Amyloidogenic Regions in bPaS1

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Bacterial S1 protein is a functionally important ribosomal protein. It is a part of the 30S ribosomal subunit and is also able to interact with mRNA and tmRNA. An important feature of the S1 protein family is a strong tendency towards aggregation.

Keywords: ribosomal S1 proteins ; amyloidogenic regions ; toxicity ; antibacterial peptides ; amyloid ; mass spectrometry

1. Introduction

The study of amyloids as ordered fibrillar protein aggregates is of great importance for elucidating their role in human pathologies, especially in neurodegenerative diseases [1][2][3]. It is known that, under certain conditions, most proteins and peptides tend not only to aggregation, but also to form amyloid-like fibrils [4][5][6]; in a particular case, the formation of amyloids of some proteins can be induced by other amyloidogenic proteins and peptides [7][8]. Currently, interest in the study of amyloids is also associated with the fact that they can be used in various nano- and bio-technological developments, including as antimicrobial agents against pathogenic microorganisms [9][10][11]. In recent reviews of scientific articles, the prospects of using antimicrobial peptides in medicine are discussed [12][13], including those acting by the mechanism of directed coaggregation with the target protein due to the interaction of amyloidogenic sites that constitute the spine of amyloid fibrils [14]. Disruption of the native structure of the most important bacterial proteins, in particular ribosomal ones, caused by directed aggregation, can be accompanied by a loss of the functional activity of the protein, which, in turn, can lead to a change in normal cellular metabolism and the death of bacteria.

The ribosomal S1 protein is the largest bacterial protein of the 30S ribosomal subunit and can perform, in addition to structural, many other functions, interacting with both RNA and other proteins [15][16][17][18]. It was shown that amber mutation and knockout of the gene encoding the bS1 protein lead to the death of bacterial cells [19][20]. The bS1 protein, which is present only in bacterial cells, contains, depending on the taxonomic affiliation of the microorganism, from one to six domains of the S1 protein (D1–D6), separated by flexible regions [21][22]. It is important that the S1 domain is a structural variant of the oligosaccharide/oligonucleotide-binding fold (OB-fold) [23][24] and can exhibit amyloidogenic properties, like another analog of the OB-fold, the cold shock domain [25]. Previously, peptides with amyloidogenic properties and antimicrobial activity against *Thermus thermophilus* were synthesized and studied based on the sequences of the S1 domains of the ribosomal S1 protein of the model organism *T. thermophilus* [26].

P. aeruginosa is a pathogenic bacterium that can cause nosocomial infections [27][28], and for which cases of multiple antibiotic resistance are increasingly being reported [29][30]. Recently, antimicrobial peptides have been considered as an alternative to classical antibiotics for the treatment of diseases caused by multidrug-resistant strains of *P. aeruginosa* [31][32][33]. Information about the amyloidogenic regions in the structure of the ribosomal S1 protein from *P. aeruginosa* (bPaS1) will allow the development of new antimicrobial peptides that specifically interact with this target protein and cause its aggregation, which will ultimately lead to disruption of the functioning of the ribosomal S1 protein and suppress the vital activity of this pathogenic bacteria.

The main contribution to the formation of amyloids is made by amino acid residues, which contribute to a denser packing of the protein structure [34][35]. Consequently, protein regions included in the spine of amyloid fibrils are characterized by high resistance to protease treatment, which is used to determine amyloidogenic regions in products of limited proteolysis of aggregates [36][37].

In the present work, amyloidogenic fragments were identified in the amino acid sequence of bPaS1, using the programs for searching and predicting amyloidogenic regions FoldAmyloid [38], Waltz [39], Pasta 2.0 [40] and AGGRESCAN [41], and experimentally by analyzing the products of limited proteolysis of bPaS1 aggregates using high performance liquid chromatography and mass spectrometry (LC-MS). The tendency to amyloid formation of peptides synthesized on the

basis of amyloidogenic regions of bPaS1 was studied by electron microscopy (EM) and fluorescence spectroscopy (using thioflavin T (ThT)), which are widely used to detect amyloids [42][43][44].

