

Comparative effects of limited tryptic hydrolysis on physicochemical and structural features of seed 11S globulins

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Abstract

The effect of the limited proteolysis by trypsin on selected seed storage 11S globulins (broad bean and pea legumins, glycinin and helianthinin) was studied by high-sensitive differential scanning calorimetry, fluorescence spectroscopy and analysis of proteolysis kinetics. Different behaviour of glycinin and helianthinin, on one hand, and broad bean and pea legumins, on the other, were observed: in the first group changes in the physicochemical characteristics of the proteins due to their limited proteolysis are more pronounced in comparison with the second one, in relation with the extent of primary structure modifications. The differences observed have been evaluated in relation with the amino acid sequence features of the four 11S globulin studied and agree with the literature data concerning the protein structural changes in the course of the limited proteolysis.

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1. Introduction

It has been shown that during the action of trypsin on glycinin, the soybean 11S globulin, two hydrolytic reactions proceed at different rates [1]. Similar results were later obtained when the tryptic hydrolysis of vetch [2] and broad bean [3] legumins, two other 11S globulins, was studied. The faster process is of limited non-cooperative (“zipper”) type, while the much slower one is of cooperative (“one by one”) type [4]. The enzymatic proteolysis of 11S seed globulins begins with non-cooperative hydrolysis leading to the formation of relatively stable high-molecular mass fragments along with a number of short peptides. It is followed by the cooperative process that brings about the degradation of proteins to small (TCA-soluble) peptides. The product resulting from the non-cooperative trypsin hydrolysis of glycinin was named legumin-T [1]. In the present work,

such designations are applied to similar derivatives of the other 11S globulins studied. The number and ratios among the high-molecular mass fragments of the still non-hydrolyzed protein remain unchanged during the course of the cooperative proteolysis. These fragments are retained by non-covalent links [4]. Studies on the high-molecular mass intermediates are especially important, since similar products are formed on the first stage of protein degradation during the seed germination and at early seedling growth [5,6].

In this paper, the results of comparative studies on 11S globulins of some crops (broad bean, soybean and sunflower) and the products of their limited proteolysis with trypsin (11S globulins-T) are presented. The following names of native and modified 11S globulins are used: legumin and legumin-T (broad bean, pea), glycinin and glycinin-T (soybean), helianthinin and helianthinin-T (sunflower). High-sensitive differential scanning calorimetry (microcalorimetry), fluorescence spectroscopy and analysis of trypsinolysis kinetics were used. Some of the results are compared with those previously obtained for pea 11S globulin [7,8].

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2. Materials and methods

2.1. Materials

Broad beans (*Vicia faba* L., var. Yantarny) were obtained from the All-Russian Scientific-Research Institute of Grain Legumes. For the legumin isolation a combined salt fractionation and isoelectric precipitation method, as previously described [9], was used.

According to the data of sedimentation analysis both legumin and legumin-T preparations contained at least 95% of the bulk substances.

Glycinin was isolated by the selective thermal denaturation [10] from soybean meal (Soya Flaw 200 W, produced by Protein Technology International). It was shown that glycinin obtained by the above method is identical to the chromatographically isolated one (unpublished results). It was also shown that the thermal denaturation cooperativity parameter of glycinin isolated by the selective denaturation coincides with that of the series of other 11S globulins obtained by various methods [11].

The isolation of helianthinin was based on the finding that the crystalloids located in the protein bodies of sunflower seeds consist of pure helianthinin and are surrounded by layers of other proteins and low-molecular mass substances [12]. Another peculiarity of sunflower seeds is the high content of chlorogenic acid that is oxidized during the extraction of the proteins. The oxidation products block the free amino groups of proteins changing the properties of the latter ones [13]. Most of chlorogenic acid was washed out from defatted sunflower meal by water adjusted to pH 5.0 containing 0.1% Na₂SO₃, 0.3% ascorbic acid and 1 mM EDTA. These chemicals prevented the oxidation of chlorogenic acid during washing. Then the meal was treated with 32% saturated solution of (NH₄)₂SO₄, which extracted all proteins but does not affect the crystalloids. The latter were solubilized by 2 M NaCl solution adjusted to pH 7.0 (ratio 10:1, v/w). The helianthinin extracted in this way was further purified by gel filtration on Sephacryl HR-300, dialyzed and freeze-dried.

