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Short communication

Size exclusion chromatography of polypeptides on Bio-Gel P2 in the dynamic axial compression mode

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Abstract

The effect of dynamic axial compression (DAC) upon the structure of a bed packed with semirigid Bio-Gel P2 and its chromatographic parameters was studied in the separation of peptides of various mass. In the studied range of DAC pressure, 0–5 bar, a decrease in the retention times of the studied substances and the standard deviation of their peaks is observed. In spite of a reduced packed bed height and an increase in the linear velocity of the eluent, the height equivalent to the theoretical plate (HETP) does not rise. The resolution of the separated substances increases throughout the studied range of DAC pressure with the Bio-Gel P2 exclusion limit extended. According to the results obtained, DAC provides more efficient optimisation of the bed parameters in columns packed with Bio-Gel P2 than in columns packed with Sephadex G-25 due to the greater rigidity of the former packing.

Keywords: Dynamic axial compression; Bio-Gel P2; Stationary phases, LC; Polypeptides

1. Introduction

DAC is known to improve the column packing quality and provide long-term stability of the packed bed in preparative HPLC [1–4]. Recently, the basic mechanics and physics of packing in the DAC mode were thoroughly investigated with experimental verification of the effect of the packing procedure on the packed bed properties [5–11]. These studies deal with rigid silica-based packing materials.

We have shown that DAC is no less favourable for chromatography on non-rigid materials in the range of compression pressure up to 5 bar [12–14]. Our results demonstrate that columns packed with materials based on Sephadex G-25 are optimised for stability and efficiency at certain values of compression pressure (different for various separated substances). This is provided by smooth consolidation of the packing material under applied pressure with continuous column volume adaptation to variations of the packing volume during operation in the DAC mode.

The aim of this paper is to investigate the DAC effect on the characteristics of a column packed with non-rigid polyacrylamide-based Bio-Gel P2 with properties differing from those of Sephadex G-25 and to compare the DAC effect on packing materials of dissimilar nature. Bio-Gel P2 seems to be of interest in such studies since softer Bio-Gel P100

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was shown to be more efficient for protein separation than Sephadex G-75 and Ultrogel AcA 54 with a pressure of 3 bar applied to its packed bed [15].

2. Experimental

The following chromatographic system was used: an HPLC pump type 2150 (LKB, Sweden), an injector MV-7 (Pharmacia, Sweden), a DAC column (1000×25 mm) with adapter-position pickup [13] from SynChro (Russia), UV detector Uvicord S-II (Pharmacia, Sweden), Shimadzu C-R6A Chromatopac integrator (Shimadzu Europe, Germany). Bio-Gel P2 (Bio-Rad, USA) with particle diameter 200 mesh was used for column packing . The peptides for trial separations (latrotoxin fragment and immunofan) were synthesised in our laboratory, and 95% purity was confirmed by HPLC and amino acid analysis. BSA and ammonium acetate were of analytical grade (Reakhim, Russia), glycyl-glycine puriss. was from Fluka AG (Switzerland).

The column was packed with a 50% (v/v) slurry of Bio-Gel P2 swollen in 0.3 *M* ammonium acetate (pH 6.4). The properties of the packed bed were assessed with a polypeptide mixture (a fragment of the latrotoxin sequence CNKVYEEKDTPPVQE, mol. wt. 1836, immunostimulator immunofan RDKVYR, mol. wt. 818 and glycyl-glycine, mol. wt. 132) and bovine serum albumin (BSA); 0.3, 0.4, 0.45 and 0.15 mg of the tested substances, respectively, in 1 ml were applied to the column. The column was tested in the DAC mode with compression pressure increasing up to 5.02 bar. The compression pressure on the moveable adapter was produced by means of detachable weights because this procedure enabled a stepwise increasing compression pressure with a high precision of 0.02 bar. The process of bed settling with increasing compression pressure is illustrated in Fig. 1. The eluate absorption was monitored at 220 nm. Data acquisition and processing is described in detail in Ref. [14].

