ELSEVIER

Journal of Chromatography A, 827 (1998) 311–318

JOURNAL OF CHROMATOGRAPHY A

Comparison of loading capacities of various proteins and peptides in culture medium and in pure state

Arne Staby*, Nina Johansen, Hanne Wahlstrøm, Inger Mollerup

Novo Nordisk A/S, Hagedornsvej 1, DK-2820 Gentofte, Denmark

Abstract

Chromatographic media suppliers most frequently state the capacities of their gels based on either static capacities or frontal analysis experiments of pure proteins, however, these capacity values are often far from the capacities experienced in the production of such proteins. In this work, static and dynamic capacities of various pure industrial proteins or peptides are compared to the capacities of the proteins or peptides under similar conditions in their natural culture medium. The results show a significant decrease in the static and dynamic capacities of the proteins or peptides when present in culture medium due to competitive binding of medium proteins. The proteins and peptides included in this study are: lipolase, glucagon-like peptide-1, truncated prothrombin, insulin precursor, and anti-Factor VII monoclonal antibody.

Keywords: Stationary phase, LC; Ion-exchange capacity; Proteins; Peptides

1. Introduction

Ion-exchange chromatography is probably the most commonly used technique for purification of proteins and practically all industrial purification processes contain at least one or several ion-exchange steps. One of the main parameters of an industrial ion-exchange step and of chromatographic steps in general, is the loading capacity of the chromatographic gel.

Chromatographic equipment and media suppliers typically state the capacities of their gels based on either static capacities or frontal analysis experiments of pure proteins, most frequently serum albumins for anion-exchangers and lysozyme for cation exchangers [1–3]. It is commonly known that a major decrease in loading capacity of a protein or peptide is obtained when present in culture medium, fermentation broth or its natural source due to competitive binding of other proteins etc. [1], however, very little information on this subject has been published in the literature.

In this work, we present some examples of capacities obtained from industrial proteins/peptides in culture medium/fermentation broth and compare them to capacities of the pure proteins or peptides at similar conditions. The comparison will include both static and dynamic capacities of: lipolase [4], glucagon-like peptide-1 (GLP-1) [5], truncated prothrombin [6], insulin precursor [7], and anti-Factor VII monoclonal antibody (anti-FVII Mab) [8].

*Corresponding author. Tel.: +45 44 439989; fax: +45 44 438400.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00764-X

2. Experimental

2.1. Materials

All pure proteins/peptides (lipolase, GLP-1, truncated prothrombin, insulin precursor and anti-FVII Mab) and protein/peptide culture medium/fermentation broth solutions were obtained from Novo Nordisk (Bagsværd, Denmark).

SP Sepharose Fast Flow beads were kindly donated by and SP Sepharose Big Beads and Q Sepharose XL beads were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Poros HQ 50 beads were kindly donated by Perseptive Biosystems (Cambridge, MA, USA).

Tris(hydroxymethyl)aminomethane (Tris, Sigma 7–9) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were analytical reagent grade and purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

A BioCAD Workstation from Perseptive Biosystems (Cambridge, MA, USA) and a fast protein liquid chromatography (FPLC) system from Amersham Pharmacia Biotech were used for dynamic capacity measurements in these studies. The standard BioCAD Workstation was equipped with a 0.3 cm flow cell and pump heads for flow-rates between 0.2 and 60 ml/min. The FPLC system was equipped with two P-500 pumps, a UV-1 detector, and a Frac-100 fraction collector. In both cases, samples were applied through the pump and UV detection was operated at 280 nm.

Chromatographic resins were packed in HR 5/2.5, HR 5/5, and HR 10/5 columns supplied by Amersham Pharmacia Biotech (Uppsala, Sweden).

Analytical RP-HPLC was performed using a System Gold analytical liquid chromatograph from Beckman Instruments (Fullerton, CA, USA) equipped with a 717plus Autosampler from Waters (Milford, MA, USA) and a HPLC column oven from Ramcon (Birkerød, Denmark).