Trypsin (Merck, Germany) with an activity 40 U/mg (benzoylarginine ethyl ester as substrate) was used. Kunitz soybean trypsin inhibitor was from Sigma, USA. All other chemical were reagent grade.

2.2. Methods

2.2.1. Preparation of 11S globulins-T

The T-derivatives of all 11S globulins studied were obtained by limited trypsinolysis. T-derivatives of both legumins and glycinin were prepared according to a method developed earlier for broad bean legumin-T [14]. Concentrated trypsin solution was added to a 1% (w/w) protein solution in 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.6, providing for an E/S ratio 1/1000 (w/w). Protein hydrolysis was carried out for 15 min at 37 °C. To stop the reaction, a solution of Kunitz soybean trypsin inhibitor was added, providing for a trypsin/inhibitor ratio 1/3 (w/w). Non-cooperative trypsinolysis of helianthinin proceeds much slower than that of legumin and glycinin (see Fig. 2).

Hence, the conditions of the helianthinin-T preparation were changed: the E/S ratio was 1/100, and the duration of the hydrolysis 3 h at 30 °C. Irrespective of the proteolysis conditions, in all cases the trypsinolysis was carried out until the completion of the non-cooperative stage. The high-molecular mass products—11S globulins-T, were separated from the peptides by means of gel-filtration through a Sephadex G-25 column (300 mm × 25 mm).

Protein concentration in solutions was determined by a micro-biuret method [15].

2.2.2. Differential scanning microcalorimetry

Calorimetric measurements were performed on a high-sensitive differential scanning microcalorimeter DASM-4A (Institute of Biological Device Construction, Russian Academy of Sciences, Pushchino/Oka, Russia) in the temperature range of 293–393 K. The protein concentration was 0.2–0.4% (w/w), the temperature-scanning rate was 2 K min^{−1}, the excess pressure in the cells of the microcalorimeter was 2 bar. In each experiment the heat capacity scale was calibrated by means of Joule–Lenz effect. For each solution studied, from three to five independent measurements were carried out, providing the mean standard deviation by the enthalpy determination to be no more than 5% of the value under measurement. Data treatment and calculation of thermodynamic denaturation parameters are described in reference [8]. Freshly prepared solutions of a protein in a 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.6, were used. Sample solutions of proteins-T were either freshly prepared or stored at −18 °C for no longer than 2 days. During this period, no changes were detected by the ultracentrifugation.

2.2.3. Fluorescence spectroscopy

Intrinsic (tryptophan) fluorescence spectra were recorded in the range of 300–500 nm, at 25 °C, with excitation wavelength at 280 nm. Proteins were dissolved in 50 mM Tris/HCl buffer, containing 0.1 M NaCl, at pH 7.4 (broad bean and soybean proteins) or 8.2 (sunflower proteins). Protein concentration was determined by the microbiuret method [15] (broad bean and soybean proteins) or from the absorbance at 280 nm using an extinction coefficient of $2.1 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (helianthinin) [16]. For fluorescence measurements protein concentration was adjusted to 0.2 mg ml^{−1}.

2.2.4. Analysis of proteolysis kinetics

Kinetics of trypsin proteolysis of broad bean legumin and helianthinin was studied under the conditions used for the preparation of helianthinin-T. As is shown below, these proteins differ in physicochemical properties. After the end of the non-cooperative stage, the proteolysis entered in the cooperative one. The reaction was prolonged till the degradation of most of the protein. Aliquots of the incubation mixture were withdrawn at specified time intervals and the residual protein (TCA-insoluble) was determined by a modification of a dye-binding method [17]. The results were plotted against the hydrolysis time in semi-logarithmic coordinates. Since, the cooperative proteolysis obeys the pseudo-first-order reaction [18], its kinetic curve was linear and the slope of the latter is equal to the rate constant, *k*, of the proteolysis.