3. Results and discussion

The distinct difference between the elution profiles obtained without pressure application (Fig. 2) and at a compression pressure of 5 bar (Fig. 3) suggests a substantial variation of the packed bed parameters in the DAC mode. This variation is illustrated in Fig. 4. While the total column volume V_c and the volume of the mobile phase V_o change practically at the same

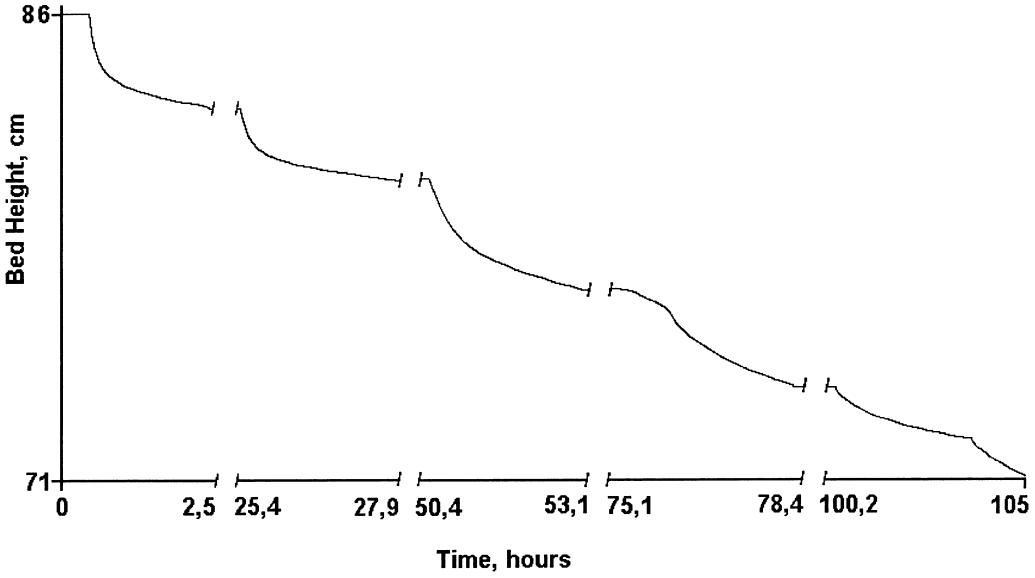

Fig. 1. Dynamics of column packing with Bio-Gel P2 with increasing compression pressure.

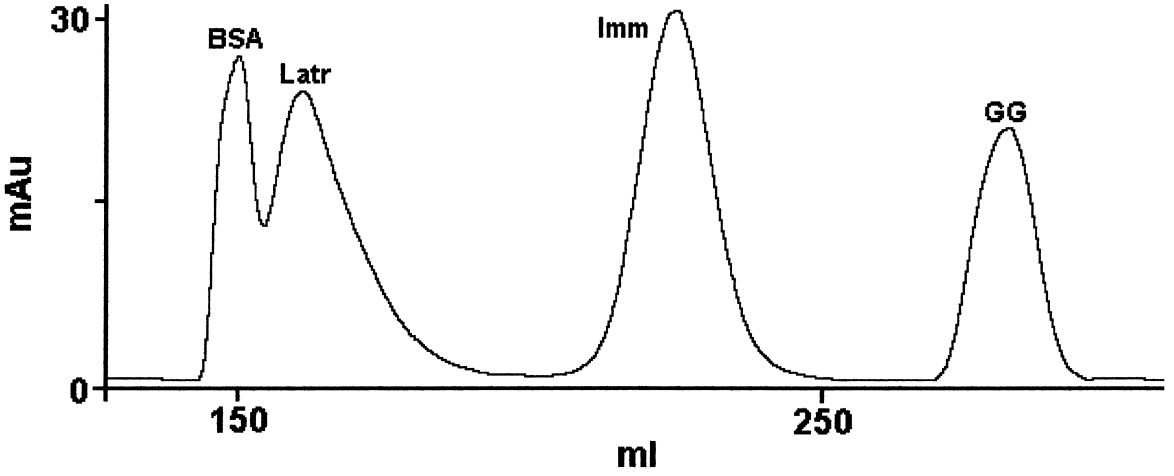

Fig. 2. Elution profile of the four-component polypeptide mixture without compression. Flow-rate, 1 ml/min; BSA, bovine serum albumin; Latr, the latrotoxin fragment; Imm, immunofan; GG, glycyl-glycine.

