Comparative modeling reveals the structure of *Staphylococcus aureus* Enterotoxin D

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**ABSTRACT**

The Staphylococcal food poisoning (SFP) is a toxin-mediated intoxication that results from the ingestion of significant amounts of bacterial enterotoxin from contaminated food. SFP symptoms emerge rapidly (2-8 hours after ingestion) and include vomiting, diarrhea, nausea and abdominal pain. Among the bacterial species that produce such toxins, *S. aureus* is one of the most relevant, being responsible for 759 SFP outbreaks in Brazil, between the years of 2000 and 2013. There are currently approximately 20 different types of *S. aureus* enterotoxins (SEs) and several molecular variants. There are total of 60 structures of SEs deposited in the Protein Data Bank (PDB), being 30 of isolated SEs and 30 in complex with other human (29 structures) or murine (1 structure) proteins. Despite the high number of representations in the PDB, most of these are redundant and the 60 deposited structures represent only 8 different SEs: SEA, SEB, SEC2, SEC3, SEG, SEH, SEI, SEK and TSST1. The revealing of the structure of the enterotoxin D (SED) of *S. aureus* represents an important breakthrough, especially since this protein is the second most prevalent in SFP cases. The structure of SED will help to elucidate its molecular function, the involvement in biochemical and physiological mechanisms. Currently, the molecular modeling of proteins allows one to obtain structural results in a short time-frame and with low cost, being considered as an important tool for bioprospecting, often anticipating and guiding laboratory experiments.

**Keywords:** Comparative modeling, Staphylococcal Enterotoxin D, Food poisoning

**RESUMO:**

A intoxicação alimentar estafilocócica (SFP) é uma intoxicação resultante do consumo de alimentos contendo quantidades significativas de enterotoxinas bacterianas. Os sintomas da SFP têm um início rápido (2-8 horas) e incluem vômitos, náuseas violentas, cólicas abdominais e diarreia. Dentre as espécies produtoras de enterotoxinas capazes de deflagrar tais sintomas, a bactéria *Staphylococcus aureus* é uma das mais relevantes, sendo responsável por 759 surtos de toxinfecção de origem alimentar ocorridos entre os anos 2000 e 2013, somente no Brasil. Existem, atualmente,
identificadas cerca de 20 tipos de enterotoxinas estafilocócicas (SEs), e algumas de suas variantes moleculares. Há um total de 60 estruturas envolvendo SEs depositadas no Protein Data Bank (PDB), sendo 30 SEs isoladas, 29 em complexos com proteínas humanas e um com uma proteína murina. Apesar do alto número de representações no PDB, muitas SEs são redundâncias e as 60 entradas representam apenas 8 SEs: SEA, SEB, SEC2, SEC3, SEG, SEH, SEI, SEK além da TSST1. A elucidação da estrutura da enterotoxina D (SED) de Staphylococcus spp representa um importante avanço, em especial por esta proteína ser a segunda mais prevalente nos casos de intoxicação alimentar. A estrutura da SED auxiliará a elucidação de suas funções moleculares e envolvimento em mecanismos bioquímicos e fisiológicos. Atualmente, a modelagem comparativa de proteínas permite a obtenção de resultados estruturais em um curto espaço de tempo e com um baixo custo associado, tornando-se uma importante ferramenta de bioprospecção que antecede e muitas vezes guiam os experimentos laboratoriais.

