant mobile phase of 21% acetonitrile in 0.05% trifluoroacetic acid. Active fractions were pooled and concentrated with a rotary evaporator. Subsequently, the concentrated sample was further purified

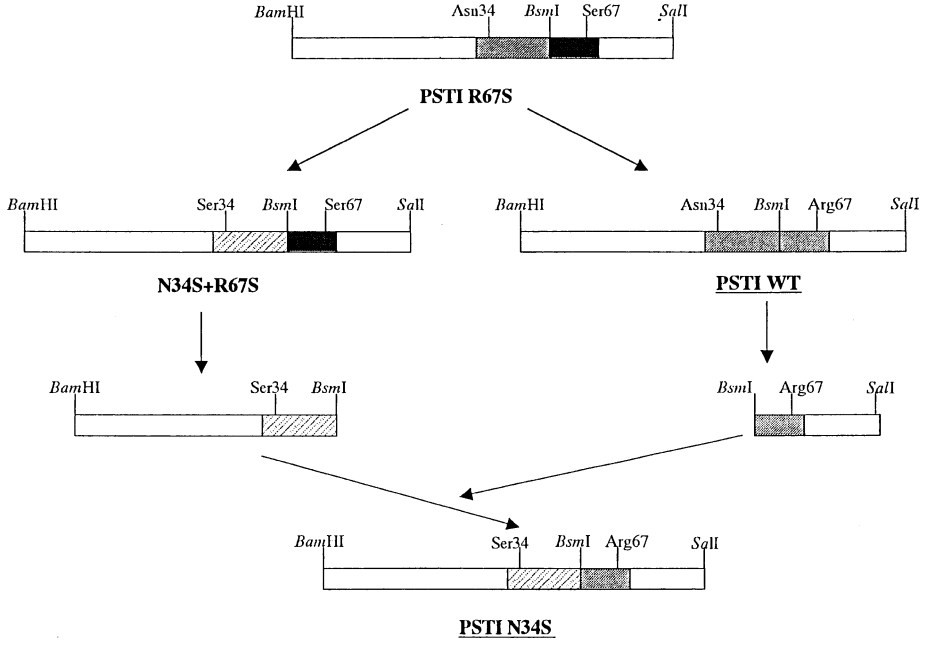

Fig. 1. Construction of expression plasmid for human *PSTI* and site-directed mutagenesis. Conditions are given under "Materials and methods". *WT*, Wild type

by a second reverse-phase HPLC, using an ODS-A column. Active fractions were pooled, concentrated, and kept at −80°C until use.

Amino-acid analysis and sequence determination

Samples were hydrolysed with 20% hydrochloric acid for 24 h at 110°C. Amino-acid analyses were performed with a Hitachi amino-acid analyzer (model L-8500; Hitachi, Tokyo, Japan) Automated Edman degradation was performed with an Applied Biosystems protein sequencer (model 492; Applied Biosystems, Tokyo, Japan) equipped with a microgradient system (model 140C) and a programmable detector (model 785A).

Determination of trypsin inhibitory activity

Trypsin inhibitory activity was determined from the residual trypsin activity after mixing human or bovine trypsin with recombinant PSTI in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and 0.01% Triton X-100 at 37°C, using L-BAPA as the substrate.[13] After 10 min of incubation at 37°C, the reaction was stopped by adding 0.5 ml of 30% acetic acid, and the absorbance at 410 nm was measured. For the estimation of the pH dependence of the trypsin inhibitory activity of PSTI, we used 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES), Tris, and 2-(N-cyclohexylamino) ethanesulfonic acid (CHES) buffers for pH 5.0–6.0, pH 7.0–8.0, and pH 9.0, respectively. For the assessment of the effect of Ca^{2+} concentration, reaction in 0.02 M $CaCl_2$ and that in 0 M $CaCl_2$ with 0.01 M ethylene diamine tetracetic acid (EDTA) were compared. The absorbance at 410 nm was measured after adjustment to pH 8.0.

