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*Biophysics*

# The Study of Transition of Giant Protein Smitin into Amiloid Form by Means of Different Methods *in vitro*

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**We studied the peculiarities of transition of smitin isolated from chicken smooth muscle (stomach) into the amyloid state *in vitro*. The protein was transferred to the amyloid state using a solution of 0.15 M glycine / KOH, pH 7.0-7.5, I = 0.1. We determined the amyloid nature of Smitin using the specific dye Congo red, which binds to amyloid protein and paints it red or pink. We studied Smitin's circular dichroism spectra before and after the formation of amyloid fibrils. Smitin, under particular conditions, in a diluted solution very quickly creates aggregates of different order and length – β sheets structures. According to the obtained results, the transition of native smitin to the amyloid state occurs rapidly and the further increase of the incubation time does not cause any change in the secondary structure. The thermal denaturation of amyloid smitin was studied by calorimetric method. The analysis of melting curves inducate that β- sheets are mainly created in the second domain of smitin native molecule and it increases its denaturation temperature by 10°C.** © *2022 Bull. Georg. Natl. Acad. Sci.*

smitin, amyloid, Congo Red, circular dichroism (CD)

In the 18-19 centuries structures were found that were called amyloids, which in Latin meant starch. These structures contained proteins and did not contain carbohydrates. These data laid foundation for the study amyloids as protein formations.

Amyloids are insoluble fibrous protein aggregates sharing specific structural features: high content of $\beta$-sheet structure, ability to bind to Congo red (CR) [1] and thioflavin T (ThT), possess a double conversion ability in a polarized ray, they are resistant to solvents and proteases. Amyloids accumulate in a different organs and tissues in the form of insoluble fibers and cause the development of pathological processes. Due to their high firmness they are capable mechanically destroy cell membrane, cause impairment of its viability and eventually its death [2].

Amyloid formation includes two periods: the period when the amyloid embryo is formed and the period when all protein monomers go into amyloid form. To get the complete description of the process of amyloid formation it is necessary to

determine all possible protein conformational states during its aggregation, as well as the study of the process of protein transition into amyloid form will not be full without determination of relevant kinetic and thermodynamic parameters [3].

Amyloid nature of proteins causes the disease – amyloidosis. It represents a big group of conformational diseases, which are characterized by precipitation of proteins and their accumulation in different organs and tissues in the form of insoluble fibers. This accumulation causes the disruption of structure and functions of organs and tissues that leads to disease and finally to disastrous result. Such diseases as second type of diabetes, Alzheimer's, Parkinson' s diseases etc. belong to amyloidosis [4]. Currently up to 50 proteins are known that form amyloidal fibers. Myoglobin, immunoglobulin, amylin, $\tau$ protein, titin family proteins (titin, smitin), alpha, beta peptides, etc. Amyloid fibers are found in skeletal muscles (striated, smooth) and blood vessels while cardiopathy, myocarditis, myositis, though their protein nature is not fully studied [5].

The speed of formation of amyloid fibers in different proteins is different. The predecessor proteins of amyloids under the influence of area conditions (low pH, high temperature, long incubation, addition of denaturizing agents etc.) undergo transformation (α-spiral, β-sheet) that generally is characteristic for amyloid fibers [6].

Structural investigations of amyloids began in the 1930 with the use of X-ray diffraction. Hence, Astbury and Dickinson [1] first noted the distinctive X-ray fiber diffraction pattern later called cross-β. Active using of the X-ray diffraction method allows to conclude that amyloids are composed of polypeptide chains extended in the so called cross-β conformation. In the cross-β structure the individual strands of each β-sheet run perpendicular to the fibril axis (4.7 A° spacing) whereas the β-sheet (∼10 A° spacing) are parallel to the fibril axis [1].

The study of the amyloid formation process is currently one of the most important tasks today.

Staining by Congo Red (CR) is one of major methods used to detect the amyloid structure of protein aggregates *in vitro*. However, a series of experiments have shown that CR staining is insufficient for confirmation of the amyloid nature of protein aggregates. [1]. There is evidence demonstrating the binding of Kongo Red to proteins with different secondary structure. This fact indicates that a specific amyloid structure is not mandatory for the binding of CR. It has also been found that the binding of CR to protein molecules leads to protein oligomerization. Based on these data, the following conclusion on the possible mechanism of CR binding to proteins was made: "It is most likely that both the hydrophobic and the electrostatic components of the structure of CR are critical for its binding to proteins" [1]. Muscle and nonmuscle contractile systems play essential role in the formation and functioning of a cell. The determination of mechanism of motility, namely – muscle contraction, is realized by highly organized apparatus, the functioning of which depends on major (myosin, actin, tropomyosin) as well as minor ( troponin, actinins) and giant (titin, smitin) proteins.

