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Article

A Study on the Three Dimensional Structure of Soybean Bowman-Birk Protease Isoinhibitor-DII Using Computer Aided Molecular Modeling

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Computer aided molecular modeling can help to predict the three dimensional structure of the polypeptide without the sample. The study on soybean Bowman-Birk protease inhibitor (SBI) is valuable, because it has been recently known that SBI possesses anticarcinogenic activities and immune-stimulating properties. SBI has several isoinhibitors, whose isolation and characterization were reported in 1990. Among these, DII inhibits trypsin only. The different inhibitory specificities cannot be explained only by their different primary sequences, but is possible with further assistance by the study on their different three dimensional structures. The study on the three dimensional structure of DII using homology method is reported in this paper.

Key words : *Bowman-Birk protease inhibitor, computer aided molecular modeling, soybean Bowman-Birk protease inhibitor.*

Protease inhibitors are commonly found in animals, plants, and microorganisms, especially rich in leguminous seeds.[1] These can be classified into two types, one of which is Kunitz soybean trypsin inhibitor and the other, BBPI.[2] BBPI was isolated by Bowman in 1946 and characterized by Birk in 1963. It consists of 71 amino acid residues and 7 disulfide bonds. BBPI can inhibit two proteases independently. The first reactive site with K16-S17 (region 1) inhibits trypsin, and the second reactive site with L43-S44 (region 2), chymotrypsin. BBPI retains two homologous regions, each of which is composed of three loops, loop I, II, III for region 1, and loop I', II', III' for region 2. The reactive sites are located in loop I and loop I', and each of them is comprised of 9 amino acid residues. The primary sequences of isoinhibitors found in the various leguminous seeds are slightly different from that of BBPI. The isoinhibitors can be classified into 4 groups. In group I, the primary sequences of the first and the second reactive sites have common sequences such as T-X-S-X-P-P and T-R-S-X-P-G, respectively. The isoinhibitors in group II have common sequences of T-K-S-X-P-P and T(A)-X-S-X-P-A. In group II, it has been known that an inhibitory specificity of BBPI for trypsin depends on K of the first reactive site, and as for chymotrypsin, L, Y or F placed on the first X position of the second reactive site. In the cases of peanut and soybean inhibitors, it is considered that the inhibitory specificity depends on the residues mentioned above as well as on the neighbouring residues. The inhibitory specificities of isoinhibitors cannot be explained based on the homologous sequences alone, but also on the three dimensional structure of the reactive sites.[3]

In order to determine the three dimensional structure of polypeptides, X-ray crystallography and NMR spectroscopy are usually used. Computer Aided Molecular Modeling can help to predict the three dimensional structure of the polypeptide. Since the three dimensional structure of BBPI obtained by NMR spectroscopy is reported in Protein Data Bank,[4] the three dimensional structures of BBPI-like isoinhibitors can be studied using the homology method. Recently, it has been known that SBIs have anticarcinogenic activities[5-11] and immune-stimulating properties.[12-16] SBI has several isoinhibitors, whose isolation and characterization were reported in 1990. Isoinhibitor A, which is BBPI, inhibits trypsin and chymotrypsin, CII elastase and trypsin,[17-20] and DII inhibits trypsin only.[3] The different inhibitory specificities cannot be explained by their different primary sequences alone, but also by the study on their different three dimensional structures. The study on the three dimensional structure of DII using the homology method

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Abbreviations: BBPI, Bowman-Birk protease inhibitor; SCRs, structurally conserved regions; SBI, soybean Bowman-Birk protease inhibitor

is reported in this paper.

Experimental Method

In order to use Homology method, firstly, a template, whose three dimensional structure had been known, was required. The primary sequence of BBPI showed the homologous sequence of DII at 61.9%, and its three dimensional structure was determined by NMR spectroscopy in 1992. The coordinate was obtained from Brookhaven Protein Data Bank. Since the arbitrary structure of the target protein, DII, was required, it was built by Viewer module of InsightII(msi). A computer used for calculations was Silicon Graphics workstation INDY R4400 and all softwares were supported by msi.