Determination of the rate of reappearance of trypsin activity after mixing with PSTI

PSTI (2 μm) was incubated with human trypsin (1 μm) in 0.8 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and 0.01% Triton X-100 at 37°C. Aliquots of 20 μl were removed at specified times, and mixed with 0.2 ml of 5 mM L-BAPA solution in the same buffer. After 10 min of incubation at 37°C, the reaction was stopped by adding 0.5 ml of 30% acetic acid, and the absorbance at 410 nm was measured.

Results

Purification and characterization of recombinant PSTI

The yields of purified recombinant PSTI from 1 liter of culture broth were 13.6 and 13.2 mg for N34S PSTI and wild-type PSTI, respectively. The amino-acid compositions of the recombinant PSTI proteins show that these proteins had the amino-acid compositions expected from the nucleotide sequence (Table 1). They had a single amino-terminal sequence of Asp-Ser-Leu, indicating that nonspecific cleavage had not occurred in their molecules.

Table 1. Amino-acid composition of N34S mutant *PSTI*

Amino acid	Wild type	N34S
Asp	7.8 (8)	6.9 (7)
Thr	3.5 (4)	3.6 (4)
Ser	2.3 (3)	3.1 (4)
Glu	6.0 (6)	6.1 (6)
Pro	2.7 (3)	2.7 (3)
Gly	5.0 (5)	5.0 (5)
Ala	0.9 (1)	1.0 (1)
1/2Cys	5.5 (6)	5.4 (6)
Val	1.9 (2)	1.9 (2)
Met	0.0 (0)	0.0 (0)
Ile	2.8 (3)	2.8 (3)
Leu	4.0 (4)	4.0 (4)
Tyr	1.7 (3)	1.8 (3)
Phe	1.0 (1)	1.0 (1)
Lys	3.8 (4)	3.7 (4)
His	0.0 (0)	0.0 (0)
Trp	0.0 (0)	0.0 (0)
Arg	2.8 (3)	2.8 (3)
Total	56	56

The numbers of residues in parentheses are from the expected sequences. Values differing from those of wild-type *PSTI* are underlined

Analysis of recombinant PSTI activity

Activity of recombinant PSTI proteins under standard conditions. Residual trypsin activity decreased with the increase in the molar ratio of PSTI to trypsin. Recombinant N34S protein showed almost the same inhibitory activity for trypsin as did wild-type PSTI (Figs. 2 and 3), although the residual activities of human trypsin with N34S appearred slightly larger than those of wild-type at pH 7 and pH 9. Furthermore, no apparent difference was found between the human and bovine types of trypsin. At a PSTI/trypsin ratio of 1, residual trypsin activity had almost disappeared.

Recombinant wild-type PSTI showed an approximate dissociation constant of 7.6×10^{-9} M, when the method of Green and Work[14] was used, whereas the dissociation constant for natural PSTI was 8.7×10^{-9} M. The concentrations of recombinant PSTI proteins determined by immunoreactivity were compatible with those determined by HPLC or amino-acid analysis. These results suggest that the recombinant wild-type PSTI shared the same higher-order structure with native PSTI, in addition to sharing the primary structure.

Effect of pH and Ca^{2+} concentration in the reaction medium. A pH-dependent decrease in the activity of