Molecule of titin contains collagen type structure ( left spiral ) that determines firmness of myofibril [7]. Titin threads of striated muscle play particular role in organization of sarcomere structure while myophibrinogenezys. Its elastic property preserves native skeletal muscle thick treads location in sarcomere center and passively resist the sarcomere stretching while contraction. Its molecular mass is 3000 kDa, it possesses immunoglobulin like repetitive sites composed by 100 amino acids (lg) and a unique section rich in proline, glutamine, valine and lysine (PEVK) [8]. During sarcomere stretching titin domains can deploy and open closed hydrophobic sections, which can cause protein aggregation and its

dysfunction. Thus, titin is a suitable model for study proteins aggregation [9].

In 2002 it was demonstrated that the smooth muscle contains unknown protein with molecular mass 2000 kDa, much resembling the striated muscle protein titin by its molecular morphology and location within the contraction apparatus. This protein was called smitin. Likewise titin it posseses immunoglobulinlike repetitive sites (lg) and a unique section rich in proline, glutamine, valine and lysine ( PEVK) [10].

While studying the secondary structure of proteins (titin, smitin), it was found that they consisted mainly of β-structure and random coil [11]. Therefore, there is a prerequisite that in physiological conditions (pH 7.0 - 7.5; I = 0.1; T = 5-30°C) in these proteins amyloidal fibers can be formed quickly.

Our departament studies muscle proteins and considering that proteins of the titin family (titin, smitn) participate in the regulation of actin-myosin interaction during muscle contraction and are characterized by amyloid nature, we considered it appropriate to study the physical-chemical properties of one of the proteins of the titin family, namely smitin, during the transition to the amyloid state.

## Materials and Methods

Smitin was isolated from the chicken smooth muscle, particularly from the stomach according to Keller with our modification [12]. Myofibrils were obtained from chicken stomach according to Wang [8]. Homogenized myofibrils (100 g) were washed three times in A buffer (2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DDT, 2 mM PMSF, 10 mM imidazole, 10 mM trypsin inhibitor, 50 mM KCl, pH 7.0) by centrifugation (5000 g, 10 min, 4°C) and resuspended in extraction buffer B (2 mM $MgCl_2$, 1 mM EDTA, 0,6 M KCl, 4 mM ATP, 0.5 mM DTT, 0.2 mM PMSF, 10 mM trypsin's inhibitor, 10 mM imidazole, pH 7.0). The extracted myofibrils were sedimentated by centrifugation (15 000 g, 30 min, 4°C) and the supernatant was applied to the Toyopearl-65 column (1.5 cm, 90 cm). The Toypearl-65 column was equilibrated with buffer (0.2 M KCl, 10 mM imidazole, 1 mM EGTA, 0.5 mM EDTA, 0.2 mM DTT, pH 7.5). The first fraction contained native smitin only, the second fraction contained smitin + myosin and the third fraction contained only myosin.

Purity of the proteins was examined by the electrophoresis method in the polyacrylamide gel gradient (3-15%) in the presence of Na-dodecyl sulfate by Laemmly method. [13]. For fixing smitin transition into amyloid form we used Congo Red dye, that stains amyloids into rose or red colors. Congo Red solution was added to amyloid protein suspension at the ratio 1: 2. The difference between absorption spectra of Congo Red and Congo Red + amyloid protein complex indicates to protein transition into amyloid form. Absorption is recorded at 450-660 nm on the spectrophotometer (Shimadzu UV-2100). Native Smitin (0,5mg/ml) in 0.6 M KCL, 30 mM $KH_2$ $PO_4$, 1 mM DTT, 0.1 M $NaN_3$ pH 7.5, was dialyzed for 20 min, 4 h, 24 h against the buffer containing 0.15 M glycine /KOH pH 7.5. Using the method of circular dichroism we studied the secondary structure of amyloid protein – smitin. The circular dichroism spectra of smitin was registered before and after the formation of amyloid fibres by means of spectropolarimeter (JASCO J-500A). The circular dichroism spectra was measured at 190-240 nM. The analysis of spectra for determination of secondary structure was carried out by means of online program (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3//).The thermal denaturation of amyloid smitin was studied by calorimetric method. This method yields information on the existence of definite thermostable sites in the macromolecule that allows to make conclusions about the structural peculiarities of macromolecules. Results obtained by using this method, were compared with data for native smitin. Thermal denaturation was determined by means of DACM-4 type microcalorimeter.