**Palavras-Chave:** modelagem comparativa, enterotoxinas estafilocócica D, intoxicação alimentar.

**INTRODUCTION**

*Staphylococcus aureus* is a gram-positive bacteria, capable of causing severe food poisoning. This bacteria has been identified as a causative agent of innumerable intoxications (staphylococcal gastroenteritis) and is probably responsible for many other non-reported cases in individuals and family groups (BENNETT, 2001). Just in the United States *S.aureus* has been one the top five pathogens contributing to domestically acquired foodborne illnesses (CDC 2011), as well as Brazil, 759 outbreaks were reported as caused by microorganism, between the years of 2000 and 2013, being the second most frequently isolated pathogen from suspected foods cause outbreaks foodborne (SINAN NET/SVS/MS 2013). The staphylococcal food poisoning (SFP), results from the ingestion of contaminated food containing significant amounts of a pre-formed bacterial enterotoxin (>200 nanograms, according to EVENSON et al., 1998). The SFP symptoms emerge rapidly (from 2 to 8 hours after ingestion) and include vomiting, nausea, abdominal pain and diarrhea (CARVALHO, 2007; Hu et al., 2007, PINCHUK et al., 2010 and DIAS et al., 2011). The manifested disease is usually self-eliminated in 24 to 48 hours. Occasionally, however, the disease is severe enough to require hospitalization, especially when the patient is a child, an elderly, HIV carrier or immunodeficient. Staphylococcal enterotoxins (SEs) are potent exotoxins, synthesized along the entire logarithmic growth phase or during the transition from the exponential growth phase and the stationary growth phase. These proteins are resistant to the cooking process and the gastrointestinal hydrolysis, thus, enabling the emergence of the symptoms triggered by the ingestion of contaminated food (CARMÓ, 2001). SEs are functional in concentrations that range from nanograms to few micrograms and are resistant to certain conditions (as extreme temperatures, pH, hydrolysis reaction and proteolytic enzymes), which are lethal to the bacteria. For these reasons, SEs are capable of conserving its native structure and, therefore, its functional activity even after ingestion and in the digestive tract (ARGUDÍN et al., 2010 e PINCHUK et al., 2010).
Referred to as superantigens, SEs can interact with MHC class II molecules (major histocompatibility complex class II) and promote T cells proliferation (PINCHUK et al., 2010). Structurally, SEs are low-weight secreted proteins, that bind metallic ions, such as zinc and may interact selectively and non-covalently with any other metallic ion. The presence of zinc ions has been related to the interaction between SEs and the MHC molecule and, therefore, has great impact as the prosthetic group of such proteins (PINCHUK, et al., 2010). SEs have a common fold pattern, formed by two distinct domains with a central alpha-helix and a beta-barrel that characterize the N-terminal domain (also known as OB-fold) and a beta-grasp motif that forms the C-terminal domain (which is structurally similar to domains from ubiquitin, 2Fe-2S ferredoxins, transcription initiation factor 3 and the immunoglobulin-binding domain). The SED protein presents 258 amino acids in its pre-processed form, presents a signal peptide and molecular weight of approximately 30kDa (UNIPROT P20723).

Until 2010, around of 20 different types of SEs had been described, namely: SEA, SEB, SEC (SEC1, SEC2 e SEC3), SED, SEG, SEH, SEI, SER, SES, SET, SEJJ, Selk, VENDER, Selm, SEIN, Selo, Selp, SEIQ, Selu, SEIU2 and SELV. In addition to the S. aureus toxic shock syndrome toxin 1 (TSST-1), which does not present emetic activity (LOIR 2003). The toxins most commonly related to food poisoning events are SEA, SEB and SED, being SEA the most frequently related to such cases, followed by SED (bioMéuriex SA 2010). There are currently 60 experimentally solved structures deposited in the Protein Data Bank (PDB), among which 30 represent isolated SEs structures and the additional 30 represent complexes with other proteins (29 human proteins and one murine protein). This set of SEs structures deposited in the PDB is highly redundant and from the 60 structures, there are only 8 distinct SEs: SEA, SEB, SEC2, SEC3, SEG, SEH, SEI, SEK and TSST1. All other experimentally solved structures are either structural variants of these SEs (for example with introduced mutations) or represent protein complexes involving these 9 toxins. The revealing of the tridimensional structure of SED can aid to elucidate its molecular functions and biochemical mechanisms in which this protein takes part.

The in silico comparative modeling of proteins represents an alternative to experimental methods as X-ray crystallography and nuclear magnetic resonance (NMR), which is capable of generating significative results in a short time-frame and with minimal resources. This Computational Biology technique has been used to anticipate experimental results, often elucidating important structural and functional properties of proteins with no experimentally solved structure. In addition, the comparative modeling of proteins can be associated to experimental analysis, as a method of initial triage, which may point out the most promising proteins for a specific study. The present work aimed to elucidate the tridimensional structure of the S. aureus enterotoxin D and to study its functional characteristics through comparative modeling and related methods.

METHODOLOGY

Retrieval and Initial Analysis of the Target-Protein Sequence

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The fasta formatted target-protein (enterotoxin D) amino acid sequence was retrieved from the National Center for Biotechnology Information (NCBI) website and used to search for a structural template. First, confirmative analyses were performed for the presence of a signal peptide (using SignalP), for the presence of transmembrane helices (using TMHMM) and for the Subcelular location (using Psort for gram-positive bacteria).