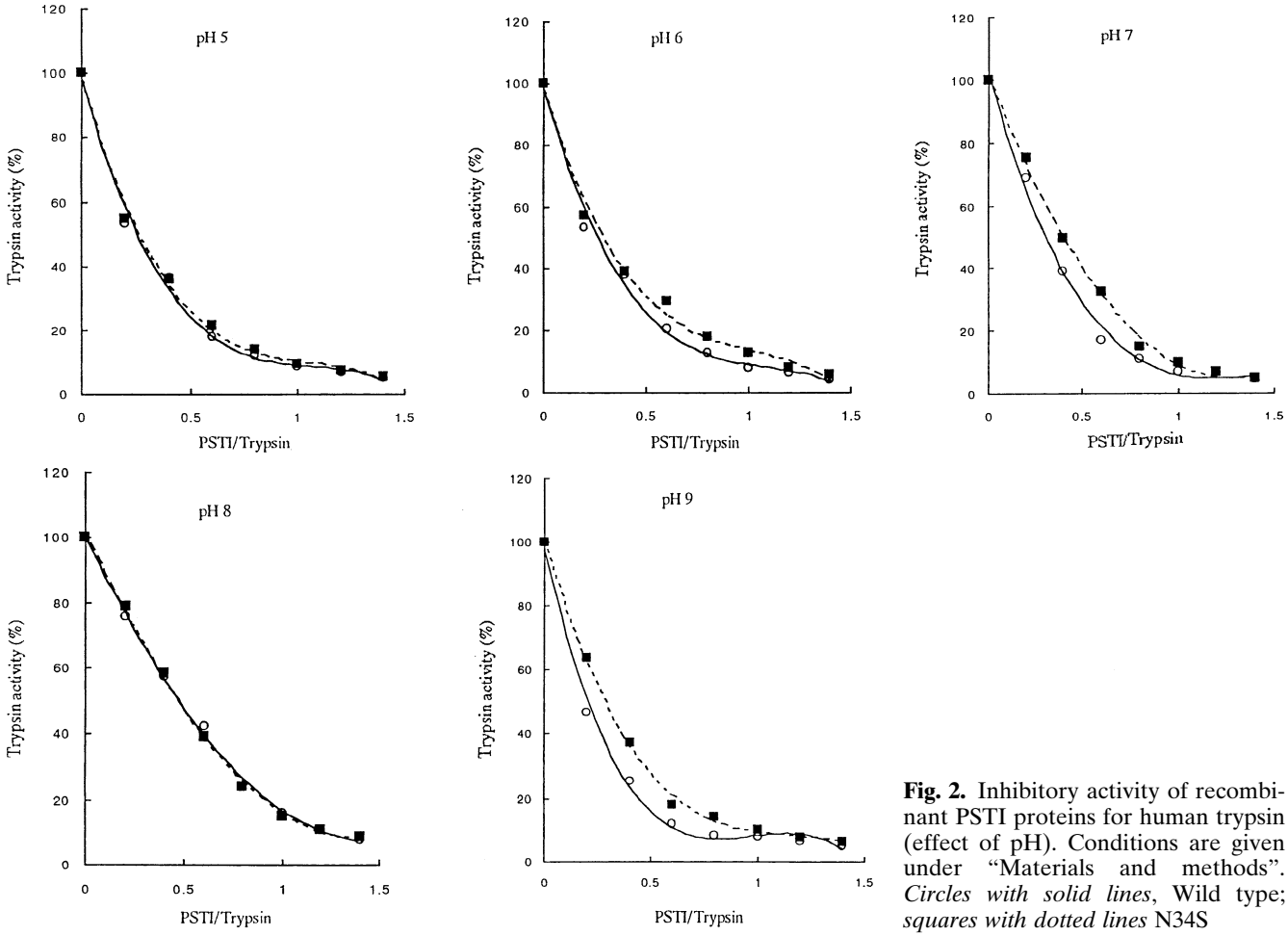

Fig. 2. Inhibitory activity of recombinant PSTI proteins for human trypsin (effect of pH). Conditions are given under "Materials and methods". *Circles with solid lines*, Wild type; *squares with dotted lines* N34S

recombinant N34S protein occurred in a fashion similar to that in the wild-type enzyme (Figs. 2 and 3). The Ca^{2+} concentration did not affect the activity of recombinant PSTI (Figs. 4 and 5).

Disappearance rate of PSTI activity by incubation with trypsin

Figure 6 shows the time course of the recovery of trypsin activity from the PSTI-trypsin complex. Both types of PSTI protein retained trypsin inhibitory activity for up to 24 h of incubation. After 120 h of incubation, the trypsin inhibitory activity of the wild-type PSTI sample was abolished. However, the same phenomenon was also found for the N34S protein.

