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Gel chromatographic analysis of ficin under native and under denaturing conditions

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Abstract. The hydrodynamic behaviour of a commercial ficin preparation was studied by analytical gel chromatography on Sephacryl S-200 HR column under native and under denaturing (in presence of 9 M urea / 6 M guanidine hydrochloride, GdnHCl) conditions. The commercial ficin preparation was fractionated into seven distinct active components (I–VII) under native conditions. The elution of the components (I–III) as compared to α-chymotrypsinogen and the elution of the components (IV–VII) at a volume exceeding the total bed volume were suggestive of the interaction between the protein molecules and the gel. Treatment of ficin with denaturants (9 M urea / 6 M GdnHCl) resulted in its elution in the form of two peaks, indicating the presence of two classes of conformers differing in their stability towards denaturants. Analysis of the chromatographic data yielded the available Stokes radii of ficin (peak I fraction) as 9.2 Å (under native conditions), 25.8 Å (in presence of 9 M urea) and 40.9 Å (in presence of 6 M GdnHCl). A comparison of Stokes radii of ficin obtained under denaturing conditions suggested complete denaturation of ficin in 6 M GdnHCl compared to 9 M urea which produced significantly less conformational alteration.

Key words: ficin, gel chromatography, guanidine hydrochloride, Stokes radius, urea

INTRODUCTION

Denaturation of a protein by chemical denaturants like urea and guanidine hydrochloride (GdnHCl) usually results in the same unfolded state of proteins [1, 2]. However, this is not always true as some proteins, *e.g.* papain, cytochrome c_{551} and stem bromelain have shown different behaviour in these denaturants [3–5]. Ficin (E.C. 3.4.22.3) is a sulfhydryl protease belonging to the papain super family based on its many properties and structural similarity to papain [6]. The enzyme, which can be naturally obtained from the latex of fig trees, is known to consist of several active components [7–15]. Occurrence of multiple molecular forms of ficin has been suggested due to variation in the folding mechanisms producing different conformers [15]. The possibility of homologous replacements of an amino acid for another in the amino acid sequence to produce these multiple forms cannot be ruled out [15]. Results on the characterization of these multiple molecular forms of ficin have shown a mixed behaviour. While several reports have suggested similarities in these components based on their molecular properties [10–15], others have shown some differences [9, 13]. Despite extensive studies on the molecular properties of these components [9–15], no attempt has been made so far to investigate the effect of denaturants (urea and GdnHCl) on their behaviour.

Both acid and chemical denaturation studies have been performed on the major ficin fraction [16, 17]. Recently, we have shown different denatured states of ficin produced by 9 M urea and 6 M GdnHCl [18]. We have also noticed differences in the denaturation behaviour of a commercial ficin preparation (consisting of several active components) when compared to that obtained with a major ficin fraction (unpublished results). This has prompted us to investigate the hydrodynamic behaviour of commercial ficin under native and under denaturing conditions using analytical gel chromatography. Here we report our data on the gel chromatographic analysis of a commercial ficin preparation both in the absence and presence of 9 M urea or 6 M GdnHCl.

EXPERIMENTAL

Materials

Ficin from fig tree latex, 2 × crystallized (Lot 058K7019), Sephacryl S-200 HR (Lot 116K0771), urea (SigmaUltra) (Lot 127K0106), GdnHCl (Lot 078K5425), blue dextran (Lot 066K1083), L-tyrosine (Lot 0001412611) and various marker proteins such as α-chymotrypsinogen A, type II

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from bovine pancreas (Lot 029K7014), carbonic anhydrase from bovine erythrocytes (Lot 99H0669) and cytochrome c from horse heart (Lot 088K7000) were procured from Sigma-Aldrich Inc., USA. Conalbumin and ovalbumin were obtained from the Gel Filtration Calibration Kit HMW (Lot 375428) supplied by GE Healthcare, UK. All other chemicals used were of analytical grade purity.

