

## Characterization of Recombinant p20 Trypsin Inhibitor, a New Protein from *Glycine max*

Akihito MATSUSHIMA, Yoshiyuki ASHIDA, Junko WATANABE, Toshifumi HIRATA\*

*Department of Mathematical and Life Science, Graduate School of Science,  
Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*

\*Corresponding author E-mail address: thirata@sci.hiroshima-u.ac.jp

Received 12 September 2002; accepted 15 October 2002

### Abstract

A 20-kDa protein (p20) having 40% sequence similarity with Kunitz-type soybean trypsin inhibitor (STI) from *Glycine max* (soybean) cultured cells was expressed in *Escherichia coli*. The recombinant p20 (rp20) inhibited the activity of trypsin at the same level as STI (rp20,  $K_i = 50$  nM; STI,  $K_i = 75$  nM), and both rp20 and STI displayed non-competitive inhibition of trypsin activity. Although STI inhibited the activity of  $\alpha$ -chymotrypsin ( $K_i = 140$  nM) and elastase ( $K_i = 207$  nM), rp20 did not inhibit the activity of  $\alpha$ -chymotrypsin and elastase. These results show that p20 is a novel type of trypsin inhibitor.

**Key words:**  $\alpha$ -Chymotrypsin, Elastase, *Glycine max* (soybean), Inhibition mechanism, Kunitz-type trypsin inhibitor.

### Abbreviations

BAPA, benzoyl-L-arginine p-nitroanilide; STI, soybean trypsin inhibitor.

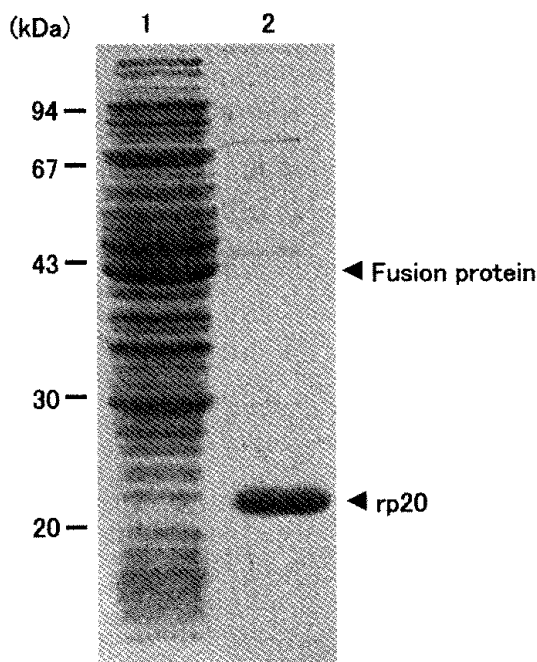
Recently we isolated from *Glycine max* (soybean) cultured cells, a 20-kDa protein (p20) having trypsin inhibitory activity on the basis of its GTP binding activity (Hirata *et al.*, 1999). The cDNA clone (accession number AB029441) of p20 was isolated and sequenced, and the putative amino acid sequence of p20 indicated an approximately 40% sequence similarity with Kunitz-type soybean trypsin inhibitor (STI) (Ashida *et al.*, 2000). Protease inhibitors belonging to the STI family has been extensively studied and at least 10 members are reported in soybean cells (Kim *et al.*, 1985; Jofuku *et al.*, 1989). The amino acid residues at reactive site of trypsin inhibitors were reported to be Arg (63)-Ile (64) in  $Ti^a$ ,  $Ti^b$  and  $Ti^c$  (Blow *et al.*, 1974; Sweet *et al.*, 1974) and His (63)-Ala (64) in  $KTi1$  and  $KTi2$  (Jofuku *et al.*, 1989). However, the corresponding amino acid residues of p20 were Lys (63)-Ile (64) (Ashida *et al.*, 2000). These facts suggested that p20 is a novel type of trypsin inhibitor. In order to clarify the inhibitory properties of p20, the recombinant p20 (rp20) was expressed and then its inhibitor activity and mechanism were

investigated.

