Direct Capture of Lactoferrin and Lactoperoxidase from Raw Whole Milk by Cation Exchange Chromatography

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Abstract

The production of high-value dairy proteins such as lactoferrin and lactoperoxidase normally requires extensive pre-treatments of milk to remove fat and caseins by centrifugation, precipitation, Ca^{2+} chelation and/or filtration. Similarly, fat and caseins are normally removed prior to capture of recombinant proteins from the milk of transgenic animals. Such pre-treatments can result in significant loss of protein yield and/or activity. In this paper we demonstrate that it is possible to pass significant quantities of raw, untreated milk through a 5 cm high chromatography column packed with SP Sepharose Big Beads™ (GE Healthcare, Uppsala, Sweden) without exceeding the maximum allowable backpressure, provided that the processing temperature is kept nominally around milking temperature (35 to 37 ^{o}C). Results show that more than 100 column volumes of raw milk could be loaded at 300 cm/hr before breakthrough of lactoperoxidase occurred. The dynamic capacity for adsorbing lactoferrin and lactoperoxidase simultaneously under these conditions was approximately 48.6 mg/mL of resin. Minor leakage (4.6% of the feed concentration) of lactoferrin occurred throughout the loading process but major breakthrough occurred only after approximately 100 column volumes was loaded.

Introduction

Over the past two decades, the dairy industry globally has moved from being based solely on commodity food production to earning a significant income from specialty proteins. The introduction of large scale membrane processing in the early 1970's made it possible not only to reduce waste but to produce new products such as lactose and whey protein concentrate. A logical extension of the latter product is whey protein isolate (WPI), produced by single-stage batch capture of proteins on anion exchange resins. WPI is a crude mixture of acidic whey proteins, containing mainly α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulins. Typical concentrations, molecular weights and isoelectric points of whey proteins are given in Table 1 [1].

Two whey proteins not captured during WPI production by anion exchange chromatography because of their high isoelectric points are lactoferrin (LF) and lactoperoxidase (LP). These basic proteins are instead captured from whey or skim milk by cation exchange chromatography and sold as specialty ingredients.

Although production of high-value whey proteins is a commercial reality, two aspects of dairy processing may not be optimal for their production. First, the proteins are subjected to a series of processing steps prior to being extracted. It is a generally accepted principle of bioseparation process design that proteins should be separated from a source material as fast and in as few steps as possible to avoid loss of activity and yield [2, 3]. Currently, high-value dairy proteins are viewed as a by-product, with the major income (dare we say, the cash cow?) of the industry coming from commodity dairy foods such as milk powder, cheese and butter. Economies of scale for production of commodity dairy products mean that centralised processing is the industry norm. Milk is typically cooled nominally to 4 ^{o}C but in practice often to only 10 ^{o}C and held in vats on the farm for up to two days before being transported to a dairy factory. There, it is pumped to holding tanks and then undergoes a series of unit operations such as cream (fat) separation, pasteurization, homogenization and blending for standardization before further processing into individual products [4]. After a number of such operations (which varies from factory to factory), LF and LP may be extracted from skim (low fat) milk or, more commonly, from whey, which is produced as permeate during membrane concentration of milk or after precipitation of caseins much further down the processing chain as a by-product of casein production or cheese making [5].

Extensive pre-treatments of milk and whey prior to ion exchange capture of proteins are by no means restricted to industry but are also used in the laboratory. Many authors have examined the capture and analysis of whey proteins by chromatography [6-28]. Three recent examples of typical pre-treatments in laboratory studies that are intended to be relevant to industrial processes are described briefly here by way of example. Hahn et al [23] examined the performance of a range of commercially available pharmaceutical grade cation exchangers for protein capture from acid whey. In their study, milk was first centrifuged at 4,420 g for 30 minutes to remove fat, then acidified to precipitate casein and centrifuged at 17,700 g for 30 minutes, diluted with distilled water and the filtered through 0.45 μm filter before being applied to the column. Doultani et al [28] used cation exchange chromatography to produce a number of protein products from mozzarella cheese whey, first adjusting the pH with H_2SO_4 and passing the whey through Whatman No. 5 filter paper before applying it to their column. Ye et al [27] used both anion and cation exchange

chromatography to isolate α-lactalbumin, β-lactoglobulin, lactoferrin and lactoperoxidase from rennet whey. The rennet whey was produced from skim milk that had first been defatted by centrifugation. After incubation for one hour with rennet, the caseins were separated by filtration and the whey thus produced was then centrifuged at 10,000 g for 25 minutes before applying it to the column.

There is, therefore, a contradiction between the normal practices of dairy production processes and the usual requirements of protein purification processes. The resulting loss in yield can be significant. For example, Nuyens and Vav Veen reported that acid precipitation of casein resulted in 4 – 8 times more LF entrapped in the casein pellet than in the whey fraction [29]. Table 2 shows the effects of various process operations on protein activity [30].

The second aspect of high-value whey protein production that may not be optimal is an economic/business problem, in that the primary producer, the farmer, is typically paid not on the basis of target protein content but on the basis of crude milk solids delivered to the factory, which is presumably the best indicator of commodity product yields. Despite the high values of LP, LF and other minor components of milk, there appears to be no particular financial advantage to an individual farmer to increase the concentrations of these components in their animals' milk, which may be possible through practices such as dietary manipulation [31], controlled frequency of milking [32], selection of high-producing animals making up the herd [33], etc.

An alternative production paradigm is for the farmer to be the primary processor of minor milk components – in effect to "harvest" high-value proteins on farm and reap the financial return for this directly before handing the milk on to the factory for production of commodities. The concept of on-farm capture of high-value milk proteins, which has been patented [34], would fit with the accepted practices of bioseparation process design i.e. fast processing with a minimum of steps, and would directly relate yield (and thus economic returns) to factors within the farmer's control. However, a dairy farmer is unlikely to be comfortable carrying out the complex set of operations required for industrial chromatography on the farm. Therefore, a simple, robust process which allows the capture ("harvesting") of the high-value components directly from raw milk without the pre-

treatments described above and without exacting buffer preparation or the complexities of standard column chromatography operations would be ideal.

A typical bovine milk contains 13% solids, with 4% fat present as an emulsion of globules with diameters up to 10 μm and caseins present as a colloidal suspension of particles with diameters up to 0.1 μm [4]. Fat globules normally cause problems for chromatographic separations, as they block packed columns as soon as the feed is introduced. Therefore, fat is removed prior to cation exchange capture of LF and LP from skim milk. Raw whole milk contains larger suspended particles than whole (full fat) processed milk, as the latter is homogenized in the factory to produce a uniform consistency. Thus, raw whole milk might be expected to cause even greater problems for chromatographic processing.

