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Prediction of Three Dimensional Model and Active Site Analysis of Inducible Serine Protease Inhibitor -2 (ISPI -2) in *Galleria Mellonella*

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Abstract

The present study undertaken to predict the three dimensional structure and active site analysis of inducible serine protease inhibitor -2(ISPI -2) known to inhibit the activity of entomopathogenic fungi *Metarhizium anisopliae* in *Galleria mellonella* a severe pest of most economic important crops. This inhibitor completely inactivates serine protease produced by *M.anisopliae* which acts as major virulent factor for *G.mellonella* and imparts natural immunity to the pest. Initially, the structural template for *G.mellonella* – ISPI-2 was identified from structural database using homology modeling or comparative modeling approach. Based on the knowledge of the template, a three-dimensional model was predicted and processed in to energy minimization, Ramachandran plot analysis, quality assessment and finally deposited into Protein Model Database. An active site of the theoretical model was analyzed and helpful to recognize the effective ligands.

Keywords: Inducible serine protease inhibitor; *Galleria mellonella*; MODELLER; Protein Model Database; RAMPAGE; Entomopathogenic fungi; Active site

Abbreviations: ISPI: Inducible serine protease inhibitor; BLAST: Basic local alignment search tool; PMDB: Protein Model Database; PIR: Protein information resource

Introduction

An entomopathogenic fungus is a one kind of fungus, which can act as a parasite of insects and now extensively used as biocontrol agents against a wide spectrum of insects and other arthropod pests that are harmful to various plants, because of their high efficacy, safe to non-target organisms and ease of multiplication (Sahayaraj and Karthick Raja Namasivayam, 2008). It infects susceptible hosts through the integument, and utilizes different proteases to carry out the digestion of cuticle proteins for colonization and to inactivate the host immune systems (Clarkson and Charnley, 1996; Frobius et al., 2000).

Insects lack the immune system of vertebrates involving antigen–antibody reactions, although they can protect themselves efficiently from entomopathogenic infections. The humoral part of the insect immune system is characterized by the rapid and transient synthesis of proteins with potent antibacterial and antifungal activity and inactivates the effect of the insect's pathogenic activity (Gillespie et al., 1997) and expressing a wide spectrum of protease inhibitors to inhibit the proteases and other lytic enzymes produced by entomopathogenic fungi. Such type of protease inhibitors was reported in many insects viz *Bombyx mori* was effective against entomopathogenic fungi *Beauveria bassiana.* Larvae of the greater wax moth, *Galleria mellonella* is very resistant to various microbes and their infections such as *Aspergillus melleus*, *Beauveria bassiana*, *Metarhizium anisopliae* etc (Eguchi et al., 1986). Because it produces various inducible serine protease inhibitors and it was identified recently such as ISPI-1, ISPI-2 and ISPI-3 in

hemolymph that are active against various toxic proteases produced from entomopathogenic fungi (Frobius et al., 2000). Based on the determined amino acid sequences, *Galleria mellonella* ISPI-2 represents a novel member of the Kunitz-type inhibitor family, whereas ISPI-1 and ISPI-3 share no similarity with other known proteins (Clermont et al., 2004).

Serine protease inhibitors are mainly classified into three basic types such as canonical, non-canonical and serpins. Canonical inhibitors are the largest family and its size ranging from 14 to 200 amino-acid residues. These are rigid, stable and regularly have beta sheet or mixed alpha-beta topologies. A few can be only alpha helical or irregular proteins that are rich in disulfide bridges (Otlewski et al., 2005). Non-canonical protease inhibitors (for eg. hirudin and haemedin) are interacting with the active site of serine proteases through their N-terminal tails (Grutter et al., 1990). Serpins are 45–55 kDa proteins that are composed of three beta sheets and eight or nine alpha helices forming a single domain and inhibit through the reactive-site loop present at their C-terminus (Gettins, 2002).

The understanding of the three-dimensional structure of a protein would be a precious aid to understand the details of a particular protein. Active site analysis is a key step for identify or design the potential molecules for the purpose of molecular docking studies followed by ligand optimization. The main objective of this study is an attempt to predict the structural as well as active site information of inducible serine protease inhibitor-2 from *Galleria mellonella*, which is helpful to identify the potential lead molecules for prevent the function of inhibitors and activate the function of fungal serine proteases.