2.3. Capacity determinations

All capacity determinations were performed employing similar conditions, i.e. the same protein/peptide concentration, pH, solution conductivity, temperature, buffer concentration, and residence time were applied in pure solution and in culture medium/fermentation broth. In the pure protein/peptide solutions, hydrochloric acid and sodium hydroxide were used to adjust pH and salt was used to adjust the conductivity to that of the culture medium/fermentation broth supplied.

For the dynamic capacity measurements, the general conditions were: The column was equilibrated with a sufficient number of column volumes (CVs) of buffer at similar conditions as the application, i.e. same pH and conductivity. The protein/peptide solutions derived from pure state or culture medium were applied to the column at the same overall conditions. The run-through during sample application was collected and fractionated, and samples were analysed as given below. Breakthrough curves of pure protein/peptide solutions were derived from both the UV detector signal and analyses, and based on these results, 10% and 50% breakthrough are presented if possible. The validity of this approach was controlled by eluting the column at normal conditions to verify the mass balance of the protein/peptide.

General conditions of measurement of static capacities were: Standing of protein/peptide solution overnight (16–20 h) with slow agitation at the same, sufficient concentration with the same amount of chromatographic ion-exchange gel to secure equilibrium between bound protein/peptide and protein/peptide in solution. Static capacity was measured by analysing the concentration of protein/peptide in the supernatant and assign the difference between this value and the initial concentration to the gel capacity. The validity of this approach was controlled by packing the loaded gel in a column and eluting it at normal conditions to verify the mass balance of the protein/peptide.

2.3.1. Lipolase

Pretreatment of the raw lipolase fermentation broth harvest covered various filtrations including diafiltration. The pure lipolase solution was made by dissolving lipolase in a 0.050 *M* Tris solution, pH 8.6, which was subsequently adjusted to a conductivity of ≈2.5 mS/cm at room temperature by

addition of sodium chloride to 0.010 *M*. The exact lipolase content of the pure solution and the fermentation broth were determined by the analytical RP-HPLC.

Analytical RP-HPLC was performed on a C_6-substituted 300 Å silica produced in house. Column dimensions were 25 cm×0.4 cm I.D. Buffer A consisted of 0.1% trifluoroacetic acid (TFA) in water, buffer B of 0.09% TFA in 80% acetonitrile. Linear gradients from 0–100% B were run with a flow-rate of 1 ml/min. The chromatographic temperature was kept at 40°C and UV detection was performed at 216 nm.

2.3.1.1. Dynamic capacity study

Pure lipolase solution and lipolase fermentation broth were applied to columns of Poros HQ 50 (2.5 cm×1 cm I.D.), which were previously equilibrated with 15 CVs of a 0.050 *M* Tris+0.010 *M* NaCl solution, pH 8.6. Non-bound material was removed by washing with 10 CVs of the equilibration solution. Bound material was eluted employing a step gradient of 1 *M* NaCl in 0.050 *M* Tris buffer, pH 8.6. The flow-rate was maintained at 1 ml/min during the experiments.

2.3.1.2. Static capacity study

Poros HQ 50 resin was equilibrated with 10 volumes of a 0.050 *M* Tris+0.010 *M* NaCl solution, pH 8.6. The resin was then added to beakers containing the pure lipolase solution and the lipolase fermentation broth, which were slowly agitated over night. A small sample of the supernatant was withdrawn and analysed by RP-HPLC for the content of lipolase. The resin was isolated using a sintered glass filter and packed in a 1 cm I.D. column. Non-bound material was removed by washing with 10 CVs of the equilibration solution. Bound material was eluted employing a step gradient of 1 *M* NaCl in 0.050 *M* Tris buffer, pH 8.6. The flow-rate during wash and elution was maintained at 1 ml/min.

2.3.2. GLP-1 (glucagon-like peptide-1)

Pretreatment of the raw GLP-1 fermentation broth harvest covered pH adjustment and filtration to clarify the solution. The pure GLP-1 solution was made by dissolving GLP-1 in a 0.300 *M* glycin solution, pH 3.0, which was subsequently adjusted to a conductivity of 15–20 mS/cm at room temperature by addition of sodium chloride to 0.175 *M*. The exact GLP-1 content of the pure solution and the fermentation broth were determined by the analytical RP-HPLC.