Total cellular RNA was isolated from soybean cultured cells using an ISOGEN kit (Nippon Gene Co.). RNA purification was then followed by single-stranded cDNA synthesis using an oligo(dT)<sub>20</sub>-M13M4 adopter primer. Two oligonucleotides, HVR-1 (+136 to +154; accession number AB029441) and HVF-1 (+674 to +693), were used as primers for polymerase chain reaction (PCR) amplification to produce the matured form p20 cDNA from single-stranded cDNA. These primers have *Bam*HI and *Xho*I restriction sequences, respectively. The amplification was done at 94 °C for 2 min for denaturation, 55 °C for 30 s for annealing, and 70 °C for 30 s for synthesis, followed by 29 cycles of these conditions. The final PCR product of 571 base pairs in length was obtained, and ligated to the pPCR-Script SK(-) vector (Stratagene) followed by transformation into *Escherichia coli* DH5  $\alpha$  competent cells. Double-stranded DNA sequencing by the dideoxynucleotide chain termination method was done using a BigDye terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM310 genetic analyzer (Applied Biosystems). Then, the cDNA clone of p20 was digested with *Bam*HI (New England Biolabs) and *Xho*I (New England Biolabs), and ligated to the pGEX-6P-1 expression vector (Amersham Bioscience), which

contains a region coding for expression as a fusion protein with glutathione *S*-transferase (GST), to construct pGEX-6P-1(p20). Finally, the pGEX-6P-1(p20) was transformed into *E. coli* BL21 competent cells. The full-length sequence of p20 cDNA cloned into pGEX-6P-1(p20) was confirmed by DNA sequencing.

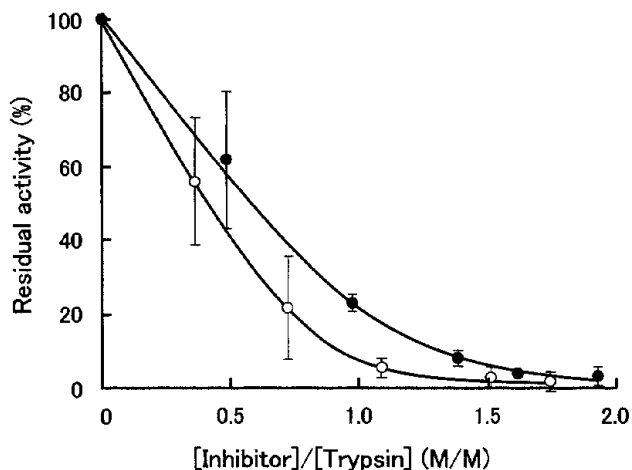
The *E. coli* strain transformed with pGEX-6P-1(p20) was cultivated at 30 °C for 6 h in LB medium (Luria *et al.*, 1960) containing 50  $\mu\text{g ml}^{-1}$  of ampicillin. After the incubation, isopropyl- $\beta$ -D-thiogalactopyranoside was added to a final concentration of 0.1 mM in the cultures and incubated at 16 °C for 16 h. After centrifugation, the *E. coli* pellet was resuspended in sonication buffer (1mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol and 51 mM NaCl) and sonicated. The sonicated mixture was centrifuged to obtain the soluble protein fraction. The GST fusion protein was purified using glutathione Sepharose 4B (Pharmacia Biotech) affinity column chromatography and digested by PreScission protease (Amersham Biosciences) to release rp20 from the GST fusion protein (Fig. 1). The purified rp20 was finally dialyzed to exchange buffers to 10 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol. The amino acid sequence at the N-terminal region of rp20 was analyzed using an Applied Biosystems model 373A protein sequencer. The analysis of amino acid sequence at the N-terminal region of rp20 (GPLGSDIVFDTEGNPIR) showed that rp20 has a N-terminal amino acid sequence of p20 (DIVFD-



**Fig. 1** Expression and purification of rp20. Lane 1, crude soluble fraction; lane 2, purified rp20.

TEGNPIR) and a sequence from part of the multiple cloning site of pGEX-6P-1 vector (GPLGS). The observed molecular mass of rp20 ( $20,495 \pm 20$ ) by TOF-MASS spectrum was in agreement with a theoretical molecular weight (20,506) of rp20 with the additional peptide (GPLGS). These results confirmed that rp20 has the same amino acid sequence as that deduced from the putative amino acid sequence of the p20 cDNA clone.