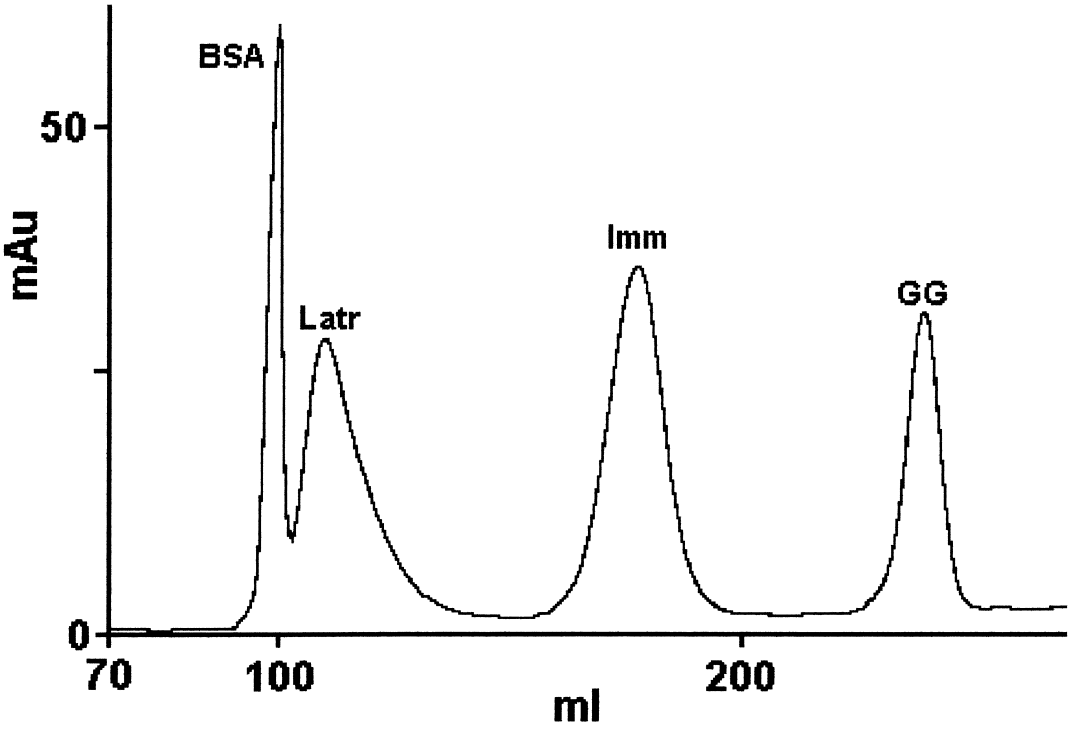

Fig. 3. Elution profile of the four-component polypeptide mixture at 5 bar compression pressure. Flow-rate, 1 ml/min; BSA, bovine serum albumin; Latr, the latrotoxin fragment; Imm, immunofan; GG, glycyl-glycine.