In order to find the three dimensional structure of the target protein, its primary sequence should be compared with that of the template in advance. After each pairwise comparison with no substitutions and no gaps, sequence matching regions showing the best scores were chosen and joined together.[21] In homology, it was supposed that the secondary structures of the inner core peptides having the identical primary sequences were conserved. In order to determine structurally conserved regions (SCRs), each interatomic distance matrix composed of α-carbons of the template and the target proteins was formed. Diagonal regions with no discrepancies were compared, and when the small rms value was found, the regions were chosen for SCRs. Subsequently, the off-diagonal regions composed of amino acids with similar chemical properties were compared, and the same procedure mentioned above was carried out. After the determination of SCRs, the alignment of two sequences was set up. If each sequence length was not identical, the gaps were inserted into the short segments.[21] In order to determine the proper coordinates for the variable regions, the method such as search loops was applied, where the Brookhaven protein database was searched for finding the regions of proteins satisfying a geometric criterion. For the side chain conformational searches, the protocol described by Ponder and Richards was used.[22] Finally, the molecular mechanics calculations including energy minimization and molecular dynamics were carried out for the refinement of the target protein.

Results and Discussion

Several isomers of SBIs have been known. Among these, CII has 76 amino acids, and its three dimensional structure was determined using Homology method based on the solution structure of BBPI.[23] According to the same method used for CII, the three dimensional structure of DII was calculated in this work. In order to apply Homology method, first of all, several SCRs should be determined. Comparing the primary sequence of DII with

DII : SDQSSSYD**DDEYSKPCCDL**CMCTRSMPPQCSCEDIRLNSCHSDC
BBPI : **DDESSKPCCDQ**CACTKSNPPQCRCSDMRLNSCHSAC
CII : SDHSSSD**DESSKPCCDL**CMCTASMPPQCHCADIRLNSCHSAC

DII : KSCMCTRSQPGQCRCLDTN**DFCYKPCKSRDD**
BBPI : KSCICALSYPAQCFCVDIT**DFCYEPCK**PSEDDKEN
CII : DRCACTRSMPGQCRCLDTT**DFCYKPCKSSDEDDD**

Fig. 1. A comparison of the primary sequences of DII and those of CII and BBPI. (SCRs are marked in bold, and loop I and loop I' are underlined.)

that of BBPI, nine SCRs are found, which are shown in Fig. 1 (marked in bold). As listed in Table 1, while DII inhibits trypsin at its first and second reactive sites, respectively, BBPI inhibits trypsin and chymotrypsin. In the case of BBPI, two reactive sites are K16-S17 and L43-S 44 which belong to loop I of region 1 and loop I' of region 2, respectively. Since the 9th residue of DII coincides with the first residue of BBPI, if the reactive sites of DII are placed in the same position as those of BBPI, R 24-S25 and R51-S52 are applicable. According to Odani and Ikenaka,[24] S17 and S44 of BBPI affect on the inhibitory activity, whereas K16 and L43, on the inhibitory specificity. Likewise, S25 and S52 of DII are considered to be the inhibitory residues, and R24 and R51, the specific inhibitory residues. The specificity for trypsin depends on R or K, and chymotrypsin depends on L, F or Y.[24] Since DII inhibits trypsin only, R24 and R51 should be considered to be the specific inhibitory residues. In addition, two reactive sites of BBPI are located in loop I and loop I', and R24-S25 and R51-S52 of DII reside in loop I and loop I'. Loop I of BBPI consists of 9 residues among C 14-C22, so that it is in a circle shape due to a disulfide bond. Likewise, loop I of DII consists of 9 residues among C22-C30. A comparison of loop I of BBPI and that of DII is shown in Fig. 2.

