

Recombinant human insulin—II. Size-exclusion HPLC of biotechnological precursors. Factors influencing on retention and selectivity

V.E.Klyushnichenko, A.N.Wulfson.

Shemyakin Institute of Bioorganic Chemistry Russian Academy of Sciences, ul.Mikluho-Maklaya 16/10, 117871 GSP Moscow, V-437 RUSSIA.

Abstract

The mechanism of exclusion-sorption interaction of insulin-containing proteins with the column support in accordance with the chemical and three-dimensional structure in native and denaturing conditions was observed. The mechanisms of weak adsorption of linear proinsulin and fusion protein presenting in native, and dynamics of formation SDS-protein complex in denaturing conditions have been revealed. The obtained results are used for SE HPLC analysis of the main and intermediate products practically at all steps of recombinant human insulin production technology.

Size-exclusion or gel-permeation liquid chromatography is one of the types of chromatography, in which separation occurs, as the result of the fact, that the specimen molecules penetrate into sorbent pores, filled with the solvent, and are retained there for different time. Molecules, having a larger size in solution, either don't penetrate at all or penetrate only into part of the gel pores and are washed out of the column earlier, than small molecules, as the results of which separation according to the size that specimen molecules having in solution, is ensured (ref. 1). SE HPLC is often used for qualitative and quantitative analysis of proteins and peptides both in wide (ref. 2,3) and narrow (ref. 4) ranges of molecular masses, and can be performed both in native (ref. 5) and denaturing (ref. 6,7) conditions. In performing analysis by means of SE HPLC, for qualitative separation of proteins, it is necessary to take into consideration many factors, such as concentration and viscosity of the applied specimen (ref. 1,8), the choice of geometric (ref. 3) and physical-chemical (ref. 9,10) parameters of column support, elution conditions within different pH limits (ref. 11), temperature (ref. 12), the value of the solution ion strength (μ) (ref. 13), concentration of organic modifier and denaturing agents (ref. 14), protein isolation source (ref. 15), stability of work and corrosive resistance of chromatographic system (ref. 16). The complicated choice of these and other parameters has been recently made with the help of computer expert system (ref. 17).

According to the classical SEC, there should no interaction between the column support and substances, which are separated, however, in practice it is difficult to achieve a purely size-exclusion effect in the separation of components. Due to a great variety of chemical structures, usually found in protein specimens, and high heterogeneity of the compound, which is present in protein chains, several mechanism can be responsible for adsorption. There are several types of interaction, mainly of ion, electrostatic, hydrophobic and mixed mode, the prevention of which reduces non-exclusion influence (ref. 10).

Note a: used abbreviations:

HPLC - high performance liquid chromatography,
SEC - size-exclusion chromatography.

Ion-exclusion effect appears in conditions of weak ion strength of the eluent ($\mu = 0,1$), when the sorbent surface has a net charge (most of column supports, as silica have weakly expressed anion properties (ref. 18)), and the solvate of the same charge is excluded from the pores because of electrostatic repelling. As ion strength increases up to $\mu=0,2$, the charges are compensated, these interaction weaken, as ion strength further increases ($\mu>0,5$), hydrophobic interaction may prevail. The net charge of given protein plays important part in non-exclusion effects. Acidic proteins (isoelectric point < 7) usually display ion exclusion at low ion strength of the mobile phase. Basic proteins (isoelectric point > 7), as, for example, lysozyme (ref. 18), usually form ion bonds between aminogroups of proteins and silanol groups on the surface of silica gel (ref. 19). In this case, one can speak about ion inclusion (ref. 13,18,19).

Intermolecular electrostatic effects of salts ions and polymer solvate repelling may appear due to the increase of ion strength. As a result of salt addition, there appears electrostatic repelling between the neighboring carboxyl groups along the polymer chain, which lead to the expansion and even depolymerization of the molecules (ref. 13).