The inability to pass raw or whole milk through a chromatography column is likely caused by low processing temperatures. Milk is processed nominally at 4 °C because of the need to minimise bacterial growth. At these temperatures, milk fat hardens or solidifies, causing column blockage. Table 3 gives the melting points and approximate compositions of the fatty acids making up the milk fat triglycerides [4]. Immediately after milking, fresh raw milk has a temperature of about 35 to 37 °C. Although this temperature is below the melting points of several fatty acids, notably myristic and palmitic acids, it is above the melting point of oleic acid, which is the most abundant fatty acid. The hardness of the fat globules will be affected by the relative fatty acid compositions of the milk fat tri-glycerides. At 35 to 37 °C the milk fat globules may be soft enough to easily deform and pass through a packed bed of chromatography resin of sufficient resin diameter. In this case, chromatographic processing should be possible. Also, the viscosity of whole milk decreases with increasing temperature, so back pressure through the column will be lower for warm milk than for cooled milk. Lower viscosity, combined with higher protein diffusivities, should also enhance the performance of chromatographic processes because of improved protein mobility compared with that at lower temperatures. If the milk can be processed quickly enough on the farm, microbial growth may not be a factor.

The purpose of this study was to demonstrate that it is possible to extract proteins by chromatography directly from untreated, raw, whole milk. SP Sepharose Big Beads™ (GE Healthcare, Uppsala, Sweden) were used for this purpose because the resin has a relatively

large particle diameter (100 to 300 μm) which should give lower column backpressures than smaller diameter media. We made no attempt to compare the performance of Big Beads with other media. A series of experiments was carried out to assess equilibrium LF and LP adsorption capacities, packed column backpressure and dynamic (breakthrough) capacities for untreated raw milk taken directly from the cow.

Materials and Methods

Raw milk was obtained from Greenfield dairy farm site (Dexcel Limited and Sensortec Limited, New Zealand), from cows milked using an automated milking system. Milk was held after collection at 37 ^{o}C under gentle stirring to prevent fat separating under the influence of gravity, until it was processed.

Chemicals were obtained from BDH Chemicals (Poole, England) unless otherwise specified. Bovine lactoferrin standards were obtained from Sigma-Aldrich (St Louis, MO, USA) and Bethyl Laboratories (Montgomery, Texas, USA). Affinity purified goat polyclonal anti-bovine lactoferrin antibody (1 mg/mL) was obtained from Bethyl Laboratories and used for both ELISA and surface plasmon resonance (SPR) analysis. Lactoperoxidase standard was obtained from Sigma-Aldrich.

SP Sepharose Big Beads™ (GE Healthcare, Uppsala, Sweden) were used to adsorb lactoferrin and lactoperoxidase proteins from raw whole milk. Resin was equilibrated before use in 10 mM phosphate buffer (10 mM mono and dibasic sodium phosphate) at pH 6.7. Protein elution was achieved in the same buffer using either gradient (0 to 1.0 M NaCl) or step elutions. Step elutions were carried out in two steps: 0.4 M NaCl to elute lactoperoxidase and 1.0 M NaCl to elute lactoferrin.

An XK16 water-jacketed chromatography column (GE Healthcare), connected to an AKTAfplc fast protein liquid chromatography system, controlled by Unicorn 4.0 (GE Healthcare, Uppsala, Sweden), was used for all column-based chromatographic milk processing. The column was packed to a height of 5 cm, following the manufacturer's instructions, giving a bed volume of 10 mL.

To determine equilibrium isotherms, 0.2 g of equilibrated, swelled, drained resin was quantitatively weighed into 10 mL centrifuge tubes. Lactoferrin and lactoperoxidase standards from samples of known purity (Tatua Dairy Cooperative Limited, Morrinsville, New Zealand) were constituted to concentrations ranging from 0.05 to 20.0 mg/mL. 5 mL of each standard solution was added to the resin and left for 24 hours on a rotating plate within an incubator at 37 ± 0.2 ^{o}C. The tubes were then centrifuged to remove the resin from suspension and the supernatant was filtered using a 5 μm filter. The equilibrium lactoferrin (C_{LF}^{*}) and lactoperoxidase (C_{LP}^{*}) concentrations of solutions were determined using the Bincinchoninic acid (BCA) protein assay (Pierce, Rockfield, IL, USA), sensitive between 20 and 1200 μg/mL. The amounts of protein bound to the resin were calculated from the differences between the initial and final solution protein concentrations and the equilibrium binding capacities for lactoferrin and lactoperoxidase, Q_{LF}^{*} and Q_{LP}^{*}, respectively, were calculated by dividing the amounts bound by the volume of the resin.

For column breakthrough studies, lactoferrin concentrations were determined using an optical biosensor analysis as described by Indyk and Filzoni [35], using a surface plasmon resonance technique (SPR) on a Biacore 3000 instrument (Biacore , Uppsala, Sweden). Raw whole milk samples were centrifuged at 4800 g (Min-Spin, Ependorf, Hamburg, Germany) for 2 minutes to remove fat and filtered using a 5 μm filter (Sartorious AG, Goettingen, Germany) before serial dilutions (to 2000x) were made in 500 mM HBS-EP buffer (10 mM HEPES, pH 7.4 with 3 mM EDTA and 0.005% (v/v) surfactant P20). The running buffer was obtained from Biacore as 150 mM HBS and NaCl concentrations were enhanced to 500 mM for sample and standard preparations to reduce non-specific interactions. Lactoferrin concentrations were also measured using a bovine lactoferrin Elisa kit (Bethyl Laboratories) with some modifications as described by Turner et al. [31].

Lactoperoxidase determinations were carried out using oxidation of synthetic substrate 2,2'-azinobis[3-ethyl-benzothiazoline-6-sulphonic] diammonium salt (ABTS) for the enzyme [36]. This assay method only measures active lactoperoxidase.

Size distributions of resin particles and fat globules were determined by laser light-scattering using a Mastersizer instrument (Malvern Instruments Ltd., Worcestershire, UK). Samples

were first diluted with distilled water to allow sufficient light transmittance. The DV0.9 (the diameter below which 90% of the volume of particles are found), DV0.5 (the diameter below which 50% of the volume of particles are found) and D[4,3] (the equivalent volume mean diameter or diameter of spheres of equivalent volume to measured particles) were determined.