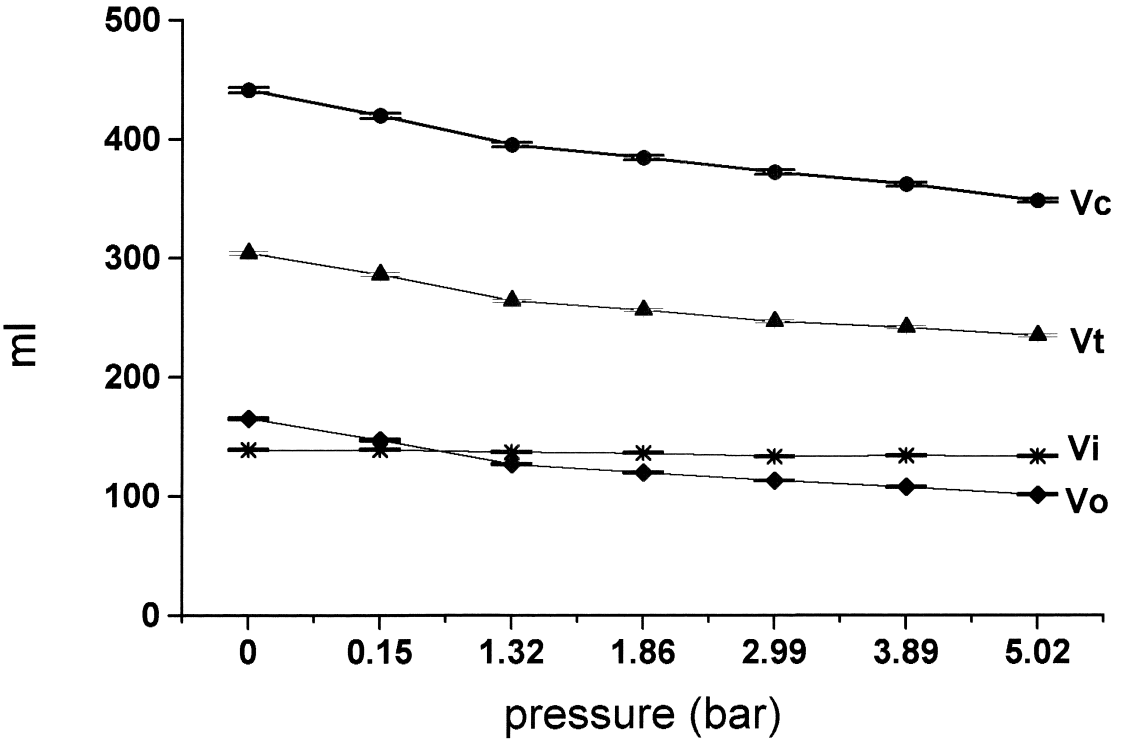

Fig. 4. Variation of the Bio-Gel P2 column volume V_c, the total volume of the liquid phase V_t (equal to the retention volume of glycyl-glycine), the volume of the mobile phase V_o (equal to the retention volume of BSA) and that of the stagnant phase V_i (derived as the difference between V_t and V_o) by the action of DAC.

rate, the volume of the stagnant phase V_i decreases only by 4%. This is in agreement with the fact that the value of the distribution constant $K_o = (V_R - V_o)/V_i$, which is a function of the ratio between the pore diameter and the eluate molecule volume, remains practically constant with increasing compression pressure (0.078 and 0.56 for the latrotoxin fragment and immunofan, respectively). The external porosity of the Bio-Gel P2 bed derived as V_o/V_c decreases from 0.37 to 0.29 at 5 bar, which is 2.6 times less than the porosity reduction measured for the Sephadex G-25 packed bed [14]. This suggests greater rigidity of Bio-Gel P2 compared to Sephadex G-25. This conclusion is supported by the fact that the stagnant phase volume of the Bio-Gel P2 bed decreases half as much as that of the Sephadex G-25 bed in the studied compression pressure range.

The sorbent rigidity is characterised by the compressibility coefficient a_ν which can be determined as the slope of the plot of the void fraction $e = \varepsilon/(1-\varepsilon)$ versus the axial compression pressure [9]. The corresponding plots for Bio-Gel P2 and Sephadex G-25 are presented in Fig. 5. The data for Sephadex G-25 were taken from Ref. [14]. The initial portion of this plot is not available since, in pressurizing a column with gas, it is difficult to obtain a pressure <1.7 bar with sufficient accuracy. It is evident from Fig. 5 that the data for Bio-Gel P2 can be approximated by two straight lines, the slope of the first steeper line ($a_\nu = 7.3 \cdot 10^{-3}$ cm^2/N) being similar to that of the plot for Sephadex G-25 ($a_\nu = 8.9 \cdot 10^{-3}$ cm^2/N). By analogy with the interpretation of the corresponding plots obtained in the packing of rigid sorbents [6], dissimilar slopes of the straight lines descriptive of the Bio-Gel P2 packing process can be assumed to stem from variations in this process. The first straight line can be associated with slippage of the particles over each other at relatively small packing density. The second straight line with smaller slope ($a_\nu = 1.7 \cdot 10^{-3}$ cm^2/N)