The rms value between the backbone of BBPI and that of DII is 1.86×10^{-6}Å. As shown in Fig. 2, seven residues of loop I except two residues are completely overlapped. K and N of BBPI are changed to R and M of DII, respectively. K and R have α, β, γ and δ carbons as well as a cation. Therefore, the change does not make a difference. The activity data of DII has not been yet known, so that the experimental comparison is not possible at present. When N18 of BBPI is changed into M26, both have α, β and δ carbons on the straight chain. However, Homology calculation shows that the case of N and M is

Table 1. The reactive sites of BBPI type protease inhibitors.

inhibitor	1st reactive site residue	inhibited enzyme	2nd reactive site residue	inhibited enzyme
DII	R	trypsin	R	trypsin
CII	A	elastase	R	trypsin
BBPI	K	trypsin	L	chymotrypsin

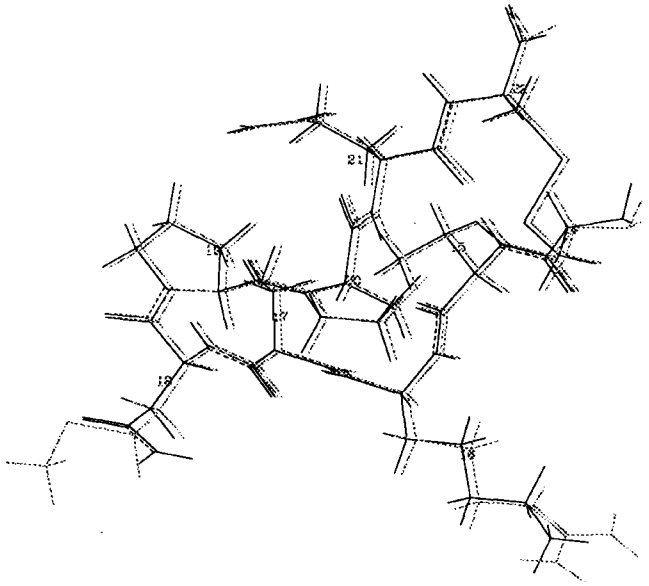

Fig. 2. A comparison of loop I of BBPI and that of DII.

different from that of K and R as shown in Fig. 2. Like K16 and R24, N18 and M26 do not affect on the inhibitory activity directly, but the conformational changes can cause the structural change of S residues. As a result, even though the activity depends on the S residue, the conformational change of the neighboring residues can produce the three dimensional structure change of the S residue, therefore it is suggested that there is a difference between the inhibitory activity caused by loop I of BBPI and that of DII.

The other reactive site of BBPI is located at loop I' which is composed of 9 residues. Like loop I, loop I' starts from C41 and ends at C49 so that it is in a round form. While 7 residues in loop I of DII are identical to those of BBPI, only 5 residues in loop I' of DII are the same. And while the second reactive site of BBPI inhibits chymotrypsin, that of DII does trypsin. In BBPI, the residue showing the inhibitory specificity is L43, and in DII, R51. The side chain of L43 is much shorter than that of R which is charged positively as shown in Fig. 3. The result of Homology calculation meets our expectation that L43 is not superimposed on R51. The rms value between the backbone of BBPI and that of DII is 0.70Å. The other three different residues are A42→T50, Y45→Q53 and

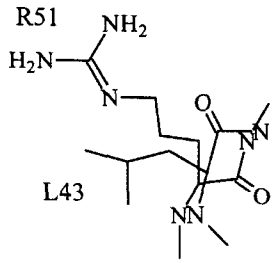

Fig. 3. A partial structure of overlapped L43 of BBPI and R 51 of DII.

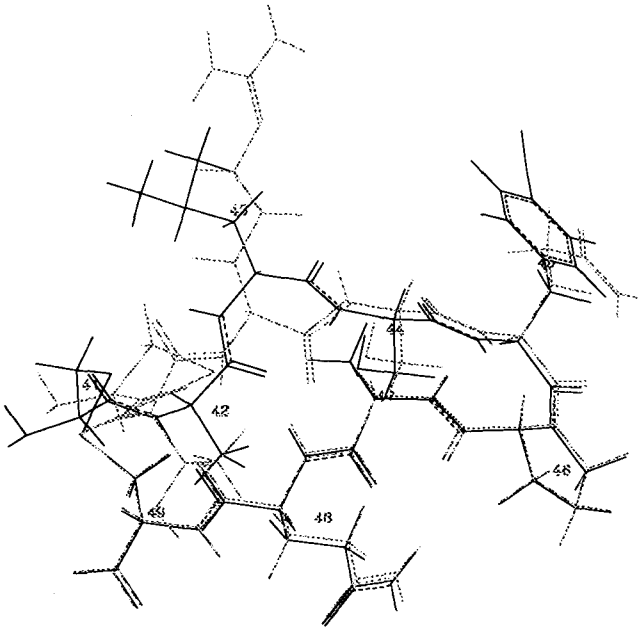

Fig. 4. A comparison of loop I' of BBPI and that of DII.