Results and Discussion

Equilibrium adsorption isotherms for LF and LP on Sepharose Big Beads are shown in Figures 1 and 2. The maximum capacities of the resin are very high for these standard solutions at high concentration but the resin capacities are highly dependent on solution concentration below 1 mg/mL. Because their concentrations in milk (Table 1) are below 1 mg/mL, the resin capacities for LF and LP will be much lower than the maximum values shown in Figures 1 and 2. It may be the case that other milk components will adversely affect the binding of LF and LP but we did not investigate this.

Figure 3 shows the backpressure exerted by a 5 cm depth packed bed of resin for raw, unfiltered milk at approximately 35° C at two flow rates. At 300 cm/hr the column backpressure remained below 0.3 MPa, the maximum allowable back pressure for the resin, for more than 100 column volumes (cv's) of loading. Figure 4 shows the effect of processing temperature on the backpressure through the bed at 300 cm/hr. Variations between individual runs may be the result of variability between milk samples collected from different animals on different days. Figure 5 shows the number of column volumes, CV*, that can be loaded before the backpressure exceeds 0.3 MPa at each temperature, T. The logarithmic regression line in Figure 5 has the formula $CV^* = 91.2 \cdot \ln(T) - 240$, and extrapolation of this to $CV^* = 0$ predicts that no flow through the column is possible below about 14 ^{o}C. This corresponds exactly with the melting point (14 ^{o}C) of the most abundant fatty acid in milk fat, oleic acid (Table 3).

Light scattering particle size measurements on 10 individual raw milk samples indicated that suspended solids had an average diameter D[4,3] = 2.91 ± 0.9 μm, Dv0.9 = 5.52 μm and Dv0.5 = 2.91 μm. These values compare well with published values for milk fat globules [4,

37]. We did not measure the size of casein micelles but published values for raw milk are around 0.15 μm [4, 37, 38]. SP Sepharose Big Beads had an average diameter D[4,3] = 154 ± 67 μm, Dv0.9 = 219 μm and Dv0.5 = 155 μm. Given the size of the milk fat globules and the strong influence of processing temperature (Figures 4 and 5) on column backpressures, we propose that the milk fat globules become more malleable as temperature increases, allowing them to pass through the bed, but that at lower temperatures they harden or solidify, preventing their passage.

Figures 6 and 7 show the breakthrough curves for LP and LF, respectively, at 300 cm/hr and 450 cm/hr. The LF level in the feed milk was determined by ELISA assay to be 550 mg/L and the level of LP was 3.94 mg/L by ABTS assay. At 300 cm/hr, more than 120 cv's of raw whole milk can be loaded before LP breakthrough occurs. Minor leakage of LF occurs throughout the loading step but there is a sharp increase in outlet LF concentration again after approximately 100 column volumes. The level of leakage in Figure 7 is 25.4 mg/L, or 4.6% of the feed LF level, in agreement with Etzel et al [21], who showed leakage of approximately 5% of the feed LF during loading in their study of LF adsorption from pre-filtered skim milk, also using SP Sepharose Big Beads.

The amount of material bound represents, for this 10 mL column, a dynamic capacity of approximately 480 mg of LF and 5.5 mg of LP bound simultaneously. The total dynamic capacity is therefore about 48.6 mg/mL under these conditions. This compares favourably with the 34 mg/mL of LF dynamic capacity of Big Beads loaded at 450 cm/hr at 10 ^{o}C at a similar starting concentration (filtered skim milk spiked to a level of 679 mg/L) reported by Etzel et al [21]. The higher dynamic capacity we obtained is probably due to our higher processing temperature and slower loading flow rate. We conclude that the presence of fat in raw whole milk does not adversely affect the dynamic loading capacity under the conditions used.

Table 4 indicates that the gross properties of milk (fat content, protein content) do not change significantly on passage through the column. The results in Table 4 and Figures 3 to 6 indicate that it should be possible to extract LF and LP from the milk in a packed column and then pass it on to normal dairy processing, with little or no change in physical milk characteristics, provided the temperature is kept sufficiently high.

As an indication of feasibility, a packed bed 5 cm in height and 36 cm in diameter has a column volume of just over 5 L. Such a bed would be capable of processing 500 L of raw milk before if the common industrial chromatography guideline of 10% breakthrough of LF is tolerated before loading is stopped. The latter volume corresponds to the milk from 33 cows, based on an average of 15 L of milk per cow per milking. According to Table 1, 33 cows will possess, on average, 275 g of LF and 28 g of LP.

At 300 cm/hr through such a column, it would take on average only 3 minutes to process the milk from each cow, which is less than the time required for milking it. The process therefore seems to fit well within the timeframe of milking. The few minutes required for processing each cow should not allow significant microbial growth, provided the column could be sanitised between milkings. Milk could be cooled in a small heat exchanger immediately upon exiting the column and sent to the holding vat to await collection.

We have not yet formally examined the cleaning, sanitisation and re-use of the resin through more than a few production cycles but we have observed empirically that cleaning with standard NaOH and isopropyl alcohol solutions, followed by regeneration with 2 M NaCl solutions allows re-use of the resin without observable decreases in performance. This aspect warrants further investigation.

The processing of raw milk need not be restricted to ion exchange chromatography but could be applied to other chromatographic techniques, particularly affinity chromatography. For example, a Protein A column could be used to recover immunoglobulins directly from standard or hyperimmune milk or from colostrum. Affinity chromatography might also be used in this mode to extract recombinant proteins directly from the milk of transgenic animals quickly and at maximum yield and activity.

Conclusions

We have demonstrated that raw, whole milk can pass through a shallow, packed-bed chromatography column in significant quantities using a commercially available resin, provided that the processing temperature is kept at or near the temperature of freshly

collected milk. Direct chromatographic capture from raw milk minimises processing time and avoids the fat and casein removal steps that are normally applied prior to capture of whey proteins, and has the potential to increase the yields and activities of high-value bioactives from milk.

This approach raises the possibility of a new business paradigm in dairy processing, in which the farmer can be a producer of crude high-value protein fractions as well as a producer of milk solids for the commodity dairy manufacturers because complex and time-consuming pre-treatments of the milk is unnecessary.

Direct chromatographic processing of raw milk may also have applications in the production of recombinant proteins from the milk of transgenic animals.