Table 1
Thermodynamic parameters of protein denaturation

Protein	Denaturation temperature (T_d) (K)	Calorimetric denaturation enthalpy ($\Delta_d h^{\text{cal}}$) (J g^{-1})	Calorimetric denaturation entropy ($\Delta_d s^{\text{cal}}$) ($10^{-2} \text{ J g}^{-1} \text{ K}^{-1}$)
Broad bean legumin	353.0 ± 0.4	17.7 ± 0.8	5.0 ± 0.22
Broad bean legumin-T	350.2 ± 0.5	21.2 ± 0.9	6.1 ± 0.26
Pea legumin ^a	356.2 ± 0.3	14.0 ± 0.7	3.9 ± 0.20
Pea legumin-T ^a	353.0 ± 0.4	15.7 ± 0.7	4.4 ± 0.20
Glycinin	355.7 ± 0.2	23.7 ± 1.5	6.7 ± 0.42
Glycinin-T	349.7 ± 0.2	1.5 ± 0.1	0.4 ± 0.04
Helianthinin	370.2 ± 0.0	25.4 ± 0.2	6.9 ± 0.04
Helianthinin-T	361.3 ± 0.0	3.3 ± 0.1	0.9 ± 0.04

^a The data are taken from reference [4].

3. Results

The enthalpy values of denaturation of native soybean glycinin and sunflower helianthinin were considerably higher than those of the two other legumins, i.e. broad bean and pea 11S globulins (Table 1). Similarly, the limited proteolysis of soybean and sunflower 11S globulins led to more profound changes in thermodynamic denaturation parameters in comparison with broad bean and pea globulins. In the latter case both the specific enthalpy and entropy of denaturation increased slightly after the limited proteolysis, while one order of magnitude drop was observed with both glycinin and helianthinin.

The two groups of 11S globulins also differed substantially in the intrinsic fluorescence spectra (Fig. 1). The limited proteolysis of broad bean legumin (Fig. 1(C)) led to a negligible red shift of the fluorescence emission maximum (3 nm). No remarkable shift of this value was previously observed also for pea legumin [6]. Contrary to these legumins, the limited trypsinolysis of glycinin (Fig. 1(B)) and, especially of helianthinin (Fig. 1(B)) resulted in a considerable red shift of the emission maximum wavelength (13 and 23 nm, respectively). The fluorescence emission decreased after the limited proteolysis of all proteins studied. This decrease was especially remarkable for helianthinin.

The kinetic curves of broad bean legumin and helianthinin trypsinolysis are shown in Fig. 2. The cooperative stage of helianthinin hydrolysis proceeded much slower than hydrolysis of broad bean legumin. The rate constant of the latter is about seven times higher than that of helianthinin. The very slow proteolysis of helianthinin occurred also under the action of a number of other proteolytic enzymes (unpublished results).

4. Discussion

The molecules of the seed 11S globulins consist of six acidic α - and six basic β -polypeptide chains. α - and β -chains form two separate domains connected by a disulfide bond and a number of non-covalent interactions. A long disordered C-terminal segment extends from the α -chain domain. Based on the prediction of the secondary structure of polypeptide chains [20] and the data of the small-angle X-ray analysis, it was suggested that the β -chains are buried in the interior, while the α -chains and especially their disordered segment are located in the outer

part of macromolecules [21,22]. This conclusion was definitely confirmed by the X-ray determination of the 3D structure of A_{1a}B_{1b} and A₃B₄ subunits of glycinin [23,24]. The disordered C-terminal segment of the α -chains, being on the surface of the domain, does not contribute substantially to the stability of protein molecules. Rather, in all 11S globulin sequences, this region represents one of the most accessible sites to proteolytic attack. Due to high content of basic amino acid residues, the C-terminal region is specially susceptible to trypsin action.

The increase of specific denaturation enthalpy and entropy values of broad bean and pea legumins can be interpreted from the above observation. Indeed, the splitting off the disordered C-terminal part of α -chains can explain the increase of both parameters observed upon the transition from 11S legumins to their corresponding T-forms. In the case of broad bean legumin, this conclusion was confirmed experimentally [25]. In contrast to both pea and broad bean legumins, glycinin and helianthinin showed one order of magnitude decrease of the specific denaturation enthalpy and entropy, evidencing deeper structural changes of these proteins brought about by the limited proteolysis. It was shown that, besides the hydrolysis of the disordered C-terminal fragment of α -chains, the limited trypsinolysis of glycinin leads also to the splitting of the α -chain domain into a number of high-molecular mass fragments [26–28]. Similar changes in the primary structure of helianthinin macromolecules occur during its limited proteolysis [29].

