J. Biochem. 2010;147(5):735–741 doi:10.1093/jb/mvq008

JB THE JOURNAL OF BIOCHEMISTRY

Effect of urea on protein separation by ion-exchange chromatography

Received November 8, 2009; accepted January 11, 2010; published online February 4, 2010

Fatemeh Khademi* and Ali Mostafaie

Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

*Fatemeh Khademi, Medical Biology Research Center, P. O. Box: 1568, Sorkheh Lizheh, Kermanshah, Iran, Tel.: 98-831-4276473, Fax: 98-831-4276471, email: fkhademi60@gmail.com

Ion-exchange chromatography (IEC) is the most frequently used chromatographic technique for the separation of proteins and peptides. In this article, the effects of urea on IEC separation of kiwifruit actinidin, egg white and urinary proteins were examined. The purity and relative amount of each protein in different conditions (in the presence or absence of urea) were compared with each other. The three parameters, including resolution, selectivity and efficiency of column in the presence of urea, were calculated and compared with the absence of urea. The results revealed that urea improved the purity of proteins and the resolution, selectivity and efficiency of IEC in separation of studied proteins.

Keywords: chromatography/efficiency/resolution/selectivity/urea.

Ion-exchange chromatography (IEC) is the most frequently used chromatographic technique for separation of proteins and peptides. The basis of IEC is the interaction between charged solute molecules and charged matrix moieties of column. The result of an ion-exchange experiment is often expressed as the resolution between the peaks of interest. The three most important parameters which affect the separation in IEC are selectivity, efficiency and capacity (*1*).

Solutions of urea in water display a number of interesting properties. Concentrated solutions of urea can be used to denature many proteins in a reversible way (*2*, *3*). Urea can affect protein structure by both direct and indirect mechanisms: an 'indirect mechanism' in which urea is presumed to disrupt the structure of water, thus making hydrophobic groups more readily solvated (*4*–*9*); and a 'direct mechanism' in which urea interacts either directly with the protein backbone, via hydrogen bonds and other electrostatic interactions with charged and polar side chains predominantly, or with the amino acids through more favourable van der Waals attractions as compared with water (*10*, *11*). As described above, indirect interaction of urea with proteins is exerted by altering water structure and dynamics. Urea is a chaotropic agent (water-structure breaker) that causes decrease of the dielectric constant of solvent (*12*, *13*). According to the principle of electrostatics, the electrostatic force exerted on ions by an electric field is inversely proportional to the dielectric constant of medium. When the dielectric constant of the solvent is decreased, the electrostatic interaction between charged groups increases *(14)*.

In this project we examined the effect of urea on the separation of major proteins of egg white, namely ovalbumin, ovotransferrin, ovomucoid and lysozyme (*15*, *16*), actinidin (a cystein protease) of kiwifruit and urine of pregnant women containing many proteins and glycoproteins such as human chorionic gonadotrophin (hCG) and its related molecules (*17*) using IEC.

Materials and Methods

Preparation of mucin-free egg-white extract

Hen eggs were purchased from a local market. Egg whites were separated from yolks and pooled. One volume of egg white was diluted with three volumes of normal saline [8.5% (w/v) of sodium chloride in water] and the mixture was stirred for 30 min at room temperature. Then the pH of mixture was adjusted to 4.5 with 0.5 N hydrochloric acid (HCl) and the solution was stirred for another 30 min. The gelatinous precipitate was extracted by 5-min centrifugation at 10,000*g* (4°C). The supernatant, called mucin-free egg white, was used in the following step. The pH of the supernatant was adjusted to the pH of the column, i.e. pH 8, by dialysing against the same buffer used to equilibrate the column.

IEC of egg-white proteins

IEC was performed on a column (2×10 cm) pre-packed with diethylaminoethyl cellulose (DEAE)–Sephacel or CM–Sepharose CL-6B (Amersham Pharmacia Biotech). The column was equilibrated with 50 mM Tris–HCl buffer at pH 8 [containing 30 mM sodium chloride (NaCl) for DEAE–Sephacel column]. The protein sample (mucin-free egg white) pre-equilibrated with the same buffer was loaded on to the column with flow rate of 20 ml/h. After loading the sample, the column was washed with three column volumes of buffer followed by elution of the DEAE–Sephacel column with a linear gradient (30–150 mM) of NaCl in the buffer. A linear gradient (0–0.5 M) of NaCl in the buffer was applied to the CM–Sepharose column for eluting bound proteins.