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Table 1. Typical concentrations of whey proteins and their isoelectric points [1].

Protein	Approx. Concentration in Whey (%)	Isoelectric point
β-Lactoglobulin	0.30	5.35-5.49
α-Lactalbumin	0.07	4.2-4.5
Immunoglobulins	0.06	5.5-8.3
Bovine serum albumin	0.03	5.13
Protease-peptones	0.14	3.3-3.7
Lactoferrin	0.003	7.8-8.0
Lactoperoxidase	0.002	9.2-9.9

Table 2. Physicochemical changes and positive (+) or negative (-) nutritional effects of process treatment and storage on proteins and amino acids [30].

Treatment/condition	Physicochemical changes	Nutritional effects
Heat treatment	Protein denaturation	Improvement of intrinsic digestibility (+)
		Reduction of trypsin inhibitor activity (-)
		Destruction of heat sensitive amino acids (-)
	Intramolecular reactions	Cross-linkages (-)
	Reaction with sugars	Destruction of lysine (-)
pH modification	Solubility	Risk of oxidation (-)
	Acid or alkaline hydrolysis	Improvement of digestibility (+)
		Unspecific peptide bond breakage (-)
		Destruction of pH-sensitive amino acids (-)
		Cross-linkages (-)
		Isomerisation (racemisation) (-)
Enzymatic hydrolysis	Reaction with proteases	Peptides (+/-)
	Reaction with oxygenases	oxidation of amino acids through lipid or polyphenol oxidation (-)
Membrane separation	Protein fractionation	Protein/peptide enrichment (+)
		Change in amino acid composition (+/-)
Storage	Reaction with sugars	Destruction of lysine (-)
	Presence of oxygen	Oxidation (-)
	Reaction with polyphenols	Oxidation (-)

Table 3. Composition and melting points of the major fatty acids in milk fat [4].

Fatty Acid	% of Total Fatty Acid Content	Melting Point ^{o}C
butyric	3.0 – 4.5	-7.9
caproic	1.3 – 2.2	-1.5
caprylic	0.8 – 2.5	16.5
capric	1.8 – 3.8	31.4
lauric	2.0 – 5.0	43.6
myristic	7.0 – 11.0	53.8
palmitic	25.0 – 29.0	62.6
stearic	3.0 – 7.0	69.3
oleic	30.0 – 40.0	14.0
linoleic	2.0 – 3.0	-5.0
linolenic	< 1.0	-5.0
arachidonic	< 1.0	-49.5

Table 4. Composition (%) of bulk raw whole milk before and after lactoferrin and lactoperoxidase extraction.

		Fat	Crude Protein	True Protein	Casein	Lactose	Total Solids
Sample 1	Feed	4.11	3.53	3.27	2.61	4.84	13.20
	Outflow	4.04	3.52	3.29	2.63	4.84	13.10
Sample 2	Feed	3.64	3.33	3.15	2.44	4.21	11.80
	Outflow	3.55	3.30	3.07	2.35	4.35	11.60

Figure Legends

Figure 1. Equilibrium isotherm for lactoferrin, measured in a standard solution. The line fitted through the points is the Langmuir isotherm.

Figure 2. Equilibrium isotherm for lactoperoxidase, measured in a standard solution. The line fitted through the points is the Langmuir isotherm.

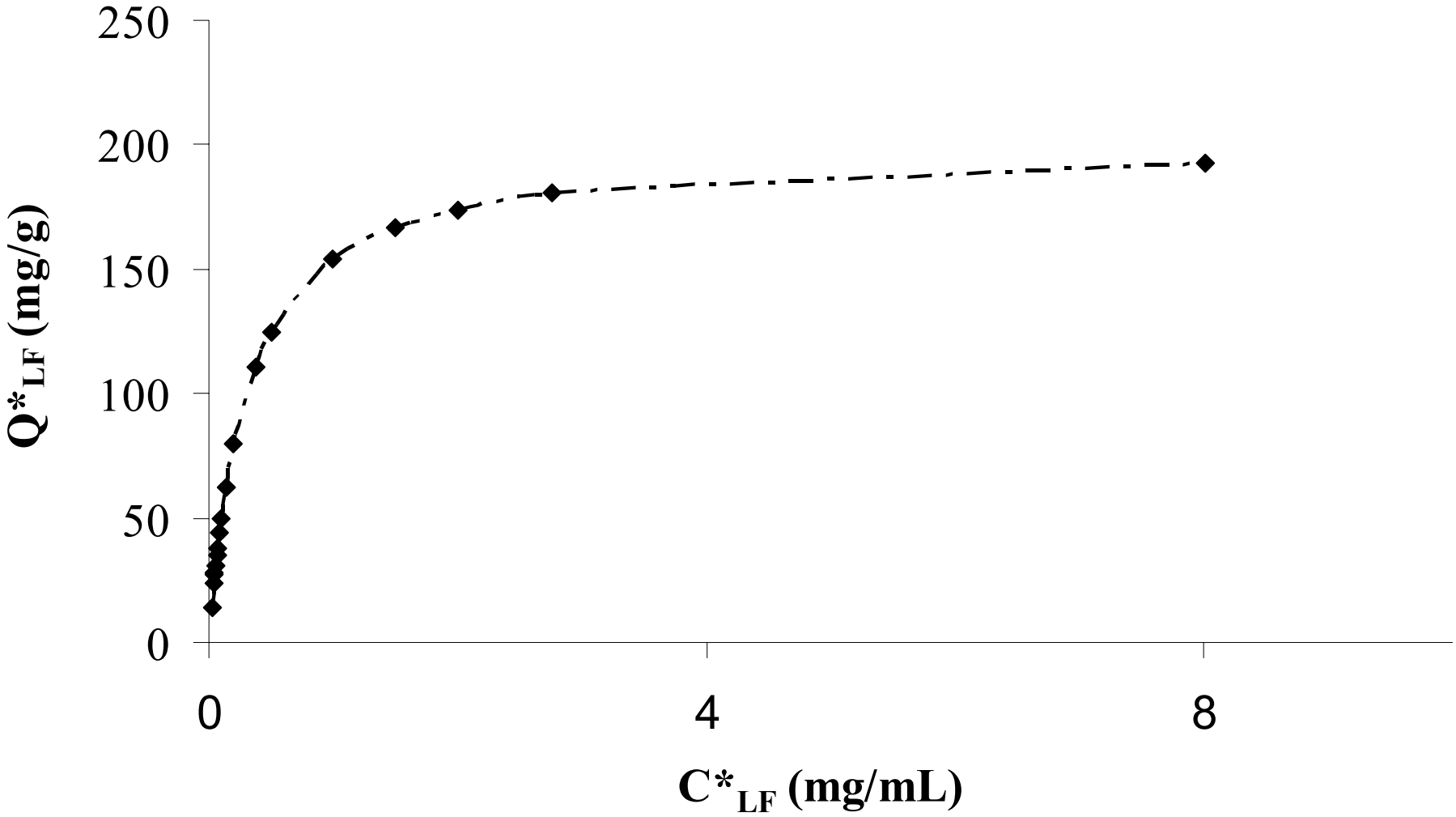
Figure 3. Backpressure exerted by flow of raw milk at approximately 35 ^{o}C through a 5 cm packed bed of SP Sepharose Big Beads at two linear flow rates.