Ion-exchange effects, which appear on nonmodified silica as a result of silanol group ionization, are weakened by the eluent pH reduction, but at $\text{pH}<4$ silica begins to dissolve. However nonmodified silica is practically not used for SE HPLC of proteins and peptides because of high sorption of the latter.

Hydrogen bonds between the column support surface and protein molecules may lead to partial adsorption, especially in case of high molecular mass or the formation of complexes of water-soluble biopolymers, for which adsorption may be sufficiently high due to a great number of contacts, in addition to secondary effects. In order to reduce the contribution of hydrogen bonds to proteins, guanidine hydrochloride and urea are used (ref. 11).

Hydrophobic-hydrophilic interactions constitute a considerable part of all non-exclusion interactions. For example, when both the column and substance have a certain hydrophobic character, the mobile phase must have an intermediate hydrophobic character for the prevention of their interaction (ref. 20). When the mobile phase is more polar than both the support surface and substance, as in case of a mobile phase with a high ion strength, hydrophobic interaction between the column and substance will take place. The opposite effects appear when the substance of lower polarity than the column support (propanol or tetrahydrofuran) and biopolymers are used as a mobile phase, the interaction between the mobile phase and substance have a "normal-phase" character (ref. 9). Hydrophobic interaction can be usually reduced by the addition to the mobile phase of denaturing agents, such as SDS (ref. 14), guanidine hydrochloride (ref. 11), urea (ref. 7), organic modifiers such as spirits and glycols (ref. 21), acetonitrile (ref. 12,22), formic acid and triethyl ammonium phosphate in organic solvents (ref. 12), or amino acids (ref. 23). Denaturing mobile phase can be used for reduction of difference of the form of proteins, for the destruction of protein association, and for the solubilization of polypeptides, low soluble in water. However, it is important to know, that guanidine hydrochloride and SDS binding themselves with globular proteins, affect their natural conformation, turning them into linear guanidine- or SDS-protein complexes (ref. 5,13), the hydration radius of which is the simple function of molecular weight. Thus, SE HPLC carried out in 6M guanidine hydrochloride or 0,1-0,2% SDS ensures the estimation of molecular weight of the proteins (ref. 24). Non-denaturing conditions must be used in the separations of enzymes and other structure-labile specimens.

Among other kind of adsorption the following types can be singled out:

- at pH of the eluent, equal to the protein isoelectric point as the result of low solubility of the given protein (ref. 11);
- non-specific adsorption at high concentration of organic modifier, for example acetonitrile (ref. 12), due to hydration of the organic solvent and affect on protein solubility;

- sorption at low temperatures (ref. 12);
- bio-affinity interaction protein - support (ref. 18).

The problems become still great when the column is anionic and hydrophobic at one at the same time. An increase of the ion strength for the reduction of ion interaction may increase of hydrophobic effects, their reduction may cause the opposite result. It should be noted that such conditions may be found, when ionic and hydrophobic interactions will result in high resolution. By means of control of the mobile phase characteristics, an even can prevent interactions between the column support and substance (ref. 25). Besides, a nonoptimum behavior of SEC columns is not always bad. The use of nonpolar eluents, which increase interactions of proteins with size-exclusion column, was effective in the purification of interferon (ref. 26) and other biopolymers (ref. 18).

Earlier, mainly two types of supports were used for SEC: inorganic (ref. 27,28,29) and hydrophilic organic polymer gel (ref. 30,31,32,33). However due to high adsorption and low resolution, the separation of biopolymers, carried out by means of these gels, left much to be desired. Inorganic supports, such as glass with controlled pore size (ref. 34) and silica (ref. 35), sustained high mechanical loads, but possessed high adsorption. SE HPLC of biopolymers became possible only after the ways of binding spherical glass particles (ref. 36) and silica with hydrophobic components has been discovered (ref. 34). In this case, column packings can be conventionally divided into protein (ref. 9) and non-protein sorbents (ref. 37-40). Among a wide range of columns for SEC of proteins, worked our earlier (ref. 13) and presented not long ago (ref. 41), one of the most stable and frequently used ones is TSK G SW (ref. 42). The chemical structure of this support is not describe in detail in literature, but it is well known, that silica partially deactivated by chemically bound hydrophobic polystyrene (ref. 33,43,1), containing some amount of free hydroxyl groups (ref. 44). Usually columns of TSK G SW used for separation of proteins in water, whereas another type, TSK G PW, is used for the measurement of biopolymers molecular weight distribution, which is carried out in less polar organic solvents (ref. 45).