## Results and Discussion

Using the specific dye Kongo Red we have shown that smitin isolated from smooth muscle (chicken stomach) in 0.15 glycine (KOH, pH 7.5, T 5-30°C) formed amorphous aggregates so called amyloid fibrils. We have studied the process of their formation depending on time (20 min, 4 h, 24 h). From the Fig. 1 can see that in 20minutes formation of amyloid fibrils already has occurred. The difference between the absorption spectra indicates to the protein transition into the amyloid form. In previous years we were studying the secondary structure of native smitin. According to our data, the secondary structure calculated from the CD spectrum of smitin after chromatography was: α-helix 2.59%, β-sheet 22.24%, random structure 75.17%. The secondary structure after the formation of aggregates was as follows: α-helix 3,2, β-sheet 30.5%. The circular dichroism spectra of smitin was registered before and after the formation of amyloid aggregates (24 h) Fig. 2 shows CD spectra of smitin before and after of aggregation. No changes were detected in secondary structure while formation of aggregates.

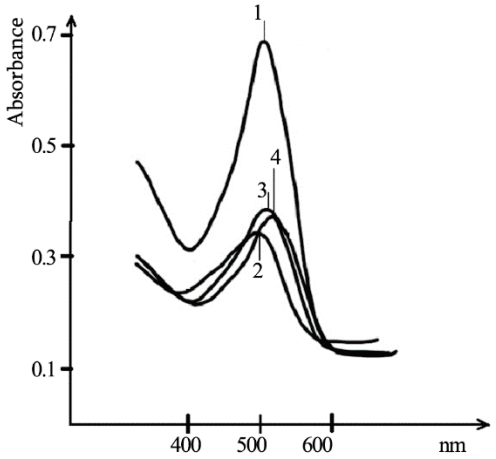


**Fig. 1.** Absorption spectra of kongo red and amyloid smitin, Gly/KOH, pH 7.5., I=0.1.
Smitin tranzition to amyloid form (20 min, 4 h, 24h): 1. Kongo red- absorption spectrum; 2. Kongo red+ native smitin-absorption spectrum (20 min. transition); 3. Kongo red + native smitin-absorption spectrum (4 h. transition); 4. Kongo red + native smitin-absorption spectrum (24 h. transition).

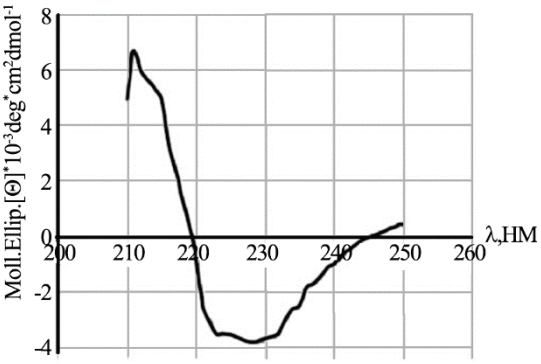


**Fig. 2.** CD- spectrum of amyloid smitin (24 h) 0.3 mg/ml, glicin/ KOH , pH 7.5.

It is known [2] that smitin, under particular conditions, in a diluted solution very quickly creates aggregates of different order and length – β-sheet structures which creates difficulties to precise CD measurements. Because of this, together with CD measurements we conducted DSC measurements.

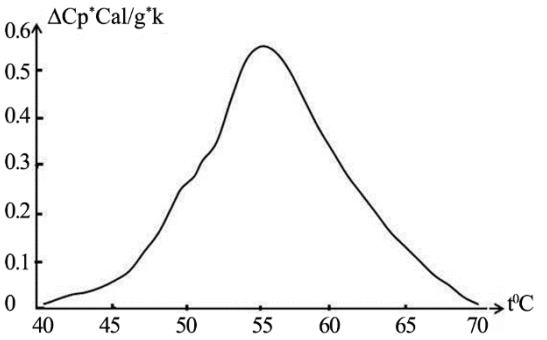


Fig. 3a. Heat absorption curve as a function of temperature on dQ/dT (J/gK) of native smitin solution, heating rate 1 degree/min, measuring volume of smitin solution 0.150 μL, smitin concentration 1.4 mg/mL, and ΔH=35.5±3.5 J/g.

The DSC method gives the possibility to explore structural transition of biopolymers as in diluted and concentrated solutions as in biopolymer suspensions.

DSC tests were conducted with diluted solutions of native smitin not containing small aggregates weighted in the solution as β-sheets and for comparison with smitin containing β-sheets, storage 24 h at 25°C.

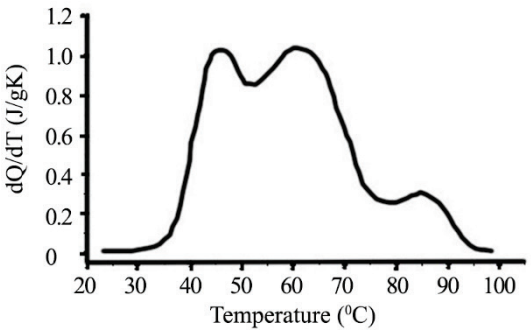


**Fig. 3b.** Heat absorption curve as a function of temperature on dQ/dT (J/gK) of treated smitin (8 h) solution, heating rate 1 degree/min, measuring volume of smitin solution 0.150 μL, smitin concentration 1.4 mg/mL, and ΔH=31.2±3.5 J/g.