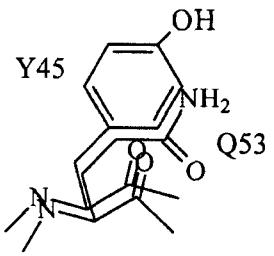

Fig. 5. A partial structure of overlapped Y45 of BBPI and Q 53 of DII.

A47→G55. The partial structure of Loop I' of DII obtained from Homology calculation, compared with that of BBPI, is shown in Fig. 4. In the case of T50 of BBPI, the hydroxyl and the methyl groups are appeared to be bulky, but as C_β has a tetrahedral structure, two groups can occupy instead of two protons of A42.

As a result, the side chains of two residues can be overlapped. Like T50, the change of A47 into G55 does not make significant differences because the methyl group of A47 can reside in one of two protons of G55. The case of Q53 is expected to differ from Y45 because of the change of the aromatic ring. However, Homology calculation shows very interesting results as shown in Fig. 5. C_β, C_γ and C_δ of Q53 are congruous with those of Y45, and the double bond of the carbonyl group is parallel to Cδ-Cε bond of the aromatic ring. Unlike a comparison of the second reactive site of DII with that of BBPI, a comparison with that of CII is expected to be worthwhile because both DII and CII inhibit trypsin at their second reactive sites. As shown in Fig. 1, loop I' of CII is comprised of CTRSMPGQC, and that of DII, CTRSQPGQC. Eight residues except Q53 or M51 are the same so that DII and CII are expected to show the same inhibitory

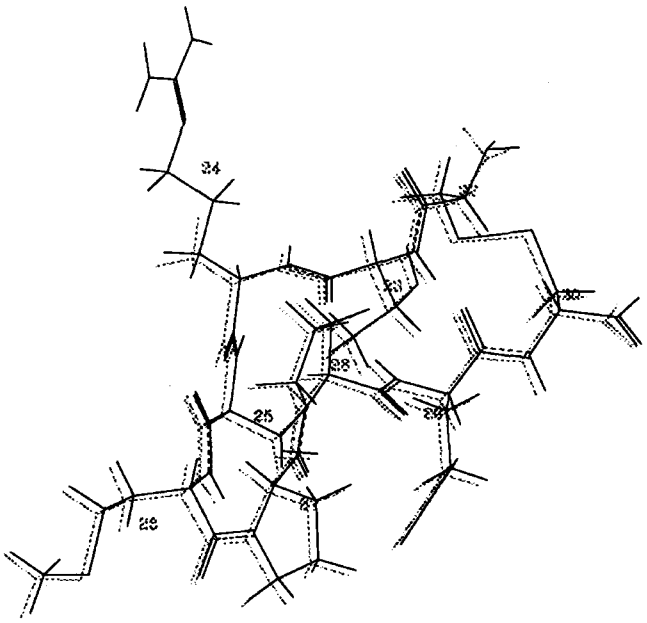

Fig. 6. A comparison of loop I' of DII and that of CII.

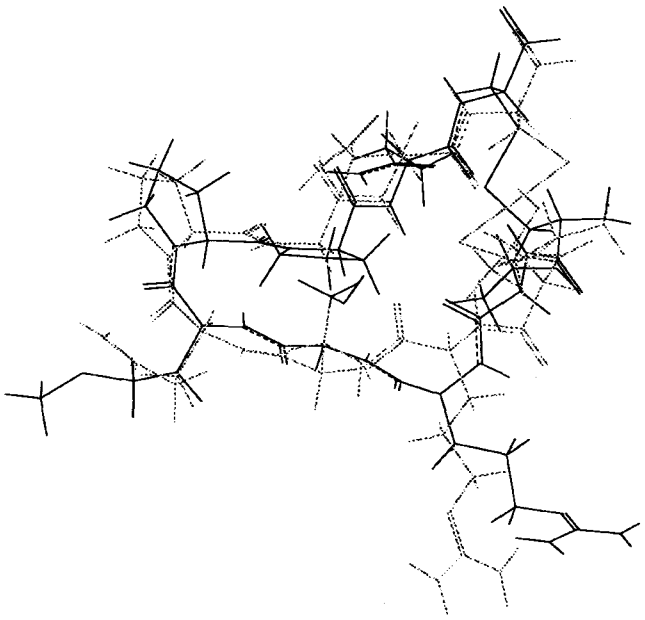

Fig. 7. A comparison of loop I (solid line) and loop I' (broken line) of DII.