Materials and Methods

Retrieval of *Galleria Mellonella* - Inducible Serine Protease Inhibitor-2 Protein Sequence

The protein sequence of inducible serine protease inhibitor-2 (ISPI-2) in *Galleria mellonella* was retrieved from the Swissprot database (http://www.expasy.org) and taken as target sequence. The main reason for choosing this protein was, it active against various serine proteases including trypsin and toxin proteases relased by several entomopathogenic fungi (Frobius et al., 2000). It was deep-rooted that the three-dimensional structure of this protein was not available in any three-dimensional structural databases. Hence, the current study of developing the three-dimensional structure of the inducible serine protease inhibitor-2 from *Galleria mellonella* was undertaken.

Selection of Structural Template

An effort was made to find a suitable structural homolog or template for the modeling of the inducible serine protease inhibitor-2 from *Galleria melonella*. In the beginning, a structural template was obtained from Protein BLAST (Altschul et al., 1990) and it is used Protein Data Bank (Berman et al., 2003) as reference data base for identify the closely related sequences.

Target – Template Alignment

The protein sequence of inducible serine protease inhibitor-2 was aligned with its corresponding template by using align-2D module in MODELLER 9V2 (Eswar et al., 2008), which required two files such as a file containing target sequence in PIR format and an another file containing structural coordinates of template. This step is essential to identify the common conserved residues or active residues present in both the sequences.

Model Building

MODELLER 9V2 was used to predict the three-dimensional structure of inducible serine protease inhibitor-2 using model-single.py based on satisfaction of spatial restraints. It is a python script, used to predict the three dimensional model from single template. Theoretical model was subjected into Swiss-PDB Viewer (Kaplan and Littlejohn, 2001) for energy minimization using the steepest descent and conjugate gradient technique to correct the stereochemistry of the model. Computational analysis was carried out in vacuo with the GROMOS96 43B1 parameters set, without reaction field in Swiss-PDB viewer.

Model Evaluation

Refined model was subjected to a series of tests for testing its internal stability and reliability. Backbone conformation of the refined model was assessed by the examination of the Psi/Phi Ramachandran plot obtained from RAMPAGE web server (Lovell et al., 2003). Errat web server (Colovos and Yeates, 1993) was used to explore the statistics of non-bonded interactions between different atom types and plots the graph. Finally, an evaluated model was deposited into Protein Model Database (www.mi.caspur.it/PMDB/).

Active Site Analysis

Potential active site and active residues was identified by CastP server (Binkowski et al., 2003) which is essential for function of serine protease inhibitor in *Galleria melonella*.

Result and Discussion

```
 _aln.pos  480       490       500       510       520       530       540
1ZRO      GIVSWGSGCAQKNKPGVYTKVCNYVSWIKQTIASN/PTGNNAEICLLPLDYGPCRALLLRYYYDRYTQ
ISPI-2    ------------------------------------LDPKCTLPLETGICRAELHRFGYDTKLK
 _consrvd                                      * ***  * *** * *  **

 _aln.pos    550       560       570
1ZRO      SCRQFLYGGCEGNANNFYTWEACDDACWRIE
ISPI-2    ECTQFVYGGCHHNENNFKKLEVCR-------
 _consrvd  * ** ****  * ***   * *
```

Figure 1: Sequence alignment of inducible serine protease inhibitor -2 [ISPI-2] with Kunitz Domain 1 of Tissue Factor Pathway Inhibitor-2 [1ZR0] using Clustalw. "*" shows conserved region between the sequences and "-"shows gaps.