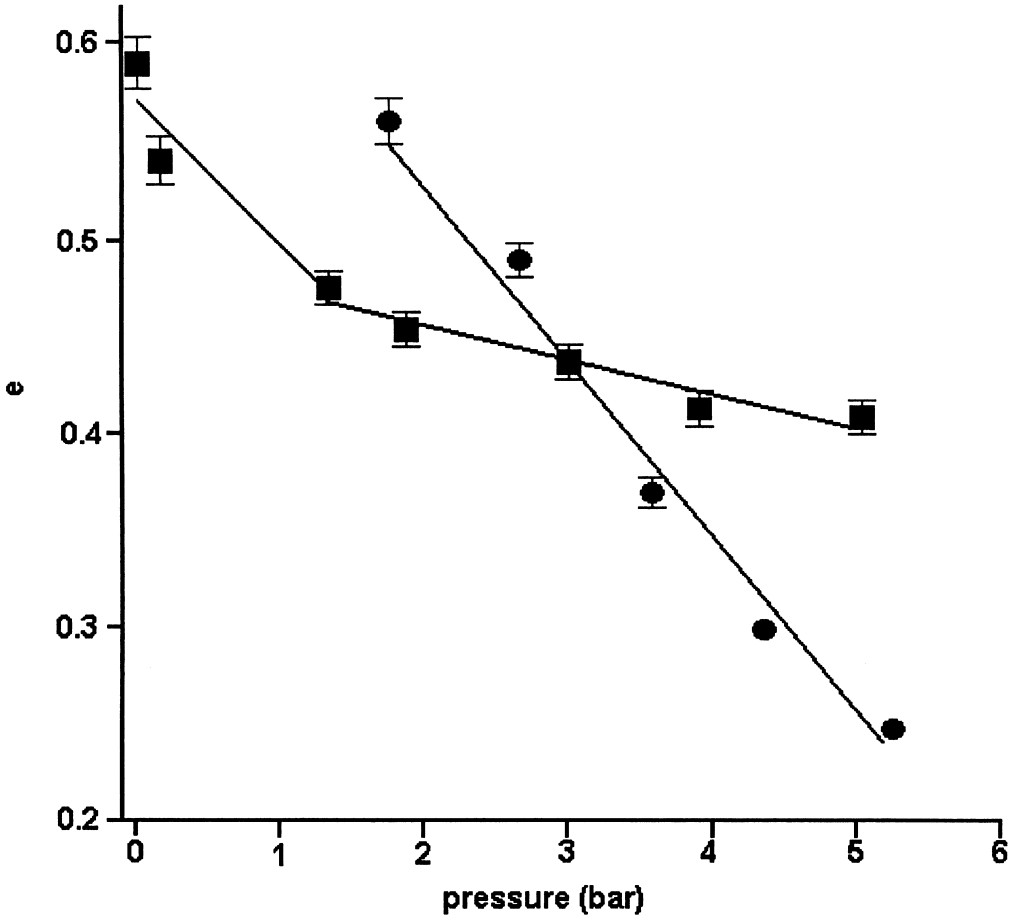

Fig. 5. Least-square fit of the void ratio vs. compression pressure for Bio-Gel P2 (■) and Sephadex G-25 (●).

suggests the formation of a denser packing structure when slippage becomes impossible and restructuring of the packed bed takes more time. In the case of Sephadex G-25, the packing process does not slow down, probably due to elastic deformation of the particles [14].

Although Bio-Gel P2 appears to be rather elastic, at least in the initial packing stage, the deformation of the particles is not permanent, and they appear to recover their shape appreciably. The increase in bed height upon instantaneous decompression is shown in Fig. 6. The residual deformation is 10.3%, whereas for Sephadex G-25 it was estimated at 50% [12].

Since SEC separation is accounted for by the different distribution of eluates in the mobile and stagnant phases, variation in the phase ratio $V_0/V_i =$ β from 1.19 to 0.76 must reflect in the peak dispersion of the separated substances as well as a decrease in the bed height. Fig. 7 illustrates the variation in the peak standard deviation σ with increasing compression pressure. It is evident that a reduction in the standard deviation of the peaks of BSA and the latrotoxin fragment is rather pronounced with minimum increasing compression pressure up to 1.3 bar and varies only slightly as it increases further. The standard deviation of the immunofan and glycyl-glycine peaks continues decreasing with increasing compression pressure up to 5 bar. Dissimilar dynamics of variation of the peak standard deviation with increasing compression pressure is obviously caused by the fact that the latter influences the retention volume and the height

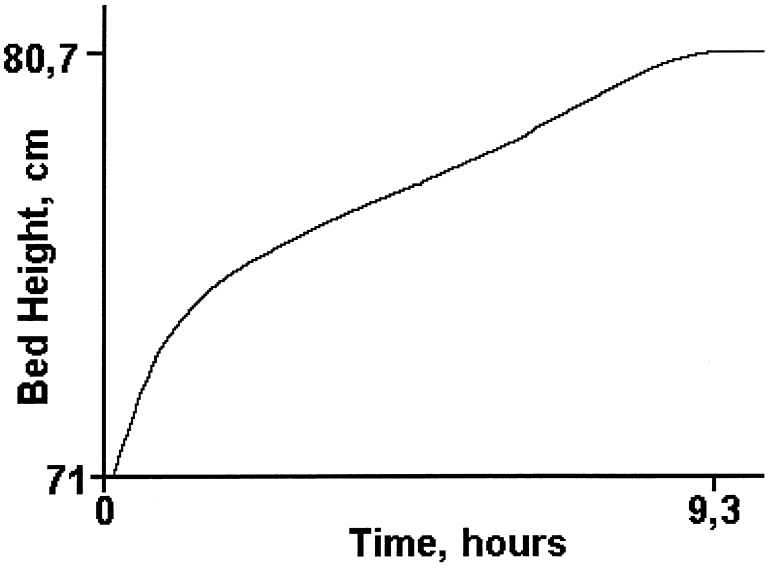

Fig. 6. Bio-Gel P2 packed bed variation upon decompression.