**Selection of a Template-Structure**

The selection of a template structure was based on the results obtained with the Basic Local Alignment Search Tool (BLAST) for searches on the PDB. The scoring matrix used was BLOSUM62 (HENIKOFF, 1992), associated with gap opening and extension costs of 11 and 1 points, respectively. Additional parameters were kept on their standard adjusts, including expected threshold of 10, word size of 3 and conditional compositional score matrix adjustment. The BLAST program used was blastp, specialized on the comparison between amino acid sequences. The subsequent selection of a template structure was based on scoring parameters of the sequence alignment (Max Score and Total Score), sequence coverage (Query Coverage), identity percentage (Max Ident) and probability value (e-value).

**Alignment Between the Sequences of the Target Protein and the Template Structure**

The next step, after the selection of the template structure that best represents the target protein is the generation of a sequence alignment between these two proteins. In the present work, all sequence alignments were performed by the program Promals3D. As inputs for this program, one needs the fasta-format sequence of the target protein (retrieved from NCBI) and the PDB identifier for the template structure (obtained as the result of BLAST searches, as detailed above). The parameters for sequence alignments were defined as follows: weight for constraints derived from sequences of 1, weight for constraints derived from homologs with structures of 1.5, weight for constraints derived from input structures of 1.5 and weight for user-defined constraints of 1.5. All other parameters were maintained as default. The obtained results were formated by Promals3D in both fasta and clustal formats. The former format was altered to the pir format and used to generate one of the inputs required for the generation of candidate structures using Modeller. The latter format was used in the process of visual inspection of the alignment and of generation of illustrative results, obtained with the DNATagger alignment colorizer.

**Required Inputs for the Generation of Candidate Structures Using Modeller**

The generation of candidate structures was performed by the comparative modeling software Modeller (version 9.10). In order to generate structures using Modeller, one will need four basic input files, containing information about the target-protein (for which one wishes to build a structure), about the template-structure (which will serve as basis for the modeling) and about the relation between both proteins (as their sequence alignment). Files needed by Modeller and its respective formats are: (i) target-protein
sequence in fasta format, (ii) structural coordinates for the template-structure in PDB format, (iii) sequence alignment between both proteins in pir format and (iv) a file (script) containing instructions for Modeller, written in the Python programming language. Using the automodel class for automatic comparative modeling using Modeller, a set of 100 candidate structures was generated for the target-protein, based on the template-structure.

**Refining the Candidate Structures**

While the first set of candidate structures was generated using the standard automated algorithm from Modeller, implemented as the automodel class, in its most basic configuration, a second step of structure generation was performed, this time considering additional parameters. At first, it was necessary to consult the information of the template-structure PDB page in order to verify the presence of prosthetic groups in this protein (one zinc ion). To correctly incorporate this heteroatom, the Modeller option env.io.hetatm was set to true in the script file. In addition to the inclusion of the zinc ion, a disulfide bridge between cysteine residues was also inserted, based on the presence of such bond on the template-structure, according to its PDB file. To include such disulfide bridges in the candidate structures generated by Modeller, the MyModel class from Modeller was used to define the cysteine residues that participate in the formation of the disulfide bridge. As an example, in order to build a bond between the cysteine residues A and B, the following commands must be specified: def special_patches(self, aln): self.patch(residue_type='DISU', residues=(self.residues['A'], self.residues['B'])). Both options (for the inclusion of heteroatoms and disulfide bridges) were inserted to the Modeller script that originated the second set of structural candidates, containing elements that confer stability and functionality to proteins. Among the 100 candidate structures, one was chosen to structurally represent the target-protein as the one with highest score in the quality assessment protocol detailed below.