Analytical procedures

Ficin concentration was determined spectrophotometrically using the specific extinction coefficient of 21.0 at 280 nm [6] on a Shimadzu double beam spectrophotometer, model UV-2450. Concentrations of urea and GdnHCl stock solutions were determined following the method suggested by Pace *et al.* [19] using the data of Warren and Gordon [20] and Nozaki [21], respectively.

Preparation of denatured protein solutions

Stock urea (10 M) and GdnHCl (6.67 M) solutions were made in 0.1 M sodium phosphate buffer, pH 7.0. To 0.5 ml stock protein solution (20 mg/ml), 4.5 ml of stock denaturant solutions were added in separate tubes in order to get the final urea and GdnHCl concentrations as 9 M and 6 M respectively. The final solution mixture (5.0 ml) was incubated for 6 h at 25°C prior to gel chromatography.

Analytical gel chromatography

Gel chromatography was performed using a Sephacryl S-200 HR column (Econo-Column, Bio-Rad Laboratories, USA) (1.5 × 16.6 cm) interfaced with AktaPrime Plus chromatographic system (GE Healthcare, UK). A 500 µl sample (2–2.5 mg protein/ml) was injected into a column pre-equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 with or without 9 M urea / 6 M GdnHCl and the flow rate was maintained at 0.3 ml/min. The column was also calibrated with standard protein markers both under native and under denaturing (in presence of 9 M urea / 6 M GdnHCl) conditions. The different marker proteins used with their known Stokes radii under native conditions and in presence of 9 M urea or 6 M GdnHCl were: conalbumin, ovalbumin, carbonic anhydrase, α-chymotrypsinogen A and cytochrome c [22–28]. Void volume, V_o of the column was determined by passing blue dextran, whereas the total volume, V_t was calculated using the formula, $\pi r^2 h$, where 'r' is the radius of the column (0.75 cm) and 'h' is the height of the gel bed in the column (16.6 cm). V_t was found to be 29.35 ml under native conditions, as well as in presence of 9 M urea. Since the height of the gel bed increased from 16.6 cm to 16.8 cm in the presence of 6M GdnHCl, the value of V_t changed from 29.35 ml to 29.7 ml. Elution volumes were determined by passing each component at least twice through the same column. Values of the elution volume of different marker proteins, as well as of ficin, peak I fraction, obtained under both native and denaturing conditions were transformed into distribution coefficient, K_d and available distribution coefficient, K_{av}, in the same way as described earlier [29]. Stokes radii of native and denatured ficin peak I fraction were determined by treating the gel chromatographic data according to Laurent and Killander [30] and Ackers [31]. Theoretical calculations were also made to determine the Stokes radii of native and denatured ficins following the method suggested by Uversky [27]. A molecular weight value of 23,800 [8] was used for ficin in these calculations.

RESULTS AND DISCUSSION

Figure 1 shows elution profiles of a commercial ficin preparation under both native (A) and denaturing (B and C) conditions when chromatographed on a Sephacryl S-200 HR column (1.5 × 16.6 cm).

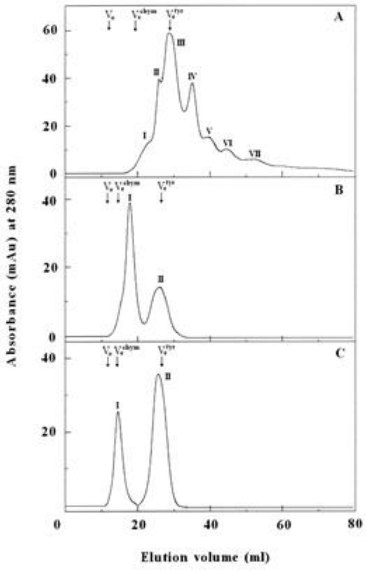

Fig. 1. Elution profiles of a commercial ficin preparation on Sephacryl S-200 HR column (1.5 × 16.6 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 (A); 0.1 M sodium phosphate buffer containing 9 M urea (B) and 0.1 M sodium phosphate buffer containing 6 M GdnHCl (C). Elution volumes of blue dextran, α-chymotrypsinogen and L-tyrosine are shown by arrows marked by V_o, V_e^{chym} and V_e^{tyr}, respectively.

