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Resolution of proteins by chromatographic cake and its application

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Introduction

Resolution of small solute separation in liquid chromatography (LC) depends on the employed column length and it is usually separated with isocratic elution mode, while for biopolymer it is almost independent of column length and the separation is accomplished by gradient elu tion mode. Thus, short column can be employed for protein separation having many advantages but it hard for preparative and productive scales. The purpose of the presentation is to solve this problem. It involves in principle, column construct, resolution and its application in both Lab. and industrial scales.

Chromatographic cake I

Chromatographic cake is also called as unit of simultaneous renaturation and purification of proteins (USRPP). A reasonable approach is to enlarge the diameter of the short column to construct a chromatographic cak e which has much diameter (1cmm~50cm) than its length (1 cm) shown in Figure 1. With

chromatographic cake, it has some advantages, protein separation can be done under a very high flow rate of mobile phase, from 1~1,000ml/min which depends on the diameter of the chromatographic cake, samples with a very high viscosity, precipitate forming during sample injection. It can be employed for protein folding liquid chromatography (PFLC) for the protein renaturation with simultaneous purification in biotechnology(J. Chromatogr B, 2007, **849:** 69; **US 7,208,085 B2; date: Apr 24/2007**). It is also the basis for buffer exchange and continuous sample injection with very high flow rate of mobile phase for on -line two-dimensional liquid chromatography by a single column, 2DLG-1C(See: HPLC 2008, P 1 3 0 1 - M) . :

Minimum column length(L_{min})

A question is that does the thickness of the chromatographic cak e have a minimum(L_{min}), or not? With the derived equation (1), it can be calculated as its resolution is defined as unity. U is the linear velocity of the mobile phase (mm/min), B is the steepness of the linear gradient (M/min).

$$L_{\min,1} = \frac{U}{B}\int_{a_{D1}}^{a_{D1}} \frac{a_D^{Z_1}}{I_1 + a_D^{Z_1}} da_D \qquad (1)$$

log I represents a constant relating to the affinity of 1 mole of solvated protein to a solvated stationary phase; Z is the corresponding moles of the displacer releasing at the contact region between the protein and the stationary phase when one mole of the solvated protein is adsorbed b y the stationary phase. The term a_D is the activity of the displacer in mobile phase. All of the foregoing three parameters are the linear parameters in stoichiometric displacement theory.

Resolution

The resolution of protein separation by the chromatographic cake with various sizes under different flow rates of mobile phase shown in Figure 2

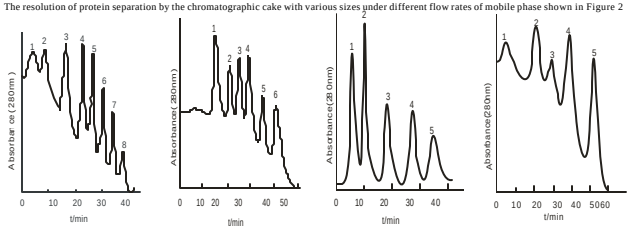

Figure 2 Chromatogram of standard proteins on chromatographic cake with various diameters (10mm in thickness, 10 to 500mm for diameter, stationary phase, HIC)

A, 10mm × 50mm, 5.0mL/min, 1, solvent; 2, cytochrome C(Cyt-C); 3,myoglobin (Myo); 4 ,ribonuclease A (RNase A); 5, lysozyme (Lys); 6, a -chmotrypsin (a -Chy); 7, a - amylase(α-Amy); 8, Ins.

B, 10mm × 00mm; 20mL/min; 1, Cyt- C; 2, Myo; 3, RNase A; 4, Lys; 5, a -Amy; 6, Ins.

C, 10mm × 200mm; 100mL/min; 1,Cyt- C; 2, Myo; 3, Lys; 4, a -Amy; 5, Ins.

D, 10 × 300mm; 120mL/min1,Cyt- C; 2, Myo; 3, RNase A; 4, Lys; 5, a -Amy;

All of the required parameters can be experimentally obtained an d the L_{min} for completely separated five-pairs of proteins with resolution,R_s = 1, which are shown in Table 1. The employed stationary phase is HIC. The shortest column here length needs 5 cm. The theoretically calculated minimum column length depends on the kinds of the pair -proteins and the difference between the partition coefficient o f the pair -protein. Figure 3 really prove it. Compared to Fig. 3, The linear velocity of mobile phase in the p acked bed of chromatographic cake shown in Fig. 2 is much lower,being favarorable to diminish band expanding in it. This is the reason why for same column length and same flow rate of mobile phase but different diameters, so long as the diameter of chromatographic cake is not too large, it has a better resolution.

