Case 7

A Storage Protein From Seeds of *Brassica nigra* is a Serine Protease Inhibitor

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Focus concept

Purification of a novel seed storage protein allows sequence analysis and determination of the protein's secondary and tertiary structure.

Prerequisites

- Protein purification techniques, particularly gel filtration and dialysis.
- Protein sequencing using Edman degradation and overlap peptides.
- Structure and mechanism of serine proteases.
- Reversible inhibition of enzymes.

Background

Seedlings use seed storage proteins as an important nitrogen source during germination. The seed storage proteins are made as large precursors, then hydrolyzed to smaller products for the seedling's use during growth. In this case, the investigators discovered a new seed storage protein, which they named BN, in the oilseed *Brassica nigra*. These seeds are important nutritionally as a source of oil as well as protein. The storage protein described here was first purified and then characterized for its important biochemical properties. The storage protein turned out to be an inhibitor of serine protease enzymes. The authors hypothesized that the purpose of serine protease inhibitors like BN is to protect the plant from proteolytic enzymes of insects and microorganisms that would damage the plant.

The protein BN likely belongs to a family of seed storage proteins called napins, which typically consist of two non-identical disulfide-bonded polypeptide chains. Napins make up as much as 20% of the protein content of seeds

Questions

1. In order to isolate the protein, seeds were ground and extracted with water. The proteins in the extract were precipitated with hydrochloric acid and the pellet isolated by centrifugation was discarded. The supernatant was heated to 70˚C to remove heat-labile proteins, then lyophilized (freeze-dried). The lyophilized powder was dissolved in a small amount of ammonium acetate buffer at pH = 5 and the sample was loaded onto a Sephadex G-25[1] gel filtration column. The elution profile showed four peaks. Most of the BN protein eluted in the first peak.
   a. On what basis is separation achieved on the Sephadex gel filtration column?
   b. Compare the BN protein to other proteins found in the *B. nigra* seeds.

2. Following gel filtration chromatography, the BN protein was further purified by dialysis using tubing with a 6000-8000 molecular weight cut-off. Analysis using SDS-PAGE (in the absence of β-mercaptoethanol) showed a single band. The results are shown in Figure 7.1.
   a. Why is the BN protein more pure after the dialysis step?
   b. Determine the molecular weight of the BN protein by constructing a standard curve (plot molecular weight of the standards vs migration distance in the gel). Why was the gel run in the absence of β-mercaptoethanol?

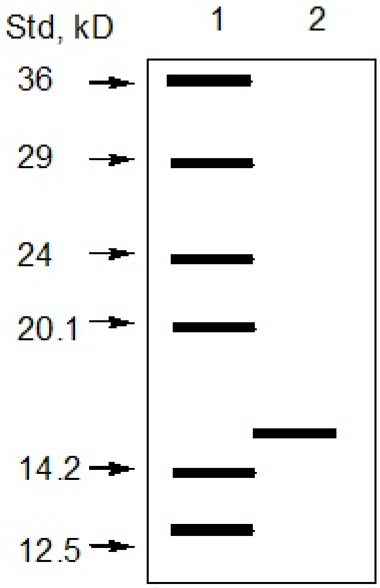

Figure 7.1: Analysis of BN protein by SDS-PAGE. The purified BN protein is shown in Lane 2; the standards in Lane 1 (modified from Genov, *et al.*, 1997).

3. Next, the investigators attempted to sequence the protein using an Edman degradation procedure. However, this was initially unsuccessful because the amino terminus was blocked. Based on comparisons with other proteins in the same family as BN whose sequences are known, the investigators hypothesized that the amino terminal amino acid was *N*-acetyl serine.
   a. Draw the structure of *N*-acetyl serine.
   b. If *N*-acetyl serine was indeed the amino terminal amino acid, why would sequencing using the Edman method be unsuccessful?

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[1] Sephadex G-25 beads exclude proteins with molecular weights greater than 25 kD.

4. The amino acid sequence of BN was finally determined in the following manner: The BN protein was first treated with β-mercaptoethanol. This treatment revealed that the BN protein consisted of two chains, a light chain and a heavy chain[2]. Next, the light chain and heavy chain were separated and then three separate samples of the purified chains were treated with three different proteases. The fragments obtained were individually sequenced using the Edman method. The protein sequence is shown in Table 7.1. The light chain is 39 amino acids long and the heavy chain is 91 amino acids long.
   a. Why was it necessary to carry out a minimum of two different proteolytic cleavages of the protein using different proteases?
   b. One of the enzymes used by the investigators was trypsin. Write the sequences of the fragments that would result from trypsin digestion.
   c. Choose a second protease to cleave the light and heavy chains. What protease did you choose, and why? Write the sequences of the fragments that would result from the digestion of the protease you chose.

Table 7.1: Amino acid sequences of the BN protease inhibitor from *Brassica nigra* seeds. Note that the first five amino acid residues of the light chain are missing due to a blocked amino terminal amino acid (based on Genov, *et al.*, 1997).