specificity and activity. It is known that their inhibitory specificities meet our expectation. As shown in Fig. 6, however, Q53 of DII and M51 of CII are not completely overlapped. The rms value between the backbone of loop I' of DII and that of CII is 0.027Å. If the conformational difference of the residue neighboring to S, which controls the inhibitory activity, affects on the three dimensional structure of the reactive residue, it is expected that the inhibitory activity against trypsin of the second reactive site of DII differs from that of CII. If the recombinant DII is obtained, another comparison would be observed. Loop I and loop I' of DII inhibit trypsin, and their primary sequences are CTRSMPPQC and CTRSQPGQC, respectively. A comparison of loop I and loop I' of DII is shown in Fig. 7. The rms value between the backbone of loop I of DII and that of loop I' of DII is 0.907Å. Because two residues differ from each other, a little larger rms value than the case of CII is considered to be observed. The rms values compared in this study are listed in Table 2.

According to the notation by Schechter *et al.*, two residues of the reactive site are named P1 and P1'.[25] The inhibitory specificity is dependent on P1, and the inhibitory activity, P1'.[24] When P1 is R or K, the reactive site inhibits trypsin. The presence of S in P1' determines the inhibitory activity. Even though P1 and P1' are the same, however, if the surrounding residues (P3P2P1P1'P2'P3'P4'P5'P6') around P1 and P1' differ from each other, the three dimensional structures of the reactive sites may not be the same based on the comparisons mentioned above.

As a result, the first and the second reactive sites of DII, the first reactive site of BBPI, and the second reactive site of CII inhibit trypsin, but it cannot be said that their inhibitory activities are the same. The evidence of this conclusion will be clarified in further studies of the recombinant DII and CII, which are undergoing by one of author's research groups.

Table 2. Lists of rms values compared in this study.

template peptide	target peptide	rms/Å
loop I of BBPI	loop I of DII	1.86×10^{-6}
loop I' of BBPI	loop I' of DII	0.70
loop I' of DII	loop I' of CII	0.027
loop I of DII	loop I' of DII	0.907

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References

1. Zimacheva, A. V. and Mosolov, V. V. (1995) Cysteine proteinase-inhibitors from soybean seeds. *Biochemistry* (Moscow) **60**, 83-87.
2. Marchetti, S., Pittoti, A., Giordano, A., Chiaba, C. and Fogher, C. (1995) Partial inactivation of microbial proteinases with soybean Kunitz and Bowman-Birk inhibitors. *J. Sci. Foods. Agric.* **69**, 423-428.
3. Ikenaka, T. and Norioka, S. (1986) In *Proteinase inhibitors: Bowman-Birk family serine proteinase inhibitors,* Ch. 9, pp. 361-374, Elsevier Pub.
4. Werner, M. H. and Wemmer, D. E. (1992) Three-dimensional structure of soybean trypsin/chymotrypsin Bowman-Birk inhibitor in solution. *Biochemistry* **31**, 999-1010.
5. Das, S. and Mukhopadhyay, P. (1994) Proteinase inhibitors in chemoprevention of cancer. *Acta Oncol.* **33**, 859-865.
6. Kollipara, K. P., Singh, R. J. and Hymowitz, T. (1995) Genomic relationships in the genus glycine. *Am. J. Bot.*

82, 1104-1111.