IEC of actinidin

IEC was performed on a column (2×10 cm) pre-packed with DEAE–Sephacel or CM–Sepharose CL-6B (Amersham Pharmacia Biotech). The column was equilibrated with 50 mM acetate buffer, pH 5.5. Purified actinidin from kiwifruit extract (prepared in Medical Biology Research Center of Kermanshah) was dialysed against 50 mM acetate buffer, pH 5.5, and then loaded on to the column. The column was washed with three column volumes of the buffer with flow rate of 20 ml/h followed by elution with linear gradient (0.1–0.4 M) of NaCl in the buffer.

IEC of urine proteins

A column (2×10 cm) was packed with DEAE–Sephacel and equilibrated with 10 mM ammonium acetate, pH 7. Urine specimens from pregnant women were pooled and concentrated 50 times using ultrafiltration method with Stirred ultra filtration cell

(Millipore Corporation Bedford, USA) using filters with the cutoff at 100 and 5 kDa. The concentrated urine was dialysed against 10 mM ammonium acetate (pH 7) and loaded on to the column. After loading the sample, the column was washed with three column volumes of the buffer with a flow rate of 20 ml/h. A linear gradient (0–0.25 M) of NaCl in the buffer was applied to the column for eluting bound proteins (*18*).

IEC of the proteins in the presence of urea

In the separate experiments, different concentrations of urea (5, 6, 7 or 8 M) were added to the protein specimens and buffers used in IEC, and the columns pre-equilibrated with the buffer containing the related concentration of urea. The concentrations of protein samples and the flow rate of buffer were similar in both conditions (presence or absence of urea). After IEC, the fractions were desalted by dialysis against deionised water or related buffers for analysis.

Calculation of the separation resolution, selectivity and - efficiency of IEC

The separation resolution (R_S), the selectivity (α) and the efficiency (N) of IEC was calculated in the presence or absence of urea from the Equations, $R_S = 2(V_{R2} - V_{R1})/W_{b1} + W_{b2}$, $\alpha = V_{R2} - V_0/V_{R1} - V_0$ and $N = 5.54\ (V_{R1}/W_{1/2})^2$, ($V_0$ = void volume, V_{R1} = elution volume for peak 1, V_{R2} = elution volume for peak 2, W_{b1} = peak width for peak 1, W_{b2} = peak width for peak 2 and $W_{1/2}$ is the peak width at half-peak height). R_S is a measure of the relative separation between two peaks. The selectivity (α) defines the ability of the system to separate peaks, i.e. the distance between two peaks. The column efficiency (N) is related to the zone broadening which occurs on the column (*1*). The data obtained are presented in Table I.

Analytical methods

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was preformed using 12.5% acrylamide separating gel and 4% stacking gel containing 0.1% SDS (*19*). Protein samples were heated in boiling water for 5 min and resolved at 50 V for 30 min and 150 V for 1 h followed by Coomassie Brilliant blue R250 or silver staining. In some cases, the stained gels were scanned with a Helena scanner and densities of protein bands were determined at 600 nm. The protein content was determined according to the method of Bradford (*20*) using bovine serum albumin (BSA) as the standard protein.

Statistical analysis

All experiments were performed triplicate in the presence or absence of urea. The mean and standard deviation of each experimental group were calculated. The significance in difference between with and without urea was assessed by two-tailed independent samples *t*-test. *P*-values <0.001 were considered significant. The data were analysed using the Statistical Package for Social Sciences (SPSS) version 11.5 software.

Table I. The effect of urea on the separation of kiwifruit actinidin, egg white and urinary proteins by anion-exchange chromatography.

Separation in different conditions	Resolution (R_S)	Selectivity (α)	Efficiency (N)
Egg white (absence of urea)	1.02 ± 0.11	2.4 ± 0.1	4.06 ± 0.4
Egg white (presence of urea)	1.58 ± 0.04	6.5 ± 0.11	8.7 ± 0.24
Actinidin (absence of urea)	–	–	272 ± 1
Actinidin (presence of urea)	–	–	411.8 ± 0.54
Urine proteins (absence of urea)	1.09 ± 0.1	1.5 ± 0.06	24.9 ± 0.85
Urine proteins (presence of urea)	1.66 ± 0.66	2.04 ± 0.08	69.7 ± 0.61

The values given are mean ± SD of three experiments.

Results

Anion-exchange chromatography elution profile of egg white, actinidin and urinary proteins in the presence or absence of urea is shown in Fig. 1A–F. Anion-exchange chromatogram of egg-white proteins showed three major peaks (Fig. 1A and B). Electrophoresis pattern of different parts of this chromatogram showed that the protein content of the first peak contains ovotransferrin with molecular weight (MW) of 78 kDa and lysozyme with MW of 14 kDa, (Fig. 2, lanes 1 and 2). The second peak contains ovomucoid with MW of 34–40 kDa, (Fig. 2, lanes 3 and 4) and the third peak that constituted a major part of this chromatogram contains ovalbumin with MW of 45 kDa, (Fig. 2, lanes 5 and 6). Ovomucoid and ovalbumin were eluted from the column under the linear gradient of NaCl. It was clearly evident from Bradford assay and electrophoresis analysis that the protein content of first peak has increased in the presence of urea (The SDS–PAGE pattern presented more intense bands of ovotransferrin and lysozyme in the presence of urea; Fig. 2, lanes 1 and 2). The purity of second and third peaks, especially the second peak containing ovomucoid, increased from about 70% in the absence of urea to near 90%, as determined by scanning of protein bands in stained gels (Fig. 2, lanes 3 and 4).