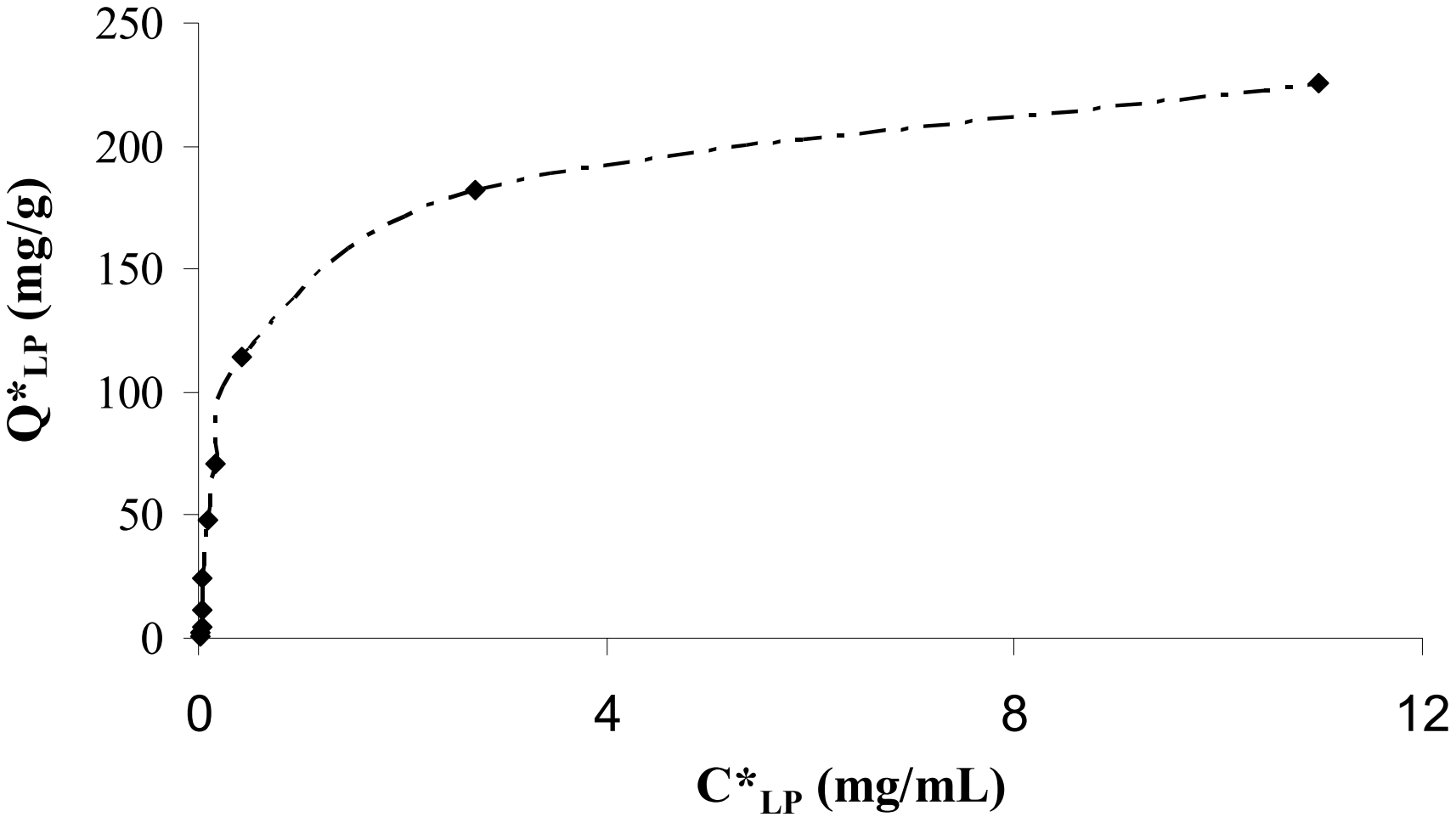
Figure 4. Effect of temperature on the backpressure for flow of raw milk at approximately 35 ^{o}C through a 5 cm packed bed of SP Sepharose Big Beads at 300 cm/hr.