**Quality Assessment of Candidate Structures**

All 100 final candidate structures were submitted to analyses of stereochemical and energetically features. To assess the stereochemical quality of each structure we have used the quantitative result of the Ramachandran plot, which exhibits the combination of phi and psi angles for each amino acid in each one of the 100 candidate structures and compares such combinations to the ones presented by native proteins. The quantitative result represents the percentage of residues in the most favorable regions of the graphic, which represent combinations of phi and psi angles that are most often found in the native proteins that served as basis to generate the reference plot. In this work, the Ramachandran plots were generated by the Procheck program. To assess the energetically features of the candidate structures we have used the ProSA protein program, which minimizes an optimization function to compare energetically properties of the candidate structures and compare these with the properties of experimentally solved structures from the PDB. The result is presented as a Z-score, representing the distance between the energy value of each candidate structure and the mean energy value for all experimentally solved structures with the same number of amino acids. In
this case, therefore, the quantitative result is represented by the Z-score for each candidate structure. A Perl language script was written and used to transform the process of retrieving the quantitative results of both Procheck and ProSA for each candidate structure and compare such results in an automated task. The given score for each candidate structure is a combination of the quantitative results obtained from stereochemical and energetical assessments, as a ration between the number representing the percentage of residues in the most favorable region of the Ramachandran plot and the absolute value of the ProSA Z-score. In order to define one unique candidate structure as the best representation of the target-protein, the structure with highest score (defined by the aforementioned ratio) was chosen and used in subsequent analyses.

**Visual Inspection of Structures**

All visual inspection of the obtained candidate structures, the structural-template and the comparison between these were performed by the PyMol program, for visual analyses of biological molecules. The candidate structures were visually explored structurally aligned to the template structure and to one another and their resemblances and discrepancies were studied. Additionally, the position of residues of special interest, prosthetic groups and disulfide bridges were observed. Finally, the software was also used to generate a homodimeric complex of the SED protein, based on the orientation of the monomer-monomer interaction of the template-structure and a SED-MHC complex, based on the orientation of the enterotoxin H and the MHC as deposited on the PDB.

**Additional Analyses**

Some additional investigations were performed, as the prediction of the prosthetic group for the SED protein, performed by the TEMSP program, the prediction of secondary structure elements for both template and target proteins by PsiPred and detailment of the structural domains of both proteins by the PDBSum tool.