The cleavage of the α -chain domain seemingly increases the exposure of hydrophobic groups to the solvent, as evidenced by the considerable greater red shift in fluorescence maximum of glycinin and helianthinin. The observed decrease of the fluorescence emission maxima at all wavelengths in each pair of samples, i.e. globulin and globulin-T, after the limited proteolysis of the proteins studied can also evidence the increase of accessibility of the hydrophobic groups to the solvent [30]. It is well known that the red shift of a fluorescence extinction maximum points to the exposure of tryptophan residues to a polar solvent. The comparison of the red shifts shows that the exposure is much more substantial in case of helianthinin and glycinin in comparison to that of broad bean and pea legumins. It is noteworthy that while helianthinin, which contains an unusual number of tryptophan residues (five in the α -chain and two in the β -chain), showed the greatest red shift, the other 11S globulins contain an identical number of tryptophan residues (2 + 1). Therefore, the

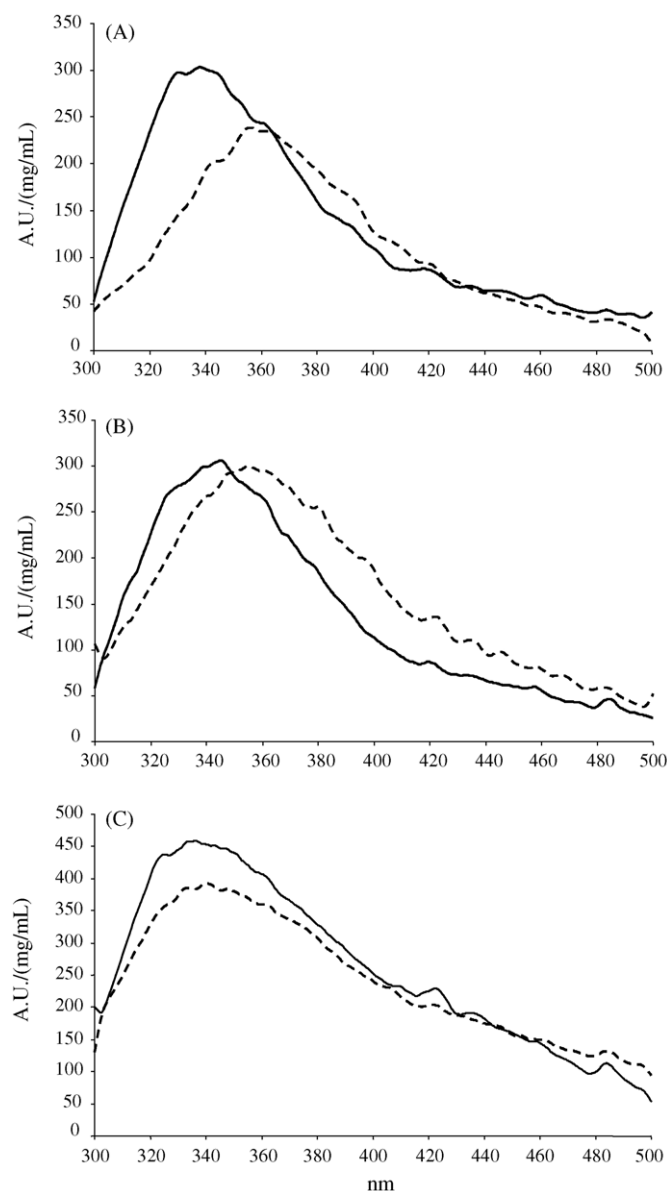


Fig. 1. Fluorescence spectra of 11S globulins both native (full lines) and modified through the limited proteolysis (dashed lines). Fluorescence is given as arbitrary units referred to the protein concentration. See Section 2.2 for details. (A) Helianthinin and helianthinin-T. Emission maximum of helianthinin: A.U. = 303, λ = 337 nm. Emission maximum of helianthinin-T: A.U. = 244, λ = 360 nm. (B) Glycinin and glycinin-T. Emission maximum of glycinin: A.U. = 302, λ = 342 nm. Emission maximum of glycinin-T: A.U. = 300, λ = 355 nm. (C) Broad bean legumin and legumin-T. Emission maximum of legumin: A.U. = 457, λ = 337 nm. Emission maximum of legumin-T: A.U. = 343, λ = 340 nm.

greater red shift measured on glycinin-T than on pea and broad bean legumins-T denotes a profound structural change taking place in this protein and confirms the conclusions drawn on the basis of the calorimetric analyses.