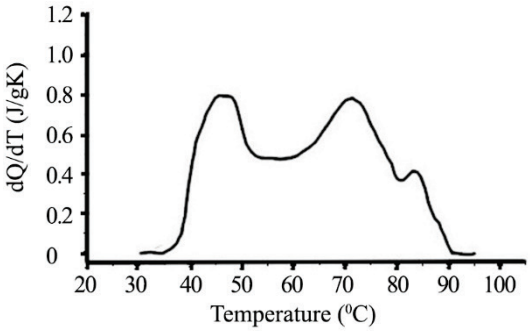


**Fig. 3c.** Heat absorption curve as a function of temperature on dQ/dT (J/gK) of treated smitin (24 h) solution, heating rate 1 degree/min, measuring volume of smitin solution 0.150 μL, smitin concentration 1.4 mg/mL, and ΔH=33.5±3.5 J/g.

Fig. 3(a, b, c) presents DSC data of native smitin solution and suspension of solutions of native and processed by gly\KOH, pH 7.5 T 25°C, which intiates quick protein sedimentacion as β-sheet structures. As we can see from Fig. 3(a) As we see, the denaturation process, consists of 3 stages with $T_d$ = 45, 60°C and 84°C.

Fig. 3 (b) Presents DSC data of denaturation of smitin stored 8 hours under 25°C denaturation process consists of 3 phases (stage) with denaturation temperature maximums around $T_d$ = 45°C, 61°C and 85°C.

Fig. 3 (c) Presents DSC data of denaturation of smitin stored 24 hours under 25°C. As we see, the denaturation process, as in the previous case fig. 3 (a, b), consists of 3 stages with $T_d$ = 45°C, 70°C and 84°C.

Comparison of Fig. 3 (a, b) and fig. 3 (c ) shows that creation of β-sheets practically has no effect on stability and intensity of first stage ΔCd = 0.8 ± 0.1 J/gK but has serious effect on second stage increasing transition temperature by 10°C. Transition temperature of the third stage is nearly the same fig. 3 (a, b) but intensity is doubled.

These data directly indicate that β-sheets are mainly created in the second domain of smitin native molecule and it increases its denaturation temperature by 10°C.

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*ბიოფიზიკა*

# გიგანტური ცილა-სმიტინის ამილოიდურ მდგომარეობაში გადასვლის შესწავლა სხვადასხვა მეთოდის გამოყენებით *in vitro*

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**ივანე ჯავახიშვილის სახ. თბილისის სახელმწიფო უნივერსიტეტის ელეფთერ ანდრონიკაშვილის სახელობის ფიზიკის ინსტიტუტი

§აკადემიის წევრი, ი. ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, გენომის სტრუქტურის და ფუნქციის ლაბორატორია, თბილისი, საქართველო

შევისწავლეთ ქათმის გლუვი კუნთიდან (კუჭი) გამოყოფილი ცილა-სმიტინის ამილოიდურ მდგომარეობაში გადასვლის თავისებურებანი *in vitro*. ცილის ამილოიდურ მდგომარეობაში გადაყვანა მოხდა 0,15 M გლიცინ/KOH, pH 7,0-7,5, I = 0,1-ის ხსნარის გამოყენებით. სმიტინის ამილოიდური ბუნების დადგენისათვის გამოყენებულია სპეციფიკური საღებავი კონგო წითელი, რომელიც უკავშირდება ამილოიდურ ცილას და ღებავს მას წითლად ან ვარდისფრად. შესწავლილია სმიტინის წრიული დიქროიზმის სპექტრები ამილოიდური ფიბრილების ჩამოყალიბებამდე და ჩამოყალიბების შემდეგ. ნატიური სმიტინი, განსაზღვრულ პირობებში, სწრაფად წარმოქმნის სხვადასხვა სტუქტურის და სიგრძის აგრეგატებს – β ხვეულებს. მიღებული შედეგებიდან გამომდინარე, ნატიური სმიტინის ამილოიდურ მდგომარეობაში გადასვლა ხდება სწრაფად და საინკუბაციო დროის შემდგომი გაზრდა არ იწვევს ცვლილებებს მეორეულ სტრუქტურაში. კალორიმეტრული მეთოდით შევისწავლეთ ამილოიდური სმიტინის სითბური დენატურაცია. ლღობის მრუდების ანალიზმა გვიჩვენა, რომ β-შრეები ძირითადად წარმოიქმნება სმიტინის ნატიური მოლეკულის მეორე დომენში, რაც იწვევს დენატურაციის ტემპერატურის ზრდას 10°C-ით.

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