The aim of this work is to study mechanism of insulin-containing proteins retaining at elution through a SE-column of TSK G SW type, revealing general laws and mechanisms of exclusion-adsorption interaction of proteins with support in accordance with three dimensional and chemical structure of insulin-containing proteins in native and denaturing conditions. The obtained results are used for SE HPLC analysis of the main and admixture products at all steps of gene-engineering human insulin production technology (ref. 46). Obtaining of this anti-diabetic substance is a vital problem of biotechnology (ref. 47). The important point is the analysis for the content of high-molecular impurities in the substance of insulin (ref. 48,49), which are formed due to polymerization and aggregate-formation of the substance molecules in the course of technology and during its storage (ref. 10). Through the range molecular mass of insulin-containing proteins and their derivatives, formed in the course of technology, is 6 - 17 kDa, a TSK G 3000 SW column with pore size 250 Å was used in the given work. This choice is explained by several reasons: - first, though in native conditions the separation range of this column is 10-100 kDa (ref. 45), in denaturing conditions (ref. 50,51) protein molecules have larger sizes and charge due to the formation of SDS-protein complex (in SDS) (ref. 5), and the separation range reduce to 2-70 kDa (ref. 52); -second, the specimens of different conformations of fusion proteins contained small impurities of its dimer and oligomers, the separation of which was desirable for verification of the obtained mechanisms. Thus, as the retaining of proteins on column support with identical chemical coating, but with different pore diameters has a slightly different character (ref. 13), a slight difference of the results, obtained from different columns, may distort the general picture of retaining dependencies. In the process of elution of all insulin-containing proteins and their

derivatives in different conditions, but on the same column, it is possible to determine in detail the mechanisms of separation and the way of its optimization.

Due to diversity of elution systems, it is more convenient to use the value of distribution coefficient K_d (1), rather than values of retention time (t_i) or volume (V_i) in processing the results:

$$K_d = \frac{V_i - V_o}{V_e - V_o} = \frac{t_i - t_o}{t_e - t_o} \quad \text{where} \quad (1)$$

V_i - specimen elution volume,

V_o - volume of unretaining peak,

V_e - total column retaining volume,

$V_e - V_o$ - inner volume of column particles pores,

t_i - specimen retaining time,

t_o - time of the elution of substances, not penetrating into the pores of column support particles, unretaining peak,

t_e - time of the elution of substances, completely penetrating into the pores of column support particles.

For SE HPLC were used column TSK G 3000 SW (300 x 7,5 mm) (TOYO SODA, Japan); elution was performed at the rate of 0,5 ml/min. Chromatography was carried out on pump Waters 510 with injector Waters U6K, spectrophotometer Waters 490E and integrator Waters 740 (Millipore, USA). For the separation we used the specimens of insulin, proinsulin, denaturated proinsulin, proinsulin-S-sulphonate, fusion protein (earlier described in (ref. 46)), as well as closed fusion protein (according to -S-S- bonds of proinsulin) and fusion protein-S-sulphonate (proinsulin SH-groups were sulphonated), for identification - human insulin standard specimen (Atlanta, cat. N 83/500, Chemie-und Handelsgesellschaft mbH, D-6900 Heidelberg 1, Germany). The following reagents were used: acetonitrile, methanol, sodium phosphate, phosphoric acid, (all esp. pure), water, purified on Milli-Q (Millipore, USA), sodium dodecylsulphate (Serva, Germany), guanidine hydrochloride (Merk, Germany). Before chromatography, the eluents were filtered through nitrocellulose and GVWP filters (pore diameter 0,45 μ , Millipore, USA) and degassed for 20 min.