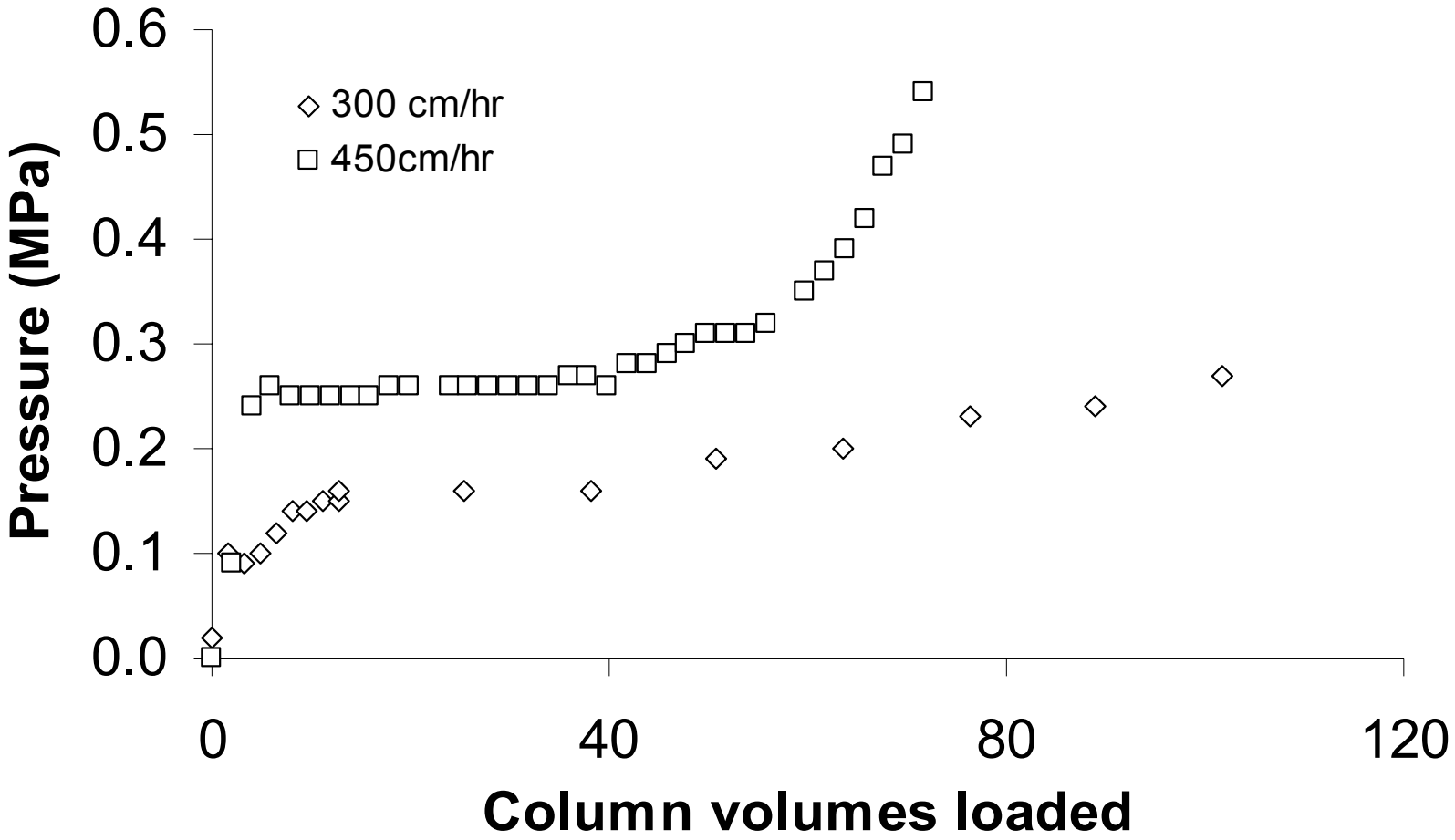
Figure 5. Number of column volumes that can be loaded before the maximum allowable resin back pressure (0.3 MPa) is exceeded as a function of temperature.

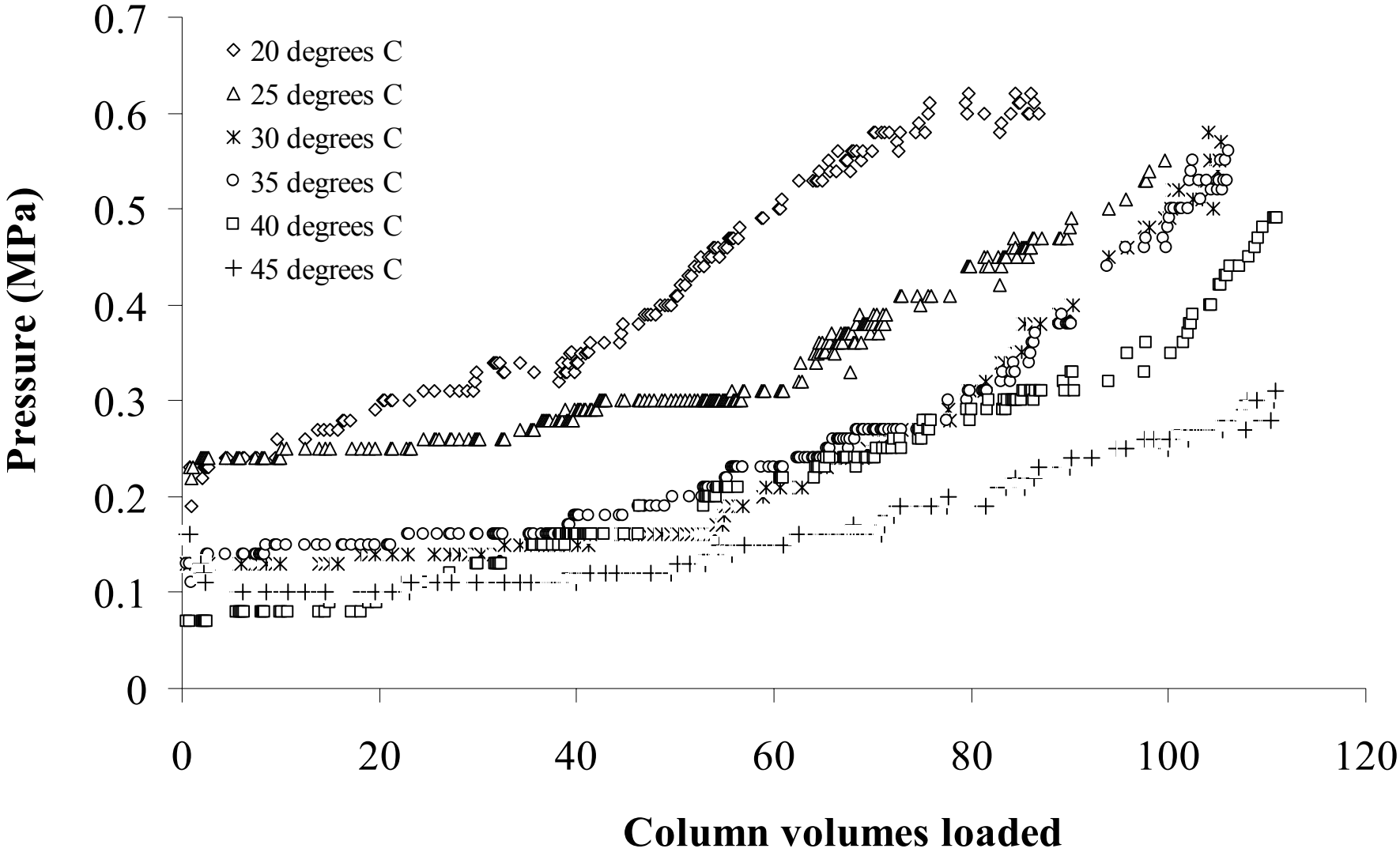
Figure 6. Breakthrough curve for lactoperoxidase at two linear flow rates. Feed concentration is 3.94 mg/L.