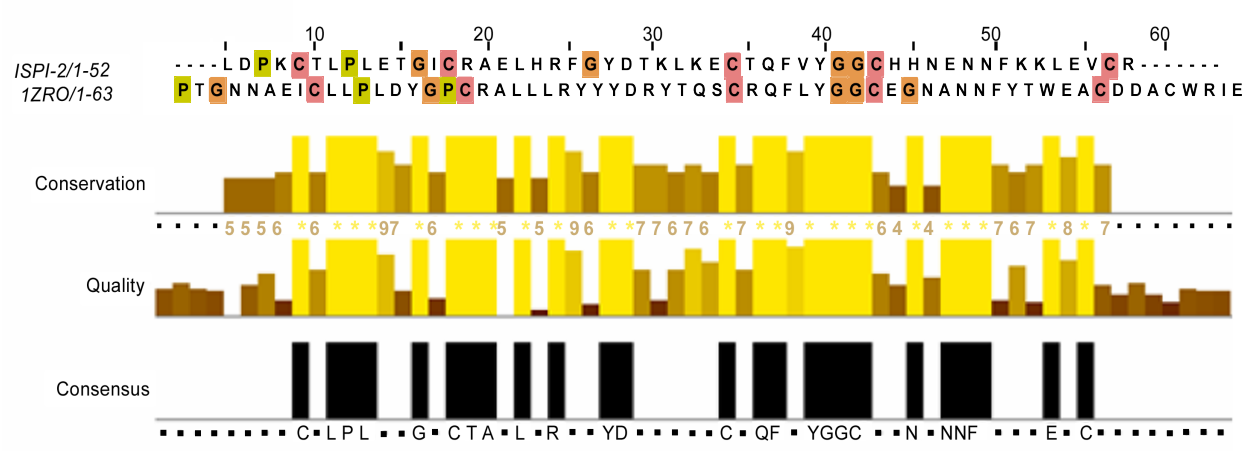

Figure 2: Graphical representation of sequence alignment of both target [ISPI-2] and template [1ZR0]. Various conserved regions with highlighted 100% conserved residues.

The amino acid sequence of inducible serine protease inhibitor-2 was retrieved from commonly used primary protein sequence database i.e. Swissprot (http://www.expasy.org) and its accession numbers is P81906 and the source was of *Galleria mellonella* in origin. The molecular weight of this inhibitor is 6.3kDa obtained from mass spectroscopy (Frobious et al., 2000). The results of Protein BLAST (http://www.ncbi.nlm.nih.gov) search for suitable template structure related to the target sequence (ISPI-2) showed the crystal structure of kunitz domain 1 of tissue factor pathway inhibitor -2 of *Bos taurus* with highest sequence similarity (53%), as the most suitable template for modeling. The alignment of inducible serine protease inhibitor-2 and its corresponding template was carefully examined and conserved regions was identified shown in Figure 1 using align-2D module in MODELLER9V2 and it was concluded that this alignment can be assisted to generate a three dimensional reliable model. Jalview (Clamp et al., 2004) was used to display the conserved regions in graphical representation shown in Figure 2.

Once target-template alignment was completed, a three dimensional structure of ISPI-2, was predicted using the program MODELLER9V2 produced three different conformations and its modeller objective functions were 262.7881, 260.3169, 275.1234. The second conformation had lowest value compared to other. Generally, the best model could be obtained from choosing the model with the lowest value of the MODELLER objective function, which is reported in the second line of the model PDB file (Eswar et al. 2008). Based on the lowest value of the modeller objective function, one predicted model was taken and processed in to Swiss-PDB Viewer for energy minimization.

An assessment of the refined model involved two independent tests. The first test was to compare the residue