Discussion

Mutation of the *PSTI* gene has been investigated in patients with chronic pancreatitis, and the association of the N34S mutation with a predisposition to pancreatitis has been suggested.[3–8] The N34S mutation of the *PSTI* gene was found frequently in patients with pancreatitis compared with nonpancreatitis subjects (83/908; 9.1% vs 11/1474; 0.7%).[3–8] In addition, N34S homozygotes showed a much higher incidence of developing chronic pancreatitis. Chou-Fasman and Robson-Garnier computer analysis of the secondary structure revealed the disappearance of the native turn structure of 34Asn-35Glu by the N34S mutation. Because this domain is adjacent to the binding site to trypsin, a possible decrease of N34S PSTI in the activity of trypsin binding was proposed.[3]

However, the present study of the recombinant PSTI proteins revealed no apparent decrease in the inhibitiory activity of the N34S mutant to trypsin. Trypsin acts preferentially in alkaline conditions. In certain conditions, however, trypsin may also act in an acidic pH, particularly after activation by lysosomal enzymes such as cathepsin B.[8,15] The activity of N34S to inhibit trypsin was shown at any pH tested in the present study, and the susceptibility of N34S to trypsin remained unchanged. These results suggest that mechanisms other than a conformational change of PSTI by amino-acid substitution may possibly be asociated with