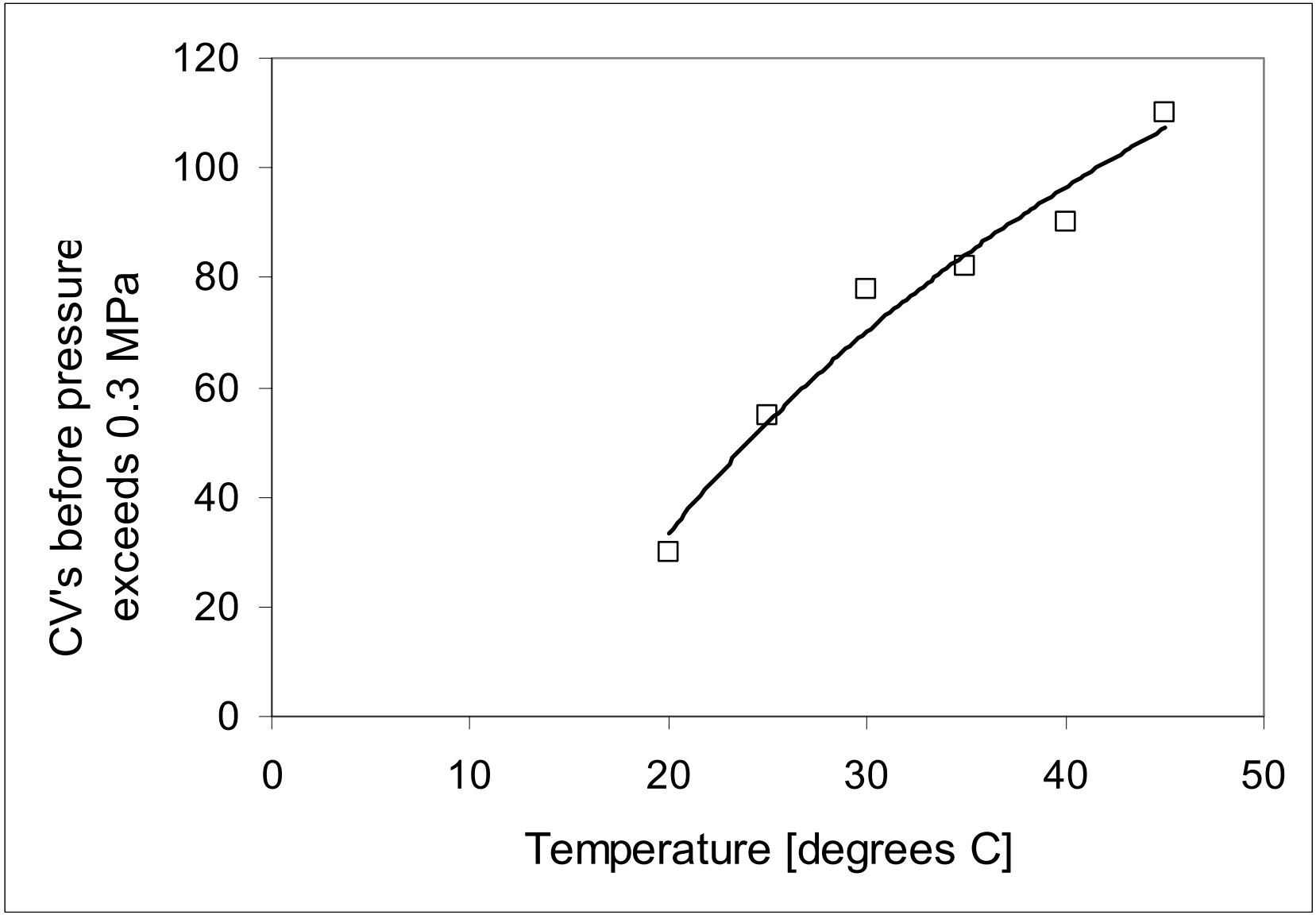
Figure 7. Breakthrough curve for lactoferrin at two linear flow rates. Feed concentration is 550 mg/L.