7. Fernandes, A. O. and Banerji, A. P. (1995) Inhibition of benzopyrene-induced forestomach tumors by field bean proteinase inhibitor. *Carcinogenesis* **16**, 1843-1846.
8. Kennedy, A. R. (1995) The evidence for soybean products as cancer preventive agents. *J. Nutr.* **125**, S733-S743.
9. Liener, I. E. (1995) Possible adverse-effects of soybean anticarcinogens. *J. Nutr.*, **125**, S744-S750.
10. Clawson, G. A. (1996) Proteinase inhibitors and carcinogenesis. *Cancer Invest.* **14**, 597-608.
11. Kennedy, A. R., Beazerbarclay, Y., Kinzler, K. W. and Newberne, P. M. (1996) Of carcinogenesis in the intestines of min mice by the soybean-derived Bowman-Birk inhibitor. *Cancer Res.* **56**, 679-682.
12. Hajos, G., Gelenscer, E., Grant, A., Sakhri, M. and Bardocz, S. (1995) Biological effects and survival of trypsin-inhibitors and the agglutinin from soybean in the small-intestine of the rat. *J. Agric. Food Chem.* **43**, 165-170.
13. Kennedy, A. R. and Manzone, H. (1995) Effects of proteinase inhibitors on levels of proteolytic activity in normal and premalignant cells and tissues. *J. Cell. Biochem.* 188-194.
14. Mcmanus, M. T., Corner, R. and Garthwaite, I. (1995) Characterization of monoclonal-antibodies that recognize the soybean (Kunitz) trypsin-inhibitor-binding to the inhibitor interrupts the formation of the trypsin-inhibitor complex. *J. Plant Physiol.* **146**, 243-248.
15. Kennedy, C. W., Donahue, J. J. and Wan, X. S. (1996) Effects of the Bowman-Birk proteinase inhibitor on survival of fibroblasts and cancer-cells exposed to radiation and cisplatinum. *Nutr. Cancer* **26**, 209-217.
16. Arentoft, A. M., Frokiaer, H., Sorensen, H. and Sorensen, S. (1994) Determination of pea proteinase-inhibitors using ELISA based on monoclonal-antibodies. *Acta Agri. Scand. Section B-Soil Plant Sci.* **44**, 236-243.
17. Gladysheva, I. P., Polekhina, O. V., Shen, W. C., Shevchenko, A. A., Kazanskaya, N. F. and Larionova, N. I. (1995) Structure and biological properties of Bowman-Birk soybean proteinase-inhibitor conjugated with block-copolymer of ethylene-oxide and propylene-oxide. *Biochemistry* (Moscow) **60**, 385-391.
18. Frokiaer, H., Mortensen, K., Sorensen, H. and Sorensen, S. (1996) Characterization of protein type proteinase-inhibitors by high-performance capillary electrophoresis. *J. Liq. Chromatog. Rel. Technol.* **19**, 57-67.
19. Mcbride, J. D., Freeman, N., Domingo, G. J. and Leatherbarrow, R. J. (1996) Selection of chymotrypsin inhibitors from a conformationally-constrainted combinatorial peptide library. *J. Mol. Biol.* **259**, 819-827.
20. Reseland, J. E., Holm, H., Jacobsen, M. B., Jenssen, T. G. and Hanssen, L. E. (1996) Proteinase inhibitors induce selective stimulation of human trypsin and chymotrypsin secretion. *J. Nutr.* **126**, 634-642.
21. Needleman, S. B. and Wunsch, C. D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* **48**, 443-453.
22. Ponder, J. W. and Richards, F. M. (1987) Tertiary templates in proteins: Use of packing criteria in the enumeration of allowed sequences for different structural classes. *J. Mol. Biol.* **65**, 775-791.
23. Lim, Y., Oh, M., Kim, J. and Kim, S. (1997) A study on the three dimensional structure of Bowman-Birk type proteinase isoinhibitor-CII using computer aided molecular modeling. *Agric. Chem. Biotechnol.* **40**, 597-599.
24. Odani, S. and Ikenaka, T. (1978) Studies on soybean trypsin inhibitors: XIV. Change of the inhibitory activity of Bowman-Birk inhibitor upon replacements of the α-chymotrypsin reactive site serine residue by other amino acids. *J. Biochem.* **84**, 1-9.
25. Schechter, I. and Berger, A. (1967) On the size of the active site in proteases: I. Papain. *Biochim. Biophy. Res. Comm.* **27**, 157-162.