Analytical RP-HPLC was performed on a C_{18}-substituted 100 Å silica produced in house. Column dimensions were 25 cm×0.4 cm I.D. Buffer A consisted of 0.15 *M* $(NH_4)_2SO_4$ in 7.8% acetonitrile, pH 2.5, buffer B of 63.4% acetonitrile. Linear gradients from 37–41% B in 12 min followed by 41–100% B in 15 min were run with a flow-rate of 1 ml/min. The chromatographic temperature was kept at 60°C and UV detection was performed at 214 nm.

2.3.2.1. Dynamic capacity study

Pure GLP-1 solution and GLP-1 fermentation broth were applied to columns of SP Sepharose BB (5 cm×1 cm I.D.), which were previously equilibrated with 15 CVs of a 0.300 *M* glycin solution, pH 3.0. Non-bound material was removed by washing with 15 CVs of a 0.300 *M* glycin+1 *M* NaCl solution, pH 3.0. Bound material was eluted employing a step gradient of 0.300 *M* glycin buffer, pH 9.0. The flow-rate was maintained at 0.8 ml/min during the experiments.

2.3.2.2. Static capacity study

SP Sepharose BB resin was equilibrated with 10 volumes of a 0.300 *M* glycin solution, pH 3.0. The resin was then added to tanks containing the pure GLP-1 solution and the GLP-1 fermentation broth, which were slowly agitated overnight. A small sample of the supernatant was withdrawn and analysed by RP-HPLC for the content of GLP-1. The resin was isolated using a sintered glass filter and packed in a 0.5 cm I.D. column. Non-bound material was removed by washing and bound material was eluted as described for the dynamic experiments at a flow-rate of 1 ml/min.

2.3.3. Truncated prothrombin

Pretreatment of the raw truncated prothrombin culture medium harvest comprised filtration to clarify the solution. The pure truncated prothrombin solution was made by dissolving truncated prothrombin in a 0.020 *M* Tris+0.120 *M* NaCl solution, pH 9.0, which subsequently had a conductivity of ≈9 mS/

cm at cold room temperature. The exact truncated prothrombin content of the pure solution and the culture medium were determined by the analytical RP-HPLC.

Analytical RP-HPLC was performed on a Deltapack C_4 column produced by Waters. Column dimensions were 15 cm×0.39 cm I.D. Buffer A consisted of 0.1% TFA in water, buffer B of 0.09% TFA in 80% acetonitrile. Linear gradients from 0 to 80% B were run with a flow-rate of 1 ml/min. The chromatographic temperature was kept at 40°C and UV detection was performed at 216 nm.

2.3.3.1. Dynamic capacity study

Pure truncated prothrombin solution and truncated prothrombin culture medium were applied to columns of Q Sepharose XL (5 cm length ×0.5 cm I.D.), which were previously equilibrated with 10 CVs of a 0.020 *M* Tris +0.120 *M* NaCl solution, pH 9.0. Non-bound material was removed by washing with 30 CVs of the equilibration solution at pH 7.4. Bound material was eluted employing a step gradient of 0.300 *M* NaCl in a 0.020 *M* Tris buffer, pH 7.4. The flow-rate was maintained at 1 ml/min during the experiments.

2.3.3.2. Static capacity study

Q Sepharose XL resin was equilibrated with 10 volumes of the equilibration solution of the dynamic experiments. The resin was then added to beakers containing the pure truncated prothrombin solution and the truncated prothrombin culture medium, which were slowly agitated over night. A small sample of the supernatant was withdrawn and analysed by RP-HPLC for the content of truncated prothrombin. The resin was isolated using a sintered glass filter and packed in a 0.5 cm I.D. column. Non-bound material was removed by washing and bound material was eluted as described for the dynamic experiments at a flow-rate of 0.5 ml/min.