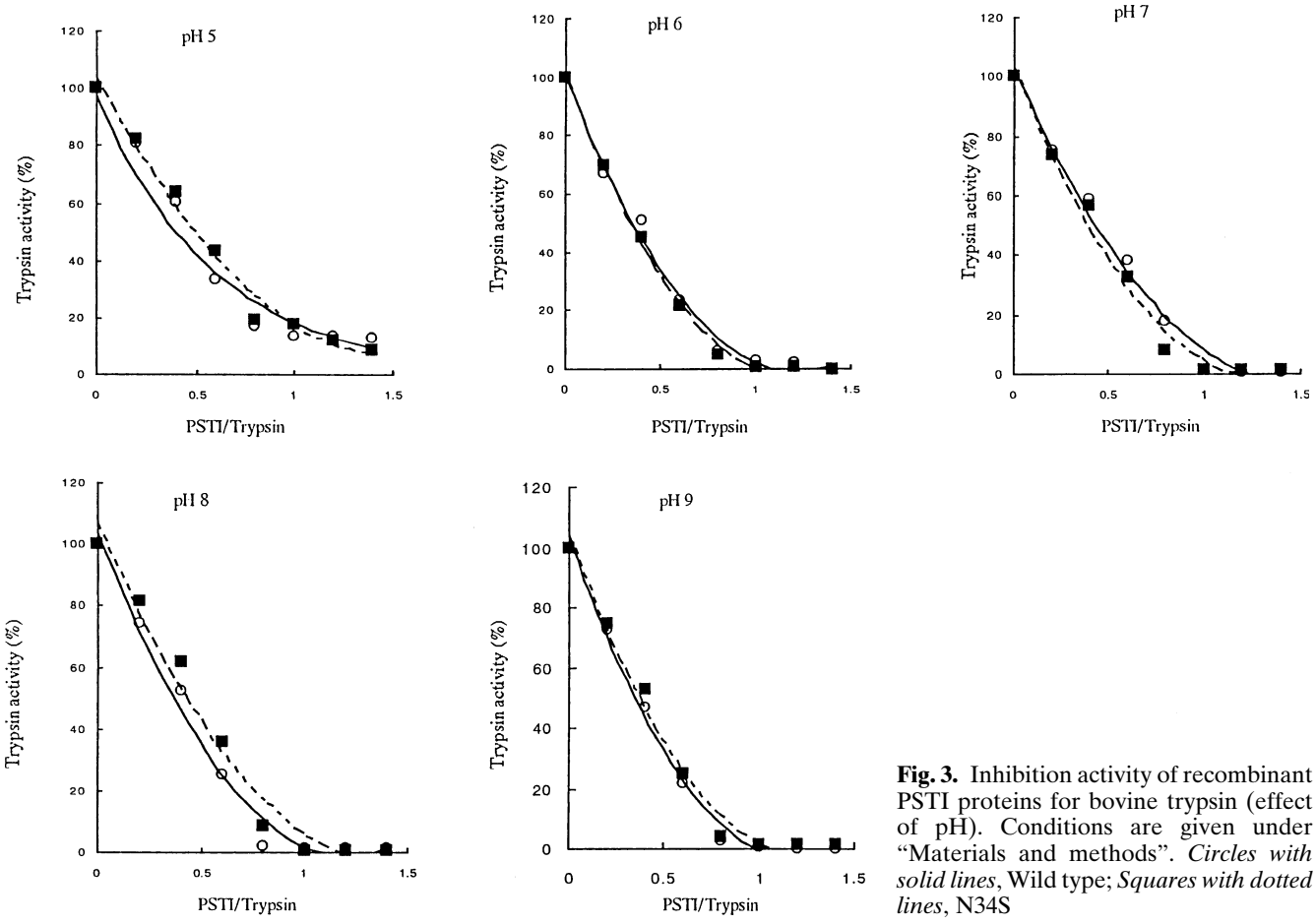

Fig. 3. Inhibition activity of recombinant PSTI proteins for bovine trypsin (effect of pH). Conditions are given under "Materials and methods". *Circles with solid lines*, Wild type; *Squares with dotted lines*, N34S

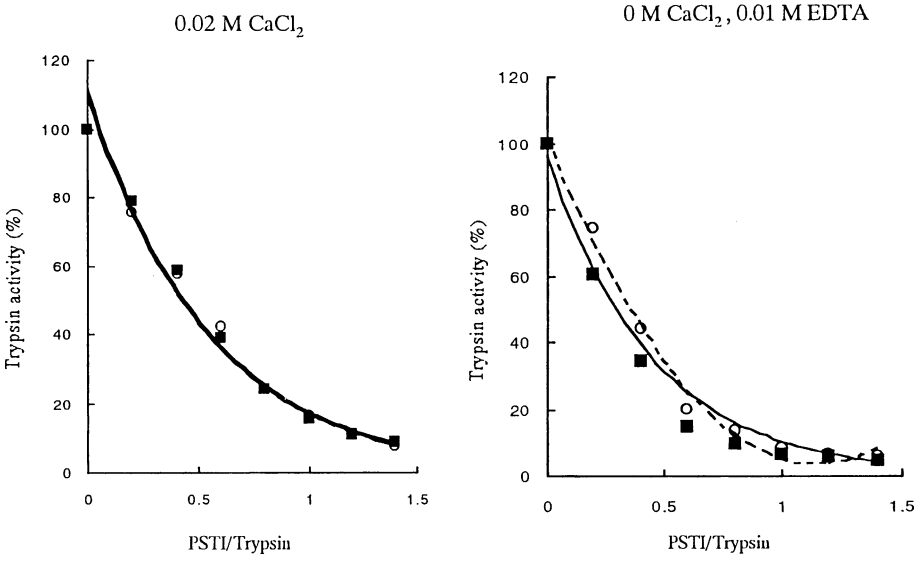

Fig. 4. Inhibitory activity of recombinant PSTI proteins for human trypsin (effect of Ca^{2+} concentration). Conditions are given under "Materials and methods". *Circles with solid lines*, Wild type; *squares with dotted lines*, N34S. *EDTA*, Ethylenediaminetetraacetie acid

a predisposition to pancreatitis. Alternatively, more sensitive methods are necessary to detect the decreased affinity of N34S PSTI to trypsin.

