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# Studies on molecular evolution and structural features of double-headed inhibitors of α-amylase and trypsin in plants

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**Abstract.** Plant seeds usually have high concentrations of proteinase and amylase inhibitors. These inhibitors exhibit a wide range of specificity, stability and oligomeric structure. In this communication, we report analysis of sequences that show statistically significant similarity to the double-headed α-amylase/trypsin inhibitor of ragi (*Eleusine coracana*). Our aim is to understand their evolutionary and structural features. The 14 sequences of this family that are available in the SWISSPROT database form three evolutionarily distinct branches. The branches relate to enzyme specificities and also probably to the oligomeric state of the proteins and not to the botanical class of the plant from which the enzymes are derived. This suggests that the enzyme specificities of the inhibitors evolved before the divergence of commercially cultivated cereals. The inhibitor sequences have three regions that display periodicity in hydrophobicity. It is likely that this feature reflects extended secondary structure in these segments. One of the most variable regions of the polypeptide corresponds to a loop, which is most probably exposed in the native structure of the inhibitors and is responsible for the inhibitory property.

**Keywords.** Inhibitors; sequence comparison; evolution; trypsin; amylase.

## 1. Introduction

Rapid advances in automated sequencing of DNA and proteins have led to an explosive growth of databases of sequences. These databases contain valuable information on the evolutionary history of proteins and of the organisms that produce them. Computer-based analysis of these sequences has become an integral part of studies in molecular evolution and structural biology. A variety of protein families have been analysed from both perspectives (Doolittle 1979; Kyte and Doolittle 1982).

Plant seeds contain a host of serine proteinase and α-amylase inhibitors that are specific for the enzymes from fungal and bacterial plant pathogens. Hence these proteins are generally believed to be part of the plant defense system. They are good candidates for studies on evolution, structure and function as they exhibit a broad range of oligomeric structure, enzyme specificity, and stability with respect to pH and temperature.

Laskowski and Kato (1980) carried out extensive studies on serine proteinase inhibitors of plants and animals and suggested a classification scheme based on all the biochemical information available at that time. In their scheme, the inhibitors from plants fall into four major classes: soybean trypsin inhibitors (Kunitz),

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Bowman–Birk inhibitors, potato inhibitor I, potato inhibitor II. A double-headed α-amylase/trypsin inhibitor, which does not belong to any of these classes, was subsequently found to occur in ragi (*Eleusine coracana*) seeds by Shivaraj and Pattabhiraman (1980, 1981; Shivaraj *et al.* 1982). This inhibitor was also found to be present in several other cereals. These inhibitors together form a new class of plant seed inhibitors generally referred to as the α-amylase/trypsin inhibitor family. Several of these proteins have been sequenced. This communication presents an analysis of these sequences with a view to understanding their possible relation to other inhibitor families and their evolutionary and structural details.

## 2. Methods of analysis

### 2.1 *Sequence sources*

The sequences of the plant seed inhibitors were obtained from SWISSPROT databank, release September 1993, available at the Bioinformatics Centre of the Indian Institute of Science.

### 2.2 *Sequence comparisons*

The sequences were compared pairwise using IALIGN from the PRONUC suite of programs (Desai and Borne 1986). This program uses the well-known Needleman and Wunsch algorithm (Needleman and Wunsch 1970) for deriving the optimal alignment of sequences. The score for alignment was evaluated using Dayhoff's log odds matrix (Schwartz and Dayhoff 1978). The matrix elements were made positive by addition of 6. A penalty of 6 was used to minimize the gaps introduced in the alignment procedure. The penalty was independent of gap length. There were 14 sequences in the databank that could be classified as belonging to the cereal α-amylase/trypsin inhibitor family. The significances of the alignments were derived by comparing pairs of sequences obtained by randomization of the original sequences. One hundred such comparisons were used to derive the mean and the standard deviation of scores for unrelated sequences. The significance of the score for alignment of the native sequences was expressed in units of standard deviation above the mean score for unrelated sequences. The sequences of the inhibitors were compared with all the entries in the SWISSPROT database using the program FASTA (Pearson and Lipman 1988; Pearson 1990). This program identified significant similarities between α-amylase/trypsin double-headed inhibitors and chloroform/methanol-soluble plant seed storage proteins.