2.3.4. Insulin precursor

Pretreatment of the raw insulin precursor fermentation broth harvest covered pH adjustment and filtration to clarify the solution. The pure insulin precursor solution was made by dissolving insulin precursor in a 0.003 *M* HCl solution, pH 2.5, which subsequently had a conductivity of ≈11 mS/cm at room temperature. The exact insulin precursor content of the pure solution and the fermentation broth were determined by the analytical RP-HPLC.

Analytical RP-HPLC was performed on a C_{18}-substituted 100 Å silica produced in house. Column dimensions were 25 cm×0.4 cm I.D. Buffer A consisted of 0.2 *M* Na_2SO_4 and 0.04 *M* H_3PO_4 in 10% acetonitrile, pH 2.3, buffer B of 50% acetonitrile. Linear gradients from 35–59% B were run with a flow-rate of 1 ml/min. The chromatographic temperature was kept at 50°C and UV detection was performed at 214 nm.

2.3.4.1. Dynamic capacity study

Pure insulin precursor solution and insulin precursor fermentation broth were applied to columns of SP Sepharose BB (5 cm×1 cm I.D.), which were previously equilibrated with 10 CVs of a 0.003 *M* HCl solution, pH 2.5. Non-bound material was removed by washing with 15 CVs of the equilibration solution. Bound material was eluted employing a step gradient of a 2.5 *M* NH_3 solution, pH 9.0. The flow-rate was maintained at 1.2 ml/min during the experiments.

2.3.4.2. Static capacity study

SP Sepharose BB resin was equilibrated with 10 volumes of the equilibration solution of the dynamic experiments. The resin was then added to tanks containing the pure insulin precursor solution and the insulin precursor fermentation broth, which were slowly agitated overnight. A small sample of the supernatant was withdrawn and analysed by RP-HPLC for the content of insulin precursor. The resin was isolated using a sintered glass filter and packed in a 0.5 cm I.D. column. Non-bound material was removed by washing and bound material was eluted as described for the dynamic experiments at a flow-rate of 1 ml/min.

2.3.5. Anti-FVII Mab

Pretreatment of the raw anti-FVII Mab culture medium harvest covered pH adjustment and filtration. The pure anti-FVII Mab solution was made by dissolving Anti-FVII Mab in a 2 g/l $NaHCO_3$+6 g/l NaCl solution, pH 4.0, which subsequently had a conductivity of ≈9 mS/cm at cold room temperature. The exact anti-FVII Mab content of the pure

solution and the culture medium were determined by anti-FVII Mab enzyme-linked immunosorbent assay (ELISA).

Anti-FVII Mab ELISA was performed with peroxidase conjugated rabbit anti-mouse immunoglobulins by a company standard operating procedure [9].

2.3.5.1. Dynamic capacity study

Pure anti-FVII Mab solution and anti-FVII Mab culture medium were applied to columns of SP Sepharose FF (5×0.5 cm I.D.), which were previously equilibrated with 10 CVs of a 2 g/l $NaHCO_3$+6 g/l NaCl solution, pH 4.0. Non-bound material was removed by washing with 20 CVs of the equilibration solution. Bound material was eluted employing a step gradient of 0.004 *M* NaCl in a 0.050 *M* NaH_2PO_4 buffer, pH 8.0. The flow-rate was maintained at 0.5 ml/min during the experiments.

2.3.5.2. Static capacity study

SP Sepharose FF resin was equilibrated with 10 volumes of the equilibration solution of the dynamic experiments. The resin was then added to tanks containing the pure anti-FVII Mab solution and the anti-FVII Mab culture medium, which were slowly agitated overnight. A small sample of the supernatant was withdrawn and analysed by ELISA for the content of anti-FVII Mab. The resin was isolated using a sintered glass filter and packed in a 0.5 cm I.D. column. Non-bound material was removed by washing and bound material was eluted as described for the dynamic experiments at a flow-rate of 0.5 ml/min.