N34S is usually associated with two intronic mutations, i.e., IVS1-37T>C and IVS3-69insTTTT.[8,16] Mutations in the intronic polypyrimidine tract, such as IVS3-69insTTTT, which converts consecutive T5 to a T9 structure, sometimes result in a splicing abnormality. The strong association of the length of the poly-T tract at the exon 9 splice branch/acceptor site (intron 8) with

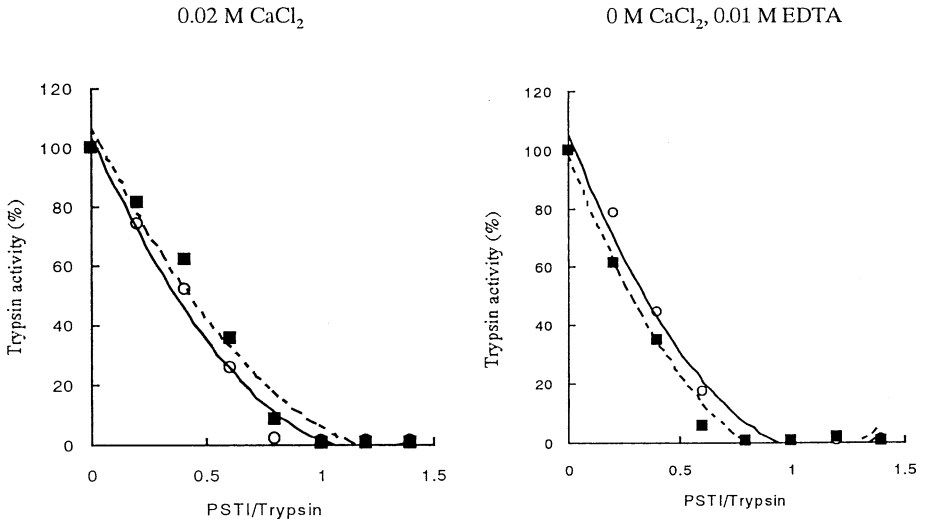

Fig. 5. Inhibitory activity of recombinant PSTI proteins for bovine trypsin (effect of Ca^{2+} concentration). Conditions are given under "Materials and methods". *Circles with solid lines*, Wild type; *squares with dotted lines*, N34S

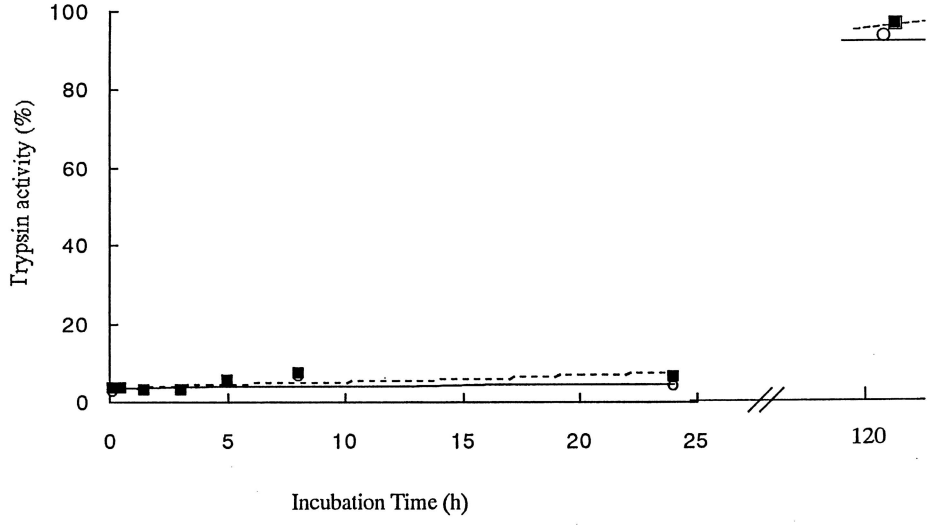

Fig. 6. Rate of reappearance of trypsin activity after incubation with PSTI. Conditions are given under "Materials and methods". *Circles with solid line*, Wild type; *squares with dotted line*, N34S.

the in-frame skipping of the exon 9 cystic fibrosis transmembrane conductance regulator (*CFTR*) gene is reported in cystic fibrosis patients.[17] Furthermore, an intronic polymorphism (variable number of a tandem repeat of intron 2) in the interleukin-1 receptor antagonist gene is known to be associated with the production of its protein.[18] Hence, intronic mutations, rather than N34S itself (IVS1-37T>C + N34S + IVS3-69insTTTT complex) may be associated with the decreased function of PSTI. The failure to exhibit function loss in the recombinant N34S PSTI protein supports this possibility. Expressional analysis of *PSTI* mRNA in patients with the N34S mutation may be necessary. Such experiments are currently in progress in our laboratory.