Anion-exchange chromatogram of purified actinidin from kiwifruit extract consisted of one protein peak which was eluted from the column using linear gradient of NaCl (Fig. 1C and D). Urea caused an increase in the protein peak content (Bradford assay) and also SDS–PAGE pattern showed more intense bands of actinidin in the presence of urea (Fig. 2, lanes 7 and 8).

Anion-exchange chromatogram of urinary proteins consisted of three major peaks called B_1, B_2 and B_3, and a minor peak at the beginning of second peak designated as B_{2S} (Fig. 1E and F). Peak B_1 was eluted from the column before NaCl gradient and the other three peaks designated as B_{2S}, B_2 and B_3 were eluted from the column under the linear gradient of NaCl. The SDS–PAGE pattern of fractionated urine proteins using IEC in the absence or presence of urea is presented in Fig. 3A and B. Peak B_1 contained at least four bands in the absence of urea and two bands with MW around 10 and 26 kDa in the presence of urea. The B_{2S} peak that was eluted from the column at the beginning of NaCl gradient mainly contained proteins with MW 21 and 28 kDa in the presence of urea (greater than 96%), compared with the purity of near 50% in the absence of urea. In the absence of urea there are contaminants with MW 35 kDa and other impurities in B_{2S}. The major part of peak B_2 protein content was proteins with the MW of 35 and 45 kDa. The purity of these protein bands was estimated greater than 96% in the presence or absence of urea. Although the purity of B_2 did not have any remarkable difference in the presence or absence of urea, the protein content of B_2 increased in the presence of urea (Bradford assay). Peak B_3 in the presence of urea contains a pure protein band with MW 32 kDa which did not appear in the absence of urea (Fig. 3A and B).