2. Isolation and Purification of bPaS1

The *E. coli* strain was obtained, the genetic construct allows us to obtain the recombinant bPaS1 with additional inserts: an N-terminal sequence with 6 His, which allows the use of affinity chromatography purification; a specific TEV protease recognition site for cleaving intact bPaS1. Nucleic acids were precipitated with streptomycin sulfate and the precipitates were removed from protein samples. The degree of purification of bPaS1 preparations was assessed by electrophoresis of samples under denaturing conditions. The resulting final preparation had a purity of at least 90%.

3. Prediction and Experimental Determination of bPaS1 Regions Prone to Aggregation

The ability of a protein to aggregate and form amyloid-like fibrils is primarily determined by the presence of amyloidogenic regions in its structure, which can be predicted using special programs developed for this purpose. Prediction of amyloidogenic sites for bPaS1 was performed using four programs: FoldAmyloid, Waltz, AGGRESCAN, and Pasta 2.0 (**Figure 1**B).

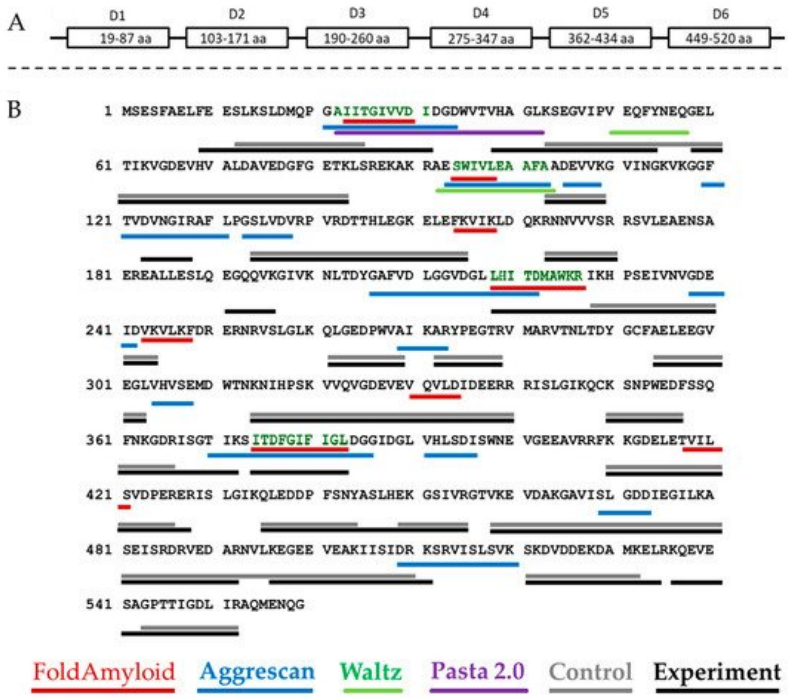

Figure 1. Schematic representation of the domain organization of bPaS1 (**A**) and comparison of predicting amyloidogenic regions using programs with the results of peptide coverage after LC-MS analysis of hydrolysates of control and experimental (aggregate) protein preparations (**B**). The peptides identified in the control and experimental samples, respectively, are underlined in gray and black. The bPaS1 sequence is taken from the UniProt database (UniProt. Available online: https://www.uniprot.org/uniprot/Q9HZ71 (accessed on 20 January 2021)). The regions of bPaS1, prototype peptide synthesis, are shown black–green color.

Each program predicts at least one region prone to amyloid formation in the bPaS1 sequence. However, the prediction results differ between different programs as they use different algorithms to find amyloidogenic regions. Subsequently, an experimental search for protein regions resistant to the action of proteases was carried out in the course of limited proteolysis and analysis of hydrolysates by LC-MS. In total, 146 significant peptides were found in the products of limited proteolysis of bPaS1 aggregates. At the same time, only 96 significant peptides were detected in the control sample without incubation for aggregation. Subsequently, significant peptides identified in the hydrolysates of control and experimental bPaS1 samples were ranked by length, the longest of them was compared with the bPaS1 sequence in order to determine the regions most protected from the action of proteases in aggregates and control preparations (**Figure 1**).

As shown in **Figure 1**B, the overall peptide coverage for protein aggregate hydrolysates and controls is similar. At the same time, additional amino acid sequences for aggregates have been identified that may play a role in the formation of

associates. LC-MS data were analyzed, and peptides with a length of at least five amino acid residues were selected (similar to the selection criterion in programs predicting amyloidogenic sequences of at least five amino acid residues), which are present only in hydrolysates of aggregates and are not observed in control samples (**Table 1**).