### 2.3 *Multiple alignment and construction of phylogenetic tree*

It is computationally prohibitive to carry out multiple alignment using dynamic programming algorithms. We therefore used the program MULTALIN (Cockerill 1993) to achieve alignment of the 14 sequences belonging to the double-headed α-amylase/trypsin inhibitor family. This program uses less rigorous procedures; nevertheless, it has been found to produce near-optimal solutions for multiple sequence alignment. In this method, initially short stretches of identity between

sequences are located, and these initial alignments are then expanded using Dayhoff's log odds matrix with a suitable gap penalty for deriving scores and combining aligned stretches. After achieving multiple alignment the sequences are hierarchically clustered. The alignment of the pair of sequences that had the highest score was initially accepted and the aligned pair was treated as a single sequence. Each further step combined either two sequences, or clusters, or a sequence and a cluster. The similarity measure was reevaluated after each combination. The procedure was continued till all the sequences were merged. After completing multiple alignment, pairwise scores were reevaluated. A binary phylogenetic tree was derived on the basis of the order in which sequences were merged. The lengths of the branches were adjusted to reflect the pairwise alignment scores.

### 2.4 *Hydrophobicity plots*

The variation of hydrophobicity along the polypeptide sequence is an important characteristic that often reflects the role of a given segment in the native structure of a protein. Hydrophobicity of different segments of the sequences in the present case is best represented as the average over the aligned residues,

$$\langle H_i \rangle = \sum_j H_{ij} / 14,$$

where $j$ is the index over sequences and $i$ the relative position of the residue in the sequence.

### 2.5 *Construction of ancestral sequences*

The aligned sequences of the 14 inhibitors were used to construct a plausible ancestral sequence based on parsimony considerations. At each position, that amino acid was assigned to the ancestral sequence which required minimum total base changes for mutation to each of the residues in the corresponding position of the 14 inhibitors. This procedure was also used to deduce ancestral sequences for subgroups of these 14 sequences. The number of base changes needed to convert any residue to a gap was assumed to be 3.

### 2.6 *Difference plots*

Plots of variation in hydrophobicity, side-chain volume, and average base change from ancestral sequence in windows of specified length placed at each residue position of the aligned set were obtained. Similar plots were also made between ancestral sequence and the 14 sequences or any subgroup of them.

## 3. Results

### 3.1 *Identification of proteins of the α-amylase/trypsin inhibitor family*

There were 170 sequences of enzyme inhibitors from plant sources in the SWISSPROT database. The length of the sequences varied from 30 residues for the short squash

inhibitor family to 250 residues for the ribosome inactivating factors and shiga-like toxins. Several entries were of partial sequence determinations. Sequences of some closely related inhibitors were found in the list. Several sequences had an N-terminal, clearly identified signal sequence.

Initially, all the sequences of plant inhibitors found in the database were used for construction of phylogeny using the MULTALIN program. The large tree so obtained suggested that 13 other sequences were likely to be related to the ragi double-headed α-amylase/trypsin inhibitor. It is possible that presence of short sequences and signal sequences might affect identification of double-headed inhibitors. We therefore eliminated small sequences corresponding to partial determinations from the list and pruned the signal sequences. The tree obtained using this truncated set of sequences was slightly different from the initial tree. However, there was no change in the list of sequences identified as related to the ragi double-headed inhibitor. Hence these core 14 sequences (table 1) were accepted as belonging to a single family and were further analysed for evolutionary and structural features.

**Table 1.** List of plant seed inhibitors used for sequence analysis.

| Sequence | Database reference | Source/details of the protein |
|---|---|---|
| S12 | IA01_WHEAT P16850 | α-Amylase/trypsin inhibitor (CM1 protein) from wheat; tetramer |
| S13 | IA02_WHEAT P16851 | α-Amylase/trypsin inhibitor (CM2 protein) from wheat |
| S14 | IA03_WHEAT P17314 | α-Amylase/trypsin inhibitor (CM3 protein) from wheat; tetramer |
| S15 | IA16_WHEAT P16159 | α-Amylase/trypsin inhibitor (CM16 protein) from wheat; tetramer |
| S18 | IAA1_WHEAT P01085 | α-Amylase inhibitor (0·19) from wheat; homodimer |
| S19 | IAA2_WHEAT P01083 | α-Amylase inhibitor (0·28) from wheat; monomer |
| S21 | IAA5_WHEAT P01084 | α-Amylase inhibitor (0·53) from wheat; homodimer |
| S22 | IAAA_HORVU P28041 | α-Amylase/trypsin inhibitor (CMa protein) from barley |
| S23 | IAAB_HORVU P13691 | α-Amylase inhibitor (BDAI protein) from barley; homodimer |
| S24 | IAAD_HORVU P11643 | α-Amylase/trypsin inhibitor (CMd protein) from barley; tetramer |
| S25 | IAAE_HORVU P01086 | Trypsin inhibitor (CMe protein) from barley |
| S29 | IAAT_ELECO P01087 | α-Amylase/trypsin inhibitor from ragi |
| S31 | IAA_HORVU P16969 | α-Amylase/trypsin inhibitor from barley |
| S115 | ITRF_MAIZE P01088 | Trypsin/factor 12A inhibitor from maize |