As is noted earlier, proinsulin, proinsulin-S-sulphonate and denaturated proinsulin (i.e. open proinsulin with free SH-groups of cysteine residues) have close molecular masses, but differ considerably in conformation and charge (ref. 44,46). Just these differences explain the possibilities of their separation by means of SE HPLC method.

Chromatography of insulin-containing proteins in non-denaturing conditions was carried out in order to study the retaining of specimens on a column, depending on the eluent ion strength, and was performed at different concentrations of sodium phosphate buffer (0,02 < μ < 0,4) (pH 7,0) (Fig.1). Figure 1 shows that, as it should be according to the classical mechanism of separation, if the eluting solution of ion strength increases, K_d as the function of specimens retaining time increases, too. However, an increase of sodium phosphate buffer ion strength introduces changes into the general picture of distribution coefficient dependence on the value of μ . Thus, it can be seen, that linear proinsulin and fusion protein are eluted latter, than their renaturated (i.e. closed) analogous, accordingly having a smaller size in solution, This is an artifact in size-exclusion chromatography. Evidently, it occurs due to the interaction of column support with SH-groups or "open" sections of these proteins, the sulphonates of which is accompanied by the opposite process. Proinsulin-S-sulphonate and fusion protein-S-sulphonate are eluted earlier, than their linear and renaturated analogous. This ion exclusion is easily explained by the interaction of weak anionic column support with negatively charged protein molecules. This effect is strong expressed at small value of $\mu = 0,02-0,1$. The time of proinsulin-S-sulphonate retaining t_i is even less than that of fusion

protein t_r at $\mu = 0,02-0,05$. As the solution ion strength increases up to $\mu = 0,4$, the difference of elution time of the corresponded sulphonates, linear and closed proteins and their resolution considerably decreases due to the display of general hydrophobic interactions. In this case, distribution curves, corresponding to proinsulin-S-sulphonate and fusion protein-S-sulphonate appear in the form of sigma-shape, whereas the curves corresponding to the rest of the proteins, are likely to take this shape in the process of further increase of μ values hydrophobic and adsorption interactions.

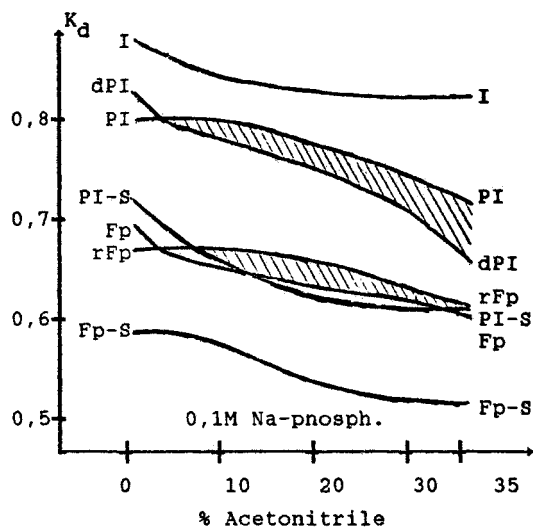
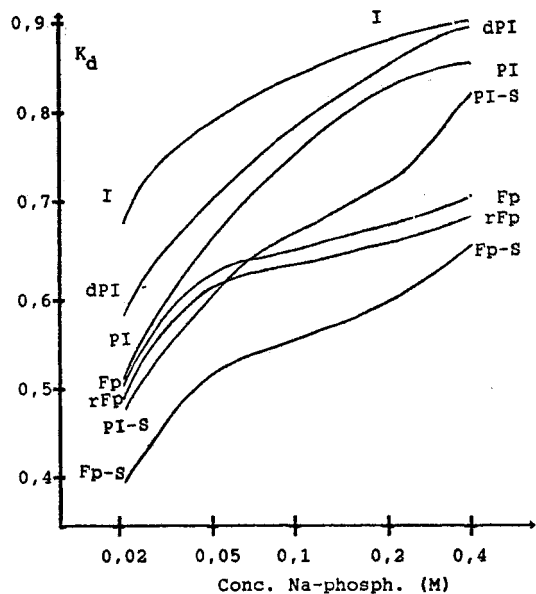


Fig. 1. Influence of sodium phosphate concentration 0.02-0.4M as the mobile phase on the K_d of insulin-containing proteins. Column TSK G 3000 SW, flow rate 0.5 ml/min.