Table 1. Unique peptides identified as a result of comparing data from LC-MS analysis of hydrolysates of bPaS1 aggregates.

Peptide	Prediction of Amyloidogenicity	Percentage of Most Non-Polar a.a. [45] (V,I,F,C,L,A,M), %	Observed Mass, Da	Theoretical Mass, Da	Measurement Error, ppm *	Molecular Ion, *m/z*	Charge (z)	Value of the Function T **
FEESLK (9–14 a.a.)	No	0	751.376	751.3752	0.5	376.6951	+2	35.71
AIITGIVVDI (22–31 a.a.)	AGGRESCAN, Pasta 2.0, partially FoldAmyloid (23–30 a.a.)	70	1012.618	1012.6168	0.9	507.3162	+2	41.51
VHAGLK (38–43 a.a.)	Pasta 2.0	50	623.374	623.3755	–1.8	312.6945	+2	17.19
DVNGIR (123–128 a.a.)	AGGRESCAN	33	672.356	672.3555	0.7	337.1852	+2	32
E (+27.99) GQQVK *** (191–196 a.a.)	No	17	715.35	715.35	–0.3	358.6822	+2	16.8
LHITDMAWKR (218–227 a.a.)	FoldAmyloid, partially AGGRESCAN (218–223 a.a.)	40	1269.666	1269.6652	0.4	635.8401	+2	114.36
ISGTIK (367–372 a.a.)	partially AGGRESCAN (370–372 a.a.)	33	617.375	617.3748	0.7	309.6949	+2	27.5
ITDFGIFIGL (374–383 a.a.)	AGGRESCAN, partially FoldAmyloid (375–382 a.a.)	60	1094.601	1094.6012	–0.1	548.3078	+2	76.43
ASLHEK (445–450 a.a.)	No	33	683.361	683.3602	1	342.6877	+2	30.93
KQEVESA (536–542 a.a.)	No	29	789.388	789.3868	1.1	395.7011	+2	41.89

*—The accuracy of molecular weight measurement of 1 ppm (parts per million) corresponds to 0.001 Da for an ion with a molecular weight of 1000 Da. **—For the PEAKS Studio 7.5 software we used (Bioinformatics Solution Inc., Waterloo, ON N2L 6J2, Canada) the value of the function T = –10 lgP, where P is the probability that a false identification of a peptide in the current search will achieve the same or better conformity score. For peptide mapping, only peptides for which a T value > 15 were used, which corresponds to the p-criterion < 0.03 [46]. ***—Mass shift (+27.99) means amino acid post-isolation modification (formylation) at the N-termini for peptide EGQQVK.

As follows from **Table 1**, the results of bioinformatic analysis and experimental determination of amyloidogenic regions in the bPaS1 sequence do not coincide for all protease-resistant peptides found in protein aggregates. At the same time, for the identified peptides FEESLK, AIITGIVVDI, DVNGIR, LHITDMAWKR, ITDFGIFIGL, ASLHEK, KQEVESA, the accuracy of molecular weight measurement was no worse than 1.8 ppm, and the T function value was at least two times higher than the threshold value, which on the whole indicates a high reliability of the experimental determination. Thus, the bPaS1 regions that overlap with the results of predicting amyloidogenicity by at least two programs, or are identified only in the products of limited proteolysis of bPaS1 aggregates, were used as prototypes for the synthesis of peptides: AIITGIVVDI, SWIVLEAAFA, ITDFGIFIGL and LHITDMAWKR (**Figure 1**B). Interestingly, the local distribution of non-polar amino acid residues, especially V, I, F, C, can be used to assess the propensity of a peptide to form amyloid structures [45]. The AIITGIVVDI, SWIVLEAAFA, ITDFGIFIGL fragments are characterized by a high percentage of nonpolar amino acid residues (70%, 70%, and 60%, respectively), in contrast to the LHITDMAWKR peptide (40%).

The bPaS1 regions, which are theoretically predicted to be amyloidogenic and experimentally resistant to the action of proteases, are of interest for further study and discussion of the prospects for using antimicrobial peptides acting on the basis of directed coaggregation in the development of antimicrobial peptides.