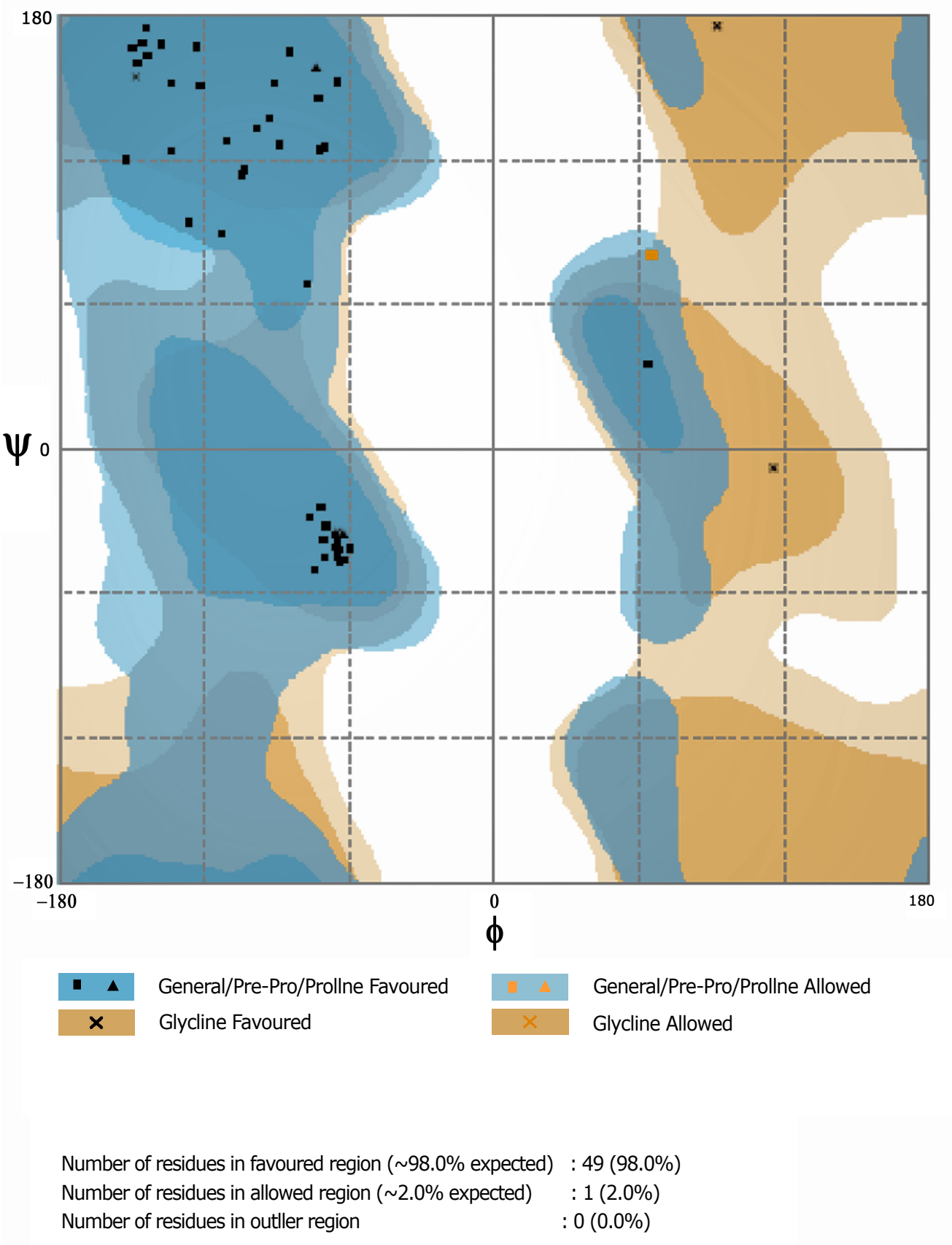

Figure 3: Ramachandran plot of theoretical model of ISPI-2.

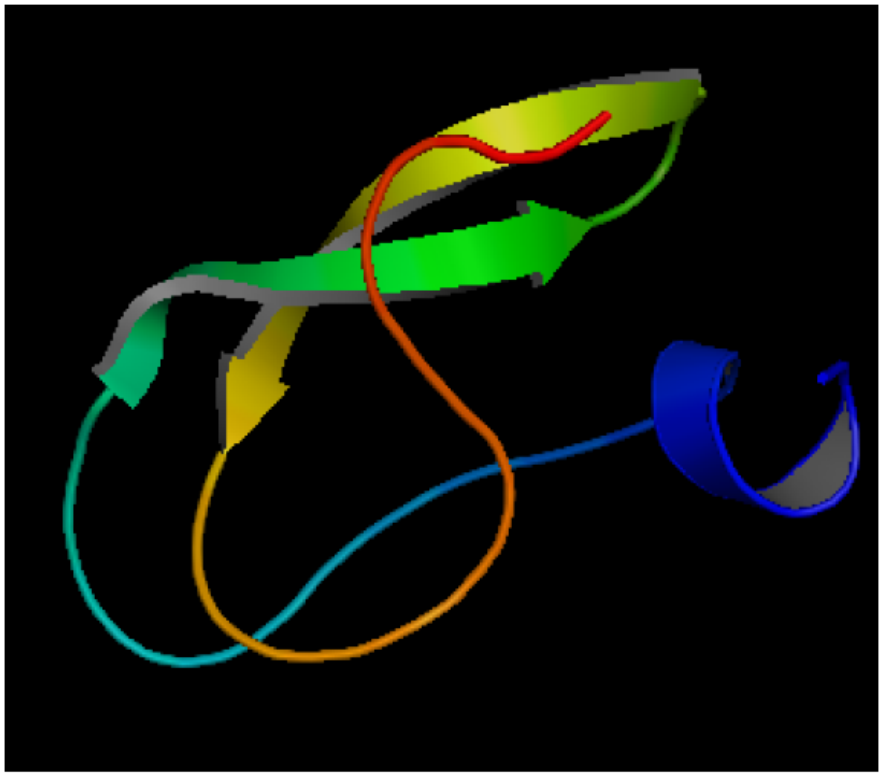

Figure 4: The final three-dimensional structural representation of ISPI-2 [PyMOL]. This model was conducted by MODELLER 9V2 Program.