### 3.2 *Relationship of double-headed inhibitor sequences to other families of plant inhibitors*

The phylogeny obtained using all the sequences suggested that the cereal double-headed inhibitors might be distantly related to the Kunitz inhibitor family, thaumatin-like proteins and Bowman–Birk inhibitors. These relationships were critically examined using the more rigorous IALIGN procedure. The alignment scores obtained in these comparisons were less than 3, indicating that the double-headed inhibitors do not have detectable sequence similarity to the other families of plant seed inhibitors.

### 3.3 *Comparison of sequences of cereal double-headed inhibitors*

The list of 14 sequences selected for comparison contains a few isoinhibitors (inhibitors with the same specificity from the same plant species). Thus S12 and S13 (table 1) are both α-amylase/trypsin inhibitors from wheat. Similarly, S18 and S21 are α-amylase inhibitors from wheat. Isoinhibitor sequences are identical at all but a few residues. The amino acid identity between sequences that are not isoinhibitors varies from 27·7% (between S18, wheat α-amylase inhibitor, and S22, barley α-amylase/trypsin inhibitor) to 82·3% (between S22 and S13, both α-amylase/trypsin inhibitors). The alignment scores for pairs of isoinhibitors are in the range of 35 to 40, the value generally obtained for self comparison of sequences that are approximately equal in length to the cereal double-headed inhibitors. For other pairs of sequences the alignment scores vary between 5 and 38. It is clear that these sequences form a family. Figure 1

| | S12 | S13 | S14 | S15 | S18 | S19 | S21 | S22 | S23 | S24 | S25 | S29 | S31 | S115 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S12 | 39.84 | 36.79 | 18.94 | 20.80 | 6.57 | 5.35 | 6.96 | 35.32 | 6.56 | 17.72 | 14.91 | 18.21 | 13.17 | 14.02 |
| S13 | | 40.74 | 18.27 | 20.39 | 6.66 | 7.42 | 5.65 | 33.79 | 6.91 | 17.70 | 14.58 | 20.43 | 12.16 | 13.34 |
| S14 | | | 44.14 | 20.64 | 7.15 | 5.48 | 6.65 | 17.66 | 5.16 | 36.80 | 14.13 | 13.10 | 12.40 | 13.24 |
| S15 | | | | 37.21 | 8.05 | 6.82 | 6.20 | 18.90 | 7.45 | 20.80 | 12.92 | 16.94 | 13.85 | 12.28 |
| S18 | | | | | 39.17 | 21.45 | 37.83 | 4.87 | 19.29 | 6.05 | 9.58 | 9.59 | 7.14 | 7.04 |
| S19 | | | | | | 34.15 | 19.80 | 6.22 | 24.32 | 5.04 | 6.20 | 8.98 | 6.57 | 5.30 |
| S21 | | | | | | | 38.10 | 5.21 | 17.92 | 5.76 | 9.15 | 9.04 | 7.67 | 7.18 |
| S22 | | | | | | | | 37.66 | 5.51 | 17.3 | 13.37 | 17.33 | 11.28 | 12.59 |
| S23 | | | | | | | | | 38.15 | 6.11 | 6.92 | 8.06 | 5.69 | 5.73 |
| S24 | | | | | | | | | | 38.71 | 14.12 | 13.58 | 14.68 | 15.7 |
| S25 | | | | | | | | | | | 38.08 | 24.81 | 19.32 | 17.75 |
| S29 | | | | | | | | | | | | 33.82 | 18.02 | 23.54 |
| S31 | | | | | | | | | | | | | 40.02 | 17.79 |
| S115 | | | | | | | | | | | | | | 40.07 |

**Figure 1.** Alignment scores for all pairs of 14 inhibitor sequences. Scores are expressed in units of standard deviation above the mean background obtained for comparison of unrelated sequences of identical length and amino acid composition.

shows the alignment scores for all pairs of sequences. Figure 2 shows the alignment of all the 14 sequences.