3. Results and discussion

The general approach of this study is to compare sorption capacities on various ion exchangers of proteins and peptides in culture medium and in pure state at similar conditions, that is, at the same pH, flow/residence time, solution conductivity, protein/peptide concentration, scale, buffer, and temperature. The experimental results are shown in Figs. 1–5 and in Table 1. Figs. 1–5 present the breakthrough curves in pure state and in culture medium/fermentation broth of lipolase, GLP-1, truncated prothrom-

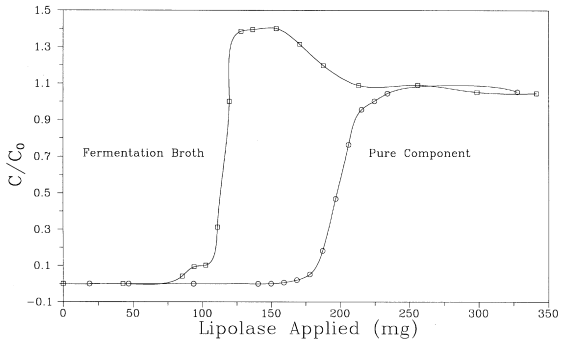

Fig. 1. Breakthrough curves of lipolase in pure state (○) and in fermentation broth (□) obtained from RP-HPLC analysis results of collected fractions.

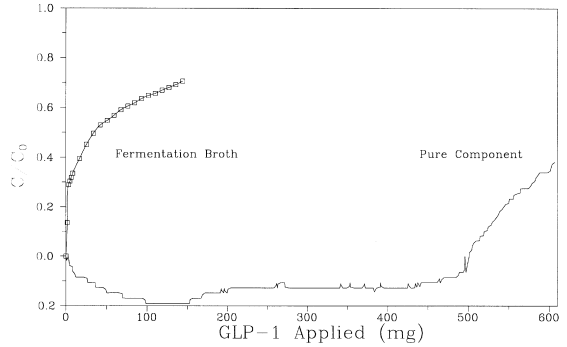

Fig. 2. Breakthrough curves of GLP-1 in pure state (no symbol) obtained from the chromatographic UV traces and in fermentation broth (□) obtained from RP-HPLC analysis results of collected fractions.

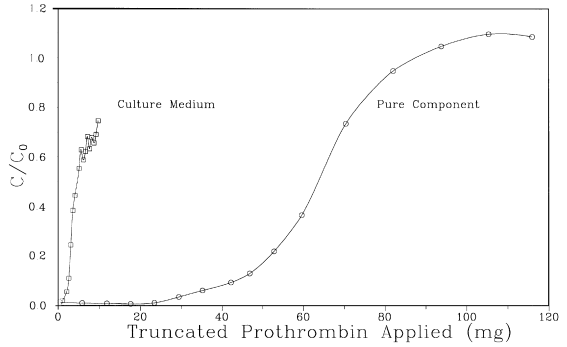

Fig. 3. Breakthrough curves of truncated prothrombin in pure state (○) and in culture medium (□) obtained from RP-HPLC analysis results of collected fractions.

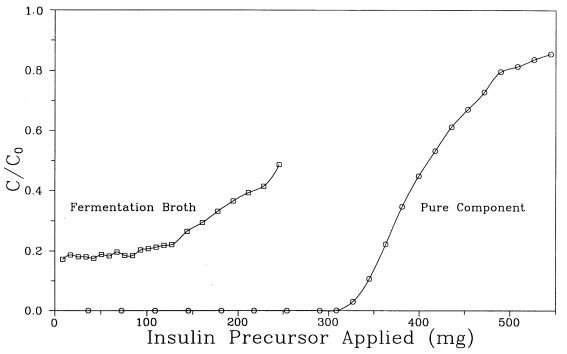

Fig. 4. Breakthrough curves of insulin precursor in pure state (○) and in fermentation broth (□) obtained from RP-HPLC analysis results of collected fractions.