The amino acid residues at the reactive site of Ti ( $\text{Ti}^a$ ,  $\text{Ti}^b$  and  $\text{Ti}^c$ ), which were most intensively studied STI, are Arg (63)-Ile (64) (Blow *et al.*, 1974; Sweet *et al.*, 1974). However, Arg(63) of Ti is replaced by Lys(63) in p20 (Ashida *et al.*, 2000). It has also been reported that enzymatic replacement of the Arg (63) of Ti with Lys conserved the trypsin inhibitor activity of Ti (Sealock *et al.*, 1969). Therefore, the trypsin inhibition activities of rp20 and STI were compared. The inhibition activity for the hydrolysis of benzoyl-L-arginine *p*-nitroanilide (BAPA) was measured by the change in absorbance at 410 nm. For double-reciprocal analysis, rp20 (37 nM) or STI (47 nM) was preincubated at 25 °C in a total volume of 1.5 ml of 100 mM Tris-HCl (pH 7.0) containing 90 nM trypsin. After 8 min incubation, 0.5–2.4 mM BAPA (Peptide Institute, Inc) was added, and the absorbance at 410 nm was continuously measured for 15 min using a UV/VIS Spectrophotometer. For the stoichiometric examination of trypsin inhibition of rp20 and STI, the increasing amount of rp20 or STI was preincubated at 30 °C in a total volume of 0.3 ml of 100 mM Tris-HCl (pH 7.0) containing 83 nM trypsin. After 4 min incubation, 333  $\mu\text{M}$  BAPA was added, and the absorbance at 410 nm was continuously measured for 6 min by a microplate reader. One unit of trypsin activity was defined as the change of *p*-nitroaniline concentration per second ( $\text{nM s}^{-1}$ ). Fig. 2 shows the

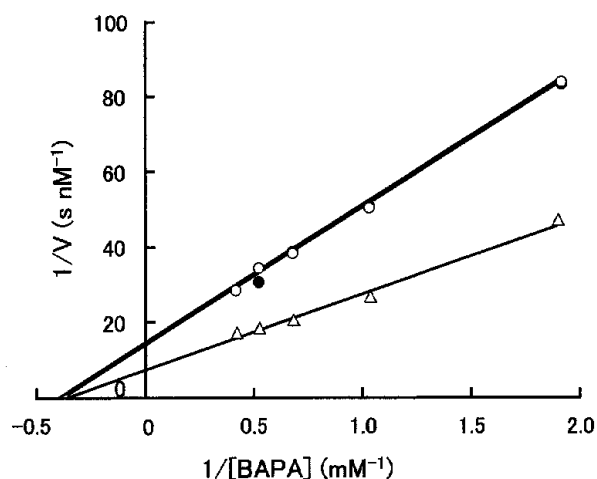


**Fig. 2** Inhibitory activity of rp20 and STI. Open circle, STI; closed circle, rp20.

**Table 1.** Comparison of the inhibitory activities of rp20 and STI

Protease	Substrate	pH	K <sub>i</sub> value (nM)	
			rp20	STI
Trypsin	Bz-Arg-pNA	7.0	50	75
$\alpha$ -Chymotrypsin	Suc-Ala-Ala-Pro-Phe-MCA	7.0	Ni <sup>1)</sup>	140
Elastase	Suc-Ala-Ala-Ala-pNA	7.0	Ni	207

<sup>1)</sup> Ni denotes no inhibition.



**Fig. 3** Double-reciprocal plot of the initial rate of trypsin activity toward BAPA in the presence and absence of inhibitors. Open triangle, no inhibitor; closed circle, rp20; open circle, STI.

stoichiometry of trypsin inhibition with rp20 and STI. Both rp20 and STI inhibited trypsin at a ratio of 1:1. The double-reciprocal plot analysis for the activity of trypsin with rp20 and STI showed that trypsin activity was non-competitively inhibited by both rp20 and STI (**Fig. 3**). The K<sub>i</sub> values of rp20 and STI (50 nM and 75 nM) were analogous, as shown in **Table 1**. These results show that both rp20 and STI interact with trypsin and trypsin-BAPA complex and inhibit trypsin activity by similar mechanisms.

It has been reported that STI can inhibit  $\alpha$ -chymotrypsin activity (Kunitz, 1947) and that the active site of STI toward trypsin and  $\alpha$ -chymotrypsin is analogous (Bidlingmeyer *et al.*, 1972). Therefore, the inhibition activities of rp20 and STI toward  $\alpha$ -chymotrypsin and elastase were measured to clarify their specificity toward other serine protease. The chymotrypsin activity was measured by the fluorimetric determination (excitation, 360 nm; emission, 465 nm) of the generation of 7-amino-4-methylcoumarin (AMC) in 96 well micro-titer plates. The rp20 (214 nM) or STI (48 nM) was preincubated at 30 °C in a total volume of 0.2 ml of 100 mM Tris-HCl (pH 7.0) containing 10 nM  $\alpha$ -chymotrypsin (Wako Pure Chemicals Ind., Ltd).