Fig. 2. Influence of concentration of the acetonitrile (0 - 35%) in the 0.1 M sodium phosphate (pH 7.0) as the mobile phase on the K_d values of insulin-containing proteins. Other conditions as in Fig. 1.

Abbreviations: I - insulin, PI - proinsulin, dPI denatured proinsulin, PI-S - proinsulin-S-sulphonate, FP - fusion protein, rFP - renatured fusion protein, FP-S - fusion protein-S-sulphonate.

In order to reduce the influence of hydrogen bonds and hydrophobic interactions and also to reduce the affinity of insulin-containing proteins to the packing surface, organic modifiers (methanol, acetonitrile) and denaturing agents in various concentrations were used for the addition to sodium sulphate buffer. With an addition to the mobile phase of 10-20% of acetonitrile (Fig. 2), relative changes of elution time of closed and linear proinsulins and fusion proteins take place against a weak net reduction of the specimen elution volumes. This process been accompanied by the reduction of peak width and increase of resolution. As it should be according to the classical mechanism of separation, closed proteins are eluted latter, than linear ones. These changes are shown in the form of cross-hatched sections in Fig. 2. It can be seen, that the distribution coefficient (as a function of retention time) of linear proinsulin and fusion protein is reduced even a few per cent of acetonitrile in the mobile phase, whereas the K_d of their closed analogues does not practically change at all. At high concentration acetonitrile (more than 30%) in condition of hydration of the organic modifier molecules, alteration of process of the protein solubilization and their conformation changes take place, sometimes leading to denaturing effects, which causes non-specific adsorption of protein molecules on the column support (ref. 12), the widening of peaks and reduction of

selectivity of their separation. In spite of small differences of the polarity of methanol and acetonitrile (ref. 53), at the elution of proteins in 0,1 M sodium phosphate, 20% methanol, takes place a weak common reduction of retention time and convergence of K_d values on conventional straight lines, corresponding to each proteins. But no relative changes of the elution time of linear and closed proinsulins and fusion proteins, as in case of acetonitrile, take place. These linear proteins possess a higher affinity to the column packing in the system with methanol than in the system with acetonitrile. The resolution does not practically increase due to the reduction of separation selectivity.

In denaturing conditions chromatography was carried out in 0,1 M sodium phosphate and with the presence of SDS and guanidine hydrochloride, which form composite complexes with proteins in the solution and considerably affect their retaining. The process of complex formation of the SDS-proteins is graphically shown in Fig. 3. At small SDS concentration 0,01-0,015%, an abrupt reduction of distribution coefficient as the function of retention time is observed. It is within this range of concentration that the main growth of SDS-protein complexes, whose hydration radius affect K_d , takes place. A further increase of SDS concentration 0,05-0,2% has a weaker effect on retaining, though the K_d function within this interval of SDS concentration is monotonously decreased. It testifies to the further complex formation. Figure 3 shows that in condition of SDS concentration 0,01 - 0,02%, the above mentioned change of elution time of linear and closed proinsulins and fusion proteins also take place, the intersection of distribution coefficient curves in the case of proinsulins taking place at a higher SDS concentration, than in the case of fusion proteins. This phenomenon is observed to a lesser degree at the protein elution with acetonitrile and guanidine hydrochloride