In 11 of the 14 sequences all the cysteine residues involved in disulphide bridges (Campos and Richardson 1983; Maeda *et al.* 1983, 1985) are conserved. Two of these cysteine residues are separated by a conserved arginine residue (Cys-Arg-Cys) in 13 sequences. Only in S21 (wheat α-amylase inhibitor), the tripeptide Pro-Arg-Cys appears in the corresponding position. This is surprising, because the Cys-Arg-Cys tripeptide is conserved in S18, and S18 and S21 are isoinhibitors. The cysteine towards the carboxyl terminal is also not conserved in all the 14 sequences. Apart from the cysteine residues, one leucine, one proline, with the exception of S31 (barley α-amylase/trypsin inhibitor), and another leucine, with the exception of S23 (barley α-amylase inhibitor), are conserved in the sequences. The total number of residues conserved in all the sequences is 10. There are seven sites where significant

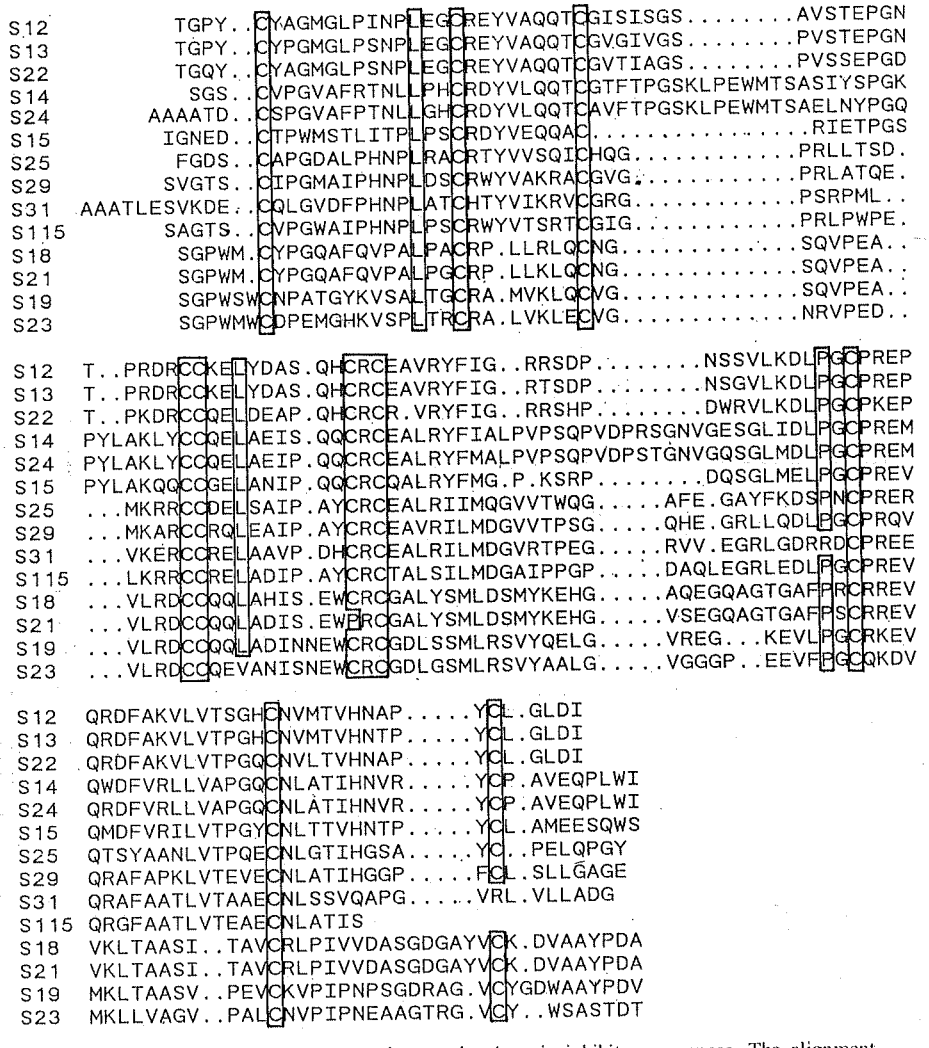


**Figure 2.** Alignment of the 14 cereal α-amylase/trypsin inhibitor sequences. The alignment was obtained using the program MULTALIN.

gaps have been introduced. Most of the gaps are short. However, sequences S14 and S24 (α-amylase/trypsin inhibitors from wheat and barley respectively) are longer by eight residues at two separate sites compared to the other sequences. Significant differences in length are observed at both amino and carboxyl ends of the aligned polypeptides.