4. Electron Microscopic Images of Aggregates

Recombinant bPaS1 was isolated, purified and analyzed using the EM method. According to EM data (**Figure 2**), bPaS1 under conditions of 50 mM TrisHCl, pH 8.0; 100 mM NaCl; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol forms disordered aggregates. That is, as in the case of the recombinant protein bS1 from *T. thermophilus* [47], bPaS1 does not form fibrils. However, it should be noted that bPaS1, in contrast to the previously studied bS1 from *T. thermophilus*, is less prone to aggregation and forms small and less dense aggregates of various sizes [47]. The images of amyloids/aggregates of peptide synthesized based on the predicted amyloidogenic regions in the bPaS1 amino acid sequence are shown in **Figure 3**.

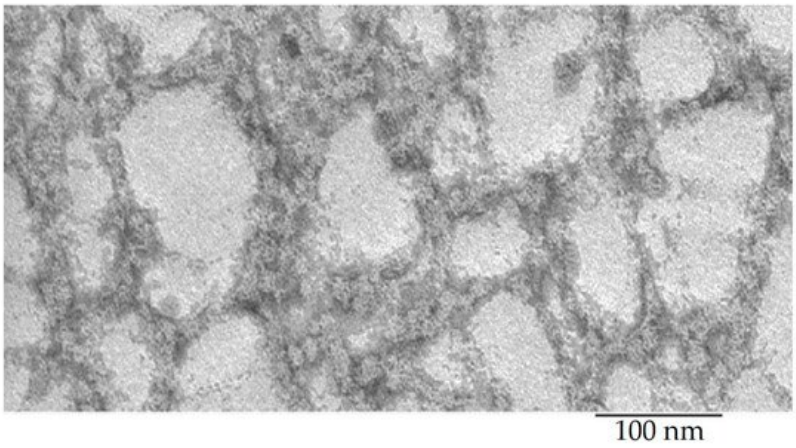

Figure 2. Electron microscopic image of the bPaS1 protein under conditions of 50 mM TrisHCl, pH 8.0; 100 mM NaCl; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol.

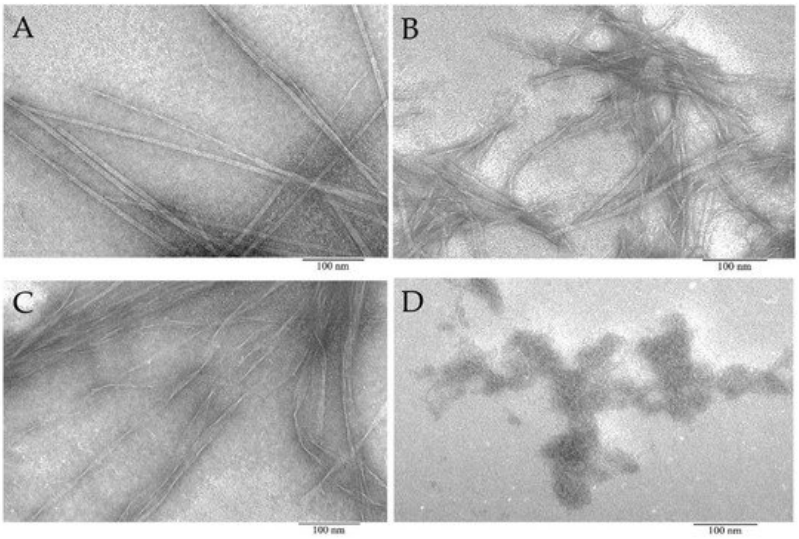

Figure 3. Electron microscopic images of aggregates formed from peptide preparations synthesized based on the bPaS1 sequence: AIITGIVVDI (**A**), SWIVLEAAFA (**B**), ITDFGIFIGL (**C**), and disordered aggregates of the LHITDMAWKR peptide (**D**).

According to EM data, it was shown that the AIITGIVVDI, SWIVLEAAFA, and ITDFGIFIGL peptides under conditions of 50 mM TrisHCl, pH 7.5; 150 mM NaCl, incubation for 5 h at 37 °C are able to form amyloid-like fibrils of various morphologies. Under the same conditions, the LHITDMAWKR peptide did not form fibrils, but only disordered aggregates.