**RESULTS AND DISCUSSION**

**S. aureus Enterotoxin D sequence**

The amino acids sequence of *S. aureus* enterotoxin D was retrieved from the Uniprot website, using the code P20723 (Fig. 1). The sequence was formatted to fasta format and used in all subsequent analyses. To confirm some of the protein features, analyses were performed regarding its amino acid sequence. The prediction of signal peptides was performed by SignalP and revealed the presence of one peptide, with predicted cleavage site between the residues 25 and 26 (Fig. 2A). In accordance, the TMHMM algorithm for transmembrane helices prediction has indicated a possible transmembrane helix in the same region, despite with low probability (Fig. 2B). Finally, the subcellular location of the protein was indicated to be on the cellular membrane, according to the Psort program, indicating the secretory character of the protein (Fig. 2C). All these
results corroborate with the expected for an enterotoxin, which has to be secreted by the bacteria in order to perform its toxic effect.

```plaintext
>sp|P20723|Enterotoxin type D|Staphylococcus aureus|
MKKFNILIALPFTSLVPLNVKANENDSVKEKELHKKSELSSTALNNMKHSYADKNP
IIIGENKSTDQFLENTLYKKFFTDLINFDLLLNFNSKEMAQHFKSKNVDVYPIRYSIN
CYGGEIDRTACTYGGPHEGNKLKERKKPIPIWINGQKEVSLDKVQTDKKNTVQEL
DAQARRYLOKDLKLYNDTLGKIQRGKIEFD5SD65KVSYDFDVCGDFPEK0LRIYSD
NKTLSTEHLHDITYEK
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**Figure 2.** A) Graphical result of SignalP, showing the signal peptide with predicted cleavage site between the residues 25 and 26. B) TMHMM result, presenting a possible transmembrane helix that coincides with the region of the predicted signal peptide. C) The subcellular localization predicted by the Psort algorithm for gram-positive bacteria points out the cellular membrane as the most probable location for the SED protein.

**Selection of a Template-Structure**

The first step for the execution of an in silico comparative modeling protocol is the selection of a structural template with a previously determined structure, solved by experimental methods, such as X-ray crystallography and NMR. As previously described, the methodology to identification a structural template is based on the search for similar sequences using the BLAST tool and the database of protein structures PDB. As a result, the PDB structure with amino acid sequence most similar to SED is the *S. aureus* enterotoxina A, of PDB code 1I4G. This protein was chosen not only based on its proximity with the SED protein, but also on good quality as a structural template, represented by an alignment score of 243, query coverage of 89% (or of 99% when the signal peptide of SED is omitted), percentage of residues identical to SED of 52%, e-value of 3e-79 and structural resolution of 2.1 angstroms. All of these values
characterize this as a good structural-template and justify its choice to base the comparative modeling of the SED protein.

**Sequence Alignment**

The next step after selecting a structural-template is the generation of a sequence alignment between the target-protein and the structural-template. In the present work, the program used to generate the sequence alignment was Promals3D. This alignment evidences important characteristics of both proteins and their correspondence. For instance, one can observe the exact correspondence between the cysteine residues 96 and 106 involved in the disulfide bridge in the template structure and cysteine residues 121 and 131 of the target protein (the numbering of residues for the SED protein refers to the pre-processed sequence). Additionally, the sequence alignment has confirmed an expected result, which the template-structure does not present a sequence complementary to the signal peptide and, therefore, the structural model that will be generated to the target-protein will be depleted of this region. It is also worth noting that the first ten amino acids of the structural template are absent from the PDB coordinate file (a very common occurrence) and were therefore not considered during the process of comparative modeling (Fig. 3). Therefore, the tridimensional model presented for the SED protein is initiated on residue 35 (given the 25 amino acids from the signal peptide and the first ten residues of the template are absent).

**Zinc Ion Coordination**

Initially, we have performed the prediction of the functional protein family, using the SVMProt program, which is available online. As a result, the predicted nature of the SED protein, with probability of 99%, is of a zinc-binding protein, corroborating what is expected, according to the features of related proteins. About the zinc coordinating residues, the structural-template of PDB code 1I4G has one mutation at the position 187, where a histidine is substituted by an alanine. This substitution has generated important alterations, as a new interaction between the zinc ion and the histidine residue on position 44. As a result, there are three residues involved in the coordination of the zinc ion, H44, H225 and D227, from which (as expected) only the last two have correspondent residues on the target-protein and the position of the H44 is occupied by a glycine residue (G69) in the target-protein (Fig. 3). Besides the H250 and D252 residues that correspond to residues H225 and H227 in the template-structure the target-protein also presents a H187 residue which is the mutated site (H187A) of the template-structure. In summary, the zinc ion in the SED protein seems to be coordinated by three amino acids, D213, H250 and D252, which is corroborated by the results of the TEMSP program (TENG et al., 2011) that identifies metal-binding sites on protein structures. The structure generated for the SED protein was submitted to the TEMSP web server and, as a result, the program has indicated exactly these three residues as metal-binding (T-deviation of 1.14). Both the disulfide bridge formed between the previously cited cysteine residues and the zinc ion were included in the final structure generated for the S. aureus SED protein.
Generation of Candidate Structures and Selection of a Structural-Template

A set of one hundred structures was generated with the Modeller program version 9.10 and analyzed according to its stereochemical and energetically features. As a result, the candidate structure number 70 was indicated as the highest quality structure, presenting a percentage of residues in the most favorable region of the Ramachandran plot of 95.1% and a Z-score value from ProSA of -6.59 (Fig. 4). The model was inspected by PyMol for the visualization of the tridimensional structures, allowing a detailed inspection of the zinc binding site and the disulfide bridge. The result is shown below (Fig. 5) that represents the superposition between the target-protein (green) and the structural-template (cyan).