The values of the elution volume of blue dextran (void volume), α-chymotrypsinogen and L-tyrosine on the same column are marked in Fig. 1 as V_o,

V_e^{chym} and V_e^{tyr}, respectively. Under native conditions, the values of V_o, V_e^{chym} and V_e^{tyr} were found to be 11.97, 19.36 and 29.0 ml, respectively (Table 1). As can be seen from Fig. 1A, the commercial ficin preparation was fractionated into seven components (I–VII) with elution volumes of 22.4, 25.48, 28.93, 34.65, 39.59, 44.16 and 52.41 ml, respectively (Table 1).

Table 1. Values of elution volume, V_e of commercial ficin, α-chymotrypsinogen, L-tyrosine and blue dextran on Sephacryl S-200 HR column (1.5 × 16.6 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 with or without 9 M urea or 6 M GdnHCl.

Protein/ Sample	Elution volume, V_e (ml)		
	0.1 M Sodium phosphate buffer, pH 7.0	0.1 M Sodium phosphate buffer, pH 7.0 containing 9 M urea	0.1 M Sodium phosphate buffer, pH 7.0 containing 6 M GdnHCl
Ficin Peak - I	22.40	17.79	14.54
- II	25.48	26.21	25.67
- III	28.93		
- IV	34.65		
- V	39.59		
- VI	44.16		
- VII	52.41		
α-Chymotrypsinogen	19.36	14.56	14.35
L-Tyrosine	29.00	26.62	26.68
Blue dextran	11.97	11.57	11.75

Interestingly, all these fractions were found active when checked for enzymatic activity. In view of this, all these components are believed to represent various isomeric forms of ficin. This was in accordance with previous reports [7–15] suggesting the presence of several components (conformers) in the ficin preparation. Four components, namely, IV, V, VI and VII, were eluted from the column with elution volumes higher than the total bed volume, V_t (29.35 ml) of the column (Table 1). Even the remaining three components (I, II and III) stayed longer in the column, as reflected by their elution volumes in relation to the total volume of the column (Table 1). This became more evident when the elution volumes of these three components were compared with the elution volume of α-chymotrypsinogen on the same column (19.36 ml). Being approximately similar in size (molecular weight = 25, 656), α-chymotrypsinogen was eluted much earlier than the three components of ficin (I, II and III). In fact, peak III had more or less the same elution volume as that obtained with L-tyrosine (Table 1). Emergence of peaks IV–VII after the total bed volume and higher elution volumes of peaks I–III compared to similar sized protein, α-chymotrypsinogen, clearly suggested an interaction between these protein components and the gel. It seems probable that ficin components reacted with the gel through non-polar interactions in the same way as adsorption of aromatic compounds takes place onto the Sephadex gel [32]. The unusual retention of ficin on Sephadex G-75 has been shown in a previous report [8], attributing it to the high content of aromatic residues. The role of both hydrophobic and ionic interactions has been suggested in the interaction of a few proteins with the gel due to the weak hydrophobic and ionic nature of gel filtration media [33]. Therefore, interaction of cationic ficin with Sephacryl gel may involve both hydrophobic and ionic interactions. A few other proteins such as lysozyme and *Bacillus licheniformis* α-amylase have also shown interaction with the gel media [34–35].