5. Thioflavin T Fluorescence Assay for Aggregation of bPaS1 and Peptides

The property of thioflavin T to bind to amyloid fibrils with a simultaneous multiple increase in fluorescence at a wavelength of ~485 nm [48] was used by us to analyze the tendency towards the formation of amyloids in bPaS1 preparations and

AIITGIVVDI, SWIVLEAAFA, ITDFGIFIGL, LHITDMAWKR peptides (**Figure 4**). In **Figure 4** (Part 1), the ThT fluorescence intensity at a wavelength of ~485 nm, exceeding the control values for free ThT by a factor of ten or more, was obtained for preparations of the AIITGIVVDI, SWIVLEAAFA, ITDFGIFIGL peptides (**Figure 4**C–E,K, Part 1), as well as in a mixture of these peptides with bPaS1 (**Figure 4**G–I,L, Part 1). At the same time, for bPaS1 preparations and the LHITDMAWKR peptide (**Figure 4**B,F, Part 1), as well as for their mixture in solution (**Figure 4**J, Part 1), a multiple increase in the ThT fluorescence intensity was not observed.

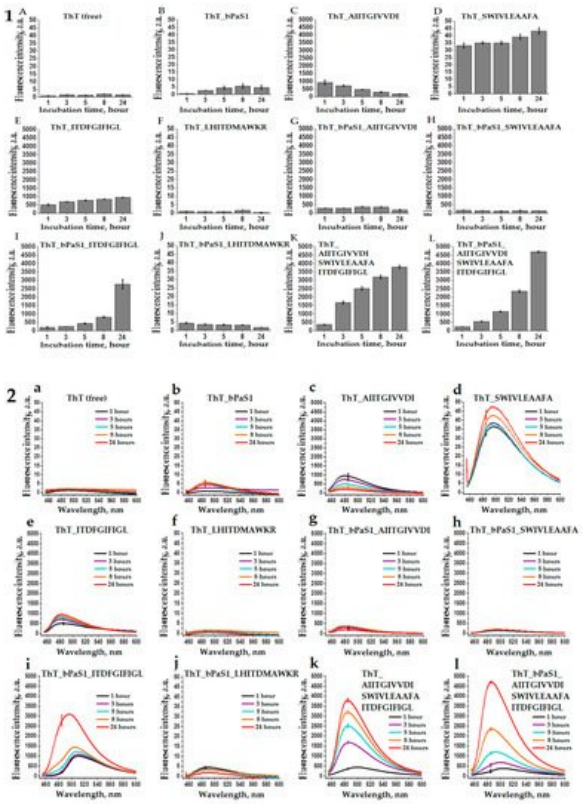

Figure 4. Histograms (**1**) and spectra (**2**) of fluorescence intensity of free thioflavin T (**A**,**a**) and in solution with bPaS1 (**B**,**b**), individual peptides AIITGIVVDI (**C**,**c**), SWIVLEAAFA (**D**,**d**), ITDFGIFIGL (**E**,**e**), LHITDMAWKR (**F**,**f**), a mixture of peptides (**K**,**k**), as well as in mixtures of bPaS1 with peptides (**G**,**g**), (**H**,**h**), (**I**,**i**), (**J**,**j**), (**L**,**l**). Error bars with standard deviations for the mean values of the measured fluorescence intensity after 1, 3, 5, 8, and 24 h of incubation are shown.

Thus, the presence of the effect of a multiple increase in the ThT fluorescence intensity upon incubation with preparations of the AIITGIVVDI, SWIVLEAAFA, ITDFGIFIGL peptides and the absence of such an effect for preparations with the LHITDMAWKR peptide is consistent with the data of electron microscopy that the AIITGIVVDI, SWIVLEAAFA, ITDFGIFIGL peptides form amyloid fibrils, while only disordered aggregates of the peptide are found in the LHITDMAWKR preparations.

It should be noted that when testing the propensity for coaggregation of individual peptides with bPaS1, the greatest increase in the ThT fluorescence intensity was observed in a mixture of the ITDFGIFIGL peptide with bPaS1 after 24 h of incubation (**Figure 4**i, Part 2).

Thus, although bPaS1 preparations do not form amyloid-like fibrils, they affect the change in the relative intensity and wavelength of the maximum intensity of ThT fluorescence in mixtures with amyloidogenic peptides. No such effects were observed in mixtures of bPaS1 with the non-amyloidogenic LHITDMAWKR peptide.

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