After 4 min incubation, 2  $\mu$ l of 1.5–10 mM succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methylcoumaryl-7-amide (Peptide Institute, Inc) was added and the changes in relative fluorescence units were continuously measured for 6 min. The elastase activity was measured by the hydrolysis of succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanilide (suc-Ala-Ala-Ala-pNA) (Peptide Institute, Inc) by a similar procedure to that described for the trypsin inhibitor activity, except for the concentration of elastase (Wako Pure Chemicals Ind., Ltd) (22 nM), rp20 (260 nM), STI (195 nM) and suc-Ala-Ala-Ala-pNA (0.24–1.308 mM).  $\alpha$ -Chymotrypsin was non-competitively inhibited by STI (K<sub>i</sub> = 140 nM), but not by rp20 (**Table 1**). Elastase was also inhibited by STI (K<sub>i</sub> = 207 nM), but not by rp20 (**Table 1**). An excessive amount of rp20 did not inhibit either serine protease. These results show that rp20 has different specificity toward serine proteases than STI.

The X-ray crystallographic analysis of a Ti-trypsin complex has indicated that Ti interacts with the trypsin at the positions of Asp (1), Asn (13), Ser (61), Tyr (62), Arg (63), Ile (64), Arg (65) and His (71) of Ti (Sweet *et al.*, 1974). However, the corresponding amino acid residues of p20 (Ashida *et al.*, 2000) were different from those of Ti at the positions of Phe (62), Lys (63), Leu (65) and Leu (71). Such differences in the amino acid residues at the positions of inhibitor-trypsin interaction may cause distinct specificity to serine proteases. On the other hand, Jofuku *et al.* (1989) reported another type of trypsin inhibitor, KT<sub>i</sub> (KT<sub>i</sub>1 and KT<sub>i</sub>2), whose corresponding amino acid residues are His (63)-Ala (64). They have reported that the KT<sub>i</sub> genes do not encode proteins with trypsin inhibitor activity, because KT<sub>i</sub> from transformed tobacco plants with KT<sub>i</sub> genes did not react with a Kunitz-type trypsin inhibitor antibody (Jofuku *et al.*, 1989). These results suggest that the STI family should be classified into three subfamilies and p20 may be a new member of the STI family.

## Acknowledgement

We thank Dr. Shunsuke Izumi of Hiroshima University for his useful discussion and the measurement of TOF-MASS spectrum.

## References

- Ashida, Y., Matsushima, A., Tsuru, Y., Hirota, T., Hirata, T., 2000. Isolation and sequencing of a cDNA clone encoding a 20-kDa protein with trypsin inhibitory activity. *Biosci. Biotechnol. Biochem.*, **64**: 1305–1309.
- Bidlingmeyer, U. D. V., Leary, T. R., Laskowski, M. Jr., 1972. Identity of the tryptic and  $\alpha$ -chymotryptic reactive sites on soybean trypsin inhibitor (Kunitz). *Biochemistry*, **11**: 3303–3310.
- Blow, D. M., Janin, J., Sweet, R. M., 1974. Mode of action of soybean trypsin inhibitor (Kunitz) as a model for specific protein-protein interactions. *Nature*, **249**: 54–57.
- Hirata, T., Izumi, S., Tsuji, S., 1999. A 20-kDa protein with the GTP-binding and trypsin inhibitory activities from *Glycine max*. *Biosci. Biotechnol. Biochem.*, **63**: 1816–1818.
- Jofuku, K. D., Goldberg, R. B., 1989. Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. *Plant Cell*, **1**: 1079–1093.
- Kim, S. H., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K., Kaizuma, N., 1985. Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors, Ti<sup>a</sup>, Ti<sup>b</sup>, and Ti<sup>c</sup>. *J. Biochem. (Tokyo)*, **98**: 435–448.
- Kunitz, M., 1947. Crystalline soybean trypsin inhibitor: II. general properties. *J. Gen. Physiol.*, **30**: 291–310.
- Luria, S. E., Adam, J. N., Teng, R. C., 1960. Transduction of lactose utilizing ability among strains of *Escherichia coli* and *Shigella dysenteriae* and the properties of the transducing phage particles. *Virology*, **12**: 348–390.
- Sealock, R. W., Laskowski, M. Jr., 1969. Enzymatic replacement of the arginyl by a lysyl residue in the reactive site of soybean trypsin inhibitor. *Biochemistry*, **8**: 3703–3710.
- Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., Blow, D. M., 1974. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-Å resolution. *Biochemistry*, **13**: 4212–4228.