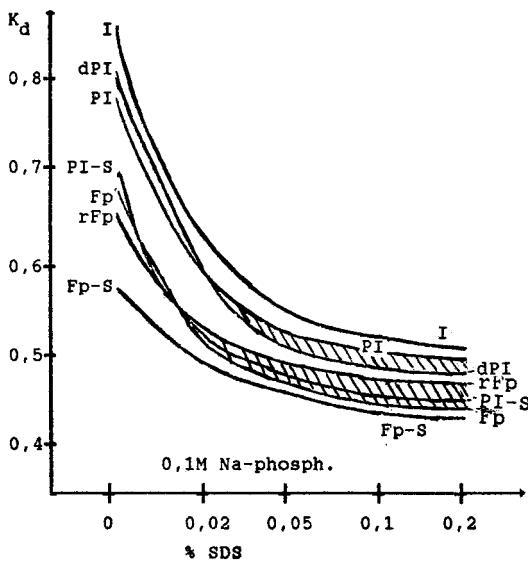


Fig. 3. Influence of the concentration of SDS (0 - 0,2 M) in the 0,1 M sodium phosphate (pH 7,0) as the mobile phase on the K_d values of insulin-containing proteins.

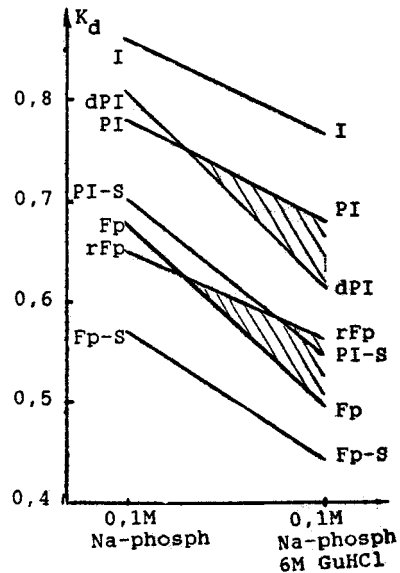


Fig. 4. Influence of the presence of 6 M guanidine hydrochloride in the 0,1 M sodium phosphate (pH 7,0) on the K_d of insulin containing proteins. Other conditions as in Fig. 1.

(Fig. 4). In other words, the contribution of hydrophobic interactions in the affinity to the column support in the case of linear proinsulin is higher, than in the case of fusion protein. As follow from the difference of amino acid sequence of these proteins (ref. 46), A-protein fragment does not enhance these interactions with the column

packing, but on the contrary reduce them. Taking into consideration particular hydrophilic properties of C-peptide (ref. 55), one can make a conclusion that weak adsorption occurs either due to specific interactions of SH-groups with support, or due to available for interactions hydrophobic amino acid residues, the content of which is high in insulin B-chain.

A successful, from the point of view of the earlier set task (ref. 46,54) chromatographic separation of insulin containing proteins was carried out in 0,1 M sodium phosphate, 6 M guanidine hydrochloride (pH 7,0). Figure 4. show that in these conditions the distance between the points, corresponding to distribution coefficient of the proteins (including hardly separable linear and closed proinsulin) is greater, than in Fig. 1-3. As the peak width is smaller, than in native conditions, the effectiveness of peak separation is higher, which corroborated by the separation of closely located pairs of linear and closed proinsulins, proinsulin and insulin. Lines connect of K_d values of the corresponding proteins in the conditions of 0,1M sodium phosphate (pH 7,0) and 0,1 M sodium phosphate, 6 M guanidine hydrochloride (pH 7,0). Other conditions as in Fig. 1. A more complete separation can be achieved on a column with a smaller diameter of pores.

As the result of the work performed, we have revealed the mechanism of exclusion-sorption interaction of insulin-containing proteins with the column support in accordance with the chemical and three-dimensional structure, carried out on TSK G 3000 SW column in native and denaturing conditions. The mechanism of weak adsorption of linear proinsulin and fusion protein presenting in native and dynamics of formation SDS-protein complex in denaturing conditions have been revealed. The obtained results are used for SE HPLC analysis of the main and admixture products practically at all steps of recombinant human insulin production technology.

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