Treatment of a commercial ficin preparation with 9 M urea for 6 h at 25ºC and its chromatographic analysis on Sephacryl S-200 HR column (1.5 × 16.6 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 containing 9 M urea showed the presence of two peaks, namely, I and II (Fig. 1B) with elution volumes of 17.79 and 26.21 ml, respectively (Table 1). Furthermore, the values of the elution volume of α-chymotrypsinogen (14.56 ml), L-tyrosine (26.62 ml) and blue dextran (11.57 ml) on the same column also changed compared to those obtained under native conditions (Table 1). Since 9 M urea denatures the globular conformation of a protein into a more extended random-coil conformation, the lower values of the elution volumes of peaks I and II compared to those obtained under native conditions are understandable. However, the emergence of seven components of ficin (under native conditions) in the form of two peaks (I and II) in presence of 9 M urea suggested the presence of two different entities differing in their denatured conformations. It seems possible that the different conformers of ficin represented by peaks I–VII under native conditions might be grouped into two classes based on their conformational stability in presence of 9 M urea. Peak I (Fig. 1B) might incorporate two other isomers in a denatured form, which were eluted as peaks I, II and III under native conditions (Fig. 1A). Similarly, peak II (Fig. 1B) might represent those conformers in a denatured form, which were eluted as peaks IV –VII under native conditions (Fig. 1A). It is noteworthy that peak II obtained in 9 M urea had a similar elution volume to that of L-tyrosine (Table 1), suggesting

relatively more compact conformation than peak I molecules in 9 M urea.

Interestingly, when the commercial ficin preparation was treated with 6 M GdnHCl at 25°C for 6 h and chromatographed on Sephacryl S-200 HR column (1.5 × 16.8 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 containing 6 M GdnHCl, the elution profile also showed the presence of two peaks (I and II) with elution volumes of 14.54 and 25.67 ml, respectively (Fig. 1C, Table 1). The values of V_o, V_e^{chym} and V_e^{tyr} changed in the same way as those obtained in presence of 9 M urea when compared to those found under native conditions (Table 1). These results further supported our hypothesis on the existence of two classes of conformers differing in structural stability against these chemical denaturants. Although peak II obtained in presence of 6 M GdnHCl showed a slight difference in elution volume when compared to that of peak II observed with 9 M urea, peak I showed a remarkable difference in elution volume under these two denaturing conditions (Table 1). Peak I obtained with 6 M GdnHCl-denatured ficin was eluted much earlier (14.54 ml) than peak I obtained with 9 M urea-treated ficin (17.79 ml) (Table 1). Such difference in the elution volumes of peak I obtained in 6 M GdnHCl as well as in 9 M urea suggested different denatured states of ficin peak I fraction, which was completely denatured in 6 M GdnHCl and partially denatured in 9 M urea. Similar denatured states of proteins have been observed after treating them with either 8 M urea or 6 M GdnHCl [1, 2]. This can be clearly seen from the elution volumes of α-chymotrypsinogen which were similar in 9 M urea and in 6 M GdnHCl (Table 1), suggesting a similar denatured conformation of the protein in these two denaturants. Interestingly, the elution volume of ficin peak I fraction in 6 M GdnHCl matched very well the elution volume of α-chymotrypsinogen in 9 M urea. This was suggestive of the completely denatured conformation of ficin peak I fraction in 6 M GdnHCl. On the other hand, a higher value of the elution volume (17.79 ml) obtained for ficin peak I fraction in 9 M urea clearly indicated the retention of some native elements in the urea-denatured state. In other words, 6 M GdnHCl was found to be the stronger denaturant compared to 9 M urea with respect to ficin peak I fraction denaturation. This agreed well with a previous report [18] in which we have shown completely different denatured states of ficin produced by 9 M urea and 6 M GdnHCl, ficin being completely denatured in 6 M GdnHCl.