The study of proteolysis kinetics confirms the existence of two groups of 11S globulins, the same as those indicated by physical methods. The lower speed of the extensive cooperative proteolysis of helianthinin in comparison with that of broad bean legumin correlates with the difference in the stability of

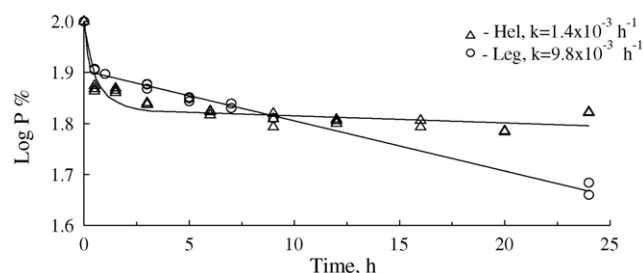


Fig. 2. Kinetics of broad bean legumin (○) and sunflower helianthinin (Δ) trypsinolysis. *P*: residual protein as percent of the initial concentration and *k*: rate constant.

their structure determined by calorimetry. In a previous semi-quantitative comparative study on a number of leguminous seed storage proteins, it was shown that the trypsinolysis of glycinin is also considerably slower than that of the other legumins [19]. More extensive protein degradation during the non-cooperative stage of helianthinin hydrolysis in comparison with broad bean legumin is most likely due to different course of their limited proteolysis. In the first group, both the cleavage of the α -subunits and the degradation of their disordered C-terminal fragment occur, while in the second one only the second process takes place.

In order to help finding the molecular reasons of the differences observed, the amino acid sequences of 11S globulins studied were aligned and their identity percent was determined using BLAST program. The following subunits were investigated (Swiss-Prot accession numbers): pea legumin P02857, broad bean legumin Q99304, glycinin P04776 and helianthinin P19084. The highest degree of identity is between pea and broad bean legumins (81%). The difference between pea legumin and glycinin is substantially greater (identity 57%). The lowest identity was between pea legumin and helianthinin (32%).

11S globulins consist of two types of subunits: A and B. The degree of identity between these two types of the same species is by far lower than that between one pair of same type subunits of different species. For example, the A and B subunits of pea legumin (accession numbers P02857 and P14594, respectively) show only 33% of identity. So, the determined identities will be significant only if the subunits of the same type will be compared. The investigated subunits, except those of helianthinin, belong to the A type. As to helianthinin, the sequence of only one of its subunits is determined and the type of the latter is unknown. Thus, a marked difference of the primary structure of glycinin from that of both legumins exists, while no definite conclusions may be drawn in case of helianthinin.

5. Conclusions

The changes of physicochemical characteristics of glycinin and helianthinin (denaturation enthalpy and entropy and fluorescence spectral properties) brought by the limited trypsin hydrolysis are more profound than those of broad bean and pea legumins. The differences between these two groups of seed storage 11S globulins are most likely due to differences in their primary structures. In the first group, both the scission of the

α -subunits and the degradation of their disordered C-terminal fragment occur, while in the second one only the second process takes place.