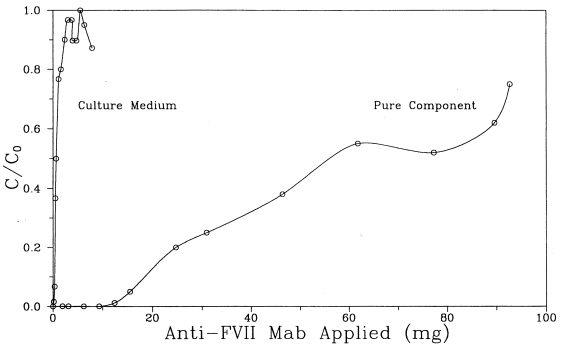

Fig. 5. Breakthrough curves of anti-FVII Mab in pure state (○) and in culture medium (□) obtained from ELISA analysis results of collected fractions.

bin, insulin precursor, and anti-FVII Mab, respectively, and Table 1 shows the static capacities obtained and the dynamic capacities at 10% and 50% breakthrough. The scatter of breakthrough curves of proteins/peptides, especially in culture medium of truncated prothrombin and anti-FVII Mab in Figs. 3 and 5, respectively, is due to uncertainty of the analytical method and variation between analysis series, while the decreasing trend in the beginning of the pure component GLP-1 curve in Fig. 2 is caused by UV monitor drifting.

Upon examination of the different figures, some well-known characteristics appear in these natural systems. In Fig. 1, a very smooth breakthrough curve is shown for the pure component. For lipolase in fermentation broth the typical picture of a compressive curve is shown of the displaced component in a competitive binary system [10], initially giving a breakthrough concentration higher than the feed concentration, C_0, and ending up at the feed concentration simultaneously with the curve of the pure component. In fact, the treated lipolase fermentation broth consisted of the product, one major impurity, and various small amount impurities, almost corresponding to a binary system. Both lipolase curves are quite steep and show that the dynamic capacity of Poros HQ 50 under these conditions are rather high. The pure component breakthrough curves in Figs. 2–5 display a less steep and thus less advantageous course indicating that the kinetics of these gels having larger particle size and smaller pore size are lower, however, the dynamic capacities are still quite high. This may also be seen in Table 1, where the difference between 10% and 50% breakthrough for pure component lipolase on Poros HQ 50 is <10%, but higher for the other proteins/peptides and gels. A similar trend is obtained for the proteins/peptides in

Table 1
Static and dynamic capacity results of various chromatographic ion exchange gels with proteins and peptides in pure state and in culture medium/fermentation broth

Chromatographic gel	Protein/peptide	Static capacity (mg/ml)		Dynamic capacity (mg/ml), % breakthrough			
		Pure state	Culture medium	Pure state		Culture medium	
				10%	50%	10%	50%
Poros HQ 50	Lipolase	96	40	91	99	50	57
SP Sepharose BB	GLP-1	125	10	125	151	<0.5	9
Q Sepharose XL	Truncated prothrombin	132	5	43	64	2.5	4.6
SP Sepharose BB	Insulin precursor	99	35	86	103	<2	>62
SP Sepharose FF	Anti-FVII Mab	42	0.43	19	57	0.33	0.63

culture medium/fermentation broth. For the components in culture medium/fermentation broth, only truncated prothrombin on Q Sepharose XL in Fig. 3 display some binding before breaking through, while GLP-1, insulin precursor, and anti-FVII Mab almost immediately runs through. Thus, even though a high pure component dynamic capacity is obtained, a very small dynamic capacity of the component situated in culture medium/fermentation broth might be experienced at the same conditions simply due to competitive binding.

Static capacity results presented in Table 1 show a pattern very similar to that of the dynamic capacities, that is high capacities of pure proteins/peptides and lower or much lower capacities when situated in culture medium/fermentation broth. A comparison of static and dynamic capacities in Table 1 shows a good concordance of pure component lipolase, partly GLP-1, and insulin precursor indicating that the flow-rate and/or steric hindrance are not limiting factors under these experimental conditions. For truncated thrombin, the static capacity is much higher than the dynamic capacity indicating that some rate limiting factors have influenced the result. The anti-FVII Mab static capacity of SP Sepharose FF is between the 10% and 50% dynamic breakthrough capacity, and we suspect that this protein would aggregate on the column during the prolonged dynamic experiments and thus are these values not directly comparable. To some extent, this may also be the case for GLP-1. By comparing static and dynamic (50%) capacities of proteins/peptides in culture medium/fermentation broth, similar results are obtained for GLP-1 and truncated prothrombin. For lipolase, insulin precursor, and anti-FVII Mab the dynamic capacities (50%) are higher than the static capacities, but whether this is due rate effects on competitive binding proteins, aggregation of the target proteins/peptide, or other effects is not known.