In order to validate the different action of these two denaturants on ficin (peak I fraction) denaturation, we determined the Stokes radii of ficin under native and under denaturing conditions using analytical gel chromatography. The same column was calibrated by passing different marker proteins with known Stokes radii (see column 2 of Table 2), *i.e.* conalbumin, ovalbumin, carbonic anhydrase, α-chymotrypsinogen and cytochrome c under native conditions, as well as in presence of 9 M urea or 6 M GdnHCl. Table 2 shows the values of the elution volume of different marker proteins, as well as the ficin peak I fraction obtained under native and denaturing (in presence of 9 M urea / 6 M GdnHCl) conditions. Transformations of V_e into K_{av} and K_d were made as described in the 'Materials and Methods' section and these values along with their other transformations, $(-\log K_{av})^{1/2}$ and $\text{erfc}^{-1}K_d$ are also given in Table 2.

Figures 2A and B show standard plots of marker proteins under native and denaturing conditions after treating the gel chromatographic data according to Laurent and Killander [30] and Ackers [31], respectively, which yielded the following linear equations:

Under native conditions:

$$(-\log K_{av})^{1/2} = 0.0110 \text{ Stokes radius, Å} + 0.374 \quad (1)$$

$$\text{Stokes radius, Å} = 59.622\ \text{erfc}^{-1}K_d - 11.689 \quad (2)$$

In presence of 9 M urea:

$$(-\log K_{av})^{1/2} = 0.0138 \text{ Stokes radius, Å} + 0.317 \quad (3)$$

$$\text{Stokes radius, Å} = 41.773\ \text{erfc}^{-1}K_d + 3.1006 \quad (4)$$

In presence of 6 M GdnHCl:

$$(-\log K_{av})^{1/2} = 0.0117 \text{ Stokes radius, Å} + 0.4218 \quad (5)$$

$$\text{Stokes radius, Å} = 50.887\ \text{erfc}^{-1}K_d - 6.4588 \quad (6)$$

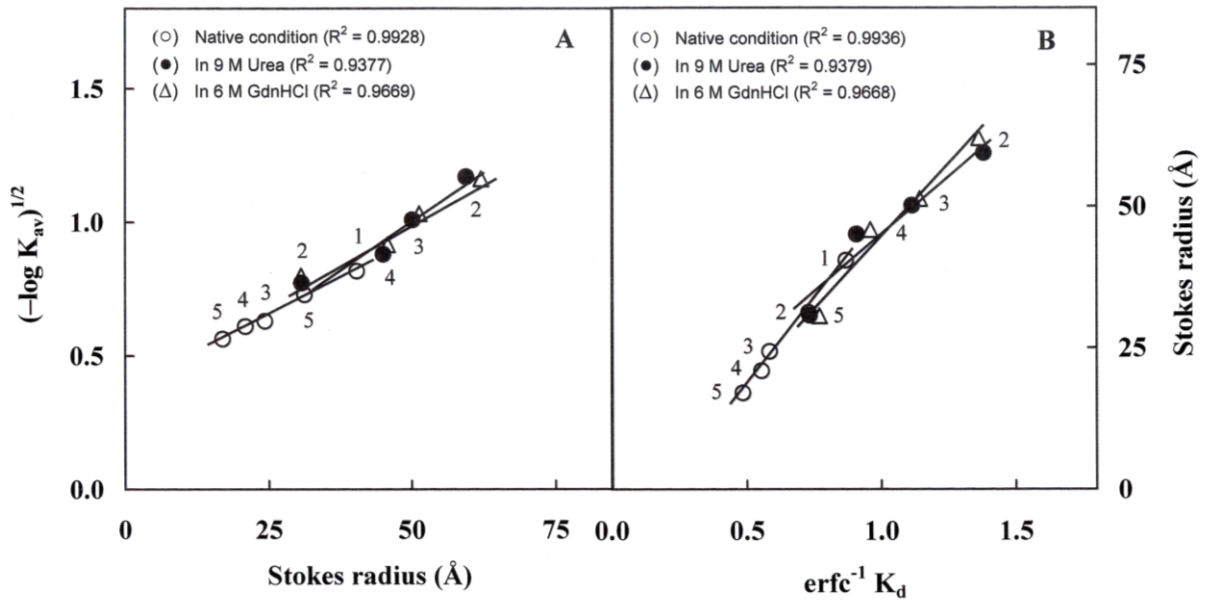

Fig. 2. Treatment of gel chromatographic data of marker proteins in the absence (○) and presence of 9 M urea (●) or 6 M GdnHCl (Δ) according to (A) Laurent and Killander [30] and (B) Ackers [31] for the determination of Stokes radii of native, urea-denatured and GdnHCl-denatured ficins (peak I fractions).