Light chain

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1						Arg	Ile	Pro	Lys	Cys	Arg	Lys	Glu	Phe	Gln
16	Gln	Ala	Gln	His	Leu	Arg	Ala	Cys	Gln	Gln	Trp	Leu	His	Lys	Gln
31	Ala	Asn	Gln	Ser	Gly	Gly	Gly	Pro	Ser						

Heavy chain

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Pro	Gln	Gly	Pro	Gln	Gln	Arg	Pro	Pro	Leu	Leu	Gln	Gln	Cys	Cys
16	Asn	Glu	Lys	His	Gln	Glu	Glu	Pro	Leu	Cys	Val	Cys	Pro	Thr	Leu
31	Lys	Gly	Ala	Ser	Lys	Ala	Val	Arg	Gln	Gln	Ile	Arg	Gln	Gln	Gly
46	Gln	Gln	Gln	Gly	Gln	Gln	Gly	Gln	Gln	Leu	Gln	Arg	Glu	Ile	Ser
61	Arg	Ile	Tyr	Gln	Thr	Ala	Thr	His	Leu	Pro	Arg	Val	Cys	Asn	Ile
76	Pro	Arg	Val	Ser	Ile	Cys	Pro	Phe	Gln	Lys	Thr	Met	Pro	Gly	Pro
91	Ser														

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[2] Separation of the two chains by disulfide bridge reduction destroyed the inhibitory capabilities of the BN protein.

5. Circular dichroism (CD) is a technique used for determining the secondary structure (α-helix and β-pleated sheet) of a protein. The authors took CD spectra of the protein at varying temperatures at various wavelengths. Then they plotted the results obtained at 220 nm. The results are an indication of the integrity of the secondary structure of the BN protein. A decrease from 100% indicates a loss of secondary structure integrity. The results are shown in Figure 7.2. What do these data tell you about the stability of the protein at various temperatures?

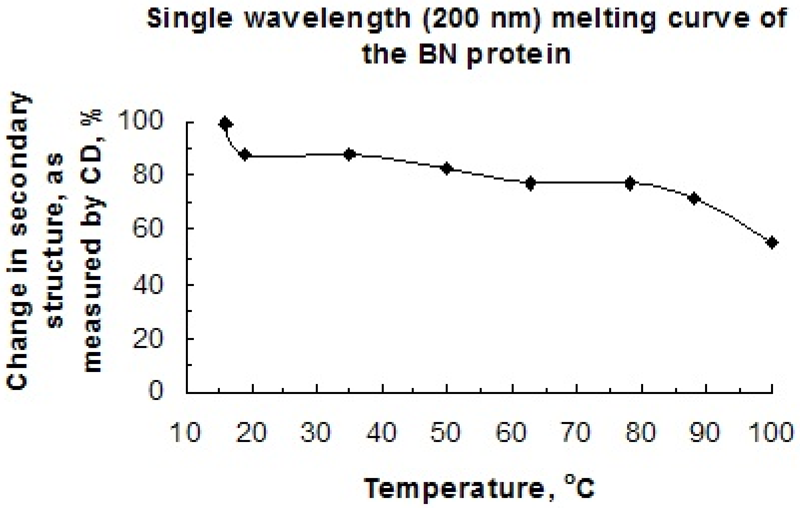


Figure 7.2: Single wavelength melting curve of the protease inhibitor BN from *Brassica nigra* (based on Genov, *et al.*, 1997).

6. The BN protein is a competitive inhibitor of the serine proteases trypsin, subtilisin and chymotrypsin. The percentage inhibition was measured for each enzyme in the presence of BN protein. The results are shown in the Table 7.2. Using what you know about enzyme inhibition, describe how BN inhibits the proteolytic activity of these serine proteases. Be sure to explain how a single inhibitor can inhibit three different enzymes.

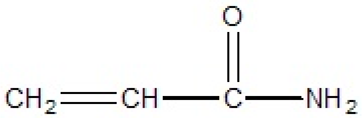
Table 7.2: The BN protein is a serine protease inhibitor (Based on Genov, *et al.*, 1997).

Enzyme	**% inhibition at [BN] = 2.0 x 10^{-6} M**
trypsin	100%
subtilisin	100%
chymotrypsin	32%

7. The authors next carried out fluorescence studies in order to obtain additional information concerning the three-dimensional structure of the BN protein. Specifically, they were trying to determine if there was an interaction between Tyr 63 and Trp 26, and for this reason, they decided to study the microenvironment of the Trp residue. Only tryptophan and tyrosine are *fluorophores*, which means they are capable of undergoing fluorescence. They found that a single tryptophan residue emits light at 330 nm upon excitation at 295 nm.

   There are several ions and small molecules that have the ability to *quench* fluorescence. This means that upon excitation at 295 nm, the tryptophan will transfer its energy to the quenching agent rather than releasing the energy in emitted light. Cesium (Cs^+) ions, iodide (I^- ions) and acrylamide (see structure in Figure 7.3) are capable of quenching the tryptophan residue's fluorescence–that is, if these quenching agents can make suitable contact with the tryptophan.

   Neither of the quenching agents worked very well. The investigators found that there was no quenching at all with cesium ions and that quenching efficiencies with iodide and acrylamide were very low. What do these observations tell you about the microenvironment of the tryptophan residue? Is an interaction between Tyr 63 and Trp 26 likely?



$CH_2=CH-C(=O)-NH_2$

Figure 7.3: Structure of acrylamide

Reference

Genov, N., Goshev, I., Nikolova, D., Georgieva, D. N., Filippi, B., and Svendsen, I. (1997) *Biochim. Biophys. Acta*, **1341**, pp. 157-164.