Table I the values for ratios of different protein pairs and minimum column length with a linear gradient

different protein pairs	ratios of partition coefficients	L_{min}(cm)
Cyt-C / Myo	1.52E-2	0.045
Myo / RNase A	9.8E-1	3.34
RNase A / Lys	2.66E-1	0.027
Lys /a -Amy	2.72E-6	3.36E-8
a -Amy / Ins	7.61E+2	4.04

Experiment test

Figure 3 shows the comparison of the resolution of same proteins under same chromatographic conditions but with various column lengths. The linear flow rate also contributes to its resolution. The across surface area of chromatographic cake is much greater than that of usual chromatographic column. Both ofthe relatively slow linear velocity and traveling distance are favorable for decreasing in Eddy diffusion term.

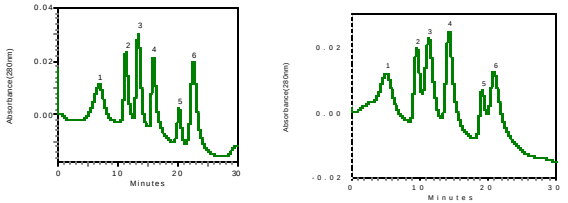

Figure 3 Comparison of resolution of the six standard proteinsby various column lengths.

HIC Columnst : A (4.6mm × 50mm); B, (4.6mm × 20 mm). Proteins: 1, Cyt-C; , Myo; 3, ase A; 4 Lys; 5, a -Amy; 6, Ins. t

Applications in biotechnology

PFLC is recognized to be a strong tool for protein folding and widely applications in molecular biology and biotechnology (J. Chromatogr B, 2007, 849: 69). Especially it can still work well, even some precipitates form on the top of the packed bed. With periodically cleaning the column with a very strong washing agen t to re-dissolve the precipitates and re-injecting the column. it can raise the recovery of mass and bioactivity. The chromatographic cake actually has four functions simultaneously. (1), Separation from denatering agent; (2), protein purification; (3), protein in unfolded state can be either partially, or completely converted into its native state; (4), Easily making waste denaturant recycling. Be cause of these advantages, the down-stream technology of therapeutic proteins in biotechnology can be significantly simplified and shorted, also the recovery of mass and bioactivity is increased very much. Figure 4 and 5 separated show the purification with simultsneous renaturation of rhG-CSF and rhCSF by SAX and HIC cakes in Lab scale. With only one step, the purity of both are g reater than 95% and required specific bioactivity by authority. Figure 6

Shows the comparison of purification procotols of rh-IFN-? in industrial scale between usual column and chromatographic cake.

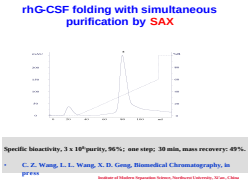

Figure 4 rhG-CSF refolding with simultaneous Purification by SAX cake (10mm × 20mm i.d)

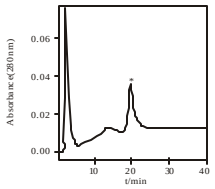

Figure 5 rhCSF refolding with simultaneous renaturation by HIC cake(10mm × 20 mm) Purification with simultaneous renaturation

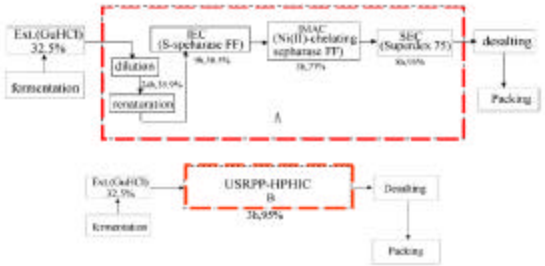

Figure 6 Scheme for the comparison of the new and usual production technologies of the rhIFN-r produced by *E. coli* with chromatographic cake.

A Usual production technology; four steps, 44 hours, purity, >95%; increases in bioactivity recovery, 1.6 folds;

B New production technology; one step, 3 hours; purity, >95%; incr ease in bioactivity recovery, 62 folds.

Conclusion

A short chromatographic column has an almost comparable resolution with a long column, a chromatographic cake with much greater diameter than its thickness even has much better resolution than that of the usual column with the same length. However, the required column length for a pair -protein is dominated by both differences of the partition coefficients and dynamics of protein transfer in packed bed. Chromatographic cake can be employed in small and industrial scales for purification with simultaneous renaturation of proteins. In many instances, the purity of the protein drugs can approach to 95% only with one step LC.