Table 2. Analytical gel chromatographic data of marker proteins and ficin peak I fraction on Sephacryl S-200 HR column (1.5 × 16.6 cm) under native (0.1 M sodium phosphate buffer, pH 7.0) and denaturing conditions (buffer containing either 9 M urea or 6 M GdnHCl).

Proteins	Stokes radius (Å)	V_e (ml)	K_{av}	K_d	$(-\log K_{av})^{1/2}$	$erfc^{-1} K_d$
A. Under native conditions						
Conalbumin	40.4 [26]	15.71	0.2152	0.2196	0.8168	0.8697
Ovalbumin	31.2 [25]	17.09	0.2946	0.3006	0.7285	0.7311
Carbonic anhydrase	24.3 [23]	18.95	0.4016	0.4099	0.6294	0.5841
α-Chymotrypsinogen	20.9 [22]	19.36	0.4252	0.4339	0.6094	0.5544
Cytochrome c	17.0 [26]	20.36	0.4827	0.4927	0.5624	0.4851
Ficin Peak - I	–	22.40	0.6001	0.6124	0.4709	0.3578
B. In presence of 9 M Urea						
Ovalbumin	59.4 [27]	12.33	0.0427	0.0505	1.1701	1.3803
Carbonic anhydrase	50.1 [27]	13.28	0.0962	0.1136	1.0084	1.1130
α-Chymotrypsinogen	45.0 [27]	14.56	0.1682	0.1987	0.8799	0.9093
Cytochrome c	30.7 [27]	16.07	0.2531	0.2990	0.7725	0.7340
Ficin Peak - I	–	17.79	0.3498	0.4133	0.6754	0.5798
C. In presence of 6 M GdnHCl						
Ovalbumin	62.0 [24]	12.55	0.0446	0.0536	1.1623	1.3619
Carbonic anhydrase	51.3 [27]	13.30	0.0864	0.1038	1.0314	1.1427
α-Chymotrypsinogen	45.8 [28]	14.35	0.1448	0.1741	0.9160	0.9603
Cytochrome c	30.6 [27]	15.88	0.2301	0.2766	0.7988	0.7707
Ficin Peak - I	–	14.54	0.1554	0.1869	0.8991	0.9334

Substitution of $(-\log K_{av})^{½}$ and $erfc^{-1}K_d$ values of ficin peak I fraction obtained under native and denaturing conditions (Table 2) into equations 1–6 yielded the values of Stokes radii of native and denatured ficins. These values obtained from two different treatments along with their mean values are given in Table 3. It should be noted that under native conditions and in presence of 9 M urea, the elution volume of ficin peak I fraction falls outside the range of standard proteins used (Table 2) due to interaction of the protein molecules with the gel. Therefore, such experimentally determined Stokes radii of ficin are referred to as 'available Stokes radii'. Stokes radii of ficin under native and denaturing conditions were theoretically calculated as well by substituting the molecular weight of ficin (23, 800 Da [8]) into different equations as suggested by Uversky [27] and these values are also listed in the last column of Table 3.