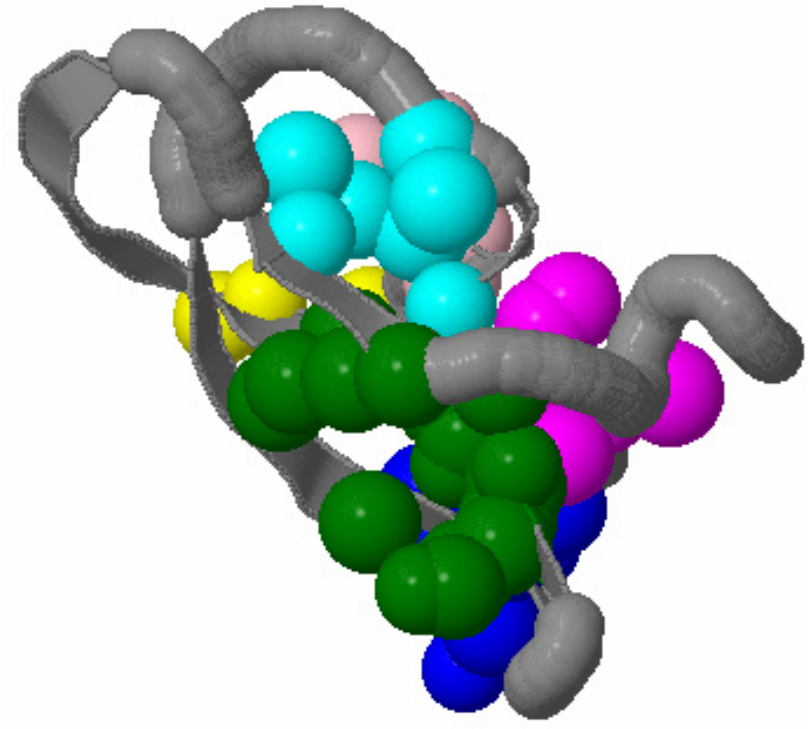

Figure 5: Cartoon display of various potential binding pockets of ISPI-2 is displayed in different colors and active site residues are highlighted [Pocket 1 – pink, Pocket 2 – magenta, Pocket 3 – yellow, Pocket 4 – cyan, Pocket 5 – blue and Pocket 6 – green].

backbone conformations in our refined model with the preferred values obtained from Protein Data Bank of known structures. The results of RAMPAGE web server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) explored that 98% residues are found to be most favoured region of the Ramachandran Plot of refined model of induced serine protease inhibitor-2 which is more then cut-off of 90% in most reliable models (Lovell et al., 2003) shown in Figure 3. The stereo chemical quality of the predicted model was found to be satisfactory and low percentage of residues having phi/psi angles in the outlier region.

The second test was carried out using Errat web server (http://nihserver.mbi.ucla.edu/ERRATv2/) for check the quality of the models. Generally, the quality factor of high resolution structures generally produce values around 90% or higher (Colovos and Yeates, 1993). Here, the overall quality factor of this refined model was 92.568 predicted from Errat. The evaluated final reliable model has been deposited in to Protein Model Database (http://mi.caspur.it/PMDB/) and is now publicly accessible [PMDB id: PM0075527]. A three dimensional structure of inducible serine protease inhibitor-2 is shown in Figure. 4.

CastP web server (http://sts-fw.bioengr.uic.edu/castp/calculation.php) was used to predict the active site of inducible serine protease inhibitor – 2 from *Galleria mellonella*. Six potential binding pockets were identified and displayed in Figure 5. The active site residues (Figure 5) obtained from CastP web server is essential for inhibition of toxic serine proteases produced from various entomopathogenic fungi (Frobius et al., 2000) and they are cysteine, leucine, glutamic acid, histidine, arginine, phenylalanine, glycine, tyrosine, aspartic acid, threonine, aspargine and lysine.

Conclusion

The understanding of the three-dimensional structure and active site of ISPI-2 is important step for identify the probable ligand candidates. However, a three dimensional structure of ISPI-2 is only a predictive, and needed to be confirmed experimentally. Further molecular docking and virtual screening approach is necessary to recognize specific ligands for ISPI-2, which will be validated by many in silico analysis such as molecular docking, QSAR (Quantitative Structure-Activity Relationship) and rule of five.

Future studies helpful to design ligand against ISPI-2 – a major immune defense factor of *G. mellonella* against *M. anisopliae* and it completely neutralizes the inhibitory activity of ISPI-2.

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