Figure 3. Sequence alignment between the target-protein and the structural-template, performed by Promals3D and colored by DNATagger (positively charged amino acids are blue, negatively charged are red, polar amino acids are green and apolar are yellow). The signal peptide region is indicated by a light blue bar, the cysteine residues of the disulfide bridge are connected by a yellow line and the zinc coordinating residues are marked by Z characters in either dark (for residues present in the target-protein) or light (residue H44, only present in the structural-template, as an effect from the mutation) grey shades.

Figure 4. A) Ramachandran plot presenting the combinations of phi and psi angles for each amino acid in the highest quality candidate structure, generated for enterotoxin D. As a result, over 95% of the residues were positioned in the most favored region of the plot. B) ProSA result showing the location of the same candidate structure in the energy plot and corresponding Z-score.
Structural Domains

The graphical representations of 1I4G structural domains were retrieved from the PDBSum. Also for the SED protein, a similar result was generated using PDBSum to represent its structural domains. As a result, one can observe a high degree of concordance between both proteins. For both proteins, the N-terminal domain is composed by seven beta-strands and one alpha-helix (with an additional alpha-helix on the SED protein, which represents the signal peptide). The C-terminal domain is structurally classified as a beta-grasp folding and composed by nine beta-strands and three alpha-helices, arranged in such a way that each alpha-helix is always preceded by two beta-strands (Fig. 6).

Figure 5. Superposition between the structures of the target protein (green) and the structural-template (cyan), evidencing the structural similarity between them and highlighting the zinc ion (grey sphere) coordination by the aspartic acid and histidine residues (red for the target-protein and yellow for the structural-template). The disulfide bridge is shown as a yellow bond connecting a beta-strand to a loop region.

Figure 6. A) Organization of the secondary structure elements predicted by the PDBSum according to structural analyses on the model generated for the enterotoxin D, showing the orientation of the beta-strand, alpha-helices and the connecting loop regions. B) Organization of the secondary structure elements for the N-terminal domain of the template-structure (1I4G). C) Organization of the secondary structure elements for the N-terminal domain of the template-structure (1I4G). It is possible to see the correspondence between the secondary structure elements of both proteins (although one needs to rotate the C-terminal domain of the template-structure in 180 degrees to better see the correspondence).
Structural Model Oligomerization

As described at the Uniprot database (code P20723), the SED protein is present in the cell as a homodimer. Similarly, the structural-template (PDB 1I4G) was also crystallized for X-ray diffraction as a homodimer. Therefore, the aforementioned structural model of the SED protein was used as the fundamental unit to generate a homodimeric molecular structure for the target-protein. Given the structural similarity between the SED protein and *S. aureus* enterotoxin A (the structural-template), the monomer-monomer connection of the latter has based the orientation of the monomers of the former to form the homodimeric complex, using PyMol. The resulting homodimer can be seen in the figure below (Fig. 7).

![Homodimeric structure generated for enterotoxin D, based on the template-structure 1I4F and represented as cartoon (A) and with a superposed surface in transparent grey (B).](image)

**Figure 7.** Homodimeric structure generated for enterotoxin D, based on the template-structure 1I4F and represented as cartoon (A) and with a superposed surface in transparent grey (B).

Structure-Function Relationship for the SED Protein

From the homodimeric structure built for the SED protein, several structural characteristics that are fundamental for its biochemical role can be explored. First, the zinc coordination was assessed and it seemed to agree with the configuration presented by SEA, used as template. In order to analyze the interaction between the enterotoxin and the MHC molecule, the SED structure generated by comparative modeling was superposed to the structure of the *S. aureus* enterotoxin H in complex with the human MHC II. In agreement with the information that the zinc ion is of fundamental importance for the SE-MHC complex formation, the interaction between these molecules is placed in this region (Fig. 8). A recent study has evidenced that the SEA D227 residue exerts important emetic activity, since its substitution by an alanine residue has resulted in a molecule with reduced emetic activity (PINCHUK, V.; BESWICK, J.; REYES, 2010). Given the importance of this residue, we have investigated the correlation between the amino acid that occupies this position in the two proteins and observed that both present an aspartic acid residue in this position.
CONCLUSION

In the present work we have generated the tridimensional structure of the *S. aureus* enterotoxin D through the comparative modeling in silico technique. As a result, we have obtained a high-quality monomeric structure that was further used to generate a homodimeric complex representative of the functional molecule. The functionally important residues from SED were investigated and compared to the corresponding residues present in the structural-template, 1I4G, which represents *S. aureus* SEA protein. The triad of amino acids that coordinate the zinc ion is conserved in the SED protein and the protein was predicted to be a zinc-binding protein. The cysteine residues that form the disulfide bridge in the structural-template are also conserved in the target-protein, indicated the presence of a similar structure, conferring stability to the SED protein. Regarding the functional domains, a comparison between the secondary structure and the predicted structural domains for both SEA and SED enterotoxins have demonstrated that the latter is structurally very similar to the chosen template-structure. In summary, the structural model generated for *S. aureus* enterotoxin D presented in this work seems to meet the structural requirements to represent a SE and may, therefore, be used to guide further studies on the dynamics and function of this protein.

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