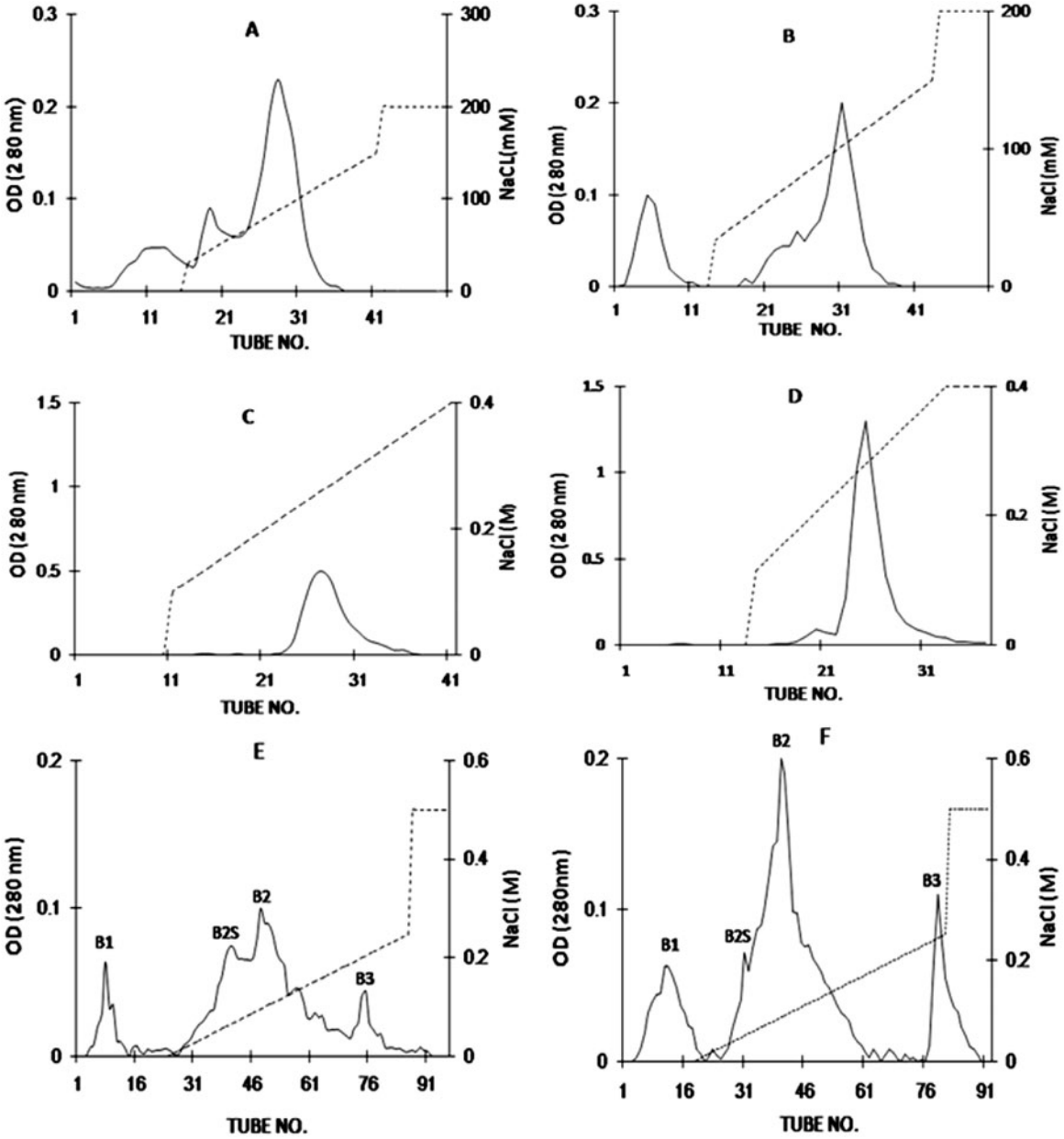

Fig. 1 Anion-exchange chromatography of egg-white proteins in the absence (A) or in the presence of urea (B), actinidin in the absence (C) or in the presence of urea (D), urinary proteins in the absence (E) or in the presence of urea (F). OD (280 nm) and NaCl (M or mM).

In our pervious study, the 28-, 32- and 35-kDa bands were identified as three major forms of β-hCG (*18*).

Cation-exchange chromatography profile of egg-white proteins showed three major peaks that were recovered separately (Fig. 4A and B). Electrophoresis pattern of different parts of this chromatogram showed that the first peak constituting a major part of this chromatogram mainly contains ovalbumin and ovomucoid (Fig. 5, lanes 1 and 2). The second peak mainly contains ovotransferrin (Fig. 5, lanes 3 and 4) and the third peak contains lysozyme (Fig. 5, lanes 5 and 6). Ovotransferrin and lysozyme were eluted from the column under the linear gradient of NaCl. Urea caused an increase in the protein content of first peak (Bradford assay) and, in addition, the SDS–PAGE profile clearly showed more intense bands of ovalbumin in the presence of urea (Fig. 5, lanes 1 and 2). The purity of the second peak containing ovotransferrin increased in the presence of urea (Fig. 5, lanes 3 and 4). The purity of the third peak containing lysozyme was estimated ~100% in the presence or absence of urea (Fig. 5, lanes 5 and 6).

Cation-exchange chromatography profile of purified actinidin from kiwifruit extract consisted of two protein peaks. The first peak containing actinidin was eluted from the column before using the linear gradient of NaCl (Fig. 4C and D). Urea caused an increase in the peak protein content (Bradford assay) and the SDS–PAGE profile showed more intense bands of actinidin in the presence of urea (Fig. 5, lanes 7 and 8).

The values obtained from the calculation of separation resolution, selectivity and efficiency of egg white

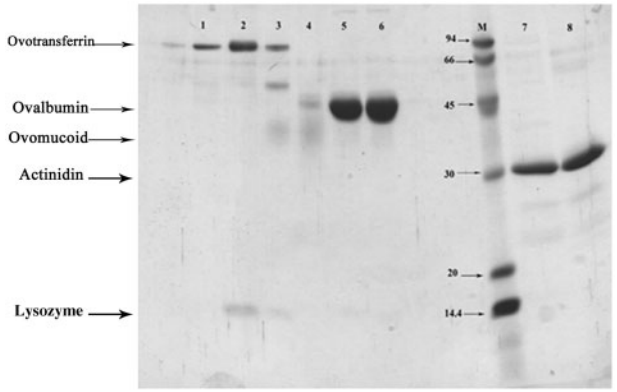

Fig. 2 SDS–PAGE analysis of fractionated egg-white proteins and actinidin using anion-exchange chromatography in the presence or absence of urea under reducing conditions followed by Coomassie blue R250 staining. Egg-white proteins (lanes 1–6), first peak containing ovotransferrin and lysozyme in the absence (lane 1) or presence of urea (lane 2), second peak containing ovomucoid fraction in the absence of urea (lane 3) or presence of urea (lane 4), third peak containing ovalbumin in the absence of urea (lane 5) or in the presence of urea (lane 6) and actinidin (lanes 7–8), absence of urea (lane 7) or presence of urea (lane 8). M shows the protein markers.

and urinary proteins and the separation efficiency of kiwifruit actinidin by anion-exchange chromatography in different conditions are presented in Table I.