### 3.4 *Phylogenetic tree*

Figure 3 shows the phylogenetic tree obtained on the basis of the multiple alignment. The tree has three major branches. Branch 1 is further divided into two distinct but closely related branches (branches 1A and 1B). These two branches include α-amylase/trypsin double-headed inhibitors from wheat and barley. Branch 2 includes α-amylase/trypsin inhibitors from barley and ragi and an α-amylase/factor 12A inhibitor from maize. Branch 3 has mainly α-amylase inhibitors from wheat and barley. It may be noted that the branches do not divide the source plants into their distinct taxonomic groups.

### 3.5 *Ancestral sequences*

Ancestral sequences were constructed for the set of all 14 sequences as well as for the three branches. The results of interbranch comparisons of these ancestral sequences are recorded in table 2. It is clear that branches 1A, 1B and 2 form a close cluster.

### 3.6 *Hydrophobicity patterns*

Figure 4 is a plot of hydrophobicity as a function of residue position averaged over the 14 aligned sequences. The plot has two prominent characteristics. It is divided into three very similar segments, and each of these segments shows periodic variation in hydrophobicity.

### 3.7 *Divergence of sequences from the ancestral sequence*

The evolutionary divergence of the sequences from the derived ancestral sequence

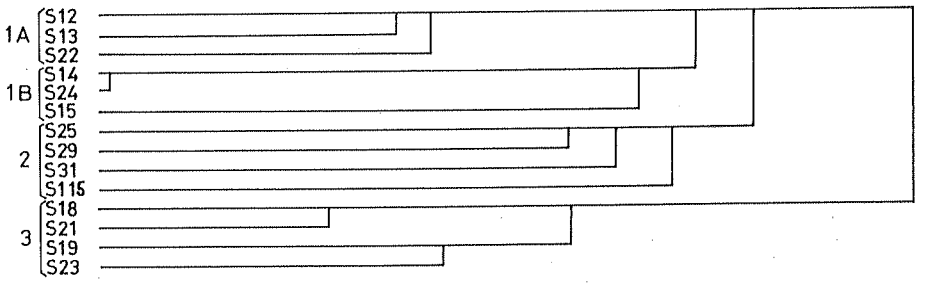


**Figure 3.** Phylogenetic tree showing the relationships of the inhibitor sequences. Branch lengths represent approximate evolutionary distances between the sequences.

**Table 2.** Alignment scores for pairs of ancestral sequences corresponding to branches 1A, 1B, 2 and 3 of figure 3. The values are scores obtained for alignment of the ancestral sequences in units of standard deviation above the mean background obtained for comparison of random sequences.

| Ancestral sequence of branch | 1A | 1B | 2 | 3 |
|---|---|---|---|---|
| 1A | 35·60 | | | |
| 1B | 18·69 | 38·43 | | |
| 2 | 16·61 | 17·48 | 36·22 | |
| 3 | 7·94 | 6·85 | 8·52 | 39·20 |

was evaluated in terms of the minimum number of base changes required for mutating a residue in the ancestral sequence to each of the residues found in the 14 sequences for that position. The resultant change in residue property, such as hydrophobicity or bulk, was also computed. The values were smoothened by averaging over windows of five residues. Figure 5 shows minimum base changes as a function of residue position, and figure 6 shows a similar plot for root mean square (RMS) hydrophobicity. These plots show remarkable relation to figure 4. The regions of maximum divergence from the ancestral sequence fall between the characteristic segments of figure 4. The most variable region of the sequences is confined to the region between the two amino-terminal domains of figure 4 that show periodic variation in hydrophobicity.