Native ficin (peak I fraction) yielded an experimentally determined value of Stokes radius of 9.2 Å compared to 23.0 Å theoretically calculated using Uversky's equation [27] (Table 3). Such a low value of experimentally determined Stokes radius of ficin can be ascribed to the possible interaction of the protein with the gel, as this value was based on the elution volume of the protein on Sephacryl S-200 HR column. Treatment of ficin with either 9 M urea or 6 M GdnHCl led to an increase in its hydrodynamic volume as revealed by the increase in its Stokes radius from 9.2 Å to 25.8 Å in presence of 9 M urea and 40.9 Å in presence of 6 M GdnHCl (Table 3).

Table 3. Stokes radii of ficin under native and under denatured conditions as determined from analytical gel chromatographic data following the methods of Laurent and Killander [30] and Ackers [31], as well as using the theoretical method of Uversky [27].

Ficin (Peak - I)	Stokes radius (Å)			
	Laurent and Killander's method [30]	Ackers' method [31]	Mean	Uversky's method [27]
Native	8.8	9.6	9.2	23.0
In 9 M urea	24.9	26.6	25.8	43.3
In 6 M GdnHCl	40.8	41.0	40.9	45.1

Both 9 M urea and 6 M GdnHCl are known to remove all kinds of non-covalent interactions present in a protein's three-dimensional structure [36–40]. Theoretical calculations of Stokes radii of ficin in 9 M urea (43.3 Å) as well as in 6 M GdnHCl (45.1 Å) following Uversky's method [27] also supported this contention, as these values were found similar to each other and much higher than that of the native ficin (Table 3). This was further supported by the Stokes radii of α-chymotrypsinogen in 9 M urea or 6 M GdnHCl (Table 2) which were similar to the theoretically calculated value of ficin (Table 3). A significant difference was noticed between the experimentally determined values of Stokes radii of ficin obtained in presence of 9 M urea (25.8 Å) and 6 M GdnHCl (40.9 Å). Furthermore, the experimentally determined value of Stokes radius of ficin in 6 M GdnHCl is close to the theoretically determined value of denatured ficin (Table 3). Although the presence of 9 M urea produced a significant change in the hydrodynamic volume of ficin, this was significantly less than the change observed with 6 M GdnHCl. These results suggested a nearly complete denaturation of ficin in presence of 6 M GdnHCl compared to 9 M urea which partially denatured it. All these results were found in agreement with our previously published report suggesting different denatured states of ficin produced in 6 M GdnHCl and in 9 M urea, being completely denatured in 6 M GdnHCl [18].

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ГЕЛ -ХРОМАТОГРАФСКИ АНАЛИЗ НА ФИЦИН ПРИ ЕСТЕСТВЕНИ УСЛОВИЯ И ПРИ ДЕНАТУРИРАНЕ

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(Резюме)

Изследвани са хидродинамичните отнасяния на търговски препарат от фицин с помощта на аналитична гел-хроматография на колона Sephacryl S-200 HR при нативни условия и при денуриране (с 9 M карбамид / 6 M гванидин хидрохлорид, GdnHCl). Търговският препарат от фицин е фракциониран на седем различни активни компоненти (I–VII) при нативниусловия. Елуирането на компонентите (I–III), сравнени с α-хемотропсиноген и елуирането на компонентите (IV–VII) в обеми, превишаващи общия обем на колоната дават сведения за взаимодействията между протеиновите молекули и гела. Третирането на фицина с денатуранти (с 9 M карбамид / 6 M гванидин хидрохлорид, GdnHCl) води до елуирането като два пика, показващо съществуването на два класа конформери различаващи се по тяхната устойчивост спрямо денатуриращите агенти. Анализът на хроматографските данни дава Стоксовите радиуси на фицина (за фракцията по пик 1) 9.2 Å (при наивни условия), 25.8 Å (в присъствие на 9 M карбамид) и 40.9 Å (в присъствие на 6 M GdnHCl). Сравнението на Стоксовите радиуси на фицина, получени при условия на денатуриране говорят за пълно денатуриране в 6 M GdnHCl в сравнение на 9 M карбамид, което води до сначително по-малко конформационни изменения.