Effect of urea on separation resolution, selectivity and efficiency of egg-white proteins

The mean separation resolution, selectivity and efficiency of three IEC experiments of egg-white proteins in the presence of urea were 1.58 ± 0.04, 6.5 ± 0.11 and 8.7 ± 0.24 compared with 1.02 ± 0.11, 2.4 ± 0.1 and 4.06 ± 0.4 in the absence of urea, indicating that urea lead to a significant increase in the separation selectivity and efficiency of IEC ($P = 0.000$). The observed increase in separation resolution by urea was not significant ($P > 0.001$). Although in the presence of urea the separation efficiency of the second peak containing ovomucoid and the third peak containing ovalbumin was not changed (data not shown), the purity of these peaks, especially the peak containing ovomucoid, increased from ~70% in the absence of urea to ~90%, as determined by scanning of stained gels (Fig. 2, lanes 3 and 4).

Effect of urea on separation resolution, selectivity and efficiency of urinary proteins

The mean separation resolution, selectivity and efficiency of three IEC experiments of urinary proteins in the presence of urea were 1.66 ± 0.66, 2.04 ± 0.08 and 69.7 ± 0.61 compared with 1.09 ± 0.1, 1.5 ± 0.06 and 24.9 ± 0.85 in the absence of urea, indicating that urea lead to a significant increase in the separation efficiency ($P = 0.000$). The increase in separation resolution and selectivity in the presence of urea was not significant ($P > 0.001$).

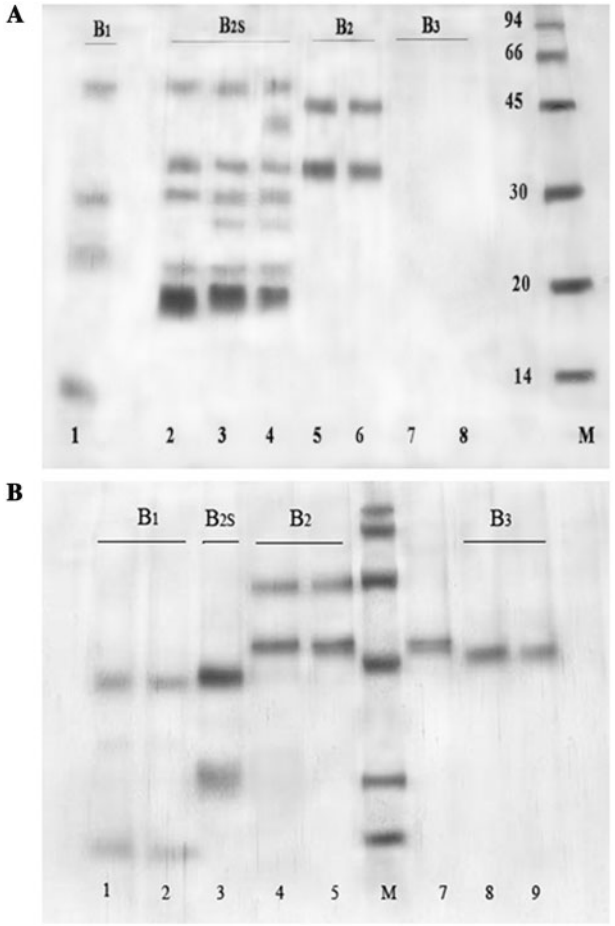

Fig. 3 SDS–PAGE pattern of fractionated urinary proteins using anion-exchange chromatography in the absence (A) or presence of urea (B) under reducing conditions followed by silver staining. A shows protein content of B_1 peak (lane 1), B_{2S} peak (lanes 2–4), B_2 peak (lanes 5 and 6), B_3 peak (lanes 7 and 8). B shows protein content of B_1 peak (lanes 1 and 2), B_{2S} peak (lane 3), B_2 peak (lanes 4, 5 and 7), B_3 peak (lanes 8 and 9). M shows the protein markers with MW of 94, 66, 45, 30, 20 and 14 kDa from top to bottom.

Effect of urea on separation efficiency of kiwifruit actinidin

The mean separation efficiency of three IEC experiments of kiwifruit actinidin in the presence of urea was 411.8 ± 0.54 compared with 272 ± 1 in the absence of urea, indicating that urea lead to a significant increase in the separation efficiency ($P = 0.000$).