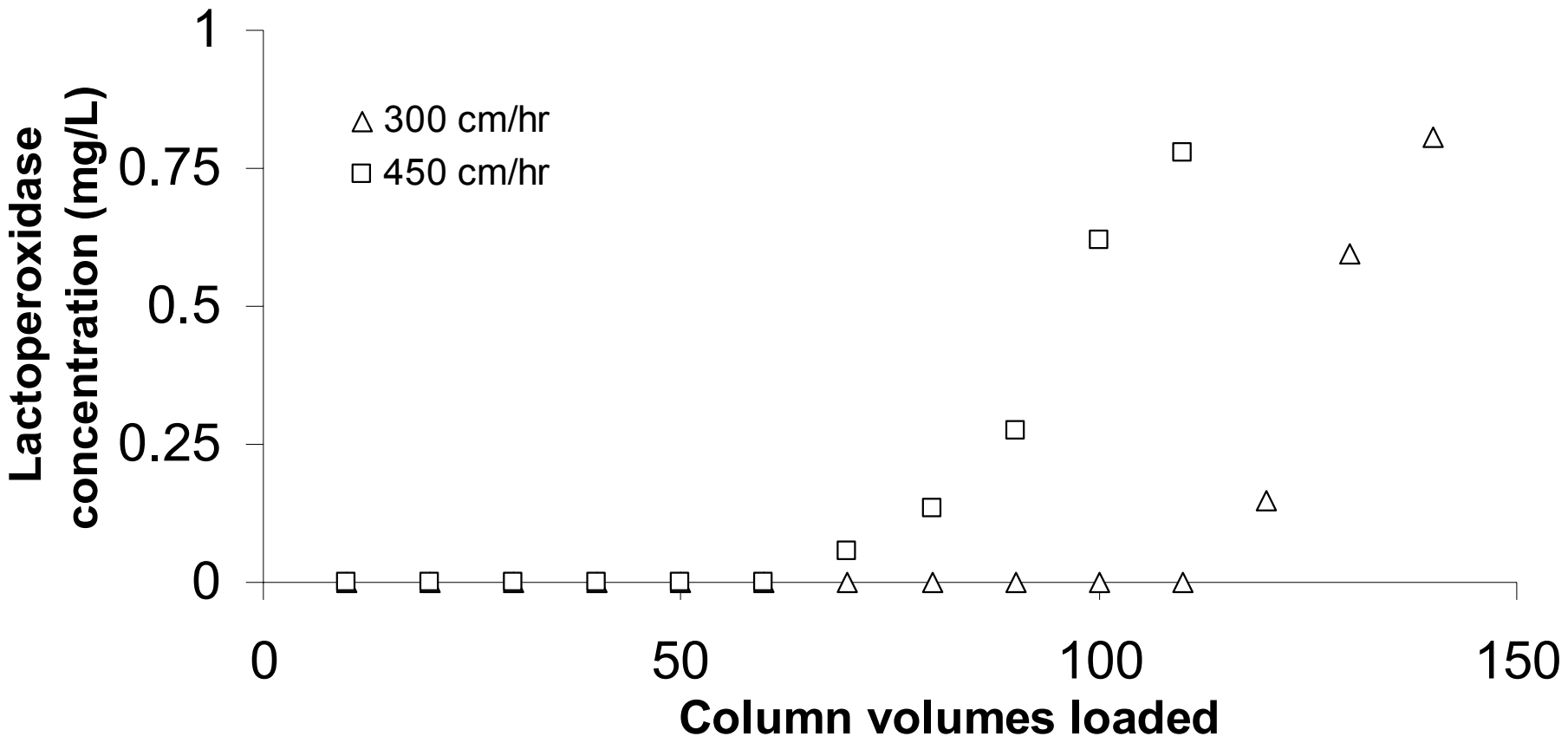

250
200
150
100
50
0
Q*LF (mg/g)
0
4
8
C*LF (mg/mL)

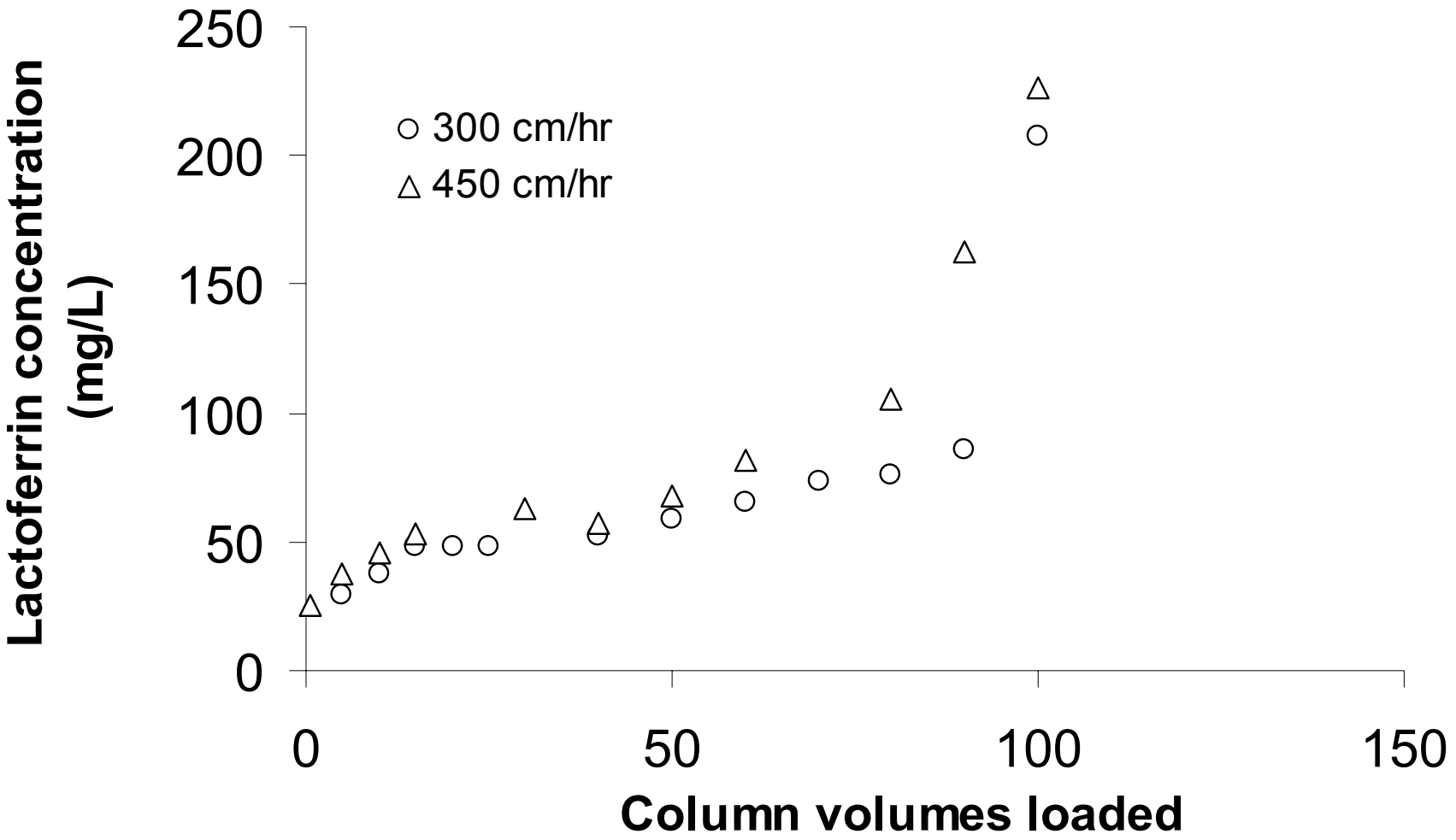

250
200
150
100
50
0
Q*LP (mg/g)
0
4
8
12
C*LP (mg/mL)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Pressure (MPa)
300 cm/hr
450cm/hr
0
40
80
120
Column volumes loaded


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pressure (MPa)
20 degrees C
25 degrees C
30 degrees C
35 degrees C
40 degrees C
45 degrees C
0
20
40
60
80
100
120
Column volumes loaded


120
100
80
60
40
20
0
CV's before pressure exceeds 0.3 MPa
0
10
20
30
40
50
Temperature [degrees C]


Lactoperoxidase concentration (mg/L)
1
0.75
0.5
0.25
0
△ 300 cm/hr
□ 450 cm/hr
0
50
100
150
Column volumes loaded


Lactoferrin concentration (mg/L)
250
200
150
100
50
0
300 cm/hr
450 cm/hr
0
50
100
150
Column volumes loaded