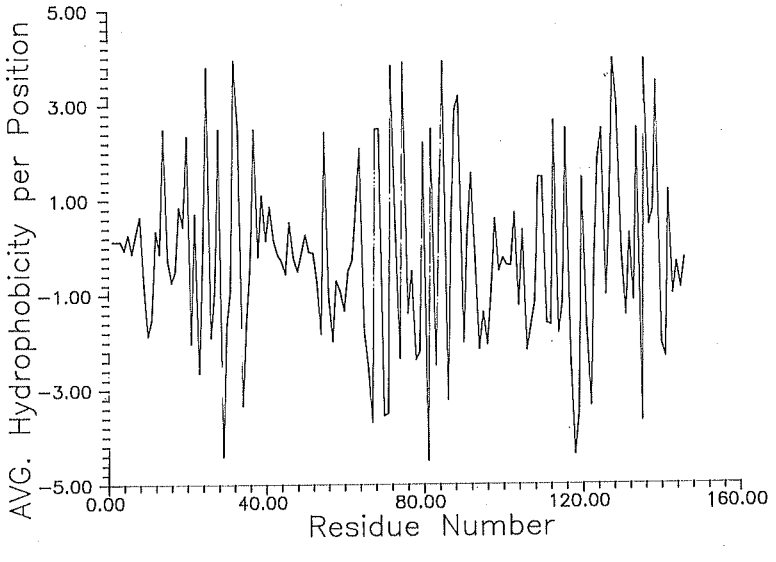


**Figure 4.** Plot of hydrophobicity along the polypeptide averaged over the 14 inhibitor sequences.

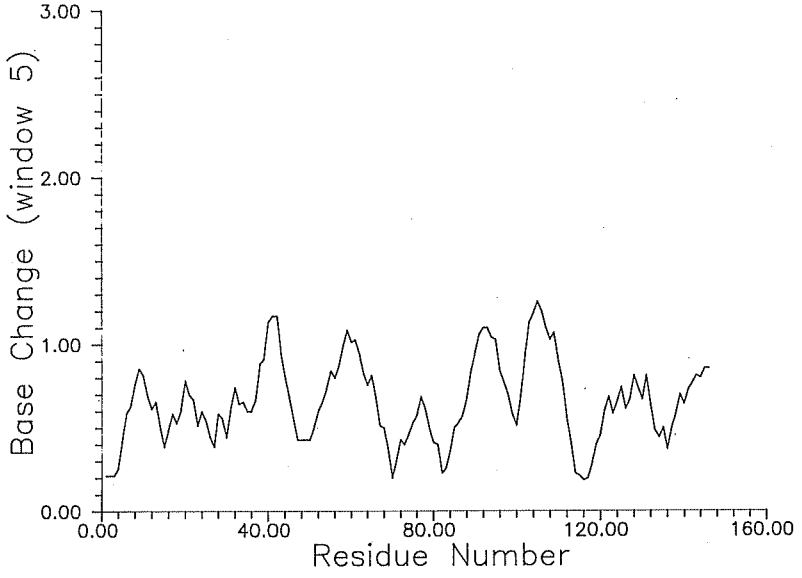


**Figure 5.** Plot of minimum base changes required to convert five consecutive residues of the derived ancestral sequence to all the residues found at the corresponding positions in the set of aligned inhibitor sequences.

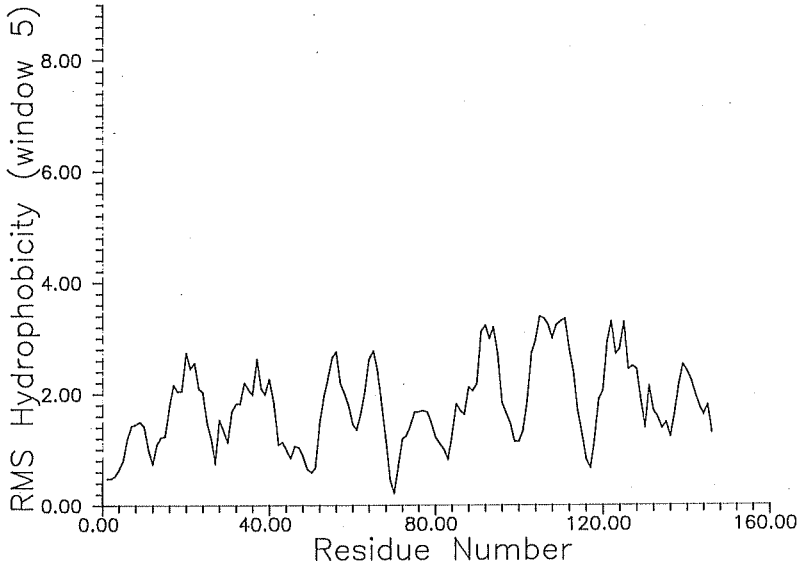


**Figure 6.** Plot of RMS change in hydrophobicity on mutating the residue in the derived ancestral sequence to the residues found at the corresponding position of the aligned inhibitor sequences. The plot is smoothened by averaging in windows of five residues.