Effect of urea on the retention times of separated peaks

The retention times of B_{2S} and B_2 peak in the case of urinary proteins and the first peak containing ovotransferrin and lysozyme in the case of egg-white proteins using anion-exchange chromatography were reduced in the presence of urea. The mean elution volumes of B_{2S}, B_2 peak and the peak containing ovotransferrin and lysozyme (obtained from three IEC experiments) were 30.66 ± 0.57, 40.66 ± 1.52 and 4.66 ± 0.57 in the presence of urea compared with 38.66 ± 0.57, 47 ± 1 and 10.66 ± 0.57 in the absence of urea. The retention time of the second peak

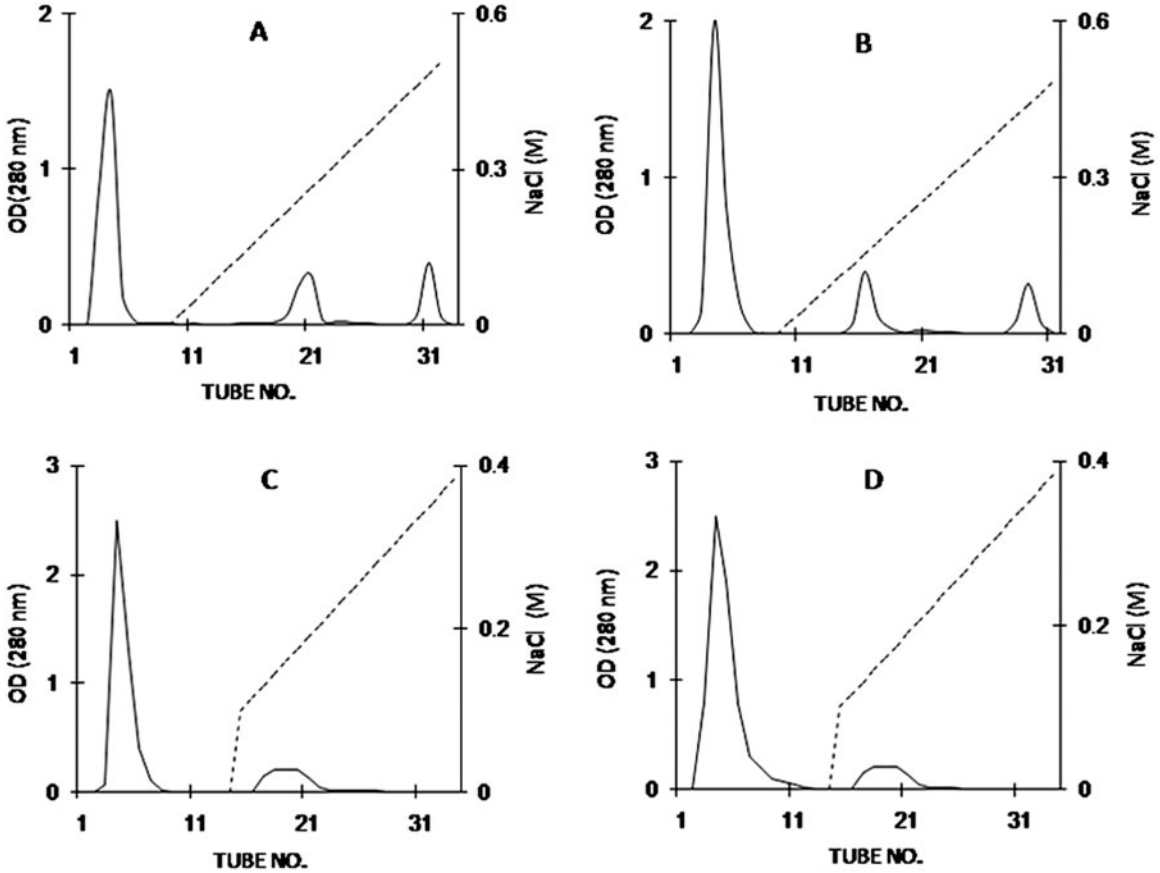

Fig. 4 Cation-exchange chromatography of egg-white proteins in the absence (A) or in the presence of urea (B), actinidin in the absence (C) or in the presence of urea (D). OD (280 nm) and NaCl (M or mM).