equivalent to theoretical plate (HETP) in a different way since $\sigma = V_R\sqrt{H/L}$. For the molecules excluded from the pores of the packing particles (BSA), the total HETP value depends only on the packing homogeneity and the particle dimensions. It can be seen from Fig. 8 that the HETP value for BSA decreases with increasing compression pressure due to optimisation of the packing structure. For the

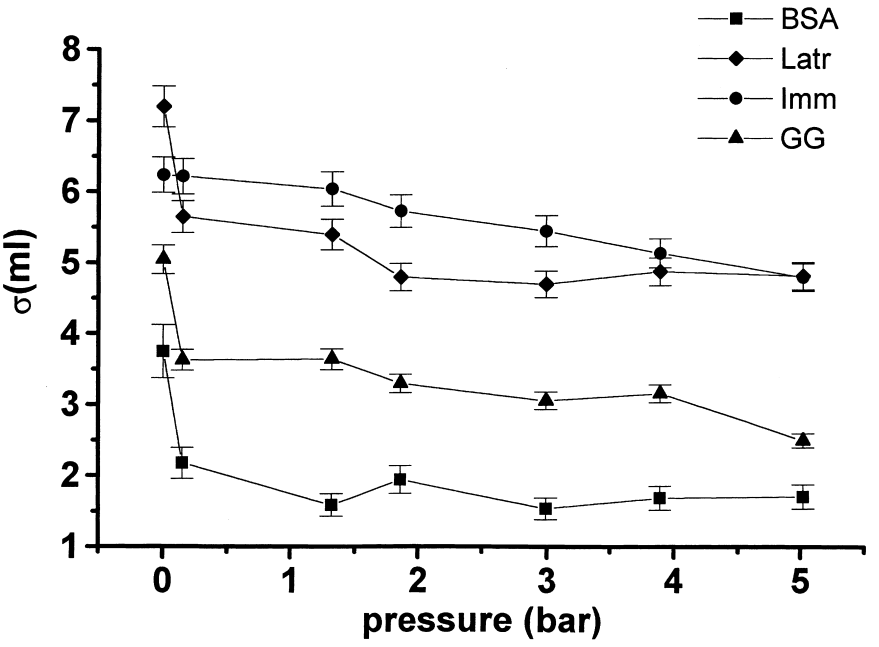

Fig. 7. Variation of the peak standard deviation on the Bio-Gel P2 column caused by DAC.