References

1. Bartelt DC, Shapanka R, Greene LJ. The primary structure of the human pancreatic secretory trypsin inhibitor. Amino acid sequence of the reduced S-aminoethylated protein. Acta Biochem Biophys 1977;179:189–99.
2. Horii A, Kobayashi T, Tomita N, Yamamoto T, Fukushige S, Murotsu T, et al. Primary structure of human secretory trypsin inhibitor (*PSTI*) gene. Biochem Biophys Res Commun 1987;149: 635–41.
3. Kuwata K, Hirota M, Sugita H, Kai M, Hayashi M, Nakamura M, et al. Genetic mutations in exons 3 and 4 of the pancreatic secretory trypsin inhibitor in patients with pancreatitis. J Gastroenterol 2001;36:612–8.
4. Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, Landt O. Mutations in the gene encoding the serine protease inhibitor, Kazal type I, are associated with chronic pancreatitis. Nature Genet 2000;25:213–6.
5. Chen JM, Mercier B, Audrezet MP, Ferec C. Mutational analysis of the human pancreatic secretory trypsin inhibitor (*PSTI*) gene in hereditary and sporadic chronic pancreatitis. J Med Genet 2000;37:67–9.
6. Pfützer RH, Barmada MM, Brunskill AP, Finch R, Hart PS, Neoptolemos J, et al. SPINK/PSTI polymorphisms act as disease modifiers in familial and idiopathic chronic pancreatitis. Gastroenterology 2000;119:615–23.
7. Kaneko K, Nagasaki Y, Furukawa T, Mizutamari H, Sato A, Masumura A, et al. Analysis of the human pancreatic secretory

trypsin inhibitor (*PSTI*) gene mutations in Japanese patients with chronic pancreatitis. J Hum Genet 2001;46:293–7.
8. Chen JM, Mercier B, Audrezet MP, Raguenes O, Quere I, Ferec C. Mutations of the pancreatic secretory trypsin inhibitor (*PSTI*) gene in idiopathic chronic pancreatitis. Gastroenterology 2001; 120:1061–3.
9. Kikuchi N, Nagata K, Shin M, Mitsushima K, Teraoka H, Yoshida N. Site-directed mutagenesis of human pancreatic secretory trypsin inhibitor. J Biochem 1989;106:1059–63.
10. Taylor JW, Schmidt W, Cosstick R, Okruszek A, Eckstein F. The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. Nucleic Acids Res 1985;13: 8749–64.
11. Taylor JW, Ott J, Eckstein F. The rapid generation of digonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res 1985;13: 8765–85.
12. Kikuchi N, Nagata K, Yoshida N, Ogawa M. The multiplicity of human pancreatic secretory trypsin inhibitor. J Biochem 1985;98: 687–94.
13. Halangk W, Lerch MM, Brabdt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, et al. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. J Clin Invest 2000;106:773–81.
14. Green NM, Work E. Pancreatic trypsin inhbitor. II. Reaction with trypsin. Biochem J 1953;54:347–52.
15. Saluja AK, Donovan EA, Yamanaka K, Yamatuchi Y, Hofbauer B, Steer ML. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. Gastroenterology 1997;113:304–10.
16. Rossi L, Pfützer RH, Parvin S, Ali L, Sattar S, Kahn AKA, Gyr N, Whitcomb DC. SPINK1/PSTI mutations are associated with tropical pancreatitis in Bangladesh. Pancreatology 2001;1:242–5.
17. Chu CS, Trapnell BC, Curristin S, Cutting GR, Crystal RG. Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. Nature Genet 1993;3: 151–6.
18. Tarlow JL, Blakemore AIF, Lennard A. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 1993;91:403–4.