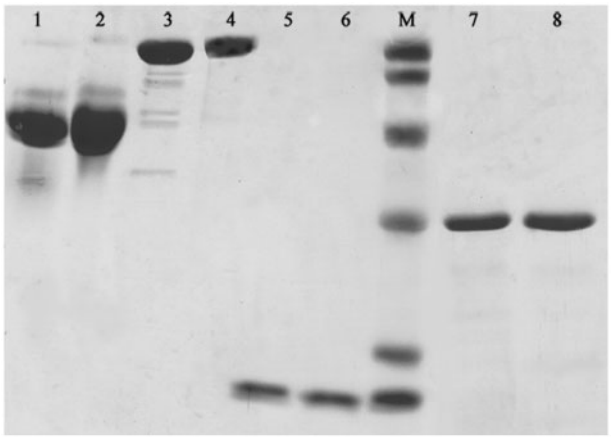

Fig. 5 SDS–PAGE profile of fractionated egg-white proteins and actinidin using cation-exchange chromatography in the presence or absence of urea under reducing conditions followed by Coomassie blue R250 staining. Egg-white proteins (lanes 1–6), first peak containing ovalbumin and ovomucoid in absence (lane 1) or presence of urea (lane 2), second peak containing ovotransferrin in the absence of urea (lane 3) or presence of urea (lane 4), third peak containing lysozyme in the absence of urea (lane 5) or in the presence of urea (lane 6) and actinidin (lanes 7–8), absence of urea (lane 7) or presence of urea (lane 8). M shows the protein markers with the MW of 94, 66, 45, 30, 20 and 14 kDa from up to down.

containing ovotransferrin using cation-exchange chromatography was reduced in the presence of urea, i.e. the mean elution volume of 15.66 ± 1.52 in the presence of urea compared with 21 ± 1 in the absence of urea. The decrease in retention times of proteins in the presence of urea was significant ($P = 0.000$).

The retention times of actinidin peak by anion- and cation-exchange chromatography were not different in the presence or absence of urea.

Effect of urea concentration on protein separation in IEC

We also examined the effect of urea concentration on protein separation in IEC. In separate experiments, different concentrations of urea (5, 6, 7 or 8 M) were used. The results revealed that 6–8 M urea had the same effects and improved the purity of proteins, the resolution, selectivity and efficiency of IEC in separation of studied proteins, but 5 M urea was not effective in protein separation on IEC (Table II).

Discussion

Herein the effect of urea was considered on the separation of proteins, i.e. egg-white proteins, actinidin and major forms of β-hCG by IEC. The results of this study indicated that urea improved the resolution, selectivity and efficiency of anion-exchange chromatography in separation of egg white and urinary proteins, and the separation efficiency of actinidin. The enhancement of chromatographic parameters by urea that observed in anion-exchange chromatography were

Table II. The effect of urea concentration on the separation of proteins by anion-exchange chromatography.

Separation in different concentrations of urea	Resolution (R_S)	Selectivity (α)	Efficiency (N)
Egg white			
(8 M urea)	1.58 ± 0.04	6.5 ± 0.11	8.7 ± 0.24
(7 M urea)	1.5 ± 0.02	6.27 ± 0.03	8.01 ± 0.29
(6 M urea)	1.39 ± 0.05	5.5 ± 0.3	7.18 ± 0.12
(5 M urea)	1.07 ± 0.11	2.62 ± 0.14	4.5 ± 0.22
Actinidin			
(8 M urea)	–	–	411.8 ± 0.54
(7 M urea)	–	–	402.3 ± 1.25
(6 M urea)	–	–	380.3 ± 2.4
(5 M urea)	–	–	279.2 ± 1.1
Urine proteins			
(8 M urea)	1.66 ± 0.66	2.04 ± 0.08	69.7 ± 0.61
(7 M urea)	1.52 ± 0.03	1.94 ± 0.03	65.14 ± 0.85
(6 M urea)	1.39 ± 0.03	1.79 ± 0.015	54.29 ± 0.83
(5 M urea)	1.14 ± 0.05	1.59 ± 0.02	28.45 ± 1.2

The values given are mean ± SD of three experiments.

not changed significantly in cation-exchange chromatography. Such an effect is probably attributable to fact that the purification of these proteins by anion exchangers is more effective than that by cation exchangers in IEC (*16*, *18*). Nevertheless, the content and purity of nearly all protein peaks were increased in both IECs. Since various glycoforms and fragments of hCG and β-hCG are present in urine, purification of various β-hCG forms is difficult and needs long consecutive purification procedures. In our pervious study (*18*), we performed IEC on a DEAE–Sephacel column in 8 M urea which allowed better separation of three major β-hCG forms with the MW 28, 32 and 35 kDa, compared with the absence of urea. Collectively, our results revealed that urea caused the greatest increase on the separation efficiency of IEC, although, in some cases, the separation resolution and selectivity increased too.

Two hypothetical mechanisms have been proposed for the effect of urea on protein structure and solubility. According to the first hypothesis, urea upsets the hydrogen-bonding network of the solvent around hydrophobic side-chains, providing a better solvation environment for non-polar side chains and non-polar amino acids. Based on the second hypothesis, urea interacts directly with the protein molecules, competing with intramolecular hydrogen bonding (*9*, *21*). Therefore, it is concluded that the observed increase in performance of IEC by urea is due to the ability of urea to alter water structure or/and to interact with protein molecules. In this regard, there is a possibility that one of the reasons for the effect of urea in IEC is the effect of this chaotropic agent on increasing the protein solubility. Urea can cause a decrease in protein–protein interactions; thus proteins act independently and the co-elution of proteins in IEC decreases causing an increase in the resolution between peaks and better separation of proteins in IEC. However, this speculation needs more accurate and quantitative studies for confirmation.