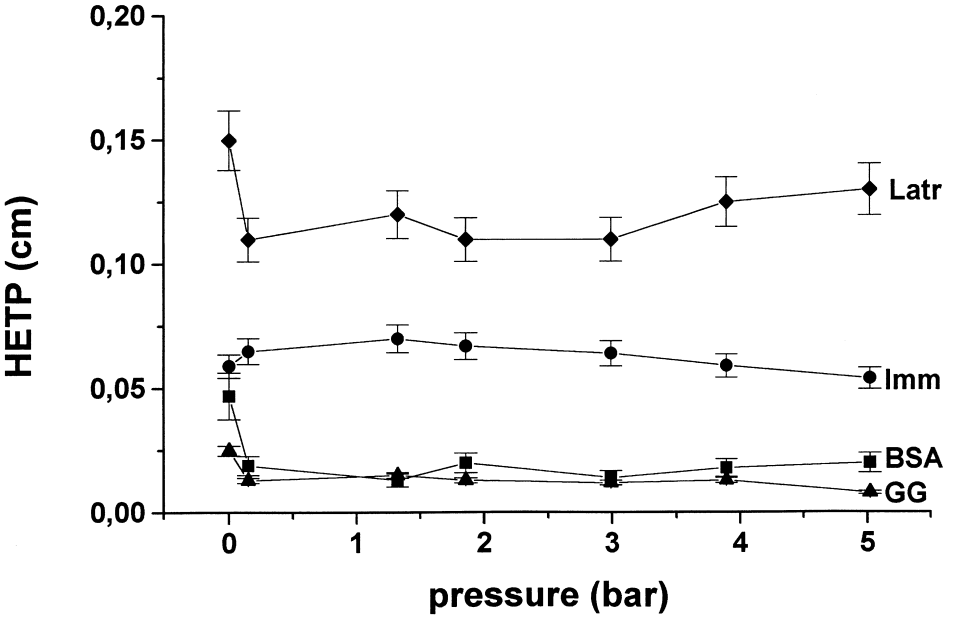

Fig. 8. HETP variation on the Bio-Gel P2 column caused by DAC.

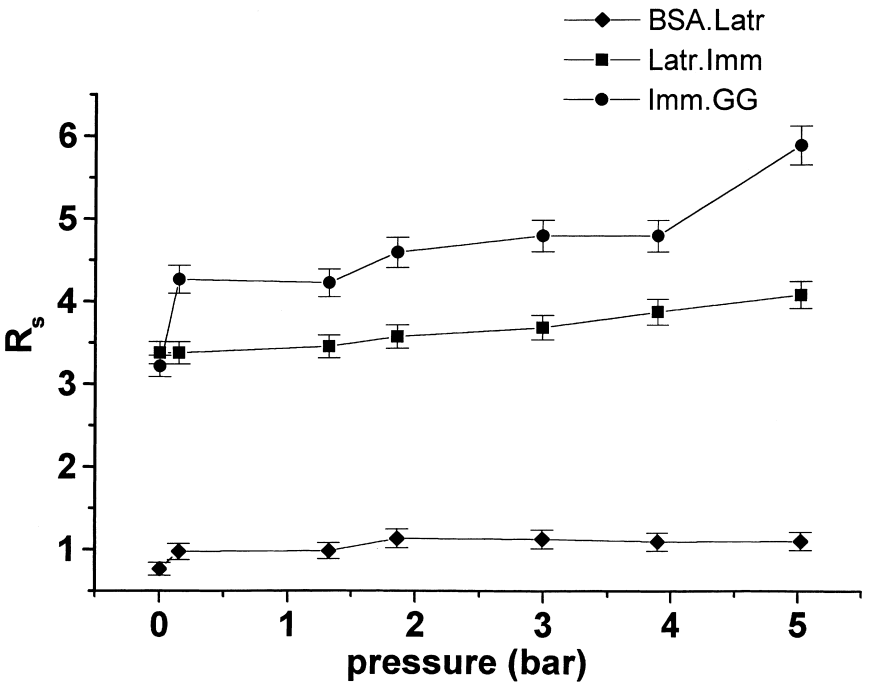

Fig. 9. DAC effect on the resolution factor for the pairs of peaks. The resolution factor R_S was estimated using $R_S \approx \{(K_{o2} - K_{o1})/[2(K_{o1} + K_{o2})]\}\sqrt{\overline{N_{eff}}}$ where $\overline{N_{eff}}$ is the mean of the N_{eff} values [$N_{eff} = (V_R - V_o)^2/\sigma^2$] of the two closely eluted peaks (see Ref. [14] for derivation of the equation).

separated substances that penetrate the particle pores, the measured total HETP values remain constant within errors or tend to decrease in the studied range of compression pressure (see Fig. 8). In the case of the less-rigid Sephadex G-25, a rise in the HETP value was observed with increasing compression pressure [14]. This must be caused by narrowing and/or closure of intraparticle pores upon packing contraction by the action of compression.

Variation of the resolution factor for the pairs of peaks with increasing compression pressure is presented in Fig. 9. The resolution factor for the pair of peaks of BSA and the latrotoxin fragment reaches a maximum at 1.8 bar and remains approximately constant with increasing compression pressure. Consequently, when applying a small compression pressure, it is possible to separate substances with a molecular weight corresponding to the exclusion limit of Bio-Gel P2 more efficiently (the molecular weight of the latrotoxin fragment is 1836, the exclusion limit of Bio-Gel P2 is 1800). The resolution factor for the remaining pairs of peaks increases throughout the studied range of compression pressure, while for the Sephadex G-25 packed bed the maximum resolution factor is achieved in the range 3–4 bar, this maximum value diminishing at higher compression pressure [14]. This suggests that the packing quality is impaired at compression pressures exceeding 4 bar because of the low rigidity of Sephadex G-25.

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