## 4. Discussion

The sequence analysis presented in this communication suggests that α-amylase/trypsin double-headed inhibitors from cereals from a distinct family of proteins. Although certain pairs of these sequences display as low an amino acid identity as 28%, the results of the multiple-alignment procedure as well as the matrix of scores in figure 1 confirm that they belong to a single family related by descent. This family of proteins is also related to the chloroform/methanol-soluble plant seed storage (CM) proteins. Hence, α-amylase/trypsin double-headed inhibitors and CM proteins form a single superfamily. The CM proteins are not known to possess inhibitory properties. This superfamily does not appear to be related to any other family of plant seed inhibitors.

The α-amylase/trypsin inhibitor sequences cluster into three major branches (figure 3). The inhibitors from branches 1A and 1B are active against both α-amylase and trypsin. Branch 1B proteins are known to be tetrameric (table 1) and one of the proteins of branch 1A is also known to be a tetramer. Branch 2 has inhibitors active against trypsin and α-amylase or α-amylase and factor 12A. The quaternary structure of these proteins is not known. The third branch is evolutionarily more distant to branches 1 and 2 than the latter two are to each other. It includes inhibitors that are active only against α-amylase. The branch 3 proteins are either monomers or homodimers. The branches therefore reflect the functional divergence of these inhibitors. In reports of earlier studies of plant seed inhibitors it was suggested that they could be classified on the basis of their quaternary structure as proteins of 12, 24 or 48 kDa (Maeda *et al.* 1985). The phylogeny we have derived may also be related to this structural classification in addition to its correlation with inhibitor specificity. The different branches do not reflect the taxonomic relationships of the plant species from which the polypeptides are derived. This suggests that cereal double-headed inhibitors are ancient proteins whose functional and possibly structural specialization occurred before the divergence of commercially cultivated cereals from their common ancestral plants.

All the 14 sequences have cysteine residues that indicate characteristic disulphide bridges, most of which have been conserved during the evolution of the sequences. Except for three sequences, the polypeptides have five disulphide bridges. It has been shown that the inhibitory activity is abolished upon reduction of the disulphides. Bowman–Birk inhibitors from plant seeds are also disulphide-rich, thermostable proteins. The three-dimensional structures of these lack a hydrophobic core, which is characteristic of other globular proteins. Cereal double-headed inhibitors may have a similar structural organization.

The periodicity in hydrophobicity (figure 4) suggests that the three-dimensional structure of these proteins is likely to be dominated by β-strands. β-Segments are likely to have alternate polar and hydrophobic residues. Figure 4 reveals three regions of remarkably similar hydrophobic profile, suggesting that the three-dimensional structure of these inhibitors may have three structurally similar motifs. Also, the polypeptide segments between these motifs are probably exposed as the hydrophobicity plot reveals clear hydrophilic segments between the periodic domains.

The weak sequence similarity that we found between double-headed inhibitors and Kunitz inhibitors in initial sequence comparisons prompted more rigorous

evaluation, but no significant similarity was found. However, examination of the hydrophobicity profile of Kunitz inhibitors showed similarities (data not shown) to figure 4 in terms of periodic variation. The three-dimensional structure of Kunitz inhibitors is known (Sweet *et al.* 1974), and contains mainly β-strands as secondary structure. This supports the observation that cereal double-headed inhibitors fold with β-structure as a major regular feature.

In some cereal double-headed inhibitors the inhibitory site has been determined as the segment around residue 35 (Mahoney *et al.* 1984). Hence the polypeptide around this residue is likely to be exposed in the three-dimensional structure. This is in conformity with the hydrophobicity profile. The proteins that inhibit only α-amylase show large differences from those that inhibit trypsin in this region (data not shown). In this respect plant seed inhibitors resemble antibody molecules, in which the antigen recognition site is the hypervariable region.

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