Acknowledgements

We thank Drs Sirous Ghobadi and Mohsen Nikoorazm for their useful suggestions, Mr Shahram Parvaneh for his technical assistance, Miss Maryam Keshavarz for statistical consultation and all our colleagues in Medical Biology Research Center, Kermanshah University of Medical Sciences.

Conflict of interest
None declared.

References

1. (1982) Ion Exchange Chromatography: Principles and Methods, Technical Booklet Series, Sweden Amersham Biosciences, Uppsala.
2. Beauregard, D.V., and Barret, R.E. (1968) Ultrasonics and water structure in urea solutions. *J. Chem. Phys.* **49**, 5241–5244
3. Soper, A.K., Castner, E.W., and Luzar, A. (2003) Impact of urea on water structure: A clue to its properties as a denaturant. *Biophys. Chem.* **105**, 649–666
4. Wetlaufer, D., Malik, S., Stoller, L., and Coffin, R. (1964) Nonpolar group participation in the denaturation of proteins by urea and guanidinium salts. Model compound studies. *J. Am. Chem. Soc.* **86**, 508–514
5. Frank, H., and Franks, F. (1968) Structural approach to the solvent power of water for hydrocarbons; urea as a structure breaker. *J. Chem. Phys.* **48**, 4746–4757
6. Finer, E., Franks, F., and Tait, M. (1972) Nuclear magnetic resonance studies of aqueous urea solutions. *J. Am. Chem. Soc.* **94**, 4424–4429
7. Hammes, G.G., and Schimmel, P.R. (1967) Water-urea and water-urea-polyethylene glycol interactions. *J. Am. Chem. Soc.* **89**, 442–446
8. Dwek, R., Luz, Z., and Shores, M. (1970) Opposite effect of urea and some of its derivatives on water structure. *J. Phys. Chem.* **74**, 2230–2232
9. Bennion, B., and Daggett, V. (2003) The molecular basis for the chemical denaturation of proteins by urea. *Proc. Natl Acad. Sci. USA* **100**, 5142–5147
10. Robinson, D., and Jencks, W. (1965) Effect of compounds of urea-guanidinium class on activity coefficient of acetyltetraglycine ethyl ester and related compounds. *J. Am. Chem. Soc.* **87**, 2462–2469
11. Wallqvist, A., Covell, D.G., and Thirumalai, D. (1998) Hydrophobic interactions in aqueous urea solutions with implications for the mechanism of protein denaturation. *J. Am. Chem. Soc.* **120**, 427–428
12. Vance, D.E., and Vance, J.E. (2008) Functional roles of lipids in membranes. *Biochemistry of Lipids, Lipoproteins and Membranes* p. 9, Elsevier Science Ltd, UK
13. Righetti, P.G., Stoyanov, A., and Zhukov, M.Y. (2001) Conversional isoelectric focussing in gel slabs and capillaries and immobilised pH gradient. *The Proteome Revisited: Theory and Practice of All Relevant Electrophoretic Steps* p. 170, Elsevier Science Ltd, UK
14. Yu, T.R. (1997) Electrostatic adsorption of cations. *Chemistry of Variable Charge Soils* p. 93, Oxford University Press, USA
15. Powire, W.D., and Nakai, S. (1985) *Characteristics of edible fluids of animal origin: eggs*. Food Chemistry, (Fennema, O.R., ed.), pp. 829–55, Marcel Dekker, New York
16. Vaysi Sh., Mostafaie, A., and Hasan, Z.M. (2008) A simple and efficient method for purification of egg white major proteins using ion exchange chromatography. *Sci. J. Hamadan Univ. Med. Sci.* **15**, 38–43

17. Stenman, U.H., Tiitinen, A., Alfthan, H., and Valmu, L. (2006) The classification, functions and clinical use of different isoforms of hCG. *Hum. Reprod. Update* **12**, 769–784
18. Khademi, F., Hamzehee, K., Mostafaie, A., and Hajihossaini, R. (2009) Purification of three major forms of β-hCG from urine and production of polyclonal antibodies against them. *Clin. Biochem.* **42**, 1476–1482
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature* **227**, 680–685
20. Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram guantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
21. Caballero-Herrera, A., Nordstrand, K., Berndt, K.D., and Nilsson, L. (2005) Effect of urea on peptide conformation in water: molecular dynamics and experimental characterization. *Biophys. J.* **89**, 842–857