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References

- [1] Y. Kamata, K. Shibasaki, *Agric. Biol. Chem.* 42 (1978) 2323.
- [2] D.N. Lanh, A.D. Shutov, I.A. Vaintraub, *Biokhim. (Biochem.)* 50 (1985) 628 (in Russian).
- [3] A.N. Danilenko, A.P. Dmitrochenko, E.E. Braudo, A.A. Bogomolov, E.G. Rozantsev, *Food/Nahrung* 37 (1993) 46.
- [4] A.D. Shutov, J. Pineda, V.I. Senyuk, V.A. Reva, I.A. Vaintraub, *Eur. J. Biochem.* 199 (1991) 539.
- [5] A.D. Shutov, I.A. Vaintraub, *Phytochemistry* 26 (1987) 1557.
- [6] J. Tiedermann, B. Neubohn, K. Müntz, *Planta* 211 (2000) 1.
- [7] K.D. Schwenke, T. Henning, St. Dudek, H. Dauzenberg, A.N. Danilenko, G.O. Kozhevnikov, E.E. Braudo, *Int. J. Biol. Macromol.* 28 (2001) 175.
- [8] G.O. Kozhevnikov, A.N. Danilenko, E.E. Braudo, K.D. Schwenke, *Int. J. Biol. Macromol.* 29 (2001) 225.
- [9] V.V. Suchkov, I.A. Popello, V.Ya. Grinberg, V.B. Tolstoguzov, *J. Agric. Food Chem.* 38 (1990) 92.
- [10] T.M. Bikbov, A.N. Danilenko, M.G. Pletenko, M.L. German, E.P. Varfolomeeva, V.Ya. Grinberg, A.L. Leontiev, V.B. Tolstoguzov, *J. Agric. Food Chem.* 34 (1987) 297.
- [11] K.D. Schwenke, V.Y. Grinberg, A.N. Danilenko, T.V. Burova, V.B. Tolstoguzov, *Food/Nahrung* 31 (1987) 183.
- [12] M.V. Alexeyeva, A.N. Cheban, *Scientific reports of high school, Biol. Sci.* (11) (1977) 24 (in Russian).
- [13] C.V. Cater, S. Gheysuddin, K.F. Matil, *Cereal Chem.* 49 (1972) 508.
- [14] A.N. Danilenko, V.T. Dianova, E.E. Braudo, T. Henning, K.D. Schwenke, *Food/Nahrung* 41 (1997) 13.
- [15] R.F. Itzhaki, D.M. Gill, *Anal. Biochem.* 9 (1964) 401.
- [16] P. Suryaprakash, V. Prakash, *Food/Nahrung* 44 (2000) 178.
- [17] I.A. Vaintraub, H.B. Yattara, *J. Agric. Food Chem.* 43 (1995) 862.
- [18] I.A. Vaintraub, *Food/Nahrung* 42 (1998) 59.
- [19] I.A. Vaintraub, R. Bassüner, A.D. Shutov, *Food/Nahrung* 20 (1976) 763.
- [20] R. Bassüner, A.V. Finkelstein, *Proceedings of 2nd International Symposium on Self-Regulation of Plant Metabolism. Bulgarian Academy of Sciences, Sofia, 1986*, p. 54.
- [21] P. Plietz, G. Damaschun, *Stud. Biophys.* 116 (1985) 153.
- [22] P. Plietz, B. Drescher, G. Damaschun, *Int. J. Biol. Macromol.* 9 (1987) 161.
- [23] M. Adachi, Y. Takenaka, A.B. Gidamis, B. Mikami, S. Utsumi, *J. Mol. Biol.* (2001) 305.
- [24] M. Adachi, J. Kanamori, T. Masuda, K. Yagasaki, K. Kitamura, B. Mikami, S. Utsumi, *Proc. Natl. Acad. Sci. U.S.A.* 100 (2003) 7395.
- [25] St. Dudek, Ch. Horstmann, K.D. Schwenke, *Food/Nahrung* 35 (1995) 193.
- [26] V.A. Senyuk, I.A. Kakhovskaya, J. Pineda, in: K.D. Schwenke, R. Mothes (Eds.), *Food Proteins. Structure and Functionality*, VCH, Weinheim, 1993, p. 140.
- [27] A.D. Shutov, V.A. Senyuk, I.A. Kakhovskaya, J. Pineda, *Biochem. Engl. Transl. Biokhim.* 58 (1993) 174.
- [28] A.D. Shutov, I.A. Kakhovskaya, A.S. Bastrygina, V.P. Bulmaga, Ch. Horstmann, K. Müntz, *Eur. J. Biochem.* 241 (1996) 221.
- [29] G.P. Artykova, *Scientific Annals of State University of Moldova. Chemistry and Biology Series*, CE USM Kishinev, 2002, p. 137.
- [30] V.N. Uversky, A.L. Fink, *Biokhim. (Biochem.)* 53 (1998) 549 (in Russian) (Obituary).