Dynamic supplier capacities at various flow-rates of the four gels Poros HQ 50, SP Sepharose BB, Q Sepharose XL and SP Sepharose FF are: 60 mg/ml bovine serum albumin (BSA), 100 mg/ml (β-lactoglobulin), >130 mg/ml (BSA), and 50 mg/ml (IgG), respectively. The capacities given by gel suppliers are in general concordance with the findings of this study with respect to pure protein/peptide solutions and in some cases values of this study are higher. Thus, the supplier data is very valuable for guidance on pure proteins/peptides. Results may, however, vary as for the dynamic capacity data of SP Sepharose FF, which are 50% higher by mass for GLP-1 compared to insulin precursor. A comparison of the general high expression level systems: *Aspergillus oryzae* for lipolase and yeast for GLP-1 and insulin precursor with the low mammalian cell expression level systems for truncated prothrombin and anti-FVII Mab does not give a clear illustration of differences/similarities. For this purpose adsorption isotherm data are necessary, which is outside the scope of this paper.

4. Conclusion

The results show a significant decrease in static and dynamic capacity of the proteins/peptides when present in culture medium/fermentation broth. Even though high dynamic capacities for pure components are obtained, very small dynamic capacities of the components situated in culture medium/fermentation broth might be experienced at the same conditions simply due to competitive binding by medium proteins. Further, sorption capacity of the target protein/peptide increases with increasing purity of the component in the culture medium/fermentation broth. The capacity is important to the industry, however, selectivity is too, therefore a gel with quite low pure component capacity may be more efficient than a high pure component capacity gel. All the gels presented in this paper are generally suitable for a capture step, however as shown, their efficiencies depend on the nature of the culture medium/fermentation broth.

Acknowledgements

The supply of culture medium/fermentation broth and pure proteins from Peter Rahbek Østergaard, Tom Chambers, Birgitte Silau and Susanne Kristensen, and the technical assistance with anti-FVII Mab ELISA of Jan Søndergård-Andersen and Susanne R. Jensen are gratefully acknowledged.

References

[1] P.R. Levison, R.M.H. Jones, D.W. Toome, S.E. Badger, M. Streater, N.D. Pathirana, J. Chromatogr. A 734 (1996) 137.
[2] J. Horvath, E. Boschetti, L. Guerrier, N. Cooke, J. Chromatogr. A 679 (1994) 11.
[3] I. Lagerlund, E. Larsson, J. Gustavsson, J. Färenmark, A. Heijbel, J. Chromatogr. A 796 (1998) 129.
[4] E. Boel, T. Christensen, E. Gormsen, B. Huge-Jensen, B.S. Olesen, in: L. Alberghina, R.D. Schmid, R. Verger (Editors), Lipases: Structure, Mechanism and Genetic Engineering, GBF Monographs 16, 1990, p. 207.
[5] R.I. Senderoff, K.M. Kontor, L. Kreilgaard, J.J. Chang, S. Patel, J. Krakover, J.K. Heffernan, L.B. Snel, G.B. Rosenberg, J. Pharm. Sci. 87 (1998) 183.
[6] R.D. Holly, D.C. Foster, Methods for Producing Thrombin, US Pat. No. 5,527 (1996) 692.
[7] I. Mollerup, S.W. Jensen, P. Larsen, O. Schou, L. Snel, in: M.C. Flickinger, S.W. Drew (Editors), The Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation, Wiley, NY, in press.
[8] L. Thim, S. Bjørn, M. Christensen, E.M. Nicolaisen, T. Lund-Hansen, A.H. Pedersen, U. Hedner, Biochemistry 27 (1988) 7785.
[9] J. Søndergård-Andersen, Anti-FVII MAb E436-056, personal communication.
[10] R.M. Nicoud, A. Seidel-Morgenstern, Isolation Purif. 2 (1996) 165.